University of Nottingham
UK | CHINA | MALAYSIA

INVESTIGATION OF PHOSPHATE-REGULATORY TRANSCRIPTION FACTORS IN WHEAT: TaPtf1 and TaMyb67

Rong Fan MSc

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

June 2018

Division of Agricultural and Environmental Sciences
School of Biosciences
University of Nottingham

Abstract

Phosphorus (P) is one of the essential nutrients for plant growth; however, it is usually in low availability to plants in most soils. P deficiency/low-P in the early growth stages of wheat can cause reductions in tiller and head formation, which poses a threat to wheat productivity and global food security. Genetic variation of phosphate use efficiency (PUE) has been documented in wheat. PUE can be improved under P deficiency/low-P by P-stress-responsive adaptation mechanisms that increase P acquisition and/or utilisation. Major regulatory components involved in PUE include P_i itself, microRNAs, hormones and sugars. In addition, several transcriptional factors (TFs) appear to play crucial roles in the regulatory complexity controlling the expression of P-stress-responsive genes. A better understanding of their roles may help to achieve favourable expression patterns of downstream genes and hence potentials to develop wheat cultivars with improved PUE in future crop improvement programme. Using bioinformatic approaches, this study identified two TFs, *TaPtf1* and *TaMyb67*, which may act as key components in PUE in wheat. Their roles in regulating PUE were investigated through molecular and transgenic approaches. Overexpression constructs for *TaPtf1* and *TaMyb67* were created and subsequently transformed into wheat by *Agrobacterium*-mediated transformation. Selected transgenic lines were studied for overexpression of these transgenes and their effects on growth-, harvest- and PUE-related plant traits in a soil-pot experiment under different P supply. The phenotypic effects of *TaPtf1* in the transgenic lines were implicated to be P-stress responsive and likely associated with plant height, biomass, grain filling and P accumulation in shoots. The results appeared to be consistent with previous studies of *TaPtf1* in wild-type wheat suggesting that *TaPtf1* has a functional divergence from *OsPtf1*/*ZmPtf1*. On the other hand, *TaMyb67* was shown to be a likely ecotypic variation of *TaPhr1-B1*. *TaMyb67* transgenic

lines gave no clear evidence of phenotypic differences, presumably due to the downstream P-stress-responsive genes regulated by *TaMyb67* being unresponsive to high-P and the low level of (or no) overexpression of *TaMyb67* under low-P in these lines.

Dedication

TO MY DEAREST PARENTS, WEI WANG AND QIXIANG FAN, WHO WERE ALWAYS THERE TO FINANCIALLY, PRACTICALLY AND MENTALLY BACK UP. I SINCERELY WISH, DEEP IN HEART, THEM A HEALTHY AND HAPPY EVERYDAY LIFE

Acknowledgement

On successful completion of this thesis, my sincere gratitude and appreciation would be extended to all the individuals who had helped me throughout the years. First, I would like to take this opportunity to express my gratitude to my supervisors Professor Chungui Lu (Nottingham Trent University), Professor Charlie Hodgman, Dr Grantley Lycett, Dr. Dr Ranjan Swarup (University of Nottingham) for their encouragement and support to my research, as well as examiners Professor Keith Edwards (University of Bristol) and Dr. Erik Murchie (University of Nottingham) for their good instruction and advice for my thesis correction.

I am also grateful to Dr Xingguo Ye, Dr Zengyan Zhang and Dr Ming Chen, as well as all their crop science research group members in the Chinese Academy of Agricultural Sciences (CAAS, Beijing), for providing the wheat transformation platform and other technical supports when I stayed over there doing my PhD project (07/2013-02/2015). Here it needs a special mention to Dr Lichao Zhang from Professor Jizeng Jia's crop science research group (CAAS, Beijing) for his providing Gateway vector for the wheat transformation.

At the same time, I am greatly indebted to the technical support offered in Sutton Bonington campus. The laboratory support was mainly provided by John Corrie, along with Saul Vazquez Reina and James Verran from Gateway Building. Additional technical support was also kindly provided by Julietta Marquez and Jayalath De Silva from South Lab, as well as Ruth Cornock and Linda Gillett from the Plant Science. During the physiological experiment, the glasshouse work was helped around by the Glasshouse office team including Mark Meacham, Sue Flint, Rosalind Beacham, Janet McLintock and Catherine Tomlinson, which I sincerely appreciated. It was also very much appreciated for the technical support provided by Dr Saul Saul Vazquez and advice provided by Dr Scott Young for the ICP analysis.

My special thanks are also reserved for Dr Lu Jiang (Jiangsu Academy of Agricultural Sciences) who greatly helped at the beginning of my laboratory experiments and Dr Quan Xie (Nan Jing Agricultural University) who gave a lot of supports in physiological experiments. I also would like to acknowledge several other colleagues for their sharing their expertise and experience that helped me to go through all the struggling stages in my PhD research. This includes Dongbing Liu, Steven Grundy, Faraz Khan, Ishan Ajmera, Martinez Martinez Manuel, Nanjun Jiang, from Professor Lu's group. Moreover, I would also like to give my appreciation to Caiyun Yang (School of Biosciences, University of Nottingham) and Lei Wang (Veterinary and Medicine Sciences, University of Nottingham) for their kindness and helping me out. During my research, I also received good advice from other colleagues from the Plant Science, including Dr Darren Wells, Dr Johnathon Atkinson, Dr Lolita Wilson, , Dr Thomas Denyer, Hongyang Yu, Wenzhe Yin, Yang Song, Nicola Leftley, Rui Mo. In addition, I also received some help from visitors to our research group, including Xiaofen Wang (China Agricultural University), Lingling Wang (Zhejiang University), Ling Zhang (Sichuan Agricultural University). Not forgetting to thank Susan Golds, Emma Hooley, Sarah Gamba for the administrative support during my PhD. Last but not the least, I gratefully acknowledge my friends Jun Zhang and Jianghai Hu who put efforts into helping me with graphics editing for the thesis.

Finally, I deeply feel indebted to my family and other good friends that I cannot name here one by one. Without their unwavering love, ceaselessly encouragements and supports, I would not be able to complete the PhD under the difficulties I had encountered.

Contents

List of Tables

List of Figures

Abbreviation

Abbreviation	Full name
°C	Centigrade degrees
µL	Microlitre
µm	Micrometre
µM	10^{-6}mol L^{-1}
ANOVA	Analysis of variance
aa	Amino acid
ADP	Adenosine diphosphate
AGP	Arabinogalactan proteins
Amp	Ampicillin
AS	Alternative splicing
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
bp	Base pair
CAAS	Chinese Academy of Agricultural Sciences
Carb	Carbenicillin
CDS	Coding DNA Sequence/coding sequence
cDNA	complementary deoxyribonucleic acid
Cef	Cefotaxime
cm	Centimetre
CTAB	Cetyltrimethylammonium bromide
Cys.	Cysteine
Dicamba	3, 6-dichloro-2-methoxy-benzoic acid
DNA	deoxyribonucleic acid
EBI	European bioinformatics Institute
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
Gent	Gentamycin
g	Gram
gDNA	Genomic DNA
Ha	Hectare
GUS	β-glucuronidase
Hyg	Hygromycin
IR	Inverted repeat
HPT	Hygromycin Phosphotransferase
Kana	Kanamycin
K	Potassium
Kg	Kilogram
LB	Luria-Bertani medium
L	Litre
LSD	Least significant difference
Mb	Million base pair
M	mol L^{-1}
MCS	Multiple cloning sites
MES	Methyl ester sulfonate

mg	Microgram
m^2	Square metre
min	Minute
mL	Millilitre
mM	10^{-3}mol L^{-1}
mRNA	Messenger ribonucleic acid
miRNA	MicroRNA
MS	Murashige and Skoog
MYB	Myeloblastosis
N	Nitrogen
NaOCl	Sodium hypochlorite
P	Phosphorus
P1BS	Phr1-specific binding sequence
PAT	Phosphinothricin acetyl transferase
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
P_i	Phosphate
PH	Potential hydrogen
PHYRE	Protein Homology/analog- Y Recognition Engine
PPT	Phosphinothricin
PSI	phosphate starvation-induced
PT	Phosphate (P_i) transporter
PTGS	Post-transcriptional gene silencing
PUE	Phosphorus Use Efficiency
qPCR	Quantitative real-time polymerase chain reaction
QTL	Quantitative trait loci
Rif	Rifampicin
RNase	Ribonuclease
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
Ser.	Serine
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SQD	Sulfoquinovosyl diacylglycerol
TGS	Transcriptional gene silencing
T0	Primary transformant generation
T1	Secondary transformant generation
Tg	Tera-gram
Tm	Annealing temperature
Tris-HCl	2-Amino-2-hydroxymethyl-propane-1,3-diol-hydrochloride
UoN	University of Nottingham
USDA	U.S. Department of Agriculture
Var.	Variety
YEP	Yeast Peptone Extract medium

Chapter 1 General introduction

1.1 Crop Species

Wheat (*Triticum spp.*) is a cereal crop cultivated for grains as a food source by humans. The earliest time of this can date back to 10,000 BC in the Neolithic period (Abbo *et al*., 2013; Lev-Yadun *et al*., 2000). During human cultivation, wild wheat was domesticated and developed into cultivated wheat through selection processes, during which favoured traits were distinguished for better harvesting performance (Salamini *et al.*, 2002). Most of contemporary wheat species (*Triticum aestivum* or *Triticum durum*) are cultivated-wheat derived from domestication and have a long evolutionary history.

1.1.1 Species

Today, about 95% the wheat grown worldwide is hexaploid bread wheat (*T. aestivum L*., 42 chromosomes) (Konvalina *et al*., 2010; Zaharieva *et al*., 2010). The remaining 5% is mainly tetraploid durum wheat (*T. durum Desf.*, 28 chromosomes), while a few other wheat species, such as ancient species of spelt, einkorn, emmer, are still grown in small-scale to meet special market requirements for traditional foods, health alternatives or organic foods, etc. (Konvalina *et al*., 2010). Bread wheat thus becomes a dominant species among the wheat genus (*Triticum spp.*) worldwide. Within the species, bread wheat encompasses a wide range of different types which are classified to various cultivars by breeders or farmers according to growth habit and functionality. By growing season, for example, there is winter wheat versus spring wheat; by gluten content, there is hard wheat (high protein content) versus soft wheat (high starch content); or by grain colour, there is red, white or amber wheat (Bridgwater and Aldrich, 1966). In the following parts of the thesis, the term “wheat” mainly refers to “bread wheat”.

1.1.2 Genetic background

Wheat is an economically important cereal crop and there has been great scientific interest in studying its genetics in the past decades, with the aim to improve its yield, grain quality and biotic or abiotic stress tolerance (Carver, 2009; Vasil, 2007). Wheat has a more complex genetic background than other domesticated crop species, e.g. rice or maize. In plant taxonomy, wheat belongs to the grass family of Poaceae that appeared from 50–70 million years ago, the sub-family of Pooideae, the tribe of Triticeae and the genus of *Triticum* comprising several wheat species (Kellogg, 2001; Van Slageren, 1994). Based on the three base genomes (A, B and D; each contains seven chromosomes, n=7) characterised for the genus of *Triticum*, these wheat species are classified into different ploidy levels: diploid (2n=14, two sets of chromosome), tetraploid (2n=28, four sets of chromosomes) and hexaploid (2n=42, six sets of chromosomes). The majority of wheat species are polyploids, while only a few are diploid (Hancock, 2004). The repetitive chromosomes result in the complexity and huge size of the wheat genome, e.g. 16,049 Mb for bread wheat; 12,377 Mb for durum wheat (Bennett and Leitch, 2011).

It is believed that the hybridisation of wild diploid wheat (*T. urantu*, 2n=14, genome AA) and goatgrass (*Ae. speltoides*, 2n=14, genome SS) initially produced the wild emmer wheat (*T. dicoccoides*, 2n=28, genome, AABB), which later became the domesticated emmer. The domesticated emmer wheat hybridised with another goatgrass (*Ae. tauschii*, 2n=14, genome DD) and produced the first spelt wheat (*T. Spelta*, 2n=42, genome AABBDD), which later became the domesticated spelt. Following an extended period of human cultivation, emmer and spelt evolved to durum wheat (*T. durum*) and bread wheat (*T. aestivum*) respectively in consequence of selection by humans and spontaneous mutation (Dvorak *et al*., 2011, 2012; Peng *et al*., 2011).

Advances in wheat genetic and genomic (or post-genomic) research have lagged behind compared to that of other major crops, mainly because the wheat genome is complex and large size. With recent developments in high-throughput technology, wealth results have been produced in wheat genetics and genomics (Feuillet and Muehlbauer, 2009). In 2010, UK scientists completed and published a draft sequence coverage of the wheat genome (Var. Chinese spring) (Wilkinson *et al*., 2012). Following this, the same research group further published their essential results of genome-wide sequencing for wheat by using random shotgun libraries of total DNA and cDNA, where almost 94,000 and 96,000 genes were identified (Brenchley *et al*. 2012). In 2014, the collective research work on the wheat genome by IWGSC (International Wheat Genome Sequencing Consortium-participated by researchers from different countries for a chromosome-based draft sequence of the bread wheat genome) was published in Science, which provided new insight into the structure, organisation and evolution of the large, complex genome of this widely grown cereal crop (Mayer *et al*., 2014).

1.1.3 Features

Wheat outperforms other crops in several aspects, such as adaptability, productivity and end-use value. Wheat can produce satisfactory yield despite being grown in a broad range of environments, such as different water availability, a wide temperature range or altitude, various soil types, etc. While the optimum growth temperature for most wheat is 25°C, the crop usually adapts to an actual temperature range between 3–4°C and 30–32°C (Briggle, 1980). Moreover, the altitude of the growth area of wheat stretches from sea level to c. 3000m (Slafer and Whitechurch, 2001). As such, wheat production expands to a large area ranging from South America and southern Oceania to North America, northern Europe and Asia (Shewry and Jones, 2005).

The desirable productivity and adaptability of wheat make it an essential constitution of daily food consumption in many countries (FAO, 2012). Ground wheat produces flour that is not only particularly suitable for making bread and biscuits but also variously usable in all kinds of processed foods. As a significant source of carbohydrate and protein, wheat provides on average 19% of the calories and 20% of the proteins consumed by humans (Braun *et al*., 2010), especially proteins of higher nutritional quality. Besides, wheat grains are composed of bran (pericarp) and germ which also contain a rich source of vitamins, minerals and fats (lipids), although they are frequently milled to primarily meet sensory expectations of consumers (Joseph, 2011). Apart from being used as a direct food source, wheat is also useful in livestock feed and biofuel (FAO, 2008). The grains can also be used by industry to make adhesives, paper additives and several other products, even the production of alcohol. In recent years, there is an increasing trend of multi-purpose use of wheat.

1.2 Wheat production and challenges

1.2.1 Significance of wheat production

Wheat production plays a crucial role in global food security to feed the world population. This can be reflected by a significant share of arable land as well as international trade among all crops in the world. Based on statistics of FAO, the global harvest area of wheat, in 2010 for example, is 217.08 million hectares, which, coupled with those of rice and maize, were the three largest (FAO, 2014). However, the arable land and production capacities of wheat are not even across countries and regions (Fig. 1). Thus, large quantities of wheat have been required by the international trade market (Fig. 2). The U.S., Canada, Argentina, EU and Australia have traditionally been the leading countries for wheat export during the 1960s to 1990s, whereas Russia, Kazakhstan and Ukraine have emerged as new competitors in recent years (USDA, 2015).

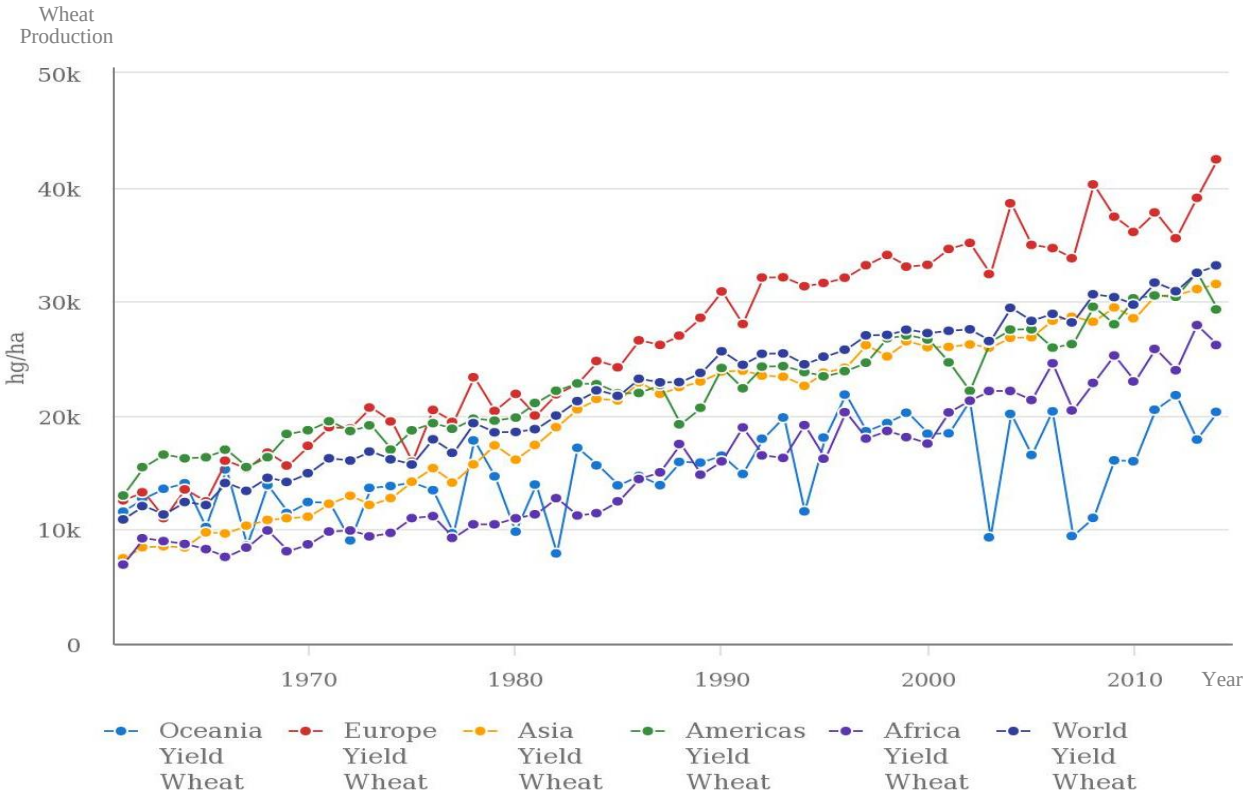


Fig. 1 Wheat production growth rates shown by regions during 1963-2014. Source: FAOSTAT 2014; retrieved 2016.

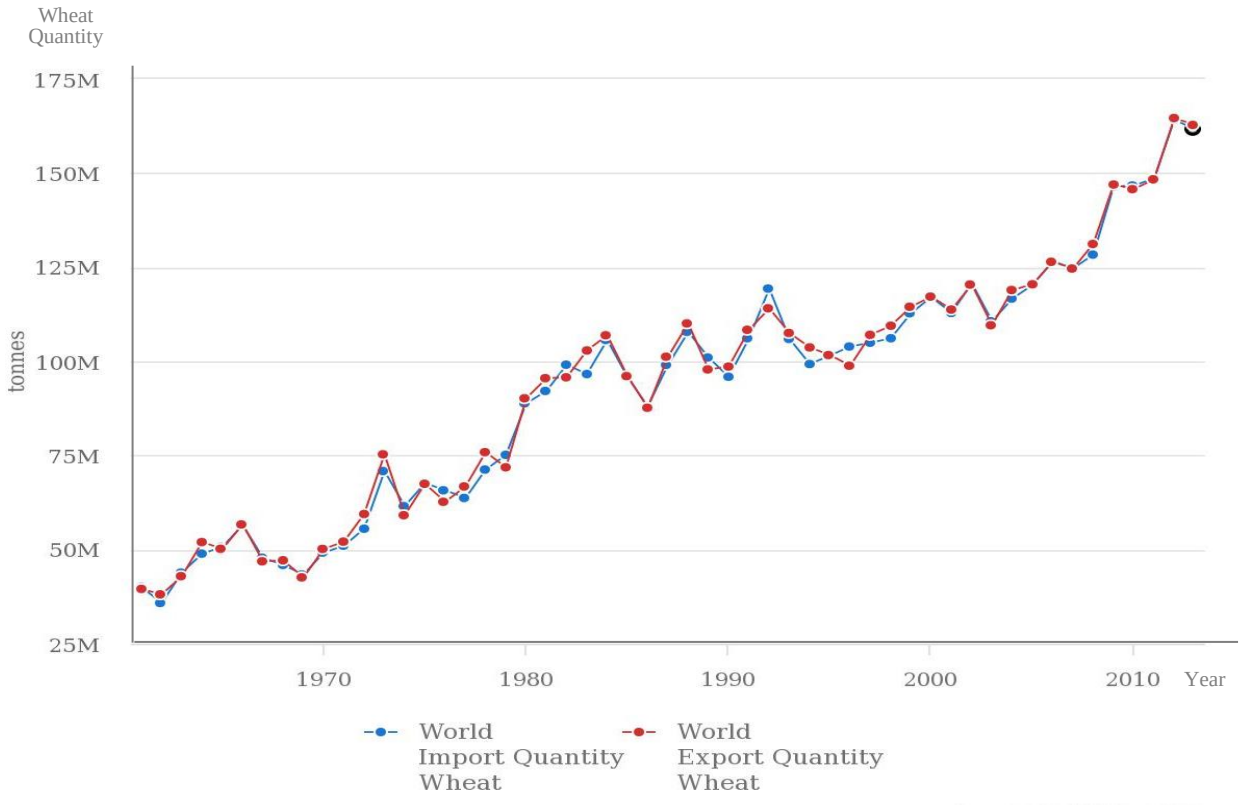


Fig. 2 World export and import quantities of wheat during 1961-2013. Source: FAOSTAT 2013; retrieved 2015.

1.2.2 Challenges for wheat production

1.2.2.1 Global food security

The world population is estimated to grow by another 3 billion and to reach 9.4 billion by 2050, which indicates an urgent demand for food consumption over the next few decades (Godfray *et al*., 2010; United Nation, 2007, 2013). The global food security is at risk of not providing enough foods for continuous growth of the world population and thus requires further progress on the yield of wheat, as well as other major crops, in agriculture (FAO, 2002). The application of high-yield wheat varieties since the Green Revolution in the mid-20th century and improved agronomic practice have led to substantial increases of yields in wheat production, whereas the growth rate has slowed down in the past few decades. An average growth rate of wheat production dropped from 3.8% between the 1960s and 1980s to 2% in the 1990s and further declined to 1.6% in the 2000s. Moreover, this trend was predicted at the beginning of this century to even drop down to 1.1% per annual in next 30–40 years, though a rise of 1.2–1.7% was expected (FAO, 2002; Rosegrant and Agcaoili, 2010). As a fact, an annual growth rate of only around 1.1% was already seen, indicating the loss is even more than that has been predicted (Reynolds *et al*., 2012).

1.2.2.2 Arable land

While wheat yields have been improved, arable land is still essential to wheat production. The global arable land area expanded between the early 1960s and late 1990s by 155 million ha to about 1.5 billion ha (FAO, 2002). Nevertheless, due to explosion of the world population, the arable land area per person during the same period declined 40% on average, from 0.43 ha to 0.26 ha (FAO,2002), which implies pressures on world arable land expansion. For wheat production, the arable land started to decline since the 1980s, despite a slight increase in recent years (FAO, 2015). It has been suggested that farmland degradation and urbanisation, especially in developing countries, are

causing the available arable land to diminish at an even faster rate, which will undoubtedly increase the competition for arable land use between wheat and other crops. Moreover, worsening tillage conditions caused by agricultural intensification, pesticide mismanagement, environmental pollution and soil contamination will also accelerate farmland degradation and thus threaten wheat production. Among different types of degradation, the loss of/decline in nutrients (e.g. P or N) is a significant factor that adversely affects wheat production. Also, new and more complex types of farmland degradation have also emerged in the past few decades (Brabant, 2010). According to World Resources Institute (2000), up to 40% of the world's arable land is facing the problem of severely getting degraded. In addition, further competition also comes from growing demands for meat and milk products, as well as biofuels, which occupy more arable land areas.

1.2.2.3 Climate change

Global atmospheric CO_2 concentration, temperature rise and changes in precipitation are demonstrated to be the most important ecological factors for agricultural production. In recent years, it is recognised more and more that extreme weather events like heat waves, droughts, storms and floods are becoming more frequent, intense and widespread (Obasi and Dowdeswell, 1998). Extreme weather events driven by climate change are the biggest threat to world farming and its ability to feed the growing world population. “The biggest uncertainty for UK agriculture is extreme weather events,” said Kendall, president of the National Farmers' Union (NFU) (Damian, 2013). Furthermore, climate change also leads to the proliferation of weeds, pests and natural disasters (Tubiello *et al*., 2007). All these factors increase difficulties in agriculture practice, especially yield decline, which is estimated to be 8–30%, and the loss of quality in the most important crops. For instance, heat waves during wheat anthesis can result in underdevelopment of the grain number and change of the grain size (Calderini *et al*., 2001; González

et al., 2011; Lizana and Calderini, 2013; Semenov *et al*., 2014).

1.2.2.4 Natural resources and environmental issues

The advent of mineral fertilisers not only compensates the shortage of nutrients in soils, but also helps to achieve crop productivity (Bennett, 2001). It therefore becomes prevalent that great inputs of fertilisers, especially nitrogen (N), phosphate (P) and potassium (K), are used in crop production (Heffer and Prud'homme, 2010). The global use of fertilisers has climbed up 6-fold between 1950 and 2000 (Heffer and Prud'homme, 2008). In Asia alone, fertiliser use has increased more than 10-fold in the past few decades (Owen *et al*., 2014). Nevertheless, mineral fertilisers are costly to apply, both financially and environmentally (Ashley *et al*., 2011; Elser and Bennett, 2011; Schröder *et al*., 2011). The production of fertilisers is responsible for the rapid depletion of relevant non-renewable resources, the scarcity of which may expose crisis to future global food security. However, the recovery of fertilisers by crops is always unsatisfactory in many cropping systems (Barber *et al*., 1963; Barber, 1995; Schenk, 2006). Estimates of overall use efficiency of applied fertilisers have been reported to be at or lower than 50% for N, less than 10% for P and about 40% for K (Baligar *et al*., 2001). Meanwhile, nutrients in the applied fertilisers could be washed or blown off from the soil surface before assimilation, which potentially contaminates an ever larger part of the planet. Adverse effects on the environment, especially the detriment of environmental quality by reactive N and P, may include pollution of water, greenhouse effect, etc. Moreover, problems with biodiversity may also be attributed to overuse of fertilisers. For instance, the discharge of excessive P from agricultural activities is likely to accelerate the irregular growth of algae bloom and other aquatic plants (Bodelier *et al*., 1999; Correll, 1998; Erisman *et al*., 1998, 2009).

1.3 Phosphate and Phosphate fertilisers in crop production

1.3.1 Essential nutrient for plant growth

Phosphorus (P) is one of the 17 essential nutrients (macro- and micro-) required by plants to grow and thrive. It makes up about 0.2 %–0.5 % of a plant's dry weight (Daniel *et al*., 1998; Raghothama, 1999). P is not only a structural element of nucleic acid but also involved in many physiological and biochemical processes in plants (Rajamanickam *et al*., 2015; Vance *et al*., 2003). P constitutes ADP and ATP, which are important organic compounds playing roles in light capture and energy storage during photosynthesis to provide a primary energy source in metabolism for all functions within a plant, such as cell division, root growth, ripening, seed and fruit development, etc. (Nilsson *et al*., 2007, 2010; Zhou *et al*., 2008a). The pivotal role of P cannot be replaced by any other nutrient in plants. Either P deficiency or excess of P harms the healthy growth of plants. Exceptionally high P appears to be poisoning and even results in necrosis or the death of a plant. In contrast, P deficiency happens when P is low or not in proper use. P deficiency can cause growth disorders as shown by growth retardation in shoots and inhibition of root development of a plant. Comparatively, quick emergence, early vigour and healthy root growth are significantly relevant to P-stress tolerance of a plant. In addition, P also influences the availability of other yield-limiting nutrients, such as nitrogen-fixing in legume species (Vance *et al*., 2003).

1.3.2 Soil P dynamics and plant uptake

Soil P exists in organic (P_o) and inorganic forms (P_i). Nevertheless, only the inorganic form of phosphate (P_i) – dihydrogen orthophosphate ions in the soil solution can be directly absorbed and used by plants. In natural soils, the fractions of P_i and P_o vary from one type to another. The fraction of P_i in the soil solution usually accounts for 35% to 70% of total soil P. The fraction of P_o constitutes 30% to 80% of total soil P, which, in soils with high (>20%) organic matter, can reach to as much as 90% (Marschner, 1995; Owen *et al*., 2014; Vance *et al*., 2003). Root surface, the root-soil interface, is the place where plants' uptake of P_i directly happens (Kochian, 2012; Marschner, 1995). Soil areas

surrounding plant roots, the rhizosphere, only have a small P_i pool immediately available to plants and sustain an initial supply, which, without being quickly replenished, will result in the rapid depletion of P_i in the rhizosphere (Bhat and Nye, 1973; Föhse and Jungk, 1983; Jungk, 1996; Kraus *et al*., 1987). This is common for various plant species. However, unlike other nutrients that can be sent to the rhizosphere mainly by mass-flow or normal diffusion, most P_i only reaches this area by slow diffusion (Lambers *et al*., 2006; McLaughlin *et al*., 1992). The diffusion coefficient of P_i appears to be much lower than those of other nutrients (Clarkson, 1981). Therefore, plant-available P_i supplied in natural soils often does not keep pace with the uptake required by plants.

Furthermore, plant-available P_i in natural soils is always less abundant (less than 10µM; 1–3µM in general) (Bieleski, 1973; Marschner, 1995). Based on the property of total P, two soil-types can be mainly distinguished: (I) Low-P soil in which total soil P is low; (II) High P-fixing soil in which total soil P is adequate but mostly fixed in the soil. The plant-available P_i is insufficient in both two soil-types (Ramaeker *et al*., 2010). In type (I) soil, the total P content inherently cannot achieve plants' requirements regardless of the availability to plants. This soil-type, either with slow diffusion or not, is unable to fast refill the P_i pool in the rhizosphere when total P is not enough. In type (II) soil, the total P content usually can exceed plants' requirements but be poorly available to plants because of high fixation (Driessen *et al*., 2001; Eswaran *et al*., 1997; Fairhust *et al*., 1999; Vance *et al*., 2003). Frequently, the dynamics tendency of soil P is to transform into less soluble and more stable forms. Most P can form either the organic (P_o) compounds or mineral P_i-complexes through conversion/ immobilisation, adsorption or precipitation and thus become immobile (Holford, 1997; Marschner, 1995; Raghothama, 1999; Richardson, 1994; Syers *et al*., 2008) (Fig. 3). Therefore, despite a considerable amount of total P present in type (II) soil, the plant-available P_i in the soil solution is usually problematic due to the binding capacity of P. Hence, P_i delivered to the rhizosphere merely

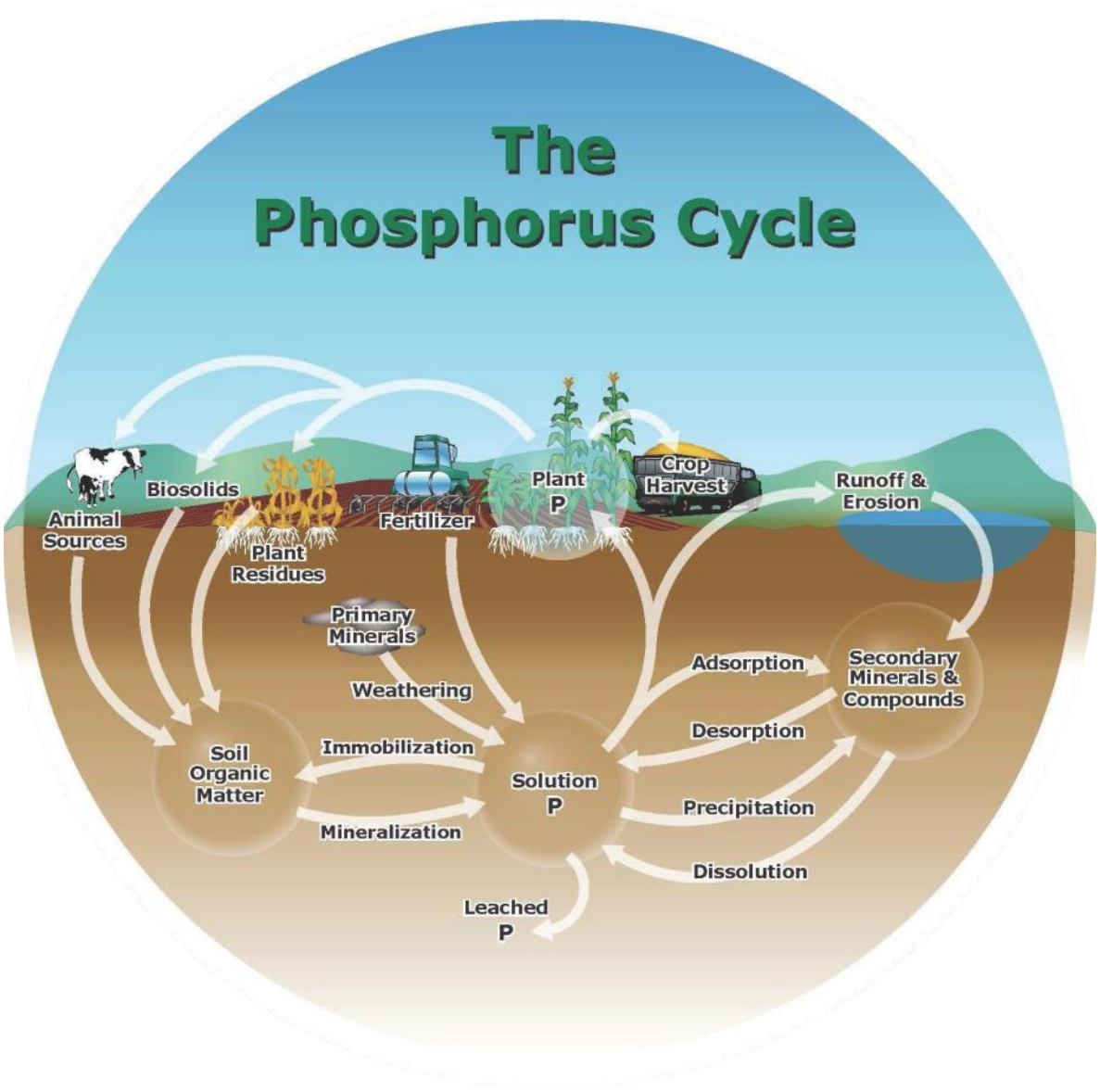


Fig. 3 The phosphorus cycle. Source: reprinted with permission by the International Plant Nutrition Institute-IPNI.

accounts for 1–5% of the plant demand. Soils with a high binding capacity of P have been identified in different areas in the world, such as southern China, Brazil and Sub-Saharan Africa (Kochian, 2012; Syers *et al*., 2008). In addition, losses of plant-available P_i caused by leaching, runoff and soil erosion, etc. even frequently happen (Pierzynski *et al*., 1994). Consequently, most soils worldwide are lack of plant-available P_i to various degrees, especially acid soils in tropical and subtropical regions (Barber, 1995; Vance *et al*., 2003). According to Batjes (1997), P availability has become a problem to an estimation of 5.7 billion hectares soil land in the world. In some extreme cases in sandy soils, alkaline soils and heavily weathered soils in tropic and subtropical regions, P_i concentration in the soil solution could be as low as <1µM (Balemi and Negisho, 2012).

1.3.3 P_i fertiliser application and use efficiency in crop production

Soils deficient in one or more plant-available essential nutrients are typical problems for cropping systems in many places of the world (Barber *et al*., 1963; Barber, 1995). As one of the least accessible nutrients, P_i, once becomes deficient, will be a major factor limiting or seriously influences crop production (Soltan *et al*., 1993; Vlek and Koch, 1992). Nevertheless, P deficiency is more difficult to diagnose than the deficiency of other nutrients in crops. There is often no apparent symptom in the early stage of P deficiency, while visual symptoms of P deficiency may not be recovered by any later remedy work. The use of mineral P_i fertilisation maintenance to overcome P deficiency and sustain high-yield in crop production has grown exponentially since the post-World War II period (Balemi and Negisho, 2012; Van de Wiel *et al*., 2016) (Fig. 4). Unfortunately, the applied mineral P_i fertilisers are also subject to soil fixation, which varies depending on the soil property (Owen *et al*., 2014). In fact, the recovery rate of P_i (P in the crop/ P_i fertilisers applied) is often low even under the optimum fertilisation (Baligar *et al*., 2001). Holford (1997) and Syers *et al*. (2008) suggested, respectively, that only 10–30% and 10–25% of the applied mineral P_i fertilisers can be recovered. In

many cropping systems, where intensive mineral P_i fertilisers are applied, a high P-input (P in fertilisers) only gives a low P-output (P in crops), which is characterised as "a low P-balance-efficiency (PBE: calculated as P-output/P-input x 100)" (Helyar, 1998; Weaver and Wong 2011). As suggested by Sattar *et al*. (2012), P-inputs on average are approximately 3-fold of the P-outputs in Western Europe during 1965–2007. Indeed, the application of mineral P_i fertilisers alone is not a cost-effective way of increasing crop production (Tilman *et al*., 2002). The low PBE indicates a requirement of annual inputs of new P_i fertilisers to sustain crop yields (Sanchez, 1976). An additional result of this is a significant amount of soil P surplus (known as legacy P), which constitutes an unused P pool in the soil (McLaughlin *et al*., 2011; Owen, 2014). The global soil P surplus continues to grow and is predicted to reach up to 18 Tg year $^{-1}$ in 2050 (Bouwman *et al*., 2011).

1.4 Status of P natural resource for crop production

The brisk demand for global food consumption has led to increased reliance of crop yields on mineral P_i fertilisers. As stated by Cordell and white (2010), food consumption could not be sustained by the global yield without continual inputs of mineral P_i fertilisers. Nevertheless, continuous P_i fertiliser maintenance is causing the rapid depletion of rock phosphate (RP) reserves. RP is the only source that mineral P_i fertilisers can be produced inorganically in a large quantity. It has been proposed that 80% of the global RP, estimated at 260 billion tonnes, is utilised to produce mineral P_i fertilisers (Owen, 2014). RP is thought to be a non-renewable resource for humans mainly because the time required for RP formation is a slow and long geological process of around 10–15 million years (Cordell *et al*., 2011; Vaccari, 2009; White, 2000). Although there are still debates among researchers as to the status of RP stock capacity or the time for peak P, a production peak of P_i mineral fertilisers (a time point after which the demand will outstrip the supply), it has been widely accepted that this natural resource is becoming more and more unavailable (Cordell and

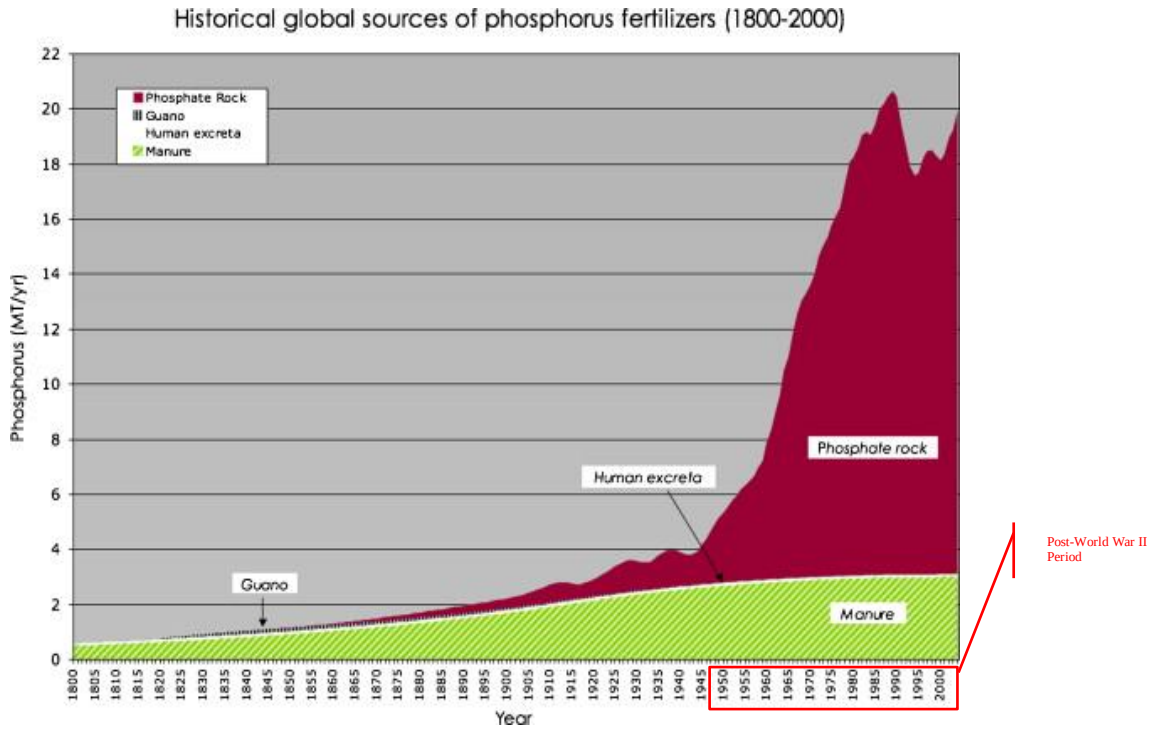


Fig. 4 Historical global source of phosphate fertilisers (1800–2000); a dramatic increase of rock phosphate fertilisers. Source: Modified from Global Environmental Change, 19 (2), Dana Cordell, Jan-Olof Drangert, Stuart White, The story of phosphorus: Global food security and food for thought, 1999, 292–305, with permission of Elsevier.

White, 2010; Kauwenbergh, 2010; Prud'Homme, 2010). Previously, a critical point of peak P has been predicted by some researchers to be around 2030–2040, but other estimations have extended this prediction to 2100 or two to three centuries beyond (Cordell *et al*., 2009; Gilbert *et al*., 2009; Sattari *et al*., 2012). While the exact timeline of this is uncertain, the future scarcity of RP is surely weighting crisis to the world, particularly to those areas with inadequate or no RP. For instance, as pointed out by The Hague Centre for Strategic Studies in 2012, mineral P_i fertilisers in Europe are largely dependent on imports (De Ridder *et al*., 2012).

Signs that suggest the future scarcity of RP also come from the pricing and protectionism of mineral P_i fertilisers in the world market. RP only distributes in limited places including the US, China, Morocco, Jordan, South Africa, etc. (Jasinski, 2010). It is believed the wealthiest RP resource hides in the North African country Morocco, involving the Western Sahara area (Kauwenbergh, 2010). Given the imbalance in RP distribution, mineral P_i fertilisers are traded in the global market and supplied from RP occupying countries to RP lacking countries. The RP reserves in the US, formerly the largest producer, consumer, importer and exporter of P_i fertilisers, have dramatically dropped after years of exercise and no longer met the demand for fertiliser consumption of the world market (Jasinski, 2010; Kauwenbergh, 2010). To efficiently protect domestic supply and to halt exports, China also imposed a 135% high export tariff on RP in 2008, leading to an unprecedented 800% price spike of RP in the world market (Sattari *et al*., 2014).

1.5 Phosphorus Use Efficiency (PUE) in plants

The above-stated critical issues of global food security, inefficient P fertilisation, depletion of RP resources and environmental pollution are closely related to P deficiency/low-P in contemporary agriculture. Published literature (Fageria *et al*., 2008; Gahoonia and Nielsen, 1996; Lynch, 2007; Ramaekers *et al*., 2010; Rao *et al*., 1999; Shenoy and Kalagudi, 2005; Syers *et al*.,

2008; Veneklaas *et al*., 2012) has suggested that one way of addressing these issues is to improve phosphorus use efficiency (PUE) in crops. Although historically there have been several definitions and calculation methods for PUE, it is now commonly accepted that PUE of plants is determined by the external PUE and internal PUE (or acquisition efficiency and utilisation efficiency respectively). The external PUE describes the ability to take up P from the soil, which is calculated as the amount of P acquired per plant. The external PUE can be further specified as the amount of P acquired per unit root length (Trolove *et al.*, 1996). The internal PUE reflects the ability to transfer and use internal P for biomass production, which is calculated as the ratio of shoot dry weight (SDW) per unit shoot P (SPU) SDW/SPU (Su *et al*., 2006). Numerous studies in the past have paid attention to the external PUE, whereas the internal PUE, by contrast, has been overlooked (Veneklaas *et al.*, 2012; Wang *et al.*, 2010). It has recently been recognised that both the external and internal PUE are important (Balemi and Negisho, 2012; Schröder *et al*., 2011; Wang *et al.*, 2010).

PUE could be highly diversified in different plant species or genotypes within a plant species (Föhse *et al.*, 1988; Vance *et al*., 2003). Variations of PUE are associated with phenotypic changes of a set of complex plant traits genetically controlled by polygenes (or QTLs) and, meanwhile, affected by environmental conditions (Gunes *et al.*, 2006; Ozturk *et al.,* 2005). Both the external and internal PUE have their breakdown component traits that are measurable to evaluate the capacity of PUE in plants (Van de Wiel *et al.*, 2016; Veneklaas *et al.*, 2012). QTLs for PUE-related component traits have been studied by using mapping populations in different plant species, e.g. rice (Ming *et al*., 2000; Ni *et al*., 1998; Wissuwa *et al*., 1998), wheat (Su *et al*., 2006, 2009; Yuan *et al*., 2017; Zhang and Wang, 2015), maize (Parentoni and Júnior, 2008; Zhu *et al*., 2005), common bean (Liao *et al*., 2004; Yan *et al*., 2004) and soybean (Li *et al*.,2005), etc. In wheat, both phenotypic and genotypic differences of PUE have been documented (Gahoonia *et al*., 1999; Manske *et al*., 2000, 2001; Osborne and

Rengel, 2002; Ozturk *et al*., 2005; Su *et al*., 2006, 2009). Plants under P deficiency/low-P can employ a cascade of different adaptation mechanisms/stress-adaptive-responses at morphological, physiological, biochemical and molecular levels to improve PUE through P acquisition, translocation and utilisation (Vance *et al*., 2003) (Fig. 5). At molecular level, these adaptation mechanisms are results of the elaborately regulated expression of P-stress-responsive genes under the control of various pathways in dynamic and intricate networks (Plaxton and Lambers, 2015; Rajamanickam *et al.*, 2015). These P-stress-responsive adaptation mechanisms are introduced from the two aspects of PUE in the following two sections.

1.5.1 Mechanisms for improving the external PUE - P acquisition efficiency

The root system of a plant undertakes an all-out role for uptake of water, P_i and other elementary nutrients to maintain the lifecycle of the plant (Cailloux, 1972; Schjorring and Nielsen, 1987). P uptake from the rhizosphere is an energy-mediated transport (cotransport) process, which mainly relies on the plasma membrane P_i transporters (PTs) (symporter pump) in roots. Once P_i is acquired, it needs to move across to the xylem of the stele in the centre of the root, which can occur either symplastically (through cytoplasm) or apoplastically (through cell wall but P_i must eventually move back into the symplasm) (Plaxton and Lambers, 2015) (Fig. 6). Through transportation in the xylem, P_i is translocated to different tissues and organs within plants. Alterations of root traits importantly contribute to scavenging plant-available P_i outside the depletion zone of the rhizosphere and therefore are closely associated with the external PUE (Gahoonia and Nielsen, 2004). These adaptation mechanisms include changes of root morphology and architecture, exudation of chemical compounds into the rhizosphere, associations of roots with mycorrhiza and production of high- affinity PTs (Föhse *et al.*, 1988; Lambers *et al.*, 2006; Raghothama, 1999; Vance *et al.*, 2003).

1.5.1.1 Root architecture and morphology

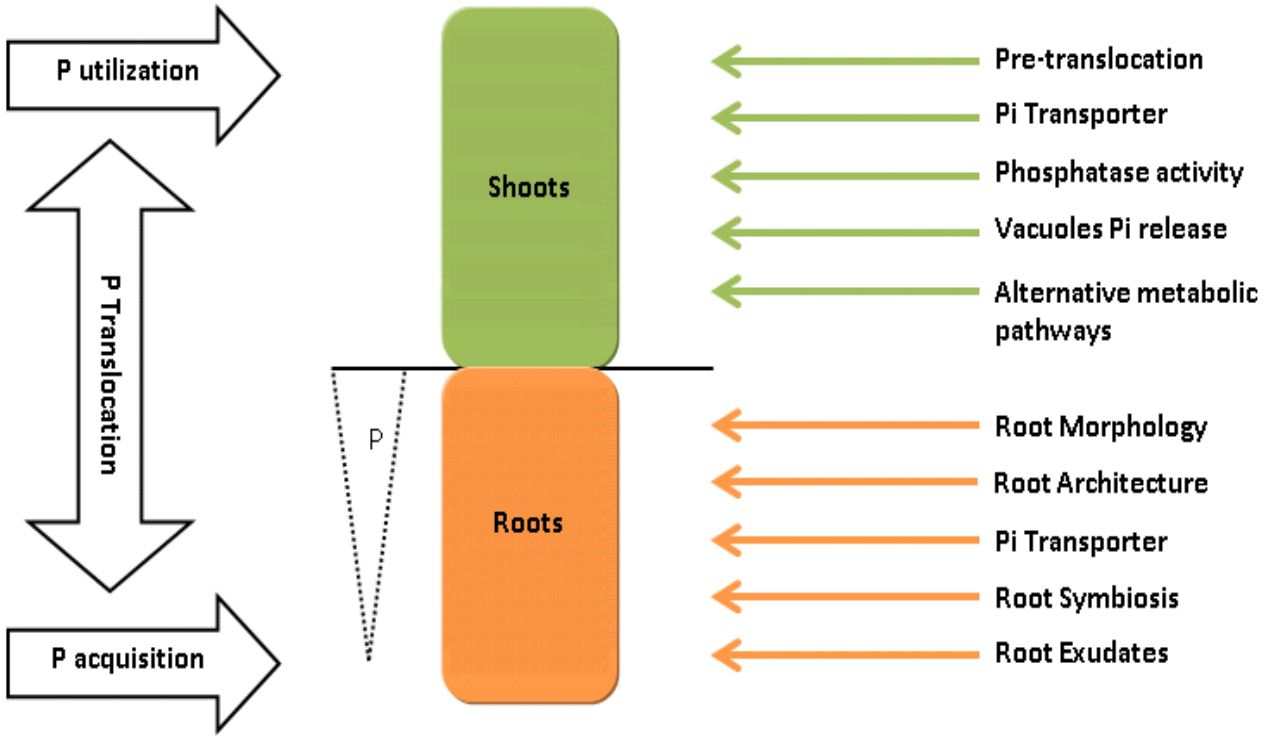


Fig. 5 Plants' adaptation mechanisms for P acquisition, P utilisation and plant growth.
P: Phosphorus levels in the soil profile.

It has been well established that root modification can occur to varying degrees under P deficiency/low-P. P_i is often unevenly distributed in the soil profile and more abundant in the topsoil than in the subsoil (Vance *et al*., 2003). Modified branching patterns of basal roots to an upward angle can increase the access to P_i in the topsoil. Moreover, increases in lateral root numbers and lengths produce a big root size with more root surface areas for P uptake (Jungk, 2001). In legumes with a tap root system, e.g. common bean or soybean, vigorous growth of lateral roots accompanied with reduced growth and shallower angle in the primary root has been shown to significantly increase P uptake (Lynch and Brown, 2001; Niu *et al.*, 2013; Zhao *et al.*, 2004). Nevertheless, trade-offs, such as developments of thinner morphology in lateral roots or less secondary growth of the stele and cortex, have also been observed and possibly explained by a strategy for carbon-cost by plants (Walk *et al*., 2006; Zhu *et al.*, 2010). In addition, cluster roots have also been found, e.g. the proteoid roots in lupin (Lambers *et al.*, 2006; Shane and Lambers, 2005; Zhou *et al*., 2008b).

Root hairs are the thin-walled unicellular outgrowth of the epidermis in the differentiation zone of roots (Dolan and Roberts, 1995) (Fig. 6). A single root may have 14 billion root hairs (Dittmer, 1937). In comparison with the thicker root cylinder, root hairs have a much smaller radius and therefore explore a larger volume of soil areas per unit of root surface area (Jungk, 2001). In crops, up to 77% of root surface area can be generated by root hairs (Parker *et al.*, 2000). Root hairs are primary places for P uptake, which can favour the P uptake rate per unit of root length, especially in lateral roots (Dolan and Costa 2001; Gahoonia and Nielsen, 1998; Marschner, 1995; Vance *et al.*, 2003). Previous studies in different species have revealed that an increased number or length of root hairs can lead to enhanced P uptake (Bates and Lynch 2000a, 2000b; Bayuelo-Jimenez *et al*., 2011; Gahoonia and Nielsen, 1997, 2003; Nestler *et al*., 2016; Yan *et al.*, 2004).

1.5.1.2 Root exudates

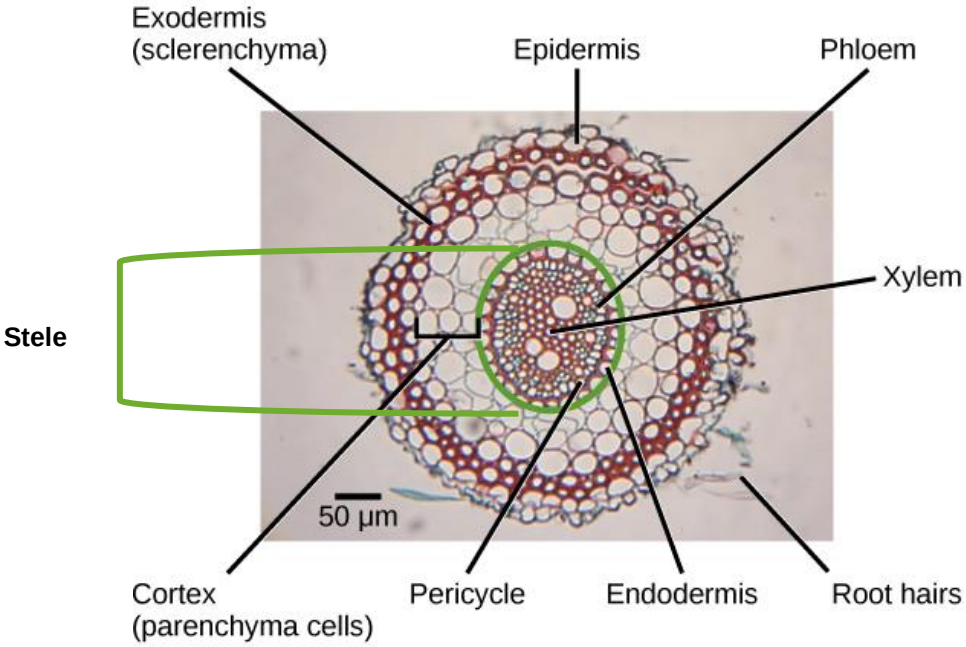


Fig. 6 Micrograph of a wheat root cross section. Source: Modified from and downloaded for free at http://cnx.org/contents/addad899-ddc7-489f-918a-30ff0b88911b@4).

Exudation of low molecular-weight organic anions (mainly organic acids), enzymes and protons have been found in many plant roots (Raghothama and Karthikeyan, 2005). Although knowledge needs to be accumulated for their molecular basis, the biochemical processes of these substances appear to help increase P solubility or release bound-P, which therefore can facilitate P uptake (Vance *et al.,* 2003). In most plants, organic acids, such as citrate and malate, are main types of organic anion exudates (Corrales *et al*., 2007; Dong *et al*., 2004; Neumann and Römheld, 1999; Yan *et al*., 2004). Organic acids can generate metal cations through ligand exchange and release inorganic P_i from mineral P_i-complexes (Raghothama and Karthikeyan, 2005). For instance, enhanced P uptake has been found in wheat plants overexpressing a malate transporter (TaALMT1) (Delhaize *et al*., 2009). It has also been demonstrated that organic acids are especially effective in plant species with cluster roots (Rath *et al.*, 2010). Enzymes like hydrolytic acid phosphatases, phytases and nucleases (RNase) secreted by roots can primarily target the organic forms of bound-P, e.g. phytin, and transform them into inorganic P_i, although a species-specific/genotypic variation of enzymic activities has been observed, such as in maize and barley (Asmar, 1997; Li *et al*., 1997; Plaxton, 2004; Vance *et al.,* 2003). Species-specific/genotypic variations have also been reported for secretion of protons in roots, which can result in acidification of the rhizosphere. Root-induced pH changes in the rhizosphere are believed to beneficial to dissolving spare P_i from the soil (Neumann and Römheld, 1999).

1.5.1.3 Association of roots with mycorrhizal fungi

In a majority of higher plants, P acquisition can also be facilitated by associations of plant roots with mycorrhizal fungi (Brundrett, 2002; Marschner and Dell, 1994). Among all types of the association with mycorrhizal fungi, the high-affinity symbiotic association with arbuscular mycorrhizal (AM) fungi is the most common and important one in many crop species (Adesemoye *et al*., 2009). Either

physically increased root exploration of the soil or an environment biologically modified by AM hyphae has the potential to improve P acquisition efficiency of associated plants, especially under P deficiency/low-P (Bolan, 1991; Smith and Read, 1997; Smith and Smith, 2011; Tinker and Nye, 2000). In maize, an increased P uptake rate under low-P has been observed, in contrast to the decreased P uptake rate under high-P (Kaeppler *et al*., 2000). Thingstrup *et al*. (2000) have proposed that the uptake rates of AM fungi associations are approximately 77% and 49% under low-P and high-P respectively.

1.5.1.4 High-affinity P_i transporters (PTs)

PTs are inbuilt proteins that can present in different locations within a plant and enable cross-membrane transportation of P_i with different uptake kinetics. They are classified into high- and low-affinity PTs (Daram *et al*., 1998; Lin *et al*., 2009; Muchhal *et al*., 1996). Both temporal and spatial expression patterns have been reported for high- and low-affinity PTs, which are influenced by changes in available P_i status (Karthikeyan *et al*., 2002; Mudge *et al*., 2002). Interestingly, most low-affinity PTs are expressed constitutively in plants, whereas most high-affinity PTs are strongly induced by P deficiency/low-P (Daram *et al*., 1998; Liu *et al*., 1998; Muchhal *et al*., 1996; Raghothama, 1999, 2000a, 2000b). The induction of high-affinity PTs in roots under P deficiency/low-P can typically facilitate P acquisition (Dong *et al*., 1999; Plaxton, 2004). It has been demonstrated that high-affinity PTs are preferentially expressed in roots but expressed in specific manners in shoots and transcriptionally regulated by P-stress-responsive pathways (Thibaud *et al*., 2010; Van de Wiel *et al*., 2016; Veneklaas *et al*., 2012). High-affinity PTs have been found to be mainly encoded by the *Pht1* (phosphate transporter 1) gene family in *Arabidopsis* as well as other crops (Rajamanickam *et al*., 2015). In *Arabidopsis*, most attention has been paid to the nine members of the *AtPHT1* gene family, *AtPHT1:1* to *AtPHT1:9*, although PTs of other gene families

(either high- or low-affinity) have also been identified, e.g. *AtPHT2:6* (Guo *et al*., 2008; Karthikeyan *et al*., 2002; Muchhal *et al*., 1996; Mudge *et al*., 2002; Okumura *et al*., 1998; Poirier and Bucher, 2002). Except for *AtPHT1:6*, which mainly expresses in anthers, expression patterns of the other eight *AtPHT1* members are all cell-type specific in roots, such as in epidermal or cortical cells (Lin *et al*., 2009; Misson *et al*., 2005; Mudge *et al*., 2002; Shin *et al*., 2004). Among these, *AtPHT1:1*, *AtPHT1:4*, *AtPHT1:8* and *AtPHT1:9* are strongly expressed in P-starved roots in contributing to P acquisition (Remy *et al*., 2012; Shin *et al*., 2004). In crops, five members in maize, 13 putative members (*Pt1*–*Pt13*) in rice, 14 members in soybean, and 12 putative members in barley (Baker *et al*., 2015) have been identified for the *Pht1* gene family. *OsPht1:6* and *OsPht1:8* have multiple roles in increasing P uptake and translocation in roots (Ai *et al*., 2009; Jia *et al*., 2011). Similarly, *HvPht1:1* is shown to relate to increased P uptake in roots (Preuss *et al*., 2011), while *TaPht1:4* is also confirmed to exclusively express in P-deficient roots (Liu *et al*., 2013). Knowledge is still expanding for understanding other high- or low-affinity PTs.

1.5.2 Mechanisms for improving the internal PUE - P utilisation efficiency

In comparison with the external PUE, the internal PUE has been less investigated and not fully rationalised so far. Nevertheless, adaptation mechanisms in several aspects of metabolic modification have been reported, which include efficient translocation of P for reuse, maintenance of cytoplasmic P_i homeostasis (a constant P_i cellular status) and lower demand for metabolic P (Plaxton and Carswell, 1999; Plaxton and Trans, 2011).

1.5.2.1 Metabolic translocation of P

Plants under P deficiency/low-P can recycle and translocate internal P from inactive sites (e.g. senescing tissues) to active sites (e.g. developing tissues), thereby leading to efficient use of P within plants. For instance, PUE-efficient *Brassica napus* (rapeseed) cultivars grown under P-deficiency have

been shown to exhibit efficient P remobilisation to developing tissues (Akhtar *et al*., 2007, 2008). In *Arabidopsis*, upregulation of a group of specific high-affinity PT genes has been detected in senescing tissues, of which *AtPHT1:5* has been further indicated to play key roles in vascular loading and unloading of P_i (Nagarajan *et al*., 2011). Moreover, similar functions have also been confirmed for *Pht1:2*, *Pht1:6* and *Pht1:8 in rice, Pht1:6* in barley, or some other high- or low-affinity PTs from the *Pht1* or *Pht2* gene family in other plants (Ai *et al*., 2009; Jia *et al*., 2011; Preuss *et al*., 2010). In the meantime, the involvement of specific phosphatases has also been found to function in P remobilisation, such as in common bean and white lupin (Duff *et al*., 1994; Kouas *et al*., 2009; Tang *et al*., 2013). However, P remobilisation only provisionally recycles very limited P due to the inhibition of photosynthesis caused by P deficiency/low-P (Richardson *et al*., 2011).

1.5.2.2 Cytoplasmic P_i homeostasis

Plaxton and Carswell (1999) have suggested that maintaining cellular P_i homeostasis is vital for healthy functioning of plants. Another remedy source for efficiently utilising internal P under P deficiency/low-P derives from vacuoles in the cytoplasm. Vacuoles can act as a storage sink when P_i is excessive of metabolic needs. A bi-directional movement of ions across the tonoplast membrane allows P_i to be selectively allocated between the cytoplasm and vacuoles. In high-P conditions, vacuoles conserve as much as 85–95% of cellular P_i (Anghinoni and Barber, 1980; Natr, 1992), whereas, in low-P conditions, vacuoles release P_i to the cytosol in a co-ordinated manner (Mimura *et al*., 1996). This has been validated by a ^{31}P nuclear magnetic resonance (NMR) study (Lauer *et al*., 1989a; Tu *et al*., 1990). The buffering effect of vacuoles can protectively maintain P_i homeostasis in the cytoplasm and therefore metabolic activities of plant cells for growth despite a fluctuation of external P_i concentration under P deficiency/low-P (Glass and Siddiqi, 1984). However, the buffering effect of vacuoles mainly depends on the permeability of the tonoplast to P_i and often

varies between species or genotypes, e.g. rapeseed (Akhtar *et al*., 2008). Other aspects related to cytoplasmic P_i homeostasis have also been occasionally reported, such as stomatal or non-stomatal induced decline of photosynthetic capacity, net carbon fixation, etc. (Brooks *et.al*., 1988; Flügge *et al*., 1980; Lauer *et al*., 1989b; Li *et al*., 2006; Rao *et al*., 1989).

1.5.2.3 Low metabolic requirements

Besides P recycling and storage, plants also employ "energy-saving patterns" under P deficiency/low-P through a lower metabolic requirement mainly achieved by a reduction of P consumption or an alternative energy source. A typical change of P-deficient plants is to increase root growth but to reduce leaf proliferation, in both size and the total number, resulting in an increased root-to-shoot ratio (Chiera *et al*., 2002; Lynch *et al*., 1991). The nature of leaf growth is determined by cell division and elongation, where P is of irreplaceable importance (Fricke, 2002; Kavanova *et al*., 2006). Fewer leaves and smaller leaf size can relieve the depletion of P_i pool in the rhizosphere. It has been suggested that the ability to maintain cell division and elongation largely influences the extent to which a plant can economise P_i under P deficiency/low-P (Assuero *et al*., 2004; Chiera *et al*., 2002; Keller-Grein *et al*., 1996; Sano *et al*., 1999). On the other hand, a bypass of P-dependence in metabolic processes, which refers to the recruitment of substitutive metabolites or structural compounds for P-saving, e.g. synthesis of enzymes without P consumption, can be employed by a plant under P deficiency/low-P. The most frequently reported examples are those in carbohydrate metabolism, e.g. glycolysis pathways. Duff *et al*. (1989) and Plaxton and Carswell, (1999) have ascertained that pyrophosphate (PP_i), an anabolic side-product from DNA, RNA, protein, lipid and polysaccharide synthesis, is an alternative energy donor of ATP for a low-P-cost purpose. Rao *et al*. (1989) and Rao and Terry (1995) have also found that some phosphorylated intermediates in carbon-fixation cycle can be replaced by other organics, e.g. starch or sucrose in replacement of

triose-P (glyceraldehyde-3-phosphate), in sugar beet, which is consistent with similar findings in *Arabidopsis* and some crop species (Sulpice *et al*., 2014). Furthermore, other adaptation mechanisms, such as (1) accumulation of anthocyanin in shoots resulting from an alternative phenylpropanoid pathway under P stress (Vance *et al*., 2003); (2) using non-phosphorus galacto and sulfonyl lipids to alternate phospholipids in cell membranes (Plaxton, 2004); (3) synthesis of P-free polysaccharides in cell walls (Byrne *et al*., 2011; Rao and Terry 1995); (4) change of P-required pathways of mitochondrial respiration (Theodorou and Plaxton, 1993; Vance *et al*., 2003); (5) low activity of rRNA (Sulpice *et al*., 2014) etc., have also been reported.

1.6 Transcription factors and transcriptional regulation in plants

Transcription factors (TFs) proteins that carry one or more DNA-binding domains. TFs, either individually or in combination with other proteins, can bind to the promoter or enhancer/silencer region upstream of a target gene and alter the binding ability of RNA polymerase to the gene, which precisely regulates the transcription from DNA to RNA of the gene (Lee and Young, 2000). The expression of a target gene may be initiated, promoted (as an activator), or repressed (as a repressor) by TFs which act in *trans* (Latchman, 1997). A TF is typically composed of a DNA-binding domain, an oligomerisation site, a transcription-regulation domain and a nuclear localisation signal (NLS). The DNA-binding domain allows a TF to bind to the target gene and frequently contains highly conserved amino acid residues for *cis*-acting element binding, as well as other non-specific binding amino acid residues (Aukerman *et al*., 1991). The oligomerisation site contributes to DNA-binding specificity and affinity, as well as the nuclear location. The transcription-regulation domain makes a TF distinct from others and directs different actions (activators or repressors) to control transcription (Yanagisawa *et al*., 1998). The NLS usually carries a significant peptide enriched in

arginine (R) and lysine (K) and navigates a TF to the nucleus, although some TFs are also located to other sub-cellular targets (Liu *et al*., 1999).

TFs are abundant in plants and account for approximately 7% coding sequences of a plant genome (Udvardi *et al*., 2007). Depending on the conserved structure of their DNA-binding domains (e.g. number and spacing) or characteristics of their functions and regulatory mechanisms, TFs can be classified into different gene families and sub-families (e.g. bHLH, MYB, WRKY, NAC, etc.). A gene family or sub-family that encode multiple TFs are either dispersed on different chromosomes or clustered on the same chromosome of a genome (Liu *et al*., 1999). It has also been indicated that larger genomes accordingly have more TF genes (Nimwegen, 2003). TF genes may express constitutively, universally or have time and tissue-specific manners (Meissner and Michael, 1997; Minami *et al*., 1993). The expression of TF genes in a plant could be highly changeable along with developmental requirements and surrounding environmental stimuli. Previous studies in various plant species have suggested that stress-adaptive-responses are transcriptionally regulated by TFs derived from different gene families, e.g. bHLH, MYB, ERF, WRKY, NAC, ZAPs, C2H2, etc. (Fowler and Thomashow, 2002; W*ang et al*., 2016a). The vital role of TFs in these responses is to dynamically and accurately harmonise signal transduction and gene expression of different pathways through complex regulatory networks, which consequently leads to changes of plant traits in response to the stress (Mitsuda and Ohme-Takagi, 2009). In *Arabidopsis*, approximately 1500 TFs thus far have been shown to function in stress-adaptive-responses (Riechmann *et al*., 2000). The myeloblastosis (MYB) and basic-helix-loop-helix (bHLH) families are the two largest TF families/ superfamilies that manipulate metabolic, physiological, and developmental processes in plants (Carretero-Paulet *et al*., 2010; Du *et al*., 2009). According to PlantTFDB-Plant Transcription

Factor Database v3.0 (Centre for Bioinformatics, Peking University, China), there are 11428 members in the bHLH superfamily, while the MYB superfamily has 8746 MYB TFs coupled with 6410 MYB-related TFs (Jin *et al*., 2014). Therefore, this study was more interested in TFs involved in P-stress responses from the bHLH or MYB family/superfamily.

1.6.1 bHLH TFs in plants

A TF belongs to the bHLH family/superfamily is recognised for a basic helix-loop-helix (bHLH) domain which contains approximately 60 amino acids (Ferré-D'Amaré *et al*., 1993). The basic region presents as an N-terminal stretch of 15 to 20 amino acids and enables DNA binding, while the HLH region at the C-terminus consists of two amphipathic α-helices connected by a diverged loop region, which increases the protein-protein interaction (Massari and Murre, 2000). With emerging knowledge in recent years, bHLH TFs in plants have been demonstrated to be more significantly diverged than it was previously thought, e.g. the identification of novel atypical bHLHs with various forms of the basic region in *Arabidopsis* (Hyun and Lee, 2006; Roig-Villanova *et al*., 2007). Genome-wide analyses have revealed that there are 167, 177, 190 and at least 191 putative bHLH TF genes in *Arabidopsis*, rice, tobacco and grapevine, respectively (Carretero-Paulet *et al*., 2010; Jaillon *et al*., 2007; Li *et al*., 2006; Rushton *et al*., 2008). Although a lot of bHLH TFs still need to be functionally studied, those with known functions so far seem to be diversified and responsive to various abiotic stresses including P stress (Carretero-Paulet *et al*., 2010). *AtbHLH112* is relevant to tolerance of salt, drought and osmotic stresses, whereas *AtbHLH17* is involved in salt, osmotic and oxidative stresses (Babitha *et al*., 2013; Liu *et al*., 2014). In rice, bhlh1 plays a role in cold-stress responses, while bhlh2 from wild rice positively regulates salt- and osmotic-stress responses (Zhou *et al*., 2009). OsPtf1 and AtbHLH32 have been validated to play roles in response to P stress (Chen *et al*.,

2007; Yi *et al*., 2005).

1.6.2 MYB TFs in plants

An MYB family/superfamily TF is characterised by the highly conserved DNA-binding domain: MYB domain, which is composed of different numbers of MYB domain repeats (R): a sequence of about 52 amino acids in the form of α-helices. According to the number of R repeats, MYB TFs are often categorised into different types, including 4R-MYB, R1R2R3-MYB (3R), R2R3-MYB (2R) and MYB-related (R or partial R) (Du *et al*., 2009; Rosinski and Atchley, 1998, Stracke *et al*., 2001). Increasing availability of plant genome sequencing has remarkably allowed more comprehensive description and comparison of plant MYB genes, especially the genome-wide analyses of MYB in model species of *Arabidopsis* and rice (Katiyar *et al*., 2012). By the year of 2010, many MYB genes have been reported, with 204 in *Arabidopsis*, 218 in rice, 157 in maize (R2R3 only), 209 in foxtail millet, 247 in soybean, 229 in apple, 279 in grapevine, 197 in poplar and 180 in *Brachypodium* (Cao *et al*., 2013; Du *et al*., 2012a, 2012b; Muthamilarasan *et al*., 2014; Zhang *et al*., 2012). In wheat, Chen *et al*. (2015) identified 127 and 128 MYB and MYB-related TFs using genome-wide analysis.

Various regulatory roles of MYB TFs in response to abiotic stress have been reported (Katiyar *et al*., 2012; Yun *et al*., 2010; Zhang *et al*., 2012). For example, *AtMYB4* and *AtMYB7* are involved in UV-B stress (Jin *et al*., 2000). *AtMYB2*, *AtMYB15, AtMYB44*, *AtMYB60*, *AtMYB61*, *AtMYB74*, *AtMYB96* and *AtMYB102* can enable tolerance to drought stress, of which *AtMYB15*, coupled with *AtMYB96*, is also found to have crosstalk with the abscisic acid (ABA)-auxin pathway (Abe *et al*., 2003; Agarwal *et al*., 2006; Cominelli *et al*., 2005; Jung *et al*., 2008; Liang *et al*., 2005; Seo *et al*., 2009). Moreover, *AtMYB15* and *AtMYB41* function in cold-stress responses (Agarwal *et al*., 2006; Lippold *et al*., 2009). In crop species like rice and wheat,

several MYB genes have been confirmed as important components in response to different abiotic stresses, including *OsMyb2* (drought, salt, cold), *OsMyb3R-2* (cold), *OsMyb4* (drought, cold), *OsMybS3* (cold), *OsMyb48-1* (drought, salt), *TaMyb2* (multiple), *TaMyb32* (salt), *TaMyb56* (cold, salt), *TaMyb30* (drought) and *TaMyb73* (salt), etc. (Roy, 2016). Abiotic stress-related MYB genes have also been reported in other crop species, such as in soybean (Liao *et al*., 2008), potato (Cheng *et al*., 2013), tobacco (Pattanaik *et al*., 2010) and sugarcane (Prabu *et al*., 2012). For P stress, a couple of MYB TFs have been identified and functionally characterised, including *Phr1*, *Myb62*, *Myb2*, *Myb1* (Dai *et al*., 2012; Devaiah *et al*., 2009; Fang *et al*., 2016; Rubio *et al*., 2001).

1.7 Regulatory roles of bHLH and MYB TFs in PUE in response to P stress

Growing evidence has indicated that the regulation of PUE in higher plants is complicated (Hammond *et al*., 2004; Su *et al*., 2009; Zhang *et al*., 2013). As revealed by a "split-root" as well as other experiments, P-stress-responsive adaptation mechanisms employed by plants to improve PUE are regulated both locally and systemically through different sensing and responding pathways, which are known as local and systemic mechanisms (Thibaud *et al*., 2010). The local mechanism appears to be separately regulated and only detects external P_i availability in the rhizosphere, which consequently leads to changes in roots (Bates and Lynch, 1996; Svistoonoff *et al*., 2007). The systemic mechanism, in contrast, perceives signals of internal P_i availability and maintains the cytoplasmic homeostasis of P_i through enhanced uptake of external and use of internal P_i, which also regulates root modification (Chiou and Lin, 2011; Franco-Zorrilla *et al*., 2005, 2007; Liu *et al*., 1999). Therefore, the external PUE (P acquisition) is mutually associated with both the local and the systemic mechanism, while the internal PUE (P utilisation) is modulated exclusively by the systemic mechanism. An integrative

understanding of the sensing and responding pathways of P-stress-responsive adaptation mechanisms, especially the interdependence between shoots and roots regulated by the systemic mechanism, is conceivably a promising strategy to improve PUE in crops (Chiou and Lin, 2011; López- Arredondo *et al*., 2014). TFs transcriptionally manipulate the expression of genes involved in regulatory networks of the different sensing and responding pathways of local and systemic mechanisms in PUE regulation (Lin *et al*., 2009; Liu *et al*., 2010a; Chiou and Lin 2011). In several plant/crop species, TFs involved in PUE regulation have been identified and studied, of which those from *Arabidopsis* and rice are better functionally characterised (Baker *et al*., 2015; Thibaud *et al*., 2010). These findings provide valuable insights to elucidate the molecular basis underlying P-stress-responsive adaptation mechanisms and hence extend the understanding of PUE regulation (Jain *et al*., 2012).

1.7.1 Regulatory roles of bHLH TFs in response to P stress

Ptf1 (P starvation induced transcription factor 1), is identified as a crucial bHLH TF involved in P-stress responses and has first been characterised in rice as OsPtf1 (Yi *et al*., 2005). The expression of *OsPtf1* remains constitutive in shoots independent of P status but can be induced by P deficiency/ low-P in roots. Overexpression of Os*Ptf1* results in extended root length and surface area, which gives rise to biomass and P accumulation in both roots and shoots. Meanwhile, a set of genes related to gluconeogenesis-controlling (phosphoenolpyruvate carboxykinase-PEPCK), glucose-6-phosphate translocation (Glc-6-P translocator), phosphohydrolase, P_i-scavenging/recycling (RNase, and vacuolar H^+-pyrophosphatase) and H^+-transporting ATPase are upregulated (Yi *et al*., 2005). In maize, the expression of *ZmPtf1* is significantly induced in roots under P deficiency/low-P. *ZmPtf1* overexpressing lines exhibit greater performance in root growth, leading to increases in biomass and P_i uptake in both roots and shoots. Meanwhile, concentrations of soluble

sugars are detected to be lower in shoots but higher in roots in *ZmPtf1* overexpressing lines, which indicates *ZmPtf1* is associated with sugar translocation from shoots to roots. Like that in rice, gluconeogenesis-controlling PEPCK and fructose-1,6-bisphosphatase, Glc-6-P translocator, P_i-scavenging/-recycling RNase and vacuolar H^+-pyrophosphatase are also regulated by *ZmPtf1*. Furthermore, sucrose synthesis synthase1 (*SPS1*), sucrose synthase1 (*Sus1*), sucrose synthase2 (*Sus2*), Invertase 1 (*Ivr1*) and Invertase 2 (*Ivr2*) are identified to be regulated by *ZmPtf1* (Li *et al*., 2011). In P-deficient soybean roots, induced expression is observed for *GmPtf1* and shown to be higher in P-tolerant variety than in P-sensitive variety. Both ZmPtf1 (DQ468654) and GmPtf1 (FJ617239) are found to have a nucleic location (Li *et al*., 2011; Li *et al*., 2014). However, the loss-of-function mutant of *ptf1* is lack of research (Jain *et al*., 2012). Evidence provided from these studies suggest *Ptf1* positively regulates P-stress responses through crosstalk with carbon metabolism, which is an important indication of crosstalk between P-stress responses and carbon metabolism under P deficiency/low-P (Baker *et al*., 2015).

A group of other bHLH TFs have been reported to regulate root hair formation. Root hair formation is important for plants' plasticity of scavenging P_i in the soil to improve P uptake under P deficiency/low-P. Among these TFs, AtbHLH32 has been validated for a functional role in regulating root hair formation. The *Atbhlh32* mutant shows significant induction of root hair growth and P uptake in normal P supply, whereas the transcriptional activity of *AtbHLH32* is repressed in P-starved plants. *AtbHLH32* negatively regulates a range of phosphoenolpyruvate carboxylase kinase (PPCK) encoding genes (Chen *et al*., 2007). These genes control the phosphorylation of phosphoenol-pyruvate carboxylase (PEPC) which is induced for the bypass of pyruvate kinase in glycolysis (Bakrim *et al*., 2001; Plaxton and Carswell, 1999). Activation of PPCK encoding genes can exert phosphorylation of PEPC and result in the release of P_i from metabolism (Hartwell *et al*., 1999). In

addition, *AtbHLH32* is also demonstrated to interconnect with regulatory components involved in epidermal cell differentiation. Apart from *AtbHLH32*, other bHLH TFs, which similarly regulate root hair formation, also potentially participate, though not yet validated, in P-stress responses. Root hairless 1 (Rhl1), a novel bHLH TF discovered in rice, has positive regulatory effects on root hair initiation and elongation. The *Osrhl1* mutant produces less and shorter root hairs. In contrast, overexpressing *OsRhl1* leads to increased root hair length and density (Ding *et al*., 2009; Heim *et al*., 2003). The functional equivalent of *OsRhl1* in lotus, *LjRhl1*, is described to show defects in root hair development when mutated (Karas *et al*., 2009). Root hairless 1-LIKE (LRL) *AtLRL1-1*, *AtLRL-2* and *AtLRL-3* are *Arabidopsis* homologues of *LjRhl1* and *OsRhl1*, which encode bHLH66*,* bHLH69 and bHLH82 respectively. The loss-of-function mutant of each of the three only leads to moderately reduced root hair lengths and numbers, while fewer and shorter root hairs are observed in the multiple mutant (Karas *et al*., 2009). Furthermore, the *Arabidopsis* root hair defective six (*RHD6*) deriving from the bHLH VIIIc subfamily positively regulates initiation of root hairs, as shown by the fewer root hairs in the *Atrhd6* mutant (Heim *et al*., 2003; Masucci and Schiefelbein, 1994). RHD6--LIKE *AtRSL1* is identified as the closest homologue of *AtRHD6* and partially shares functional redundancy (Menand *et al*., 2007). *AtRSL2* and *AtRSL4*, another two RDH6-LIKE homologues, also belong to the bHLH VIIIc subfamily and express concurrently in the root elongation zone to stimulate root hair initiation and elongation (Yi *et al*., 2010). Recently, the rice orthologue of *AtRSL4* is revealed to be able to recover the root hair growth in the hairless mutant of *Atrsl4* (Hwang *et al*., 2017)

1.7.2 Regulatory roles of MYB TFs in response to P stress

MYB TFs such as AtPHR1, AtMYB62, OsPhr2, OsMyb2P-1 and TaMyb1 have been functionally characterised for their different roles in response to P stress.

1.7.2.1 Phr1: central TF and its regulatory network

Phr1 (phosphate starvation response 1), an MYB-related TF, has been the most well-studied MYB TF, which plays a central and systemically regulatory role in maintaining P_i homeostasis within plants. Phr1 is a nuclear-localised TF, which encodes a DNA-binding protein as a dimer, targetting micro RNAs (miRNA) or *cis*-elements (P1BS: GNATATNC, *Phr1*-specific binding sequence) in the promoter region of P-stress-responsive genes (also known as phosphate-starvation-induced -PSI gene) (Bustos *et al*., 2010; Rubio *et al*., 2001; Zhou *et al*., 2008a). It has been shown Phr1 in *Arabidopsis* and its equivalents in other plant species have positive regulatory effects on root modification, P uptake and translocation and other aspects of P metabolism under P-deficiency/low-P (Rubio *et al*., 2001; Van de Wiel *et al*., 2016). The *Atphr1* mutant exhibits significant reduction in plant growth and P_i level in shoots under normal P supply, while under P deficiency it shows inabilities to produce long root hairs and defective expression of PSI genes up to 60% and 20% in shoots and roots, respectively (Bari *et al*., 2006; Nilsson *et al.*, 2007). Similar changes have also been observed in the mutant of *AtPHL1*, a function redundant PHR1-like homologue (At5g29000) (Bustos *et al*., 2010; Rubio *et al*., 2001). Overexpression of *AtPHR1* (At4g2860) significantly enhances P_i uptake irrespective of P status and upregulates the expression of PSI genes. Meanwhile, evidence from proteomics supports that *AtPHR1* is post-translationally regulated by AtSIZ1 (or SUMO E3 ligase), a small ubiquitin-like modifier, through sumoylation (Miller *et al*., 2010; Miura *et al*., 2005). In rice, *OsPhr1* (AK063486) and *OsPhr*2 (AK100065) are both identified as the homologue of *AtPHR1*, whereas only the function of *OsPhr2* is consistent with that of *AtPHR1*. Equivalents of *AtPHR1* that have similar functional roles have also been found in other plant/crop species, e.g. maize - *ZmPhr1* (Wang, *et al*., 2013a), rapeseed - *BnPhr1* (Ren *et al*., 2012), common bean - *PvPhr1* (Valdés-López *et al*., 2008), soybean - *GmPhr1* (Li *et al*., 2017), wheat - *TaPhr1* (Wang *et al*., 2013b).

The regulatory network of *Phr1* is by far best described in *Arabidopsis* and rice, although other plant species have also been studied to validate this (Fig. 7). *Phr1* is demonstrated to mainly work through three major pathways to regulate the expression of other PSI genes by either binding straight to the destination gene or targetting other regulatory components upstream of the destination genes (Guo *et al*., 2015). P_i transporters (PTs) seem to be involved in all the three pathways, suggesting that P_i transportation is an essential aspect of maintaining P_i homeostasis. In the *Atphr1* mutant, the expression of several high-affinity PTs of the *Pht1* gene family, including *PHT1:2*, *PHT1:3*, *PHT1:4*, *PHT1:6*, *PHT1:7*, *PHT1:8* and *PHT1:9*, is extensively inhibited (Bari *et al*., 2006; Nilsson *et al*., 2007).

(1) Phr1, miRNA399/phosphate 2 (Pho2) -mediated pathway

In the most important pathway of the three, the miRNA399/Pho2-mediated pathway, Phr1 maintains P_i homeostasis by regulating the expression of miRNA399, which targets and directs the cleavage of *Pho2* mRNA (Aung *et al*., 2006; Bari *et al*., 2006; Fujii *et al*., 2005). *Pho2* encodes a ubiquitin-conjugating E2 enzyme (UBC24) located at the endoplasmic reticulum (ER)/Golgi, which can mediate protein degradation of PTs and other P_i carriers, such as phosphate 1 (Pho1) and phosphate transporter traffic facilitator 1 (Phf1) (Bari *et al*., 2006; Hamburger *et al*., 2002; Huang *et al*., 2013; Liu *et al*., 2012). The abundance of PTs and other P_i carriers at the plasma membrane is highly regulated to maintain P_i homeostasis. PTs act as the primary cross-membrane transporter of P_i in P acquisition and translocation. Pho1, destined for the early endosomes/trans-Golgi network, is implicated to be a translocator of P_i from root cortical cells to the xylem and thus plays a crucial role in P_i homeostasis (Arpat *et al*., 2012; Poirier *et al*., 1991). The *Arabidopsis* mutant *pho1*/*pho1:h1* (*Pho1:h1*, homologue of *Pho1*) has defective P_i translocation under P deficiency/low-P (Hamburger *et al*., 2002; Stefanović *et al*., 2007). Phf1, located in the

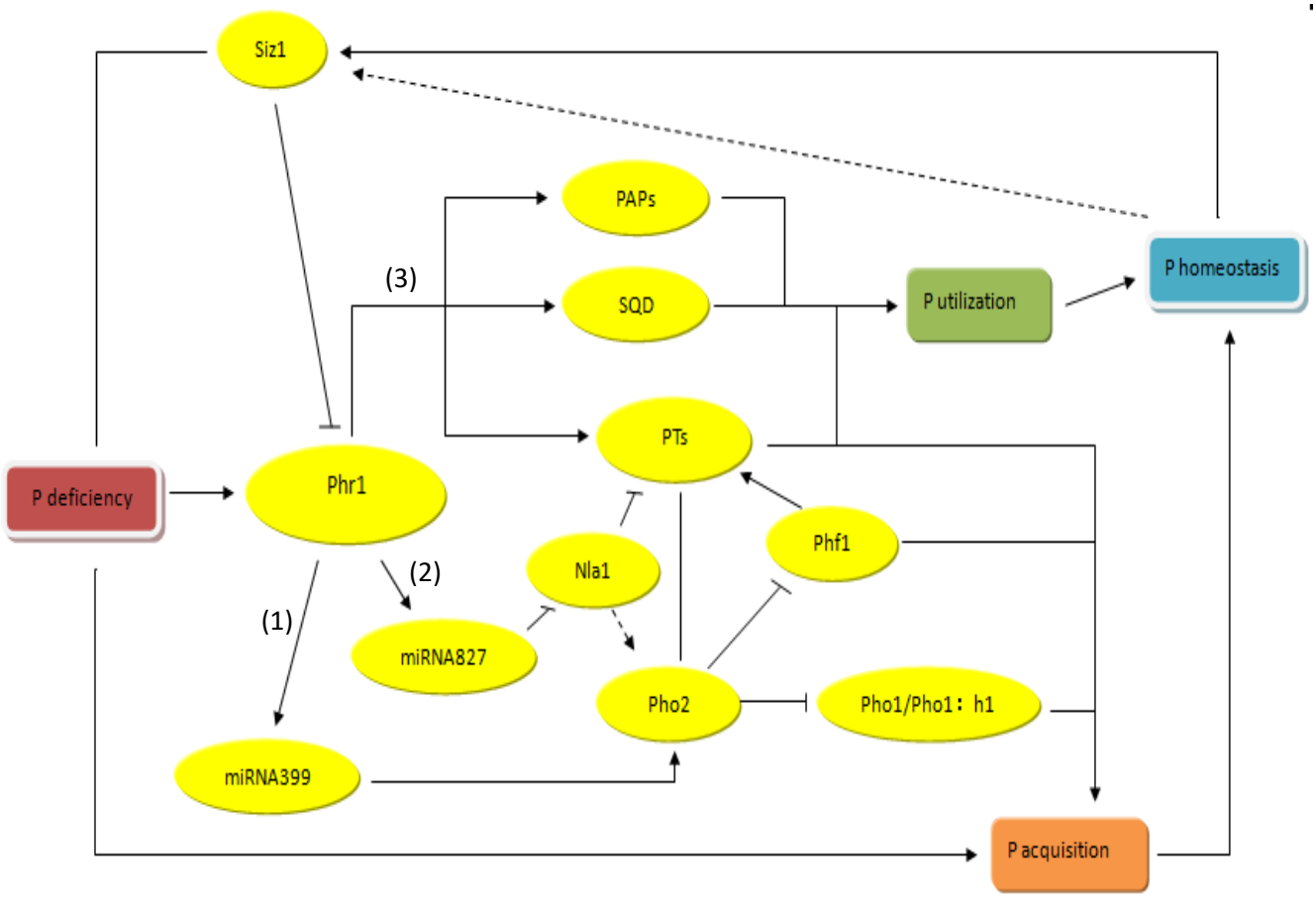


Fig. 7 Phr1 regulatory pathways and key components in response to P stress in plants. The threes pathways are shown as (1) Phr1, miRNA399/Pho2--mediated pathway; (2) Phr1, miRNA827/Nla1-mediated pathway; (3) Phr1, PT/ PAPP/SQD pathway.

endoplasmic reticulum (ER), is suggested to facilitate induction and regulation of both high- and low-affinity PTs (Chen *et al*., 2011). The *Arabidopsis* mutant *phf1* exhibits interrupted P acquisition and translocation under P deficiency/low-P (González *et al*., 2005). On the other hand, the loss-of-function mutant of *Pho2,* in either *Arabidopsis* or rice, results in overaccumulation of P_i in shoots under normal P supply, which is toxic to plant growth (Bari *et al*., 2006; Delhaize *et al*., 1995; Dong *et al*., 1998; Fujii *et al*., 2005). Therefore, Phr1 regulates P_i homeostasis through suppression of *Pho2* by miR399 to enable P acquisition and translocation under P deficiency/low-P, while *Pho2* benefits P_i homeostasis by avoiding P_i to be overloaded in plants (Huang *et al*., 2013; Liu *et al*., 2012).

(2) Phr1, miRNA827/nitrogen limitation adaptation (Nla1)-mediated pathway

P_i homeostasis is also regulated by Phr1 through the miRNA827/Nla1-mediated pathway. Phr1 alters the expression of miRNA827, following which miRNA827 targets and represses the mRNA of *Nla1*, a gene encoding RING FINGER-type ubiquitin E3 ligase and mainly responding to nitrogen-stress responses (Hsieh *et al*., 2009; Kant *et al*., 2011). Interestingly, Nla1 participates in ubiquitination and subsequent protein degradation of PTs, especially high-affinity PTs, in the vacuole (Lin *et al*., 2013). Meanwhile, Nla1 can also recruit Pho2 to achieve protein degradation of PTs (Park *et al*., 2014). The *Atnla1* mutant shows overaccumulation of shoot P_i and plant toxicity (Kant *et al*., 2011). Overexpression of miRNA827 similarly leads to P_i accumulation in shoots (Wang *et al*., 2012a) and downregulation of *Nla1* (Hsieh *et al*., 2009).

(3) Phr1, PT/purple acid phosphatase (PAP)/sulfoquinovosyl diacylglycerol (SQD) pathway

In the PT/PAP/SQD pathway, Phr1 regulates the expression of PSI genes, which carry a P1BS motif in their promoters, and subsequently instructs downstream activities in regulating P_i homeostasis. The P1BS motif has been found in the promoter of PT genes in different plant/

crop species, e.g. tomato (Chen *et al*., 2014a), barley (Schünmann *et al*., 2004) and rice (Liu *et al*., 2010b). OsPhr2, the rice equivalent of Phr1, directly modulates OsPht1:2 and other downstream PSI genes through the P1BS motif. Some PAPs in rice, which function in P recycling from organic compounds, are also demonstrated to be targeted by Phr1 due to containing P1BS motifs in their gene promoters (Tran *et al*., 2010a, 2010b; Wu *et al*., 2013). A group of SQD2s, which serve for membrane phospholipids remodelling during P recycling, are similarly shown to incorporate the P1BS motif in their gene promoters as the target of Phr1 (Yu *et al*., 2002; Wu *et al*., 2013).

1.7.2.2 Other MYB TFs in response to P stress

In addition to Phr1, other MYB TFs involved in P-stress responses are also emerging from *Arabidopsis*, rice and wheat. AtMYB62 is a nuclear-localised TF with multiple roles in response to P stress. It seems that *AtMYB62* is not related to the local mechanism but likely to be a systematic negative regulator of root modification in response to P stress (Devaiah *et al*., 2009). Overexpression of *AtMyb62* results in decreased length in primary root but increased root P uptake, P accumulation and acid phosphatase activity under high-P. Furthermore, it leads to growth repression of lateral roots and deficient P translocation from roots to shoots, as well as reduced expression of an array of PSI genes under both high-P and low-P (Devaiah *et al*., 2009). Another *AtMYB2* is initially described to function in drought and salt stresses and correlate with several phytohormone pathways. Nevertheless, it is recently revealed that *AtMYB2* can be induced by P deficiency/low-P, especially in vascular tissues as shown by tissue-specific expression patterns, and transcriptionally regulate miRNA399 (Baek *et al*., 2013). Overexpression of *AtMYB2* shows impaired primary root growth but increased root hair proliferation. In rice, Myb2P-1 is identified as a nuclear localised R2R3-MYB protein (Dai *et al*., 2012). Inhibited and promoted primary root growth is observed in *OsMyb2P-1*

overexpressing plants under high-P and low-P respectively, implying a positive regulatory effect of *OsMyb2P-1* on P-stress-responsive root modification (Dai *et al*., 2012). Overexpression of *OsMyb2P-1* causes strong upregulation of a group of PSI genes, including miRNA399 and several specific PTs (e.g. OsPht1:2 under high-P; OsPht1:6, OsPht1:8, OsPht1:10), which suggests *OsMyb2P-1* may also recruit the miRNA399 pathway to regulate PTs. In wheat, a recent study on gene expression profiling of 42 MYB genes under P deficiency/low-P is reported by Fang *et al*. (2016). Their observations suggest the upregulated and suppressed expression of several MYB genes in association with P-stress responses. Of these MYB genes, *TaMyb1* is further studied and shown to increase plant biomass and P accumulation when overexpressed in tobacco under P deficiency/low-P.

1.8 Translational genomics for crop improvement

Abiotic stress causes considerable loss of crop yields and constraints crop production (Ahmad and Prasad, 2012; Sah *et al.*, 2016). For major crops, it has been estimated that up to 70% yield loss worldwide can be attributed to multiple abiotic stresses including P deficiency/low-P (Kaur *et al*., 2008; Thakur *et al*., 2010; Vorasoot *et al*., 2003). Improving the abilities of crops in resistance to abiotic stress is vital to achieving the objective of productivity. However, most stress-resistant traits in crops are genetically and physiologically complex, which is time-consuming and difficult for breeding. Therefore, to link between gene(s) and phenotype(s) has become the core concept of genetics in modern plant breeding. Advances in molecular genetics and genomics, especially the recent application of high-throughput DNA sequencing platforms, supported by the co-development of different bioinformatics tools (e.g. large-scale database with direct links to data, Local Alignment Search Tool - BLAST, sequence alignment, genome browser, protein structure analysis, etc.) as well as other modern molecular biology

techniques over the past few decades, have significantly enhanced understandings of the structural and functional aspects of plant genomes as well as the association between genotype and phenotype, e.g. fine-scale genetic mapping or association study (Flint-Garcia *et al*., 2003; Pop, 2009; Varshney *et al.* 2009; Zhou *et al*., 2015). It thus opens new opportunities for these resources being utilised accurately and efficiently as genomic tools for breeding crops in resistance to abiotic stress (known as genomics-assisted breeding) (Klug *et al*., 2014). Due to their smaller but integrative genomes, model plant species have been more intensively studied than others. Wealth results have been generated, such as the availability of whole genome sequences of *Arabidopsis* (The *Arabidopsis* Genome Initiative, 2000), *Oryza sativa* (Goff *et al*., 2002) and *Nicotiana benthamiana* (Bombarely *et al*., 2012), characterisation of genes and gene functions at transcript and protein levels, e.g. genome-wide characterisation of wheat TFs (Chen *et al*., 2015), or characterisation of key developmental processes (Meyers *et al*., 2004).

Plant translational genomics combines the perspectives of comparative genomics, systemic genomics and evolutional genomics (Kang *et al.,* 2016). It has emerged as a field with the aim to provide applications of the quickly expanding knowledge of model plant species in crop improvement. Among plant families, shared synteny and orthology of conserved functionally essential genomic regions have been observed, which provides a basis for extrapolation of gene functions between species through translational genomics (Laurie, 2004). The candidate gene approach established from translational genomics is especially useful to rapidly identify trait-specific major/minor genes (QTL) in target crops (Salentijn *et al*., 2007). It could be reasonably assumed that a candidate gene with proven/predicted functions present in a model species may similarly exist and have identical functions or control similar traits in related crop

species (Byrne and McMullen, 1996). A functional candidate gene (functionally characterised gene based on functional genomics), with validated functional information for a trait from physiological studies, microarray expression analysis, or gene-function studies using transgenics or mutants, can be selected from a model plant species (Salentijn *et al*., 2007). Following this, the orthologue of the candidate gene needs to be identified in a target crop species, which can be achieved by sequence-based homology. Sequence homology can reflect the conservation of genomic features and suggests the possibility of common phenotype contribution (Cannon *et al*., 2009). The target crop species thus can be annotated by translating the knowledge of model plant species for an enhanced prediction of phenotypes to assist breeding. The cross-species translation of genomic information from model species to underutilised and poor-resourced crop species is likely to be crucial to investigating these crops for breeding. However, loss of genes due to extensive rearrangement and duplications may occur during evolution (Lai *et al*., 2006). Moreover, regulatory pathways and genes involved in pathways may also have diverged by speciation especially in distantly related species (Kato *et al*., 2002). Therefore, the actual involvement or function of an identified orthologue of the candidate gene in the target crop species remains to be validated before the application in breeding. Various genetic manipulation approaches are available to complement this.

1.9 Project aims, hypothesis and objectives

A broad goal of this study was to understand the regulatory components involved in P-stress-responsive adaptation mechanisms associated with PUE in bread wheat. As discussed in 1.6, bHLH and MYB TFs constitute important regulatory components in plants. In dicot and monocot model species of *Arabidopsis* and rice, several bHLH and MYB TFs have been

validated to have vital roles in regulating PUE , e.g. AtPHR1 (Bari *et al*., 2006; Nilsson *et al*., 2007), OsPhr1 and OsPhr2 (Zhou *et al*., 2008a), AtMYB2 (Devaiah *et al*., 2009), OsPtf1 (Yi *et al*., 2005), etc. A few other bHLH TFs have also been indicated to have important functions in root development and thus have potentials of improving PUE (reviewed in section 1.7). However, their orthologous TF genes were not well understood in bread wheat. This study therefore aimed to identify two (bHLH and/or MYB) orthologous TF genes in wheat by using two candidate TFs related (or potentially related) to PUE in model species, based on the candidate gene approach established from translational genomics (reviewed in section 1.8), and to further investigate how the functions of the identified wheat TFs can affect PUE and other aspects of growth or yield.

The shared synteny (or conserved synteny) found between two genomes is indicative of orthology and a clue of functional conservation in different species (Salentijn *et al*., 2007). Rice, emerging as a monocot model crop, has significantly shared synteny with other taxonomically-close neighbouring crop species such as wheat, maize and barley according to comparative studies of grass genomes (AKhunov *et al.,* 2013; Goff *et al*., 2002; Krutovsky *et al*., 2004). Meanwhile, the dicot model, *Arabidopsis,* has been shown to have much less synteny when compared with rice (Salentijn *et al*., 2007). Hence, using a rice candidate may increase the possibility of identifying the wheat orthologue and translating the genomic information to wheat. In line with this, OsRhl1, a bHLH TF, and OsPhr2, an MYB TF (to be detailed in Chapter 3), were selected out for seeking the wheat orthologues that presumably bear similar roles. It was hypothesised that 1) there are *OsRhl1*/*OsPhr2* orthologous TF genes in wheat which play crucial roles in PUE; 2) altering expression of these TF genes in wheat may lead to changes of growth-, yield- and PUE -related phenotypes which are consistent with observations in model

species, e.g. improvements of PUE.

Sequential work steps of this study were accomplished with specific objectives including:

1) Identify the orthologue of *OsRhl1*/*OsPhr2* in *Triticum aestivum* as the target TF gene through a bioinformatic approach;
2) Obtain and analyse sequence information of the target TF gene;
3) Alter expression of the target TF gene and generate wheat plants in which the gene is overexpressed through a transgenic approach;
4) Provide evidence for the transgene effects by assessing the association between the target TF gene and growth-, yield- and PUE-related traits in transgenic wheat plants under different P supply;
5) Conclude the study and propose future work;

Thesis structure:

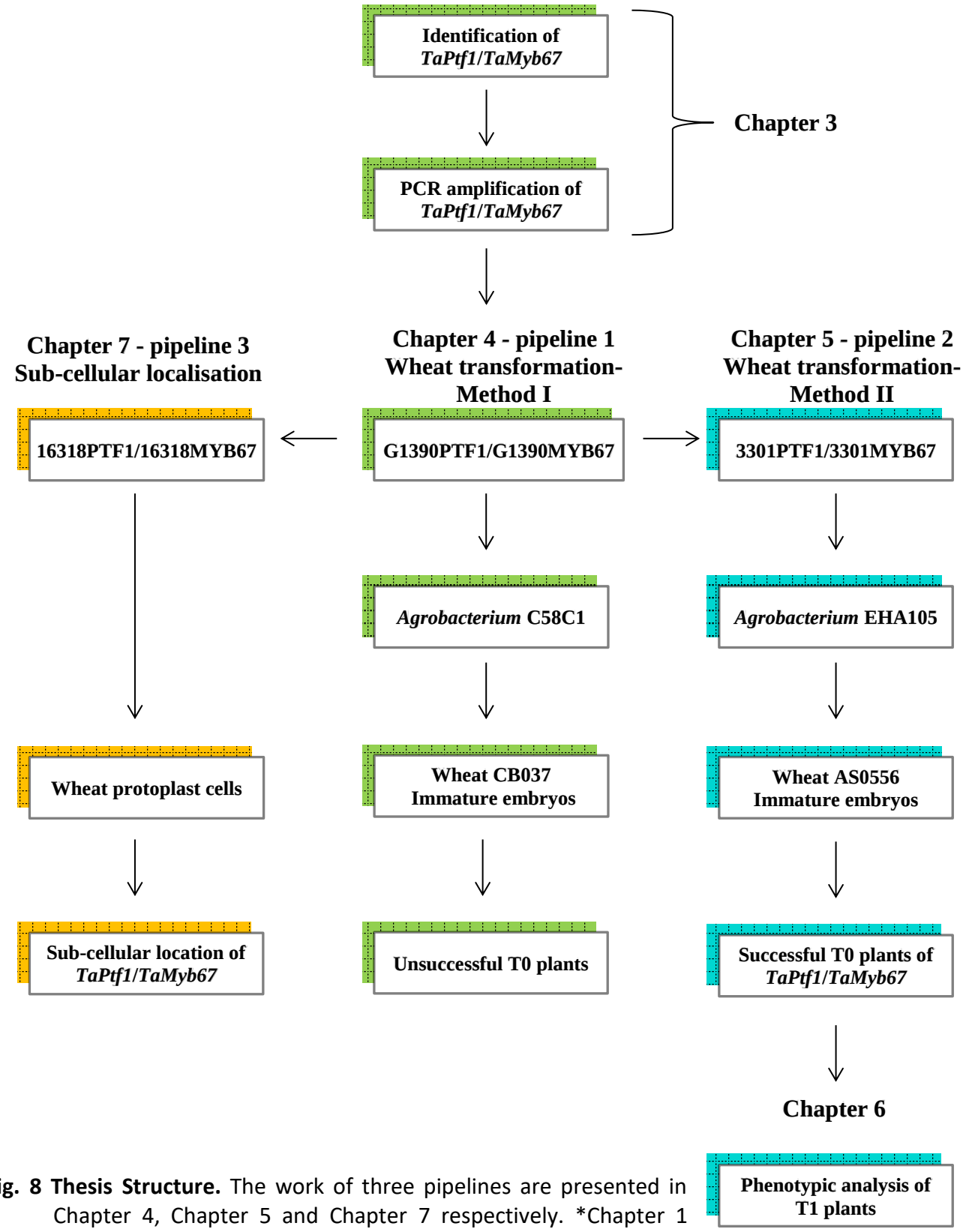


Fig. 8 Thesis Structure. The work of three pipelines are presented in Chapter 4, Chapter 5 and Chapter 7 respectively. *Chapter 1 (General introduction) and Chapter 8 (General discussion and future work) are not included in the figure.

Table 1. Summary of key elements used in experiments

	Vector Name	Cloning method and Features	*Agrobacterium* Strain	Transformation	Plant receptor
-	pMBW006	Restriction digestion and ligation	n/a	Biolistic particle bombardment	Wheat immature embryos
Chapter4	pGWCUbi1390	Gateway Recombination	C58C1	*Agrobacterium*-mediated transformation	Wheat immature embryos
Chapter 5	pCambia3301	Restriction digestion and ligation	EHA105	*Agrobacterium*-mediated transformation	Wheat immature embryos
-	pBI121	Infusion Recombination	C58C1	*Agrobacterium*-mediated transformation	Wheat immature embryos
Chapter 7	P16318GFP	Restriction digestion and ligation	n/a	PEG-mediated transformation	Wheat protoplast

* "n/a" indicates not applicable
* "-" indicates the experiment was not successful and not included in the thesis.

Chapter 2 General materials and methods

The work of three pipelines shown in Fig. 8 was accomplished in both UK and China using different facilities and protocols. When different methods for the same procedure were used, they were described as Method 1) and Method 2) respectively in the following sections.

2.1 Laboratory chemicals and reagents

Chemicals and reagents for the work based in University of Nottingham (UoN), UK were supplied as follows:

DNeasy Plant Mini Kit, RNeasy Plant Mini Kit and RNase-free DNase Set were supplied by QIAGEN (QIAGEN Ltd. Skelton House Lloyd Street North, Manchester, M15 6SH, UK). DNase I (RNase-free) Kit and SuperScript® III Reverse Transcriptase Kit were supplied by Invitrogen-Thermo Fisher Scientific (Thermo Fisher Scientific Inc. 81, Wyman Street, Waltham, MA, USA). Taq polymerase with standard Taq buffer, high-fidelity Phusion polymerase with compatible buffer were supplied by New England Biolabs - NEB (New England Biolabs (UK) Ltd., 75-77 Knowl Piece Wilbury Way Hitchin, Herts SG4 0TY, UK). Gateway® BP Clonase™ II Enzyme Mix and Gateway® LR Clonase™ II Enzyme Mix were supplied by Invitrogen (USA). SensiMix TM SyBr Green®Hi-ROX Kit for qPCR analysis was supplied by Bioline (Bioline USA Inc, 305 Constitution Drive, Taunton, MA, USA). QuickStix Kit for LibertyLink (bar) was supplied by Envirologix (Envirologix, 500 Riverside, Industrial Parkway, Portland, ME 04103-1486, USA).

Antibiotics of Zeocin and Kanamycin-Kana sulfate powder were supplied by Invitrogen (USA) and Fisher Scientific (Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, LE11 5RG, UK). Kana sulfate powder was diluted (with sterile ddH_2O, in Lamina flow hood) to a stock concentration of 10mg mL^{-1} according to manufacturer's instructions and dispensed into 1.5mL sterile microcentrifuge tubes to be stored at -20°C for later use. Other chemicals and

reagents were supplied by Fisher Scientific (UK) or Sigma-Aldrich (Sigma-Aldrich Corporation, 3050 Spruce Street, St. Louis, MO, USA) if not otherwise stated. All plates, falcon tubes, microcentrifuge tubes and pipettes were sterile.

Chemicals and reagent for the work based in Chinese Academy of Agricultural Sciences (CAAS), China were supplied as follows:

PurePlasmid Plasmid Mini Kit, Gel Extraction Kit, Nuclean PlantGen DNA Kit and EndoFree Plasmid Kit were supplied by CWBio (Beijing CW Biotech Co., Ltd., Beijing, China). QuickStix Kit for LibertyLink (bar) was supplied by Envirologix (Envirologix, USA).

Restriction enzymes and buffers were supplied by TAKARA (Takara Biomedical Technology (Beijing) Co., Ltd., Beijing, China). T4 DNA ligase (5U μL^{-1}) and buffer, restriction enzymes BstXI (10U μL^{-1}) and BstEII (or EcoR91I, 10U μL^{-1}) (with 10x buffer "O") were supplied by Thermo Fisher Scientific (China Branch, Shanghai, China). High-fidelity KOD-Plus PCR polymerase (1U μL^{-1}) and reagents were supplied by ToYoBo (C310, 188, Zhangyang Road, New Pudong District, 200122, Shanghai).

Antibiotics of Kanamycin (Kana), Ampicillin (Amp), Rifamycin (Rif), Gentamycin (Gent), Carbenicillin (Carb), Cefotaxime (Cef) were supplied by Beijing BioDee (Beijing BioDee Biotechnology Co. Ltd., Beijing, China), which were diluted (with sterile ddH_2O, in Lamina flow hood) respectively to stock concentrations as Kana (50mg mL^{-1}), Amp (100mg mL^{-1}), Rif (50mg mL^{-1}), Gent (100mg mL^{-1}), Carb (100mg mL^{-1}) and Cef (100mg mL^{-1}) according to manufacturer's instructions and dispensed into 2mL sterile microcentrifuge tubes to be stored at -20°C for later use. Additional antibiotics of hygromycin B (Hyg, Roche/Cellgro-branded) and Phosphinothricin (PPT, Sigma-branded) for plant selection were also supplied by BioDee (Beijing, China) as stock solutions and used directly. Phytohormones, including Vitamin B1

(VB1/Thiamin), Vitamin B6 (VB6), Vitamin C, glycine, inositol and niacin, sucrose, asparagine, Dicamba, Arabinogalactan proteins (AGP) and phytagel for plant tissue culture were supplied by BioDee (Beijing, China).

Murashige and Skoog basal salt mixture with vitamins (M519:100L) and Murashige and Skoog basal salt mixture (M524: 100L) for MS medium were supplied by SeajetSci (Beijing Seaject Scientific Co., Ltd, Beijing, China). Individual basal salt for modified MS medium and additional medium-required chemicals (Sigma-branded) were supplied by BioDee (Beijing, China).

Other chemicals and reagents, such as ethanol, sodium hypochlorite (NaOCl), isopropanol, sodium chloride (NaCl), agarose, X-Gluc, etc., unless otherwise specified, were supplied from BioDee (Beijing, China), TianGen (TianGen Biotech Co. Ltd., Beijing, China), or Sinopharm (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China). All plates, falcon tubes, microcentrifuge tubes and pipettes were sterile.

2.2 Primers

Primers were designed by using Primer Premier 5 (PREMIER Biosoft, 3786 Corina Way, Palo Alto CA 94303-4504, USA) and supplied by Method 1): Eurofins MWG Operon (Anzingerstr. 7a, 85560 Ebersberg, Germany) or Method 2): Sangon Biotech (Sangon Biotech Co., Ltd., Shanghai, China). Primers were diluted according to manufacturer's instructions to a stock concentration of 100pmol μL^{-1} and further prepared to a working concentration of 10pmol μL^{-1} with sterile, DNase-free ddH_2O before use.

2.3 Medium for cell culture

LB/YEP medium (aliquot): LB (or YEP) medium was made by mixing 10g Tryptone, 10g NaCl and 5g Yeast Extract (or 10g Tryptone, 5g NaCl and 10g Yeast Extract) into deionised, distilled water to a final volume of 1L with pH=7.5 (or 7.0). The medium was autoclaved at 121°C for

15–20 minutes and cooled to 55°C, after which the medium was stored at 4°C for up to 4 weeks.

LB/Low-salt LB plates: LB (or Low-salt LB) medium was made by mixing 5g Tryptone, 5g (or 2.5g) NaCl and 2.5g Yeast Extract into 475mL deionised, distilled water, which was made to 500mL (pH=7.5) before adding 1.5% agar. The medium was autoclaved at 121°C for 15–20 minutes and cooled to 55°C, after which appropriate antibiotics were added and mixed evenly before spreading 25mL per plate (in Lamina flow hood).

YEP plates: YEP medium was made by mixing 10g Tryptone, 5g NaCl and 10g Yeast Extract into 975mL deionised, distilled water, which was made to a final volume of 1L (pH=7.0) before adding 1.5% agar. The medium was autoclaved at 121°C for 15–20 minutes and cooled to 55°C, after which appropriate antibiotics were added to the medium and mixed evenly before spreading 25mL per plate (in Lamina flow hood).

Method 1):

Low-salt LB+Zeocin (50μg mL^{-1}) plates were prepared with Zeocin (Invitrogen, USA) which was a 100mg mL^{-1} solution in deionised, sterile water stored at -20°C and protected from light. Autoclaved low-salt LB medium was added with 250μL of Zeocin (100mg mL^{-1}). The plates were stored at 4°C in the dark for up to 2 weeks.

LB+Kana (10μg mL^{-1}) plates were prepared with a Kana stock solution (10mg mL^{-1}) thawed from -20°C. Autoclaved LB medium was added with 500μL of Kana (10mg mL^{-1}). The plates were stored at 4°C in the dark for up to 4 weeks.

Method 2):

LB+Amp (100μg mL^{-1})/Kana (50μg mL^{-1}) plates: autoclaved LB medium was added with 500μL of an Amp (100mg mL^{-1}) or a Kana (50mg mL^{-1}) stock solution thawed from -20°C. The plates were stored at 4°C in the dark for up to 4 weeks.

YEP+Rif (50µg mL^{-1})+*Gent (100µg mL^{-1})+Kana (50µg mL^{-1}) plates: autoclaved YEP medium was added with 1mL of each Rif (50mg mL^{-1}), Gent (100mg mL^{-1}) and Kana (50mg mL^{-1}) stock solutions thawed from -20°C. The plates were stored at 4°C in the dark for up to 4 weeks. *Gent (100mg mL^{-1}) was used where appropriate.

2.4 *Escherichia coli* (*E.coli*) transformation and culture

Method 1):

Chemically competent cells of *E.coli* DB3 and DH5α strains, as well as methods of transformation, were provided by Plant Science (School of Biosciences, University of Nottingham - UoN). Each preparation of competent *E.coli* DB3 or DH5α cells, 25µL per stock, was thawed from -80°C on ice and gently mixed with 1µL (<5%) of the target plasmid DNA, with the control being added with no DNA. This was followed by incubation on ice for 30 minutes before the tube was placed in a 42°C water bath for 60–90 seconds' heat-shock and placed back on ice for 5 minutes. Subsequently, 900µL of S.O.C. medium (2% Tryptone, 0.5% Yeast Extract, 0.4% glucose, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$ and 10mM $MgSO_4$, autoclaved) was gently mixed with the competent cells and transferred to a 15mL falcon tube, which was incubated in a 37°C shaker-incubator at 180rpm for 60 minutes. A volume of 100µL out of 925µL cell culture was spread using a sterilised "hockey puck" spreader onto a LB (or Low salt LB) plate plus appropriate antibiotics. The remaining 825µL cell culture was centrifuged at 5,000rpm for 2 minutes with the supernatant discarded. The culture was re-suspended to 100µL with deionised, sterile water and spread in the same way onto a separate plate. Same operations were applied to the control. After 5 minutes, the plates were sealed and incubated inverted at 37°C for 16 hours.

Method 2):

Chemically competent cells of an *E.coli* TOP10 strain (CWBio, Beijing, China) were supplied as 50µL per stock in a 1.5mL microcentrifuge tube. Each preparation of competent *E.coli* TOP10 cells was thawed from -80°C on ice and gently mixed with 1µL (<1ng) of the target plasmid DNA. This was followed by incubation on ice for 30 minutes before the tube was placed in a 42°C water bath for 45 seconds' heat-shock and placed back on ice for 3 minutes. Subsequently, 450µL of LB medium (aliquot) was gently mixed with the competent cells in the microcentrifuge tube, which was incubated in a 37°C shaker-incubator at 160rpm for 45 minutes. A volume of 200µL out of 500µL cell culture was spread using a sterilised "hockey puck" spreader onto a LB plate plus appropriate antibiotics. After 5 minutes the plate was sealed and incubated inverted at 37°C for 16 hours.

For both methods, single colonies selected from LB plates were used to separately inoculate 10mL of LB medium (aliquot) plus appropriate antibiotics in 15mL falcon tubes/100mL flasks (autoclaved), which were incubated in a 37°C shaker-incubator at 180rpm for 12 hours before plasmid extraction. Spare transformed *E.coli* cultures were individually incorporated into *Escherichia coli*-glycerol stock by mixing each cell culture with an equal volume of 50% glycerol (autoclaved) in a 1.5mL/2mL microcentrifuge tube and stored at -80°C.

2.5 Plasmid DNA miniprep

Method 1):

Null plasmid vectors (pDNOR/Zeo or pGWCUbi1390) were recovered separately from transformed *E.coli* DB3 cultures using a GeneJET plasmid miniprep kit (supplied by Fermentas GmbH-Thermo Fisher Scientific, USA) prior to Gateway reactions. Differently, constructs, pDNOR/Zeo or pGWCUbi1390 plasmid vectors cloned with the target DNA, were propagated through transformed *E.coli* DH5α cultures and similarly recovered using the GeneJET plasmid

miniprep kit. Plasmids were extracted form 5mL transformed *E.coli* DB3/DH5α cultures by following the procedure provided in manufacturer's protocol (Birnboim and Doly, 1979) and eluted with 50µL ddH_2O (DNase-free) into a 1.5mL microcentrifuge tube to be stored at -20°C for later use.

Method 2):

Plasmids were prepared from 5mL transformed *E.coli* TOP10 cultures by using PurePlasmid Mini Kit (CWBio, Beijing, China), with the method adapted from manufacturer's protocol (Appendix 1) and eluted with 50µL ddH_2O (DNase-free) into a 1.5mL microcentrifuge tube to be stored at -20°C for later use.

2.6 Spectrophotometric analysis

Concentrations of DNA/RNA samples were quantified by Method 1): a NanoDrop1000 spectrometer machine (Thermo Fisher Scientific, USA) or Method 2): a NanoDrop2000 spectrometer machine (Thermo Fisher Scientific, USA) according to manufacturer's instructions. For each measurement, a 2µL sample was loaded onto the Nanodrop sensor which was calibrated before loading. The sensor was cleaned between samples to avoid contamination. Readings of concentration were measured with two or three repeats for each sample. Ratios of 260/280 and 260/230 were recorded to indicate contamination and purity of the measured DNA/RNA samples.

2.7 Polymerase Chain Reaction-PCR

Method 1):

A standard PCR reaction system, composed of relevant DNA templates and primers, 10x Taq PCR buffer (Fisher Scientific, UK), *dNTPs (Fisher Scientific, UK), ddH_20 and Taq DNA polymerase (5U μL^{-1}), in a volume of 50µL (or 20µL) was set up in each 0.2mL standard PCR

tube as in Table 2. Following brief centrifugation, PCR amplifications were performed on an Applied Biosystem-ABI GeneAmp PCR System 9700 Thermal Cycler (Thermo Fisher Scientific, USA). The PCR products were assessed by agarose gel electrophoresis and the results were photographed (see later section). *The dNTPs were made to 2.5mM per dNTP (or 10mM total).

Method 2):

When the PCR amplification was performed for cloning, high-fidelity KOD-Plus (1U μL^{-1}) and reagents* (ToYoBo, Shanghai, China) were used for each sample in a 2x50µL PCR reaction system (Table 3). *The dNTPs were supplied as 2mM per dNTP for direct use. When the PCR amplification was performed to validate the presence of a target gene (screening PCR), a 1x20µL PCR reaction system was set up for each sample using 2x Taq MasterMix-Dye (CWBio, Beijing, China) (Table 4). Primers used in PCR amplifications include M13, hybridised primers of Pubi on the vector and PTF(20)/MYB(18) on the target gene, CDS primers of PTF(27)/PTF(20) and MYB(23)/MYB(18) both on the target gene, or hybridised primers on the target gene and the promoter/terminator (see 2.11 for primer sequences).

For both, a premix (with or without templates) was prepared in 1.5mL microcentrifuge tubes and then dispensed into 0.2mL separate or 8-stripe PCR tubes before brief centrifugation. PCR amplifications were performed on a Bio-Rad T100TM/C1000TM Thermal Cycler (Bio-Rad, USA) by using different thermal profiles depending on experiments. The PCR products were assessed by agarose gel electrophoresis and the results were photographed (see later section).

2.8 Agarose gel electrophoresis

Method 1):

Agarose gel electrophoresis was carried out on a standard system supplied by Bio-Rad (Bio-Rad Laboratories Inc., 2000 Alfred Noble Drive, Hercules, CA 94547, USA), for which a 1% or 2%

Table 2. PCR reaction system using Taq polymerase

PCR reagents	volume	volume
10x Taq PCR buffer (µL)	2.4	6
dNTPs (2.5mM) (µL)	0.4	1
Forward primer (10pmol μL^{-1}) (µL)	0.4	1
Reverse primer (10pmol μL^{-1}) (µL)	0.4	1
DNA template (µL)	2	2
Taq (µL)	0.1	0.25
ddH_2O (µL)	14.3	38.75
Total (µL)	20	50

Table 3. PCR reaction system using high-fidelity KOD-Plus

PCR reagents	Volume
10x KOD-Plus buffer (µL)	5
dNTPs (2mM each) (µL)	5
DNA template (µL)	5
Forward primer (10pmol μL^{-1}) (µL)	2
Reverse primer (10pmol μL^{-1}) (µL)	2
$MgSO_4$ (25mM) (µL)	3
KOD-Plus polymerase (1U μL^{-1}) (µL)	1
ddH_2O (µL)	27
Total (µL)	50

Table 4. PCR reaction system using Taq MasterMix

PCR reagents	Volume
2x Taq MasterMix (Dye) (µL)	10
Forward primer (10pmol μL^{-1}) (µL)	0.8
Reverse primer (10pmol μL^{-1}) (µL)	0.8
DNA template (µL)	1
ddH_2O (µL)	7.4
Total (µL)	20

gel was made with molecular biology grade agarose (Fisher Scientific, UK) and 1x TAE buffer diluted from 10x TAE buffer (Bio-Rad, USA). Once the dissolved agarose cooled to 55°C after heating in a microwave, an appropriate volume of 0.01% v/v SYBR™ Safe™ DNA Gel Stain (10000x, in DMSO; Fisher Scientific, UK)/or Ethidium Bromide (10mg/mL, Sigma-Aldrich) was carefully mixed into the 150mL liquid. The gel was made in a casting tray with combs in appropriate sizes and stood until complete solidification. This was then placed in an electrophoresis bath to be submerged in 1x TAE buffer for electrophoresis. Next, 1µL of 6x loading dye (Fisher Scientific, UK) was mixed with each 5µL DNA/RNA sample before loading into each experiment well. A volume of 3µL 1kb or 100bp DNA ladder (Fisher Scientific, UK) was loaded to each marker well. Once samples and markers were loaded, the gel was run at 10V cm^{-1} for 0.5–1 hour prior to getting photographed under ultraviolet light. The results were analysed by SnapGene Viewer v3.1.4 (GSL Biotech LLC, Chicago).

Method 2):

DNA ladders BM2000 (90ng μL^{-1}), BM5000 (110ng μL^{-1}), 100bp (120ng μL^{-1}) and 1Kb (90ng μL^{-1}) were supplied by BioMed (Beijing BioMed Technology Co., Ltd, Beijing, China). Nucleic acid dye Genecolour gel stain (10000X, in ddH_2O) was supplied by JinBoYi Biotech (Beijing JinBoYi Biotechnology Co., Ltd., Beijing, China). Agarose gel electrophoresis was carried out by using a standard system supplied by LIUYI Biotech (Beijing LiuYi Biotechnology Co., Ltd., Beijing, China), for which a 1% gel was made with molecular biology grade agarose and 1x TAE buffer diluted from 10x TAE buffer (10x TAE buffer: 53g L^{-1} Tris base, 27.5g L^{-1} boric acid, 10mM L^{-1} EDTA, made up to 1L with H_2O). Once the dissolved agarose cooled to 55°C after heating in a microwave, a volume of 15µL Genecolour gel stain was carefully mixed into the 150mL liquid. The gel was made in a casting tray with combs in appropriate sizes and stood until complete

solidification. This was then placed in an electrophoresis bath to be submerged in 1x TAE buffer for electrophoresis. Next, a 1/10-volume of 10x loading dye (5% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) was mixed with each DNA sample, if required, before loading into each experiment well. A volume of 5µL appropriate DNA ladder was loaded to each marker well. Once samples and marks were loaded, the gel was run at 10V cm^{-1} for 0.5 hour prior to getting photographed by Bio-Rad ChemiDoc MP (Bio-Rad, USA). The results were analysed by ImageLab v5.0 (Bio-Rad, USA). If purification was required, DNA bands were sliced out and recycled in 1.5mL centrifuge tubes and stored at -20°C for later use.

2.9 Restriction enzyme digestion

Restriction enzyme digestion sites of a target plasmid DNA were analysed by NEBcutter v2.0 (New England Biolabs Inc., 240, country Road, Ipswich, MA 01938-2723, USA). Up to 1µg plasmid DNA was examined according to different restriction enzyme sites of the plasmid. Single digestion or double digestion was carried out with selected restriction enzymes and appropriate buffers in a one-step or two-step digestion reaction by referring to manufacturer's instructions (Takara http://www.takara.com.cn/; Thermo Fisher Scientific-China branch https://www.thermofisher.com/uk/en/home.html). The reaction products were assessed by agarose gel electrophoresis and the results were analysed.

2.10 Purification of DNA

Method 1):

DNA samples were purified by using 30% PEG 8000/30mM $MgCl_2$ (Invitrogen, USA), with the method adapted from manufacturer's protocol (Appendix 2). Briefly, the DNA was initially diluted 4-fold with 150µL of TE buffer (10mM Tris-HCl with pH=7.5, 1mM EDTA) in a 1.5mL microcentrifuge tube and mixed with 100µL of 30% PEG8000/30mM $MgCl_2$ thoroughly by

vortex. The mixture was centrifuged for 15 minutes at 9,500rpm at room temperature, followed by the supernatant being carefully removed without interrupting the DNA pellet. The pelleted DNA was re-suspended in 20µL of TE buffer before Gateway cloning.

Method 2):

DNA samples were purified from agarose gel by using Gel Extraction Kit (CWBio, Beijing, China), with the method adapted from manufacturer's protocol (Appendix 2). Briefly, gel slices collected in a 1.5mL centrifuge tube were thawed at room temperature from -20°C and dissolved in an equivalent volume of buffer PG by heating at 50°C. The DNA was recovered by using a Spin Column DM and wash buffers (PS and PW), after which the DNA was eluted with 50µL ddH_2O (DNase-free) into a 1.5mL microcentrifuge tube and stored at -20°C for later use.

2.11 DNA sequencing

Plasmid DNA samples (constructs) were prepared by following manufacturer's instructions and sequenced by Method 1): Eurofins MWG Operon (Germany) using either universal or compatible gene-specific sequencing primers; or Method 2): Sangon Biotech (China) using either universal or gene-specific sequencing primers. Sequencing results were analysed by the European Bioinformatics Institute (EBI) online tools (http://www.ebi.ac.uk/Tools/psa/) or DNAMAN v7.0 (Lynnon Biosoft, USA). Primers used for DNA sequencing are listed below:

M13-uni (-21): 5´-TGTAAAACGACGGCCAGT-3'
M13-rev (-29): 5'-CAGGAAACAGCTATGACC-3'

Pubi: 5'-TTTGTCGATGCTCACCCTG-3'
Tnos: 5'-TTGCCAAATGTTTGAACGA-3'

PTF (27): 5'-ATGGACTACTCTAATGGTTCTTTCTTT-3'
PTF (20): 5'-TCACCTTTCAGGAGGGATTG-3'

MYB (23): 5'-TGAGGAGGTGTGATCTGAGACA-3'
MYB (18): 5'-TCAGCGCTTCTCTTGCG-3'

2.12 Extraction of genomic DNA

Genomic DNA was extracted from no more than 100mg fresh leaf tissues using Method 1): DNeasy Plant Mini Kit (QIAGEN, UK); or Method 2): Nuclean PlantGen DNA Kit (CWBio, Beijing, China) according to manufacturer's instructions (Appendix 3).

2.13 Extraction of total RNA and genomic DNA digestion

For RNA extraction, each sample of no more than 100mg fresh Method 1): root tissues; or Method 2): leaf tissues from wheat plants were collected and immediately frozen in liquid nitrogen on collection. Each sample was thoroughly disrupted by a mortar and pestle or a Bullet blender (Next Advance, Inc., 1548, Burden Lake Road, Averill Park, NY 12018-2818) into a fine powder and transferred into a 2mL microcentrifuge tube before the sample thawed.

Method 1):

For miniprep of total RNA, each sample was homogenised in RTL lysis buffer (provided in RNA RNeasy Plant Mini Kit and added with 10µL β-mercaptoethanol (β-ME) per mL RTL buffer). RNA extraction and on-column DNA digestion were subsequently carried out by using RNA RNeasy Plant Mini Kit and RNA-free DNase Set (QIAGEN, UK), with the method adapted from manufacturer's protocol (Jin and Bian, 2004; Kunitz, 1950). The purified RNA was eluted with 50µL RNase-free ddH_2O into a 1.5mL microcentrifuge tube (RNase-free) and stored in -80°C for later use.

Method 2):

For midiprep of total RNA, each sample was homogenised in 1mL of Ambion Trizol (Thermo Fisher Scientific, USA). RNA extraction was subsequently carried out using the protocol described by Chomczynski (1993). The purified RNA was dissolved in 50µL RNase-free ddH_2O in a 1.5mL microcentrifuge tube (RNase-free) and stored in -80°C for later use. Before downstream applications, the purified RNA was treated with RNase-free DNase I (1U μL^{-1},

Thermo Fisher Scientific, USA). Briefly, 1µg total RNA was added with 1µL of 10x DNase I reaction buffer (containing $MgCl_2$), 1µL DNase I and an appropriate volume of ddH_2O (calculated according to RNA concentration determined by Nanodrop as previously described in 2.6) to make a 10µL reaction system in a 1.5mL microcentrifuge tube (RNase-free). The reaction was conducted by incubating the mixture at 37°C for 30 minutes and inactivated by adding 1µL of 50mM EDTA before additional incubation at 65°C for 10 minutes.

2.14 cDNA synthesis

For each reaction, 1µg total RNA (DNase I-treated) was used to reversely transcribe mRNA into the first-strand cDNA on a 9700 Thermal Cycler (Thermo Fisher Scientific, USA) following the procedure provided in manufacturer's protocol (Kotewicz *et al*., 1985). Briefly, a premix was prepared by using total RNA, $OligodT_{12\text{-}18}$ (0.5 µg μL^{-1}, Fisher Scientific, UK), dNTPs (2.5mM per dNTP or 10mM in total, Fisher Scientific, UK) and ddH_2O as described by step 1 in Table 5, which was dispensed into 0.2mL standard PCR tubes. The tubes were heated at 65°C for 5 minutes and immediately cooled on ice, followed by the addition of Superscript III transcriptase and appropriate reagents (Invitrogen, USA) in the reaction as described by step 2 to step 5 in Table 5. The reaction was conducted at 50°C for 45 minutes and inactivated at 70°C for 15 minutes. The first-strand cDNA was stored at -20°C for later use.

2.15 Quantitative real-time polymerase chain reaction-qPCR analysis

Relative quantification of gene expression levels was determined by real-time quantitative PCR (qPCR) analysis, which was first described by Heid *et al*. (1996). Appropriate reference genes were employed in the qPCR for normalisation of gene expression levels. Three technical replicates were set up for each cDNA template that was tested by each primer pair. For each sample well of a 384-well PCR plate that was to be used, a mixture consisted of an appropriate

volume of forward and reverse primers, SyBr Green Hi-Rox Mixture (Bioline, USA) and the diluted first-strand cDNA was prepared, as shown in Table 6, and this was made up to a 12µL reaction system with ddH_2O. The 384-well PCR plate was loaded onto a Roche LightCycler480, which was performed following the thermal profile as one cycle at 95°C for 10 minutes; 50 cycles of 95°C for 10 seconds, 56°C for 30 seconds and 72°C for 20 seconds; one cycle of 95°C for 5 seconds; one cycle of 65°C for 60 seconds. LightCycler480 software (Roche) was used to process the outputs and calculate Ct values for each cDNA template assessed by each primer pair for results.

Table 5. Preparation of reverse transcription reactions

Procedure		**FScDNA**
1. Premix	OligodT $_{12\text{-}18}$ (0.5 μg μL^{-1}) (μL)	1
	dNTPs (10mM) (μL)	1
	RNA (μL)	3
	RNase-free ddH_2O (μL)	8
2. Mix well and incubate at 65°C for 5 minutes		
3. Cool on ice for 2–3 minutes		
4. Add	First Strand buffer (5x) (μL)	4
	RNaseout (μL)	1
	0.1 DTT (μL)	1
	SuperScript III Reverse Transcriptase (μL)	1
5. Mix well and incubate at 50°C for 45 minutes		
6. Incubate at 70°C for 15 minutes		

* FScDNA: first-strand cDNA

Table 6. Preparation of quantitative real-time PCR

Procedure		**Volume**
1. Premix	SyBr Green®Hi-ROX Mixture	6
	Forward primer (100pmol μL^{-1}) (μL)	0.1
	Reverse primer (100pmol μL^{-1}) (μL)	0.1
	ddH_2O (μL)	0.8
	Total (μL)	7
2. Mix well and dispense 7μL into each qPCR sample well		
3. Add 5μL cDNA template (100 times diluted by using the FScDNA from reverse transcription)		
5. Mix well and proceed to qPCR steps		

Chapter 3 Identification and PCR amplification of transcription factors *TaPtf1* and *TaMyb67*

3.1 Overview

P acquisition and P utilisation are two essential aspects of PUE and regulated both locally and systemically within plants (Chiou and Lin, 2011; Svistoonoff *et al*., 2007; Thibaud *et al*., 2010). Soil P_i deficiency triggers local sensing and response, resulting in characteristic differentially controlled changes in root development. Systemic sensing responds to internal P_i deficiency and works through various pathways to co-ordinate an increase in P uptake and translocation, as well as P utilisation, within the plant. This is crucial to adapting to P deficiency/low-P and maintaining P_i homeostasis. Considerable progress has been made in model species of rice and *Arabidopsis* suggesting that major regulatory components for PUE include P_i, microRNAs, hormones, sugars and transcriptional factors (TFs) (Franco-Zorrilla *et al*., 2005; Jain *et al*., 2012; Lin *et al*., 2009; Vance, 2010). Among these, TFs are important to the positive or negative modulation of P-stress-responsive genes in determining PUE. Despite the advances made in *Arabidopsis* and rice, the regulatory basis of P_i homeostasis and TFs involved in PUE is poorly understood for polyploidy crop species, such as wheat (Bovill *et al*., 2013).

This study has made attempts to investigate the regulatory role of TFs in PUE in wheat by exploiting the concept of translational genomics. Two key TFs in rice, *OsRhl1* and *OsPhr2*, were chosen as candidate genes for the identification of wheat orthologues and further investigation of their gene functions and roles in PUE in wheat (described in 1.9). OsRhl1 is a novel bHLH TF and has been reported to regulate root hair initiation and elongation (Ding *et al*., 2009). The *Osrhl1* mutant shows short root hairs, while overexpression of *OsRhl1* results in abundant growth of root hairs. Evidence collected in *Arabidopsis* and *Lotus japonicus* also

suggests that closely related homologues of *OsRhl1* are involved in root hair formation (Karas *et al*., 2009; Schneider *et al*., 1998). Root hair formation (and growth) is a key adaptation mechanism employed by plants under P deficiency/low-P and significantly promotes P acquisition (reviewed in section 1.5). It is therefore likely that Rhl1 acts as a regulatory component of PUE. In contrast to OsRhl1, the role of OsPhr2 in PUE is clear. OsPhr2 is an MYB-CC TF and has been identified as a rice equivalent of AtPhr1 that systemically regulates P acquisition as well as utilisation and therefore contributes to PUE. Overexpression of *AtPhr1* has been shown to respond to P deficiency/low-P through an increase in shoot P_i concentration, which results from upregulation of PSI genes that encode phosphate transporters (PTs), phosphatase and RNase, while impaired root development, expression of PSI genes and P allocation can be observed in the *Arabidopsis phr1* mutant (Bari *et al*., 2006; Nilsson *et al*., 2007). Similarly, overexpression of *OsPhr2* results in altered root growth, elevated expression of a range of PSI genes including PTs and increases of P redistribution from roots to shoots under P deficiency/low-P (Zhou *et al*., 2008a). Furthermore, *OsPhr2* equivalents studied in other crop plant species, such as maize (Wang *et al*., 2013a), rapeseed (Ren *et al*., 2012), common bean (Valdés-López *et al*., 2008), soybean (Li *et al*., 2017), have also validated the role of *OsPhr2* as a crucial regulator in resistance to P stress (reviewed in section 1.7). The key aim of the research presented in this chapter was therefore to investigate and identify wheat orthologues of OsRhl1 and OsPhr2 using a bioinformatic approach and to design strategic experiments to confirm/obtain their coding DNA sequences (CDSs) through DNA cloning.

3.2 Materials and methods

3.2.1 Bioinformatics tools and analysis

Information (name/accession) for candidate genes (*OsRhl1* and *OsPhr2*) was obtained from relevant publications. Sequence data including protein sequences and associated genomic or coding sequences (CDSs) was collected by searching the NCBI (National Centre for Biotechnology Information) protein or nucleotide database and saved in FASTA format for subsequent BLAST or sequence alignment analyses (Geer *et al*., 2010).

For selection of orthologues that may have same gene functions with candidate genes, the orthology assignment usually derives directly from cross-species sequence similarity searching (Salentijin *et al*., 2007). Sequence similarity searching statistically assesses the match of nucleotide/protein sequences from a target database with a query sequence using alignments. The degree of match theoretically reflects the structural and functional conservation (or the likelihood of homology) between two or more cross-species sequences. In practice, BLAST search of nucleotide sequences (DNA/RNA) or protein sequences is commonly employed and the resulting sequence with the most significant homology is likely to be the orthologue (Salentijin *et al*., 2007). In this study, BLAST search was achieved by using the NCBI online bioinformatics search engine, BLAST, with different criteria applied. A BLASTP search of the wheat non-redundant protein database (BLASTP; taxid: 4565)/rice non-redundant protein database (BLASTP; taxid: 4530)/non-redundant protein database with no restriction to any specific organism (BLASTP) was performed by using a query protein sequence (Johnson *et al*., 2008). A tBLASTn search of the wheat translated nucleotide database (tBLASTn: taxid: 4530) was performed by also using a query protein sequence. In the output, target DNA or protein sequences were sought according to the similarity (identical and coverage) to the query

sequence and their sequence data were collected by following corresponding links to the NCBI entries and saved in FASTA format. A BLAST search of the wheat genome was achieved by using the Ensembl Plants online bioinformatics search engine, BLAST, during which a CDS was submitted as a query to search the wheat genome database. The resulting genomic sequences were downloaded and saved in FASTA format.

Multiple and pairwise sequence alignment analyses were carried out with the EBI online sequence alignment analysis tools, Clustal Omega and EMBOSS Needle, respectively. Sequence data for analyses were collected either from the BLAST search already performed or using name/accession. In a multiple sequence alignment analysis using Clustal Omega, three or more DNA/protein sequences were entered sequentially and submitted to generate alignments. In a pairwise sequence alignment analysis using EMBOSS Needle, sequences were entered in pairs and submitted separately to obtain the optimum alignment (including gaps) of each pair of sequences. The Protein Homology/analog- Y Recognition Engine v2.0 (PHYRE2.0) was used for protein structure analysis, where a target protein database is searched using a query protein sequence.

3.2.2 Plant materials

A popular commercial wheat cultivar assumed to have high PUE, Battalion (winter wheat, 2012 harvest; fresh seeds were available at the time of the experiment), was used to obtain the target DNA. Wheat seeds were supplied by Plant Science and surface sterilised in a Lamina flow hood according to the method adapted from an experimental protocol provided by Duncan Scholefield from Plant Science. Briefly, approximately 20 seeds were sterilised in a 50mL falcon tube by rinsing in 20mL of 70% (v/v) aqueous ethanol (prepared from 96% ethanol and sterile distilled water, Lamina hood) for 3 minutes, followed by soaking in 20mL of 2% (v/v)

NaOCl (prepared from 10% NaOCl and sterile distilled water, Lamina hood) for 30 minutes and rinsing thoroughly with three changes of 20–30mL sterile distilled water. The sterilised seeds were germinated for 3 days in a polystyrene petri dish (90x15mm) filled with two layers of moist and Whatman filter paper (Ø 85-90mm, sterile) in a growth room (B05, Gateway Building, Sutton Bonington campus, School of Biosciences, UoN; day/night temperature: 20°C/16°C). The germinated seeds were hydroponically grown in a plastic box (18L, dark) containing the Long Ashton solution, which was prepared with Mili-Q water (Mili-Q water purification system, Merch, UK) as the formula described by Hewitt (1966), with the pH adjusted to 5.8 at room temperature (Appendix 4). The germinated seeds were grown in the same growth room for 2–3 weeks and the hydroponic solution was refilled every 2–3 days.

3.2.3 Extraction of total RNA and DNA digestion

Root tissues (<100mg) of wheat plants grown in hydroponics were sampled. Total RNA extraction and on-column DNA digestion were performed according to Method 1) as described in 2.13. The total RNA was eluted with 50µL RNase-free ddH_2O into a 1.5mL microcentrifuge tube (RNase-free) and assessed by spectrophotometric analysis and agarose gel electrophoresis according to Method 1) as described in 2.6 and 2.8.

3.2.4 cDNA synthesis

The first-strand cDNA was synthesised in reverse transcription using the total RNA from the previous step according to Method 1) as described in 2.14, after which the first-strand cDNA was used for the downstream PCR amplification.

3.2.5 PCR amplification

For quality control of cDNA synthesis, a pair of wheat Tubulin (reference gene) primers, as described by Zang *et al*. (2010), were generated according to Method 1) as described in 2.2. By

using the first-strand cDNA from the previous step as templates and Tubulin primers in each 50µL standard PCR reaction system (Table 7), PCR amplifications were performed according to Method 1) as described in 2.7. The thermal profile was at 94°C for 5 minutes; 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds; at 72°C for 7 minutes. The PCR products were assessed by agarose gel electrophoresis as previously described.

Primers used to obtain the full-length CDS of *TaPtf1*/*TaMyb67* were designed and modified manually to increase specificity, considering Tm and GC content, and were generated according to Method 1) as described in 2.2 (the forward primer begins with the first base of the start codon and the reverse primer ends at the last base of the stop codon in *TaPtf1*/*TaMyb67* CDS). The primers were unable to detect the individual contribution of each gene copy from the three sub-genomes on this occasion. The PCR amplification of *TaPtf1*/*TaMyb67* was performed by using a 50µL standard PCR reaction system as described for Tubulin, except for replacement with *TaPtf1*/*TaMyb67* gene-specific primers and appropriate annealing temperatures (*TaPtf1*: 57°C *TaMyb67*: 59°C). The PCR products were assessed by agarose gel electrophoresis as previously described.

Table 7. PCR amplification of Tubulin

PCR	Tubulin-1	Tubulin-2	Tubulin-3	Tubulin-4
10x Taq PCR buffer (µL)	6	6	6	6
dNTP(2.5mM) (µL)	1	1	1	1
Tubulin forward primer (10pmol μL^{-1}) (µL)	1	1	1	1
Tubulin reverse primer (10pmol μL^{-1}) (µL)	1	1	1	1
FScDNA (µL)	2	2	2	2
Taq (µL)	0.25	0.25	0.25	0.25
ddH_2O (µL)	38.75	38.75	38.75	38.75
Total (µL)	50	50	50	50

* FScDNA: first-strand cDNA

3.3 Results

3.3.1 Identification and sequence analysis of TaPtf1

3.3.1.1 BLAST search of OsRhl1 homologues in wheat

To find wheat homologues of OsRhl1 (GenBank accession No. BAD72512.1), a direct BLASTP search of the wheat protein database using OsRhl1 as a query was performed as described in method section 3.2.1. Surprisingly, no significant results were returned. Hence, a tBLASTn search by using OsRhl1 as a query was conducted alternatively to explore the wheat translated nucleotide database, which resulted in the CDS entry of "*Triticum aestivum* cultivar PH85-16 PTF1 mRNA" (complete cds, GenBank accession No. DQ979392) as being the most similar wheat homologue to OsRhl1 (red frame, Fig. 9). Its protein ID was shown as TaPtf1-"PTF1 [*Triticum aestivum*]" (GenBank accession No. ABI95371), indicating TaPtf1 is the most similar homologue of OsRhl1 in wheat. TaPtf1 was found to be a protein of 480aa encoded by a CDS of 1443bp, whereas no genome location or a gene function has been described for TaPtf1. As the most similar homologue in the BLAST search could be an orthologue candidate (Salentijin *et al*., 2007), TaPtf1 was initially identified as a putative orthologue of OsRhl1. Conversely, to investigate whether OsRhl1 is the most similar homologue of TaPtf1 in rice (or the orthology between OsRhl1 and TaPtf1), TaPtf1 was used as a query to search back through the rice protein database using a BLASTP search. This showed OsPtf1 (GenBank accession No. AAO73566) and not OsRhl1 to be the rice homologue that was most similar to TaPtf1 (Fig. 10). The result suggests TaPtf1 might not be the orthologue of OsRhl1 and was therefore subjected to further analysis.

3.3.1.2 Homology of OsRhl1, OsPtf1 and TaPtf1 by alignment

To compare the similarities and differences between TaPtf1, OsRhl1 and OsPtf1 in more detail, multiple sequence alignment was performed as described in method section 3.2.1. The output (Fig. 11) indicated that all the three sequences were highly conserved in the bHLH regions. However, TaPtf1 and OsPtf1 appeared to be more similar to each other than OsRhl1. OsRhl1 only shared 38.2% and 30.5% homology with OsPtf1 and TaPtf1, respectively. Except for the aligned bHLH region and the nearby regions bordering the bHLH region on each side, homology was rarely found among the sequences of OsRhl1 and the other two. In contrast, OsPtf1 and TaPtf1 showed 82% similarity, covering not only the functionally important bHLH region but also other more extensive parts out of the sequence. The result suggests that TaPtf1 is more likely to be the orthologue of OsPtf1 than of OsRhl1, while the wheat orthologue of OsRhl1 seemed to be absent. In the absence of an OsRhl1 orthologue, TaPtf1 was used for subsequent study (to be discussed in section 3.4.1).

Description	Max score	Total score	Query cover	E value	Ident	Accession
Triticum aestivum cultivar PH85-16 PTF1 mRNA, complete cds	114	114	34%	3e-27	44%	DQ979392.1
Triticum aestivum cDNA, clone: WT003_F15, cultivar: Chinese Spring	40.8	40.8	8%	0.003	51%	AK332182.1
Triticum aestivum cDNA, clone: WT012_N15, cultivar: Chinese Spring	38.5	38.5	8%	0.013	53%	AK335416.1
Triticum aestivum bHLH transcription factor (bHLH94) mRNA, partial cds	33.5	33.5	9%	0.34	41%	AY625684.1

Fig. 9 TaPtf1 identified as the most similar homologue to OsRhl1. tBLASTn search to the NCBI translated nucleotide database was performed by using OsRhl1 protein sequence as the query and the organism was restricted to *Triticum aestivum* (taxid: 4565). The result shown in the red frame is the DNA sequence that is most similar to the query.

Query ID ABI95371.1
Description PTF1 [Triticum aestivum]
Molecule type amino acid
Query Length 480

Database Name nr
Description All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects
Program BLASTP 2.6.1+ Citation

Other reports: Search Summary [Taxonomy reports] [Distance tree of results] [Multiple alignment]

Alignments Download GenPept Graphics Distance tree of results Multiple alignment

Description	Max score	Total score	Query cover	E value	Ident	Accession
bHLH transcription factor PTF1 [Oryza sativa]	663	663	100%	0.0	72%	AAQ73566.1
hypothetical protein OsI_21996 [Oryza sativa Indica Group]	663	663	100%	0.0	73%	EAY99993.1
PREDICTED: transcription factor SPATULA isoform X1 [Oryza sativa Japonica Group]	662	662	100%	0.0	72%	XP_015644031.1
bHLH transcription factor [Oryza sativa Japonica Group]	661	661	100%	0.0	72%	ACF60480.1
PREDICTED: uncharacterized protein LOC4340382 isoform X2 [Oryza sativa Japonica Group]	591	591	100%	0.0	67%	XP_015644034.1
Os06g0193400 [Oryza sativa Japonica Group]	441	494	74%	7e-153	74%	BAS96597.1
PREDICTED: transcription factor bHLH82 isoform X3 [Oryza sativa Japonica Group]	440	560	83%	5e-152	74%	XP_015644035.1
Transcription factor [Oryza sativa Japonica Group]	310	310	97%	1e-99	51%	CDN24603.1
Transcription factor [Oryza sativa Indica Group]	297	297	97%	1e-94	49%	CDN24604.1
PREDICTED: transcription factor bHLH68 [Oryza sativa Japonica Group]	229	229	34%	2e-68	69%	XP_015623831.1
hypothetical protein OsI_07700 [Oryza sativa Indica Group]	229	229	34%	2e-68	69%	EAY86326.1
PREDICTED: transcription factor UNE12 isoform X2 [Oryza sativa Japonica Group]	187	187	31%	1e-52	66%	XP_015610785.1

Fig. 10 Search of rice protein database by using TaPtf1 as a query. BLASTP search to the NCBI protein database was performed by using TaPtf1 protein sequence as the query and the organism was restricted to *Oryza sativa* (taxid: 4530). The result shown in the red frame is the protein sequence that is most similar to the query.

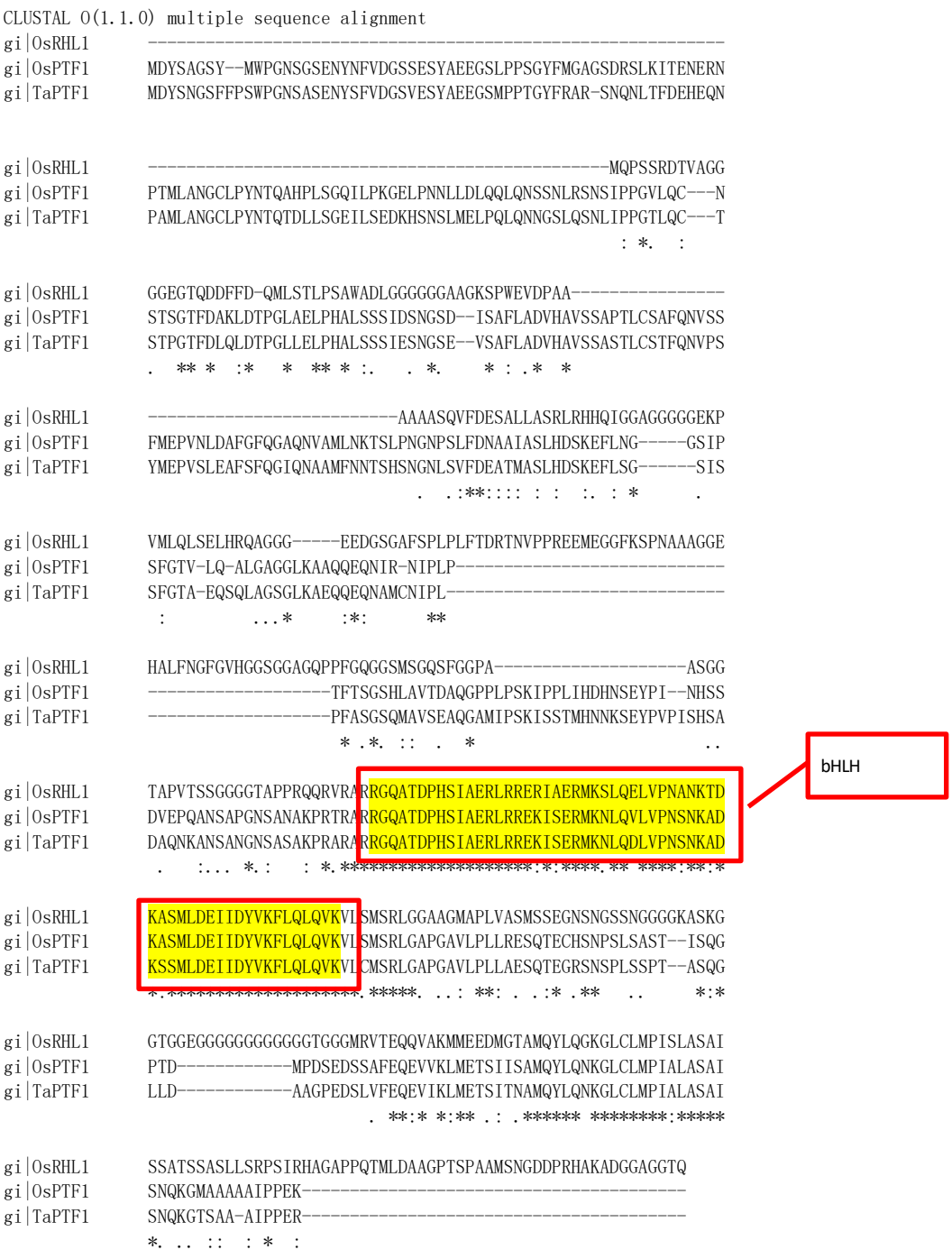


Fig. 11 Multiple sequence alignment analysis of OsRhl1, OsPtf1 and TaPtf1 protein sequences. The result was produced from the EBI online analysis tool-Clustal Omega. OsRhl1 GenBank accession: No. BAD72512. OsPtf1 GenBank accession: No. ABI95371. TaPtf1 GenBank accession: No. ABI95371. The bHLH functional regions are highlighted in yellow and shown in red frame.

3.3.1.3 Genome location and sequence structure of *TaPtf1*

The genome location and genome sequence structure of *TaPtf1* were further analysed by wheat genome BLAST and pairwise alignment, as described in method section 3.2.1. According to the results of genome BLAST using the full-length *TaPtf1* CDS (GenBank accession No. DQ979392) as a query, *TaPtf1* was mapped to three homoeoloci on 7AS (Location: Scaffold TGACv1_scaffold_569918_7AS: 72116–77788), 7BS (Location: Scaffold TGACv1_scaffold_591870_7BS: 77047–84446) and 7DS (Location: Scaffold TGACv1_scaffold_624291_7DS: 18777–24175) (S=short arm; 7=chromosome 7; A/B/D=sub-genome). Splice variants predicted by the Ensembl database for the three homoeoloci are shown in Table 8. At least one splice variant responsible for a protein of 480aa (TaPtf1) was found in each homoeolocus.

A conserved gene structure of *TaPtf1* on three sub-genomes, composed of eight exons and seven introns as the lengths and structure shown in Fig. 12, was indicated by the pairwise alignments between the query *TaPtf1* and its three genome sequences. The genome sequence derived from 7DS shared the most significant homology, implying genome D might be the origin for the query *TaPtf1* CDS, whereas the genome sequences on 7AS and 7BS showed more changes in single nucleotides when compared with *TaPtf1* CDS. Aligned regions on each genome sequence (1443bp) were equivalent to the query of full-length *TaPtf1* CDS (Fig. 13), suggesting that the CDS was a full-length product.

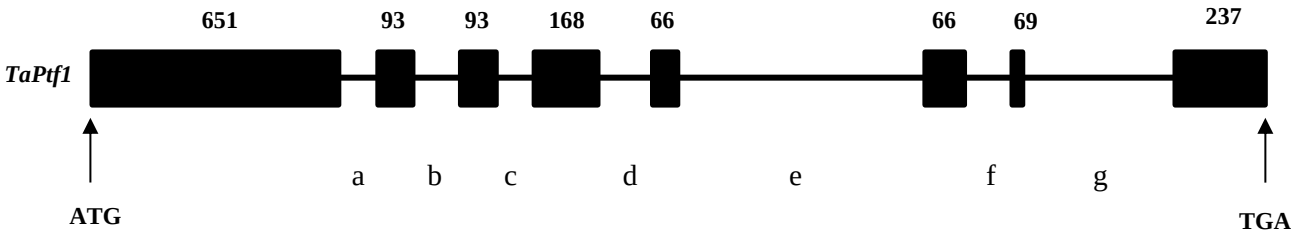


Fig. 12 *TaPtf1* genome sequence structure. Black box: exon (translated). Numbers above the black box indicate the length of the exon in bp. Black string: intron. Letters indicate the lengths of introns in bp (a: 93; b:99; c: 95; d: 129; e: 773; f: 103; g: 362). The arrow ATG indicates the start codon of the gene. The arrow TGA indicates the stop codon.

Gene ID and location on the physical map (Ensembl wheat genome):

TaPtf1-A1: Gene ID: TRIAE_CS42_7AS_TGACv1_569918_AA1826920;
Genome location: scaffold_569918_7AS: 72116–77788.
TaPtf1-B1: Gene ID: TRIAE_CS42_7BS_TGACv1_591870_AA1923970;
Genome location: scaffold_591870_7BS: 77047–84446.
TaPtf1-D1: Gene ID: TRIAE_CS42_7DS_TGACv1_624291_AA2060360;
Genome location: scaffold_624291_7DS: 18777–24175.

Table 8. Splice variants from *TaPtf1* homoeoloci

Genome	Number of splice variant	Transcript ID	Transcript length (bp)	Protein (aa)	Biotype (Y/N)
7AS	4	7AS_TGACv1_569918_AA1826920.1	2466	480[(a)]	Y
		7AS_TGACv1_569918_AA1826920.2	2471	480[(a)]	Y
		7AS_TGACv1_569918_AA1826920.3	2304	460[(b)]	Y
		7AS_TGACv1_569918_AA1826920.4	2364	480[(a)]	Y
7BS	4	7BS_TGACv1_591870_AA1923970.1	2250	478[(d)]	Y
		7BS_TGACv1_591870_AA1923970.2	2256	480[(e)]	Y
		7BS_TGACv1_591870_AA1923970.3	2141	431[(c)]	Y
		7BS_TGACv1_591870_AA1923970.4	2132	480[(e)]	Y
7DS	2	7DS_TGACv1_624291_AA2060360.1	2213	478[(f)]	Y
		7DS_TGACv1_624291_AA2060360.2	2219	480[(g)]	Y

* bp: base pair. aa: amino acid. Y: Protein coding. N: Non-protein coding.
* Letters represent Ensembl accessions of proteins. (a): A0A1D6BW60; (b): A0A1D6BW59; (c): A0A1D6CDV5; (d): A0A1D6CDV3; (e): W5HSP1; (f): A0A1D6D511; (g): W5I7Y6

>*TaPtf1*_TRIAE_CS42_7DS_TGACv1 _624291_AA2060360

cataatatttctgagcagaggttggggagtcaagttgtaagggtgtaaagATGGACTACTCTAATGGTTCTTTCTTTCCTTCAT
GGCCTGGCAATTCCGCTTCCGAGAATTATAGCTTTGTTGATGGTTCAGTGGAATCATATGCAGAAGAAGG
AAGTATGCCACCTACAGGCTATTTCAGAGCTAGATCAAATCAGAATTTAACATTTGATGAGCATGAACAGA
ACCCTGCTATGCTTGCAAATGGGTGCTTGCCGTACAACACCCAGACTGATCTATTATCTGGTGAGATTCTGT
CAGAGGACAAACATTCCAACAGCCTTATGGAGCTTCCACAACTTCAGAACAATGGCAGTCTGCAAAGTAAT
TTAATCCCACCAGGGACTCTTCAGTGCACTTCAACACCTGGAACATTTGACCTGCAGTTGGATACCCCTGGC
CTTCTAGAACTTCCTCATGCCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGAAGTTTCAGCTTTTCTTGCT
GATGTACATGCTGTTTCTTCAGCCTCAACTCTGTGCTCGACATTCCAAAATGTTCCTTCCTACATGGAACCA
GTAAGCCTAGAAGCTTTCAGTTTTCAAGGGATACAAAATGCTGCTATGTTCAACAATACAAGTCATTCAAA
TGGGAACCTGTCAGTATTTGATGAGGCAACCATGGCATCACTACATGtaaggctcatgacaaagatataactataac
atattggtactactgttacctatttctcattcacttaactattcattcttgtggtaatacaggATAGCAAAGAATTTCTCAGTGGTAG
CATCTCATCTTTTGGTACGGCCGAGCAGTCACAACTAGCTGGTAGTGGTTTGAAGGCTGAACAACAGGtttt
aattttggaactctgccactaaacagcaataactacacgttatttttcattcaatactgtactggtaaattaacttcttttgtttgtattgcaggA
ACAAAATGCGATGTGCAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGATGGCAGTGAGTGAAGCACAAG
GGGCAATGATTCCTTCGAAGgtattctttagcatttgttacatatttagtgtttcgttttattctctttctttggattcatatctaactgag
tttgactgtttgcaaatgttcagATAAGCTCAACGATGCATAACAATAAAAGTGAGTACCCTGTCCCTATCAGCCAT
TCTGCTGATGCGCAGAACAAGGCAAATTCAGCTAATGGAAACAGTGCCAGTGCTAAGCCACGAGCAAGG
GCTCGTCGTGGACAGGCAACTGACCCTCATAGTATTGCTGAACGGgtgagttatcttgtttggtaaattcactctagata
aatgaaatttttttcataacttgggttggttgaattttatctatgtgttcatgccctatcatatatttctaactggaacttgcttaatactgcagCT
TCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCTCCAAGACCTTGTACCAAACTCAAATAAGGtattatt
ttagtgtttccatttatattggtacaaaaaatgcaggggtatttctttttttccctattcaacatgcagtacatttatattagtccaccgaaatatc
ttttattacttccatataacgaaatcagaagagaatgacatatggtaataacgaatcatatatgtggatacatatgtgctgaagtattgtttta
aaattggcccagcaataactgaagcaagtagttgtttggattttatgacttgagaagtgcaaataagatttgtcccaaatagttaccagttag
gggtagcctaccccaacttgcttgggacaaaatgccttgctgttgttgtagttatcagttaggggtaacctaagccatcacaagtcatagtttc
agttccttttatgatctgcttaatgttttaggcatgtttgttttgtagccacacttcggcaaactagagtttgacaagttagagcttttctcgccac
tgtttggttagtgcctagcgccatacttgcagggcatcacacttctctagtacttatgcatcatgcttgacgtgtcgtatgcacaagccaaattt
gcatttgttgtggcgggcaggcaccggattcgggcacaacaaaatgtgtgccacctatactgttatccatgcctggtttagaacattgcaaag
ttaataaaattaacttttccttaagtttattggtcactctcgaataaaatagtataacaatgaaatttggaattcctaaccaatagtctttttat
gtgagcaggCAGATAAATCATCAATGCTCGATGAAATAATTGATTATGTGAAATTTCTTCAGCTTCAGGTGAA
GGTgcgtccacaaacatgttcatgagtagtaactttccctgttggttgtcattctccttcatttcctttctctgaaacaaatcgccctggtttca
acattacaggtCTTAAGCATGAGTAGGCTAGGAGCTCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAA
CTGAGGtacgaaaacttttctgtttgtataaagtgaatagctccttgttgtgactataggcataaaaaaaattggtagacaatgacgtgttt
tttcgttgttgcatttctacagttggttacactacttttatgaccataatatgcttcgtgattattcaggagtcttagagctccacttgcattgtag
ttagaatttatattagattcttttgtatttttcatggctttaatttggttgaatgtaaccaaatttaatttaccaataccagtactgatgaataaac
acttcattcaagtcaagtccttgctttttctctagaatgcccgtgtactcaatttttattgcactgtcgaactttccacaggGCCGTAGCAA
TTCACCTCTATCATCTCCAACCGCTTCACAAGGGCTTCTGGACGCAGCAGGCCCAGAAGACAGCTTGGTCT
TTGAGCAAGAAGTTATAAAGCTGATGGAAACAAGCATCACAAATGCAATGCAGTACCTTCAGAACAAGGG
CCTCTGCCTGATGCCTATCGCTCTTGCTTCAGCCATATCCAACCAGAAAGGCACTTCTGCAGCTGCAATCCC
TCCAGAAAGGTGAaaacagaaagcacatcatgctcctcccatcggcccgcggaaacaactgtc

Fig. 13 *TaPtf1* aligned regions on 7DL (long arm of chromosome 7 on the wheat sub-genome D). Gene ID: TRIAE_CS42_7DS_TGACv1_624291_AA2060360**.** Uppercase under-lined: exons. Lowercase: introns. The start codon and stop codon of *TaPtf1* are highlighted in yellow.

3.3.2 Identification and sequence analysis of TaMyb67

3.3.2.1 BLAST search of OsPhr2 homologues in wheat

To find wheat homologues of OsPhr2 (GenBank accession No. BAF21429.1), a direct BLASTP search of the wheat protein database using OsPhr2 as a query was conducted as described in the method section 3.2.1. TaMyb67 (GenBank accession No. AEV91190.1) was identified as the wheat homologue of OsPhr2 that had the greatest similarity [database accessed on 08/2012; *TaPhr1* as described by Wang *et al*. (2013b) was published later]. TaMyb67 was indicated to be a protein of 441aa encoded by a CDS of 1326bp (complete cds, GenBank accession No. JF951950).

3.3.2.2 Comparison of TaMyb67 cross-species homologues

Sequence data of TaMyb67 cross-species homologues was generated from a BLASTP search by using TaMyb67 as a query as described in method section 3.2.1. As expected, OsPhr2 and its close homologue OsPhr1 were both pulled out in the result of the BLAST. In addition, homologous sequences with the top similarity from other model plants/closely-related crops, including *Arabidopsis thaliana*, *Zea mays* (maize) and *Hordeum vulgare* (barley), were also chosen and aligned together (multiple sequence alignment analysis) or separately in pairs (pairwise alignment analysis) to assess the relationship in a group or between individuals.

The output of multiple sequence alignment analysis suggested that these sequences contained highly conserved MYB-LIKE DNA-binding domains (MYB_SHAQKYF and MYB_CC_LHEQLE), which are commonly characterised by MYB proteins (Fig. 14). With the exception of AtPHR1, which appeared to be very different, other crop species had good coverage of consensus in their aligned sequences. TaMyb67, ZmPhr and HvPhr seemed to have higher similarities to each other than to rice; although HvPhr from barley displayed low identical scores, presumably due to its shorter and incomplete sequence (307aa) when compared to other

sequences (over 400aa) (Table 9).

An identity matrix (Table 10) was generated by pairwise alignment analysis for a more detailed description of similarity. The matrix shows that almost all identity scores (%) were relatively high, implying that all the proteins have similar 3D folds (Sander and Schneider, 1991). The lowest identity score of 30.6% was between barley and *Arabidopsis*, while the highest was between wheat and barley (64.3%). Moreover, the identity score between ZmPhr/HvPhr and TaMyb67 (63.2%/64.3%) was found to be relatively higher than that between OsPhr1/OsPhr2 and TaMyb67 (60.1%/40.1%).

Table 9. TaMyb67 homologous protein sequences from different species

NCBI entry	Name and plant species	Accession	Protein length
TaMyb67	MYB-related protein [*Triticum aestivum*]	AEV91190.1	441aa
AtPHR1	phosphate starvation response 1 protein [*Arabidopsis thaliana*]	AEE85512.1/NP_194590.2	409aa
OsPhr1	Os03g0329900 [*Oryza sativa* Japonica Group]	BAF11920.1/NP_001050006.1	428aa
OsPhr2	Os07g0438800 [*Oryza sativa* Japonica Group]	BAF21429.1/NP_001059515.2	426aa
ZmPhr	phosphate starvation protein [*Zea mays*]	AEH96380.1	449aa
HvPhr	phosphate starvation regulator protein-like protein [*Hordeum vulgare*]	ACT34981.1	307aa

Table 10. Identity matrix for pairwise alignments between TaMyb67 homologous protein sequences

Identity matrix						
	AtPHR1	OsPhr1	OSPhr2	TaMyb67	ZmPhr	HvPhr
AtPHR1	100.00%	-	-	-	-	-
OsPhr1	38.10%	100.00%	-	-	-	-
OsPhr2	37.30%	41.80%	100.00%	-	-	-
TaMyb67	38.10%	60.10%	40.10%	100.00%	-	-
ZmPhr	38.80%	61.30%	41.10%	63.20%	100.00%	-
HvPhr	30.60%	48.70%	35.80%	64.30%	46.90%	100.00%

* Identity scores were produced from the EBI online analysis tool-EMBOSS Needle

Fig. 14 Multiple sequence alignment analysis of TaMyb67 homologous protein sequences. The result was produced from the EBI online analysis tool-Clustal Omega. MYB functional regions are highlighted in yellow. The two different MYB-like DNA-binding domains, MYB-like SHAQKY and MYB_CC_LHEQLE, are shown in red and blue frames.

3.3.2.3 Genome location and sequence structure of *TaMyb67*

The genome location and genome sequence structure of *TaMyb67* were further analysed by wheat genome BLAST and pairwise alignment, as described in method section 3.2.1. Based on the results of genome BLAST using the full-length *TaMyb67* CDS (GenBank accession No. JF951950) as a query, *TaMyb67* was mapped to three homoeoloci on 4AS (Location: Scaffold TGACv1_ scaffold_308022_4AS: 10713–18566), 4BL (Location: Scaffold TGACv1_scaffold_321722_4BL: 33591–42579) and 4DL (Location: Scaffold TGACv1_scaffold_343590_4DL: 33506–41010) (L/S=long/short arm; 4=chromosome 4; A/B/D=sub-genome). Splice variants predicted by the Ensembl database for the three homoeoloci are shown in Table 11. Among these, 12 predicted splice variants were found from 4BL, which indicates this homoeolocus may be highly spliced.

A conserved gene structure of *TaMyb67* on all sub-genomes, containing seven exons and six introns as the lengths and structure shown in Fig. 15, was suggested by the pairwise alignments between the query *TaMyb67* and its three genome sequences. The most significant homology resulted from the genome sequence on 4BL, implying genome B might be the origin for the query *TaMyb67*. Aligned regions on each genome sequence (1323bp) were equivalent to the coding region of the query *TaMyb67* CDS (Fig. 16A).

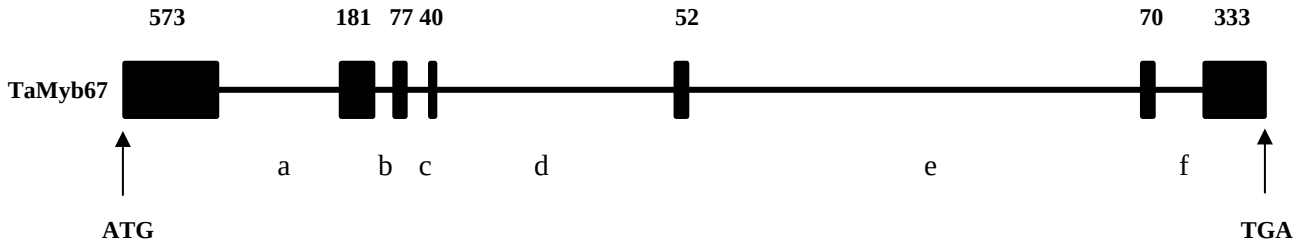


Fig. 15 *TaMyb67* genome sequence structure. Black box: exon (translated). Numbers above the black box indicate the length of the exon in bp. String: intron. Letters indicate the lengths of introns in bp (a: 693; b: 87; c: 94; d: 1299; e: 2517; f: 276). The arrow ATG indicates the start codon. The arrow TGA indicates the stop codon.

Gene ID and location on the physical map (Ensembl wheat genome):

Genome A: Gene ID: TRIAE_CS42_4AS_TGACv1_308022_AA1025320;
Genome location: scaffold_308022_4AS: 10713–18566.
Genome B: Gene ID: TRIAE_CS42_4BL_TGACv1_321722_AA1064640;
Genome location: scaffold_321722_4BL: 33591–42579.
Genome D: Gene ID: TRIAE_CS42_4DL_TGACv1_343590_AA1137010;
Genome location: scaffold_343590_4DL: 33506–41010.

Table 11. Splice variants from *TaMyb67* homoeoloci

Genome	Number of splice variant	Transcript ID	Transcript length (bp)	Protein (aa)	Biotype (Y/N)
4AS	6	4AS_TGACv1_308022_AA1025320.1	3461	451[(a)]	Y
		4AS_TGACv1_308022_AA1025320.2	2201	451[(a)]	Y
		4AS_TGACv1_308022_AA1025320.3	1924	490[(c)]	Y
		4AS_TGACv1_308022_AA1025320.4	2084	451[(a)]	Y
		4AS_TGACv1_308022_AA1025320.5	2019	451[(a)]	Y
		4AS_TGACv1_308022_AA1025320.6	3369	470[(b)]	Y
4BL	12	4BL_TGACv1_321722_AA1064640.1	2698	437[(e)]	Y
		4BL_TGACv1_321722_AA1064640.2	2591	447[(g)]	Y
		4BL_TGACv1_321722_AA1064640.3	2590	447[(g)]	Y
		4BL_TGACv1_321722_AA1064640.4	2579	447[(g)]	Y
		4BL_TGACv1_321722_AA1064640.5	2715	439[(f)]	Y
		4BL_TGACv1_321722_AA1064640.6	2566	439[(f)]	Y
		4BL_TGACv1_321722_AA1064640.7	2740	447[(g)]	Y
		4BL_TGACv1_321722_AA1064640.8	2704	439[(f)]	Y
		4BL_TGACv1_321722_AA1064640.9	2728	447[(g)]	Y
		4BL_TGACv1_321722_AA1064640.10	3995	439[(f)]	Y
		4BL_TGACv1_321722_AA1064640.11	5318	319[(d)]	Y
		4BL_TGACv1_321722_AA1064640.12	2606	447[(g)]	Y
4DL	6	4DL_TGACv1_343590_AA1137010.1	2138	450[(i)]	Y
		4DL_TGACv1_343590_AA1137010.2	1991	451[(j)]	Y
		4DL_TGACv1_343590_AA1137010.3	1980	490[(h)]	Y
		4DL_TGACv1_343590_AA1137010.4	2129	451[(j)]	Y
		4DL_TGACv1_343590_AA1137010.5	2141	451[(j)]	Y
		4DL_TGACv1_343590_AA1137010.6	3437	451[(j)]	Y

* bp: base pair. aa: amino acid. Y: Protein coding. N: Non-protein coding.
* Letters represent Ensembl accessions of proteins. (a): W5DXY7; (b): A0A1D5XDW3; (c): A0A1D5XDW0; (d): A0A1D6RXS1; (e): A0A1D6RXR9; (f): A0A1D6RXS9; (g): A0A1D6RXS8; (h): A0A1D5Y0S2; (i): A0A1D5Y0S0; (j): A0A1D5Y0S3

A

>*TaMyb67*_ TRIAE_CS42 _4BL_TGACv1_321722_AA1064640

atatcaaaatcaatttatgggaaagacgcaacgcttgtccacaattcttgATGAGGAGGTGTGATCTGAGACAGTCTCACAA
CAGCAGGGTTTCTGGAGGAATGTCATCCTCTTTACCTATTCTGCCAAATTCTCTGAAAGAAACCTTCCATGG
GCCTTACAATCCGCAGCTCACTCCGATGCAAAGGCAACTGACGAGTGATCTTGTGCCCTTACATCAGAGTG
CACTTCCGTCTGCTACTTTGCACCCAAGAGCTGGGGCTATGAGATCATCATATGCAGCCTCATTAGGATAC
TCACCTAATCCTCTTGATTCTGCGCTTAACCATGAGAGGCAGTCTATGGTTGCTCCTTTTGCTCCTCAGCCAT
CAAATATTGAAGTATTTCAGACCTTATCTAATAATATCCCTGGAGGACACACTGAGGCAACTTGGTTCCCA
GGTTCGGCTGATGGTTTATCAGATTACAGGGATAACATCCCTGCTTCTGGTAGTCAGATCCAGAATGGCG
GTCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATGAATGGTGGGCAGAGATAATGAATGATGATTG
GAGAGATATTCTAGATGCAACGGCTGCTGATCCCCAGTCAAAGgtatgcaaataaaacagcataaagagtcattgtag
tagctctgtcttgggttctgatagtttagaagtaatctgtaatttatttgttaatttcacgtaagttctttgccagtgcacatactggtattatgat
atcatttttgtgtggccaacctagataccttaatgtatgtcaattttaaagttaagaggaagttttgacttactgtatacttctcgggccatactt
ccatttacaaattgggccatacttcttgggcctctttaaacagtgtaggtaactatcccttcctcttattagaagtttaaaactgatgtgtagta
ggttttgttagagtgatggttagggcaccagtaaatgatttcttgtttgcattgtttaaagagggtcgttattagattgactaccagagcttaca
ttagaatcttgtaatgaatgttcatgacttgatatgagtgtctctctccgcaaattcttctaagggatatatgctgcttcttctctaattctcgttg
gcacaaaatagaaatatgcggtccacttaattttaaggtggaggtctcttaacttgttggagttgtagtttctagagtgatatttgtttctcact
gtgatgcttttggtaagcccattcgatgtgtttcttttatgaccaaatgtttgttgtcacctttgttgtaatccatggttcagCCTTCCAATTC
GTCTGCATCACAGCCTGCTGTCAACCAGCCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAGCTAGTC
CTCCCAATAGCAACTCTGCAGCCAAACAACGGATGAGGTGGACTCCAGAACTCCATGAATGCTTCGTAGA
CTCTGTAAATAAGCTTGGTGGTAGTGAAAgtatgtctgcagtgccattgattcccaaaagtgcattttagatatgaatcatgttc
taatgcttgtaatttttgtcttgtccttcagAAGCTACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTTGACGGTTTGA
CAATATATCATGTGAAAAGCCATCTGCAGgtttgccattagctttctaaattctagttctaaacaatagtgttgcccacaagaga
tttgagttctcacttacctttttctggtttgtgttgcagAAGTACCGAACAGCTCGCTATAAGCCAGACCTAACGGAAGGTtt
gtctggattagatttttttgcatgtattttgtatagatcttccctgcacaggtgcatagttgatattattattcatgacccatgatgcgatgctatt
gagttgagtacatttttatgataatggttagggctgatgctttttttatggacgccgataagtgccacacgtgtgggcgttagggagactgtcc
acacattttgtgtggtgtataagaggaacagcccacacacctacgtgtgggcaaaacaagtaatgcccacacacctctttttttcctcccgat
ccctctcacacgcgtgcgtgtgggcgaagtagataacgcccacacgccccgcatgtcaggcctcgtacctcgtggtcccgcacgccacgcgt
gacagtcaccgcgcgcccgcagttgccatggtccagaccctcgtccatgttcgtttaactgcagttgccatgtcgctgaactacggttgccat
gtcggacaactgcagttgccatggttgctcaactgcagttgccatgtatggtctggtctactgtaagttgtcatgatttcaaaactttaggagtt
gccacatactaacactaggcagttgagagagttgccatgtgctcacaagcatgctagggcgttgccatgtaaaaaggaagagttgccatct
gcttacgtgcacgttaaggcagttgccatgtaccatgcaaaaacacatggcaactcggtaaagagagagttgccatctgcttacatgcaca
ctaggcagttgccgcgtaccctgcaaaacagatggcaactcggataaaaagagagttgtcaccttcttataaagcacactagggcagttgc
catgtgcgctgcaaaacatatggcaactagcagcttgggtgtgggagaggagacgggcgtgtgggcgagatggcaaatgcccacacacta
gcccttgtgcgtgcatggaaagtggcgtgtgggcgaactgctgaacgcccacacaccagcccctcccgtgtggtgaaacggccgtgtgggc
gaccgggtgaacgcccacacaccaggccagtcctacgtggtactgaaaacatgccaagattcgtgcgctcacgaacggacgcggatccac
gcgtgtgggcgagatgcaaacgcccacacgtgtgggcgttaacatttccgtttttttatatatatgtaaatcttctcaggacgcgatattattttg
gtaaggttccattattgcatagaattcatgcatctacattgtgtttggtataattggtaacataacataaccattccataccaaattatcaggt
ACAGCAGAAAAAAGGACTACCACCGAAGAGTTGACTCTAGACCTGAAATCGtgagttggataatttttttacttattat
ctattattctgttcaattgtactctagcatgatacccatctctcatgatgcttattggtagctggttactgtgcaatgtgacatccatcaactatt
aaggctaaaaccaatacttaagttgtagttaagatggtggtttagtttgtaaaactgaattccaactaatgctcagtaattacattgagaaaa
ctgatgctgatataagtttgtgattcaactgcattttaatggggattagaaaagatagcttcaatgtaggcaattgcccaaaatggatttttctt
ccatatatacatcctggtattcaccttctgatcaaaatcataaataaaaagataattaacatgggatataacccaggaaaactttgaaggaa
cttacatgcccaccattaaatgtcatgtggtgggccttggaggaaacacaaagccccaacaaagtacattaccctcatcaaggacatgtac
gataatgttgtgacaagtgttcgaacaagtgatggcgacactgatgacttcctgattaataggattgcagcaagggtcagctttgagccctta
tctttttgctttggtgacggatgaggtcacaaggggtatacaaggagatatcccatggtgtatgctctttgctgatgatgtggtgctagtcaa

tgatagttggatgggggttaatagaaagttagagctatggagtcaaactttggaatcgaaaggttttagtcttagtagaactaaaactgagt
acatgaggtgcggtttcagtactgctaagcactaggaggaggttagccttgatgtgcaggtggtacctcagaaggacacctttcgatatttg
gggtcaatgcaatgctgcaaaaaggatggtgatatcaatgaagatgtgagccatcgaatcaaagccggatggatgaagtggcgccaagct
tcaggcgttctctatgacaagagagtaccacaaaagctaaaaggcaggttctgtaggacggcgattcgactcgcaatgttgtatggtgctgg
gtgttggcctactaaaaggcgacatgtgcaacagttaggtgtagcggagatgcgcatgttgaaatggatgtgtgatcacccaagaaaggat
tgggtctggaatgatgatatacaatgatgatatacaggagagagttggggtagcaccgattgaagagaagcttgtccaacatcgtttgaga
tggtttgggcatatacagcagtggcctccagaagctccagtgcatagtggatggttgaagcgtgccgataatgttaagagaggtcggggta
gaccaaactggacatgggaagagtccataaagagagatctgaaggactggtatgtcacgaaatgactagccatggacaggggtgcgtgg
aaccatgagttagttttgttgttgtcgtcgtcgttgtcgttgtcattcatcgcatagttggcacgttcagttatgatatcaagacagctctattaa
acttggtcctgtcaaatcaatttagtggcttggaacttttctcaattatgtgagattttggaagtgcaagtagtagtagaacctaatattatttt
gcttcccatgaagctgttttgcctcttaatgagaaggggaatgatttatgttttgacaatacaggattctactacttgcagttttaacatctgac
atatttcaaaaaatgttttattagcccttcagcctcttcagaagcgaaaccagtaattttattttgctgcactaagtttcatgtagctagattga
ttttggaattctgtgcaccagttatgagcagattgatattcttataggtgaattagcataaatatcagatggtgggatgcaaattcctttgtttt
tcttgtgttacctttggtcttgatgttttcagtgtctcttaacaagaggttgggtacatttcgcaaacacttagctgactggaaagatataacgc
gttttccggagcttatgtctaggttactagcactgtcaagattcctggttactcaaccaacttcctcaggtgttattacttgaggaagatgctat
tcttttgatagttatgttgacgtgtgtctatgcacacacattttattggcactggagcgttacattttctgtctgttctaaatccatgctgtacaga
cctagcaagcactgcgaattgccttgcagttaatccatttgttttgaaacagtcgttatttgtagctatgatataaacaatatgctcacagttat
gttctttgatgtcgagctcacaattaagaagtgttgttgtttgtgctcccaagtcttggtcatgctgtaaatgtggatctctcaaatcctccctct
gttatgagagtttgatgagtcctgttgaacccaatttatattcacttcctgttgggccaggaaaaactatttttgaggattcattgtgagacagt
gcattctcttgctttgtcagccatgattaacactatacatgttatttttcttatacaggAGCATGGATCTTACTGAAGCGCTGCGTC
TTCAGATGGAAGTTCAGAAACGTCTTCATGAACAACTTGAGgtactagtccaaaagaacatagactggttcttaatttag
gtgatgtctaggtgaccgaataaaaaaagttgcatgcgaaacataatagacttcaaatgtgaggaaacattgttctttttgcacacctataa
atcaaattttgactgcagtgatactctacaaaagaaacatccacgtaattgcattggtctttacatgatataggcccggctcagtcagactat
taccatgatgaaacacacatagtctgaacttgattccatgtttctatgacagACCCAGAGAAAGTTGCAATTGCGAATTGAAG
AACAAGGGAAGTATCTTCAGATGATGTTTGAAAAGCAGTCTAAATCCAATACGGAGAAGGTGCAGGATCT
ATCCTCGGGAGCTACAACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGAAATAGGGGTGATGAT
GCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGTGAGTGCCGAAATAGGAGAAACTTTGATGCAT
GCAGGTGGCAACCAGGAGATGGCAGAAAGCGAGTCTTCTGAGCCCCTTGCAAATACTAATGATGGTTCCA
AGGCCCCGCAAGAGAAGCGCCGAagggtgcatgatagttaaccatcagtttggtaattcagatgatagcagtt

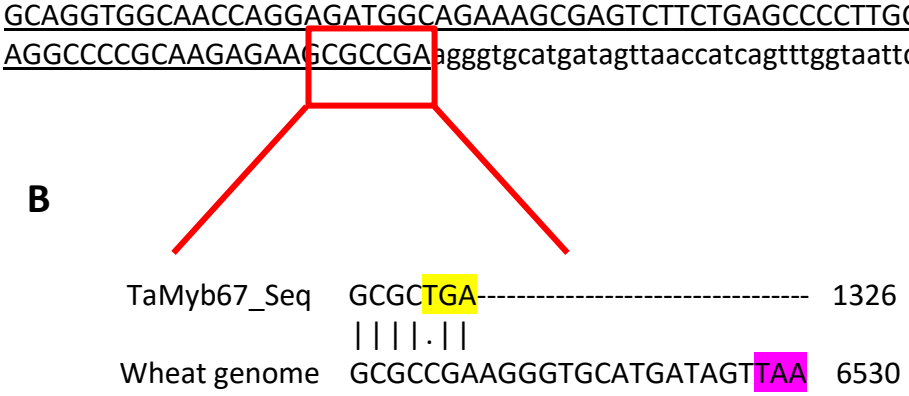


Fig. 16 *TaMyb67* aligned regions on 4BL (long arm of chromosome 4 on the wheat sub-genome B). (A) *TaMyb67* aligned regions. Gene ID: TaMyb67_TRIAE_CS42_4BL _TGACv1_321722 _AA1064640. Uppercase underlined: exons. Lowercase: introns. (B) The region of the stop codon on the alignment between the CDS of *TaMyb67* and the genome sequence on 4BL. The stop codon region is shown in the red frame. The start codon and stop codon of *TaMyb67* are highlighted in yellow. The stop codon of the genome sequence on 4BL is highlighted in pink.

3.3.2.4 Comparison of proteins encoded by *TaMyb67* and other splice variants

Based on the results from the previous step, *TaMyb67* CDS was aligned to the three homoeoloci on A, B and D sub-genomes, respectively. These gene loci were annotated as described by Wang *et al*. (2013b), in which three *TaPhr1* genes (deduced protein: 451aa) were reported. They studied the function of *TaPhr1-A1* and showed that it had a positive regulatory role in PUE. Multiple protein sequence alignment, as described in method section 3.2.1, was used to investigate the difference between TaMyb67 (GenBank accession No. JF951950), TaPhr1s as described by Wang *et al*. (2013b)(GenBank accession No. TaPhr1-A1: KC218925; TaPhr1-B1: KC286910; TaPhr1-D1: KC286911) and all proteins encoded by the three homoeoloci of *TaMyb67* found in the Ensembl wheat genome in this study (Table 11; identical proteins used only once). From the alignment (Fig. 17), it was likely that the two 451aa proteins, W5DXY7 from 4AS and A0A1D5Y0S3 from 4DS, were equivalent to the TaPhr1-A1 and TaPhr1-D1 (451aa) described by Wang *et al*. (2013b). No protein from 4BL was found to be 451aa, whereas a shorter protein with only 447aa (A0A1D6RXS8) seemed to be the equivalent of TaPhr1-B1. Moreover, alterations of amino acids between the two 451aa sequences and the 447aa sequence were also observed.

Interestingly, the TaMyb67 (441aa) used in this study was not identical to the TaPhr1 sequences reported by Wang *et al*. (2013b) or those from the Ensembl wheat genome. It lacked one oligopeptide of 4aa (“SMVQ”) and one oligopeptide of 6aa (“RRVHDS”) in the middle and at the C-terminus of the sequence, respectively, when compared to the 451aa protein. When compared to the 447aa protein, it only lacked 6aa at the C-terminus of the sequence (Fig. 17). The reason for this discrepancy in size is not clear, but it is possible that the wheat cDNA used for *TaMyb67* found in this study may represent another allele of *TaPhr1*.

This hypothesis is further supported by the fact that both missing peptides of “SMVQ” and “RRVHDS” were not located in the known functional regions, as inferred from a wide range of protein modelling in PHYRE2.0 protein structure analysis (Fig. 18), described in method section 3.2.1, using a query of TaPhr1-D1 (A0A1D5Y0S3, 451aa). In addition, there was no agreement between the stop codon (TGA) on *TaMyb67* CDS and the genome, where the normal amino acid codon was present (Fig. 16B). *TaMyb67* CDS incorporates the stop codon of “TGA” at 1324–1326, whereas the genome sequence at this position has “CGA” (incorporates a non-stop codon of “CGA”) and the open reading frame continues with 15 additional nucleotides before encountering a stop codon “TAA” later.

(** e.g. 4AS-451-W5DXY7:4AS-name of chromosome; 451-number of amino acids; W5DXY7-Ensembl accession)

```
TaMyb67                 ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
TaPhr1-A1               ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
TaPhr1-B1               ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
TaPhr1-D1               ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
4AS-451-W5DXY7          ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
4AS-470-A0A1D5XDW3      ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
4AS-490-A0A1D5XDW0      MVRWGCAVRLPPATGGRRPPLEHIKINLWERHNACAQFLMRRCDLRQSHNSRVSGGMSSS
4DS-451-A0A1D5Y0S3      ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
4DS-450-A0A1D5Y0S0      ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
4DS-490-A0A1D5Y0S2      MVRWGCAVRLPPATGGRRPPLEHIKINLWERRNACPQFLMRRCDLRQSHNSRVSGGMSSS
4BL-319-A0A1D6RXS1      ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
4BL-437-A0A1D6RXR9      ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
4BL-439-A0A1D6RXS9      ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
4BL-447-A0A1D6RXS8      ----------------------------------------MRRCDLRQSHNSRVSGGMSSS
                                                                ********************

TaMyb67                 LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
TaPhr1-A1               LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
TaPhr1-B1               LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
TaPhr1-D1               LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
4AS-451-W5DXY7          LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
4AS-470-A0A1D5XDW3      LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
4AS-490-A0A1D5XDW0      LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
4DS-451-A0A1D5Y0S3      LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
4DS-450-A0A1D5Y0S0      LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
4DS-490-A0A1D5Y0S2      LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
4BL-319-A0A1D6RXS1      LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
4BL-437-A0A1D6RXR9      LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
4BL-439-A0A1D6RXS9      LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
4BL-447-A0A1D6RXS8      LPILPNSLKETFHGPYNPQLTPMQRQLTSDLVPLHQSALPSATLHPRAGAMRSSYAASLG
                        ************************************************************

TaMyb67                 YSPNPLDSALNHERQSMVAPFAPQPSNIEVFQTLSNNIPGGHTEATWFPGSADGLSDYRD
TaPhr1-A1               YSPNPLDSAPNHERQSIVAPFAPQPSDIEVFQTLSNNIPGGHTGATWFPGSADGLSDYRD
TaPhr1-B1               YSPNPLDSALNHERQSMVAPFAPQPSNIEVFQTLSNNIPGGHTEATWFPGSADGLSDYRD
TaPhr1-D1               YSPNPLDSAPNHERQSMVAPFAPQPSDIEVFQTLSNNIPGGHTGATWFPGSADGLSDYRD
4AS-451-W5DXY7          YSPNPLDSAPNHERQSMVAPFAPQPSDIEVFQTLSNNIPGGHTGATWFPGSADGLSDYRD
4AS-470-A0A1D5XDW3      YSPNPLDSAPNHERQSMVAPFAPQPSDIEVFQTLSNNIPGGHTGATWFPGSADGLSDYRD
4AS-490-A0A1D5XDW0      YSPNPLDSAPNHERQSMVAPFAPQPSDIEVFQTLSNNIPGGHTGATWFPGSADGLSDYRD
4DS-451-A0A1D5Y0S3      YSPNPLDSAPNHERQSMVAPFAPQPSDIEVFQTLSNNIPGGHTGATWFPGSADGLSDYGD
4DS-450-A0A1D5Y0S0      YSPNPLDSAPNHERQSMVAPFAPQPSDIEVFQTLSNNIPGGHTGATWFPGSADGLSDYGD
4DS-490-A0A1D5Y0S2      YSPNPLDSAPNHERQSMVAPFAPQPSDIEVFQTLSNNIPGGHTGATWFPGSADGLSDYGD
4BL-319-A0A1D6RXS1      YSPNPLDSALNHERQSMVAPFAPQPSNIEVFQTLSNNIPGGHTEATWFPGSADGLSDYRD
4BL-437-A0A1D6RXR9      YSPNPLDSALNHERQSMVAPFAPQPSNIEVFQTLSNNIPGGHTEATWFPGSADGLSDYRD
4BL-439-A0A1D6RXS9      YSPNPLDSALNHERQSMVAPFAPQPSNIEVFQTLSNNIPGGHTEATWFPGSADGLSDYRD
4BL-447-A0A1D6RXS8      YSPNPLDSALNHERQSMVAPFAPQPSNIEVFQTLSNNIPGGHTEATWFPGSADGLSDYRD
                        ********* ******:*********:**************** ************** *

TaMyb67                 NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSK----PSNSSA
TaPhr1-A1               NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSKSMVQPSNSAA
TaPhr1-B1               NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSKSMVQPSNSSA
TaPhr1-D1               NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSKSMVQPSNSAA
4AS-451-W5DXY7          NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSKSMVQPSNSAA
4AS-470-A0A1D5XDW3      NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSKSMVQPSNSAA
4AS-490-A0A1D5XDW0      NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSKSMVQPSNSAA
4DS-451-A0A1D5Y0S3      NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSKSMVQPSNSAA
4DS-450-A0A1D5Y0S0      NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSKSMVQPSNSAA
4DS-490-A0A1D5Y0S2      NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSKSMVQPSNSAA
4BL-319-A0A1D6RXS1      NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSK----PSNSSA
4BL-437-A0A1D6RXR9      NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQS----------
4BL-439-A0A1D6RXS9      NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQS----------
4BL-447-A0A1D6RXS8      NIPASGSQIQNGGPAVTSDVVAKQNEWWAEIMNDDWRDILDATAADPQSK----PSNSSA
                        ************************************************
```

SMVQ

```
TaMyb67             SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
TaPhr1-A1           SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
TaPhr1-B1           SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
TaPhr1-D1           SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMMWTPELHECFVDSVNKLGGSEKATPKG
4AS-451-W5DXY7      SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
4AS-470-A0A1D5XDW3  SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
4AS-490-A0A1D5XDW0  SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
4DS-451-A0A1D5Y0S3  SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
4DS-450-A0A1D5Y0S0  SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSE-TTPKG
4DS-490-A0A1D5Y0S2  SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
4BL-319-A0A1D6RXS1  SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
4BL-437-A0A1D6RXR9  -KPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
4BL-439-A0A1D6RXS9  -KPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
4BL-447-A0A1D6RXS8  SQPAVNQPASSHGGEICNVASPPNSNSAAKQRMRWTPELHECFVDSVNKLGGSEKATPKG
                     :****************************** ****************** :****

TaMyb67             VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTEG--------------------TAEKRTT
TaPhr1-A1           VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTEG--------------------TAEKRTT
TaPhr1-B1           VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTEG--------------------TAEKRTT
TaPhr1-D1           VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTEG--------------------TAEKRTT
4AS-451-W5DXY7      VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTEG--------------------TAEKRTT
4AS-470-A0A1D5XDW3  VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTEGLSGLDFFALLFYIDLPCTGTAEKRTT
4AS-490-A0A1D5XDW0  VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTEG--------------------TAEKRTT
4DS-451-A0A1D5Y0S3  VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLMEG--------------------TAEKRTT
4DS-450-A0A1D5Y0S0  VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLMEG--------------------TAEKRTT
4DS-490-A0A1D5Y0S2  VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLMEG--------------------TAEKRTT
4BL-319-A0A1D6RXS1  VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTEGLSGLDFFACILYRSSLHRCIVDIII-
4BL-437-A0A1D6RXR9  VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTE----------------------AEKRTT
4BL-439-A0A1D6RXS9  VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTEG--------------------TAEKRTT
4BL-447-A0A1D6RXS8  VLKLMKVDGLTIYHVKSHLQKYRTARYKPDLTEG--------------------TAEKRTT
                    ****************************** *                    .:

TaMyb67             TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
TaPhr1-A1           TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
TaPhr1-B1           TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
TaPhr1-D1           TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
4AS-451-W5DXY7      TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
4AS-470-A0A1D5XDW3  TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
4AS-490-A0A1D5XDW0  TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
4DS-451-A0A1D5Y0S3  TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
4DS-450-A0A1D5Y0S0  TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
4DS-490-A0A1D5Y0S2  TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
4BL-319-A0A1D6RXS1  -----HDP-----------------------------------------------------
4BL-437-A0A1D6RXR9  TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
4BL-439-A0A1D6RXS9  TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
4BL-447-A0A1D6RXS8  TEELTLDLKSSMDLTEALRLQMEVQKRLHEQLETQRKLQLRIEEQGKYLQMMFEKQSKSN
                          *

TaMyb67             TEKVQDLSSGATTTLSSEPSHPATRNRGDDAADDLNRTGENPVSAEIGETLMHAGGNQEM
TaPhr1-A1           TEKVQDLSSGATTTLSSEPSHPATRNRGDDAADDLNRTGENPVSAEIGETLMHAGGNQEM
TaPhr1-B1           TEKVQDLSSGATTTLSSEPSHPATRNRGDDAADDLNRTGENPVSAEIGETLMHAGGNQEM
TaPhr1-D1           TEKVQDLSSGATTTLSSEPSHPATRNRSNDAADDLNRTGENPVSAEIGETLMHAGGNQEM
4AS-451-W5DXY7      TEKVQDLSSGATTTLLSEPSHPATRDRDNDAADDLNRTGENPVSAEIGETSMHAGGNQEM
4AS-470-A0A1D5XDW3  TEKVQDLSSGATTTLLSEPSHPATRDRDNDAADDLNRTGENPVSAEIGETSMHAGGNQEM
4AS-490-A0A1D5XDW0  TEKVQDLSSGATTTLLSEPSHPATRDRDNDAADDLNRTGENPVSAEIGETSMHAGGNQEM
4DS-451-A0A1D5Y0S3  TEKVQDLSSGATTTLSSEPSHPATRNRDNDAADDVNRTGENPVSAEIGETLMHAGGNQEM
4DS-450-A0A1D5Y0S0  TEKVQDLSSGATTTLSSEPSHPATRNRDNDAADDVNRTGENPVSAEIGETLMHAGGNQEM
4DS-490-A0A1D5Y0S2  TEKVQDLSSGATTTLSSEPSHPATRNRDNDAADDVNRTGENPVSAEIGETLMHAGGNQEM
4BL-319-A0A1D6RXS1  ------------------------------------------------------------
4BL-437-A0A1D6RXR9  TEKVQDLSSGATTTLSSEPSHPATRNRGDDAADDLNRTGENPVSAEIGETLMHAGGNQEM
4BL-439-A0A1D6RXS9  TEKVQDLSSGATTTLSSEPSHPATRNRGDDAADDLNRTGENPVSAEIGETLMHAGGNQEM
4BL-447-A0A1D6RXS8  TEKVQDLSSGATTTLSSEPSHPATRNRGDDAADDLNRTGENPVSAEIGETLMHAGGNQEM
```

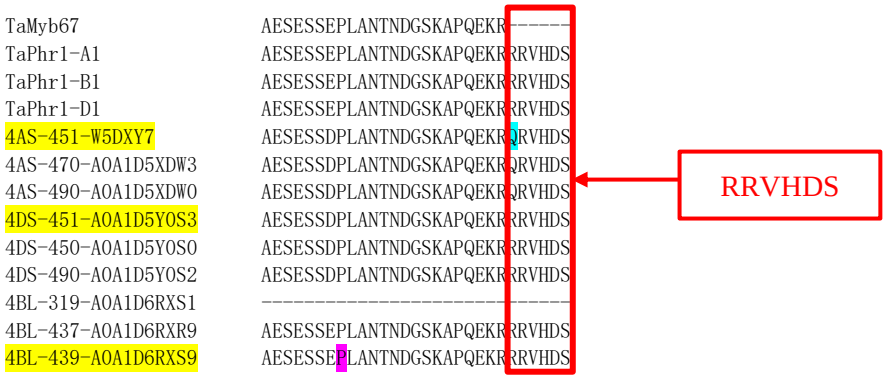


Fig. 17 Comparison of TaMyb67, TaPhr1s [deduced protein sequences described by Wang *et al*. (2013b)] and protein sequences encoded by the three homoeoloci of *TaMyb67* on 4AS, 4BL and 4DS of the wheat genome (Ensembl). Differences in sequences in the middle and at the C-terminus between TaMyb67 and other proteins are shown in red frames. TaPhr1-A1, TaPhr1-B1 and TaPhr1-D1 represent the three deduced Phr1 proteins described by Wang *et al*. (2013b). 4AS-451-W5DXY7 highlighted in yellow is the equivalent of TaPhr1-A1 on sub-genome A. 4DS-451-A0A1D5Y0S3 highlighted in yellow is the equivalent of TaPhr1-D1 on sub-genome D. 4BL-447-A0A1D6RXS8 highlighted in yellow is the equivalent of TaPhr1-B1 on sub-genome B. Amino acids highlighted in blue, pink and green indicate the variation between the three protein sequences derived from the wheat A, B and D sub-genomes (Ensembl).

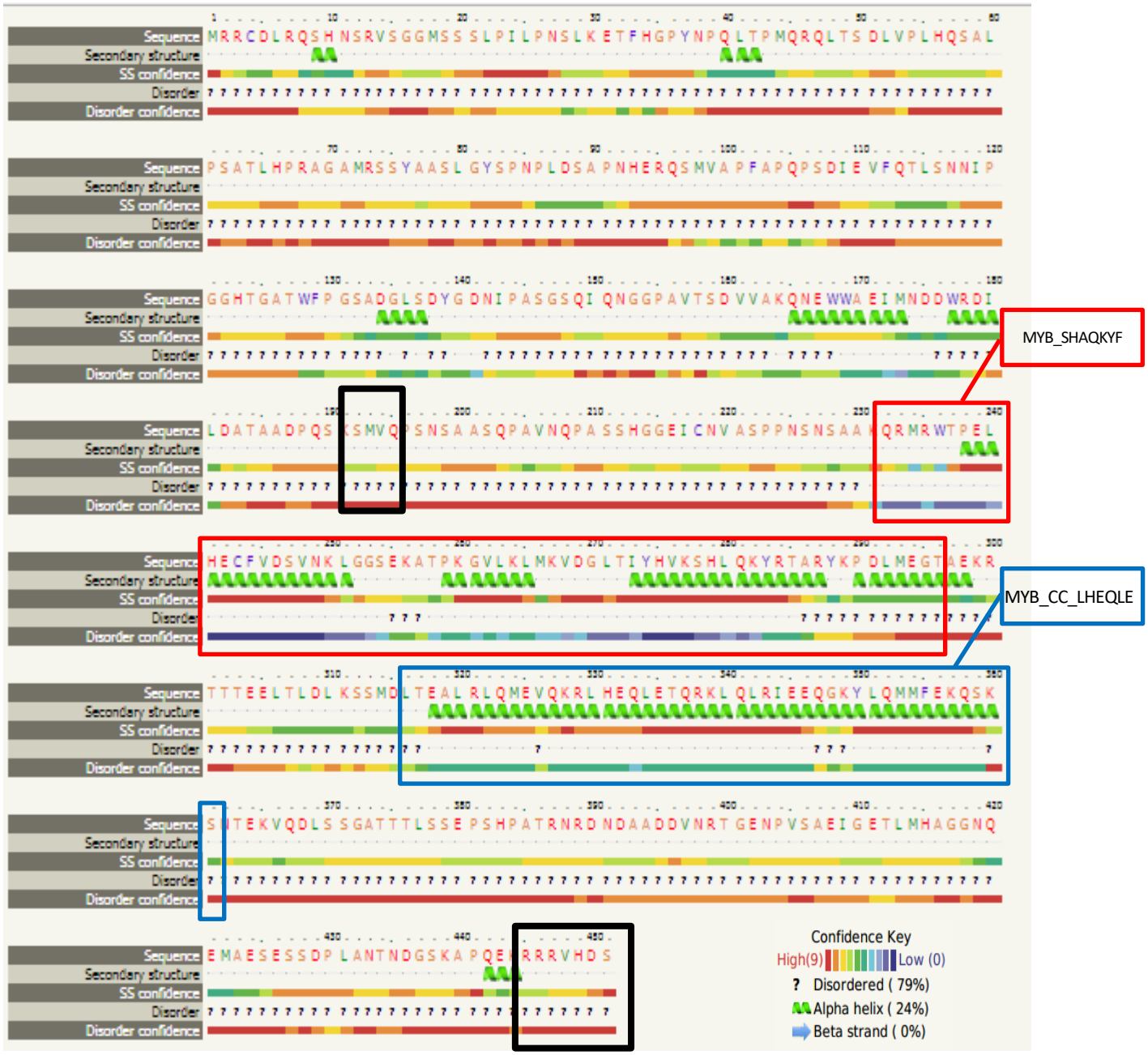


Fig. 18 Prediction of protein secondary structure and disorders produced from PHYRE2.0. The TaPhr1-D1 protein sequence, which is identical to TaPhr1-B1 at the C-terminus, was used as a query to explore the PHYRE2.0 database. Letters in the figure represent amino acids. Black frames show the oligopeptides of SMVQ and RRVHDS on TaPh1-D1 that are different from the TaMyb67 protein sequence. Red and blue frames indicate the MYB-binding functional domains.

3.3.3 PCR amplification of *TaPtf1*/*TaMyb67* CDS

Following analyses of *TaPtf1* and *TaMyb67* using bioinformatics tools as described in previous sections, the two identified wheat targets were obtained by PCR amplification for wheat transformation to investigate their functions. For simplicity and ease of transformation, CDSs of *TaPtf1* and *TaMyb67* were used. The *TaPtf1*/*TaMyb67* CDS was obtained by RT-PCR using a wheat root cDNA library, where these targets might be more abundant as predicted from their orthologues in the model crop rice (Yi *et al*., 2005; Zhou *et al*., 2008a).

3.3.3.1 Isolation of total RNA

Two replicates of total RNA, designated as Root 1 and Root 2 (Fig. 19A), were isolated from wheat roots, as described in method section 3.2.3. They were assessed by agarose gel electrophoresis and spectrophotometry to determine yield and quality. As the gel result in Fig. 19A shows, two clear RNA bands were obtained in each lane, suggesting a satisfactory quality of RNA for cDNA synthesis. The RNA quantification was also satisfactory (Appendix 5).

3.3.3.2 cDNA synthesis and PCR amplification of Tubulin

The total RNA from the previous step was used for cDNA synthesis and the quality of cDNA was assessed by using Tubulin primers (Table 12) in the PCR amplification, as described in method section 3.2.4 and 3.2.5. All four replicate PCR products of Tubulin in the gel result were found to be positive, with the expected size at around 500bp, and of sufficient intensity (Fig. 19B), which suggested successful cDNA synthesis.

3.3.3.3 PCR amplification of *TaPtf1*/*TaMyb67* CDS

The full-length CDS of *TaPtf1*/*TaMyb67* was amplified using the cDNA from the previous step and gene-specific primers (Table 12), as described in method section 3.2.5. This resulted in two relatively clear bands at the expected sizes of 1443bp for *TaPtf1* and 1326bp for *TaMyb67*,

respectively, when assessed on a gel with no significant evidence of contamination from a heterogeneous band (Fig. 19C). The single DNA band represented the overall accumulation of *TaPtf1* (or *TaMyb67*) transcripts from A, B and D sub-genomes, but did not distinguish between the three gene copies. While the PCR products were not assessed by sequencing here, the downstream work of Gateway cloning (see Chapter 4) further confirmed the products amplified by these gene-specific primers to be *TaPtf1* and *TaMyb67*. As such, the CDSs of *TaPtf1* and *TaMyb67* were successfully obtained by RT-PCR.

Table 12. Primers for PCR amplification of Tubulin and the full-length CDS of *Tatf1*/*TaMyb67*

Tubulin-F	**5'-AGAACACTGTTGTAAGGCTCAAC-3'**
Tubulin-R	**5'-GAGCTTTACTGCCTCGAACATGG-3'**
PTF(27)-F	**5'-ATGGACTACTCTAATGGTTCTTTCTTT-3'**
PTF(20)-R	**5'-TCACCTTTCAGGAGGGATTG-3'**
MYB(23)-F	**5'-ATGAGGAGGTGTGATCTGAGACA-3'**
MYB(18)-R	**5'-TCAGCGCTTCTCTTGCG-3'**

*F: forward primer 5'–3'; R: reverse primer 5'–3'

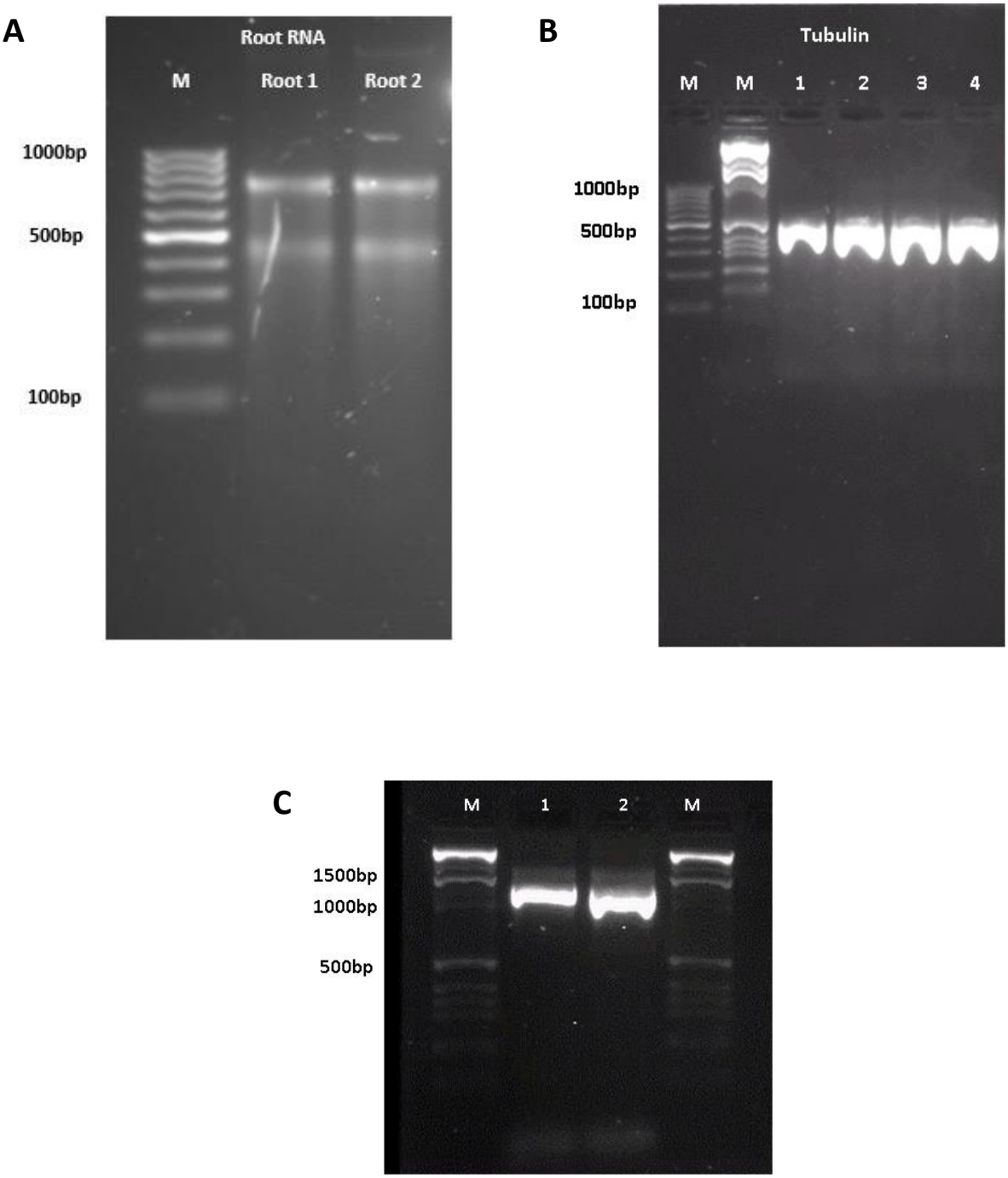


Fig. 19 RNA extraction and PCR amplification of *TaPtf1* and *TaMyb67*. (A) Purified total RNA extracted from wheat roots (two replicates: Root 1 and Root 2). (B) PCR products of amplified Tubulin (lanes 1–4). (C) PCR products of amplified full-length CDSs of *TaPtf1* (lane 1) and *TaMyb67* (lane 2). M: 1kb or 100bp DNA ladder.

3.4 Discussion

Through bioinformatic, genetic and genomic approaches, studies mainly in model plants rice and *Arabidopsis* have identified several genes that appeared to play crucial roles in regulating PUE (Bovill *et al*., 2013). Using the concept of translational genomics, this study has identified orthologues of two key rice TFs and their regulatory roles in PUE were investigated in wheat.

3.4.1 TaPtf1

3.4.1.1 TaPtf1 as a diverged homologue of OsRhl1

In this study, TaPtf1 was identified using a tBLASTn search starting with OsRhl1 (Fig. 11). OsRhl1 is a bHLH TF that regulates root hair formation and it was reasoned that this TF plays a regulatory role in PUE. Surprisingly, the orthologue of OsRhl1 in wheat seemed to be absent, which may be explained by limited gene entry numbers in the NCBI wheat database or a loss of genomic synteny/orthology as a result of speciation or genome duplication during grass genome evolution (AKhunov *et al.,* 2013; Schnabel *et al*., 2012). The wheat chromosome 3B studied by Glover *et al*. (2015), for example, was found to have 27% predicted genes non-syntenic with the orthologous chromosomes of the model grass, rice. More detailed results of the BLASTP search (Fig. 12) and sequence alignment (Fig. 13) revealed that TaPtf1 has a closer homology to OsPtf1 than to OsRhl1. Therefore, OsRhl1 may simply be a diverged homologue of the bHLH family/superfamily, while TaPtf1 is more likely to be the wheat orthologue of OsPtf1. Previous reports in rice and maize have suggested that Ptf1 is a P-responsive positive regulator for plant growth and regulates genes associated with carbon metabolism under P deficiency/low-P, which provides important implications of carbohydrate supply to maintain plant growth under P deficiency/low-P (Li *et al*., 2011; Yi *et al*., 2005). Overexpression of Os*Ptf1* or *ZmPtf1* exclusively results in P-stress-resistant phenotypes of greater root growth and increases of biomass and P accumulation in both shoots and roots compared with the wild-

type, suggesting a positive regulatory role of *Ptf1* for PUE under P deficiency/low-P (Li *et al*., 2011; Yi *et al*., 2005).

In wheat, whether *TaPtf1* bears similar roles to *OsPtf1*/*ZmPtf1* is not yet evident through mutant or overexpression studies. Evidence provided by Espindula *et al*. (2009) indicated that a putative wheat *Ptf1* was induced early (1–5 days) in P-deficient roots but the expression reduced through time (10 days). This early induction of *TaPtf1* in roots was consistent with the expression profile as shown by expVIP (Borrill, 2015; see chapter supplement 1). More recently, Aziz *et al*. (2014) characterised the tissue-specific expression pattern of *TaPtf1* in PUE efficient and less-efficient wheat cultivars under different P treatments (for an extended period of 18 days). Their results showed *TaPtf1* maintained similar expression levels in leaves in both PUE efficient and less-efficient cultivars. In the stem, the expression of *TaPtf1* was higher in a PUE efficient cultivar than a PUE less-efficient cultivar, suggesting a positive effect of *TaPtf1* on PUE in the stem. Interestingly, the plasma-membrane-localised H^+-transporting ATPase previously reported to be positively regulated by *OsPtf1* in rice shoots (Yi *et al*., 2005) was coincidently induced by P deficiency in the leaf base and the stem in a PUE efficient wheat cultivar and is likely to be co-regulated with *TaPtf1* (Aziz *et al*., 2014). The ATPase antiporter-mediated proton transport mechanism has been demonstrated to be important in phloem loading of photosynthetic sugars (Giaquinta, 1979; Plaxton and Carswell, 1999). Photosynthetic sugars have been shown to be lower in shoots but higher in roots in *ZmPtf1* overexpressing lines, which suggests a functional role of *ZmPtf1* in translocating photosynthetic sugars from shoots to roots through phloem (Li *et al*., 2011). Furthermore, observations in a PUE efficient wheat cultivar also found that a plastid enzyme that breaks down transient starch in the primary carbon metabolism, *TaGPho1* (α-1,4-glucan phosphorylase), had an expression profile correlated with

TaPtf1 in the stem and was also elevated in young leaves (Aziz *et al*., 2014; Tiwari and Kumar, 2012). It is possible that the PUE efficient cultivar has benefitted from accelerated starch degradation in shoots, which can provide more sugars to be translocated through phloem for root development. Together, these imply that a similar shoot-to-root sugar translocation mechanism subject to the regulation of *TaPtf*1 might also exist in wheat. However, the expression of *TaPtf1* in later roots in a PUE efficient cultivar, when compared with that in a PUE less-efficient cultivar, was slightly suppressed under high-P and significantly suppressed under low-P (Aziz *et al*., 2014). In both cultivars, the expression was lesser in low-P than in high-P in a similar manner. Besides *TaPtf1*, strong suppression of a fructose 1,6-bisphosphate aldolase encoding gene *TaALD*, which is associated with both glycolysis and glycolytic bypass reactions, was also detected in roots of the PUE efficient cultivar but not in those of a PUE less-efficient cultivar (Aziz *et al*., 2014; Gregory *et al*., 2010). The combined results of different studies indicate the likely involvement of *TaPtf1* in carbon metabolism in wheat, where it may share some conserved essential functions with *OsPtf1*/*ZmPtf1* in shoots but seems to be a P-stress-responsive negative regulator of PUE in roots, despite early induction. The functions of *TaPtf1* in roots appeared to contrast with previous findings in other species and might be characterised as species-specific features. Both Os*Ptf1* and *ZmPtf1* were induced in P-deficient roots and promoted root growth when overexpressed, as supported by a GUS staining assay in lateral roots for *OsPtf1* and the significant increases in lateral root length and number for *ZmPtf1* (Yi *et al*., 2005; Yi *et al*., 2011). A root induction of *GmPtf1* under P deficiency/low-P in haploid soybean was also proposed by Li *et al*. (2014). There seems a functional divergence of *Ptf1* in the more complex crop species of wheat, which is therefore worth studying.

3.4.1.2 *TaPtf1* from three homoeoloci

The triplicate genome of hexaploid wheat is evolutionarily merged from three diploid subunits of different sources and underwent long-term human cultivation (reviewed in section 1.1). *TaPtf1* was mapped to the three homoeoloci on 7AS, 7BS and 7DS. Previous QTL studies in Chinese spring wheat have described 1A, 4A, 7A, 3B, 5B and 7D as being more closely related to P-deficiency/low-P resistance (Li *et al*., 1999). In another study of a double haploid wheat population under P-sufficient and P-deficient conditions, Su *et al*. (2009) also identified three QTLs on 7D between two SSR markers WMC436 and WMC463 (based on Wheat Microsatellite Consortium; prefix: WMC), which are associated with tiller number, biomass yield and total P accumulation per plant. It was interesting that the location of *TaPtf1* on 7DS found in this study maps closely to QTLs of tiller number and biomass yield and may also overlap that of P uptake, which might indicate a link between *TaPtf1-D1* and PUE (see chapter supplement 2). Therefore, *TaPtf1-D1* was chosen for the downstream experiment.

The CDSs of the three *TaPtf1* homoeologues from A, B and D sub-genomes are highly similar to each other, except for a few single nucleotide changes. Nevertheless, the abundance of RNA transcripts for each *TaPtf1* homoeologue could vary when they are differentially regulated or when there is monoallelic expression, as occurs in wheat flour genes (Dopunt *et al*., 2011). Although the primers used to amplify the CDS of *TaPtf1* were not able to distinguish the individual contribution of each homoeologue to the overall transcript pool, this should not make a significant difference to obtaining the CDS of *TaPtf1-D*, considering the similarity of the three loci. In addition, sequencing the PCR products (or qPCR analysis) could have helped to indicate the differential expression level of each *TaPtf1* homoeologue and identify other splice variants concomitantly amplified in RT-PCR.

3.4.2 TaMyb67

3.4.2.1 TaMyb67 as a wheat orthologue of OsPhr2

This study identified TaMyb67 as a wheat orthologue of OsPhr2. *TaMyb67* was mapped to the three homoeoloci on 4AS, 4BL and 4DL, respectively. Interestingly, *TaPhr1* previously described by Wang *et al*. (2013b) was also located at the same gene loci on these locations. Wang *et al*. (2013b) showed that overexpression of *TaPhr1-A1* upregulated a subset of P-starvation-responsive genes and improved root development as well as biomass and P accumulation, which validated a positive regulatory role of *TaPhr1* in promoting plant growth in resistance to P stress. Moreover, based on an SSR marker system developed by Róder *et al*. (1998), the homoeolocus on 4A maps to the QTL near Xgwm262, which is associated with P acquisition and utilisation (Su *et al*., 2006). It is likely that *TaMyb67* might just be *TaPhr1*. This is further supported by the most significant homology between *TaMyb67* and the homoeolocus on 4BL. This homoeolocus maps between Xgwm251-Xgwm6 QTL region, which was shown to contain five QTLs controlling tiller number, P acquisition and P utilisation (Su *et al*., 2006) (see chapter supplement 3). In agreement with Su *et al*. (2009), Zhang and Wang (2015) also confirmed these QTLs on 4BL later. These results seem to imply that *TaMyb67*/*TaPhr1* gene loci are important for PUE in wheat.

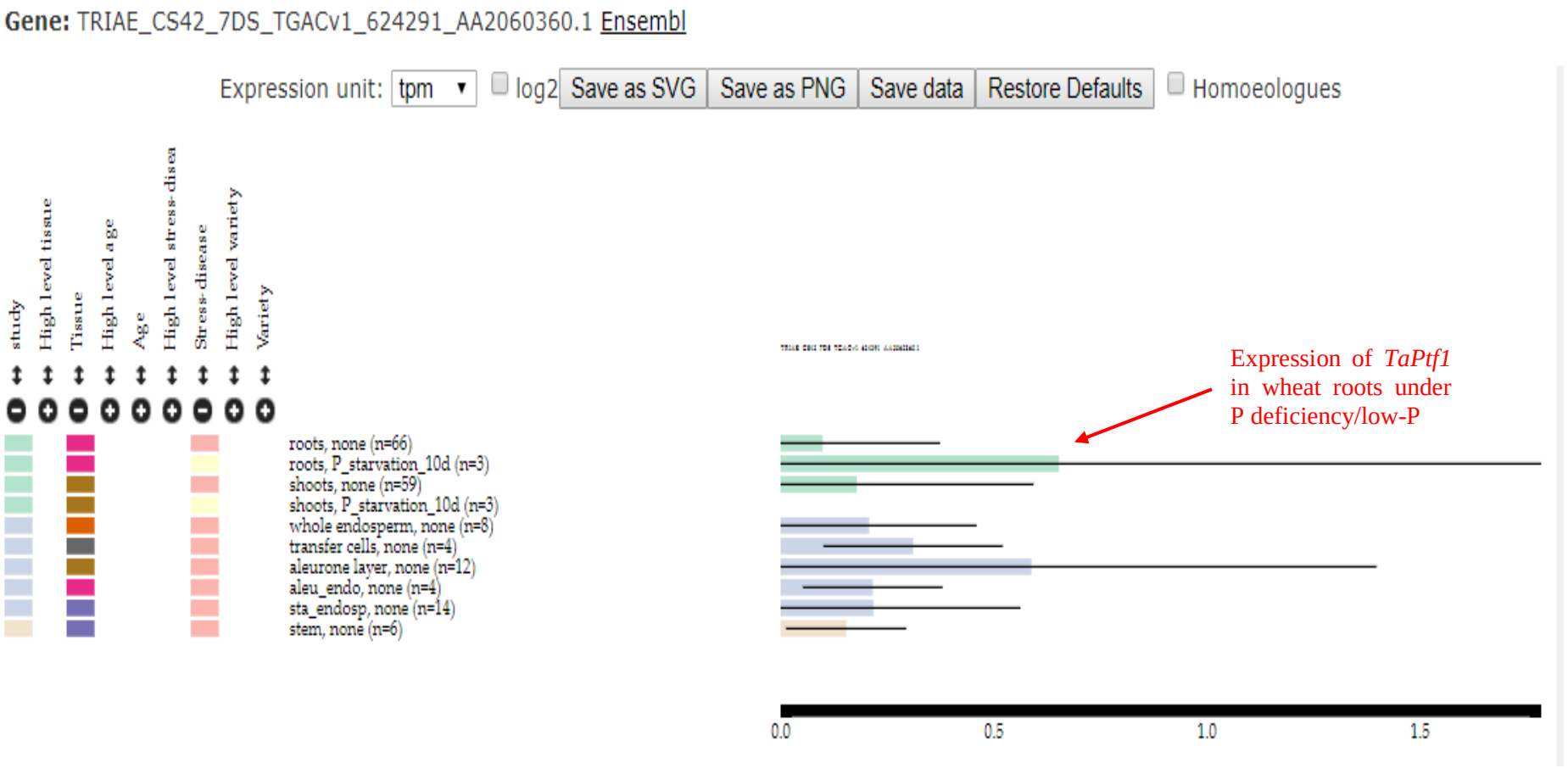
3.4.2.2 Comparison of *TaMyb67* and *TaPhr1*

While *TaMyb67* maps together with *TaPhr1*, comparisons of the *TaMyb67* protein sequence derived from its CDS to *TaPhr1* protein sequences suggested TaMyb67 (441aa) was shorter than TaPhr1-A1, B1, D1 (Fig. 17). The closest protein sequence to TaMyb67, as supported by the greatest homology, was the 447aa protein from 4BL recognised as TaPhr1-B1, which has an additional 6aa-oligopeptide at the C-terminus compared with TaMyb67. The results are rather unexpected but indicate a sequence discrepancy between TaMyb67 and TaPhr1-B1. One possibility initially

considered is that the discrepancy in the protein sequence has resulted from alternative splicing (AS). *TaMyb67* may exist as a splice variant of *TaPhr1-B1* from a novel AS event in the wheat cultivars used by Zhang *et al*. (2012). Although AS is not well defined, it has been well documented in model and other plants. With advances in sequencing technology in recent years, it is becoming clear AS is more prevalent than it was previously thought (Filichkin *et al*., 2015; Staiger and Brown, 2013), for instance, Filichkin *et al*.'s (2010) study on the genome-wide mapping of AS in *Arabidopsis*. Observations in *Arabidopsis* have also demonstrated that plant cells typically contain one major plus multiple minor splice variants (Li *et al*., 2016). The homoeolocus on 4BL is implicated to be highly spliced, perhaps reflecting that a cultivar-specific AS event might occur and lead to the production of *TaMyb67* as a splice variant. During AS, pre-mRNA transcripts spliced in multiple ways will undergo differentially selective inclusion or exclusion of coding (exon) or non-coding (intron) sequences through alternative donors and acceptors (Lareau *et al*., 2004; Staiger and Brown, 2013; Stamm *et al*., 2005). AS constitutes the main source of proteome diversity in eukaryotes, while multiple protein isoforms produced from AS may exhibit differences in their lengths, structures and functions (Kriventseva *et al*., 2003; Swarup *et al*., 2016). AS has also been reported for the important MYB family/superfamily, e.g. an R2R3 type MYB gene alternatively spliced into four different variants leading to either an MYB-related type or an R2R3 type MYB protein in *Arabidopsis* and rice (Li *et al*., 2006; O'Rourke *et al*., 2008). Nevertheless, considering probabilities based on positions of exon-intron boundaries and the size of exons, it seems unlikely, unless as a rare case of cryptic splicing, that the lack of 6aa-oligopeptide in the TaMYB67 protein sequence can be attributed to AS.

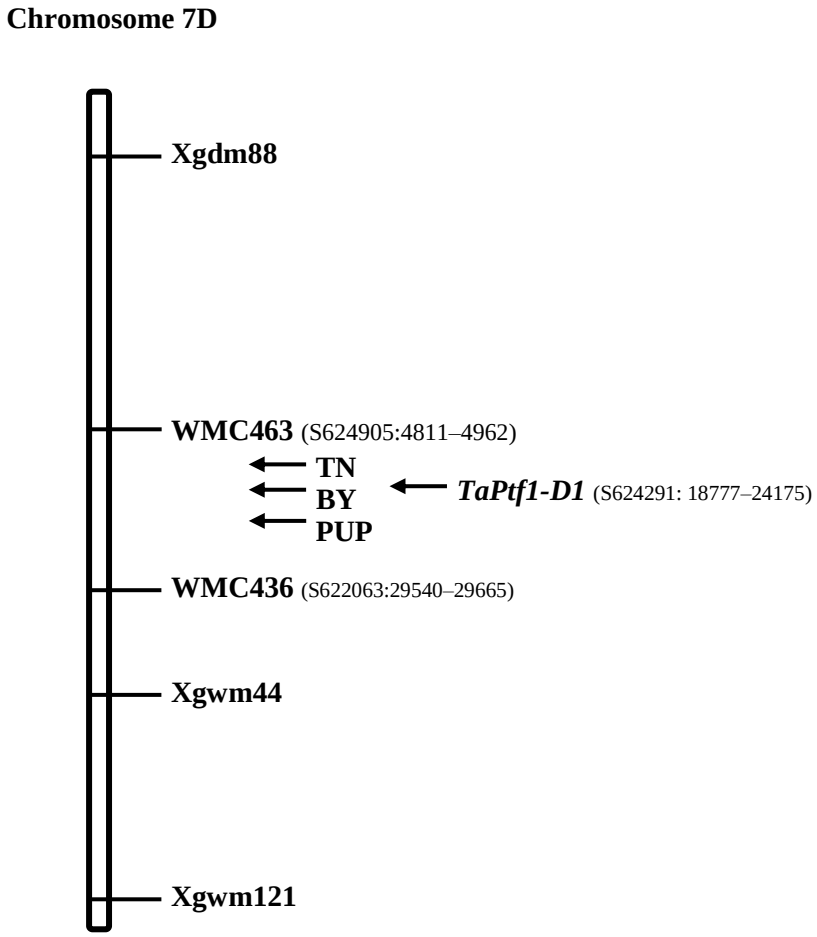
It is more likely that the discrepancy in the protein sequence can be explained by existing genetic diversity in the form of genotypic variation of TaPhr1-B1, presumably due to different cultivars used. The CDS of *TaMyb67* theoretically encoding a 441aa protein was originally identified in a study using mixed wheat samples (Zhang *et al*., 2012), which were different from the wheat cultivar studied for the wheat genome (Ensembl). Genome sequencing projects are now shedding more light on genotypic variations. These variations are presumably caused by the diverse selection pressures under different environments and geographical locations during evolution or human cultivation, as well as spontaneous mutations (Meyer and Purugganan, 2013). Extended data from wheat genome sequencing in the Ensembl database also elucidated that the nucleobase "T" of the stop codon "TGA" on the *TaMyb67* sequence, which is inconsistent with "C" of the amino acid codon "CGA" on the *TaPhr1-B1* sequence, is a stop gained SNP allele (SNP accession: BA00613289), as analysed by markers from SNP arrays (KASP, Axiom 820k and Axiom 35k) in sequencing (Wilkinson *et al*., 2016). This suggests that both "TGA" and "CGA" potentially exist as natural variations (see chapter supplement 4), which strongly points to the likelihood that *TaMyb67* is naturally an ecotypic variation of *TaPhr1-B1* (a "full-length" product). Furthermore, the ecotypic variation hypothesis can also be supported by the fact that the missing sequence at the C-terminus of TaMyb67 protein sequence does not reside in a known functional region in the MYB protein and is thus not under selection pressure (Fig. 18). Therefore, the amplified CDS of *TaMyb67* in this study should represent a full-length product of *TaPhr1-B1* and may function in a similar manner with *TaPhr1-A1* in regulating PUE.

Chapter supplement 1



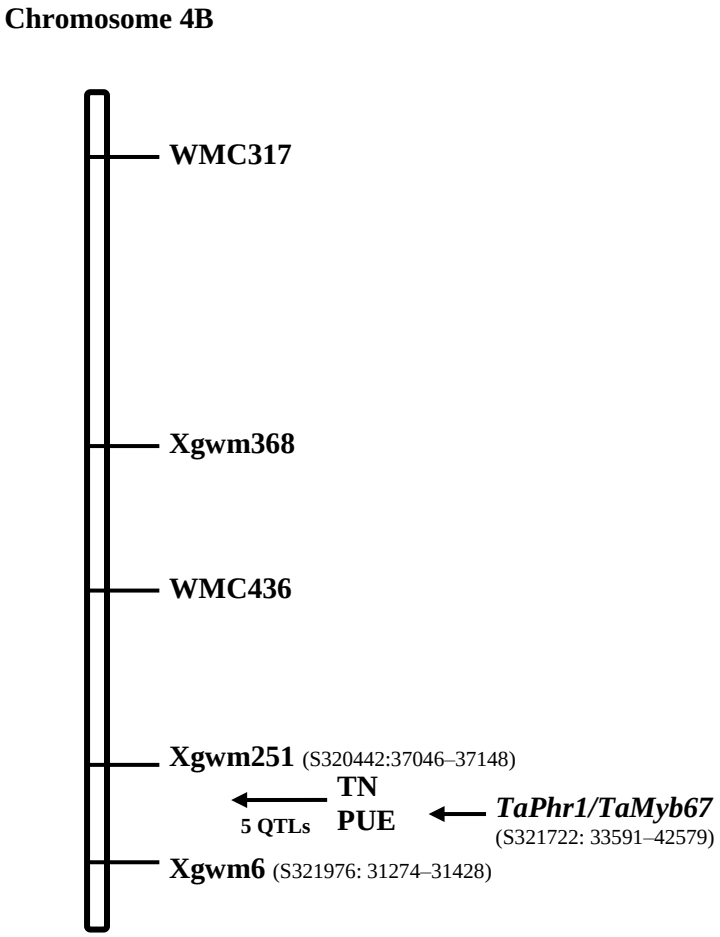
Supp. Fig. 1 Expression profile of *TaPtf1* **in** expVIP **database.** The database is supported by John Innes Centre (Borrill, 2016). The expression levels of *TaPtf1* are derived from three different studies under the option of "study" and described in green, light blue and pale pink. Different colours under the option of "Tissue" indicate the expression levels of ***TaPtf1*** in different tissues. Yellow and pink under the option of "Stress/disease" indicate the expression levels of ***TaPtf1*** in different abiotic/biotic stress treatments or none-treatment.

Chapter supplement 2



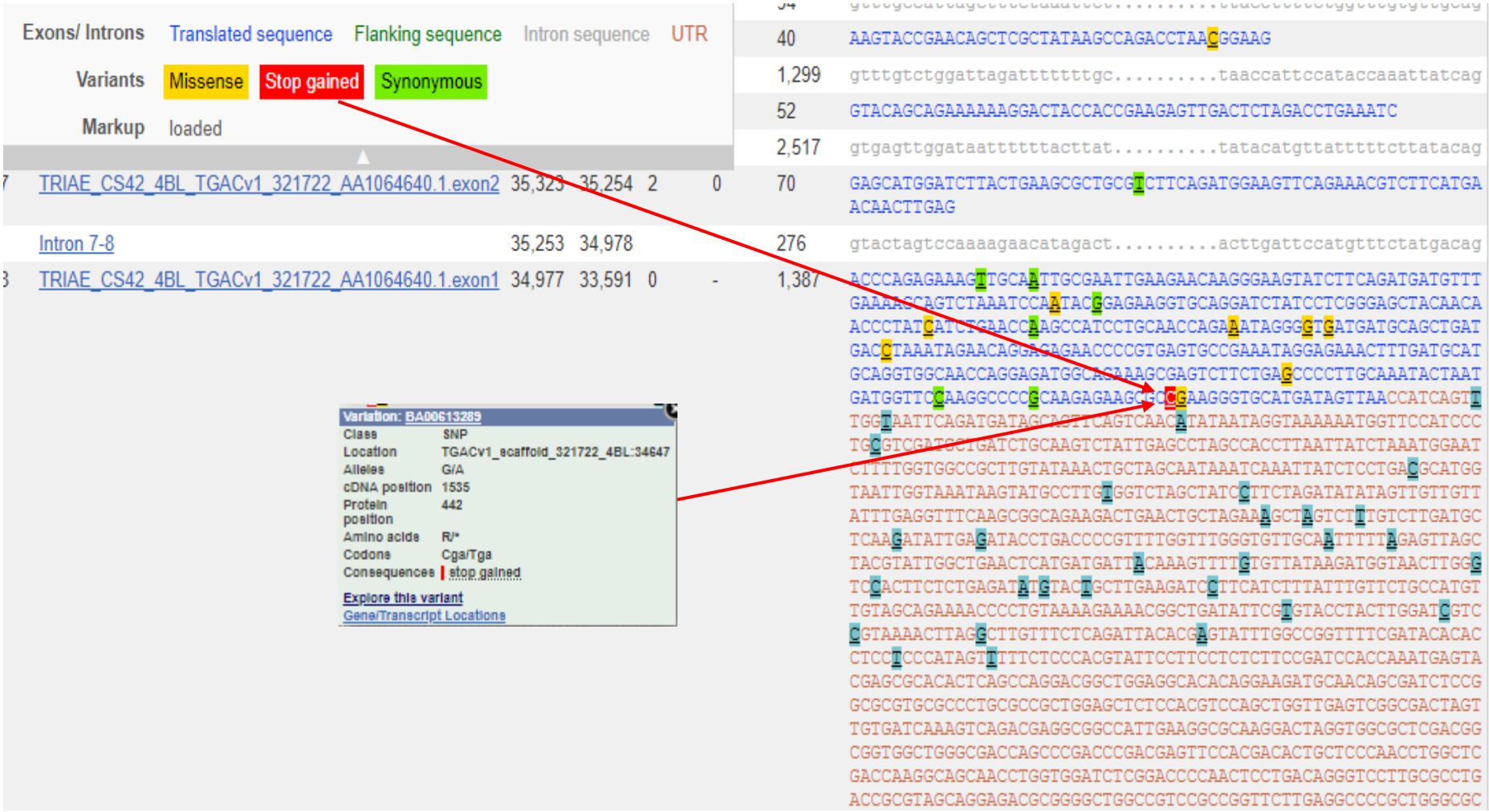
Supp. Fig. 2 Demonstration of *TaPtf1* location and QTLs on 7D. Locations of three QTLs were described by Su *et al*. (2009). TN: QTL of tiller number. BY: QTL of biomass yield. PUP: QTL of total P accumulation per plant. Xgdm: SSR maker system developed by Pestsova *et al*. (2000). Xgwm: SSR marker system developed by Róder *et al*. (1998). WMC: SSR marker system developed by Wheat Microsatellite Consortium. Numbers in brackets indicate locations of markers or *TaPtf1-D1* on the physical map (Ensembl wheat genome) with a start of "S" referring to the Scaffold in sequencing.

Chapter supplement 3



Supp. Fig. 3 Demonstration of *TaPhr1*/*TaMyb67* location and QTLs on 4B. Locations of five QTLs related to tiller number and PUE were described by Su *et al*. (2006). TN: QTL of tiller number. PUE: QTLs of P acquisition and utilisation. Xgwm: SSR marker system developed by Róder *et al*. (1998). WMC: SSR marker system developed by Wheat Microsatellite Consortium. Numbers in brackets indicate locations of markers or *TaMyb67* on the physical map (Ensembl wheat genome) with a start of "S" referring to the Scaffold in sequencing.

Chapter supplement 4



Supp. Fig. 4 Demonstration of the stop gained SNP allele on *TaPhr1-B1* (*TaMyb67*). *TaPhr1-B1* gene sequence shown as exons and introns in the Ensembl wheat genome database. Letters in blue: translated region of *TaPhr1-B1*. Letters in pink: untranslated region of *TaPhr1-B1*. Letters in grey: introns. The arrow-pointed letter "C" highlighted in red on the sequence indicates the stop-gained SNP allele; SNP accession: BA00613289 supported by sequencing using markers from SNP arrays (KASP, Axiom 820k and Axiom 35k) (Wilkinson *et al.*, 2016).

Chapter 4 Generation of transgenic wheat with *TaPtf1* and *TaMyb67* overexpression constructs – Method I (pipeline 1)

4.1 Overview

A prerequisite for transgenic manipulation is a target DNA to be cloned into plasmid vectors as workable constructs that allow genetic modification and introduction into plants. Overexpression constructs are frequently used in plants to characterise gene functions and particularly effective in discovering the underlying molecular basis of phenotypes, which might remain undetected using traditional loss-of-function analysis (Chiou and Lin, 2011; Prelich, 2012). TFs involved in PUE regulatory mechanisms have been studied through the overexpression approach in various plant/crop species, with well-studied examples being *OsPhr2* (Zhou *et al*., 2008a), *TaPhr1* (Wang *et al*., 2013b), *TabHLH1* (Yang *et al*., 2016), *BnPhr1* (Ren *et al*., 2012) and *ZmPhr1* (Wang *et al*., 2013a).

The soil bacterium *Agrobacterium tumefaciens* carries a tumour-inducing Ti-plasmid which is capable of delivering the T-DNA into the nucleus of a host cell, thereby permanently altering a plant genome (Gelvin, 2003). For this reason, *Agrobacterium*-mediated transformation has been established for many plants since the first successful example in *Tabacum* as described by Zambryski *et al*. (1983). There are several advantages of the method, such as being active, low-cost, achieving low-gene copy, simple and silencing in operation. The method frequently uses a binary vector system (a Vir-helper plasmid derived from the Ti-plasmid and contained in a given *Agrobacterium* strain; a genetically modified T-DNA construct that carries a target DNA). During transformation, the Vir gene from the helper plasmid can work in *trans* on the T-DNA to be inserted to plant genome (Gelvin, 2003). So far, *Agrobacterium*-mediated transformation is more efficient and reliable than other methods for plant transformation,

although there are still challenges for the transformation of wheat (Jones *et al*., 2005). Wheat transformation is often limited by the large genome size (17 Gb), highly repetitive DNA sequences, low regeneration ability and other difficult transformation features of this crop species (Bhalla, 2006).

For *Agrobacterium*-mediated transformation, an important factor that decides the success of transformation is the explant . Wheat transformation has been investigated through various explants (e.g. immature embryos, mature embryos, young ear, shoot tip, root, microspore, anther, inflorescences and protoplast, etc.) (Caswell *et al*., 2000; Chin and Scott, 1977; Delporte *et al*., 2001; Folling *et al*., 2001; Harris *et al*., 1988; Harvey *et al*., 1999; Jones, 2005; Rajyalakshmi *et al*., 1991; Trione *et al*., 1968; Viertel and Hess, 1996). However, the transformation efficiency appeared to be often low and genotype-dependent. Since Shimada (1978) who first succeeded in regenerating wheat plants from immature embryos, wheat immature embryos have become the mainstream and most commonly used explants for wheat transformation.

Following successful confirmation of the CDS of *TaPtf1*/*TaMyb67* by PCR amplification as described in Chapter 3, the work of this study subsequently moved on to the transgenic manipulation of *TaPtf1*/*TaMyb67* in wheat in order to obtain successful transformants in which *TaPtf1*/*TaMyb67* are overexpressed. The aims of chapter 4 were therefore to show the work of pipeline 1 (Fig. 8), where two overexpression constructs, G1390PTF1 and G1390MYB67, were generated and transformed, mediated by *Agrobacterium*, into wheat immature embryos. Possible reasons causing the consequence of no successful transformants were discussed in the chapter.

4.2 Materials and methods

4.2.1 Generation of G1390PTF1/G1390MYB67 construct

To generate the G1390PTF1/G1390MYB67 construct, Gateway cloning, which realizes site-specific recombination and allows efficient cloning of DNA sequences into multiple plasmid vectors by utilising properties of bacteriophage lambda, was employed (Landy, 1989). Standard Gateway cloning has two reactions - BP and LR reactions (Katzen, 2007) (Fig. 20).

4.2.1.1 Vector for cloning

The pDNOR/Zeo vector was supplied by Invitrogen (USA) and served as an entry vector for Gateway BP reaction (Fig. 21). It contains *att*P sites that allow recombination with the *attB*-flanked target DNA to establish an entry clone. It carries a zeocin-resistance gene (*ble* gene) as the bacteria selection marker and a *ccd*B gene for the negative control selection. The protein of the *ccd*B gene interferes with *E.coli* DNA gyrase (Bernard and Couturier, 1992), thereby inhibiting the growth of most *E.coli* strains (e.g. DH5α). The propagation of pDNOR/Zeo therefore requires competent *E.coli* DB3 cells which survive the *ccd*B. Two priming sites of a pair of M13-uni (-21) and M13-rev (-29) primers flanking outside *att*P sites can be used for sequencing the DNA fragment between the two sites.

The pGW-CUbi1390 vector was provided by Dr Lichao Zhang (CAAS, China) and served as a destination vector for Gateway LR reaction (Fig. 22A). It is an overexpression vector modified from the commercial vector pCambia1390 and constructed with a maize ubiquitin promoter and a Gateway cassette C.1 (Fig. 22B). The Gateway cassette C.1 contains *att*R sites that allow recombination with *att*L sites on an entry clone to generate a destination clone. Given the *ccd*B gene carried by the Gateway cassette C.1, the propagation of pGW-CUbi1390 similarly needs competent *E.coli* DB3 cells which survive the *ccd*B. The vector employs a kanamycin

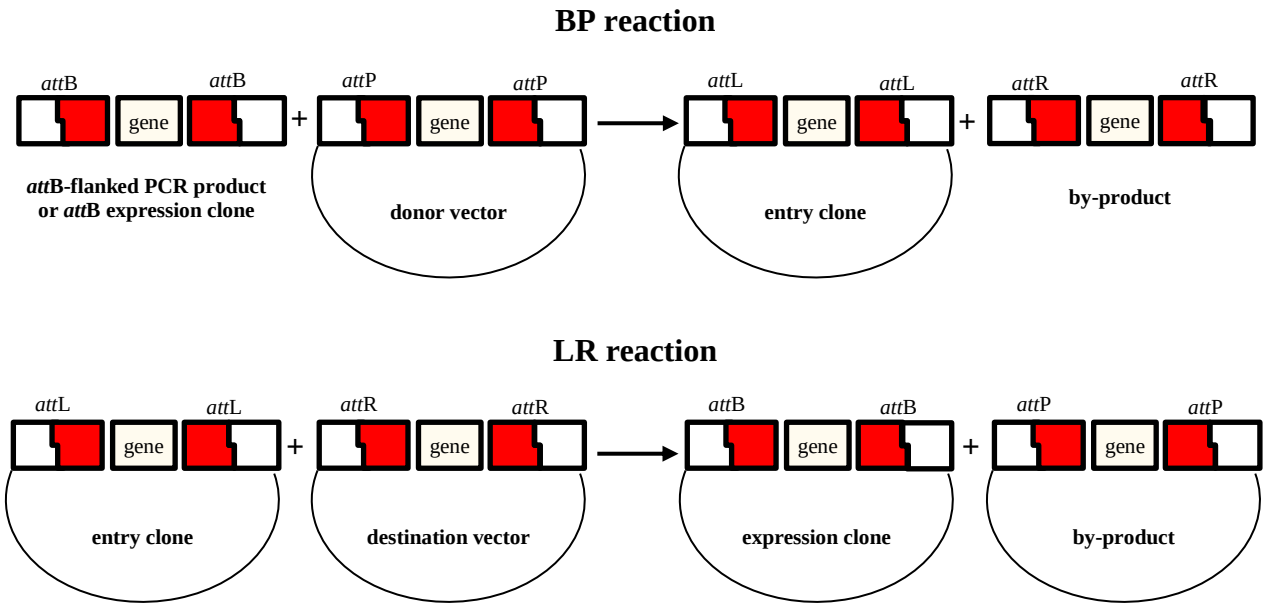
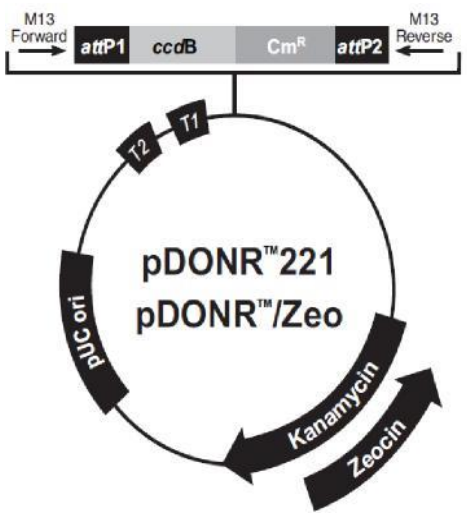


Fig. 20 Gateway cloning - BP and LR reactions. Source: reprinted from Gateway® Technology (2010) with permission by Thermo Fisher Scientific Inc. (USA).

A

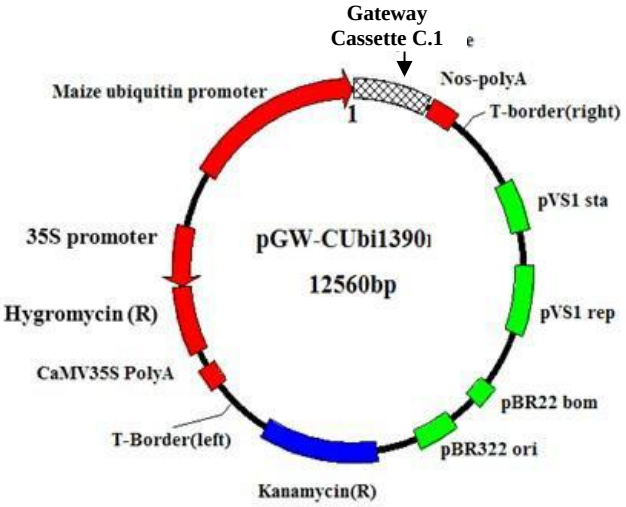


B

Feature	Benefit
*rrn*BT1 and T2 transcription termination	Protects the cloned gene from expression vector-encoded promoters, thereby reducing possible toxicity (Orosz et al., 1991).
M13 forward (-20) priming site	Allows sequencing in the sense orientation.
*att*P1 and *att*P2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from an *att*B containing expression clone or *att*B-PCR product (Landy, 1989).
*ccd*B gene	Allows negative selection of the plasmid.
Chloramphenicol resistance gene (Cm^R)	Allows counterselection of the plasmid.
M13 reverse priming site	Allows sequencing in the anti-sense orientation.
Kanamycin resistance gene (pDNOR™ 221 only)	Allows selection of the plasmid in *E. coli*.
EM7 promoter (pDNOR™/Zeo only)	Allows expression of the Zeocin™ resistance gene in *E. coli*.
Zeocin™ resistance gene (pDNOR™/Zeo only)	Allows selection of the plasmid in *E. coli*.
pUC origin and replisome assembly site	Allows high-copy replication and maintenance of the plasmid in *E. coli*.

Fig. 21 Structure and features of pDONR/Zeo entry vector. (A) Vector map and *att*P recombination sites. (B) Elements of the vector. Source: reprinted from Gateway® Technology (2010) with permission by Thermo Fisher Scientific Inc. (USA).

A



B

Reading Frame Cassette:	A (1711bp)	B(1713bp)	C.1 (1714bp)
*att*R1 site	4-128	5-129	6-130
Primer 1	163-185	164-186	165-187
Chloramphenicol resistance gene (Cm^2)	237-896	2380897	239-919
*ccd*B gene	1238-1543	1239-1544	1239-1544
Primer 2	1444-1466	1445-1467	1445-1467
*att*R2 site	1564-1708	1585-1709	1585-1709

Fig. 22 Structure of pGW-CUbi1390 destination vector and Gateway cassettes. (A) Vector map of pGW-CUbi1390. (B) Elements of Gateway cassettes. Source: (A) provided by Dr Lichao Zhang (CAAS, China). (B) reprinted from Gateway®Technology (2010) with permission by Thermo Fisher Scientific Inc. (USA).

-resistance gene (*nptII* gene) as the bacteria selection marker and hygromycin (*hpt* gene) for plant selection. Two priming sites of a pair of primers (Pubi as the forward primer; Tnos as the reverse primer) flanking outside *att*R sites can be used for sequencing the DNA fragment between the two sites (Appendix 6 for priming site information).

4.2.1.2 Cloning method - Gateway

To clone the target DNA into the pDNOR/Zeo entry vector, primers (described as *att*B primers) were designed by adding a Gateway *att*B-adaptor (25bp plus) onto the 5' end of each CDS primer used to amplify the target DNA [PTF(27)/PTF(20) and MYB(23)/MYB(18) as described in Chapter 3]. This was completed according to manufacturer's instructions (Weisberg and Landy, 1983; Landy, 1989) and generated according to Method 1) as described in 2.2. The PCR amplification was prepared by using the *att*B-primers, high-fidelity Phusion DNA polymerase (2U μL^{-1}) with buffer and the first-strand cDNA (described in Chapter 3) as templates in a 50µL reaction system, where either a routine 3-step thermal profile or a combination of 2-step and 3-step thermal profile was performed (Table 13). To achieve high-quality cloning, the PCR products were purified according to Method 1) as described in 2.10 and assessed by spectrophotometric analysis as well as agarose gel electrophoresis according to Method 1) as described in 2.6 and 2.8. The BP reaction, mediated by Gateway BP Clonase II enzyme mix (Invitrogen, USA), was performed by using a 10µL reaction system (Table 14) that included the recommended 100fmol target DNA and 150ng pDONR/Zeo vectors (150ng μL^{-1}), the amounts of which were calculated according to manufacturer's instructions as by the formula below (Appendix 7):

-*Taptf1* CDS (1443bp): $(100\text{fmol})(1443\text{bp})\left(\frac{660\text{fg}}{\text{fmol}}\right)\left(\frac{1\text{ng}}{10^6\text{fg}}\right)$ ***≈95.2ng***

-*TaMyb67* CDS (1326bp): $(100\text{fmol})(1326\text{bp})\left(\frac{660\text{fg}}{\text{fmol}}\right)\left(\frac{1\text{ng}}{10^6\text{fg}}\right)$ ***≈87.5ng***

Table 13. Amplification of Gateway adaptors *att*B -flanked *TaPtf1* and *TaMyb67*

High Fidelity PCR	***att*B-*TaPtf1***	***att*B-*TaMyb67***
5x Phusion HF buffer (µL)	10	10
dNTP(10mM) (µL)	1	1
***att*B-forward primer (µL)**	2.5	2.5
***att*B-reverse primer (µL)**	2.5	2.5
cDNA (µL)	4	4
ddH_2O (µL)	29.5	29.5
HF Phusion (µL)	0.5	0.5
total (µL)	50	50
Thermal profile	Pre-denaturation: 98°C 30 seconds 5 cycles Denaturation: 98°C 15 seconds Annealing: 59°C 30 seconds Extension: 72°C 45 seconds 35 cycles Denaturation: 98°C 15 seconds Elongation: 72°C 60 seconds Final Extension: 72°C 7 minutes	Pre-denaturation: 98°C 30 seconds 35 cycles Denaturation: 98°C 15 seconds Annealing: 62°C 30 seconds Elongation: 72°C 45 seconds Final Extension: 72°C 7 minutes

On completion of the BP reaction, competent *E.coli* DH5α cells were transformed by using 1μL BP reaction products and selected on Low-salt LB+Zeocin (50μg mL^{-1}) plates according to Method 1) as described in 2.3 and 2.4. Positive clones were selected by a screening PCR using M13-uni (-21) and M13-rev (-29) primers as well as a further screening PCR using hybridised primers of M13-uni (-21) as the forward primer and PTF(20)/MYB(18) as the reverse primer according to Method 1) as described in 2.7 and assessed by agarose gel electrophoresis. Plasmids of positive clones were also extracted and assessed by DNA sequencing with M13 primers according to Method 1) as described in 2.5 and 2.11 to confirm a successful entry clone.

To further clone the target DNA into the pGW-CUbi1390 destination vector, successful entry clones were used in the LR reaction which was mediated by Gateway LR Clonase II enzyme mix (Invitrogen, USA) and performed by using a 10μL reaction system (Table 15) that included the recommended 150ng entry clones and 150ng pGW-CUbi1390 vectors according to manufacturer's instructions. On completion of the LR reaction, competent *E.coli* DH5α cells were transformed by using 1μL LR reaction products and selected on LB+Kanamycin (10μg mL^{-1}) plates according to Method 1) as described in 2.3 and 2.4. To obtain positive clones, plasmid extraction was directly conducted and a screening PCR was performed by using hybridised primers of Pubi as the forward primer and PTF(20)/MYB(18) as the reverse primer, as well as plasmids of transformants as templates. These were followed by the PCR products being assessed by agarose gel electrophoresis before positive clones were sent for DNA sequencing with primers of Pubi and Tnos to confirm successful destination clones.

4.2.1.3 Restriction endonuclease analysis of G1390PTF1/G1390MYB67

Prior to restriction endonuclease analysis, successful destination clones (G1390PTF1/

1390MYB67) were propagated by using competent *E.coli* TOP10 cells according to Method 2) as described in 2.4 and 2.5. Based on restriction enzyme sites analysed, the constructs were examined by appropriate restriction endonucleases according to the method described in 2.9 (Appendix 7) and assessed by agarose gel electrophoresis.

Table 14. BP reaction mediated by Gateway BP Clonase II enzyme mix

	TaPtf1	*TaMyb67*
***att*B-*TaPtf1* (µL)**	4	-
***att*B-*TaMyb67* (µL)**	-	1.5
pDONR/Zeo (µL)	1	1
ddH_2O (µL)	1.5	2
TE buffer(pH=8.0) (µL)	3	5

Table 15. LR reaction mediated by Gateway LR Clonase II enzyme mix

	TaPtf1	*TaMyb67*
***TaPtf1* entry clone (µL)**	4	-
***TaMyb67* entry clone (µL)**	-	5
pGW-CUbi1390 (µL)	1	1
ddH_2O (µL)	1.5	1.5
TE buffer(pH=8.0) (µL)	3	2

4.2.2 Transfer constructs to *Agrobacterium* by tri-parental mating

4.2.2.1 Tri-parental mating

Tri-parental mating employs bacteria conjugation to enable efficient plasmid mobilisation from a donor bacteria strain to a recipient bacteria strain with the help of an assistant-plasmid present in a third bacterial strain. The Tri-parental mating in this study was performed according to the protocol provided by Dr Xingguo Ye (CAAS, China) (Appendix 8) by using a disarmed-Ti *Agrobacterium* strain C58C1 and an *E.coli* strain PRK2013, which were also provided by Dr Xingguo Ye. The *E.coli* strain DH5α containing the G1390PTF1/G1390MYB67 construct acted as a donor bacteria strain to offer the T-DNA, while the disarmed-Ti *Agrobacterium* strain C58C1 carrying the Vir gene acted as a recipient bacteria strain. PRK2013 was employed to provide the assistant-plasmid to help transfer a construct from *E.coli* DH5α to *Agrobacterium* C58C1. Positive clones were screened by a PCR using hybridised primers of Pubi and PTF(20)/MYB(18) and were assessed by agarose gel electrophoresis. They were also individually incorporated into *Agrobacterium*-glycerol stock by mixing each cell culture with an equal volume of 50% glycerol (autoclaved) and stored at -80°C.

4.2.2.2 Extraction of *Agrobacterium* plasmid and restriction endonuclease analysis

Plasmids of a selected *Agrobacterium* positive clone were prepared from a volume of 5mL *Agrobacterium* C58C1 cultures by using EndoFree Plasmid Mini Kit (CWBio, Beijing, China). The method was adapted from manufacturer's protocol (Appendix 9). For restriction endonuclease analysis, plasmids were digested with XhoI and HindIII respectively according to the method described in 2.9 and were assessed by agarose gel electrophoresis.

4.2.3 Generation of transgenic plants with G1390PTF1/G1390MYB67

4.2.3.1 Medium for plant tissue culture

Medium plates were prepared based on Murashige and Skoog (MS) by mixing a specific number of medium-required powders with sucrose in distilled water to a final volume of up to 1L (pH=5.8) before 2.4g phytagel/appropriate agarose being added. The medium was sterilised at 121°C for 15 minutes and cooled to 55°C. Following this, appropriate phytohormones, medium supplements (prepared to stock solutions with sterile ddH_2O and sterilised by syringe and filter-membrane strainer in a Lamina flow hood) and antibiotics were added to the medium and mixed well before spreading plates (90x20mm)/boxes (78x78x95mm) in a Lamina flow hood. These were then stored at 4°C for up to 4 weeks (see Appendix 10 for protocol).

4.2.3.2 Replacement of medium

All procedures of plate transfer were carried out in a Lamina flow hood which was sterilised by ultraviolet for 30 minutes before use. The medium plates for replacement were thawed from 4°C with the condensation eliminated in the hood. The embryos/calli/sprouts were removed to the replacement medium plates by using sterile forceps and evenly distributed in the plate depending on the growth rate and size.

4.2.3.3 Preparation of *Agrobacterium* culture and spectrometer analysis

Approximately 2–3 days prior to transformation (inoculation step), G1390PTF1/G1390MYB67-contained *Agrobacterium* C58C1 was revived from glycerol stock by streaking on a YEP+Rif (50mg L^{-1})+Gent(100mg L^{-1})+Kana (50mg L^{-1}) plate to be incubated at 28°C for 48–72 hours. Each selected single colony was carefully scraped off from the plate using a transferring loop and used to inoculate 10mL of YEP medium plus Rif (50mg L^{-1}), Gent (100mg L^{-1}) and Kana (50mg L^{-1}) in a 15mL falcon tube and incubated in a shaker-incubator (28°C+180rpm; 12–16

hours). More YEP medium (plus described antibiotics) was added into the culture to a volume of up to 50mL in a 100mL flask (autoclaved) for an expanded culture of another few hours. To monitor the growth rate of *Agrobacterium*, a volume of 1.5–2mL culture was filled into a cuvette, with the control filled with YEP medium, and examined for OD600 in a calibrated spectrometer (PERSEE, Beijing, China). The *Agrobacterium* was cultured till OD600=0.5, which was subsequently transferred into a 15mL/50mL falcon tube according to the volume required for inoculation and spun down at 3,500rpm for 10 minutes, followed by re-suspension with an equal volume of the 1/10WCC inoculation buffer.

4.2.3.4 Immature embryos

Wheat plants (Var. CB037) were grown in a CAAS glasshouse (18–28°C, supplemental lighting outside of summertime) or field sites, with unified management of compost, watering, insect and pest control, or other growth conditions being carried out by technicians from Dr Xingguo Ye's lab (CAAS, China). Wheat spikes were collected at different time points according to the required size of immature embryos (1.0–1.2mm) during the post-anthesis stage. Following collection, fresh caryopses (immature seeds) were manually and carefully removed from panicles without injury to the germ area (Fig. 23).

4.2.3.5 Seed surface sterilisation, isolation and pre-incubation

All procedures, if not specified, were performed in a Lamina flow hood which was sterilised by ultraviolet for 30 minutes before use. Approximately 100 healthy caryopses were selected and surface sterilised by rinsing in 1.5 volume of 70% (v/v) aqueous ethanol (prepared from 99.5% ethanol and sterile distilled ddH_2O) for 1–2 minutes and then soaking in 1.5 volume of 1.5%~ (v/v) NaOCl (prepared from 8–13% NaOCl and sterile distilled ddH_2O) for 15 minutes, followed by rinsing thoroughly with four or five changes of sterile distilled water. The sterilised seeds

were transferred to a polystyrene petri dish (90x20mm) filled with two layers of filter paper (Ø 85-90mm). Immature embryos were manually and carefully isolated from the germ using sterile forceps and knife (surgical grade) and placed in pre-incubation medium plates, with the scutellum orientated upward and the radicle (embryo axis) placed downward to contact with the medium (Fig. 23). The plates were incubated at 25°C in the dark in an incubator (Shaoguan Taihong Medical Apparatus and Instruments Co., Ltd., Guangdong, China) for 4 days prior to inoculation (see Appendix 10 for protocol).

4.2.3.6 Transformation, selection and regeneration of transformants

Inoculation, co-cultivation, callus induction, selection, differentiation and rooting were undertaken as the steps shown in Table 16. The protocols were provided by Dr Xingguo Ye (CAAS, China) (see Appendix 10 for protocol). Briefly, immature embryos were separated from wheat seeds and pre-incubated on appropriate medium plates as described in the previous section. The pre-incubated immature embryos were inoculated with *Agrobacterium* C58C1 containing appropriate constructs (as prepared in 4.2.3.3) for 30 minutes in either a 2mL centrifuge tube or a polystyrene petri dish (30x15mm) as demonstrated by Fig. 24, which was followed by dry co-cultivation for 3 days and callus induction for 5 days. The selection of immature embryos and regeneration of shoots took place on selection medium plates with an appropriate concentration of hygromycin for 3 weeks and differentiation medium plates with an appropriate concentration of hygromycin for 2 weeks prior to extended differentiation for another 3 weeks. The regenerated-shoots were removed to rooting medium plates/boxes to grow a root system with a suitable size. Numbers of calli and regenerated-shoots were recorded and analysed during tissue culture.

Fig. 23 Flowchart of plant collection and isolation of immature embryos from wheat.

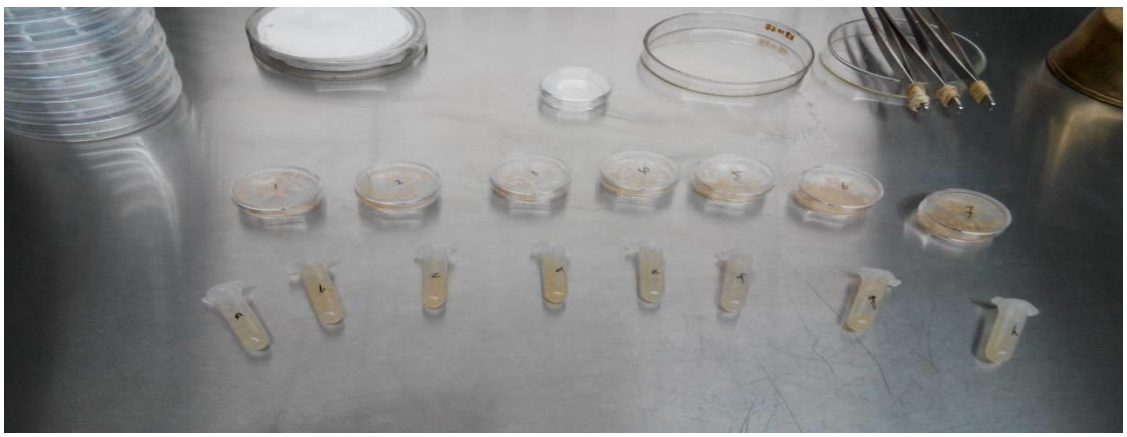


Fig. 24 Demonstration of inoculation in wheat transformation. The tools shown are inoculation containers of polystyrene petri dish or microcentrifuge tube.

4.2.3.7 Analysis of inoculation efficiency by GUS290 histochemical staining assay

The GUS290 histochemical staining assay was carried out as a trial of inoculation efficiency according to the protocol provided by Dr Xingguo Ye (CAAS, China) (see Appendix 10 for protocol). Briefly, the isolated immature embryos were pre-incubated and inoculated with GUS290-contained *Agrobacterium* C58C1 (provided by Dr Xingguo Ye), followed by dry co-cultivation and callus induction at 25°C in the dark using the method described in the previous section. The co-cultivated immature embryos were randomly selected from each plate and placed into a 2mL microcentrifuge tube, following which a volume of 1mL buffer X-Gluc was added. The tube was incubated at 37°C overnight (12–16 hours), after which the buffer was replaced with 70% (v/v) ethanol to stand for another 48 hours.

4.2.3.8 *Agrobacterium*-mediated transformation with G1390PTF1/G1390MYB67

The G1390PTF1/G1390MYB67-contained *Agrobacterium* C58C1 culture, with OD600=0.5 as already described, was used to transform wheat immature embryos using the methods described in previous sections (Table 16). The selection and regeneration of transformants were achieved by employing a low-to-high gradient concentration of hygromycin as the screening mechanism. The selection medium supplement with hygromycin at a start concentration of 10mg L^{-1} was followed by the differentiation medium supplement with hygromycin at 20mg L^{-1} before two higher concentrations of hygromycin at 35mg L^{-1} and 50mg L^{-1} being attempted in the downstream differentiation and the removal of hygromycin in the rooting medium.

4.2.3.9 Data collection and analysis

Results of DNA sequencing for constructs were analysed according to the method described in 2.11. Callus induction and shoot regeneration rates were analysed in Excel 2010 (Microsoft Limited,

Microsoft Campus, Thames Valley Park, Reading, Berkshire, RG6 1WG).

Table 16. *Agrobacterium*-mediated transformation, selection and regeneration - Method I

	Method I
Constructs	G1390PTF1/G1390MYB67
Selection mechanism	Hyg (hygromycin)
Wheat variety of Immature embryos	CB037
Inoculation efficiency analysis	Histochemical staining assay
Isolation	60–70 embryos per plate
Pre-incubation	Pre-incubation medium 4 days; 25°C; dark
Inoculation	1/10 WCC buffer; 30 minutes
Co-cultivation	Dry co-cultivation 3 days; 25°C (axis removed after); dark
Callus induction	Callus induction medium 5 days; 25°C; dark
Selection	Selection medium + Hyg (10mg L^{-1}) 21 days; 25°C; dark
Differentiation-1	Differentiation medium + Hyg (20mg L^{-1}) 14 days; 24°C; light
Differentiation-2	Differentiation medium + Hyg (35mg L^{-1}/50mg L^{-1}) 21 days plus; 24°C; light
Rooting	Rooting medium; 24°C; light

4.3 Results

4.3.1 Generation of G1390PTF1 and G1390MYB67 constructs

4.3.1.1 Amplification of *att*B-*TaPtf1*/*att*B-*TaMyb67*

With the aim of generating G1390PTF1/G1390MYB67 constructs, the planned cloning approach was to introduce the full-length CDS of *TaPtf1*/*TaMyb67* into pDNOR/Zeo (entry vector) through Gateway BP reaction, followed by a further delivery of *TaPtf1*/*TaMyb67* into pGWUbi1390 (destination vector) through Gateway LR reaction. The *att*B-primers designed to amplify and add Gateway *att*B-adaptors onto the ends of *TaPtf1*/*TaMyb67* are shown in Table 17. The purpose of the adaptors is to enable Gateway BP reaction, during which the *att*B sites recombine with the *att*P sites on pDNOR/Zeo and incorporate into new *att*L sites on the entry clone. The PCR amplification and purification of the PCR products were performed as described in method section 4.2.1.2. As displayed in Fig. 25, two clear DNA bands at the expected sizes of approximately 1443bp and 1326bp (*att*B-adaptors: 50bp) were separately obtained for *TaPtf1* (Fig. 25, lane 1) and *TaMyb67* (Fig. 25, lane 2). Primers and primer-dimers did not appear in the gel due to the removal of DNA <300bp by purification. In addition, the concentration of the purified *TaPtf1*/*TaMyb67* (added with *att*B-adaptors) determined by spectrophotometric analysis was also shown to be satisfactory (>10ng μL^{-1}) for a downstream BP reaction (Appendix 11).

4.3.1.2 Gateway BP reaction and PCR screening

Once the site-specific recombination occurs during Gateway BP reaction, the target DNA may be able to be introduced into pDNOR/Zeo (Bushman, 1985). By using the purified *TaPtf1*/*TaMyb67* (added with *att*B-adaptors) from the previous step and pDNOR/Zeo, BP reactions were performed and the reaction products were used to transform *E.coli* individually for

Table 17. The *att*B-primers for amplification of *TaPtf1*/*TaMyb67*

*att*B-primers	*att*B-adaptor	CDS primer
*att*B-F PTF(27)	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGC→	ATGGACTACTCTAATGGTTCTTTCTTT-3'
*att*B-R PTF(20)	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTA→	TCACCTTTCAGGAGGGATTG-3'
*att*B-F MYB(23)	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGC→	ATGAGGAGGTGTGATCTGAGACA-3'
*att*B-R MYB(18)	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTA→	TCAGCGCTTCTCTTGCGG-3'

**att*B-F: *att*B forward primer; *att*B-R: *att*B reverse primer

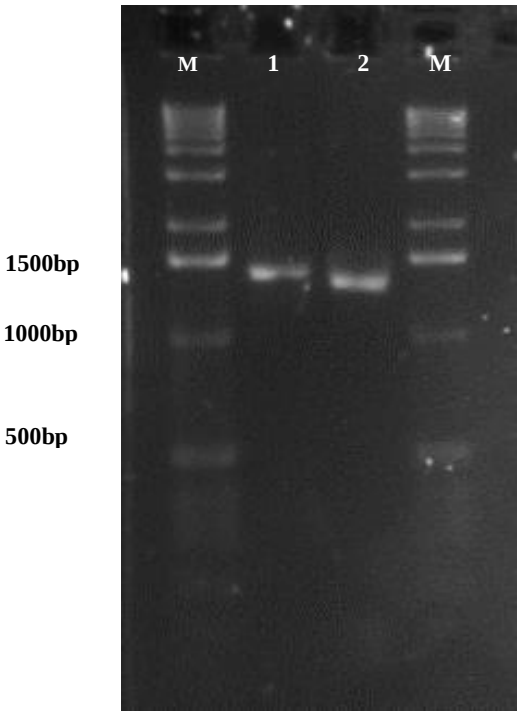


Fig. 25 Purified PCR products of *att*B-*TaPtf1* (lane 1) and *att*B-*TaMyb67* (lane 2). M: 1kb DNA ladder.

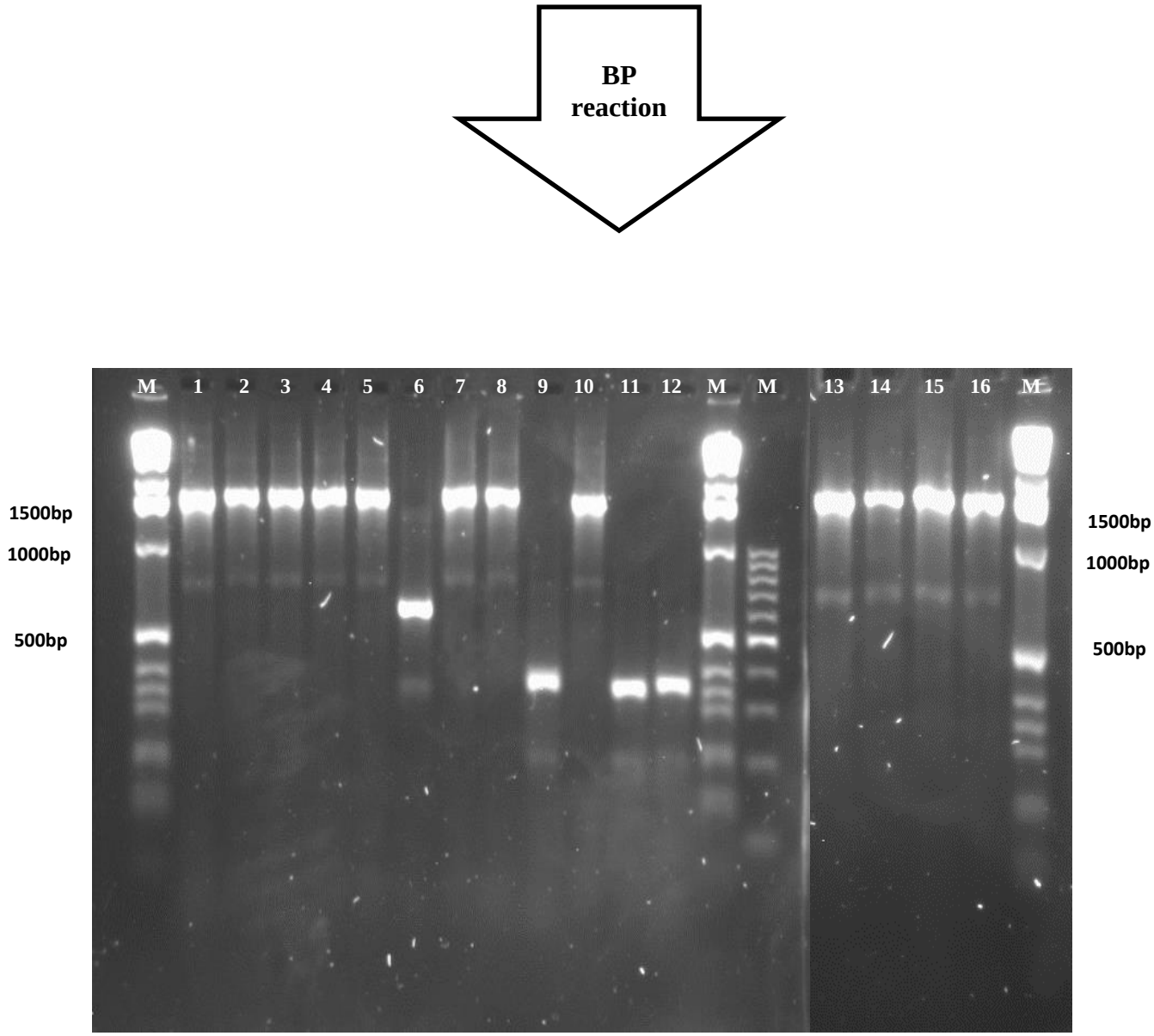


Fig. 26 Result of PCR screening for BP transformants of *TaPtf1* and *TaMyb67.* The screening PCR was performed by using M13-uni (-21) and M13-rev (-29) universal primers**.** Lanes 1–8: *TaPtf1* transformants BPPTF1–8. Lanes 9–16: *TaMyb67* transformants BPMYB1–8. M: 1Kb or 100bp DNA ladder**.**

selection of successful entry clones. Fig. 26 shows the gel result of single colony screening PCR using M13-uni (-21) and M13-rev (-29) primers, where eight *E.coli* transformants (BPPTF1–8: Fig. 26, lanes 1–8) were tested for *TaPtf1* and another eight *E.coli* transformants were tested for *TaMyb67* (BPMYB1–8: Fig. 26, lanes 9–16). As the two priming sites for M13 flank outside the *att*L sites on the entry clone and extend the region of amplification (an addition of 130bp before and 160bp after), the anticipated product sizes are at approximately 1730bp for *TaPtf1* and 1615bp for *TaMyb67*, respectively. For *TaPtf1*, except lane 6 (Fig. 26) which displayed a lower band at 700bp, all the other seven lanes obtained bands at expected size and therefore were justified as *TaPtf1* BP clones. For *TaMyb67*, lane 9, lane 11 and lane 12 (Fig. 26) showed much lower bands near to 300bp, while the other five lanes resulted in bands at expected size and therefore were recognised as *TaMyb67* BP clones. A consistent result was also produced from a further screening PCR using hybridised primers of M13-uni (-21) and PTF(20)/MYB(18), where the gene-specific reverse primer was used instead of M13-rev (-29).

4.3.1.3 Sequencing result of successful BP clones

Plasmids of justified BP clones were prepared and subsequently assessed by repeated DNA sequencing, as described in method section 4.2.1.2, through which correct recombination sites were confirmed in all sequenced BP clones (Fig. 27 and Fig. 28). The BP clones detected with a further number of mismatches on the target DNA were discarded, although these mismatches could have derived from the unexpected inaccuracy of DNA sequencing.

For *TaPtf1,* the successful BP clone - BPPTF2 was confirmed to carry the sequence equivalent to the CDS of *TaPtf1-D1* and therefore was chosen. The sequenced *TaPtf1* on BPPTF2 was compared in alignment with the CDS of *TaPtf1* from GenBank and the CDS of *TaPtf1* from 7DS of the wheat genome (as described in Chapter 3, Fig. 13) and was shown to be significantly

consistent with the CDS of *TaPtf1* from 7DS of the wheat genome, except for one single-nucleotide alteration at 1434bp. This alteration, however, did not affect the amino acid of the resulting protein (Fig. 27). The result confirmed the cloned *TaPtf1* to be suitable for a downstream LR reaction. By contrast, four single nucleotides differing from the CDS of *TaPtf1* in GenBank were identified at places of 180bp, 498bp, 594bp and 1144bp respectively in the sequenced *TaPtf1* on BPPTF2. According to the amino acid codon, the nucleotides altered at 180bp, 498bp and 594bp would not lead to the conversion of amino acids, whereas a change of the nucleotide from "T" to "A" at 1144bp in the sequenced *TaPtf1* would turn Cys. to Ser.. Nevertheless, a variation of such a single amino acid appeared to frequently happen among a group of homologous proteins as suggested by an alignment produced from position Specific Itemed (PSI)-BLAST by PHYRE2.0 protein analysis (Altschul *et al*., 1997) (Appendix 12). These observed differences could have derived from the unexpected inaccuracy of DNA sequencing in the CDS of *TaPtf1* in GenBank, but seemed more likely to be natural SNPs due to different wheat cultivars used and thus reasonably imply the genetic variability of *TaPtf1* among wheat cultivars.

For *TaMyb67*, the successful BP clone - BPMYB6 was confirmed to have an identical sequence to the CDS of *TaMyb67* from GenBank (Fig. 28). This qualified the cloned *TaMyb67* as a satisfactory result for a downstream LR reaction. The sequenced *TaMyb67* on BPMYB6 was also shown to be significantly consistent, except for the C-terminus, with the CDS of *TaPhr1* from 4BL of the wheat genome (as described in Chapter 3, Fig. 16) when compared in alignment with the three CDSs of *TaPhr1* from the wheat genome (Ensembl) (Fig. 28). Interestingly, another sequence detected in a different sequenced BP clone - BPMYB5 appeared to contain an extra 12bp-nucleotide that encodes 4aa of "SMVQ" in the middle of

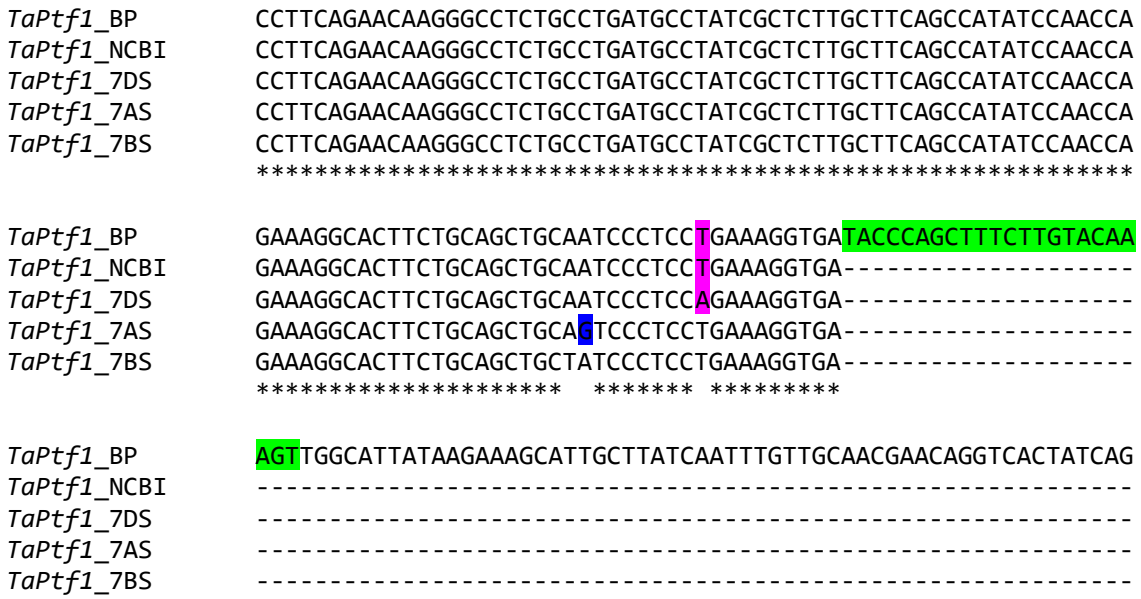
the sequence when compared to *TaMyb67*. As indicated by findings of the differences between the three TaPhr1s and TaMyb67 (as described in Chapter 3, Fig. 17), the 12bp-nucleotide found in BPMYB5 here, by which "SMVQ" is encoded, coincides with that appearing in *TaPhr1-A* or *TaPhr1-D1*. Meanwhile, the 12bp-nucleotide is similarly missing in *TaPhr1-B1.* Moreover, this sequence on BPMYB5 was found to be a better match with *TaPhr1-A1* or *TaPhr1-D1*, than that with *TaPhr1-B1*. It was therefore presumed that either a *TaPhr1-A1* or *TaPhr1-D1* sequence co-existent with *TaMyb67* was accidentally amplified due to the homology of its sequence with the primer binding sites.

(I) Sequencing result of *TaPtf1* BP clone

```
TaPtf1_BP       TATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTT
TaPtf1_NCBI     ------------------------------------------------------------
TaPtf1_7DS      ------------------------------------------------------------
TaPtf1_7AS      ------------------------------------------------------------
TaPtf1_7BS      ------------------------------------------------------------

TaPtf1_BP       TTTTATAATGCCAACTTTGTACAAAAAAGCAGGCTGCATGGACTACTCTAATGGTTCTTT
TaPtf1_NCBI     -----------------------------------ATGGACTACTCTAATGGTTCTTT
TaPtf1_7DS      -----------------------------------ATGGACTACTCTAATGGTTCTTT
TaPtf1_7AS      -----------------------------------ATGGACTACTCTAATGGTTCTTT
TaPtf1_7BS      -----------------------------------ATGGACTACTCTAATGGTTCTTT
                                                   **********************

TaPtf1_BP       CTTTCCTTCATGGCCTGGCAATTCCGCTTCCGAGAATTATAGCTTTGTTGATGGTTCAGT
TaPtf1_NCBI     CTTTCCTTCATGGCCTGGCAATTCCGCTTCCGAGAATTATAGCTTTGTTGATGGTTCAGT
TaPtf1_7DS      CTTTCCTTCATGGCCTGGCAATTCCGCTTCCGAGAATTATAGCTTTGTTGATGGTTCAGT
TaPtf1_7AS      CTTTCCTTCATGGCCTGACAATTCCGCTTCTGAGAATTATAGCTTTGTTGATGGTTCAGT
TaPtf1_7BS      CTTTCCTTCATGGTCTGGCAATTCCGCTTCCGAGAGGTATAGCTTTGTTGATGGTTCAGT
                ************* *** ************ ****  **********************

TaPtf1_BP       GGAATCATATGCAGAAGAAGGAAGTATGCCACCTACAGGCTATTTCAGAGCTAGATCAAA
TaPtf1_NCBI     GGAATCATATGCAGAAGAAGGAAGTATGCCACCTACAGGCTATTTCAGAGCTAGATCAAA
TaPtf1_7DS      GGAATCATATGCAGAAGAAGGAAGTATGCCACCTACAGGCTATTTCAGAGCTAGATCAAA
TaPtf1_7AS      GGAATCATATGCAGAAGAAGGAAGTATGCCACCTGCAGGCTATTTCAGAGCTAGATCAAA
TaPtf1_7BS      GGAATCATATGCAGAAGAAGGAAGTATGCCACCTACAGGCTATTTCAGAGCTAGATCAAA
                ********************************* ************************

TaPtf1_BP       TCAGAATTTAACATTTGATGAGCATGAACAGAACCCTGCTATGCTTGCAAATGGGTGCTT
TaPtf1_NCBI     TCAGAATTTAACATTTGATGAGCATGAACAGAACCCGGCTATGCTTGCAAATGGGTGCTT
TaPtf1_7DS      TCAGAATTTAACATTTGATGAGCATGAACAGAACCCTGCTATGCTTGCAAATGGGTGCTT
TaPtf1_7AS      TCAGAATTTAACATTTGATGAGCATGAACAGAACCCTGCTATGCTTGCAAATGGGTGCTT
TaPtf1_7BS      TCAGAATTTAACATTTGATGAGCATGAACAGAACCCTGCTATAATTGCAAATGGGTGCTT
                ********************************** *****  ****************

TaPtf1_BP       GCCGTACAACACCCAGACTGATCTATTATCTGGTGAGATTCTGTCAGAGGACAAACATTC
TaPtf1_NCBI     GCCGTACAACACCCAGACTGATCTATTATCTGGTGAGATTCTGTCAGAGGACAAACATTC
TaPtf1_7DS      GCCGTACAACACCCAGACTGATCTATTATCTGGTGAGATTCTGTCAGAGGACAAACATTC
TaPtf1_7AS      GCCGTACAACACCCAGACTGATCTATTATCTGGTGAGATTCTGTCAGAGAACAAACCTTC
TaPtf1_7BS      GCCGTACAACGCCCAGACTGATCTATTATCTGGTGAGATTCTGTCAGAGGACAAACCTTC
                ********** ************************************ ****** ***

TaPtf1_BP       CAACAGCCTTATGGAGCTTCCACAACTTCAGAACAATGGCAGTCTGCAAAGTAATTTAAT
TaPtf1_NCBI     CAACAGCCTTATGGAGCTTCCACAACTTCAGAACAATGGCAGTCTGCAAAGTAATTTAAT
TaPtf1_7DS      CAACAGCCTTATGGAGCTTCCACAACTTCAGAACAATGGCAGTCTGCAAAGTAATTTAAT
TaPtf1_7AS      CAACAGCCTTATGGAGCTTCCACAACTTCAGAACAATGGCAGTCTGCAAAGTAATTTAAT
TaPtf1_7BS      CAATAGCCTTATGGAGCTTCCACAACTTCAGAACAATGGCAGTCTGCAAAGTAATTTAAT
                *** ************************************************************

TaPtf1_BP       CCCACCAGGGACTCTTCAGTGCACTTCAACACCTGGAACATTTGACCTGCAGTTGGATAC
TaPtf1_NCBI     CCCACCAGGGACTCTTCAGTGCACTTCAACACCTGGAACATTTGACCTGCAGTTGGATAC
TaPtf1_7DS      CCCACCAGGGACTCTTCAGTGCACTTCAACACCTGGAACATTTGACCTGCAGTTGGATAC
TaPtf1_7AS      CCCATCAGGGACTCTTCACTGCACTTCAACACCTGGAACATTTGACCTGCAGTTGGATAC
TaPtf1_7BS      CCCACCAGGGACTCTTCAGTGCACTTCAACACCTGGAACATTTGACCCGCAGTTGGATAC
                **** ************ ************************** ************
```

```
TaPtf1_BP       CCCTGGCCTTCTAGAACTTCCTCATGCCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGA
TaPtf1_NCBI     CCCTGGCCTTCTAGAACTTCCTCATGCCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGA
TaPtf1_7DS      CCCTGGCCTTCTAGAACTTCCTCATGCCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGA
TaPtf1_7AS      CCCTGGCCTTCTAGAACTTCCTCATGCCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGA
TaPtf1_7BS      CCCTGGCCTTCTAGAACTTCCTCATGCCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGA
                ************************************************************

TaPtf1_BP       AGTTTCAGCTTTTCTTGCTGATGTACATGCTGTTTCTTCAGCCTCAACTCTGTGCTCGAC
TaPtf1_NCBI     AGTTTCAGCTTTTCTTGCTGATGTACATGCTGTTTCTTCAGCCTCAACTCTGTGTTCGAC
TaPtf1_7DS      AGTTTCAGCTTTTCTTGCTGATGTACATGCTGTTTCTTCAGCCTCAACTCTGTGCTCGAC
TaPtf1_7AS      AGTTTCAGCTTTTCTTGCTGATGTACATGCTGTTTCTTCAGCCTCAACTCTGTGTTCGAC
TaPtf1_7BS      AGTTTCAGCTTTTCTTGCTGATGTACATGCTGTTTCTTCAGCCTCAACTCTCTGTTCGAC
                ************************************************** ** *****

TaPtf1_BP       ATTCCAAAATGTTCCTTCCTACATGGAACCAGTAAGCCTAGAAGCTTTCAGTTTTCAAGG
TaPtf1_NCBI     ATTCCAAAATGTTCCTTCCTACATGGAACCAGTAAGCCTAGAAGCTTTCAGTTTTCAAGG
TaPtf1_7DS      ATTCCAAAATGTTCCTTCCTACATGGAACCAGTAAGCCTAGAAGCTTTCAGTTTTCAAGG
TaPtf1_7AS      ATTCCAAAATGTTCCTTCCTACATGGAACCAGTAAGCCTAGAAGCTTTCAGTTTTCAAGG
TaPtf1_7BS      ATTCCAAAATGTTCCTTCCTACATGGAGCCAGTAAGCCTAGAAGCTTTCAGTTTTCAAGG
                *************************** ********************************

TaPtf1_BP       GATACAAAATGCTGCTATGTTCAACAATACAAGTCATTCAAATGGGAACCTGTCAGTATT
TaPtf1_NCBI     GATACAAAATGCTGCTATGTTCAACAATACGAGTCATTCAAATGGGAACCTGTCAGTATT
TaPtf1_7DS      GATACAAAATGCTGCTATGTTCAACAATACAAGTCATTCAAATGGGAACCTGTCAGTATT
TaPtf1_7AS      GATACAAAATGCTGCTATGTTCAACAATACAAGTCATTCAAATGGGAACCTGTCAGTATT
TaPtf1_7BS      GATACAAAATGCTCCTATGTTCAACAATACAAGTCATTCAAATGGAAACCTGTCAGTATT
                ************* **************** ************** **************

TaPtf1_BP       TGATGAGGCAACCATGGCATCACTACATGATAGCAAAGAATTTCTCAGTGGTAGCATCTC
TaPtf1_NCBI     TGATGAGGCAACCATGGCATCACTACATGATAGCAAAGAATTTCTCAGTGGTAGCATCTC
TaPtf1_7DS      TGATGAGGCAACCATGGCATCACTACATGATAGCAAAGAATTTCTCAGTGGTAGCATCTC
TaPtf1_7AS      TGATGAGGCAACCATGGCATCACTACATGATAGCAAAGAATTTCTCAGTGGTGGCATCTC
TaPtf1_7BS      TGATGAGGCAACCATGGCATCACTACATGATAGCAAAGAGTTTCTCAGTGGTAGCATCTC
                ************************************* ************ *******

TaPtf1_BP       ATCTTTTGGTACGGCCGAGCAGTCACAACTAGCTGGTAGTGGTTTGAAGGCTGAACAACA
TaPtf1_NCBI     ATCTTTTGGTACGGCCGAGCAGTCACAACTAGCTGGTAGTGGTTTGAAGGCTGAACAACA
TaPtf1_7DS      ATCTTTTGGTACGGCCGAGCAGTCACAACTAGCTGGTAGTGGTTTGAAGGCTGAACAACA
TaPtf1_7AS      ATCTTTTGGTACGGCCGAGCAGTCTCAACTAGCTGGTAGTGGTTTGAAGGCTGAACAACA
TaPtf1_7BS      ATCTTTTGGTATGGCCGAGCAGTCACAACTAGCTGGTAGTGGTTTGAAGGCTGAACATCA
                *********** ************ ******************************** **

TaPtf1_BP       GGAACAAAATGCGATGTGCAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGATGGCAGT
TaPtf1_NCBI     GGAACAAAATGCGATGTGCAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGATGGCAGT
TaPtf1_7DS      GGAACAAAATGCGATGTGCAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGATGGCAGT
TaPtf1_7AS      GGAACAAAATGTGATGTGCAATATTCCGCTCCCTTTCGCTTCTGGTAGTCAGATGGCAGT
TaPtf1_7BS      GGAGCAAAATGCGATGTGCAATATTCCGCTCCCTTTCGCTTCTGGTAGTCAGATGGCAGT
                *** ******* *************** ******************************

TaPtf1_BP       GAGTGAAGCACAAGGGGCAATGATTCCTTCGAAGATAAGCTCAACGATGCATAACAATAA
TaPtf1_NCBI     GAGTGAAGCACAAGGGGCAATGATTCCTTCGAAGATAAGCTCAACGATGCATAACAATAA
TaPtf1_7DS      GAGTGAAGCACAAGGGGCAATGATTCCTTCGAAGATAAGCTCAACGATGCATAACAATAA
TaPtf1_7AS      GAGTGAAGCACAAGGGACACTGATTCCTTCAAAGATAAGCTCAACGATCCATAACAATAA
TaPtf1_7BS      GAGTGAAGCACAAGGGGCACTGAATCCTTCAAAGATAGGGTCAACGATCCATAACAATAA
                *************** ** *** ****** ****** * ******** **********
```

```
TaPtf1_BP       AAGTGAGTACCCTGTCCCTATCAGCCATTCTGCTGATGCGCAGAACAAGGCAAATTCAGC
TaPtf1_NCBI     AAGTGAGTACCCTGTCCCTATCAGCCATTCTGCTGATGCGCAGAACAAGGCAAATTCAGC
TaPtf1_7DS      AAGTGAGTACCCTGTCCCTATCAGCCATTCTGCTGATGCGCAGAACAAGGCAAATTCAGC
TaPtf1_7AS      AAGTGAGTACCCTGTCCCTATCAGCCATTCTGCTGATGCGCAGAACAAGGCAAATTCAGC
TaPtf1_7BS      AAGTGAGTACCCTGTCCCTATCAGCCATTCTGCTGATGCGCAGAACAAGGCAAATTCAGC
                ************************************************************

TaPtf1_BP       TAATGGAAACAGTGCCAGTGCTAAGCCACGAGCAAGGGCTCGTCGTGGACAGGCAACTGA
TaPtf1_NCBI     TAATGGAAACAGTGCCAGTGCTAAGCCACGAGCAAGGGCTCGTCGTGGACAGGCAACTGA
TaPtf1_7DS      TAATGGAAACAGTGCCAGTGCTAAGCCACGAGCAAGGGCTCGTCGTGGACAGGCAACTGA
TaPtf1_7AS      TAATGGAAATAGTGCCAGTGCTAAGCCACGAGCAAGGGCTCGTCGTGGACAGGCAACTGA
TaPtf1_7BS      TAATGGAAACAGTGCCGGTGCTAAGCCACGAGCAAGGGCTCGTCGTGGACAGGCAACTGA
                ********* ****** ****************************************

TaPtf1_BP       CCCTCATAGTATTGCTGAACGGCTTCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCT
TaPtf1_NCBI     CCCTCATAGTATTGCTGAACGGCTTCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCT
TaPtf1_7DS      CCCTCATAGTATTGCTGAACGGCTTCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCT
TaPtf1_7AS      CCCTCATAGTATTGCTGAACGGCTTCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCT
TaPtf1_7BS      CCCTCATAGTATTGCTGAGCGGCTTCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCT
                ***************** ****************************************

TaPtf1_BP       CCAAGACCTTGTACCAAACTCAAATAAGGCAGATAAATCATCAATGCTCGATGAAATAAT
TaPtf1_NCBI     CCAAGACCTTGTACCAAACTCAAATAAGGCAGATAAATCATCAATGCTCGATGAAATAAT
TaPtf1_7DS      CCAAGACCTTGTACCAAACTCAAATAAGGCAGATAAATCATCAATGCTCGATGAAATAAT
TaPtf1_7AS      CCAAGACCTTGTACCAAATTCAAATAAGGCAGATAAATCATCAATGCTTGATGAAATAAT
TaPtf1_7BS      CCAAGACCTTGTACCAAACTCAAATAAGGCAGATAAATCATCAATGCTCGATGAAATAAT
                ***************** ***************************** ***********

TaPtf1_BP       TGATTATGTGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGCATGAGTAGGCTAGGAGC
TaPtf1_NCBI     TGATTATGTGAAATTTCTTCAGCTTCAGGTGAAGGTCTTATGCATGAGTAGGCTAGGAGC
TaPtf1_7DS      TGATTATGTGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGCATGAGTAGGCTAGGAGC
TaPtf1_7AS      TGATTATGTGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGCATGAGTAGGCTAGGAGC
TaPtf1_7BS      TGATTATGTGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGCATGAGTAGGCTAGGAGC
                ************************************ ******************

TaPtf1_BP       TCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAACTGAGGGCCGTAGCAATTCACC
TaPtf1_NCBI     TCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAACTGAGGGCCGTAGCAATTCACC
TaPtf1_7DS      TCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAACTGAGGGCCGTAGCAATTCACC
TaPtf1_7AS      TCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAACTGAGGGCCATAGCAATTCATC
TaPtf1_7BS      CCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAACTGAGGGCCGTAGCAATTCACC
                 ******************************************** ********** *

TaPtf1_BP       TCTATCATCTCCAACCGCTTCACAAGGGCTTCTGGACGCAGCAGGCCCAGAAGACAGCTT
TaPtf1_NCBI     TCTATCATCTCCAACCGCTTCACAAGGGCTTCTGGACGCAGCAGGCCCAGAAGACAGCTT
TaPtf1_7DS      TCTATCATCTCCAACCGCTTCACAAGGGCTTCTGGACGCAGCAGGCCCAGAAGACAGCTT
TaPtf1_7AS      TCTATCACCTCCAACCGCTTCACAAGGGCTTCTGGATGCAGCAGGCCCAGAAGACAGCTT
TaPtf1_7BS      TCTATCATCTCCAACTGCTTCACAAGGGCTGCTGGATGCAGCAGGCCCAGAAGACAGCTT
                ******* ******* ************** ***** ************************

TaPtf1_BP       GGTCTTTGAGCAAGAAGTTATAAAGCTGATGGAAACAAGCATCACAAATGCAATGCAGTA
TaPtf1_NCBI     GGTCTTTGAGCAAGAAGTTATAAAGCTGATGGAAACAAGCATCACAAATGCAATGCAGTA
TaPtf1_7DS      GGTCTTTGAGCAAGAAGTTATAAAGCTGATGGAAACAAGCATCACAAATGCAATGCAGTA
TaPtf1_7AS      GGTCTTTGAGCAAGAAGTTATAAAGCTGATGGAAACAAGCATCACAAATGCAATGCAGTA
TaPtf1_7BS      GGTCTTTGAGCAAGAAGTTATAAAGCTGATGGAAACAAGCATCACCAAGGCAATGCAGTA
                ****************************************** ** ***********
```



** *TaPtf1*_BP: sequenced *TaPtf1* on the BP clone - BPPTF2; *TaPtf1*_NCBI: CDS of *TaPtf1* in GenBank; *TaPtf1*_7AS/BS/DS: CDS of *TaPtf1* from the wheat sub-genome A, B or D (Ensembl).

** The places highlighted in yellow are differences at places of 180, 498, 594 and 1144 between *TaPtf1*_BP and *TaPtf1*_NCBI; The place highlighted in pink is the difference at the place of 1434 between *TaPtf1*_BP and *TaPtf1*_7DS; The places highlighted in blue are the differences between *TaPtf1*_BP and *TaPtf1*_7AS or 7BS. Except for these places, the remaining parts of these *TaPtf1* sequences are identical to each other (full-length:1443bp).

** Sequences highlighted in green indicate the correct recombined *att*B sites.

Fig. 27 Alignment between the sequenced *TaPtf1* clone on BPPTF2, the CDS of *TaPtf1* in GenBank and the CDS of *TaPtf1-A1, TaPtf1-B1* and *TaPtf1-D1* from the three wheat sub-genomes.

(II) Sequencing result of *TaMyb67* BP clone

```
TaMyb67_BP          TTTATAATGCCAACTTTGTACAAAAAAGCAGGCTGCATGAGGAGGTGTGATCTGAGACAG
TaMyb67_NCBI        -----------------------------------ATGAGGAGGTGTGATCTGAGACAG
TaPhr1_4BL          -----------------------------------ATGAGGAGGTGTGATCTGAGACAG
TaPhr1_4DL          -----------------------------------ATGAGGAGGTGTGATCTGAGACAG
TaPhr1_4AS          -----------------------------------ATGAGGAGGTGTGATCTGAGACAG
                                                        ***********************

TaMyb67_BP          TCTCACAACAGCAGGGTTTCTGGAGGAATGTCATCCTCTTTACCTATTCTGCCAAATTCT
TaMyb67_NCBI        TCTCACAACAGCAGGGTTTCTGGAGGAATGTCATCCTCTTTACCTATTCTGCCAAATTCT
TaPhr1_4BL          TCTCACAACAGCAGGGTTTCTGGAGGAATGTCATCCTCTTTACCTATTCTGCCAAATTCT
TaPhr1_4DL          TCTCACAACAGCAGGGTTTCTGGAGGAATGTCATCCTCTTTACCTATTCTGCCAAATTCT
TaPhr1_4AS          TCTCACAACAGCAGGGTTTCTGGAGGAATGTCATCCTCTTTACCTATTCTGCCAAATTCT
                    ************************************************************

TaMyb67_BP          CTGAAAGAAACCTTCCATGGGCCTTACAATCCGCAGCTCACTCCGATGCAAAGGCAACTG
TaMyb67_NCBI        CTGAAAGAAACCTTCCATGGGCCTTACAATCCGCAGCTCACTCCGATGCAAAGGCAACTG
TaPhr1_4BL          CTGAAAGAAACCTTCCATGGGCCTTACAATCCGCAGCTCACTCCGATGCAAAGGCAACTG
TaPhr1_4DL          CTGAAAGAAACCTTCCATGGGCCTTACAATCCACAGCTCACTCCGATGCAAAGGCAACTG
TaPhr1_4AS          CTGAAAGAAACCTTCCATGGGCCTTACAATCCGCAGCTCACTCCGATGCAAAGGCAACTG
                    ******************************** ***************************

TaMyb67_BP          ACGAGTGATCTTGTGCCCTTACATCAGAGTGCACTTCCGTCTGCTACTTTGCACCCAAGA
TaMyb67_NCBI        ACGAGTGATCTTGTGCCCTTACATCAGAGTGCACTTCCGTCTGCTACTTTGCACCCAAGA
TaPhr1_4BL          ACGAGTGATCTTGTGCCCTTACATCAGAGTGCACTTCCGTCTGCTACTTTGCACCCAAGA
TaPhr1_4DL          ACGAGTGATCTTGTGCCCTTACATCAGAGTGCACTTCCGTCTGCTACTTTGCACCCAAGA
TaPhr1_4AS          ACGAGTGATCTTGTGCCCTTACATCAGAGTGCACTTCCATCTGCTACTTTGCACCCAAGA
                    ************************************ ********************

TaMyb67_BP          GCTGGGGCTATGAGATCATCATATGCAGCCTCATTAGGATACTCACCTAATCCTCTTGAT
TaMyb67_NCBI        GCTGGGGCTATGAGATCATCATATGCAGCCTCATTAGGATACTCACCTAATCCTCTTGAT
TaPhr1_4BL          GCTGGGGCTATGAGATCATCATATGCAGCCTCATTAGGATACTCACCTAATCCTCTTGAT
TaPhr1_4DL          GCTGGTGCTATGAGATCATCATATGCAGCCTCATTAGGATACTCACCTAATCCTCTTGAT
TaPhr1_4AS          GCTGGTGCTATGAGATCATCATATGCAGCCTCATTAGGATACTCACCTAATCCTCTGGAT
                    ***** ************************************************ ***

TaMyb67_BP          TCTGCGCTTAACCATGAGAGGCAGTCTATGGTTGCTCCTTTTGCTCCTCAGCCATCAAAT
TaMyb67_NCBI        TCTGCGCTTAACCATGAGAGGCAGTCTATGGTTGCTCCTTTTGCTCCTCAGCCATCAAAT
TaPhr1_4BL          TCTGCGCTTAACCATGAGAGGCAGTCTATGGTTGCTCCTTTTGCTCCTCAGCCATCAAAT
TaPhr1_4DL          TCTGCACCTAACCATGAGAGGCAGTCTATGGTTGCTCCTTTTGCTCCTCAGCCATCAGAT
TaPhr1_4AS          TCTGCGCCTAACCATGAGAGGCAGTCTATGGTTGCTCCTTTTGCTCCTCAGCCATCAGAT
                    ***** * ********************************************** **

TaMyb67_BP          ATTGAAGTATTTCAGACCTTATCTAATAATATCCCTGGAGGACACACTGAGGCAACTTGG
TaMyb67_NCBI        ATTGAAGTATTTCAGACCTTATCTAATAATATCCCTGGAGGACACACTGAGGCAACTTGG
TaPhr1_4BL          ATTGAAGTATTTCAGACCTTATCTAATAATATCCCTGGAGGACACACTGAGGCAACTTGG
TaPhr1_4DL          ATCGAAGTATTTCAGACCTTATCTAATAATATCCCTGGAGGTCACACTGGGGCAACTTGG
TaPhr1_4AS          ATCGAAGTATTTCAGACCTTATCTAATAATATCCCTGGAGGTCACACTGGGGCAACTTGG
                    ** ********************************** ******* **********

TaMyb67_BP          TTCCCAGGTTCGGCTGATGGTTTATCAGATTACAGGGATAACATCCCTGCTTCTGGTAGT
TaMyb67_NCBI        TTCCCAGGTTCGGCTGATGGTTTATCAGATTACAGGGATAACATCCCTGCTTCTGGTAGT
TaPhr1_4BL          TTCCCAGGTTCGGCTGATGGTTTATCAGATTACAGGGATAACATCCCTGCTTCTGGTAGT
TaPhr1_4DL          TTCCCAGGTTCGGCTGATGGTTTATCAGATTACGGGGATAACATCCCTGCTTCTGGTAGT
TaPhr1_4AS          TTCCCAGGTTCGGCTGATGGTTTATCAGATTACAGGGATAACATCCCTGCTTCTGGTAGT
                    ******************************** *************************
```

```
TaMyb67_BP        CAGATCCAGAATGGCGGTCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATGAATGG
TaMyb67_NCBI      CAGATCCAGAATGGCGGTCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATGAATGG
TaPhr1_4BL        CAGATCCAGAATGGCGGTCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATGAATGG
TaPhr1_4DL        CAGATCCAGAATGGCGGTCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATGAATGG
TaPhr1_4AS        CAGATCCAGAACGGCGGTCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATGAATGG
                  ********** ****************************************************

TaMyb67_BP        TGGGCAGAGATAATGAATGATGATTGGAGAGATATTCTAGATGCAACGGCTGCTGATCCC
TaMyb67_NCBI      TGGGCAGAGATAATGAATGATGATTGGAGAGATATTCTAGATGCAACGGCTGCTGATCCC
TaPhr1_4BL        TGGGCAGAGATAATGAATGATGATTGGAGAGATATTCTAGATGCAACGGCTGCTGATCCC
TaPhr1_4DL        TGGGCAGAGATAATGAATGATGATTGGAGAGATATTCTAGATGCAACAGCGGCTGATCCC
TaPhr1_4AS        TGGGCAGAAATAATGAATGATGATTGGAGAGATATTCTAGATGCAACGGCTGCTGATCCC
                  ******** ************************************ ** *********

TaMyb67_BP        CAGTCAAA------------GCCTTCCAATTCGTCTGCATCACAGCCTGCTGTCAACCAG
TaMyb67_NCBI      CAGTCAAA------------GCCTTCCAATTCGTCTGCATCACAGCCTGCTGTCAACCAG
TaPhr1_4BL        CAGTCAAA------------GCCTTCCAATTCGTCTGCATCACAGCCTGCTGTCAACCAG
TaPhr1_4DL        CAGTCAAAGTCCATGGTTCAGCCTTCCAATTCGGCTGCGTCACAGCCTGCTGTCAACCAG
TaPhr1_4AS        CAGTCAAAGTCCATGGTTCAGCCTTCCAATTCGGCTGCATCACAGCCTGCTGTCAACCAG
                  ********            ************* **** ********************

TaMyb67_BP        CCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAGCTAGTCCTCCCAATAGCAACTCT
TaMyb67_NCBI      CCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAGCTAGTCCTCCCAATAGCAACTCT
TaPhr1_4BL        CCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAGCTAGTCCTCCCAATAGCAACTCT
TaPhr1_4DL        CCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAGCTAGTCCTCCCAATAGCAACTCT
TaPhr1_4AS        CCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAGCTAGTCCTCCCAATAGCAACTCT
                  ************************************************************

TaMyb67_BP        GCAGCCAAACAACGGATGAGGTGGACTCCAGAACTCCATGAATGCTTCGTAGACTCTGTA
TaMyb67_NCBI      GCAGCCAAACAACGGATGAGGTGGACTCCAGAACTCCATGAATGCTTCGTAGACTCTGTA
TaPhr1_4BL        GCAGCCAAACAACGGATGAGGTGGACTCCAGAACTCCATGAATGCTTCGTAGACTCTGTA
TaPhr1_4DL        GCAGCCAAACAACGGATGAGGTGGACTCCAGAACTCCATGAATGCTTCGTAGACTCTGTA
TaPhr1_4AS        GCAGCCAAACAACGGATGAGGTGGACTCCAGAACTCCATGAGTGCTTCGTAGACTCTGTA
                  ************************************** ******************

TaMyb67_BP        AATAAGCTTGGTGGTAGTGAAAAAGCTACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTT
TaMyb67_NCBI      AATAAGCTTGGTGGTAGTGAAAAAGCTACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTT
TaPhr1_4BL        AATAAGCTTGGTGGTAGTGAAAAAGCTACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTT
TaPhr1_4DL        AATAAGCTTGGTGGTAGTGAAAAAGCTACTCCCAAGGGTGTCCTGAAGCTTATGAAAGTT
TaPhr1_4AS        AATAAGCTTGGTGGTAGTGAAAAAGCTACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTT
                  ************************************** ******************

TaMyb67_BP        GACGGTTTGACAATATATCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGCTAT
TaMyb67_NCBI      GACGGTTTGACAATATATCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGCTAT
TaPhr1_4BL        GACGGTTTGACAATATATCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGCTAT
TaPhr1_4DL        GATGGTTTGACAATATATCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGCTAT
TaPhr1_4AS        GACGGTTTGACAATATATCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGCTAT
                  ** ********************************************************

TaMyb67_BP        AAGCCAGACCTAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGAAGAGTTGACTCTA
TaMyb67_NCBI      AAGCCAGACCTAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGAAGAGTTGACTCTA
TaPhr1_4BL        AAGCCAGACCTAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGAAGAGTTGACTCTA
TaPhr1_4DL        AAGCCAGACCTAATGGAAGGTACAGCAGAGAAAAAGGACTACCACCGAAGAGTTGACTCTA
TaPhr1_4AS        AAGCCAGACCTAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGAAGAGTTGACTCTA
                  ************* *************** ******************************
```

```
TaMyb67_BP        GACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGCGTCTTCAGATGGAAGTTCAGAAA
TaMyb67_NCBI      GACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGCGTCTTCAGATGGAAGTTCAGAAA
TaPhr1_4BL        GACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGCGTCTTCAGATGGAAGTTCAGAAA
TaPhr1_4DL        GACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGCGCCTTCAGATGGAAGTTCAGAAA
TaPhr1_4AS        GACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGCGCCTTCAGATGGAAGTTCAGAAA
                  ************************************ ********************

TaMyb67_BP        CGTCTTCATGAACAACTTGAGACCCAGAGAAAGTTGCAATTGCGAATTGAAGAACAAGGG
TaMyb67_NCBI      CGTCTTCATGAACAACTTGAGACCCAGAGAAAGTTGCAATTGCGAATTGAAGAACAAGGG
TaPhr1_4BL        CGTCTTCATGAACAACTTGAGACCCAGAGAAAGTTGCAATTGCGAATTGAAGAACAAGGG
TaPhr1_4DL        CGTCTTCATGAACAACTTGAGACCCAGAGAAAGTTGCAGTTGCGAATTGAAGAACAAGGG
TaPhr1_4AS        CGTCTTCATGAACAACTTGAGACCCAGAGAAAGCTGCAGTTGCGAATTGAAGAACAAGGG
                  ******************************** **** ********************

TaMyb67_BP        AAGTATCTTCAGATGATGTTTGAAAAGCAGTCTAAATCCAATACGGAGAAGGTGCAGGAT
TaMyb67_NCBI      AAGTATCTTCAGATGATGTTTGAAAAGCAGTCTAAATCCAATACGGAGAAGGTGCAGGAT
TaPhr1_4BL        AAGTATCTTCAGATGATGTTTGAAAAGCAGTCTAAATCCAATACGGAGAAGGTGCAGGAT
TaPhr1_4DL        AAGTATCTTCAGATGATGTTTGAAAAGCAGTCTAAATCCAATACTGAGAAGGTGCAGGAT
TaPhr1_4AS        AAGTATCTTCAGATGATGTTTGAAAAGCAGTCTAAATCCAATACTGAGAAGGTGCAGGAT
                  ****************************************** **************

TaMyb67_BP        CTATCCTCGGGAGCTACAACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGAAAT
TaMyb67_NCBI      CTATCCTCGGGAGCTACAACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGAAAT
TaPhr1_4BL        CTATCCTCGGGAGCTACAACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGAAAT
TaPhr1_4DL        CTATCCTCGGGAGCTACAACAACCCTATCATCTGAACCGAGCCATCCTGCAACCAGAAAT
TaPhr1_4AS        CTATCCTCGGGAGCTACAACAACCCTATTATCTGAACCGAGCCATCCTGCAACCAGAGAT
                  **************************** ********* ****************** **

TaMyb67_BP        AGGGGTGATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGTGAGTGCCGAA
TaMyb67_NCBI      AGGGGTGATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGTGAGTGCCGAA
TaPhr1_4BL        AGGGGTGATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGTGAGTGCCGAA
TaPhr1_4DL        AGGGATAATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGTGAGTGCCGAA
TaPhr1_4AS        AGGGATAATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGTGAGTGCCGAA
                  **** * ***************** ***********************************

TaMyb67_BP        ATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGGAGATGGCAGAAAGCGAGTCTTCT
TaMyb67_NCBI      ATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGGAGATGGCAGAAAGCGAGTCTTCT
TaPhr1_4BL        ATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGGAGATGGCAGAAAGCGAGTCTTCT
TaPhr1_4DL        ATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGGAGATGGCAGAAAGCGAGTCTTCT
TaPhr1_4AS        ATAGGAGAAACTTCCATGCATGCAGGTGGCAACCAGGAGATGGCAGAAAGCGAGTCTTCT
                  ************  ******************************************

TaMyb67_BP        GAGCCCCTTGCAAATACTAATGATGGTTCCAAGGCCCCGCAAGAGAAGCGCTGATACCCA
TaMyb67_NCBI      GAGCCCCTTGCAAATACTAATGATGGTTCCAAGGCCCCGCAAGAGAAGCGCTGA------
TaPhr1_4BL        GAGCCCCTTGCAAATACTAATGATGGTTCCAAGGCCCCGCAAGAGAAGCGCCGAAGGGTG
TaPhr1_4DL        GACCCCCTTGCAAATACTAATGATGGTTCTAAGGCCCCACAAGAGAAGCGCCGAAGGGTG
TaPhr1_4AS        GACCCCCTTGCAAATACTAATGATGGTTCTAAGGCCCCGCAAGAGAAGCGCCAAAGGGTG
                  ** ************************ ******** ************  *

TaMyb67_BP        GCTTTCTTGTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAA
TaMyb67_NCBI      ------------------------------------------------------------
TaPhr1_4BL        CATGATAGTTAA--------------------------------------------------
TaPhr1_4DL        CATGATAGTTAA--------------------------------------------------
TaPhr1_4AS        CATGATAGTTAA--------------------------------------------------
```

** *TaMyb67*_BP: sequenced *TaMyb67* on the BP clone - BPMYB6; *TaMyb67*_NCBI: CDS of *TaMyb67* in GenBank; *TaPhr1*_4AS/BL/DL: CDS of *TaPhr1* from the wheat sub-genome A, B or D (Ensembl).

** The sequenced *TaMyb67* on *TaMyb67*_BP is identical to the CDS of *TaMyb67* in GenBank (full length: 1326bp); The places highlighted in blue are the differences between *TaMyb67*_BP and *TaMyb67*_4BL/4DL/4AS. Except for these places, the remaining parts of these sequences are identical to each other.

** Sequences highlighted in green indicate the correct recombined *att*B sites.

Fig. 28 Alignment between the sequenced *TaMyb67* clone on BPMYB6, the CDS of *TaMyb67* in GenBank and the CDS of *TaPhr1-A1*, *TaPhr1-B1* and *TaPhr1-D1* from the three wheat sub-genomes.

4.3.1.4 Gateway LR reactions

Following successful BP clones, LR reactions were performed with BPPTF2 and BPMYB6 from the previous step and the reaction products were used to transform *E.coli* individually for selection of successful destination clones. This used hybridised primers of Pubi and PTF(20)/ MYB (18) in the screening PCR, where six out of eight *E.coli* transformants obtained for *TaPtf1* (G1390PTF1: 1–6) and four out of five *E.coli* transformants obtained for *TaMyb67* (G1390MYB67: 1–4) were examined to select LR clones (other transformants failed in cell culture). The priming site for Pubi flanks almost just outside the target sequence on the destination clone (extends approximately 50bp). The hypothesised product sizes at 1493bp for *TaPtf1* and 1376bp for *TaMyb67*, respectively, were confirmed in all transformants as the gel results shown in Fig. 29A (lanes 1–6) and Fig. 29B (lanes 1–4).

4.3.1.5 Sequencing result of G1390PTF1 and G1390MYB constructs

Furthermore, the results of DNA sequencing for justified LR clones (G1390PTF1 and G1390MYB67) also agreed with those of the successful BP clones, with no further mismatch, insertion or deletion found in the sequences (Fig. 30 and Fig. 31). As an outcome of the Gateway cloning, the full-length CDS of *TaPtf1*/*TaMyb67* was successfully introduced into the overexpression vector pGWCUbi1390.

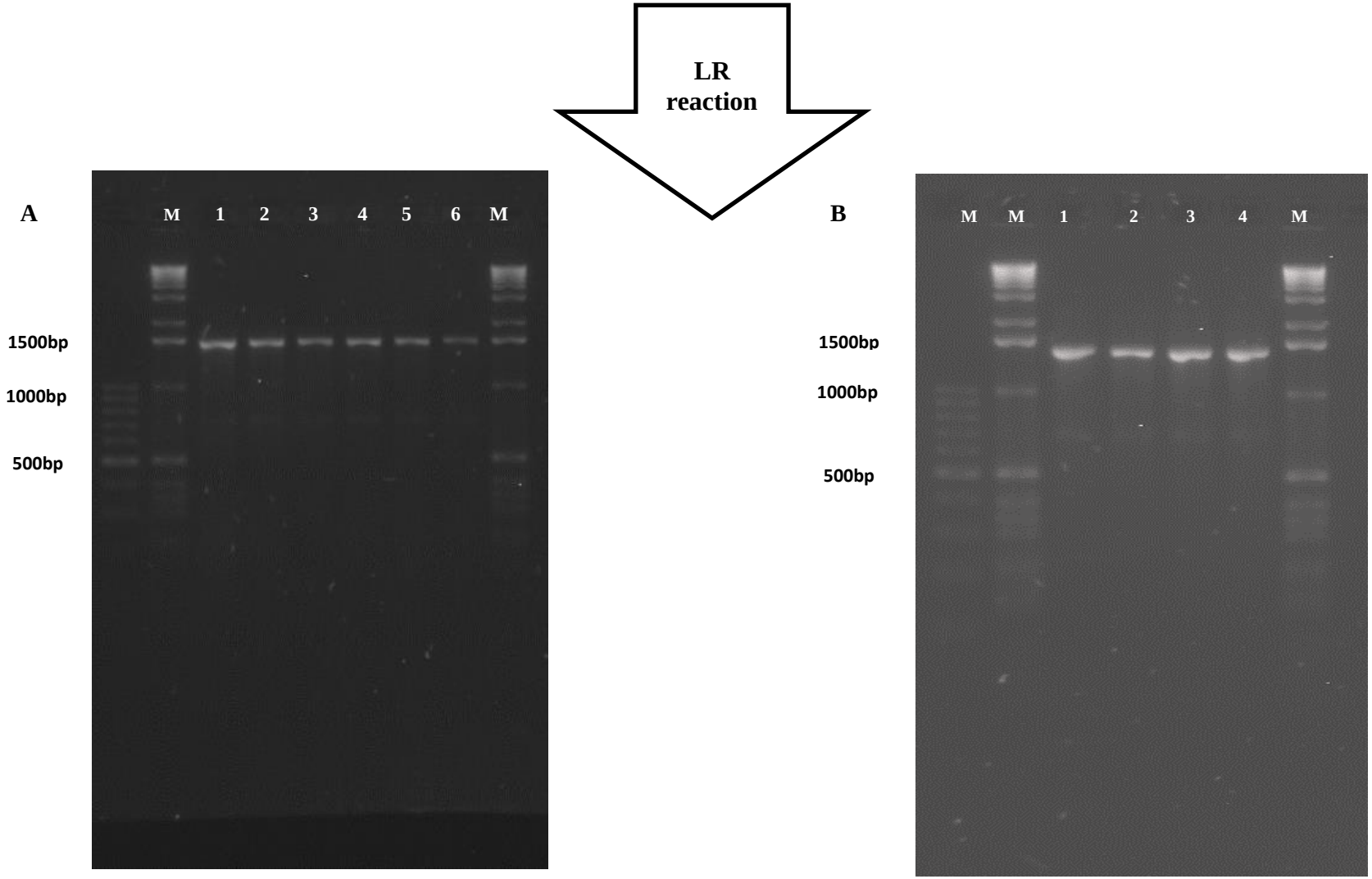
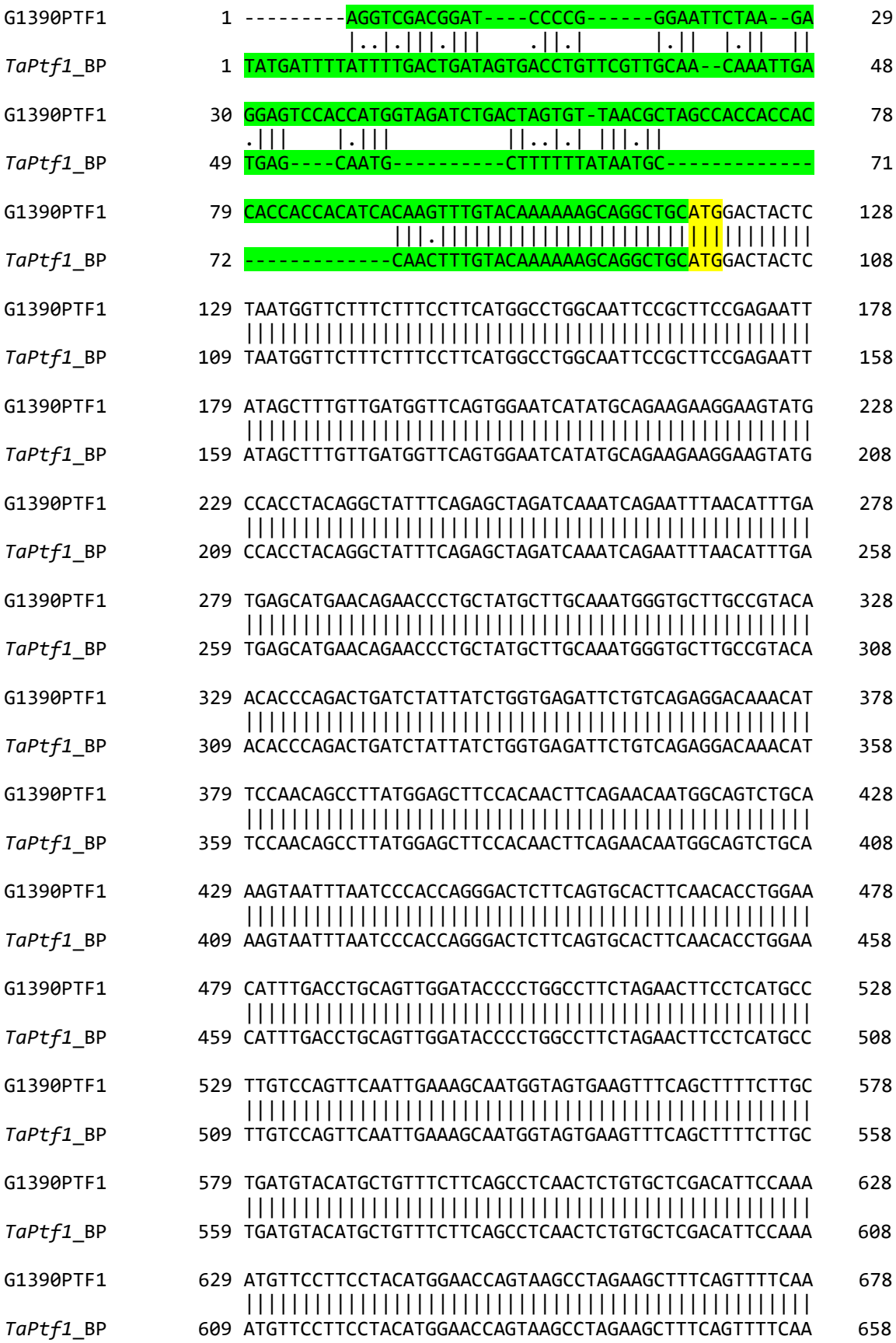
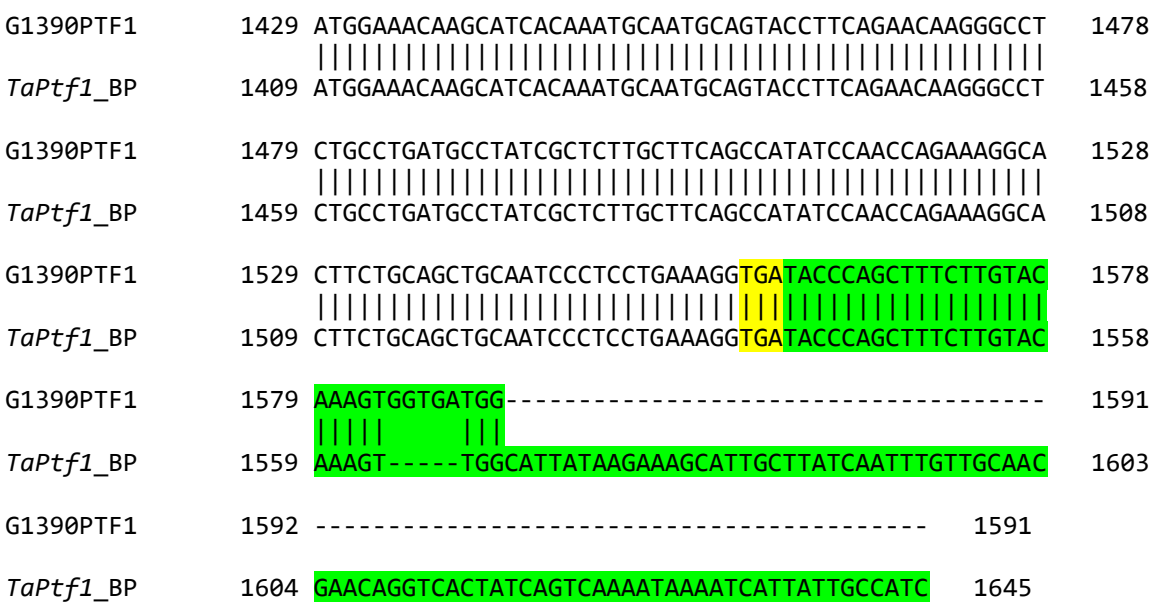


Fig. 29 Results of PCR screening for LR transformants of *TaPtf1* and *TaMyb67*. The screening PCR was performed by using plasmid DNA as templates and hybridised primers of Pubi and PTF(20)/MYB(18). (A) Lanes 1–6: G1390PTF11–6. (B) Lanes 1–4: G1390MYB67 1–4. M: 100bp or 1kb DNA ladder.

(III) Sequencing result of G1390PTF1



```
G1390PTF1       679 GGGATACAAAATGCTGCTATGTTCAACAATACAAGTCATTCAAATGGGAA   728
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP       659 GGGATACAAAATGCTGCTATGTTCAACAATACAAGTCATTCAAATGGGAA   708
G1390PTF1       729 CCTGTCAGTATTTGATGAGGCAACCATGGCATCACTACATGATAGCAAAG   778
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP       709 CCTGTCAGTATTTGATGAGGCAACCATGGCATCACTACATGATAGCAAAG   758

G1390PTF1       779 AATTTCTCAGTGGTAGCATCTCATCTTTTGGTACGGCCGAGCAGTCACAA   828
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP       759 AATTTCTCAGTGGTAGCATCTCATCTTTTGGTACGGCCGAGCAGTCACAA   808

G1390PTF1       829 CTAGCTGGTAGTGGTTTGAAGGCTGAACAACAGGAACAAAATGCGATGTG   878
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP       809 CTAGCTGGTAGTGGTTTGAAGGCTGAACAACAGGAACAAAATGCGATGTG   858

G1390PTF1       879 CAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGATGGCAGTGAGTGAAG   928
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP       859 CAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGATGGCAGTGAGTGAAG   908

G1390PTF1       929 CACAAGGGGCAATGATTCCTTCGAAGATAAGCTCAACGATGCATAACAAT   978
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP       909 CACAAGGGGCAATGATTCCTTCGAAGATAAGCTCAACGATGCATAACAAT   958

G1390PTF1       979 AAAAGTGAGTACCCTGTCCCTATCAGCCATTCTGCTGATGCGCAGAACAA  1028
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP       959 AAAAGTGAGTACCCTGTCCCTATCAGCCATTCTGCTGATGCGCAGAACAA  1008

G1390PTF1      1029 GGCAAATTCAGCTAATGGAAACAGTGCCAGTGCTAAGCCACGAGCAAGGG  1078
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP      1009 GGCAAATTCAGCTAATGGAAACAGTGCCAGTGCTAAGCCACGAGCAAGGG  1058

G1390PTF1      1079 CTCGTCGTGGACAGGCAACTGACCCTCATAGTATTGCTGAACGGCTTCGC  1128
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP      1059 CTCGTCGTGGACAGGCAACTGACCCTCATAGTATTGCTGAACGGCTTCGC  1108

G1390PTF1      1129 AGAGAGAAGATCTCAGAGAGGATGAAAAATCTCCAAGACCTTGTACCAAA  1178
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP      1109 AGAGAGAAGATCTCAGAGAGGATGAAAAATCTCCAAGACCTTGTACCAAA  1158

G1390PTF1      1179 CTCAAATAAGGCAGATAAATCATCAATGCTCGATGAAATAATTGATTATG  1228
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP      1159 CTCAAATAAGGCAGATAAATCATCAATGCTCGATGAAATAATTGATTATG  1208

G1390PTF1      1229 TGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGCATGAGTAGGCTAGGA  1278
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP      1209 TGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGCATGAGTAGGCTAGGA  1258

G1390PTF1      1279 GCTCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAACTGAGGGCCG  1328
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP      1259 GCTCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAACTGAGGGCCG  1308

G1390PTF1      1329 TAGCAATTCACCTCTATCATCTCCAACCGCTTCACAAGGGCTTCTGGACG  1378
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP      1309 TAGCAATTCACCTCTATCATCTCCAACCGCTTCACAAGGGCTTCTGGACG  1358

G1390PTF1      1379 CAGCAGGCCCAGAAGACAGCTTGGTCTTTGAGCAAGAAGTTATAAAGCTG  1428
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
TaPtf1_BP      1359 CAGCAGGCCCAGAAGACAGCTTGGTCTTTGAGCAAGAAGTTATAAAGCTG  1408
```



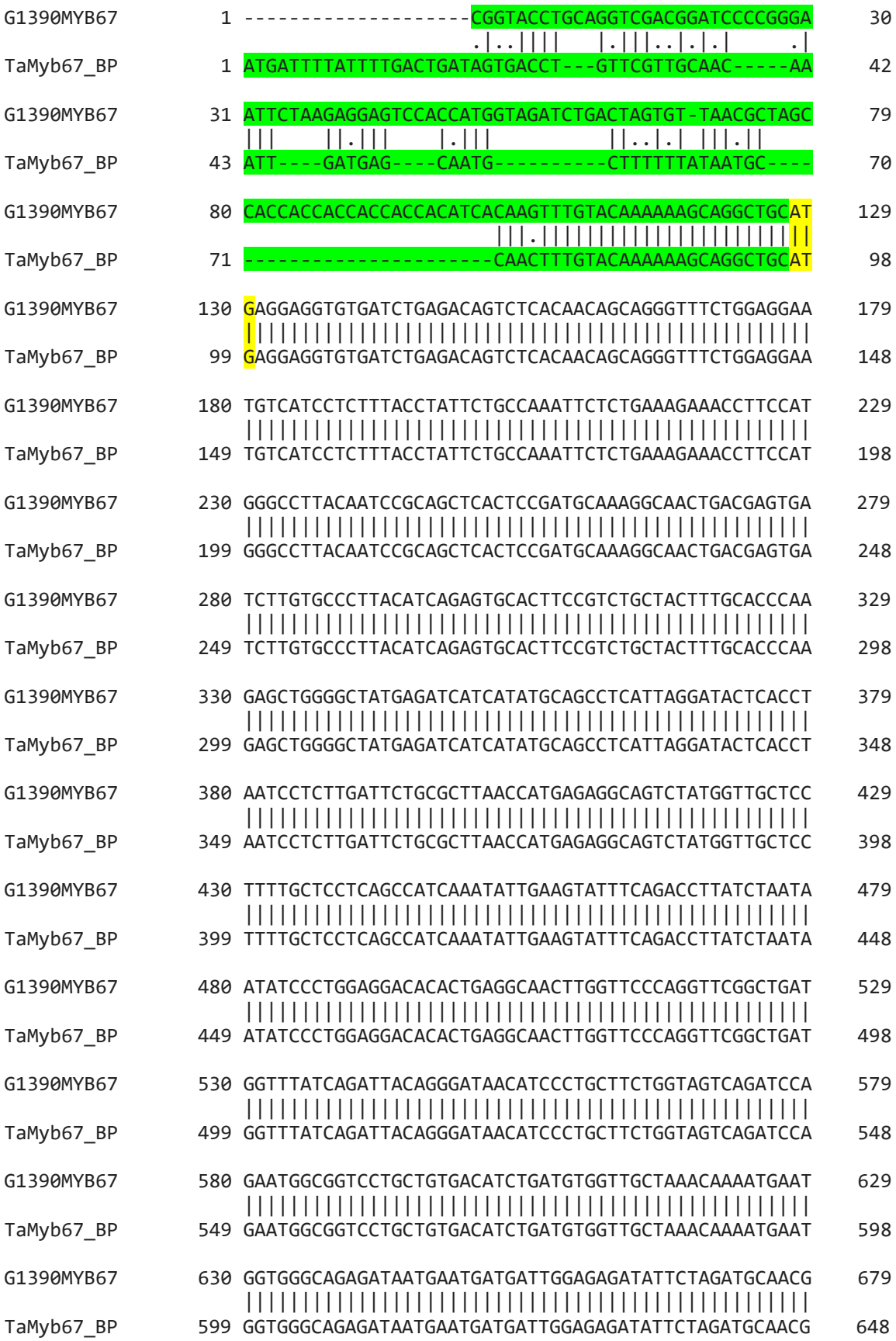
** G1390PTF1: sequenced *TaPtf1* on the G1390PTF1 construct (Gateway LR clone).
*TaPtf*1_BP: sequenced *TaPtf1* on BPPTF2 Gateway BP clone.

** The start codon and stop codon are highlighted in yellow. The sequenced *TaPtf1* on G1390PTF1 is identical to the sequenced *TaPtf1* on the BP clone (1443bp).

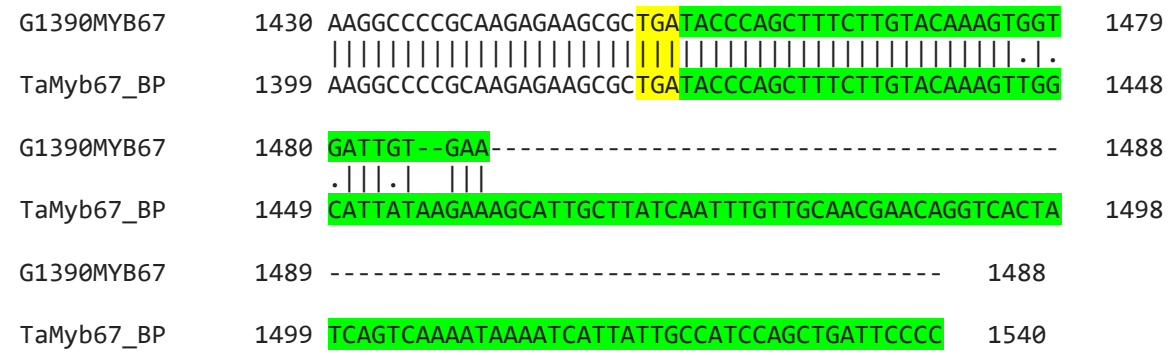
** Sequences highlighted in green are sequenced vector regions.

Fig. 30 Sequencing result for successful *TaPtf1* LR clone. The sequenced *TaPtf1* clone on the G1390PTF1 construct (Gateway LR clone) was compared with that on BPPTF2 (Gateway BP clone) by alignment.

(I) Sequencing result of G1390MYB67



```
G1390MYB67       680 GCTGCTGATCCCCAGTCAAAGCCTTCCAATTCGTCTGCATCACAGCCTGC    729
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP       649 GCTGCTGATCCCCAGTCAAAGCCTTCCAATTCGTCTGCATCACAGCCTGC    698

G1390MYB67       730 TGTCAACCAGCCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAGCTA    779
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP       699 TGTCAACCAGCCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAGCTA    748

G1390MYB67       780 GTCCTCCCAATAGCAACTCTGCAGCCAAACAACGGATGAGGTGGACTCCA    829
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP       749 GTCCTCCCAATAGCAACTCTGCAGCCAAACAACGGATGAGGTGGACTCCA    798

G1390MYB67       830 GAACTCCATGAATGCTTCGTAGACTCTGTAAATAAGCTTGGTGGTAGTGA    879
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP       799 GAACTCCATGAATGCTTCGTAGACTCTGTAAATAAGCTTGGTGGTAGTGA    848

G1390MYB67       880 AAAAGCTACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTTGACGGTTTGA    929
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP       849 AAAAGCTACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTTGACGGTTTGA    898

G1390MYB67       930 CAATATATCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGCTAT    979
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP       899 CAATATATCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGCTAT    948

G1390MYB67       980 AAGCCAGACCTAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGAAGA   1029
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP       949 AAGCCAGACCTAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGAAGA    998

G1390MYB67      1030 GTTGACTCTAGACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGCGTC   1079
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP       999 GTTGACTCTAGACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGCGTC   1048

G1390MYB67      1080 TTCAGATGGAAGTTCAGAAACGTCTTCATGAACAACTTGAGACCCAGAGA   1129
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP      1049 TTCAGATGGAAGTTCAGAAACGTCTTCATGAACAACTTGAGACCCAGAGA   1098

G1390MYB67      1130 AAGTTGCAATTGCGAATTGAAGAACAAGGGAAGTATCTTCAGATGATGTT   1179
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP      1099 AAGTTGCAATTGCGAATTGAAGAACAAGGGAAGTATCTTCAGATGATGTT   1148

G1390MYB67      1180 TGAAAAGCAGTCTAAATCCAATACGGAGAAGGTGCAGGATCTATCCTCGG   1229
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP      1149 TGAAAAGCAGTCTAAATCCAATACGGAGAAGGTGCAGGATCTATCCTCGG   1198

G1390MYB67      1230 GAGCTACAACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGAAAT   1279
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP      1199 GAGCTACAACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGAAAT   1248

G1390MYB67      1280 AGGGGTGATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGT   1329
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP      1249 AGGGGTGATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGT   1298

G1390MYB67      1330 GAGTGCCGAAATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGGAGA   1379
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP      1299 GAGTGCCGAAATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGGAGA   1348

G1390MYB67      1380 TGGCAGAAAGCGAGTCTTCTGAGCCCCTTGCAAATACTAATGATGGTTCC   1429
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
TaMyb67_BP      1349 TGGCAGAAAGCGAGTCTTCTGAGCCCCTTGCAAATACTAATGATGGTTCC   1398
```



** G1390MYB67: sequenced *TaMyb67* on the G1390MYB67 construct (Gateway LR clone). *TaMyb67*_BP: sequenced *TaMyb67* on BPMYB6 Gateway BP clone.

** The start codon and stop codon are highlighted in yellow. The sequenced *TaMyb67* on G1390MYB67 is identical to the sequenced *TaMyb67* on the BP clone (1326bp).

** Sequences highlighted in green are sequenced vector regions.

Fig. 31 Sequencing result for successful *TaMyb67* LR clone. The sequenced *TaMyb67* on the G1390MYB67 construct (Gateway LR clone) was compared with that on BPMYB6 (Gateway BP clone) by alignment.

4.3.1.6 Restriction endonuclease analysis

The segment of T-DNA on the construct plays a vital role in transformation (Gelvin, 2003), while the transgene and its promoter are key components within this region. Depending on the property of their different restriction enzyme sites, G1390PTF1 and G1390MYB67 from the previous step were assessed for a check of these two components by restriction endonucleases as described in method section 4.2.1.3.

G1390PTF1 and G1390MYB67, double digested with XhoI+KpnI, both resulted in four DNA bands, of which the third top band near to 1300bp represented a partial fragment of the ubiquitin promoter (Fig. 32C, P1 and M1 lanes, the band pointed by white arrow). This is because one of the XhoI sites sits in the middle of the ubiquitin promoter while the KpnI site flanks outside the 5' end of the ubiquitin promoter on the construct (Fig. 32B). In lane P2 (Fig. 32C), three DNA bands with sizes of approximately 750bp, 2000bp (Ubi) and 9700bp resulting from the cleavage sites of Kpnl and two HindIII on G1390PTF1 were in agreement with the hypothesis. In lane M2 (Fig. 32C), three DNA bands with sizes of approximately 650bp, 1400bp and 10300bp were also obtained as expected following the cleavage of G1390MYB67 at KpnI and two SphI sites. In lanes P2 and M2, the smallest band, at around 750bp and 650bp respectively, indicates the presence of *TaPtf1* and *TaMyb67* and hence the correctness of the constructs. As such, the overexpression constructs of G1390PTF1 and G1390MYB67 were validated to be eligible for wheat transformation.

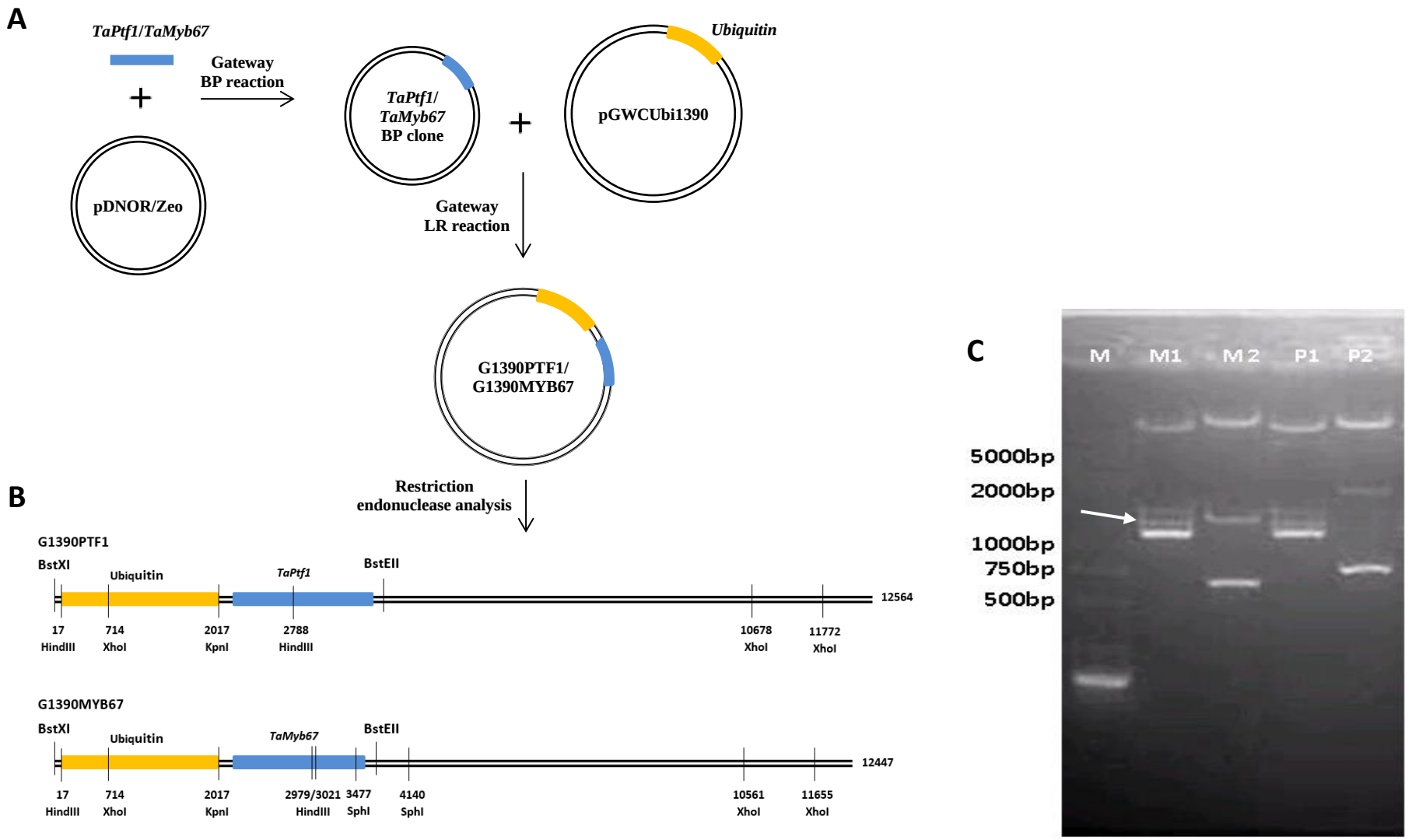


Fig. 32 Restriction endonuclease analysis of G1390PTF1 and G1390MYB67. (A) Generation of G1390PTF1/G1390MYB67. (B) Enzyme sites for restriction endonuclease analysis of G1390PTF1/G1390MYB67. Positions of enzyme sites on the construct are shown in numbers (** The counting of 0 starts from the BstXI restriction enzyme site on the construct). (C) Gel result of restriction endonuclease digestion of G1390PTF1/G1390MYB67. P1: digested G1390PTF1 by XhoI+KpnI. P2: digested G1390PTF1 by KpnI+HindIII. M1: digested G1390MYB67 by XhoI+KpnI. M2: digested G1390MYB67 by KpnI+SphI. M: DNA ladder BM5000.

4.3.2 Transferring G1390PTF1/G1390MYB67 to *Agrobacterium*

The successful constructs of G1390PTF1/G1390MYB67 from the previous step were transferred from *E.coli* to *Agrobacterium* C58C1 by Tri-parental mating as described in method section 4.2.2.1, through which a binary vector system (T-DNA construct and Vir-helper plasmid) was established for *Agrobacterium*-mediated wheat transformation.

Fig. 33 shows the gel result of single colony screening PCR using hybridised primers of Pubi and PTF(20)/MYB(18) when G1390PTF1 and G1390MYB67 were respectively transferred to *Agrobacterium* C58C1. No product was observed in the two negative control lanes (Fig. 33, CK1 and CK2), which provided evidence that there was no contamination in the PCR system. The six single colonies tested for G1390PTF1 (Fig. 33, lanes 1–6) and the other six single colonies tested for G1390MYB67 (Fig. 33, lanes 7–12) were suggested to be positive with the DNA bands at expected sizes of 1493bp for *TaPtf1* and 1376bp for *TaMyb67*, respectively. Furthermore, plasmids of positive *Agrobacterium* clones were selectively prepared (two out of six positive clones) for restriction endonuclease analysis with XhoI and HindIII, as described in method section 4.2.2.2. The digestion with XhoI led to three DNA bands for both G1390PTF1 (Fig. 34, lanes 1–2) and G1390MYB67 (Fig. 34, lanes 3–4), which consisted with the hypothesis that the cleavage of XhoI on the constructs would result in three fragments at 1094bp, 1506bp and 9964bp for G1390PTF1 (or 9847bp for G1390MYB67). The two DNA bands obtained following the digestion with HindIII were also in line with the expected sizes at 2771bp and 9793bp for G1390PTF1 (Fig. 35, lanes 1–2), or 2962bp and 9485bp for G1390MYB67 (Fig. 35, lanes 3–4). These results verified the presence of G1390PTF1/G1390MYB67 in *Agrobacterium* C58C1 for the downstream wheat transformation.

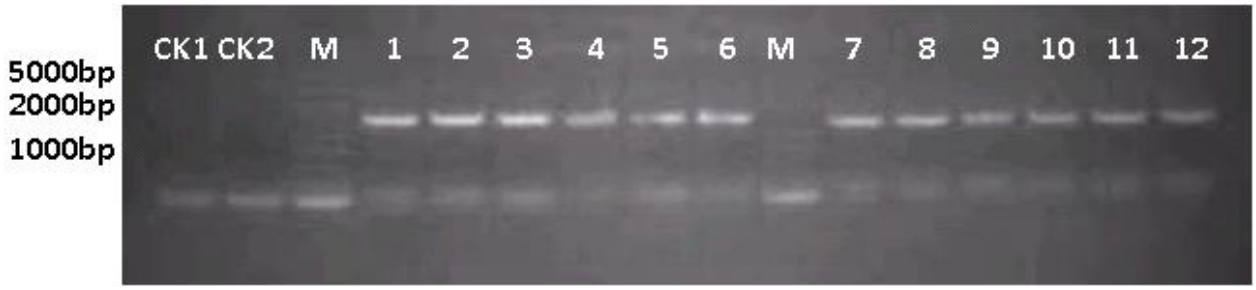


Fig. 33 Screening PCR of *Agrobacterium* single colonies for G1390PTF1 (lanes 1–6) and G1390MYB67 (lanes 7–12). The screening PCR was performed by using hybridised primers of Pubi and PTF(20)/MYB(18). CK1: negative control for G1390PTF1. CK2: negative control for G1390MYB67. M: DNA ladder BM5000.

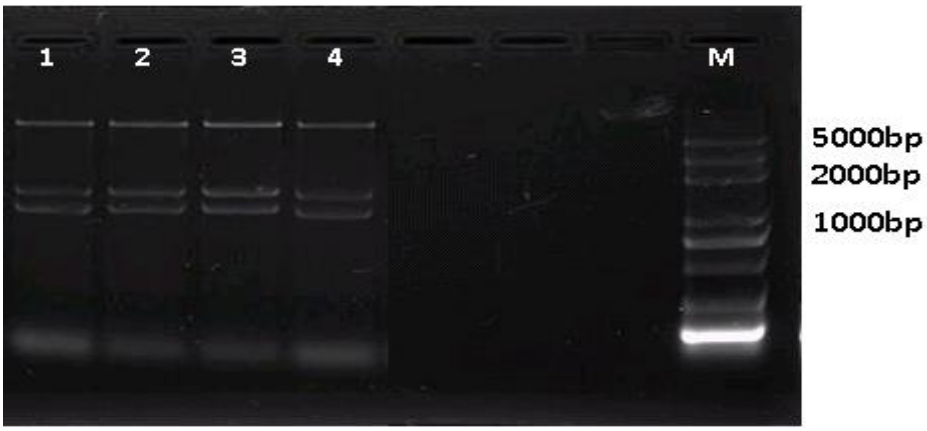


Fig. 34 Restriction endonuclease analysis of G1390PTF1 and G1390MYB67 extracted from *Agrobacterium* by XhoI (XhoI restriction sites, see Fig. 32B). Lanes 1–2: plasmid DNA of G1390MYB67 digested with XhoI. Lanes 3–4: plasmid DNA of G1390MYB67 digested with XhoI. M: DNA ladder BM5000.

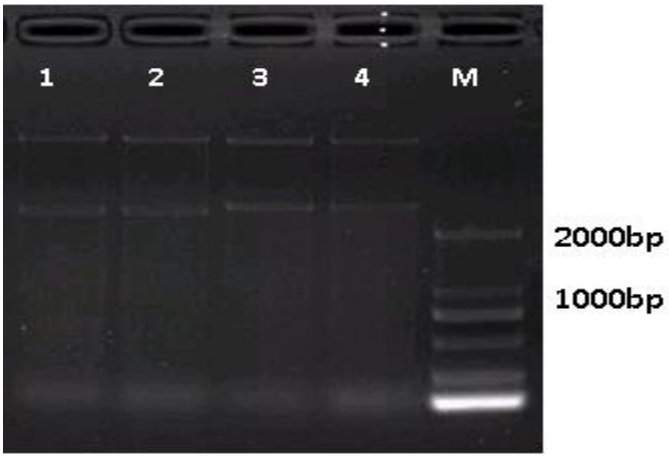


Fig. 35 Restriction endonuclease analysis of G1390PTF1 and G1390MYB67 extracted from *Agrobacterium* by HindIII (HindIII restriction sites, see Fig. 32B). Lanes 1–2: plasmid DNA of G1390PTF1 digested with HindIII. Lanes 3–4: plasmid DNA of G1390MYB67 digested with HindIII. M: DNA ladder BM2000.

4.3.3 Generation of transgenic plants with G1390PTF1/G1390MYB67

4.3.3.1 Analysis of inoculation efficiency by GUS290 histochemical staining assay

Prior to the transformation of G1390PTF1/G1390MYB67, inoculation efficiency was assessed by a histochemical staining assay as described in method section 4.2.3.7, with the *Agrobacterium* C58C1 strain that contains a blank GUS290 vector constructed with a GUS/β-glucuronidase reporter gene driven by a CAMV35S promoter (without a transgene linked to GUS on this occasion). In Fig. 36, several blue-stain areas were observed on immature embryos in each tube as indicated by outcomes of the assay. The production of visible blue colour was due to the transient expression of β-glucuronidase, enabled by the GUS reporter gene, in reaction with X-Gluc as a substrate. This reflected a successful entry of the GUS reporter gene (the vector) into embryo cells after inoculation (Fig. 36) and thus suggested the method of inoculation was effective for the first step of wheat transformation.

4.3.3.2 *Agrobacterium*-mediated transformation with G1390PTF1/G1390MYB67

Following the analysis of inoculation efficiency, the G1390PTF1/G1390MYB67-contained *Agrobacterium* C58C1 culture was prepared to transform the immature embryos pre-incubated before inoculation. The inoculation, co-cultivation and callus induction were performed as described in method section 4.2.3.8, where untransformed immature embryos were used as the negative control. High rates of callus induction resulting from the conducted transformation were observed, with 316 embryonic calli out of 365 being obtained for G1390PTF1-transformation while 311 embryonic calli out of 328 being obtained for G1390MYB67-transformation (Table 18). Full induction of all embryos into embryonic calli was even seen in few plates.

In subsequent steps, culturing the transformed immature embryos on the selection medium and differentiation medium supplement with a low concentration of hygromycin, at 10mg L^{-1} and 20mg L^{-1} respectively, as described in method section 4.2.3.8, resulted in the sprouting of numerous regenerated-shoots in G1397PTF1/G1390Myb67-transformed embryonic calli (Fig. 37). An average of up to 89% induced embryonic calli differentiated for G1390MYB67-transformation (Table 18). Fewer numbers of induced embryonic calli were found to differentiate in G1390PTF1-transformation, where a sharp reduction of rates by nearly 30% to 55% occurred when compared with the callus induction rates (Table 18). Comparatively, the induced calli in the negative control were also observed to partially survive the low concentration of hygromycin with the growth of regenerated-shoots, indicating the selection pressure might need to be enhanced. However, further screening by an increased concentration of hygromycin (35mg L^{-1} or 50mg L^{-1}) resulted in severe browning or necrosis of the embryonic calli as well as growth suppression of the regenerated-shoots, especially under the highest concentration of hygromycin at 50mg L^{-1} (Fig. 37), where growth retardation and declines in greenness were apparent. Meanwhile, the calli in the negative control appeared to struggle to survive. These observations indicated the ineffectiveness of the calli and regenerated-shoots in resistance to the selection by hygromycin, neither in the control nor in the transformation plates. A remedy work attempted by an immediate reduction of the selection pressure (30mg L^{-1}) was not successful to help survive the regenerated-shoots. In consequence of the transformation with G1390PTF1/G1390MYB67, there was no production of transformants, implying that the methods described in this chapter were not effective enough to deliver *TaPtf1*/*TaMyb67* into wheat immature embryos.

Fig. 36 GUS histochemical staining assay with inoculated wheat immature embryos. Pre-incubated immature embryos were inoculated by a GUS290-contained *Agrobacterium* C58C1 provided by Dr Xingguo Ye (CAAS, China) and assessed by GUS staining.

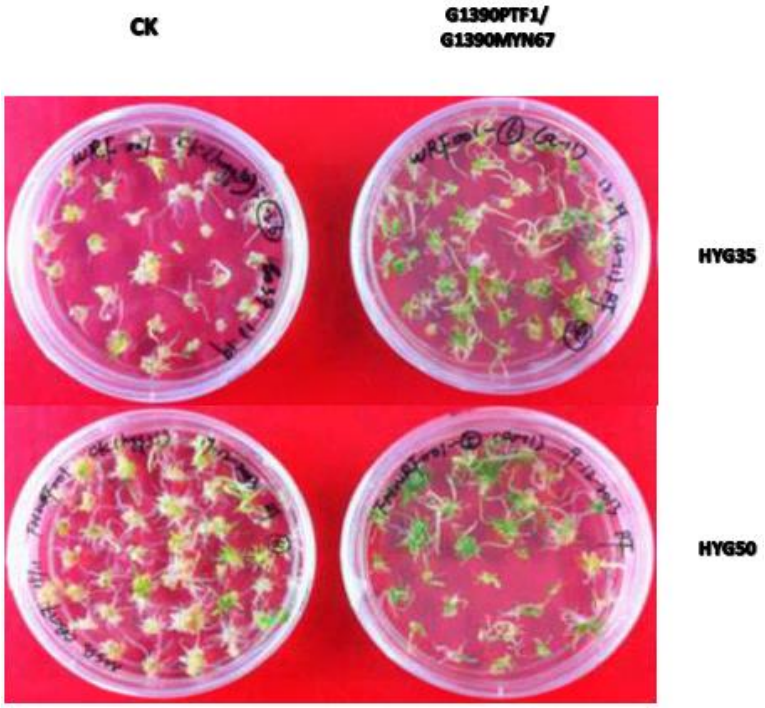


Fig. 37 Shoot regeneration on differentiation medium supplement with hygromycin at two concentrations. CK: negative control. HYG35: hygromycin at 35mg L^{-1}. HYG50: hygromycin at 50mg L^{-1}.

Table 18. Callus induction and regeneration rates of transformation of G1390PTF1 and G1390MYB67 to wheat CB037

Gene/ Plate No.	Immature embryo	Embryonic calli	Differentiated calli	Induction rate	Differentiation rate
G1390TaPTF1					
1	33	24	24	72.73%	72.73%
2	62	62	41	100.00%	66.13%
3	54	38	27	70.37%	50.00%
4	58	40	20	68.97%	34.48%
5	45	44	22	97.78%	48.89%
6	28	28	21	100.00%	75.00%
7	31	31	17	100.00%	54.84%
8	54	49	32	90.74%	59.26%
Total/Average	365	316	204	87.57%	55.89%
G1390TaMYB67					
1	24	22	22	100.00%	91.67%
2	32	30	28	93.75%	87.50%
3	45	43	43	95.56%	95.56%
4	46	42	38	91.30%	82.61%
5	36	35	35	97.22%	97.22%
6	47	41	35	87.23%	74.47%
7	38	38	32	100.00%	84.21%
8	30	29	29	96.67%	96.67%
9	30	29	29	96.67%	96.67%
Total/Average	328	311	291	95.38%	89.62%

**The counting was based on numbers in different callus induction plates

4.4 Discussion

4.4.1 No production of transformant by *Agrobacterium-mediated* transformation with G1390PTF1/G1390MYB67

Drawing on the *Agrobacterium-mediated* transformation to wheat immature embryos as described in this chapter, neither *TaPtf1* nor *TaMyb67* was successfully transformed by the G1390-constructs. So far, the genotype-independent wheat transformation system remains to be challengeable to achieve. Thus, the option of genotypes for good regeneration capability and genotype-specific selection sensitivity is a significant factor for success in wheat transformation (Harwood, 2012). During the experiment of this study, satisfactory callus induction and shoot regeneration were observed in the wheat cultivar CB037, suggesting the capabilities of embryogenesis (embryonic calli that can differentiate) in this wheat genotype were desirable and suitable to be used as the donor of immature embryos for transformation. Besides limits by variations in regeneration ability, the genotypic difference of selection-marker-resistance is also crucial to the outcome of transformation. Hygromycin-resistance as an effective selection marker for wheat transformation has been reported in early studies (Ortiz *et al*., 1996), whereas the sensitivity in response to hygromycin could vary among wheat genotypes. Similar genotypic dependency is also observed in other crops, e.g. rice (Sultana *et al*., 2014). In this study, the induced embryonic calli and regenerated-shoots successfully survived low concentration of hygromycin nevertheless followed by a spread of severe tissue damage and growth suppression happening to the regenerated-shoots during differentiation, where the concentration of hygromycin was 1.5-fold (and 2-fold) higher than the start concentration. Such a result might be attributed to several factors. The selection concentration of hygromycin for wheat cultivar CB037 was not well-established in the existing literature or practice by Xingguo Ye's lab in CAAS and therefore seemed to be uncertain or

need further investigation in terms of an appropriate threshold of concentration to be used for screening transformants. The background of host genetics is important and therefore the tissue damage and growth suppression derived from this study may be a consequence of the determined use of high hygromycin concentrations, which could be an overuse of selection pressure.

Agrobacterium-mediated transformation is to a large extent affected by the susceptibility of a host to the infection with "viral DNA". Historically, studies on the genetic basis of this were more inclined to attribute the most influential effects to the Ti-plasmid as a determinant for the capacity of a target DNA to be preferentially taken up by a host, hence the overall transformation efficiency (Thomashow *et al*., 1980; Yanofsky *et al*., 1985). With accumulation of evidence, however, it is now better understood that the complicated process of infection is genetically controlled by multiple factors that interact with each other within both the *Agrobacterium* strain (e.g. *Agrobacterium* genome) and the plant host (Gelvin, 2003). The binary vector system used for *Agrobacterium*-mediated transformation consists of both the Vir-helper plasmid contained in a given *Agrobacterium* strain and the genetically modified T-DNA construct (e.g. G1390-constructs). Therefore, different *Agrobacterium* strains incorporated with distinct T-DNA constructs may trigger various viral susceptibilities in different hosts. In the experiment of this study, the result of a negative transformation is indicative of the bias away from the infection by the wheat host and may be associated with the establishment of a successful delivery route. Although the inoculation efficiency demonstrated by GUS histochemical staining assay showed that a construct could successfully enter embryo cells, the effectiveness for entry of the T-DNA into the nucleus or its further integration into the wheat genome, which is likely to have a more decisive impact on a successful transformation, was unknown. Whereas the pGW-

CUbi1390 vector had previously been approved in rice for successful transformation (CAAS, unpublished), the *Agrobacterium* strain C58C1 and the wheat cultivar CB037 have different or comparatively more complex genetic backgrounds, which may impact the way they work, which, in turn, make them less effective. Additionally, the loss of constructs in *Agrobacterium* possibly caused by the instability of DNA plasmids or contaminations during cell culture could also increase the risk of a negative transformation. In that case, the tissue damage and growth suppression resulting from this study would be explained by the inabilities to resist to hygromycin in the absence of the resistant construct.

Chapter 5 Generation of transgenic wheat with *TaPtf1* and *TaMyb67* overexpression constructs – Method II (pipeline 2)

5.1 Overview

Given there was no production of transformants in consequence of the conducted transformation described in Chapter 4, the protocols used for transformation were improved. Immature embryos were still used as the explants in the subsequent wheat transformation for a target of obtaining successful transformants. The improvements included a reconstructed vector for overexpression with the selection marker being replaced, a changed wheat cultivar, improved growth conditions, pre-treatment of immature embryos, an altered *Agrobacterium* strain with a modified method for inoculation, optimised medium formulas for tissue culture, etc. The aims of chapter 5 were therefore to show the work of pipeline 2 (Fig. 8), where two new overexpression constructs, 3301PTF1 and 3301MYB67, were generated and transformed into wheat immature embryos. Consequently, successful transformants were obtained and the presence of the *TaPtf1*/*TaMyb67* transgene was confirmed in T0 plants.

5.2 Materials and methods

5.2.1 Generation of 3301PTF1/3301MYB67 construct

The 3301PTF1/3301MYB67 construct was reconstructed from G1390PTF1/G1390MYB67 (described in Chapter 4) through methods of traditional restriction enzyme digestion and ligation using appropriate restriction enzyme sites.

5.2.1.1 Vector for cloning

The pCambia3301 vector used for cloning was provided by Dr Xingguo Ye (CAAS, China). The vector employs a kanamycin-resistant gene (*nptII* gene) as the bacteria selection marker and phosphinothricin (PPT)/glufosinate for plant selection. The *bar* gene (PAT) on the vector confers phosphinothricin (PPT)-resistance. Multiple cloning sites (MCS) are provided on the vector to allow insertion of genes or promoters. Furthermore, the vector is also constructed with GUS/β-glucuronidase reporter gene driven by a CAMV35S promoter for histological staining analysis (GUS reporter gene was removed in this study) (Fig. 38).

5.2.1.2 Cloning method

For reconstruction, plasmids of pCambia3301 and G1390PTF1/G1390MYB67 were prepared to up to 1µg based on concentrations determined by spectrophotometric analysis according to Method 2) as described in 2.5 and 2.6. A two-step reaction of restriction enzyme digestion with BstEII (or EcoR91I, 10U μL^{-1}) and BstXI (10U μL^{-1}), as shown in Table 19, was performed according to the method described in 2.9. Insert (contains*TaPtf1*/*TaMyb67*) and vector fragments resulting from the digestion were assessed by, and purified from, agarose gel electrophoresis according to Method 2) as described in 2.8 and 2.10, followed by the concentrations being adjusted for ligation. The ligation mediated by T4 ligase was conducted as in Table 20, following which competent *E.coli* TOP10 cells were transformed by the ligation

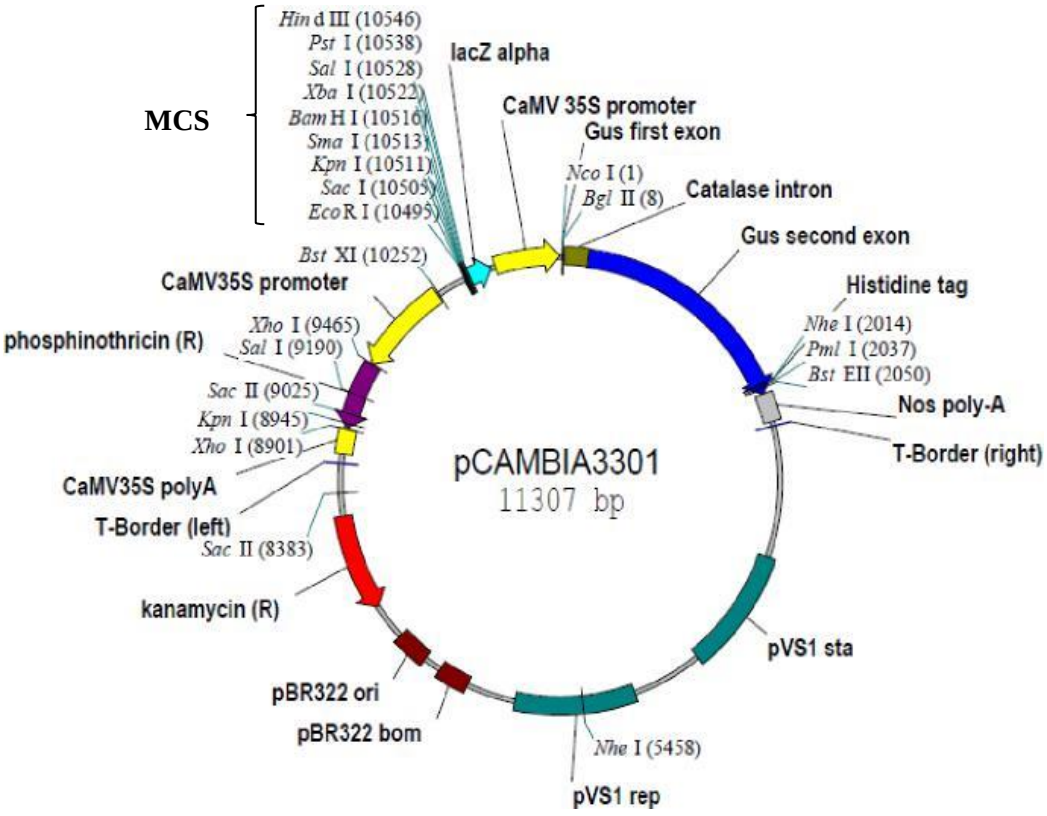


Fig. 38 Vector map of pCambia3301 and the MCS. Source: provided by Dr Xingguo Ye (CAAS, China).

products and selected on LB+Kanamycin (50mg L^{-1}) plates according to Method 2) as described in 2.3 and 2.4.

Positive clones were selected from single colonies picked from each plate by a screening PCR using hybridised primers of Pubi and PTF(20)/MYB(18) according to Method 2) as described in 2.7 and assessed by agarose gel electrophoresis. Plasmids of positive clones (3301PTF1/3301MYB67) were selectively extracted as previously described and assessed by restriction endonuclease analysis with XhoI (Appendix 13) and agarose gel electrophoresis. These plasmids were also assessed by DNA sequencing with primers of Pubi and Tnos according to Method 2) as described in 2.11 to further confirm the clones.

Table 19. Restriction enzyme digestion of pCambia3301, G1390PTF1 and G1390MYB67

Reaction/Sample	G1390PTF1	G1390MYB67	pCambia3301
Plasmid (µL)	42.5	42.5	42.5
10x Orange buffer (µL)	5	5	5
BstEII /EcoR91(µL)	1	1	1
The reaction was incubated at 37°C for 4 hours			
BstXI (µL)	1.5	1.5	1.5
The reaction was incubated at 55°C for 4 hours and inactivated at 80°C for 20 minutes			

Table 20. Ligation of pCambia3301 vector and insert fragments

	3301PTF1	3301MYB67
TaPtf1+Ubi insert fragment (µL)	2	-
TaMyb67+Ubi insert fragment (µL)	-	2
Pcambia3301 vector fragment(µL)	0.5	0.5
ddH_2O (µL)	6	6
T4 Ligation BF (µL)	1	1
T4 Ligase(µL)	0.5	0.5
Total(µL)	10	10
The reaction was incubated at 22°C for 1 hour		

5.2.2 Transfer constructs to *Agrobacterium* competent cells

5.2.2.1 *Agrobacterium* competent cell transfer

Upon successful construct generation, 3301PTF1/3301MYB67 was transferred to competent cells of a disarmed-Ti *Agrobacterium* strain EHA105 according to the protocol provided by Dr Yi Sui (CAAS, China) (Appendix 14). Briefly, 50µL competent cells of the disarmed-Ti *Agrobacterium* strain EHA105 were gently mixed with 3µL (<1ng) of the target plasmid DNA, followed by incubation on ice for 30 minutes. The mixture was frozen in liquid nitrogen for 2 minutes before being incubated at 37°C for 3 minutes and on ice for 2 minutes. The competent cells were mixed with 450µL of YEP medium and cultured in a 28°C shaker-incubator at 150rpm for 3 hours. The cultured cells were spun down and re-suspended to 100µL, which was subsequently used to spread a YEP+Rif (50mg L^{-1})+Kana (50mg L^{-1}) plate to be incubated at 28°C for 48–72 hours. Positive clones were screened by a PCR using hybridised primers of Pubi and PTF(20)/MYB(18) and were assessed by agarose gel electrophoresis. They were also individually incorporated into *Agrobacterium*-glycerol stock according to the method previously described in 4.2.2.1.

5.2.2.2 Extraction of *Agrobacterium* plasmid and restriction endonuclease analysis

Plasmids of a selected *Agrobacterium* positive clone were prepared from a volume of 5mL *Agrobacterium* EHA105 cultures by using EndoFree Plasmid Mini Kit (CWBio, Beijing, China). The method was adapted from manufacturer's protocol (Appendix 9). For restriction endonuclease analysis, plasmids were digested with XhoI according to the method described in 2.9 and were assessed by agarose gel electrophoresis.

5.2.3 Generation of transgenic plants with 3301PTF1/3301MYB67

5.2.3.1 Medium for plant tissue culture

Medium plates were prepared and stored according to the method previously described in 4.2.3.1, but the formula of the medium was modified with additional or different amounts of phytohormones and medium supplements being used to improve transformation efficiency (Appendix 15 for protocol).

5.2.3.2 Replacement of medium

All procedures for plate transfer were carried out according to the method previously described in 4.2.3.2.

5.2.3.3 Preparation of *Agrobacterium* culture and spectrometer analysis

Approximately 2–3 days prior to transformation (inoculation step), 3301PTF1/3301MYB67-contained *Agrobacterium* EHA105 was revived from glycerol stock and its culture was prepared according to the method previously described in 4.2.3.3, except that Gent (100mg $^{L-1}$) was not applied in the YEP medium and the modified-1/10WCC inoculation buffer was used instead for re-suspension.

5.2.3.4 Immature embryos

Wheat plants (Var. AS0556) were grown in CAAS growth room (15–25°C) with unified management in the same manner as previously described in 4.2.3.4. Wheat spikes were collected at different time points according to the required size of immature embryos (2.0–2.2mm) during the post-anthesis stage. Following collection, fresh caryopses (immature seeds) were manually and carefully removed from panicles without injury to the germ area.

5.2.3.5 Seed surface sterilisation and isolation

Approximately 100 healthy caryopses were selected and surface sterilised according to the

method previously described in 4.2.3.5, except that the time for soaking in NaOCl was reduced to 10 minutes with the flask capped and whirled by a shaker-incubator at 150–160rpm, 20–25°C. The sterilised seeds were transferred to a polystyrene petri dish (90x20mm) filled with two layers of filter paper (Ø 85-90mm). Immature embryos were manually and carefully isolated from the germ using sterile forceps under a microscope and placed into a 2mL microcentrifuge tube containing the modified-1/10WCC inoculation buffer (to prevent dehydration) prior to inoculation (Appendix 15 for protocol).

5.2.3.6 Transformation, selection and regeneration of transformants

Inoculation, co-cultivation, callus induction, selection, differentiation and rooting were undertaken as the steps shown in Table 21. The protocols were modified from those previously described in 4.2.3.6. The selection mechanism was replaced by PPT (phosphinothricin) and the pre-incubation was not used (Appendix 15 for protocol). Briefly, immature embryos were separated from wheat seeds as described in the previous section and treated by centrifugation at 7,500rpm for 10 minutes at 4°C, followed by the buffer being pipetted and discarded. The treated immature embryos were directly inoculated with *Agrobacterium* EHA105 containing appropriate constructs (prepared in 5.2.3.3) for 10 minutes in the tube before they were placed in co-cultivation medium plates, with the scutellum orientated upward and the radicle (embryo axis) downward to contact with the medium (Fig. 23). The inoculated immature embryos were co-cultivated for 2 days, followed by callus induction for 5 days. The selection of immature embryos was extended to 4 weeks but divided into to two steps on modified-selection medium plates with appropriate PPT, whereas the regeneration of shoots on modified-differentiation medium plates with appropriate PPT was shortened to 2 weeks. The regenerated-shoots were transferred to rooting medium plates/boxes to grow a root system with a suitable size. Numbers of calli and regenerated-shoots were recorded and

analysed during tissue culture.

5.2.3.7 Analysis of transformation efficiency by GFP fluorescence imaging

A GFP fluorescence imaging assay was performed for the check of inoculation efficiency. The protocol and a blank GFP vector 16318GFP were provided by Dr Xinguo Ye and Dr Zengyan Zhang (CAAS, China), respectively. Briefly, the vector was transferred to competent cells of disarmed-Ti *Agrobacterium* EHA105 as previously described. The isolated immature embryos were inoculated and co-cultivated with 16318GFP-contained *Agrobacterium* EHA105 using the method described in the previous section and cultured in modified-callus induction medium plates at 25°C in the dark for 5 days. The induced calli were selectively used to prepare samples for fluorescence imaging observation, where the calli were cut into thin pieces and placed onto a microscope slide under a cover slice before being examined with a Zeiss LSM700 confocal laser scanning microscope according to manufacturer's instructions.

5.2.3.8 *Agrobacterium*-mediated transformation with 3301PTF1/3301MYB67

The 3301PTF1/3301MYB67-contained *Agrobacterium* EHA105 culture, with OD600=0.5 as already described, was used for transformation to wheat immature embryos. The transformation was performed using the methods described in previous sections (Table 21), where the selection of transformants was carried out by using a low-to-high concentration of PPT. The modified-selection medium supplement with PPT at a start concentration of 5mg L^{-1} was followed by the modified-differentiation medium supplement with PPT at 10mg L^{-1} for further selection. However, the concentration of PPT was reduced to 5mg L^{-1} in the downstream differentiation before the removal of PPT in the modified rooting medium.

5.2.3.9 Growing successful transformants

When the regenerated shoots formed into plantlets on the rooting medium with a reasonable root system

established, these primary transformants were transferred to the soil. In summary, the plantlets were carefully removed from the rooting medium and briefly rinsed with water to remove gel residues. These were then potted into 1–1.5L plastic pots filled with "CAAS prescription mix" compost made of Klasman base substrate (Klasmann-Delimann GmbH, Georg-Klasmann-Str. 2-10, 49744, Germany) blended with Everris Osmocote EC fertiliser NPK blend (Everris International B.V., 4190, CA Geldermalsen, Netherlands). To acclimatise from the tissue culture, the plantlets were protected by a plastic cover in humidity for 1–2 weeks and grown under illumination in a growth room (15–20°C, 45µmol m^{-2} $^{-s}$, 16 hours d^{-1}). Later, all plants were moved to a different growth room (18–28°C, supplemental lighting) for normal growth till full maturation at which point they were harvested.

5.2.3.10 Data collection and analysis

Results of DNA sequencing for constructs were analysed according to the method described in 2.11. Callus induction and shoot regeneration rates were analysed in Excel 2010.

Table 21. *Agrobacterium*-mediated transformation, selection and regeneration - Method II

	Method II
Constructs	3301PTF1/3301MYB67
Selection mechanism	PPT (phosphinothricin)
Wheat variety of Immature embryos	AS0556
Inoculation efficiency analysis	GFP fluorescence imaging analysis
Isolation	50–60 embryos per tube
Pre-incubation	n/a
Inoculation	Pre-treatment by centrifugation; Modified 1/10 WCC buffer; 10 minutes
Co-cultivation	Co-cultivation medium 2 days; 25°C (axis removed before); dark
Callus induction	Modified callus induction medium 5 days; 25°C; dark
Selection	Modified selection medium + PPT (5mg L^{-1}) 14 days; 25°C; dark
Further selection	Modified differentiation medium + PPT (10mg L^{-1}) 14 days; 25°C; dark
Differentiation	Modified differentiation medium +PPT (5mg L^{-1}) 14 days; 25°C; light
Rooting	Modified rooting medium; 25°C; light

5.2.4 Confirmation of the transgene in T0 plants

Once the primary transformants were suitably established (three to four leaves), leaf tissues were sampled to assess the presence of the transgene using two different approaches, the results of which were compared and T0 plants were tagged accordingly.

5.2.4.1 Test by Bar assay to confirm the transgene

A fast-screening assay (Bar assay) using LibertyLink (bar) QuickStix Kit (Envirologix, USA) was individually applied to each leaf sample at room temperature by following the procedure provided in manufacturer's instructions (Appendix 16). For each sample, a piece of 3cm young wheat leaf collected in a 1.5mL extraction tube was manually ground by twisting a disposable plastic pestle (provided in the kit) around at the bottom of the tube or by a Bullet blender after immediate freezing in liquid nitrogen. This was followed by adding 0.5mL of extraction buffer (warmed to room temperature before use) into the sample and mixing well. A QuickStix strip with an absorbent pad attached at the bottom was removed from canisters (warmed to room temperature before use) and placed into the extraction tube to allow the sap of the sample to travel up. The extraction tube was stood for 10 minutes' reaction-time before the assay result was recorded. In the results, a sample showing two red bands (upper and lower) on the QuickStix stripe resulting from the sap travelling up the strip was recorded as a Bar-positive; otherwise, it was recorded as Bar-negative when only one upper red band presented. Any strip that displayed a weak lower band was considered as a debatable position between Bar-positive and Bar-negative.

5.2.4.2 Test by screening PCR to confirm the transgene

Different hybridised primers were designed and generated according to Method 2) as described in 2.2, with the forward/reverse primer annealing to the transgene (*TaPtf1*/

TaMyb67) while its paired primer annealing to either the ubiquitin promoter or the Tnos terminator. Genomic DNA of each leaf sample (<100mg) was extracted individually according to Method 2) as described in 2.12. For each sample, a 20µL PCR reaction system was set up by using 2x Taq MasterMix according to Method 2) as described in 2.7. A blank control was included by using ddH_2O as the template. Two templates of genomic DNA from wild-type wheat AS0556 were used as the negative control while the plasmid DNA of 3301PTF1 and 3301MYB67 were employed as the positive control respectively. The screening PCR was optimised by testing various combinations of different forward and reverse primers along with different amplification conditions. A touchdown PCR was finally used following the thermal profile as one cycle at 94°C for 5 minutes; 20 touchdown cycles of 94°C for 55 seconds, 65–55°C for 45 seconds (annealing temperature reduced by 0.5°C after each cycle) and 72°C for 60 seconds; 30 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 60 seconds; 72°C for 7 minutes. The PCR products were assessed by agarose gel electrophoresis and the results were compared with those results from Bar assay.

5.2.5 Harvesting of T0 plants

Seedlings of the primary transformants were grown in the described growth room (CAAS, China) and harvested at maturity for T1 seeds by Ms Na Li from Dr Xingguo Ye's lab (CAAS, China). Seeds of an individual spike on each single T0 plant were collected into a single bag and all spikes were collected separately. As seeds from different spikes were distinguished, every single bag of seeds made up one transformant subline.

5.3 Results

5.3.1 Generation of 3301PTF1 and 3301MYB67 constructs

5.3.1.1 Restriction enzyme digestion, ligation and PCR screening

The strategy used for reconstruction was to remove the fragment of ubiquitin promoter linked with *TaPtf1*/*TaMyb67* from the G1390PTF1/G1390MYB67 constructs (described in Chapter 4) and re-ligate it to the main skeleton of the pCambia3301 vector, which would lead to the generation of 3301PTF1/3301MYB67 constructs. This strategy was chosen due to a lack of built-in promoter on pCambia3301. Plasmids of pcambia3301 and G1390PTF1/G1390MYB667 were digested with BstXI and BstEII (EcoR91I) as described in method section 5.2.1.2 to obtain corresponding insert and vector DNA fragments as demonstrated in Fig. 39. For pCambia3301 (Fig. 39, lanes 1–2), the lower and upper DNA bands at two sizes supported the hypothesis of approximately 3100bp and 8200bp. The upper DNA bands (vector fragment) were therefore recycled for the downstream ligation. For the digested G1390PTF1 (Fig. 39, lane 3) and G1390MYB67 (Fig. 39, lane 4), the insert fragments, expected to be 3800bp and 3700bp respectively for the two constructs, were obtained as the two smaller DNA bands shown in the gel and therefore recycled separately for the downstream ligation.

Following ligations of the recycled insert and vector fragments as well as *E.coli* transformation as described in method section 5.2.1.2, the results of single colony screening PCR for positive clones of 3301PTF1/3301MYB67 were observed to be in accordance with those previously shown for G1390-constructs (described in Chapter 4), suggesting the insert fragment to be correct. This was due to the use of G1390-constructs as the donor of the insert fragment, although this did not reflect the correctness of the vector fragment. To assess the vector fragment, restriction endonuclease analysis was

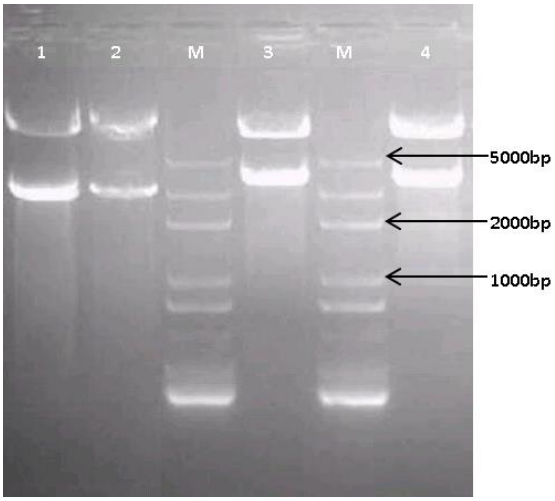


Fig. 39 Digestion products of pCambia3301, G1390PTF1 and G1390MYB67 by BstXI and BstEII (EcoR91I). Lanes 1–2: digestion of pCambia3301. Lane 3: digestion of G1390PTF1. Lane 4: digestion of G1390MYB67. M: DNA ladder BM5000.

subsequently carried out.

5.3.1.2 Restriction endonuclease analysis

The vector fragments were confirmed by restriction endonuclease analysis with XhoI, as described in method section 5.2.1.2, for the obtained three positive clones of 3301PTF1 and two positive clones of 3301MYB67 respectively. Plasmids of pCambia3301, 3301PTF1 and G1390PTF1 (described in Chapter 4) were simultaneously digested for comparison (Fig. 40). In comparison with the digested 3301PTF1, the digested pCambia3301 lacked a middle band, which was consistent with the hypothesis and owing to 3301PTF1 being introduced with an additional XhoI cleavage site on the ubiquitin promoter by the insert fragment. Of the three DNA bands in each lane with expected sizes at approximately 10000bp, 1500bp and 570bp obtained from the digested 3301PTF1 (Fig. 40C, lanes 2–4), the bottom band (570bp), representing the *bar* gene (PAT) between two XhoI cleavage sites, was comparable to the lower band resulting from the digested pCambia3301 (Fig. 40C, lane 1). This was attributed to the role of pCambia3301 as a donor of the vector fragment. In contrast, G1390PTF1 digested with XhoI displayed a bottom band in a different size at around 1100bp, suggesting the expected discrepancy between the vector fragment on G1390PTF1 and that on 3301PTF1 (Fig. 40C, lane 5). Whereas the digested G1390PTF1 had a different bottom band, its middle band was shown to be the same with that in the digested 3301PTF1 due to its role as a donor of the insert fragment. These results together imply the vector fragment originally on pCambia3301 has been successfully reconstructed with the insert fragment originally on G1390PTF1. Likewise, 3301MYB67 examined by XhoI also led to the generation of three DNA bands at approximately 9900bp, 1400bp and 570bp, which was assumed to be a result in agreement with the expectation (Fig. 40D).

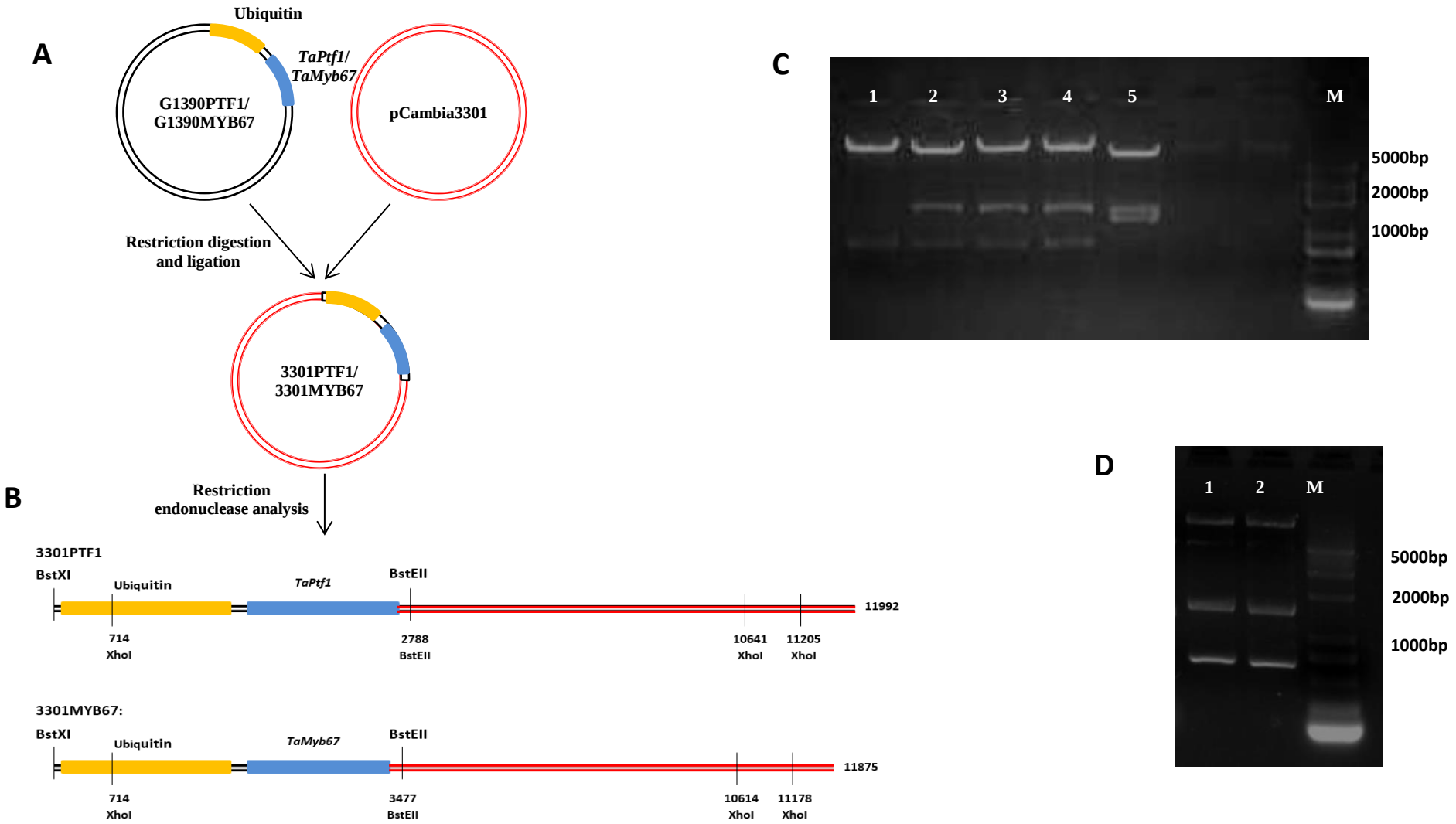


Fig. 40 Restriction endonuclease analysis of 3301PTF1 and 3301MYB67. (A) Generation of 3301PTF1/3301MYB67. (B) Enzyme sites for restriction endonuclease analysis of 3301PTF1/3301MYB67. Positions of enzyme sites on the construct are shown in numbers (**The counting of 0 starts from the BstXI restriction enzyme site on the construct). (C) Gel result of restriction endonuclease digestion of 3301PTF1, pCambia3301 and G1390PTF1 with XhoI. Lane 1: digested pCambia3301. Lanes 2–4: digested 3301PTF1. Lane 5: digested G1390PTF1. (D) Gel result of restriction endonuclease digestion of 3301MYB67 with XhoI. Lanes 1–2: digested 3301MYB67. M: DNA ladder BM5000.

5.3.1.3 Sequencing result of 3301PTF1/3301MYB67 construct

Plasmids of justified 3301PTF1 and 3301MYB67 clones were prepared and subsequently assessed by DNA sequencing, as described in method section 5.2.1.2. The sequenced *TaPtf1* and *TaMyb67* on 3301PTF1 and 3301MYB67, respectively, were found to be identical sequences when compared with those of G1390-constructs (described in Chapter 4) as shown in alignments (Fig. 41 and Fig. 42). Based on such outcomes, the 3301PTF1/ 3301MYB67 overexpression constructs cloned with the full-length CDS of *TaPtf1*/*TaMyb67* were successfully generated and further confirmed as satisfactory for wheat transformation.

(I) Sequencing result of 3301PTF1

```
Length: 1649
# Identity:    1589/1649 (96.4%)
# Similarity:  1589/1649 (96.4%)
# Gaps:          59/1649 (3.6%)
# Score: 9344.0

G1390PTF1          1 ----------------------------AGGTCGACGGATCCCCGGGAA     21
                                                 ||||||||||||||||||| |
3301PTF1           1 CTTGCTTCCTTTACGTTCAACGGTACTGCAGGTCGACGGATCCCCGGG-A     49

G1390PTF1         22 TTCTAAGAGGAGTCCACCATGGTAGATCTGACTAGTGTTAACGCTAGCCA     71
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1          50 TTCTAAGAGGAGTCCACCATGGTAGATCTGACTAGTGTTAACGCTAGCCA     99

G1390PTF1         72 CCACCACCACCACCACATCACAAGTTTGTACAAAAAAGCAGGCTGCATGG    121
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         100 CCACCACCACCACCACATCACAAGTTTGTACAAAAAAGCAGGCTGCATGG    149

G1390PTF1        122 ACTACTCTAATGGTTCTTTCTTTCCTTCATGGCCTGGCAATTCCGCTTCC    171
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         150 ACTACTCTAATGGTTCTTTCTTTCCTTCATGGCCTGGCAATTCCGCTTCC    199

G1390PTF1        172 GAGAATTATAGCTTTGTTGATGGTTCAGTGGAATCATATGCAGAAGAAGG    221
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         200 GAGAATTATAGCTTTGTTGATGGTTCAGTGGAATCATATGCAGAAGAAGG    249

G1390PTF1        222 AAGTATGCCACCTACAGGCTATTTCAGAGCTAGATCAAATCAGAATTTAA    271
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         250 AAGTATGCCACCTACAGGCTATTTCAGAGCTAGATCAAATCAGAATTTAA    299

G1390PTF1        272 CATTTGATGAGCATGAACAGAACCCTGCTATGCTTGCAAATGGGTGCTTG    321
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         300 CATTTGATGAGCATGAACAGAACCCTGCTATGCTTGCAAATGGGTGCTTG    349

G1390PTF1        322 CCGTACAACACCCAGACTGATCTATTATCTGGTGAGATTCTGTCAGAGGA    371
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         350 CCGTACAACACCCAGACTGATCTATTATCTGGTGAGATTCTGTCAGAGGA    399

G1390PTF1        372 CAAACATTCCAACAGCCTTATGGAGCTTCCACAACTTCAGAACAATGGCA    421
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         400 CAAACATTCCAACAGCCTTATGGAGCTTCCACAACTTCAGAACAATGGCA    449

G1390PTF1        422 GTCTGCAAAGTAATTTAATCCCACCAGGGACTCTTCAGTGCACTTCAACA    471
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         450 GTCTGCAAAGTAATTTAATCCCACCAGGGACTCTTCAGTGCACTTCAACA    499

G1390PTF1        472 CCTGGAACATTTGACCTGCAGTTGGATACCCCTGGCCTTCTAGAACTTCC    521
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         500 CCTGGAACATTTGACCTGCAGTTGGATACCCCTGGCCTTCTAGAACTTCC    549

G1390PTF1        522 TCATGCCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGAAGTTTCAGCTT    571
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         550 TCATGCCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGAAGTTTCAGCTT    599
```

```
G1390PTF1        572 TTCTTGCTGATGTACATGCTGTTTCTTCAGCCTCAACTCTGTGCTCGACA    621
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         600 TTCTTGCTGATGTACATGCTGTTTCTTCAGCCTCAACTCTGTGCTCGACA    649

G1390PTF1        622 TTCCAAAATGTTCCTTCCTACATGGAACCAGTAAGCCTAGAAGCTTTCAG    671
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         650 TTCCAAAATGTTCCTTCCTACATGGAACCAGTAAGCCTAGAAGCTTTCAG    699

G1390PTF1        672 TTTTCAAGGGATACAAAATGCTGCTATGTTCAACAATACAAGTCATTCAA    721
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         700 TTTTCAAGGGATACAAAATGCTGCTATGTTCAACAATACAAGTCATTCAA    749

G1390PTF1        722 ATGGGAACCTGTCAGTATTTGATGAGGCAACCATGGCATCACTACATGAT    771
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         750 ATGGGAACCTGTCAGTATTTGATGAGGCAACCATGGCATCACTACATGAT    799

G1390PTF1        772 AGCAAAGAATTTCTCAGTGGTAGCATCTCATCTTTTGGTACGGCCGAGCA    821
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         800 AGCAAAGAATTTCTCAGTGGTAGCATCTCATCTTTTGGTACGGCCGAGCA    849

G1390PTF1        822 GTCACAACTAGCTGGTAGTGGTTTGAAGGCTGAACAACAGGAACAAAATG    871
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         850 GTCACAACTAGCTGGTAGTGGTTTGAAGGCTGAACAACAGGAACAAAATG    899

G1390PTF1        872 CGATGTGCAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGATGGCAGTG    921
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         900 CGATGTGCAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGATGGCAGTG    949

G1390PTF1        922 AGTGAAGCACAAGGGGCAATGATTCCTTCGAAGATAAGCTCAACGATGCA    971
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1         950 AGTGAAGCACAAGGGGCAATGATTCCTTCGAAGATAAGCTCAACGATGCA    999

G1390PTF1        972 TAACAATAAAAGTGAGTACCCTGTCCCTATCAGCCATTCTGCTGATGCGC   1021
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1000 TAACAATAAAAGTGAGTACCCTGTCCCTATCAGCCATTCTGCTGATGCGC   1049

G1390PTF1       1022 AGAACAAGGCAAATTCAGCTAATGGAAACAGTGCCAGTGCTAAGCCACGA   1071
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1050 AGAACAAGGCAAATTCAGCTAATGGAAACAGTGCCAGTGCTAAGCCACGA   1099

G1390PTF1       1072 GCAAGGGCTCGTCGTGGACAGGCAACTGACCCTCATAGTATTGCTGAACG   1121
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1100 GCAAGGGCTCGTCGTGGACAGGCAACTGACCCTCATAGTATTGCTGAACG   1149

G1390PTF1       1122 GCTTCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCTCCAAGACCTTG   1171
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1150 GCTTCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCTCCAAGACCTTG   1199

G1390PTF1       1172 TACCAAACTCAAATAAGGCAGATAAATCATCAATGCTCGATGAAATAATT   1221
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1200 TACCAAACTCAAATAAGGCAGATAAATCATCAATGCTCGATGAAATAATT   1249

G1390PTF1       1222 GATTATGTGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGCATGAGTAG   1271
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1250 GATTATGTGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGCATGAGTAG   1299

G1390PTF1       1272 GCTAGGAGCTCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAACTG   1321
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1300 GCTAGGAGCTCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAACTG   1349
```

```
G1390PTF1       1322 AGGGCCGTAGCAATTCACCTCTATCATCTCCAACCGCTTCACAAGGGCTT   1371
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1350 AGGGCCGTAGCAATTCACCTCTATCATCTCCAACCGCTTCACAAGGGCTT   1399

G1390PTF1       1372 CTGGACGCAGCAGGCCCAGAAGACAGCTTGGTCTTTGAGCAAGAAGTTAT   1421
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1400 CTGGACGCAGCAGGCCCAGAAGACAGCTTGGTCTTTGAGCAAGAAGTTAT   1449

G1390PTF1       1422 AAAGCTGATGGAAACAAGCATCACAAATGCAATGCAGTACCTTCAGAACA   1471
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1450 AAAGCTGATGGAAACAAGCATCACAAATGCAATGCAGTACCTTCAGAACA   1499

G1390PTF1       1472 AGGGCCTCTGCCTGATGCCTATCGCTCTTGCTTCAGCCATATCCAACCAG   1521
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1500 AGGGCCTCTGCCTGATGCCTATCGCTCTTGCTTCAGCCATATCCAACCAG   1549

G1390PTF1       1522 AAAGGCACTTCTGCAGCTGCAATCCCTCCTGAAAGGTGATACCCAGCTTT   1571
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301PTF1        1550 AAAGGCACTTCTGCAGCTGCAATCCCTCCTGAAAGGTGATACCCAGCTTT   1599

G1390PTF1       1572 CTTGTACAAAGTGGTGATGG----------------------------   1591
                     |||||||||||||||||.|
3301PTF1        1600 CTTGTACAAAGTGGTGATTGGAATACAGTAGCAGTCGACGGAGGCGAAC   1648
```

** 3301PTF1: sequenced *TaPtf1* on the 3301PTF1 construct.
G1390PTF1: sequenced *TaPtf1* on the G1390PTF1 construct.

** The start codon and stop codon are highlighted in yellow. The sequenced *TaPtf1* on 3301PTF1 is identical to the *TaPtf1* sequence on G1390PTF1 (1443bp).

** Sequences highlighted in green are sequenced vector regions.

Fig. 41 Sequencing result for successful *TaPtf1* clone on the 3301PTF1 construct. The sequenced *TaPtf1* clone on 3301PTF1 was compared with that on G1390PTF1 (Chapter 4) by alignment.

(II) Sequencing result of 3301MYB67

```
# Length: 1519
# Identity:    1487/1519 (97.9%)
# Similarity:  1487/1519 (97.9%)
# Gaps:          32/1519 (2.1%)
# Score: 7425.0

G1390MYB67         1 --------------CGGTACCTGCAGGTCGACGGATCCCCGGGAATTCTA     36
                                    ||||| ||||||||||||||||||||||||||||||
3301MYB67          1 TACGGGTCTCGATTCGGTA-CTGCAGGTCGACGGATCCCCGGGAATTCTA     49

G1390MYB67        37 AGAGGAGTCCACCATGGTAGATCTGACTAGTGTTAACGCTAGCCACCACC     86
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67         50 AGAGGAGTCCACCATGGTAGATCTGACTAGTGTTAACGCTAGCCACCACC     99

G1390MYB67        87 ACCACCACCACATCACAAGTTTGTACAAAAAAGCAGGCTGCATGAGGAGG    136
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67        100 ACCACCACCACATCACAAGTTTGTACAAAAAAGCAGGCTGCATGAGGAGG    149

G1390MYB67       137 TGTGATCTGAGACAGTCTCACAACAGCAGGGTTTCTGGAGGAATGTCATC    186
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       150 TGTGATCTGAGACAGTCTCACAACAGCAGGGTTTCTGGAGGAATGTCATC    199

G1390MYB67       187 CTCTTTACCTATTCTGCCAAATTCTCTGAAAGAAACCTTCCATGGGCCTT    236
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       200 CTCTTTACCTATTCTGCCAAATTCTCTGAAAGAAACCTTCCATGGGCCTT    249

G1390MYB67       237 ACAATCCGCAGCTCACTCCGATGCAAAGGCAACTGACGAGTGATCTTGTG    286
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       250 ACAATCCGCAGCTCACTCCGATGCAAAGGCAACTGACGAGTGATCTTGTG    299

G1390MYB67       287 CCCTTACATCAGAGTGCACTTCCGTCTGCTACTTTGCACCCAAGAGCTGG    336
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       300 CCCTTACATCAGAGTGCACTTCCGTCTGCTACTTTGCACCCAAGAGCTGG    349

G1390MYB67       337 GGCTATGAGATCATCATATGCAGCCTCATTAGGATACTCACCTAATCCTC    386
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       350 GGCTATGAGATCATCATATGCAGCCTCATTAGGATACTCACCTAATCCTC    399

G1390MYB67       387 TTGATTCTGCGCTTAACCATGAGAGGCAGTCTATGGTTGCTCCTTTTGCT    436
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       400 TTGATTCTGCGCTTAACCATGAGAGGCAGTCTATGGTTGCTCCTTTTGCT    449

G1390MYB67       437 CCTCAGCCATCAAATATTGAAGTATTTCAGACCTTATCTAATAATATCCC    486
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       450 CCTCAGCCATCAAATATTGAAGTATTTCAGACCTTATCTAATAATATCCC    499

G1390MYB67       487 TGGAGGACACACTGAGGCAACTTGGTTCCCAGGTTCGGCTGATGGTTTAT    536
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       500 TGGAGGACACACTGAGGCAACTTGGTTCCCAGGTTCGGCTGATGGTTTAT    549

G1390MYB67       537 CAGATTACAGGGATAACATCCCTGCTTCTGGTAGTCAGATCCAGAATGGC    586
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       550 CAGATTACAGGGATAACATCCCTGCTTCTGGTAGTCAGATCCAGAATGGC    599
```

```
G1390MYB67       587 GGTCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATGAATGGTGGGC    636
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67        600 GGTCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATGAATGGTGGGC    649

G1390MYB67       637 AGAGATAATGAATGATGATTGGAGAGATATTCTAGATGCAACGGCTGCTG    686
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67        650 AGAGATAATGAATGATGATTGGAGAGATATTCTAGATGCAACGGCTGCTG    699

G1390MYB67       687 ATCCCCAGTCAAAGCCTTCCAATTCGTCTGCATCACAGCCTGCTGTCAAC    736
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67        700 ATCCCCAGTCAAAGCCTTCCAATTCGTCTGCATCACAGCCTGCTGTCAAC    749

G1390MYB67       737 CAGCCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAGCTAGTCCTCC    786
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67        750 CAGCCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAGCTAGTCCTCC    799

G1390MYB67       787 CAATAGCAACTCTGCAGCCAAACAACGGATGAGGTGGACTCCAGAACTCC    836
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67        800 CAATAGCAACTCTGCAGCCAAACAACGGATGAGGTGGACTCCAGAACTCC    849

G1390MYB67       837 ATGAATGCTTCGTAGACTCTGTAAATAAGCTTGGTGGTAGTGAAAAAGCT    886
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67        850 ATGAATGCTTCGTAGACTCTGTAAATAAGCTTGGTGGTAGTGAAAAAGCT    899

G1390MYB67       887 ACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTTGACGGTTTGACAATATA    936
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67        900 ACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTTGACGGTTTGACAATATA    949

G1390MYB67       937 TCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGCTATAAGCCAG    986
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67        950 TCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGCTATAAGCCAG    999

G1390MYB67       987 ACCTAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGAAGAGTTGACT   1036
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       1000 ACCTAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGAAGAGTTGACT   1049

G1390MYB67       1037 CTAGACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGCGTCTTCAGAT   1086
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       1050 CTAGACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGCGTCTTCAGAT   1099

G1390MYB67       1087 GGAAGTTCAGAAACGTCTTCATGAACAACTTGAGACCCAGAGAAAGTTGC   1136
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       1100 GGAAGTTCAGAAACGTCTTCATGAACAACTTGAGACCCAGAGAAAGTTGC   1149

G1390MYB67       1137 AATTGCGAATTGAAGAACAAGGGAAGTATCTTCAGATGATGTTTGAAAAG   1186
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       1150 AATTGCGAATTGAAGAACAAGGGAAGTATCTTCAGATGATGTTTGAAAAG   1199

G1390MYB67       1187 CAGTCTAAATCCAATACGGAGAAGGTGCAGGATCTATCCTCGGGAGCTAC   1236
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       1200 CAGTCTAAATCCAATACGGAGAAGGTGCAGGATCTATCCTCGGGAGCTAC   1249

G1390MYB67       1237 AACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGAAATAGGGGTG   1286
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       1250 AACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGAAATAGGGGTG   1299
```

```
G1390MYB67      1287 ATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGTGAGTGCC   1336
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       1300 ATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGTGAGTGCC   1349
G1390MYB67      1337 GAAATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGGAGATGGCAGA   1386
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       1350 GAAATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGGAGATGGCAGA   1399

G1390MYB67      1387 AAGCGAGTCTTCTGAGCCCCTTGCAAATACTAATGATGGTTCCAAGGCCC   1436
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       1400 AAGCGAGTCTTCTGAGCCCCTTGCAAATACTAATGATGGTTCCAAGGCCC   1449

G1390MYB67      1437 CGCAAGAGAAGCGCTGATACCCAGCTTTCTTGTACAAAGTGGTGATTGTG   1486
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301MYB67       1450 CGCAAGAGAAGCGCTGATACCCAGCTTTCTTGTACAAAGTGGTGATTGTG   1499

G1390MYB67      1487 AA----------------   1488
                     ||
3301MYB67       1500 AATACAGTATAGCCACGTT   1518
```

** 3301MYB67: sequenced *TaMyb67* on the 3301MYB67 construct.
G1390MYB67: sequenced *TaMyb67* on the G1390MYB67 construct.

** The start codon and stop codon are highlighted in yellow. The sequenced *TaMyb67* on 3301MYB67 is identical to the *TaMyb67* sequence on G1390MYB67 (1326bp).

** Sequences highlighted in green are sequenced vector regions.

Fig. 42 Sequencing result for successful *TaMyb67* clone on the 3301MYB67 construct. The sequenced *TaMyb67* clone on 3301MYB67 was compared with that on G1390MYB67 (Chapter 4) by alignment.

5.3.2 Transferring 3301PTF1/3301MYB67 to *Agrobacterium*

The successful 3301PTF1/3301MYB67 constructs from the previous step were transferred from *E.coli* to *Agrobacterium* using competent cells of disarmed-Ti *Agrobacterium* EHA105, with positive clones being selected as described in method section 5.2.2.1. The gel result of single colony screening PCR showed no evidence of contamination, as suggested by the negative control (Fig. 43A, lane 7), confirming the DNA bands detected in other lanes to be valid. The three DNA bands obtained from selected single colonies for 3301PTF1 (Fig. 43A, lanes 1–3) and the other three DNA bands obtained from selected single colonies for 3301MYB67 (Fig. 43A, lanes 4–6) agreed with the hypothesised sizes at 1493bp for *TaPtf1* and 1376bp for *TaMyb67*, respectively, and therefore were positive.

Plasmids extracted from selected positive *Agrobacterium* clones were further analysed by restriction endonuclease analysis using XhoI, as described in method section 5.2.2.1. As XhoI was previously used to examine these plasmids in *E.coli*, the results were expected to be consistent with those tested in *E.coli.* In Fig. 43B, the three DNA bands presented in each lane were identical to the results obtained from their *E.coli* plasmids (Fig. 40C and Fig.40D). However, the intensities of the lowest DNA bands (in white frame, Fig. 43B) were significantly undermined, presumably caused by the low copies of *Agrobacterium* plasmids and less dye binding to short lengths of DNA. Thus, *Agrobacterium* EHA105 carrying 3301PTF1 or 3301MYB67 were qualified for the downstream wheat transformation.

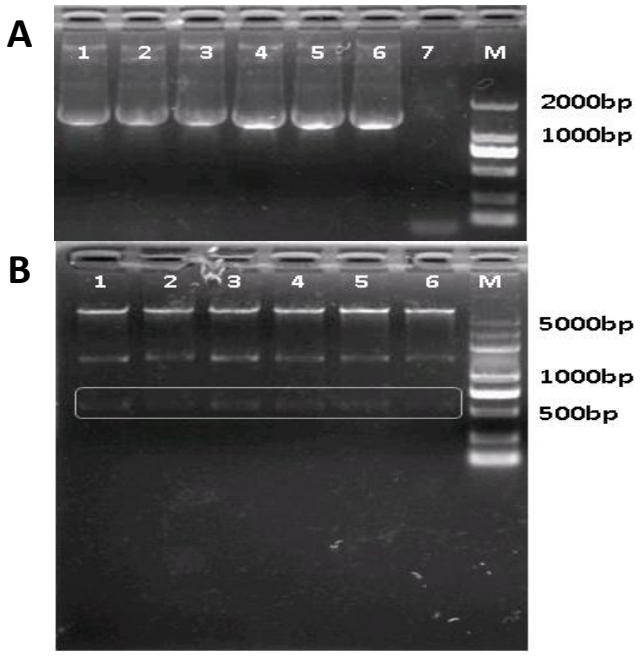


Fig. 43 Screening PCR and restriction endonuclease analysis of 3301PTF1 and 3301MYB67 extracted from *Agrobacterium*. (A) Screening PCR of *Agrobacterium* single colonies for 3301PTF1 (lanes 1–3) and 3301MYB67 (lanes 4–6). The screening PCR was performed by using hybridized primer of Pubi and PTF(20)/MYB(18). Lane 7: Blank control. (B): Restriction endonuclease analysis of 3301PTF1 and 3301MYB67 by XhoI (XhoI restriction sites, see Fig. 40B). Lanes 1–3: plasmid DNA of 3301PTF1 digested with XhoI. Lanes 4–6: plasmid DNA of 3301MYB67 digested with Xho. M: DNA ladder BM2000 or BM5000.

5.3.3 Generation of transgenic plants with 3301PTF1/3301MYB67

5.3.3.1 Analysis of inoculation efficiency by GFP fluorescence imaging

Prior to the transformation of 3301PTF1 and 3301MYB67, inoculation efficiency was analysed with the blank 16318GFP vector as described in method section 5.2.3.7, which provided side evidence that the inoculation method used was effective for further transformation steps. The result was detected by fluorescence imaging; green fluorescence signals exhibited in several areas on the sampled calli. One image, displayed as an example in Fig. 44, showed intense green fluorescence enabled in a small area of calli. The enlarged image suggested the green fluorescence was carried individually by many callus cells, resulting from the transient expression of *hGFP* on 16318GFP contained by *Agrobacterium* EHA105. Since the observed green fluorescence derived from the callus cells which already underwent a brief co-cultivation following inoculation, the result thus may reasonably be indicative of subsequent chances for an entry of the transgene to the nucleus. This would promote the successful integration of the transgene into the wheat genome.

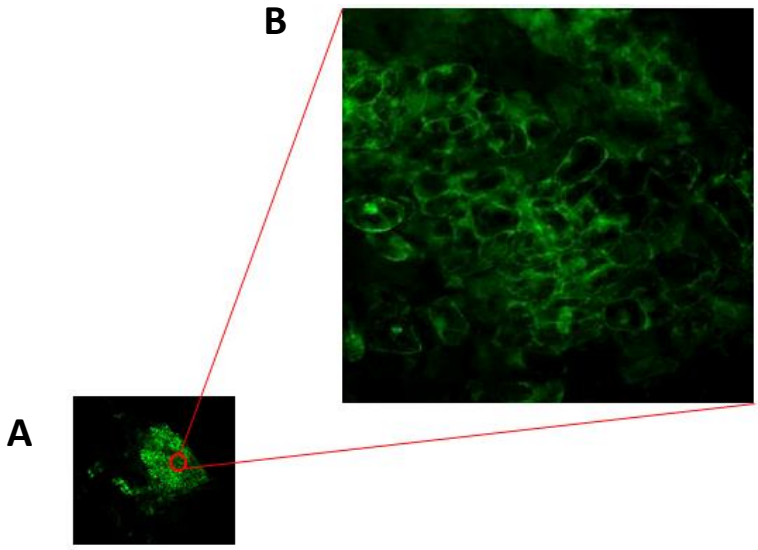


Fig. 44 Image of 16318GFP transformed callus under LSM700. (A) captured image of callus sample. (B) enlarged image of spotted area of the callus sample. Immature embryos were inoculated by 16318GFP-contained *Agrobacterium* EHA105 provided by Dr Yi Sui (CAAS, China) and observed under a LSM700 microscope.

5.3.3.2 *Agrobacterium*-mediated transformation with 3301PTF1/3301MYB67

Following the analysis of inoculation efficiency, the transformation of 3301PTF1/3301MYB67 was conducted as described in method section 5.2.3.8, with the aim to achieve a successful transformation using the improved protocols. Satisfactory production of induced embryonic calli was observed despite limited availability of wheat materials for immature embryos in the experiment (two plates for 3301PTF1-transformation; one plate for 3301MYB67 transformation). According to Table 22, the callus induction rate ranged from 50% to 65%, although this was not as high as the transformation rates of G1390-constructs (described in Chapter 4). For 3301PTF1, the callus induction rate was obtained as 54.84%, where 68 embryonic calli were induced from 124 embryos. For 3301MYB67, 37 embryonic calli grown from 51 embryos led to a slightly higher callus induction rate of 64.7%.

The induced embryonic calli turned out well when they were cultured on the selection medium supplement with a start concentration of PPT as described in method section 5.2.3.8., without their growth tendency being largely affected by a subsequent increase of the selection pressure to a doubled concentration of PPT (10mg L^{-1}). No observation of severe browning/necrosis for an impressive number of embryonic calli reflected that the selection marker *bar* gene (PAT) was expressed in these calli in resistance to the high concentration of PPT, which was an indication of the stably introduced transgene. Although shoots were only partially regenerated, the culture on the differentiation medium with a reduced concentration of PPT (5mg L^{-1}) resulted in a differentiation rate of 22% on average for 3301PTF1-transformation and a slightly higher rate for 3301MYB67-transformation, which was 47% (Table 22).

Whereas the callus induction and differentiation rates appeared not to be dramatically outstanding, the embryonic calli and regenerated-shoots did not overwhelmingly undergo tissue damage or

show growth suppression (Fig. 45). Therefore, those regenerated-shoots in strong resistance to the selection pressure could survive and grow into seedlings. As a result, 26 and seven primary transformants, obtained from the transformation with 3301PTF1 and 3301MYB67 respectively, were believed to have *TaPtf1* and *TaMyb67* successfully delivered into the plants. These might benefit from the effectiveness of improved methods described in this chapter.

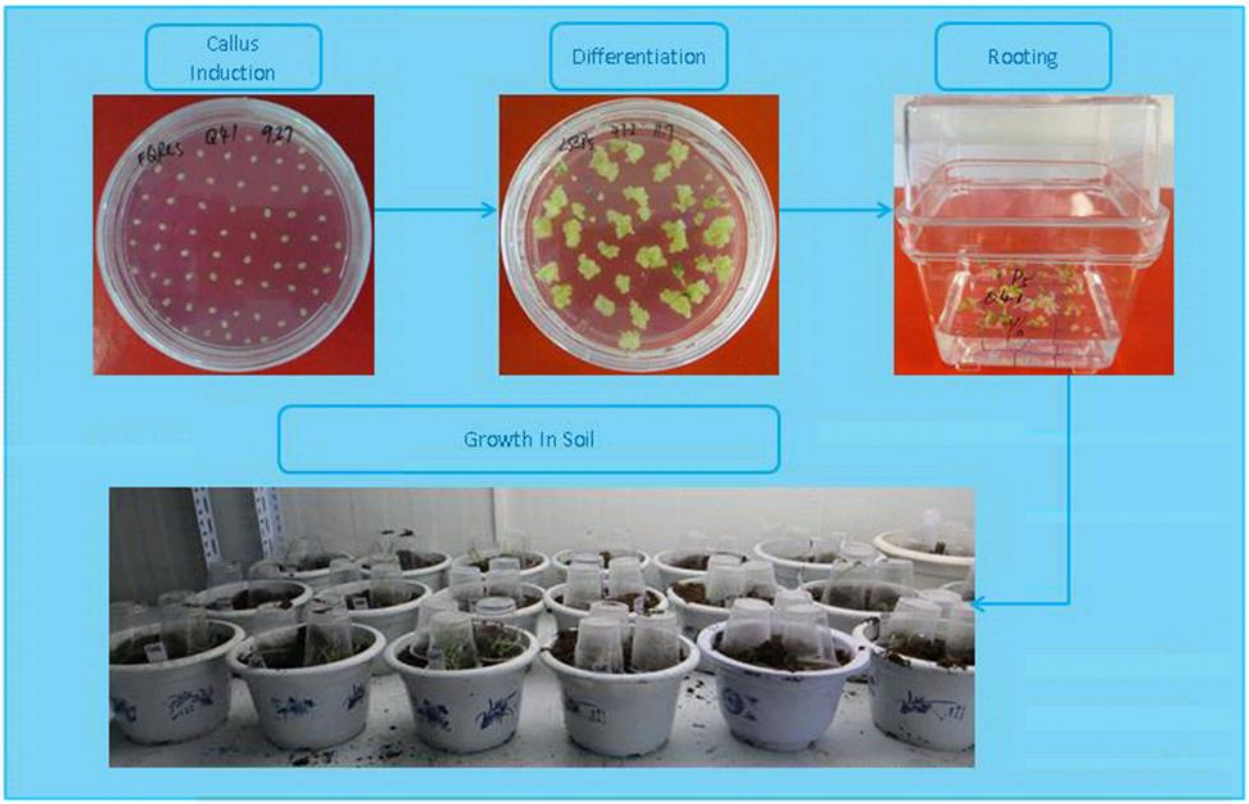


Fig. 45 Key stages of *Agrobacterium*-mediated wheat transformation with 3301PTF1 and 3301MYB67, including callus induction, differentiation, rooting and seedling growth.

Table 22. Callus induction and regeneration rates of transformation of 3301PTF1 and 3301MYB67 to wheat AS0556

Gene/ Plate No.	Immature embryo	Embryonic calli	Differentiated calli	Induction rate	Differentiation rate
3301PTF1					
1	66	33	13	50.00%	20.00%
2	59	35	15	59.32%	25.42%
Total/Average	124	68	28	54.84%	22.58%
3301MYB67					
1	51	33	24	64.71%	47.05%
Total/Average	51	33	24	64.71%	47.05%

**The counting was based on numbers in different callus induction plates

5.3.4 Confirmation of the transgene in T0 plants

The presence of the *TaPtf1*/*TaMyb67* transgene in the primary transformants was assessed by two approaches, Bar assay and PCR screening, as described in method section 5.2.3.10. The assessment at protein level by Bar assay examined the expression of the selection marker *bar* gene (PAT) which is closely linked and co-transformed with *TaPtf1*/*TaMyb67* when the T-DNA integrates into the host genome. Recombination is extremely unlikely between the transgene and the selection marker gene co-transformed in *cis* simultaneously (Kempken and Jung, 2010). Hence, a positive result of Bar assay could report the presence of the transgene. Two consequences were expected on the QuickStix strip during the Bar assay, with the presence of both an upper and a lower red band to be Bar-positive while the presence of a single lower red band to be Bar-negative. The assessment at DNA level using traditional PCR directly tested the presence of the *TaPtf1*/*TaMyb67* transgene. Table 23 shows the hybridised primers used for screening *TaPtf1* transformants (P2 and NosR2) and *TaMyb67* transformants (Ubi1922 and M2718) respectively. The priming sites of these primers on the T-DNA region of the constructs are illustrated in Fig. 47 and Fig. 50 (Appendix 6 for sequence information). A conclusive result for the presence of *TaPtf1*/*TaMyb67* in a sample was determined by a combination of the two assessments, where the sample with positive results in both Bar assay and PCR screening would be counted as transgene positive; otherwise, the sample would be discounted. To prevent the damage caused by sampling, only wheat seedlings with healthier growth were selectively sampled.

Table 23. Primers for PCR screening of the *TaPtf1*/*TaMyb67* transgene

TaPtf1
F: **P2: 5'-ATGGGAACCTGTCAGTATTTG-3'** R: **NosR2: 5'-TGTATAATTGCGGGACTCTAATC-3'**
TaMyb67
F: **Ubi1922: 5'-GCCCTGCCTTCATACGCTATTT-3'** R: **M2718: 5'-TGGAGTCCACCTCATCCGTTGT-3'**

*F: forward primer 5'–3'; R: reverse primer 5'–3'

5.3.4.1 Confirming the transgene of *TaPtf1* in T0 transformants

From the 19 *TaPtf1* transformants (nine from plate-1 and 10 from plate-2 of transformation by 3301PTF1, numbered as sample 1-19) selected and examined by Bar assay, 17 samples were recognised as positive, with an upper and a lower red band both being observed on the QuickStix strip, while sample 15 and 16 displayed the uncertain result of a weak lower band on the QuickStix strip (Fig. 46).

The subsequent PCR screening performed by using P2 and NosR2 primers was expected to amplify a DNA sequence with a product size of approximately 1050bp, which overlaps both the *TaPtf1* transgene and Tnos terminator (Fig. 47; Appendix 6 for full sequence information). In the gel result, the blank control (Fig. 48, lane 20) and negative control lanes (Fig. 48, lanes 21–22) were found to be blank, which qualified the transgenic lanes to be valid. Except for the weak DNA bands observed in lane 7 and lane 11, all the remaining 17 transgenic lanes showed strong DNA bands at the expected size, which was the same as that detected in the positive control (Fig. 48, lane 23), indicating the presence of *TaPtf1* in these samples. Although the insufficient intensity of the two contentious samples was probably attributed to a low concentration of genomic DNA templates, they were counted as transgene negative, while the other 17 samples were accepted as transgene positive.

The comparison between Bar assay and PCR screening resulted in 15 samples (combined transgene-positive rate: 78%) being confirmed as transgene positive while sample 7, 11, 15 and 16 were accordingly recorded as transgene negative.

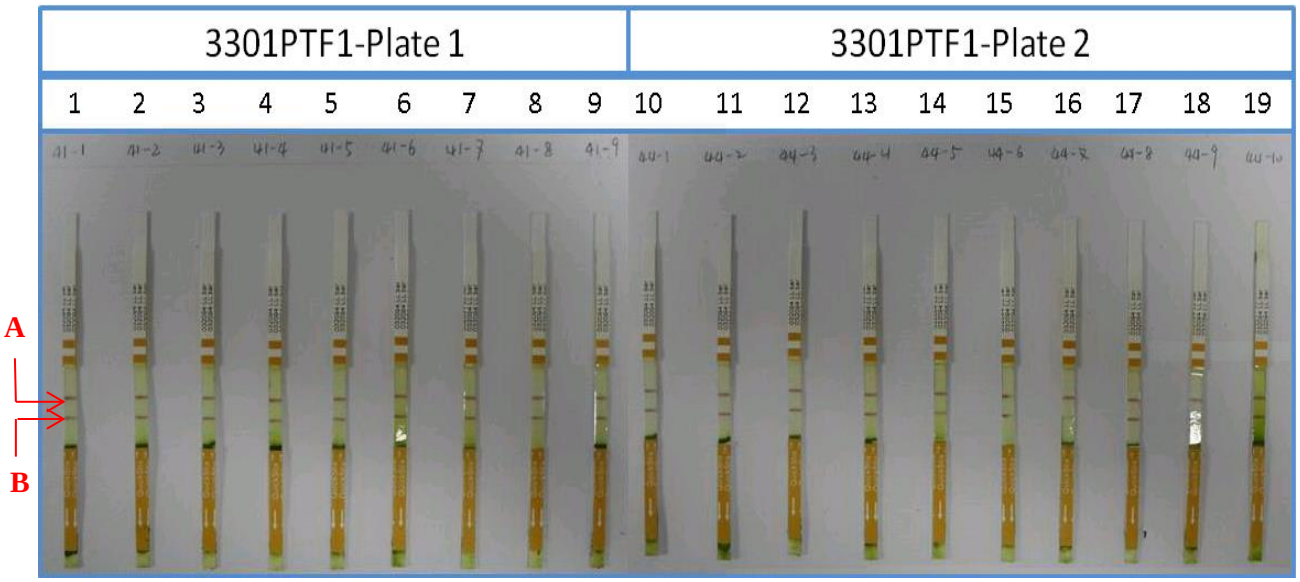


Fig. 46 Results of Bar assay for transformants of 3301PTF1. The results were from two plates of transformation with 3301PTF1 respectively. The Bar-positive rate is 89%. A: upper red band. B: lower red band. **presence of A and B indicates Bar-positive; presence of A only indicates Bar-negative.

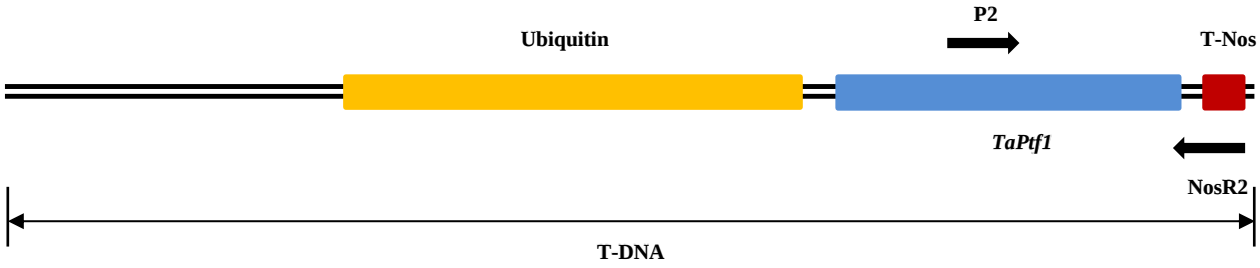


Fig. 47 Priming sites of P2 and NosR2 on the T-DNA region of 3301PTF1 construct.
P2: 5'-ATGGGAACCTGTCAGTATTTG-3'
NosR2: 5'-TGTATAATTGCGGGACTCTAATC-3'

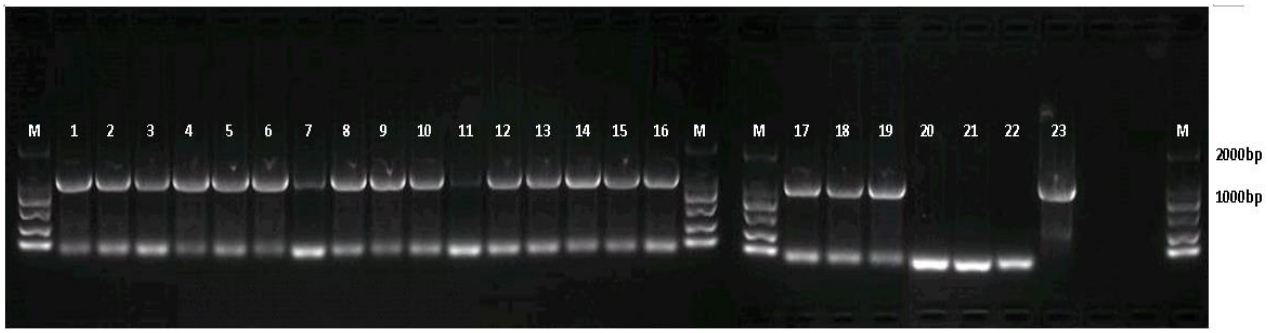


Fig. 48 Results of PCR screening for 3301PTF1 transformants. The transgene-positive rate is 89%. The PCR was performed by using P2 and NosR2 primers. Lanes 1–19: transgenic lanes for transformants of 3301PTF1 (1–9 from plate 1; 10–19 from plate 2). Lanes 20–21: negative control lanes for wild-type wheat AS0556 (genomic DNA of AS0556 as PCR template). Lane 22: blank control lane (ddH_2O as PCR template). Lane 23: positive control lane (plasmid DNA of 3301PTF1 as PCR template). M: DNA ladder BM2000.

5.3.4.2 Confirming transgene of *TaMyb67* in T0 transformants

The test with Bar assay on the six sampled *TaMyb67* transformants (six from the one plate of transformation by 3301MYB67) detected no negative results, with all the six QuickStix strips showing both the upper and lower red bands (Fig. 49). Consistent with this, the gel result of PCR screening obtained strong DNA bands with the expected size at 800bp (Fig. 51, lanes 1–6) in each transgenic lane when the blank control (Fig. 51, lane 7) and negative control lanes (Fig. 51, lanes 8–9) were blank. The Ubi1922 forward primer used for amplification resides at the Ubiquitin promoter, while the M2718 reverse primer anneals to the *TaMyb67* transgene (Fig. 50; Appendix 6 for full sequence information), which led to the product size in these samples to be equivalent to that detected in the positive control (Fig. 51, lane 10). This further validated the presence of *TaMyb67* in the six transformants. Based on the results from the two assessments, the six samples were thus all confirmed as transgene positive.

In addition, the outcomes of Bar assay and PCR screening were demonstrated to be greatly consistent with each other, implying that Bar assay could be used for reporting the presence of the transgene in fast screening of transformants in the downstream experiment.

5.3.5 Growing and harvesting

As a result, T0 plants of 26 and seven primary transformants for *TaPtf1* and *TaMyb67* respectively were grown to maturity, as described in method section 5.2.3.9, and harvested as described in method section 5.2.5 (by individual spike) for T1 seeds from CAAS (China), with 88 *TaPtf1* transformant sublines and 29 *TaMyb67* transformant sublines being obtained.

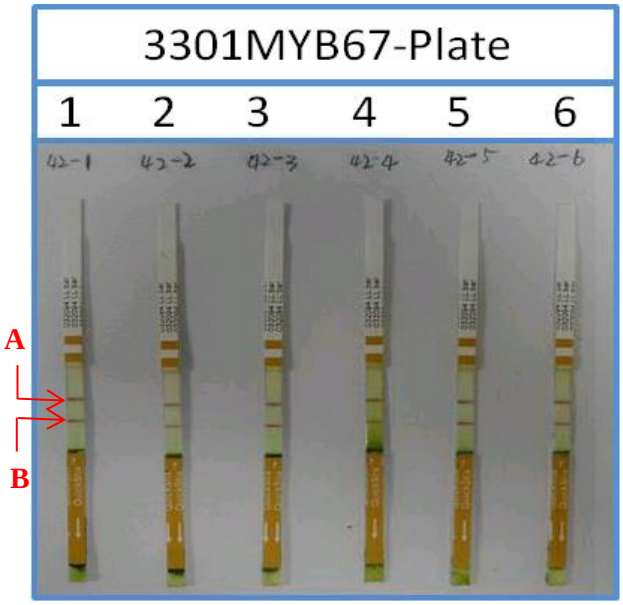


Fig. 49 Results of Bar assay for transformants of 3301MYB67. All the six samples tested were Bar-positive. A: upper red band. B: lower red band. **presence of A and B indicates Bar-positive; presence of A only indicates Bar-negative.

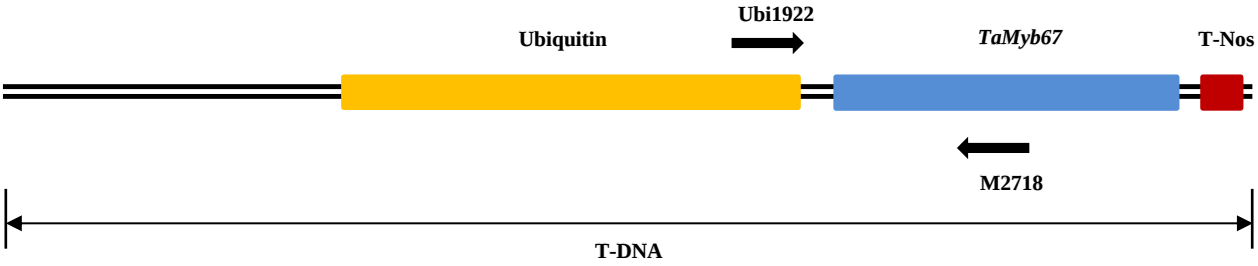


Fig. 50 Priming sites of Ubi1922 and M2718 on the T-DNA region of 3301MYB67 construct.
Ubi1922: 5'-GCCCTGCCTTCATACGCTATTT-3'
M2718: 5'-TGGAGTCCACCTCATCCGTTGT-3'

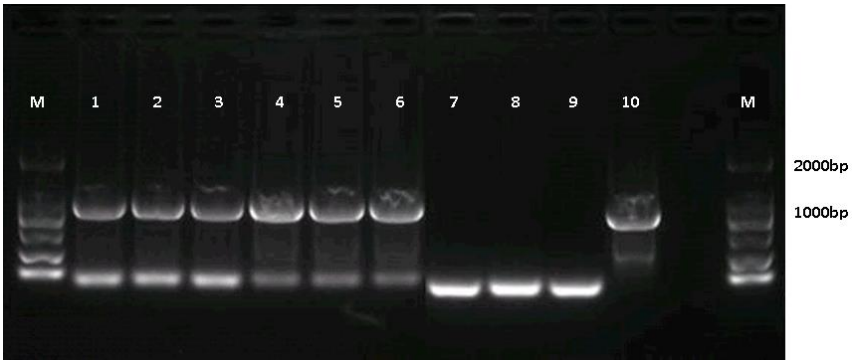


Fig. 51 Results of PCR screening for 3301MYB67 transformants. All the six samples tested were transgene positive. The PCR was performed by using Ubi1922 and M2718 primers. Lanes 1–6: transgenic lanes for transformants of 3301MYB67. Lanes 7–8: negative control lane for wild-type wheat AS0556 (genomic DNA of AS0556 as PCR template). Lane 9: blank control lane (ddH_2O as PCR template). Lane 10: positive control lane (plasmid DNA of 3301MYB67 as PCR template). M: DNA ladder BM2000.

Chapter 6 Characterisation and phenotypic analysis of *TaPtf1*/*TaMyb67* transgenic wheat

6.1 Overview

P deficiency/low-P drastically affects various aspects of plant growth and metabolism (De Groot *et al*., 2001) including photosynthesis (Warren *et al*., 2002), respiration (Marschner, 1995) and biomass partitioning (Li *et al*., 2006). Plants under P deficiency/low-P can optimise P utilisation and maximise P acquisition to achieve a more efficient PUE through P-stress-responsive adaptation mechanisms, which are locally and systemically regulated by TFs (and other regulators) of the underlying molecular pathways in complex networks, leading to phenotypic changes of component traits and plant growth. In P-deficient plants, root growth is favoured over shoot growth, resulting in a typical increase in the root-to-shoot ratio (Lynch and Brown, 2001). The adaptation mechanisms for P acquisition include: (1) Reduced growth of primary root, lateral root branching and elongation, and formation of longer, denser root-hairs or cluster roots (Jungk, 2001; Lambers *et al*., 2006; Lynch and Brown, 2001); (2) Mycorrhizal associations (Smith *et al*., 2011); (3) exudation of organic acids, nucleases/phosphatases and protons (Neumann and Römheld, 1999; Plaxton, 2004; Vance *et al.,* 2003); and (4) expression of high-affinity PTs (Dong *et al*., 1999; Plaxton, 2004). The adaptation mechanisms for P utilisation include: (1) vacuolar P_i storage (Mimura *et al*., 1996); (2) enhanced P recycling (Akhtar *et al*., 2007, 2008); (3) reduced metabolic requirement (Lynch, 1991); and (4) use of P-bypass in the primary carbon or phospholipid metabolism (Plaxton and Carswell, 1999). These were reviewed in section 1.7.

Among these mechanisms, many architectural and morphological changes in roots correlate with local P_i sensing and responding pathways independent of internal P-deficient status

(Svistoonoff *et al*., 2007). Systemic P_i sensing and responding pathways, where interdependent communications between roots and shoots allow P supply and partitioning of the carbon source (photosynthetic sugars) to be regulated between sink and source tissues, are complicated and not well-understood (Thibaud *et al*., 2010). A model describing this suggested that the primary systemic signals (e.g. strigolactone, cytokinin), generated when internal P-deficient status is sensed locally in root tips, are transmitted to shoots through the xylem; this triggers systemic P_i sensing in shoots (Chiou and Lin, 2011). Photosynthetic sugars, the essential carbon source for initiation of cell division and organ growth, are preferentially allocated from shoots to roots through the phloem under the regulation of systemic responding pathways (Li *et al*., 2011). This appears to be crucial to P-stress responses in roots. At the same time, sugars (most likely sucrose), as shoot-derived signalling metabolites, coupled with other secondary systemic signals (e.g. microRNA) travelling from shoots to roots, can further activate systemic P_i sensing in roots and elicit downstream systemic P-stress responses that subsequently enhance P uptake and translocation (Jain *et al*., 2007; Karthikeyan *et al*., 2007). The enhanced P uptake and translocation, in turn, can support photosynthesis in shoots. Furthermore, phytohormones are likely associated with both the sugar signalling and regulatory processes of P-stress-responsive adaptation mechanisms (Franco-Zorrilla *et al*., 2005; Rubio *et al*., 2009).

A positive regulatory role of Os*Ptf1*/*ZmPtf1* for PUE and plant growth under P deficiency/low-P has been discussed in Chapter 3. However, pathways and gene functions involved in a pathway may have diverged and be differentially regulated in wheat due to speciation (Salentijn *et al*., 2007). Initial evidence provided by Aziz *et al*. (2014) has indicated potential functional divergence of *TaPtf1*. Therefore, overexpressing *TaPtf1* in wheat may exhibit species-specific

phenotypes, which have not yet been studied. On the other hand, *TaMyb67* has been presumed to be an ecotypic variation of *TaPhr1-B1*. Whether overexpression of *TaMyb67* can result in the same phenotypes as those observed for overexpression of *TaPhr1-A1*, as described by Wang *et al*. (2013b), requires investigation. Therefore, after obtaining T1 seeds from successful *TaPtf1*/*TaMyb67* transformants as described in Chapter 5, this chapter investigates the expression pattern and phenotypic effects of the *TaPtf1*/*TaMyb67* transgene in selected transgenic lines based on a soil-pot experiment in a glasshouse. The aim is to provide evidence for their functions in PUE and other aspects of wheat growth. As there is more interest in yield and P utilisation, the experiment focused more on the above-ground phase.

6.2 Materials and methods

6.2.1 Soil-pot experiment site and plant management

The experimental site, facilities and support were provided by the Glasshouse office (School of Biosciences, UoN). Seed germination and plant growth were carried out in a Genetic Modification (GM) containment glasshouse (Sutton, Bonington campus, conditions: venting at 25°C, day heating at 20–22°C, night heating at 18–20°C, 16-hour day length, supplemental lighting outside of British Summer Time). Plants were watered daily. Depending on requirements, pest/pathogen management was occasionally applied during plant growth.

6.2.2 Soil-pot experiment compost

Two types of compost for high-P and low-P treatments were recommended by Mr Mark Mecheam from the Glasshouse office and were prepared respectively. Briefly, a total amount of 66L (66x 1L) P-Zero compost was made (by Mr Mark Mecheam) based on a formula used for practical crop research (per 100L compost: 25L silver sand from J. Arthur Bowers, 75L sphagnum peat from Shamrock, 10g NH_4NO_3, 150g KNO_3, 250g ground limestone, 250g magnesium limestone, 40g fritted trace elements). The P-Zero compost was divided into two halves, followed by appropriate amounts of superphosphate (J. Arthur Bowers, Ltd UK, approximately 15.5% P_2O_5, 25kg) being added into each half. For the high-P compost that requires a full-rate of P (1500g/m^3 superphosphate, equals to P=100mg/L), 49.5g superphosphate was mixed into 33L P-Zero. For the low-P compost that requires a 1/10-full-rate of P (150g/m^3 superphosphate, equals to P=10mg/L), 4.95g superphosphate was mixed into the remaining 33L P-Zero. The high-P/low-P compost was filled into 1L plastic pots (with drain-hole at the bottom) and labelled.

6.2.3 T1 Seed germination and elimination of null plants

Before the soil-pot experiment, a screening trial by Bar assay, as the same as previously

described in 5.2.4.1, was performed to re-test the 88 *TaPtf1* transformant sublines and 29 *TaMyb67* transformant sublines harvested from CAAS. Following the trial, five independent *TaPtf1*/*TaMyb67* transformant sublines were selected from the sublines showing positive results in the trial and derived from those T0 plants that tested positive by both Bar assay and PCR screening. The selected transgenic lines, coupled with one wild-type line as the control (wild-type wheat AS0556), led to 11 wheat lines in total for germination of T1 seeds in the soil-pot experiment. For each selected transgenic or the control line, 10 wheat seeds were sown in a 308-well plastic tray (tray: 540x280 mm, 50mm depth, 22x14 wells per tray, long and narrow cube with drain hole at bottom) filled with Levington F2+S compost (Scotts, 14111 Scottslawn Road, Marysville, OH 43041, USA). The compact germination took place in the described glasshouse. After germination, seedlings of the 11 wheat lines were grown for up to 3 weeks (21 days) until null plants being eliminated.

6.2.3.1 Bar assay to eliminate null plants from T1 plants

Once seedlings of the sown 10 seeds of each line were suitably established (three to four leaves), Bar assay was performed as previously described, with the aim to screen six positive wheat seedlings out of 10 for each transgenic line. The test was initially carried out on the first six seedlings. If any negative result appeared, the test was repeated on the seventh to tenth seedlings until six positive seedlings were obtained for each transgenic line.

6.2.3.2 Confirmation of random samples by PCR

Leaf tissues of one wheat seedling randomly chosen from the six wheat seedlings in each transgenic line were sampled to re-confirm the presence of the transgene by PCR amplification. Genomic DNA of each leaf sample (<100mg) was extracted individually according to Method 1) as described in 2.12, which was then used in a 20µL PCR reaction system set up by using Taq

polymerase according to Method 1) as described in 2.7. The blank control, hybridised primers, and touchdown thermal profile were the same as previously described in 5.2.4.2. The PCR products were assessed by agarose gel electrophoresis according to Method 1) as described in 2.8 and the results were compared with those from the Bar assay.

6.2.4 Soil-pot experiment design - P treatments

The soil-pot experiment was designed as shown in Table 24. Immediately following the elimination of null plants upon 3 weeks of pre-growth in normal P supply, as described in the previous section, seedlings of the 11 wheat lines (60 transgenic and six wild-type plants) were individually potted to each 1L plastic pot filled with high-P or low-P compost previously described. For the six plants of each line, three (designated as plant 1 to 3, Fig. 24) were randomly selected and transferred to high-P pots, while the other three (designated as plant 4 to 6, Fig. 24) were moved to low-P pots. The soil pots were randomised on the bench in the described glasshouse, with their locations being rotated on a weekly basis. The plants were grown until full maturation for harvest. The above-ground phase of each plant was collected for the downstream measurement.

6.2.5 Expression analysis by qPCR

The expression levels of *TaPtf1*/*TaMyb67* in the transgenic and control plants were determined by qPCR analysis. However, due to technical limits, only two samples from each treatment of each transgenic or control line were analysed (samples x treatments x lines = 2 x 2 x 11 = 44).

6.2.5.1 Primer design

Wheat actin was used as a reference gene in the qPCR. A pair of wheat actin primers (TaActinF: 5'-ACCTTCAGTTGCCCAGCAAT-3'; TaActinR: 5'-CAGAGTCGAGCACAATACCAGTTG-3'; product size 156bp) were provided by Dr Jose Fernandez from Plant Science. Gene-specific primer pairs used to detect *TaPtf1*/*TaMyb67* in the qPCR were generated according to Method 1) as

Table 24. Soil-pot experiment design

	Group	M1	M2	M3	M4	M5	Control
Treatment	H	M1-1	M2-1	M3-1	M4-1	M5-1	AS-1
		M1-2	M2-2	M3-2	M4-2	M5-2	AS-2
		M1-3	M2-3	M3-3	M4-3	M5-3	AS-3
	L	M1-4	M2-4	M3-4	M4-4	M5-4	AS-4
		M1-5	M2-5	M3-5	M4-5	M5-5	AS-5
		M1-6	M2-6	M3-6	M4-6	M5-6	AS-6

*[group factor] M1–M5: *TaMyb7* transgenic lines; AS: wild-type wheat AS0556 as the control line.

*[treatment factor] H: high-P treatment; L: low-P treatment. For each transgenic or control line, three plants were randomly chosen, designated as 1–3, for high-P treatment; the other three plants, designated as 4–6, were randomly chosen for low-P treatment.

*N=3 in each “group” x “treatment” block; heightened in yellow.

*The same design applies to *TaPtf1* transgenic lines with the same wild-type line.

described in 2.2. Each primer pair was designed to span the corresponding transgene regions that are moderately conserved with the three endogenous gene copies and it was unable at this time to detect the individual gene copy of the sub-genome.

6.2.5.2 Extraction of total RNA and DNase treatment

Leaf tissues (<100mg) from each wheat plant were individually sampled following a 21-day P treatment in the soil-pot experiment. Total RNA was purified and dissolved in 50µL RNase-free ddH_2O in a 1.5mL microcentrifuge tube (RNase-free) according to Method 2) as described in 2.13. Concentrations of the total RNA were determined by spectrophotometric analysis according to Method 1) as described in 2.6. The RNA samples were normalised to 1µg before a DNase treatment was performed according to Method 2) as described in 2.13.

6.2.5.3 cDNA synthesis

Reverse transcription was performed by using the total RNA from the previous step to synthesise the first-strand cDNA according to Method 2) as described in 2.14. For quality control, the first-strand cDNA samples were selectively assessed by PCR amplification using the wheat actin primers and corresponding annealing temperature (56.5°C) following the same method previously described in 3.2.5. A “no-RT” negative control, in which RNase-free ddH_2O was added instead of the reverse transcriptase in the reaction, was also used in the RT-PCR to check genomic DNA contamination. The cDNA samples were diluted by 100 folds for the downstream qPCR.

6.2.5.4 qPCR

The amplification efficiency of each primer pair to be used was determined by a calibration curve generated from a qPCR using five cDNA templates, with concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, prepared from the serial dilution of a mixture of several cDNA samples. The

primer pair was only accepted for qPCR analysis when the amplification efficiency was between 90%–120%. The expression levels of *TaPtf1*/*TaMyb67* were quantified by qPCR analysis, as described in 2.15, using the cDNA samples from the previous step as templates and gene-specific primers for *TaPtf1*/*TaMyb67*, while wheat actin was the reference gene for normalisation. The outputs were processed as described in 2.15.

6.2.5.5 Analysis of qPCR data

The data of Ct values obtained from qPCR analysis was imported to Microsoft Excel 2010 to calculate fold-changes of gene expression normalised to wheat actin and to generate relevant tables and graphs. The fold-change was calculated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen (2001) when the difference of amplification efficiency between primers of the target gene and wheat actin was <5%. Alternatively, the fold-change was calculated by using the Pfaffl method (Pfaffl, 2001) when the difference of amplification efficiency between primers of the target gene and wheat actin was >5%.

6.2.6 Measurement of growth-related traits

Observations were taken in the glasshouse according to the wheat growth stages (GS) described by Bradley *et al*. (2008). Several growth-related traits were surveyed based on measurements. For the main spike, the flag leaf (named as leaf area 1), the first (named as leaf area 2) and second (named as leaf area 3) leaves below the flag leaf were assessed for leaf area by multiplying leaf length (cm) and width (cm) after the flag leaf was fully extended between GS47 (flag leaf sheath opening) and GS51 (first spikelet of ear just visible above flag leaf ligule) (Bradley *et al*., 2008). Chlorophyll concentrations were non-destructively measured for each flag leaf of the main spike at GS51 (Bradley *et al*., 2008) using a chlorophyll meter (SPAD-502, Minolta, USA) provided by South Lab (School of Biosciences, UoN). Measurements

were taken along the leaf at three points (one third, half and two-thirds; avoiding the midrib and major veins). The average of the three point-readings was recorded and designated as chlorophyll concentration index (CCI; ranging from 0 to 99.9). The plants were studied for anthesis date (main spike) at GS61 (Bradley *et al*., 2008), with the flowering of a spike being identified as the day of the first anther exserting from the middle spikelet. The anthesis date was transformed into the number of days by counting from the sowing day to the anthesis day. Plant height was measured with appropriate rulers and tiller numbers (fertile and infertile) were recorded during the post-anthesis (ripening) stage (GS91-GS93) (Bradley *et al*., 2008).

6.2.7 Measurement of yield-related traits after harvest

The plants were grown to full maturity and harvested. The above-ground phase of each wheat plant was collected and measurements of harvest-related traits were carried out following collection. For each wheat plant, the main spike was measured for weight, height and spikelet numbers with appropriate tools (ruler or scale) prior to all seeds being threshed from spikes and the non-seed portion being collected. Seeds and the non-seed portion of each wheat plant were separated in different bags. The seeds were measured for grain numbers and fresh weight, followed by five regular-sized seeds being sampled for the downstream P content analysis. The sampled seeds were measured for fresh weight and, along with the non-seed portion, oven-dried at 80°C for 48 hours for measurements of dry weight (DW). The DW of the sampled seeds were measured to calculate water loss (%). The DW of all seeds, designated as seed biomass, was converted from fresh weight according to the water loss in the sampled seeds. The thousand-grain weight was evaluated based on seed biomass. The DW of the non-seed portion was designated as shoot biomass. The DW of the above-ground phase, designated as above-ground biomass, was calculated as shoot biomass plus seed biomass.

6.2.8 Study of P-related traits

P-related traits, including shoot, seed and above-ground P accumulation, shoot, seed and above-ground P utilisation efficiency as well as the soil P/plant P ratio, were studied based on P content analysis. The experiment of P content analysis was implemented by using an inductively coupled plasma mass spectrometry (ICP-MS, Thermo Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany) and supported by Dr Saul Vazquez Reina from AES (School of Biosciences, UoN). The method was adapted from a protocol provided by Dr Scott Young from AES (School of Biosciences, UoN). Due to facility limitations, only selected wheat lines (3 lines of *TaPtf1*/*TaMyb67*) were analysed. For each wheat plant, the oven-dried sampled-seeds and non-seed portion were measured separately. The P content of all seeds, designated as seed P, was calculated based on the P content analysed for the sampled-seeds, while the P content of the non-seed portion was designated as shoot P. The P content of the above-ground phase, designated as above-ground P, was calculated as shoot P plus seed P. P utilisation efficiency was calculated as shoot/seed/above-ground biomass per mg shoot/seed/aboveground P (biomass/P content) as described by Su *et al*. (2006). The soil P/plant P ratio was calculated based on the remaining soil P estimated from the total amount of compost P.

6.2.8.1 Sampling

Each bag of the oven-dried sampled-seeds was manually and individually ground by mortars and pestles, while each bag of the oven-dried non-seed portion was ground individually by a mill machine. A normalised amount of 200mg powdered samples from each bag was measured and used for microwave digestion and ICP-MS analysis.

6.2.8.2 Hot acid microwave digestion

The normalised seed/non-seed portion samples were individually transferred into vessels

(with smart vent) and digested in 6mL of Conc. HNO_3 (for seeds: 3mL Conc. HNO_3, 3mL Milli-Q H_2O and 2mL 30%; 100 Vols H_2O_2) in a fume cupboard. Each digested sample was made up to a volume of 20mL (for seeds: 15mL) and transferred to a universal tube individually (Appendix 17 for protocol). All samples were diluted five times before ICP-MS analysis.

6.2.8.3 ICP-MS analysis

ICP-MS analysis allows concentrations of major and trace elements in samples to be measured. The analysis of P contents by the ICP-MS was carried out in B06, Gateway Building by Dr Saul Vazquez Reina with equipment and method described in the following. The diluted samples were placed in order on a 4x12 sample rank and introduced by an autosampler (Cetac ASX-520, Omaha, NE, USA) incorporating an ASXpress™ rapid uptake module through a Burgener UMira Mist PEEK nebuliser (Burgener Research International, 89 Latchmere Rd., Kingston Upon Thames, Surrey, England, UK). Two sets of external multielement calibration standards (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA; PlasmaCAL, SCP Science, France) were used for both major elements (concentrations ranged from 0 –30mg L^{-1}) and trace elements (concentrations ranged from 0 –100µg L^{-1}). The calibration of P utilised in-house standard solutions (KH_2PO_4), while in-sample switching was used to measure P in STD mode. Processing of the outputs was undertaken by using Qtegra™ software (Thermo Fisher Scientific, USA) with external cross-calibration between pulse-counting mode and analogue detector modes when required, where all elemental concentrations were converted to mg kg^{-1} for results (by Dr Scott Young).

6.2.9 Statistical analysis

The raw data of measured plant traits for the wheat lines grown in the soil-pot experiment, as the P treatments and randomisation described in 6.2.4 (three in high-P and three in low-P),

was manually inputted into Microsoft Excel 2010, with high-P and low-P groups in each wild-type or transgenic line being distinguished when necessary. The data was initially prepared in Excel, which included the calculations of leaf area, total tiller number, anthesis date, seed water loss, seed dry weight, thousand-grain weight, shoot/seed/above-ground biomass, shoot/seed/above-ground P, shoot/seed/above-ground P utilisation efficiency, soil remaining P (estimated by using the total amounts of compost P) and the P plant/P soil ratio, etc. Each of these measurements/calculations was designated as a single phenotypic variable. Meanwhile, two more variables, named as “group” and “treatment”, were introduced to distinguish different transgenic or control lines and P treatments for the downstream statistical analysis. Also, the transgene expression levels obtained as fold-changes analysed for selected wheat plants by qPCR analysis were included and designated as a variable of “expression” where necessary.

The statistical analysis was performed with Genstat (17th Edition, VSN International Ltd, 2 Amberside House, Wood Lane, Hemel Hempstead, HP2 4TP, UK), where spreadsheets were created by using the data imported from Excel. The variables of “group” and “treatment” were defined as “factors”, while all the other phenotypic variables remained the same; to be “variables”. For optimisation of the data, the variables were initially assessed by normality test (Shapiro- Wilk or W-test) as well as homogeneity test for equality of variance and then transformed by the logarithmic function to improve normality and homogeneity where necessary. Tests and analyses, involving summary statistical analysis, independent two-sample T-tests (Student’s), non-parametric U-tests (Mann-Whitney or M-W), two-way ANOVA and correlation analysis, were undertaken with the outputs further processed in Excel for tables or graphs. Three strategies were used for examination of the transgene effects in T1 plants as

below. The statistical significance was determined at the 5% and 1% confidence limits ($P<0.05$ and $P<0.01$) where necessary.

1) Exploration analysis of phenotypes

Based on the overall six plants of each line, phenotypic variables of each transgenic or control line were described by statistical summary analysis as means and standard errors of means (S.E.M.). To identify significant differences between each transgenic and the control line, variables satisfactory for homogeneity and normality were parametrically assessed by independent two-sample T-tests (Student's, significant levels of 5% or 1%). Variables not qualified for the T-test were nonparametrically assessed by M-W tests instead (significant levels of 5% or 1%). The results were summarised in descriptive tables.

2) Phenotypic analysis

Phenotypic variables in high-P and low-P treatments of each transgenic and the control line were described separately as means and standard errors of means (S.E.M) for each P treatment (three plants) by statistical summary analysis. Two-way ANOVA (analysis of variance) analyses were performed to assess the significance of the difference in combination of both the "group" and "treatment" factors, where multiple comparisons (post-hoc test Fisher's unprotect LSD) were carried out to determine significant differences of each transgenic line under high-P or low-P in comparison with the relevant control (significant levels of 5% or 1%). Based on the outputs of statistical summary analysis, clustered column (mean values) charts were plotted and error bars (standard error of means) were specified on each column in Excel. For presentation of the two-way ANOVA results, statistically significant differences ($P<0.05$ and $P<0.01$) were marked by asterisks over columns in the charts.

3) Correlation analysis

With the aim of further assessing the relationship between the expression levels of *TaPtf1*/ *TaMyb67* and other phenotypic variables, correlation analyses were performed separately for high-P and low-P using data of the two transgenic plants in each treatment of each transgenic line analysed for expression levels by qRT-PCR. The resulting correlation coefficients (r) were tested by Genstat (two-sided test) according to the hypothesis H0: r=0 (no correlation); H1: r=1 (correlation) (statistical significance levels of 5% or 1%; $P<0.05$ or $P<0.01$). The "r" was validated to be true (accept H0) when $P<0.05$. Otherwise, it was false (reject H0; accept H1). The "r" in minus was interpreted as a negative correlation, while it was otherwise justified as a positive correlation. The outputs of correlation coefficients and P values were demonstrated in a matrix table.

6.3 Results

6.3.1 Results for *TaPtf1* transgenic wheat

6.3.1.1 Confirmation of the transgene in *TaPtf1* T1 plants

Five *TaPtf1* independent T1 transgenic lines that were confirmed as positive by the screening trial were chosen from the 88 *TaPtf1* transformant sublines collected from CAAS for the soil-pot experiment (*only from the sublines derived from those T0 plants that tested positive in both Bar assay and PCR screening). After seed germination, the 10 seedlings in each line were fast screened by Bar assay as described in method section 6.2.3.1, prior to six positive plants of each line being potted onto P treatments. According to the results, the first six samples were revealed to be positive in transgenic lines P1, P2, P4 and P5, while samples 1–2 and 6–9 were positive in the transgenic line P3 (Fig. 52).

The five *TaPtf1* transgenic lines were assessed for the presence of the *TaPtf1* transgene by sampling one of the six plants (Bar-positive) in each line by PCR amplification subsequently carried out as described in method section 6.2.3.2. The gel result showed no product in the blank control (Fig. 53, lane 1), which suggested no contamination in the PCR system and confirmed the validity of the other lanes (Fig. 53). The five transgenic lanes (Fig. 53, lanes 2–6) all resulted in DNA bands with expected product size at approximately 1050bp, as seen in the positive control (Fig. 53, lane 8). The outcome agreed with the results of the Bar assay thus supporting their validity.

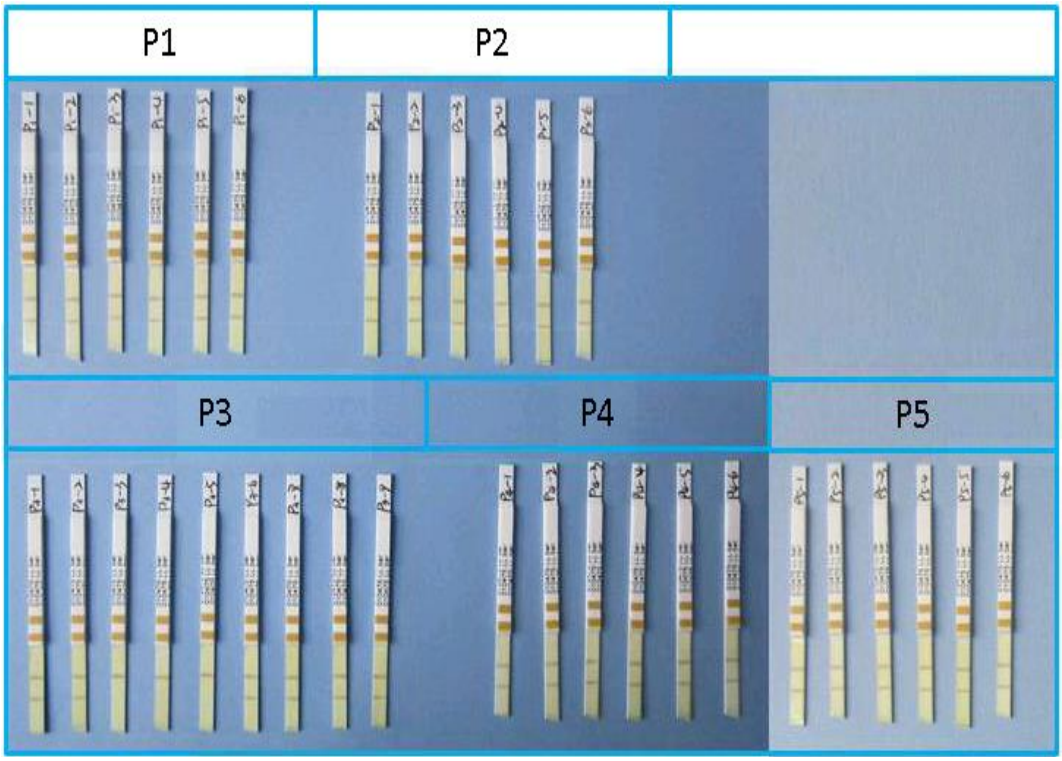


Fig. 52 Results of Bar assay applied to T1 plants of *TaPtf1* transgenic lines. P1: P1-1 to P1-6 are Bar-positive. P2: P2-1 to P2-6 are Bar-positive. P3: P3-1, P3-2, P3-6 to P3-9 are Bar-positive. P4: P4-1 to P4-6 are Bar-positive. P5: P5-1 to P5-6 are Bar-positive.

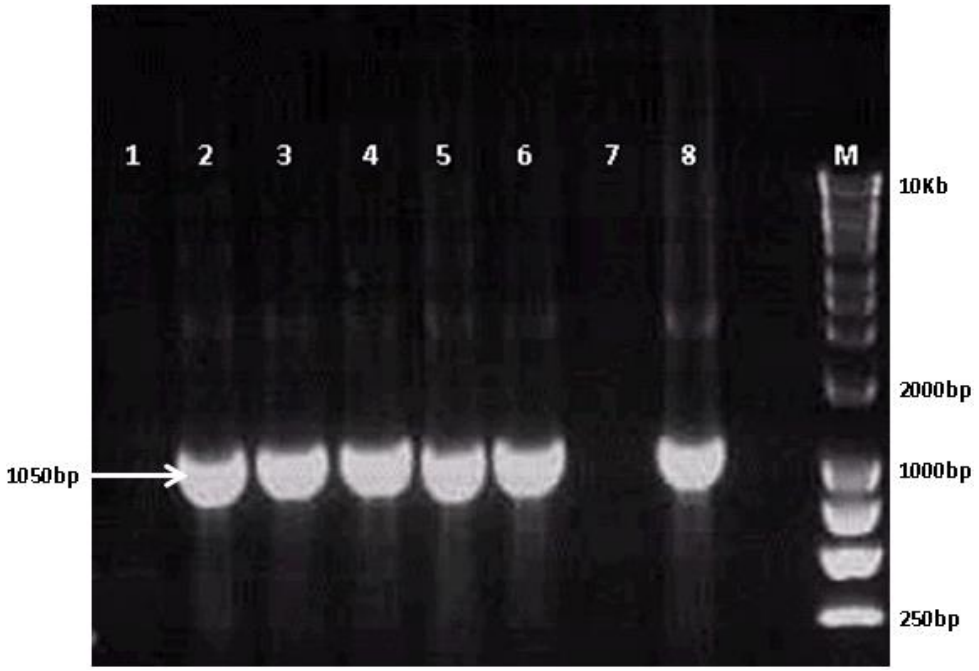


Fig. 53 Confirmation of the transgene in *TaPtf1* transgenic lines by random sample PCR. The PCR was performed by using P2 and NosR2 primers. Lane 1: blank control (ddH_2O as PCR template). Lanes 2–6: transgenic lanes (samples from each *TaPtf1* transgenic line of P1–P5). Lane 7: a blank lane with no sample. Lane 8: positive control (plasmid DNA of 3301PTF1 as PCR template). M: 1Kb DNA ladder.

6.3.1.2 Expression analysis of *TaPtf1* in transgenic wheat

Following the elimination of null plants, a total of 30 *TaPtf1* transgenic plants were grown in high-P and low-P treatments in the glasshouse, as described in 6.2.4. After 3 weeks of P treatment, leaf tissues were sampled individually for each transgenic and control plant, followed by RNA extraction and expression analysis using a qRT-PCR, as described in method section 6.2.5; however, samples were only partially analysed due to technical limits, with two samples (biological replicates) from each treatment of each transgenic or control line (total = 24). The primers used to detect *TaPtf1* are shown in Fig. 54.

The expression levels of *TaPtf1* mRNA for the 24 samples are depicted as columns as the fold-change relative to the control in normal P in Fig 55. The expression of *TaPtf1* in the control was not greatly changed between high-P and low-P, with the latter being 18% on average higher than the former, indicating that *TaPtf1* was not induced by P deficiency in leaves. In the transgenic lines, expression patterns appeared to vary from one line to another and to change between the two P treatments of each transgenic line. Furthermore, differential expression was also observed between the two replicate plants in the same P treatment of each transgenic line.

Under high-P, successful overexpression of *TaPtf1* was found in all transgenic lines except P3. P3 displayed a much lower *TaPtf1* mRNA level than other transgenic lines and, in the meantime, tended to be not higher than the control, which might be characterised as co-ordinated suppression (co-suppression) to an extended capacity of both the transgene of *TaPtf1* and other endogenous genes homologous to *TaPtf1*. The maximum expression was in P4, with a fold-change of 8.24, which was also the highest of all.

Under low-P, most transgenic lines expressed either moderately or marginally higher levels of *TaPtf1* mRNA than the low-P-treated control, particularly P1, P2 and P3, where the fold-change of

overexpression was less than 1.5-fold in one of the two replicates used. The maximum expression was in P5, while the minimum expression was in P1, compared to the low-P-treated control. Moreover, the mRNA levels of *TaPtf1* in P2 and P4 in low-P were found to be greatly reduced in contrast to those in high-P, whereas P3 and P5 showed opposite trends, though the increases seemed to be small.

Partial CDS of *TaPtf1*:

AATGCGATGTGCAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGATGGC 800
AGTGAGTGAAGCACAAGGGGCAATGATTCCTTCGAAGATAAGCTCAACGA 850
TGCATAACAATAAAAGTGAGTACCCTGTCCCTATCAGCCATTCTGCTGAT 900
GCGCAGAACAAGGCAAATTCAGCTAATGGAAACAGTGCCAGTGCTAAGCC 950
ACGAGCAAGGGCTCGTCGTGGACAGGCAACTGACCCTCATAGTATTGCTG 1000
AACGGCTTCGCAGAGAAGAAGATCTCAGAGAGGATGAAAAATCTCCAAGAC 1050
CTTGTACCAAACTCAAATAAGGCAGATAAATCATCAATGCTCGATGAAAT 1100
AATTGATTAT 1110

TaPtf1-F: 5'-ACCCTGTCCCTATCAGCCATTC-3'

TaPtf1-R: 5'-CTGCGAAGCCGTTCAGCAATAC-3'

*DNA product size 142bp

Fig. 54 Primers used for analysis of *TaPtf1* mRNA expression in qRT-PCR analysis. Primers and priming sites are highlighted in blue. The primers were designed to span the transgene (*TaPtf1* CDS) regions, which had moderately conserved consensus with the three endogenous gene copies of *TaPtf1*.

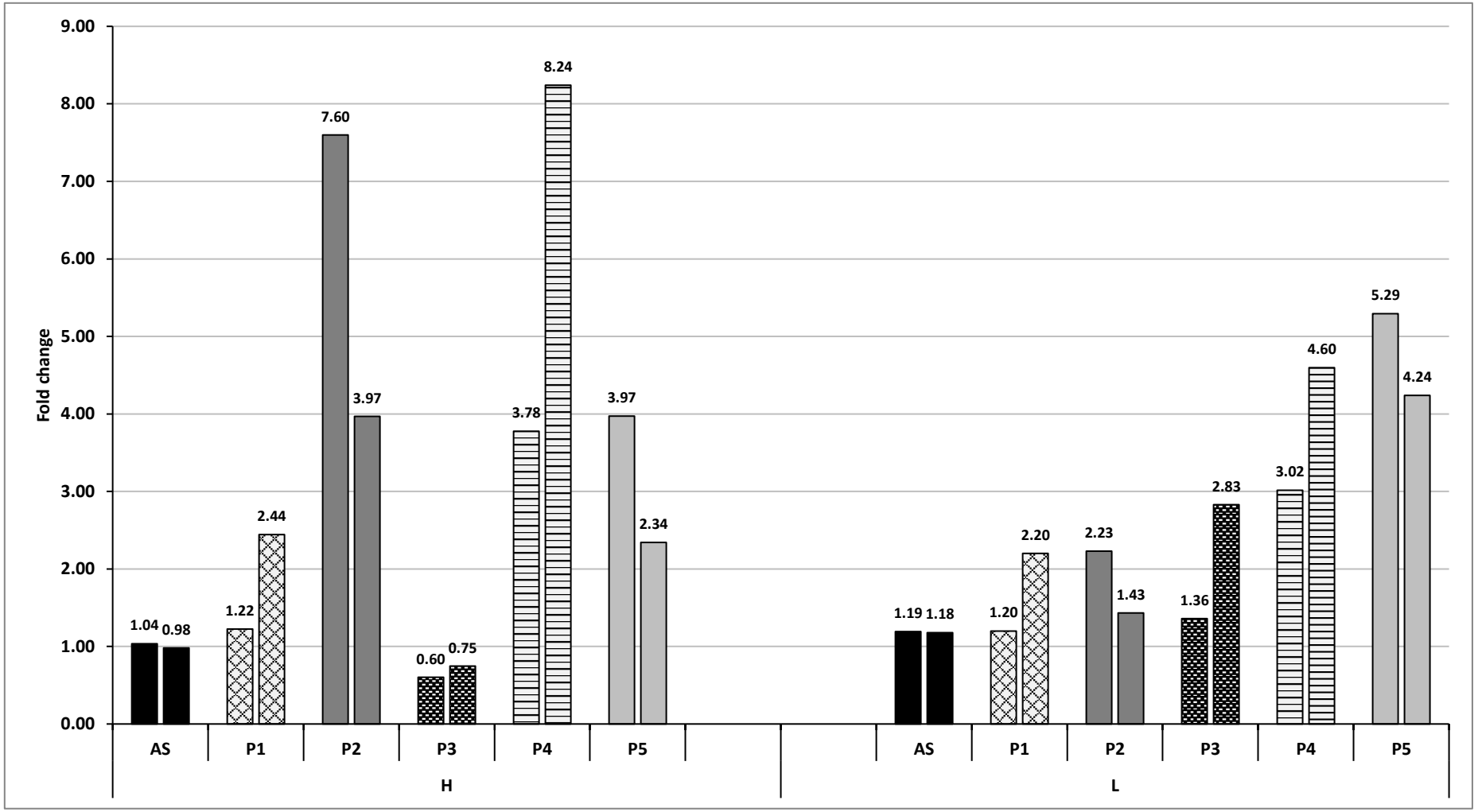


Fig. 55 Analysis of *TaPtf1* mRNA expression in transgenic plants in comparison to the control plants under high-P and low-P. The results shown are *TaPtf1* mRNA expression levels analysed by qRT-PCR for leaf samples collected from wheat plants of the *TaPtf1* transgenic or control line on 21-day high-P or low-P treatment. The Ct values were analysed and converted to fold-changes using the Pfaffl method (Pfaffl, 2001). Each sample was compared with the average of the two control plants under high-P. AS: the wild-type control. P1–P5: *TaPtf1* transgenic lines. H: high-P treatment. L: low-P treatment. The number over each column indicates the fold-change in an individual plant.

6.3.1.3 Phenotypic analysis of *TaPtf1* transgenic wheat

To investigate variations in phenotypes, the phenotypic data was collected as described in 6.2.6–6.2.9. Following this, an exploratory analysis was performed based on the six plants of each line, irrespective of P treatment, to statistically compare phenotypic variables of *TaPtf1* transgenic lines with the control line as described in method section 6.2.9. As shown in Table 25, each transgenic line seemed to have its own characteristics in terms of the overall differences, with P3 appearing to differ the most from the control. For different variables, significant differences higher (+) or lower (-) than the control were detected (highlighted in the table). In summary, four of the five lines were associated with significant differences in leaf area 3 (+), anthesis date (+) and seed biomass (-) compared to the control. Three of the five lines were significantly different from the control in chlorophyll concentration (-), valid/total tiller number (-) and spikelet number (+). Two lines exhibited statistically significant differences in leaf area 1 (-), plant height (+), grain number (-), thousand-grain weight (-) and above-ground biomass (-) compared to the control. Only one line significantly differed from the control in leaf area 2 (+), seed P content (-), main-spike height (+) and weight (+). These outcomes suggested the inconsistency among the five *TaPtf1* transgenic lines used in this study.

Table 25. Exploration analysis of phenotypes in *TaPtf1* transgenic lines

TaPtf1			AS			P1				P2				P3				P4				P5			
	Test	Equality	Mean	S.E.M	Normality	Mean	S.E.M	Normality	P value	Mean	S.E.M	Normality	P value	Mean	S.E.M	Normality	P value	Mean	S.E.M	Normality	P value	Mean	S.E.M	Normality	P value
CCI	T	0.596	45.100	1.353	0.795	34.830	2.535	0.395	0.005**	34.730	1.697	0.985	<0.001**	35.680	1.319	0.932	<0.001**	44.620	1.321	0.996	0.830	40.930	1.398	0.064	0.058
Leaf area 1 (cm^2)	T	0.144	21.820	1.148	0.990	19.140	1.125	0.481	0.126	26.150	3.124	0.940	0.250	14.370	2.927	0.923	0.039*	20.150	2.200	0.307	0.517	14.560	2.677	0.070	0.032*
Leaf area 2 (cm^2)	M-W	0.003	16.140	0.799	0.927	18.780	1.421	0.015	0.132	24.030	1.090	0.913	0.002**	19.350	2.670	0.337	0.394	18.240	4.529	0.016	0.589	18.480	2.078	0.032	0.818
Leaf area 3 (cm^2)	M-W	<0.001	9.640	0.491	0.816	18.420	2.025	0.211	0.002**	23.070	1.509	0.233	0.002**	20.620	1.529	0.188	0.002*	13.440	5.042	0.001	0.699	21.230	2.671	0.992	0.002**
Total leaf area	M-W	0.005	47.600	1.589	0.938	56.340	4.202	0.071	0.065	73.260	4.143	0.483	0.002**	59.060	6.922	0.258	0.394	51.830	11.620	0.011	0.394	54.270	5.153	0.042	0.589
Height (cm)	T	0.055	51.330	1.153	0.469	49.930	3.413	0.476	0.710	56.680	2.154	0.544	0.013*	59.870	1.750	0.577	0.002**	49.200	4.222	0.414	0.664	60.470	4.332	0.227	0.090
Valid tiller	M-W	0.068	6.500	0.500	0.101	5.500	0.224	0.004	0.232	5.167	0.307	0.212	0.102	3.000	0.730	0.167	0.006**	5.000	0.258	0.101	0.045*	4.500	0.619	0.389	0.035*
Invalid tiller	M-W	0.008	1.000	0.258	0.101	0.667	0.211	0.001	0.636	1.167	0.167	<0.001	1.000	1.667	0.211	0.001	0.177	0.833	0.401	0.035	0.602	1.500	0.719	0.111	0.835
Total tiller	M-W	0.205	7.500	0.428	0.820	6.167	0.307	0.212	0.065	6.333	0.211	0.001	0.080	4.667	0.667	0.505	0.015*	5.833	0.307	0.212	0.028*	6.000	0.447	0.004	0.043*
Main-spike height (cm)	M-W	0.017	6.500	0.183	0.775	5.783	0.422	0.261	0.240	7.217	0.282	0.238	0.052	7.017	0.128	0.548	0.063	6.200	0.627	<0.001	0.069	7.983	0.471	0.110	0.030*
Main-spike weight (g)	M-W	0.002	1.100	0.058	0.960	0.900	0.169	0.055	0.394	1.167	0.049	0.415	0.517	1.267	0.021	0.001	0.045*	0.900	0.124	0.007	0.076	1.167	0.092	0.121	0.667
Anthesis date (day)	M-W	<0.001	43.670	0.211	0.001	53.830	2.104	0.231	0.002**	53.670	1.801	0.062	0.002**	50.830	0.703	0.210	0.002**	46.830	2.442	0.077	0.450	62.670	4.660	0.517	0.002**
Spikelet number	M-W	0.069	11.670	0.422	0.473	14.170	1.014	0.033	0.089	15.830	0.833	0.926	0.004**	15.500	0.342	0.006	0.002**	13.330	1.202	0.020	0.219	17.500	1.147	0.062	0.002**
Grain number	M-W	0.924	111.300	6.391	0.939	91.170	7.769	0.029	0.093	104.000	6.885	0.837	0.515	47.670	10.080	0.180	0.002**	79.330	6.616	0.960	0.011*	93.170	7.947	0.734	0.132
Thousand-grain weight (g)	M-W	0.091	30.060	0.917	0.946	27.340	1.265	0.182	0.180	27.490	1.195	0.850	0.132	32.630	1.332	0.958	0.180	26.730	0.615	0.085	0.015*	20.640	2.401	0.024	0.002*
Shoot biomass (g)	M-W	0.534	3.411	0.228	0.412	3.143	0.359	0.271	0.485	3.826	0.283	0.400	0.394	2.145	0.482	0.285	0.065	2.580	0.507	0.024	0.065	4.050	0.433	0.692	0.310
Seed biomass (g)	T	0.682	3.330	0.162	0.960	2.515	0.271	0.228	0.027*	2.864	0.238	0.925	0.137	1.528	0.308	0.041	<0.001**	2.122	0.184	0.970	<0.001**	1.940	0.311	0.928	0.003**
Above-ground biomass (g)	T	0.585	6.741	0.338	0.999	5.658	0.615	0.222	0.154	6.690	0.479	0.587	0.932	3.673	0.772	0.117	0.005**	4.702	0.675	0.118	0.022*	5.990	0.475	0.094	0.227
Shoot P content (mg)	T	0.725	5.576	1.966	0.064	-	-	-	-	5.946	2.199	0.168	0.903	4.162	2.032	0.075	0.628	-	-	-	-	8.475	3.091	0.233	0.447
Seed P content (mg)	T	0.911	17.370	2.358	0.120	-	-	-	-	14.960	2.972	0.058	0.541	9.020	2.262	0.062	0.029*	-	-	-	-	11.340	2.229	0.732	0.097
Above-ground P content (mg)	M-W	0.965	22.940	4.223	0.189	-	-	-	-	20.910	5.136	0.066	0.589	13.180	4.223	0.044	0.093	-	-	-	-	19.820	4.852	0.058	0.485
Shoot utilisation efficiency	M-W	0.984	1.581	0.591	0.122	-	-	-	-	1.631	0.585	0.118	0.818	1.522	0.501	0.022	1.000	-	-	-	-	1.334	0.542	0.140	0.394
Seed utilisation efficiency	M-W	0.683	0.205	0.023	0.259	-	-	-	-	0.220	0.030	0.154	0.699	0.184	0.020	0.004	0.818	-	-	-	-	0.192	0.033	0.391	0.589
Above-ground utilisation efficiency	T	0.272	0.342	0.058	0.033	-	-	-	-	0.424	0.091	0.231	0.464	0.348	0.064	0.071	0.947	-	-	-	-	0.458	0.131	0.084	0.436
P plant/ P soil	M-W	0.103	0.063	0.019	0.023	-	-	-	-	0.046	0.011	0.108	0.589	0.026	0.006	0.501	0.240	-	-	-	-	0.043	0.011	0.161	0.485

P1–P5: *TaPtf1* transgenic lines. AS: the control line (wild-type wheat AS0556). Mean: mean of each transgenic or control line. S.E.M.: standard error of the mean of each transgenic or control line. Equality: probability of homogeneity test among lines. Normality: probability of W-test for normality of each line. T: T-test. M-W: Mann-Whitney test. P value: probability of T-test or M-W test of the difference between the transgenic and control lines. *: A difference significant at P<0.05. **: A difference significant at P<0.01. P-values were highlighted in yellow or blue respectively when the means of the transgenic line is higher or lower than the control.

To further investigate *TaPtf1* transgenic wheat in response to P supply, summary statistical analysis was used to separate means and S.E.M.s. A two-way ANOVA, as described in method section 6.2.9, was employed to identify statistically significant differences between each *TaPtf1* transgenic line and the control under the same P treatment (high-P or low-P). The results are illustrated in Figs. 56–58 with significant differences (either higher or lower than the control as described below) being marked with asterisks.

A) Growth-related variables

Based on the analysis of CCI for the flag leaf of the main stem (Fig. 56A), significant decreases of CCI were observed in P1 and P2 under high-P, but in all transgenic lines, except P4, under low-P ($P<0.05$ or 0.01). The observation probably indicates the reduction of chlorophyll is related with the transgene effects of *TaPtf1*.

Under high-P and low-P, the increases of anthesis date in P2 and P5 were shown to be significant ($P<0.05$) and extremely significant ($P<0.01$)(Fig. 56B). In addition, P1 under high-P was also detected to produce an extremely high increase ($P<0.01$). Other transgenic lines under high-P and low-P only had minor increases or almost equalled the control. It seems uncertain whether the transgene effects of *TaPtf1* are the cause of the affected developmental stage.

The results for plant height (Fig. 56C) suggested a spread pattern of variations under high-P, while the control was mid-range. The transgenic lines under low-P seemed to show a tendency to increased height compared to the control, although only the increases in P3 ($P<0.05$) and P5 ($P<0.01$) were statistically significant. The result is presumably indicative of an association between overexpression of *TaPtf1* and the promotion of plant height under P stress.

With respect to total tiller number (Fig. 56D), the transgenic lines under high-P tended to be similar to the control. Under low-P, P3 displayed a statistically significant ($P<0.01$) and the greatest reduction, while reductions, to a lesser extent, in other transgenic lines were also statistically significant ($P<0.05$ or 0.01). It was interesting that the control under low-P was slightly higher than that under high-P.

Comparisons of leaf areas for the flag leaf (leaf area 1) on the main stem and the first and second leaves beneath the flag leaf (leaf area 2 and leaf area 3) showed that leaf area 1 and leaf area 2 in the transgenic lines under both high-P and low-P had varying patterns, but only the increase in P2 under low-P was significantly different from the control ($P<0.05$)(Figs. 56E and 56F). Comparatively, increases observed for leaf area 3 in the transgenic lines, excluding P4, were significantly different to the control ($P<0.05$ or 0.01)(Fig. 56G). For the total leaf area, only P2 under low-P increased significantly ($P<0.05$)(Fig. 56H). The transgene effects of *TaPtf1* in these lines perhaps have some effect on leaf growth.

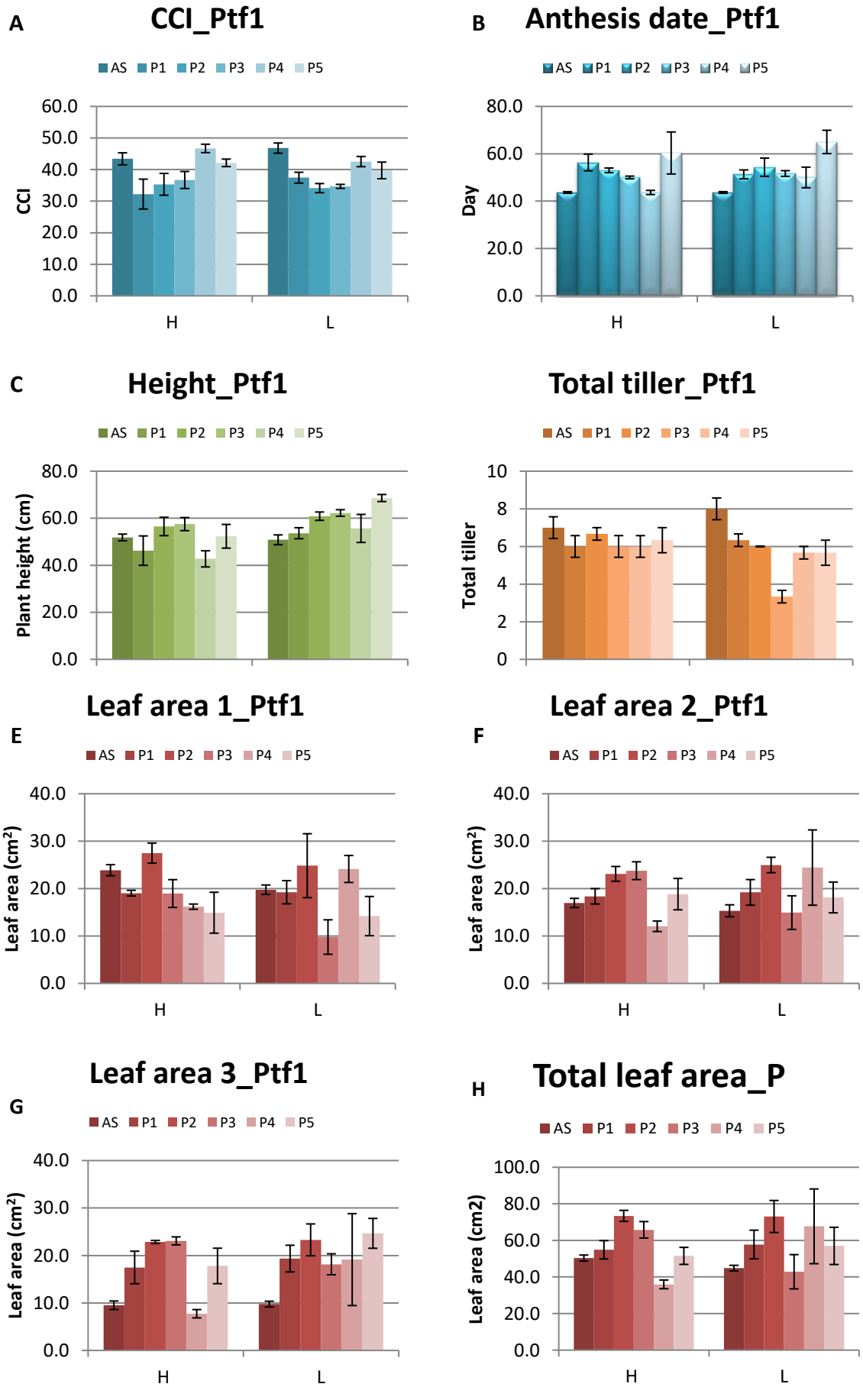

A
CCI_Ptf1
AS P1 P2 P3 P4 P5
CCI
60.0
50.0
40.0
30.0
20.0
10.0
0.0
H
L
B
Anthesis date_Ptf1
AS P1 P2 P3 P4 P5
Day
80.0
60.0
40.0
20.0
0.0
H
L
C
Height_Ptf1
AS P1 P2 P3 P4 P5
Plant height (cm)
80.0
60.0
40.0
20.0
0.0
Total tiller_Ptf1
AS P1 P2 P3 P4 P5
Total tiller
10
8
6
4
2
0
E
Leaf area 1_Ptf1
AS P1 P2 P3 P4 P5
Leaf area (cm²)
40.0
30.0
20.0
10.0
0.0
H
L
F
Leaf area 2_Ptf1
AS P1 P2 P3 P4 P5
Leaf area (cm²)
40.0
30.0
20.0
10.0
0.0
H
L
G
Leaf area 3_Ptf1
AS P1 P2 P3 P4 P5
Leaf area (cm²)
40.0
30.0
20.0
10.0
0.0
H
L
H
Total leaf area_P
AS P1 P2 P3 P4 P5
Leaf area (cm2)
100.0
80.0
60.0
40.0
20.0
0.0
H
L

Fig. 56 Phenotypic analysis of *TaPtf1* transgenic and the control lines under high-P and low-P treatments. H: High-P treatment. L: Low-P treatment. AS: the control line (wild-type wheat AS0556). P1–P5: *TaPtf1* transgenic lines. In each line of each treatment, n=3. Differences between the transgenic and control lines under high-P and low-P were analysed by two-way ANOVA respectively. *: A difference significant at P<0.05. **: A difference significant at P<0.01. Growth-related variables include A: CCI of flag leaf on the main spike (CCI: chlorophyll concentration index); B: Anthesis date; C: Plant height; D: Total tiller number; E: Leaf area 1; F: Leaf area 2; G: Leaf area 3; H: Total leaf area.

B) Harvest-related variables

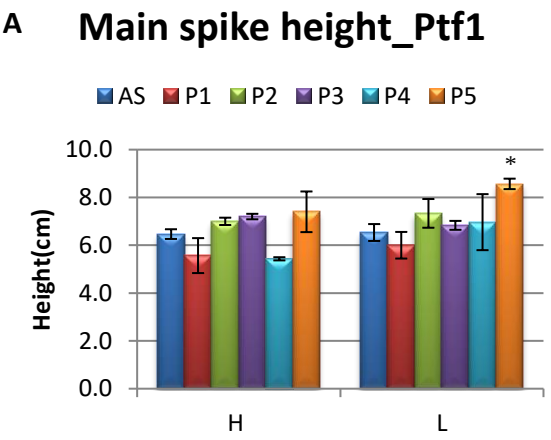
Studies on spike height and spike weight of the main stem have rarely generated results that had implications of the transgene effects of *TaPtf1*. As for the main-spike height (Fig. 57A), only P5 under low-P was significantly higher than the control ($P<0.05$). As for the main-spike weight (Fig. 57B), no significant differences were found, although the transgenic lines varied from the control to different extents. As suggested by the analysis for spikelet number (Fig. 57C), all transgenic lines, except P1, under low-P and P5 under high-P significantly increased in comparison to the control ($P<0.05$ or 0.01), though several other transgenic lines also tended to increase to higher ranges than the control.

Compared with the grain number of the control, most transgenic lines in both high-P and low-P, excluding P2 in high-P, exhibited reduced levels (Fig. 57D). However, significant reductions were only found in a few lines; these include P3 and P4 under high-P, as well as P3 under low-P ($P<0.01$). For thousand-grain weight (Fig. 57E), only the declines in P5 under high-P and low-P ($P<0.01$) were detected to be significant.

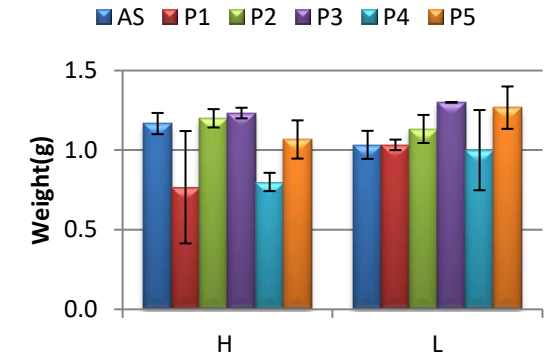
From the study of shoot and seed biomass (Figs. 57F and 57G), the shoot biomass in the transgenic lines under high-P varied, where only P4 showed a statistically significant reduction ($P<0.05$). Under low-P, P1, P2 and P5 all showed a higher range. P4 remained close to the control with a large error bar and was possibly affected by pathogen infection. It was unclear why P3 displayed a significant reduction ($P<0.01$). A climbing trend, though without statistical significance, might exist for the shoot biomass under low-P if the negative effects from P3 are discounted (Fig. 57F). The seed biomass was significantly reduced in all transgenic lines, except for P2, in high-P ($P<0.05$ or 0.01), whereas in low-P a significant reduction was shown by P3 and P5 ($P<0.01$)(Fig. 57G). In addition, P4 under high-P and P3 under low-P also displayed significant reductions in

above-ground biomass ($P<0.01$). Together, these results may imply that the transgene effects of *TaPtf1*, to some extent, are correlated with shoot and seed biomass under both high-P and low-P.

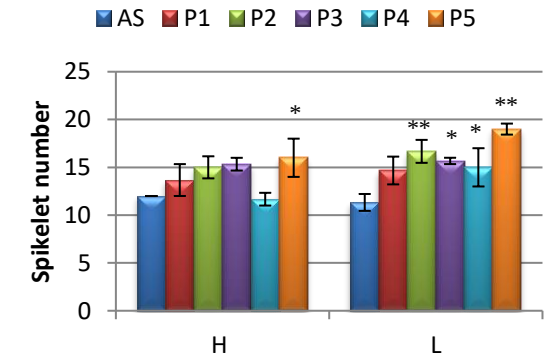
A


Main spike height_Ptf1
AS P1 P2 P3 P4 P5
Height(cm)
10.0
8.0
6.0
4.0
2.0
0.0
H
L
*

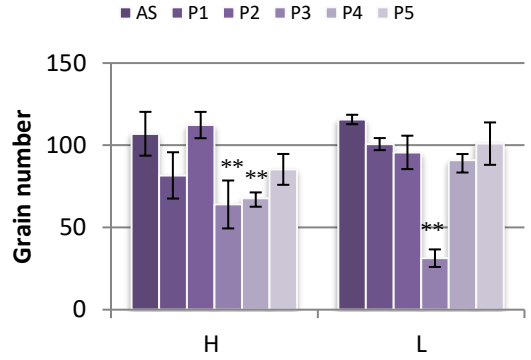
B


Main spike weight_Ptf1
AS P1 P2 P3 P4 P5
Weight(g)
1.5
1.0
0.5
0.0
H
L

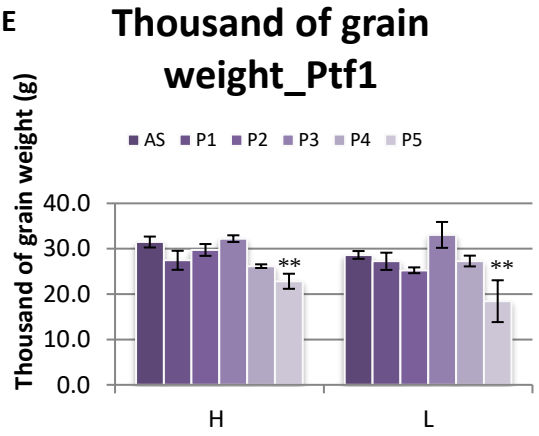
C


Spikelet number_Ptf1
AS P1 P2 P3 P4 P5
Spikelet number
25
20
15
10
5
0
H
L
*
**
**
*
*

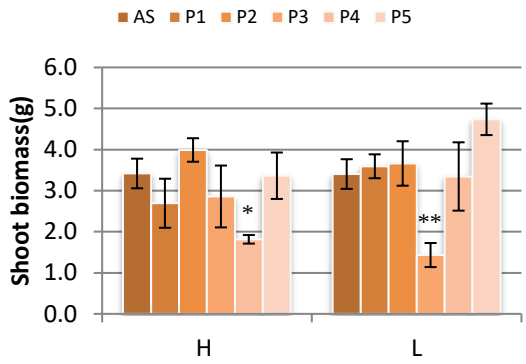
D


Grain number_Ptf1
AS P1 P2 P3 P4 P5
Grain number
150
100
50
0
H
L
**
**
**

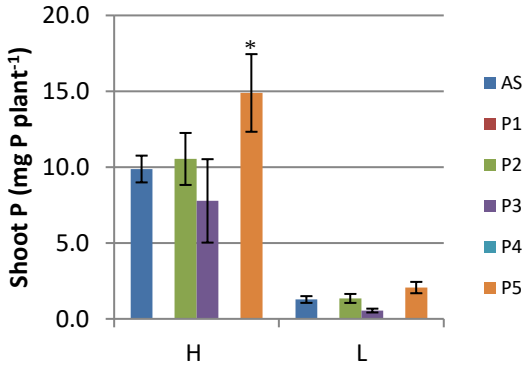
E


Thousand of grain weight_Ptf1
AS P1 P2 P3 P4 P5
Thousand of grain weight (g)
40.0
30.0
20.0
10.0
0.0
H
L
**
**

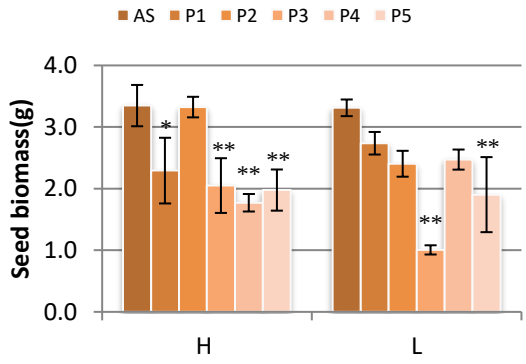
F Shoot biomass_Ptf1



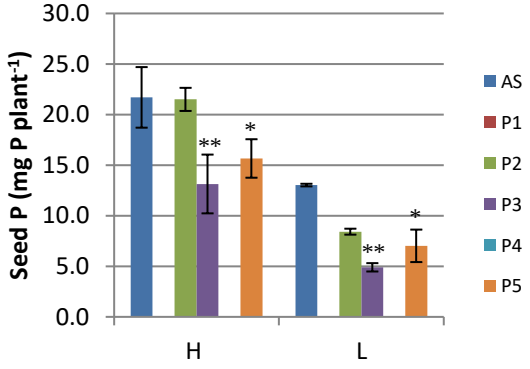
I Shoot P content_Ptf1



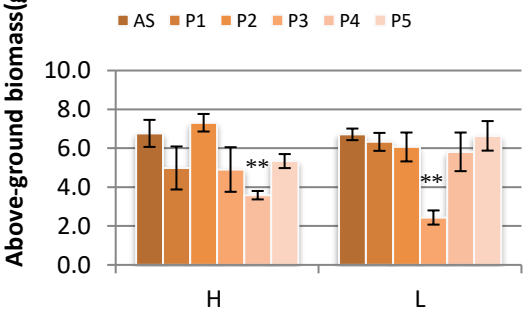
G Seed biomass_Ptf1



J Seed P content_Ptf1



H Above-ground biomass_Ptf1



K Above-ground P content_Ptf1

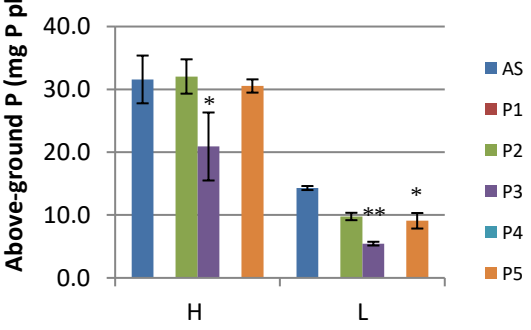


Fig. 57 Phenotypic analysis of *TaPtf1* transgenic and the control lines under high-P and low-P treatments. H: High-P treatment. L: Low-P treatment. AS: the control line (wild-type wheat AS0556). P1–P5: *TaPtf1* transgenic lines. In each line of each treatment, n=3. Differences between the transgenic and control lines under high-P and low-P were analysed by two-way ANOVA respectively. *: A difference significant at P<0.05. **: A difference significant at P<0.01. Harvest-related variables include A: Main-spike height; B: Main-spike weight; C: Spikelet number of the main spike; D: Grain number; E: Thousand-grain weight; F: Shoot biomass; G: Seed biomass; H: Above-ground biomass. P-related variables include I: Shoot P content; J: Seed P content; K: Above-ground P content. Note: “Shoot” represents the non-seed portion after harvest.

C) P-related variables

Due to technical limitations, only three transgenic lines of P2, P3 and P5 were selected to proceed to P content and P utilisation efficiency analyses. P4, which showed a relatively high overexpression, was not analysed due to pathogen infection. Influenced by P availability in the compost for P treatments, the high-P groups resulted in overall higher P accumulation than the low-P groups, in both the transgenic and control lines. Each transgenic line seemed to demonstrate its own pattern (Figs. 57I, 57J and 57K).

Among these, P5 under high-P showed a significant increase in shoot P accompanied with a significant decrease in seed P. Similar patterns of change were maintained in P5 under low-P, but only the decrease in seed P was significant. The above-ground P in P5 under low-P also significantly declined. Meanwhile, P3 similarly showed significant reductions in seed P and above-ground P under both high-P and low-P. The results of P3 were likely to be correlated with the severe reductions of tiller number and grain yield in this line as previously described. In addition, changes in P2 did not result in any statistical significance, though they seemed to tend towards higher and lower ranges in shoot and seed P, respectively. The statistical significance was not consistent in the three lines, though there might be a tendency to increased shoot P or to declined seed or above-ground P. Furthermore, no significant difference was found in either shoot or seed P utilisation efficiency under both high-P and low-P, but significantly increased above-ground utilisation efficiency was detected in P2 and P5 under low-P (Figs. 58A, 58B and 58C). In addition, the P plant/P soil ratio in all the three lines under low-P significantly declined (Fig. 58D).

Together, the results suggest that the growth of *TaPtf1* transgenic wheat was subject to high-P and low-P treatments. High-P-treated *TaPtf1* transgenic plants were more similar to the

control plants in most aspects but showed a few phenotypic differences, such as an increase in leaf area 3 and reduction of seed biomass. Compared to the control, low-P-treated *TaPtf1* transgenic plants resulted in phenotypic differences in several aspects. There seem indications that overexpressing *TaPtf1* might be associated with decreased chlorophyll concentration, tiller number, seed biomass and seed P, increased leaf area 3, plant height, spikelet number, shoot biomass, shoot P and above-ground P utilisation, and delayed anthesis. However, the overall phenotypes were not apparent or weak (in terms of comparison with the control) and there was also insufficient consistency across the transgenic lines. This weak nature of phenotypes may partially be explained by the minor to moderate change in mRNA expression of *TaPtf1* in these lines (especially under low-P). Their correlation with the transgene effects therefore requires further investigation.

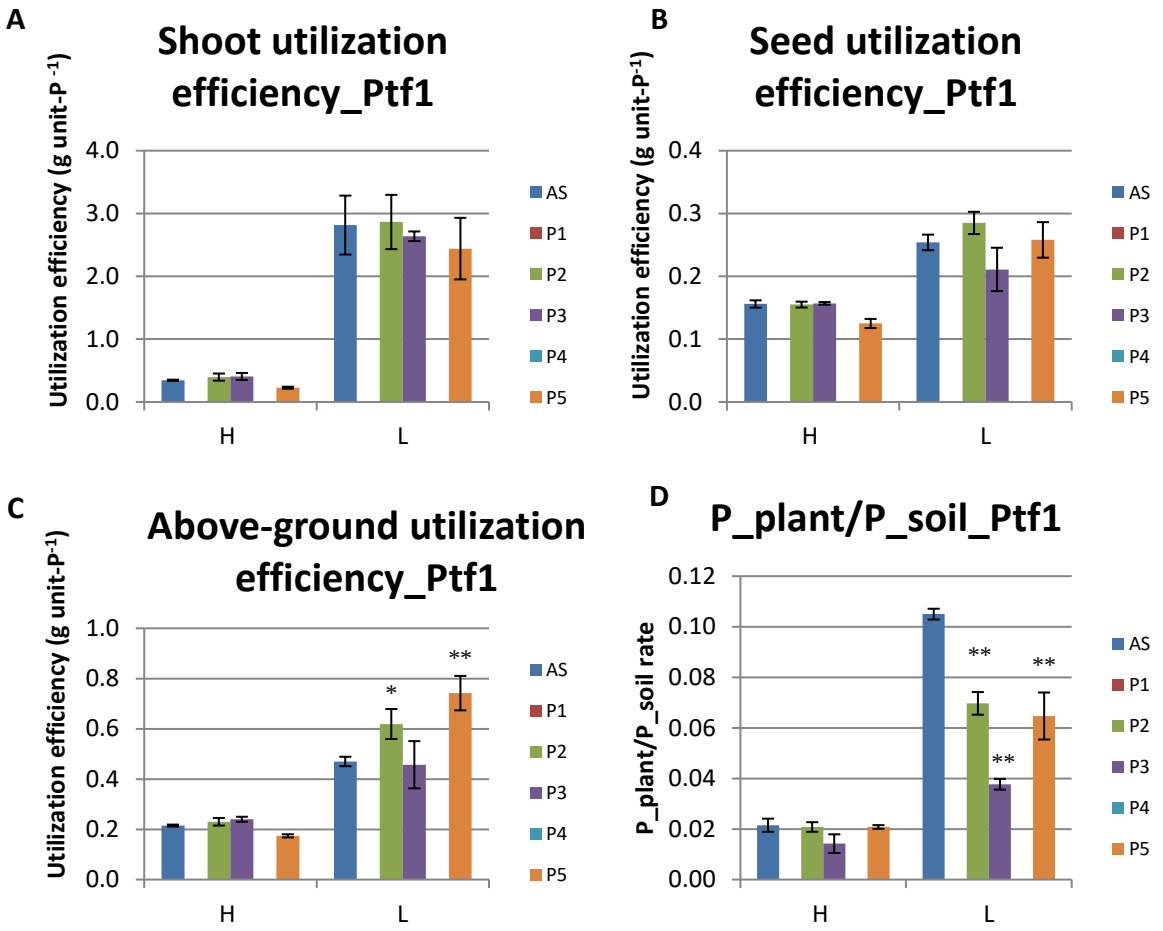


Fig. 58 Phenotypic analysis of *TaPtf1* transgenic and the control lines under high-P and low-P treatments. H: High-P treatment. L: Low-P treatment. AS: the control line (wild-type wheat AS0556). P1–P5: *TaPtf1* transgenic lines. In each line of each treatment, n=3. Differences between the transgenic and control lines under high-P and low-P were analysed by two-way ANOVA respectively. *: A difference significant at P<0.05. **: A difference significant at P<0.01. P-related variables include A: Shoot P utilisation efficiency ("Shoot" represents the non-seed portion after harvest); B: Seed P utilisation efficiency; C: Above-ground P utilisation efficiency; D: P_plant/P_soil ratio.

6.3.1.4 Correlation analysis of *TaPtf1* expression and phenotypes

Correlation analysis was further carried out as described in method section 6.2.9, using data of the 20 transgenic plants that were analysed for *TaPtf1* expression from the five *TaPtf1* transgenic lines. The purpose was to assess further the link between the *TaPtf1* transgene expression and phenotypes observed in each transgenic plant, which might help to account for the phenotypic effects contributed by the transgene.

Given the phenotypes in *TaPtf1* transgenic plants appeared to respond to different P supply, the analysis was carried out for high-P and low-P, respectively. The results expressed as correlation coefficients in the matrix table are given in Tables 26 and 27. Under high-P, no correlation was found between *TaPtf1* expression and other phenotypic variables (Table 26). Under low-P, *TaPtf1* expression was shown to positively or negatively correlate with several phenotypic variables (Table 27, the column highlighted in yellow). Interestingly, a positive correlation between *TaPtf1* expression and chlorophyll concentration was detected, which disagreed with the evidence collected in phenotypic analysis in the previous section that suggested an adverse effect of *TaPtf1* overexpression on chlorophyll. Moreover, it was further confirmed that *TaPtf1* expression positively contributed to plant height, as observed in phenotypic analysis. There was also a positive correlation deriving from the analysis of *TaPtf1* expression and shoot biomass, which appeared to support the observation in phenotypic analysis. As indicated by the correlation coefficients obtained from the analyses for shoot P and shoot P utilisation efficiency, *TaPtf1* expression was positively correlated with the former but negatively correlated with the later. Additionally, correlations were partially observed between different phenotypic variables.

Table 26. Correlation analysis of *TaPtf1* transgenic wheat under high-P

Name of variables	r	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
TaPtf1 expression	A	-																									
Chlorophyll	B	0.104	-																								
Leaf area 1	C	0.330	-0.493	-																							
Leaf area 2	D	-0.059	-0.551	*0.6524	-																						
Leaf area 3	E	-0.277	**-0.8508	0.434	*0.7072	-																					
Total leaf area	F	-0.004	*-0.7555	**0.8147	**0.9027	**0.8468	-																				
Height	G	-0.452	*-0.6878	0.162	0.286	*0.7361	0.483	-																			
Valid tiller	H	0.039	-0.233	0.227	0.039	0.095	0.148	-0.186	-																		
Invalid tiller	I	0.160	0.253	-0.212	-0.499	-0.209	-0.340	0.138	*-0.6897	-																	
Total tiller	J	0.196	-0.095	0.123	-0.393	-0.057	-0.101	-0.132	*0.7608	-0.055	-																
Anthesis date	K	-0.310	**-0.8778	0.340	0.557	*0.7605	*0.6541	0.557	0.505	-0.583	0.174	-															
Main-spike height	L	-0.273	*-0.7001	0.374	*0.7004	**0.8583	*0.7575	**0.8062	-0.029	-0.282	-0.293	*0.6458	-														
Main-spike weight	M	-0.283	**-0.8863	0.394	0.559	**0.9312	*0.7522	**0.8512	-0.064	-0.049	-0.132	*0.6927	**0.8486	-													
Spikelet number	N	-0.262	**-0.8376	0.209	0.484	*0.7343	0.565	**0.7971	-0.155	-0.156	-0.353	*0.6878	**0.7973	**0.8778	-												
Grain number	O	0.180	-0.585	*0.6615	0.393	0.383	0.569	0.065	**0.8278	*-0.6386	0.569	*0.6868	0.268	0.295	0.189	-											
Thousand-grain weight	P	-0.402	-0.573	0.211	0.396	*0.7253	0.533	**0.779	-0.514	0.376	-0.372	0.312	0.610	**0.8257	*0.6695	-0.172	-										
Shoot biomass	Q	0.062	**-0.7922	0.593	0.427	*0.6502	*0.6687	0.401	*0.6881	-0.510	0.492	**0.8072	0.546	0.612	0.478	**0.9141	0.131	-									
Seed biomass	R	0.088	*-0.7458	*0.7345	0.478	0.572	*0.7093	0.279	*0.6621	-0.493	0.471	*0.7481	0.417	0.534	0.379	**0.9515	0.128	**0.9536	-								
Above-ground biomass	S	0.074	**-0.7813	*0.6611	0.454	0.624	*0.694	0.353	*0.6848	-0.508	0.489	**0.7909	0.496	0.585	0.440	**0.9409	0.131	**0.9915	**0.9847	-							
Shoot P	T	0.694	-0.130	0.335	0.036	-0.291	0.114	-0.622	**0.9324	-0.795	0.687	0.549	-0.642	-0.434	-0.448	*0.886	*-0.8321	0.737	0.708	0.733	-						
Seed P	U	0.711	-0.506	0.684	0.145	0.077	0.505	-0.278	*0.8324	-0.491	0.781	0.652	-0.222	0.021	-0.170	**0.9984	-0.462	**0.9383	**0.9656	**0.9617	*0.8667	-					
Above-ground P	V	0.728	-0.362	0.557	0.103	-0.078	0.354	*-0.4348	*0.9036	-0.638	0.767	0.630	-0.410	-0.173	-0.295	**0.9842	-0.637	*0.8838	*0.8878	*0.8962	**0.9534	**0.9768	-				
Shoot utilisation efficiency	W	-0.494	-0.412	-0.123	-0.230	0.695	0.119	*0.9265	-0.658	**0.9358	-0.198	-0.062	**0.9413	*0.8286	0.682	-0.548	**0.9308	-0.261	-0.306	-0.284	*-0.8283	-0.524	-0.672	-			
Seed utilisation efficiency	X	-0.308	-0.503	0.302	0.091	0.779	0.509	**0.8247	-0.567	*0.8797	-0.114	-0.099	**0.9526	*0.9134	0.578	-0.276	**0.967	-0.039	0.026	-0.011	-0.681	-0.234	-0.434	*0.8862	-		
Above-ground utilisation efficiency	Y	-0.435	-0.507	-0.011	-0.218	0.787	0.228	0.936	-0.555	**0.9215	-0.066	0.010	**0.9768	*0.9085	0.668	-0.417	**0.9364	-0.113	-0.155	-0.133	-0.745	-0.388	-0.555	**0.9852	**0.9291	-	
Plant/soil P ratio	Z	0.730	-0.365	0.558	0.103	-0.076	0.355	-0.432	*0.9033	-0.636	0.769	0.632	-0.408	-0.171	-0.294	**0.9844	-0.635	*0.8848	*0.8883	*0.897	**0.953	**0.9771	**1	-0.670	-0.432	-0.552	-

"r" =correlation coefficient. Correlation analysis was carried out by using the expression and phenotypic data of *TaPtf1* transgenic plants which were analysed by qRT-PCR from the five *TaPtf1* transgenic lines under high-P. The capital letter A indicates the variable of *TaPtf1* expression levels in *TaPtf1* transgenic lines under high-P. The capital letters B to Z indicate other phenotypic variables in *TaPtf1* transgenic lines under high-P, as described in the first column of the table. Each number in a cell indicates the "r" produced from the correlation analysis for the two variables in such a row and such a column. The column highlighted in yellow shows the "r"s between *TaPtf1* expression (A) and another phenotypic variable (B to Z). The "r" was validated to be true when P<0.05 (shown in red, *significant at P<0.05. **significant at P<0.01.). Otherwise, it was false (as tested by correlated analysis). The "r" in minus indicates a linear negative correlation, otherwise it indicates a positive linear correlation.

Table 27. Correlation analysis of *TaPtf1* transgenic wheat under low-P

Name of variables	r	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
TaPtf1 expression	A	-																									
Chlorophyll	B	*0.7127	-																								
Leaf area 1	C	-0.0641	0.0556	-																							
Leaf area 2	D	0.1768	0.0356	**0.7927	-																						
Leaf area 3	E	0.3239	-0.0623	0.4056	**0.8252	-																					
Total leaf area	F	0.1444	0.0166	**0.8578	**0.9833	**0.8094	-																				
Height	G	*0.6579	0.1875	-0.1358	0.1153	0.4939	0.1469	-																			
Valid tiller	H	0.0159	0.2666	0.5642	0.462	0.1301	0.4568	-0.5556	-																		
Invalid tiller	I	0.1744	0.0812	-0.4004	-0.5012	-0.1947	-0.4218	0.6262	-0.6748	-																	
Total tiller	J	0.1504	0.4167	0.4578	0.2465	0.0297	0.2983	-0.279	0.8373	-0.1614	-																
Anthesis date	K	0.5762	0.3967	-0.2511	0.0276	0.3919	0.0311	0.6165	-0.0482	0.5056	0.3101	-															
Main-spike height	L	0.5857	0.1725	0.1421	0.5533	**0.8355	0.5411	0.8054	-0.1636	0.2205	-0.0554	*0.6833	-														
Main-spike weight	M	0.1321	-0.2751	-0.1298	0.3231	*0.7145	0.2989	0.6243	-0.5172	0.1374	-0.5898	0.2494	*0.6797	-													
Spikelet number	N	0.4996	-0.0168	-0.0458	0.3855	*0.7858	0.3817	0.7862	-0.2294	0.2337	-0.1337	*0.7013	**0.9332	*0.7122	-												
Grain number	O	0.2369	0.2761	0.5061	0.562	0.4362	0.5697	-0.1979	**0.9014	-0.4962	*0.8377	0.3081	0.2162	-0.2304	0.1893	-											
Thousand-grain weight	P	-0.5707	-0.5949	-0.1441	-0.1211	-0.1613	-0.1601	-0.2497	-0.4683	-0.2359	**-0.8009	*-0.76	-0.3488	0.3303	-0.3062	*-0.6602	-										
Shoot biomass	Q	*0.6386	0.5105	0.5209	0.5193	0.5018	0.582	0.3587	0.5108	-0.0429	*0.6512	0.4537	0.4451	-0.0372	0.3716	*0.7078	-0.7383	-									
Seed biomass	R	-0.0988	0.0471	*0.7428	0.6254	0.3229	*0.6578	-0.4144	**0.7694	-0.7576	0.4675	-0.3778	-0.1151	-0.1354	-0.1713	*0.6795	-0.0306	0.486	-								
Above-ground biomass	S	0.4334	0.3972	*0.6845	0.6361	0.5016	*0.6956	0.0991	*0.6877	-0.3364	*0.6704	0.1842	0.2835	-0.082	0.2065	**0.7976	-0.5592	**0.9363	*0.7621	-							
Shoot P	T	*0.8735	0.9007	0.071	0.0412	0.1915	0.0977	0.837	0.531	0.658	0.7797	*0.8242	0.6607	-0.5664	0.5012	0.6277	**-0.9451	*0.8959	0.0223	0.7242	-						
Seed P	U	-0.4055	-0.2605	0.6972	0.7393	0.637	0.7723	-0.3136	0.4864	-0.686	0.2271	-0.3032	0.1773	0.2918	0.0976	0.4083	0.1291	0.1972	**0.9553	0.488	-0.19	-					
Above-ground P	V	0.0507	0.2043	0.7081	0.7336	0.711	0.794	0.1209	0.7375	-0.3286	0.6132	0.1245	0.5051	-0.005	0.3476	0.711	-0.3534	0.6432	**0.9323	*0.8367	0.3224	*0.8681	-				
Shoot utilisation efficiency	W	*-0.8561	-0.7593	0.714	0.6427	0.2062	0.6493	*-0.8094	0.1409	-0.6678	-0.1115	-0.7515	-0.426	0.0685	-0.458	-0.071	0.5183	-0.2534	0.628	0.0145	-0.6468	0.6662	0.3152	-			
Seed utilisation efficiency	X	-0.038	0.1504	*0.8807	0.7984	0.5881	*0.8814	0.0323	0.7377	-0.2298	0.6508	0.0039	0.3261	-0.2339	0.1404	0.6438	-0.3447	0.6839	**0.9503	*0.8755	0.304	*0.8224	**0.9467	0.4603	-		
Above-ground utilisation efficiency	Y	0.6473	0.7271	0.4625	0.3663	0.292	0.4421	0.6207	0.7145	0.5222	*0.9119	0.6231	0.5586	-0.7308	0.3372	0.7142	*-0.9113	**0.9827	0.3147	*0.8947	**0.9135	0.0564	0.5163	-0.287	0.5905	-	
Plant/soil P ratio	Z	0.0473	0.2024	0.7096	0.7329	0.7082	0.7939	0.1186	0.7342	-0.3294	0.6097	0.1195	0.5001	-0.0036	0.3432	0.707	-0.3491	0.6412	**0.934	*0.8357	0.319	*0.8698	**1	0.3188	**0.9476	0.5139	-

"r" =correlation coefficient. Correlation analysis was carried out by using the expression and phenotypic data of *TaPtf1* transgenic plants which were analysed by qRT-PCR from the five *TaPtf1* transgenic lines under low-P. The capital letter A indicates the variable of *TaPtf1* expression levels in *TaPtf1* transgenic lines under low-P. The capital letters B to Z indicate other phenotypic variables in *TaPtf1* transgenic lines under low-P, as described in the first column of the table. Each number in a cell indicates the "r" produced from the correlation analysis for the two variables in such a row and such a column. The column highlighted in yellow shows the "r"s between *TaPtf1* expression (A) and another phenotypic variable (B to Z). The "r" was validated to be true when P<0.05 (shown in red, *significant at P<0.05. **significant at P<0.01.). Otherwise, it was false (as tested by correlated analysis). The "r" in minus indicates a negative linear correlation, otherwise it indicates a positive linear correlation.

6.3.2 Results for *TaMyb67* transgenic wheat

6.3.2.1 Confirmation of the transgene in *TaMyb67* T1 plants

Five *TaMyb67* independent T1 transgenic lines detected to be positive in the screening trial were chosen from the 26 *TaMyb67* transformant sublines collected from CAAS for the soil-pot experiment (*only from the sublines derived from those T0 plants that tested positive in both Bar assay and PCR screening). After seed germination, the 10 seedlings in each line were fast screened by Bar assay as previously described, prior to six positive plants of each line being potted on to P treatments. The results showed the first six samples to be positive in transgenic lines M2, M4 and M5. In transgenic lines M1 and M3, samples 1–5 and 7 were found to be positive, but sample 6 tested negative (Fig. 59).

The PCR amplifications took place in succession using the method previously described. The five *TaMyb67* transgenic lines were assessed for the presence of the *TaMyb67* transgene by sampling one plant of the six (Bar-positive) for each line. This generated the results consistent with those of the Bar assay. For the gel (Fig. 60), no product was observed in the blank control (Fig. 60, lane 9), which indicated no contamination of the PCR system, confirming the other lanes were valid. As in the positive control (Fig. 60, lane 16), the DNA bands obtained in the five transgenic lanes (Fig. 60, lanes 10–13 and 15) had a product size of approximately 800bp as expected.

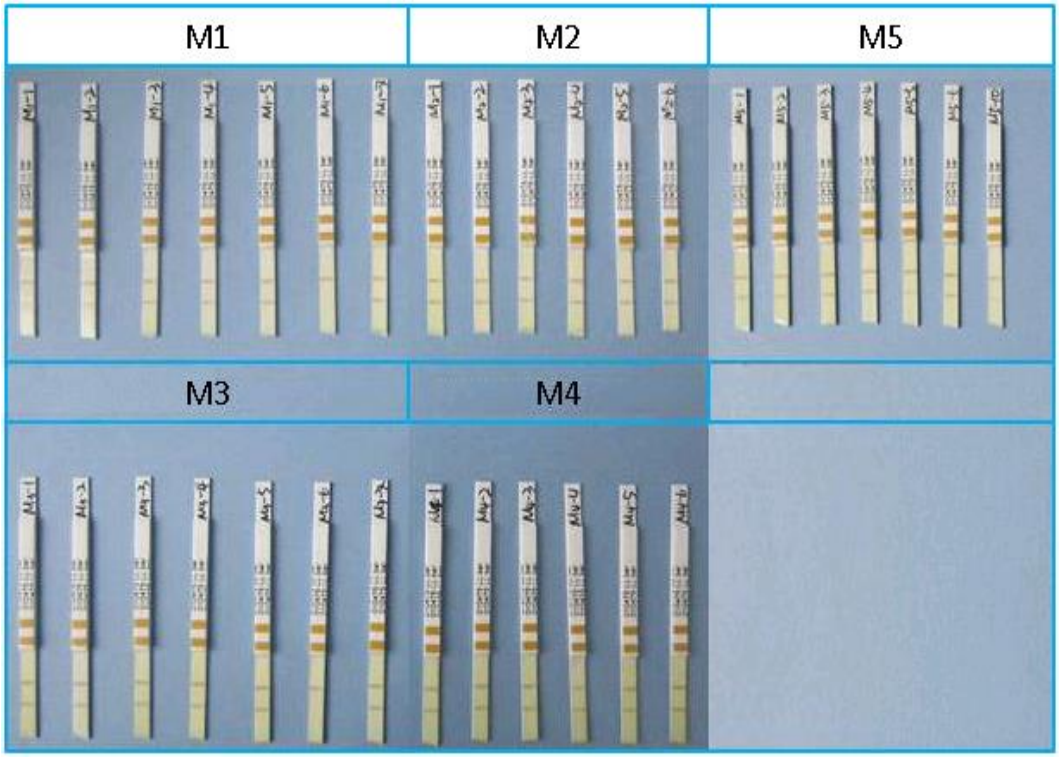


Fig. 59 Results of Bar assay applied to T1 plants of *TaMyb67* transgenic lines. M1: M1-1 to M1-5 and M7 are Bar-positive. M2: M2-1 to M2-6 are Bar-positive. M3: M3-1 to M3-5 and M3-7 are Bar-positive. M4: M4-1 to M4-6 are Bar-positive. M5: M5-1 to M5-6 are Bar-positive.

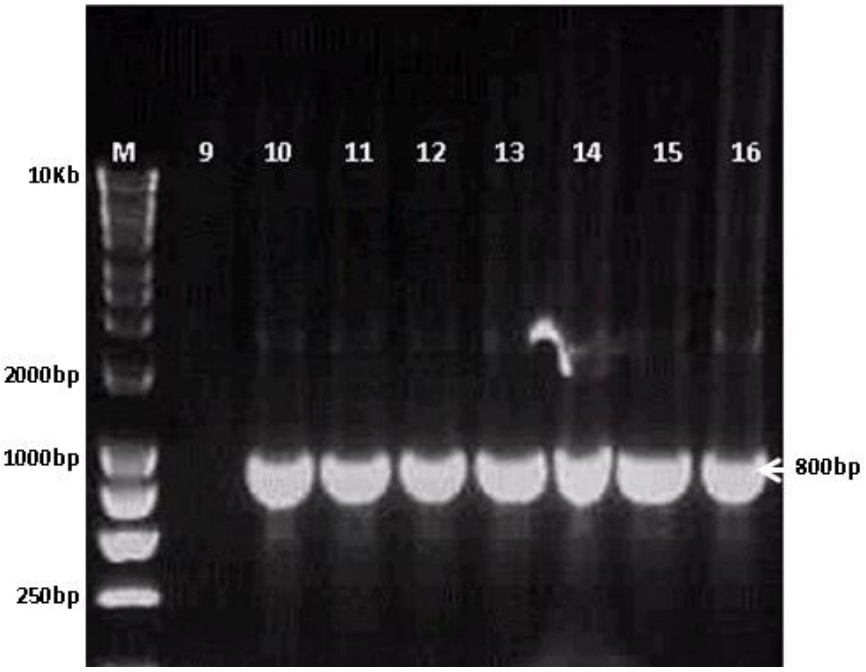


Fig. 60 Confirmation of the transgene in *TaMyb67* transgenic lines by random sample PCR. The PCR was performed by using Ubi1922 and M2718 primers. Lane 9: blank control (ddH_2O as PCR template). Lanes 10–16: transgenic lanes (samples from each *TaMyb67* transgenic line of M1–M5). Lane 14 and 15 are duplicate loading of the same sample M4). Lane 16: positive control (plasmid DNA of 3301MYB67 as PCR template). M: 1Kb DNA ladder.

6.3.2.2 Expression analysis of *TaMyb67* in transgenic wheat

After screening out null plants, a total of 30 *TaMyb67* transgenic plants were grown in high-P and low-P treatments, as previously described. Following a 3-week P treatment, leaf tissues were sampled individually for each transgenic and control plant, followed by RNA extraction and expression analysis using a qRT-PCR as previously described, with two samples from each treatment of each transgenic or control line (total = 24). The primers used to detect *TaMyb67* are shown in Fig. 61.

Fig. 62 shows the expression levels of *TaMyb67* mRNA in the 24 samples, with each plant being depicted in a column as the fold-change relative to the corresponding control in the same treatment, either high-P or low-P. Differential expression was detectable between the two replicate plants (biological replicates) in the same treatment of each transgenic line, but the difference seemed to be small in most lines.

In high-P, excluding M4 which exhibited suppressed expression of *TaMyb67* mRNA, the other four transgenic lines were all detected for successful overexpression of *TaMyb67*, of which M1 and M2 had higher and much higher mRNA levels than M3 and M5. In low-P, an apparent trend of suppressed expression of *TaMyb67* mRNA, in contrast to the low-P-treated control, was observed in more than half of the transgenic lines (M1, M4, M5), which might be indicative of co-suppression of both the transgene and its homologous endogenous genes in these lines. Besides M4, which demonstrated the lowest *TaMyb67* mRNA level, M1 and M5 also resulted in suppression to a lesser extent. On the other hand, although minor overexpression of *TaMyb67* was observed in M2 and M3, the mRNA levels were not much higher than the control, especially in M2, where the fold-changes were less than double in the two replicate plants assessed. In addition, M1 and M2 in low-P displayed strong declines in *TaMyb67* mRNA levels compared to those in high-P.

Partial CDS of *TaMyb67*:

AACTTGAGACCCAGAGAAAGTTGCAATTGCGAATTGAAGAACAAGGGAAG 1035
TATCTTCAGATGATGTTTGAAAAGCAGTCTAAATCCAATACGGAGAAGGTG 1085
CAGGATCTATCCTCGGGAGCTACAACAACCCTATCATCTGAACCAAGCCAT 1135
CCTGCAACCAGAAATAGGGGTGATGATGCAGCTGATGACCTAAATAGAAC 1185
AGGAGAGAACCCCGTGAGTGCCGAAATAGGAGAAACTTTGATGCATGCAG 1235
GTGGCAACCAGGAGATGGCAGAAAGCGAGTCTTCTGAGCCCCTTGCAAAT 1285
ACTAATGATGGTTCCAAGGCCCCGCAAGAGAAGCGCTGA 1326

TaMyb67-F: 5'- CGGGAGCTACAACAACCCTA -3'

TaMyb67-R: 5'- GACTCGCTTTCTGCCATCTC -3'

*DNA product size 169bp

Fig. 61 Primers used for the analysis of *TaMyb67* mRNA expression in qRT-PCR analysis. Primers and priming sites are highlighted in blue. The primer pairs were designed to span the transgene (*TaMyb67* CDS) regions which had moderately conserved consensus with the endogenous genes derived from the three homoeoloci of *TaMyb67* on the wheat genome (Ensembl).

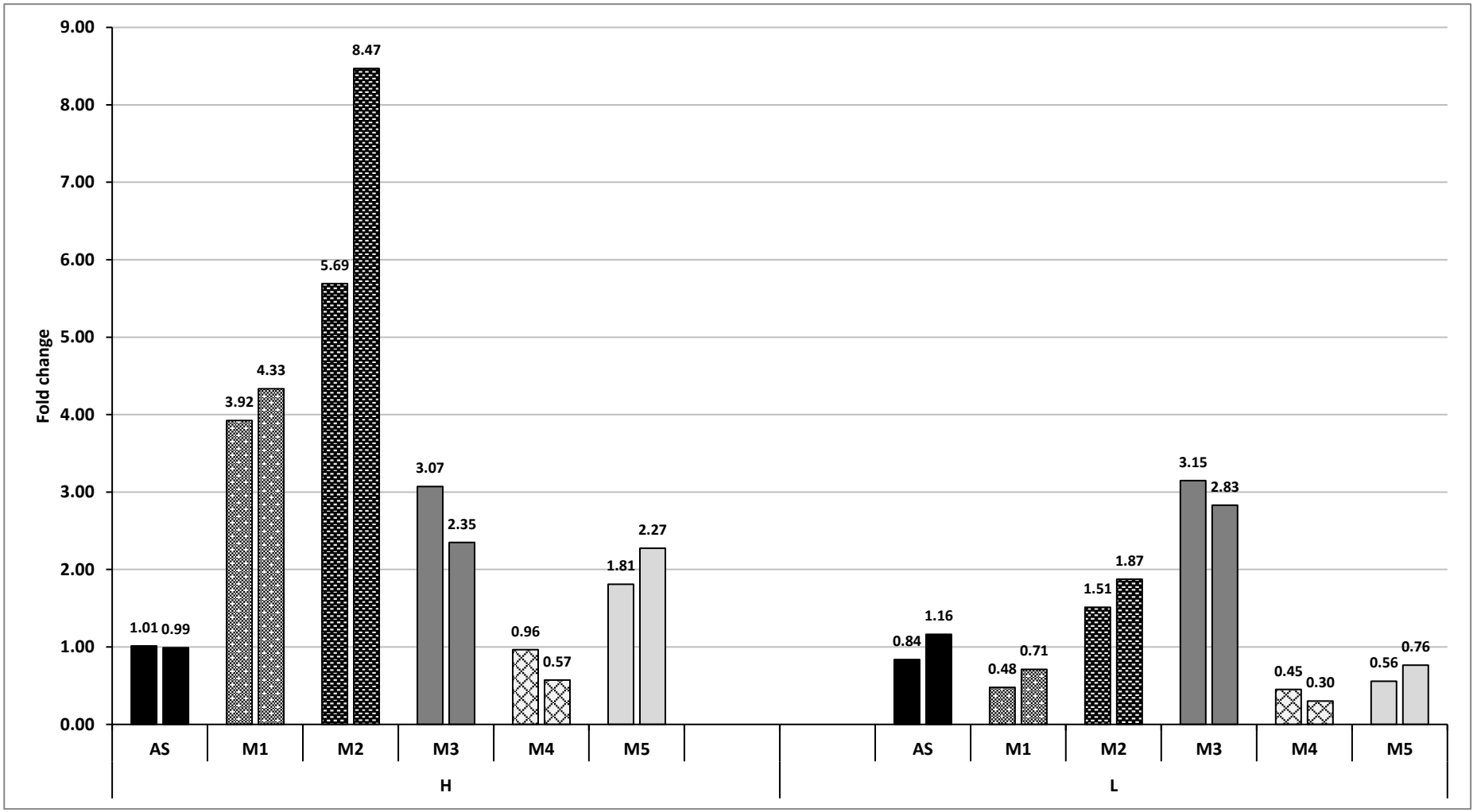


Fig. 62 Expression analysis of *TaMyb67* mRNA expression in transgenic plants in comparison to the control plants under high-P and low-P. The results shown are expression levels of *TaMyb67* mRNA analysed by qRT-PCR for leaf samples collected from wheat plants of the *TaMyb67* transgenic or control line on 21-day high-P or low-P treatment. The Ct values were analysed and converted to fold-changes using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Each sample was compared with the average of the two control plants in the same P treatment, either high-P or low-P. AS: the wild-type control. M1–M5: *TaMyb67* transgenic lines. H: high-P treatment. L: low-P treatment. The number over each column indicates the fold-change in an individual plant.

6.3.2.3 Phenotypic analysis of *TaMyb67* transgenic wheat

Following data collection, which was the same as previously described, phenotypic variables of each *TaMyb67* transgenic line were statistically analysed against the control line by using the six plants of each line irrespective of P treatment. According to the results shown in Table 28, phenotypes observed in most *TaMyb67* transgenic lines were generally very weak, although M4 seemed to be more varied from the control. Together, three of the five lines showed statistically significant differences in total tiller number (-) and spikelet number (+) compared with the control. Two of the five lines were significantly different from the control in chlorophyll concentration (-), leaf area 1 (-), anthesis date (+), grain number (-), shoot biomass (-), seed biomass (-) and above-ground biomass (-). Only one line significantly differed from the control in leaf area 2 (+), leaf area 3 (+), plant height (+), main-spike height (+) and weight (-).

Table 28. Exploration analysis of phenotypes in *TaMyb67* transgenic lines

TaMyb67			AS			M1				M2				M3				M4				M5			
		Equality	Mean	S.E.M	Normality	Mean	S.E.M	Normality	P value	Mean	S.E.M	Normality	P value	Mean	S.E.M	Normality	P value	Mean	S.E.M	Normality	P value	Mean	S.E.M	Normality	P value
CCI	M-W	0.007	45.100	1.353	0.795	43.200	1.125	0.055	0.240	43.820	0.893	0.924	0.331	43.430	1.868	0.097	0.818	35.680	2.506	0.062	0.015*	30.080	4.316	0.599	0.015*
Leaf area 1 (cm^2)	T	0.313	21.820	1.148	0.990	21.660	1.407	0.140	0.932	21.640	1.848	0.309	0.936	16.550	1.544	0.227	0.021*	24.500	2.250	0.259	0.315	17.850	0.752	0.179	0.016*
Leaf area 2 (cm^2)	M-W	0.175	16.140	0.799	0.927	19.870	2.704	0.535	0.468	18.670	1.551	0.855	0.132	13.600	1.451	0.370	0.240	25.410	1.554	0.038	0.004**	18.740	2.466	0.620	0.589
Leaf area 3 (cm^2)	M-W	<0.001	9.640	0.491	0.816	10.770	1.778	0.094	0.937	10.990	1.701	0.037	1.000	11.000	1.240	0.551	0.732	28.590	4.563	0.038	0.002**	15.040	2.749	0.711	0.132
Total leaf area	M-W	0.071	47.600	1.589	0.938	52.300	4.540	0.092	0.937	51.300	2.581	0.401	0.310	41.150	3.186	0.498	0.180	78.500	6.518	0.515	0.004**	51.630	5.504	0.833	0.589
Height (cm)	M-W	0.082	51.330	1.153	0.469	51.280	1.455	0.672	0.842	49.120	0.763	0.438	0.240	46.550	2.221	0.872	0.102	57.030	0.980	0.018	0.015*	48.480	2.536	0.677	0.485
Valid tiller	M-W	0.112	6.500	0.500	0.101	5.667	0.211	0.001	0.394	5.167	0.167	<0.001	0.054	6.000	0.258	0.101	0.545	5.333	0.211	0.001	0.123	6.667	0.422	0.473	0.673
Invalid tiller	M-W	0.949	1.000	0.258	0.101	0.500	0.342	0.006	0.232	0.500	0.342	0.006	0.232	1.500	0.428	0.820	0.429	0.500	0.342	0.006	0.232	0.667	0.333	0.091	0.494
Total tiller	M-W	0.502	7.500	0.428	0.820	6.167	0.167	0.001	0.041*	5.667	0.333	0.091	0.022*	7.500	0.428	0.820	1.000	5.883	0.307	0.212	0.028*	7.333	0.333	0.091	0.989
Main-spike height (cm)	T	0.858	6.500	0.183	0.775	6.033	0.255	0.241	0.168	6.717	0.158	0.644	0.391	6.067	0.239	0.682	0.180	7.367	0.173	0.064	0.006**	6.417	0.168	0.052	0.744
Main-spike weight (g)	M-W	0.011	1.100	0.058	0.960	0.950	0.056	0.191	0.132	1.083	0.079	0.004	0.879	0.833	0.152	0.405	0.188	1.283	0.477	0.421	0.063	0.717	0.182	0.036	0.080*
Anthesis date	M-W	0.007	43.670	0.211	0.001	45.330	1.022	0.148	0.206	46.830	1.537	0.217	0.097	47.830	1.740	0.068	0.097	53.500	0.719	0.505	0.002**	53.500	1.232	0.118	0.002**
Spikelet number	M-W	0.586	11.670	0.422	0.473	13.500	0.806	0.060	0.080	15.000	0.516	0.101	0.004**	13.000	0.516	0.101	0.056	16.330	0.422	0.001	0.002**	15.170	0.749	0.158	0.006**
Grain number	M-W	0.045	111.300	6.391	0.939	101.500	5.334	0.267	0.240	94.330	4.216	0.232	0.041*	80.000	8.299	0.408	0.026*	112.800	6.750	0.602	0.937	86.000	15.900	0.166	0.329
Thousand-grain weight (g)	M-W	<0.001	30.060	0.917	0.946	29.110	0.651	0.951	0.485	26.730	1.109	0.885	0.065	30.370	2.554	<0.001	0.310	29.210	0.818	0.367	0.818	29.810	3.426	0.122	0.589
Shoot biomass (g)	T	0.069	3.411	0.228	0.412	2.840	0.144	0.632	0.543	2.630	0.123	0.185	0.013*	2.431	0.281	0.148	0.022*	4.017	0.246	0.246	0.101	3.348	0.458	0.458	0.905
Seed biomass (g)	M-W	0.017	3.330	0.162	0.960	2.954	0.169	0.590	0.093	2.506	0.086	0.665	0.004**	2.252	0.252	0.466	0.015*	3.274	0.132	0.173	0.937	2.530	0.214	0.355	0.240
Above-ground biomass (g)	M-W	0.044	6.741	0.338	0.999	5.794	0.303	0.998	0.132	5.136	0.207	0.296	0.004**	4.683	0.530	0.233	0.026*	7.291	0.346	0.156	0.310	5.878	0.827	0.231	0.589
Shoot P content (mg)	T	0.940	5.576	1.966	0.064	6.927	2.433	0.152	0.481	7.151	2.542	0.214	0.635	-	-	-	-	6.827	2.568	0.057	0.707	-	-	-	-
Seed P content (mg)	M-W	0.781	17.370	2.358	0.120	17.870	2.257	0.727	0.937	15.13	1.633	0.180	0.589	-	-	-	-	15.820	2.658	0.044	0.699	-	-	-	-
Above-ground P content (mg)	T	0.957	22.940	4.223	0.189	24.790	4.502	0.275	0.646	22.280	4.129	0.279	0.913	-	-	-	-	22.640	5.206	0.052	0.966	-	-	-	-
Shoot utilisation efficiency	M-W	0.357	1.581	0.591	0.122	1.581	0.591	0.116	0.240	0.973	0.347	0.178	0.240	-	-	-	-	1.924	0.732	0.031	0.818	-	-	-	-
Seed utilisation efficiency	T	0.590	0.205	0.023	0.259	0.178	0.023	0.324	0.413	0.178	0.025	0.358	0.443	-	-	-	-	0.239	0.038	0.065	0.472	-	-	-	-
Above-ground utilisation efficiency	M-W	0.422	0.342	0.058	0.033	0.280	0.055	0370	0.240	0.285	0.063	0.432	0.394	-	-	-	-	0.441	0.104	0.036	0.589	-	-	-	-
P plant/ P soil	M-W	0.794	0.063	0.019	0.023	0.068	0.021	0.074	0.699	0.060	0.018	0.114	0.818	-	-	-	-	0.052	0.013	0.052	0.699	-	-	-	-

M1–M5: *TaMyb67* transgenic lines. AS: the control line (wild-type wheat AS0556). Mean: mean of each transgenic or control line. S.E.M.: standard error of the mean of each transgenic or control line. Equality: probability of homogeneity test among lines. Normality: probability of W-test for normality of each line. T: T-test. M-W: Mann-Whitney test. P value: probability of T-test between the transgenic and control lines. *: A difference significant at P<0.05. **: A difference significant at P<0.01. P-values were highlighted in yellow or blue respectively when the means of the transgenic line is higher or lower than the control.

Further information on the phenotypes of *TaMyb67* transgenic wheat in high-P or low-P treatment were obtained by separating means and S.E.M.s. A two-way ANOVA, as previously described, was then performed to statistically assess each *TaMyb67* transgenic line in comparison with the control under the same P treatment (high-P or low-P) (asterisks in relevant charts standing for significant differences higher or lower than the control, Figs. 63–65).

A) Growth-related variables

According to the analysis of CCI for the flag leaf of the main stem (Fig. 63A), transgenic lines of M1, M2, M3 and M4 (high-P) displayed little variation from the control, whereas M5 and M4 (low-P) showed significant declines in CCI levels ($P<0.01$). It seems that the reduction of chlorophyll only happens on rare occasions.

Regarding anthesis date (Fig. 63B), significant increases were detected in M4 and M5 in both high-P and low-P ($P<0.01$), as well as M3 in high-P ($P<0.05$), while other transgenic lines showed minor increases or almost equalled the control. The result probably implies the developmental stage has been affected in *TaMyb67* transgenic lines.

As suggested by the analysis for plant height (Fig. 63C), no significant difference was found between the transgenic and control lines. Interestingly, M4 appeared to be in a slightly different pattern of change from other transgenic lines.

The total tiller number in the transgenic lines in low-P partially showed significant reductions (M1, M2 and M4, $P<0.05$ or 0.01), while those in high-P were only slightly or moderately varied (Fig. 63D).

Studies on leaf areas of the flag leaf (leaf area 1) and the first and second leaves beneath the flag leaf on the main stem (leaf area 2 and leaf area 3) indicated that M4 was distinctive from other transgenic lines and the control. Significant increases of leaf area 1 and leaf area 2 were

observed in M4 under low-P (P<0.01)(Figs. 63E and 63F). Highly significant increases of leaf area 3 and the total leaf area were also found in M4 under both high-P and low-P (P<0.01)(Figs. 63G and 63H). In addition, M3 under high-P had a significant reduction in leaf area 1 (P<0.01).

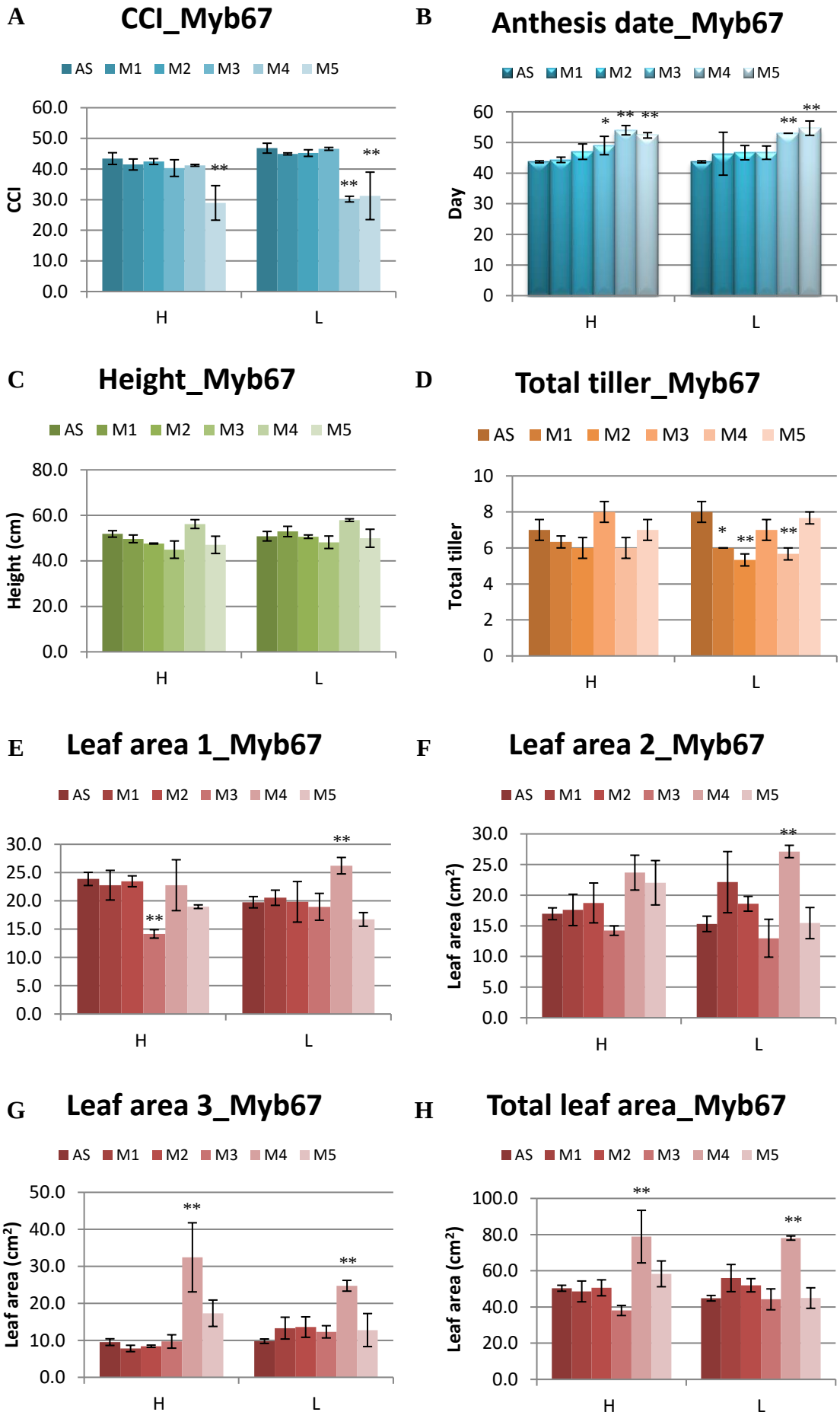

A
CCI_Myb67
AS M1 M2 M3 M4 M5
CCI
60.0
50.0
40.0
30.0
20.0
10.0
0.0
H
L
B
Anthesis date_Myb67
AS M1 M2 M3 M4 M5
Day
60
50
40
30
20
10
0
H
L
C
Height_Myb67
AS M1 M2 M3 M4 M5
Height (cm)
80.0
60.0
40.0
20.0
0.0
H
L
D
Total tiller_Myb67
AS M1 M2 M3 M4 M5
Total tiller
10
8
6
4
2
0
H
L
E
Leaf area 1_Myb67
AS M1 M2 M3 M4 M5
Leaf area (cm²)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
H
L
F
Leaf area 2_Myb67
AS M1 M2 M3 M4 M5
Leaf area (cm²)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
H
L
G
Leaf area 3_Myb67
AS M1 M2 M3 M4 M5
Leaf area (cm²)
50.0
40.0
30.0
20.0
10.0
0.0
H
L
H
Total leaf area_Myb67
AS M1 M2 M3 M4 M5
Leaf area (cm²)
100.0
80.0
60.0
40.0
20.0
0.0
H
L

Fig. 63 Phenotypic analysis of *TaMyb67* transgenic and the control lines under high-P and low-P treatments. H: High-P treatment. L: Low-P treatment. AS: the control line (wild-type wheat AS0556). M1–M5: *TaMyb67* transgenic lines. In each line of each treatment, n=3. Differences between the transgenic and control lines under high-P and low-P were analysed by two-way ANOVA respectively. *: A difference significant at $P<0.05$. **: A difference significant at $P<0.01$. Growth-related variables include A: CCI of flag leaf on the main spike (CCI: chlorophyll concentration index); B: Anthesis date; C: Plant height; D: Total tiller number; E: Leaf area 1; F: Leaf area 2; G: Leaf area 3; H: Total leaf area.

B) Harvest-related variables

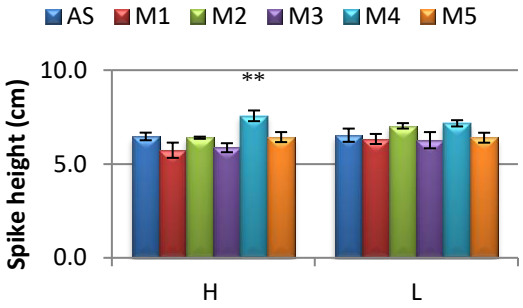
Observations of the main-spike height revealed that only the increase detected in M4 under high-P was significant ($P<0.01$), while other transgenic lines were more close to the control (Fig. 64A). The main-spike weight of the transgenic lines showed considerable variation, but only the reduction in M3 under high-P was significant compared with the control ($P<0.05$)(Fig. 64B).

In comparison with the control, the spikelet number of the transgenic lines, except M3, under low-P, increased significantly, whereas under high-P a significant increase was only found in M4 and M5 ($P<0.05$ or 0.01)(Fig. 64C), though other transgenic lines also tended to increase to higher ranges than the control.

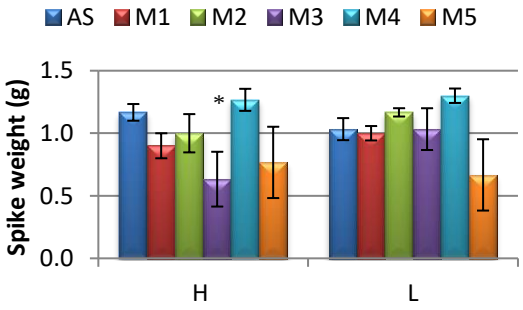
From the study of grain number, none of the changes in the transgenic lines, except for the reduction of M3 under high-P ($P<0.05$), was found to be statistically significant (Fig. 64D). For thousand-grain weight, there was no significant difference for all transgenic lines under either high-P or low-P (Fig. 64E).

In the results for biomass, the lowest shoot or seed biomass resulted from M3 under high-P, where the differences were statistically significant compared with the control ($P<0.05$). The shoot and seed biomass in other transgenic lines demonstrated no significant difference but exhibited various patterns. Similarly, the above-ground biomass in M3 also significantly declined ($P<0.05$)(Fig. 64H). Interestingly, it looked M4 had a different pattern of change from other transgenic lines (Figs. 64F and 64G).

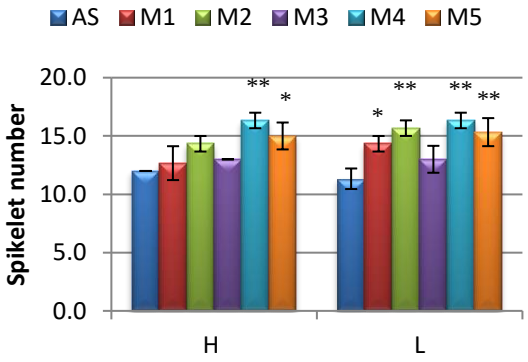
A Main spike height _Myb67



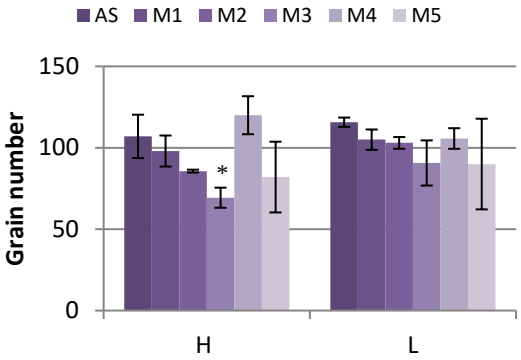
B Main spike weight_Myb67



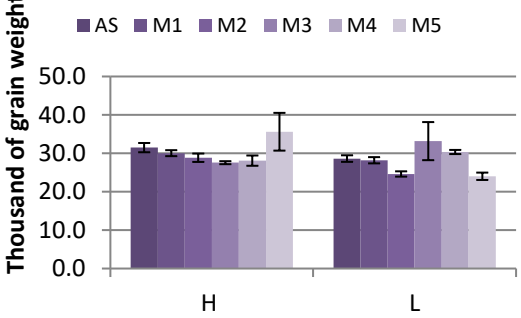
C Spikelet number_Myb67



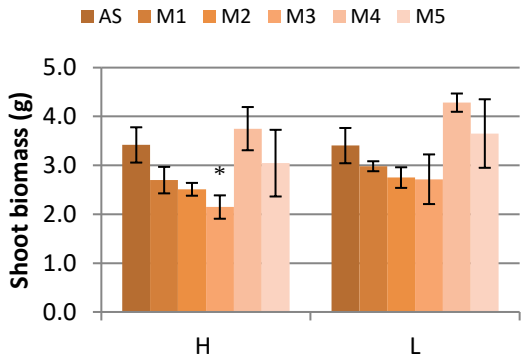
D Grain number_Myb67



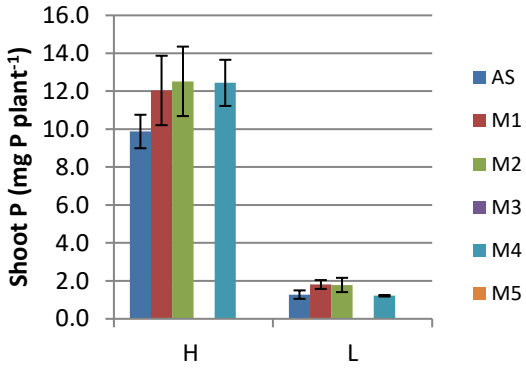
E Thousand of grain weight_Myb67



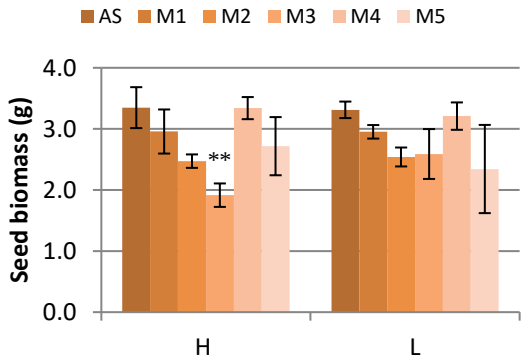
F Shoot biomass_Myb67



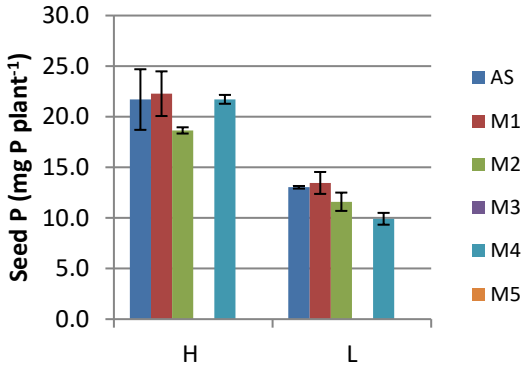
I Shoot P content_Myb67



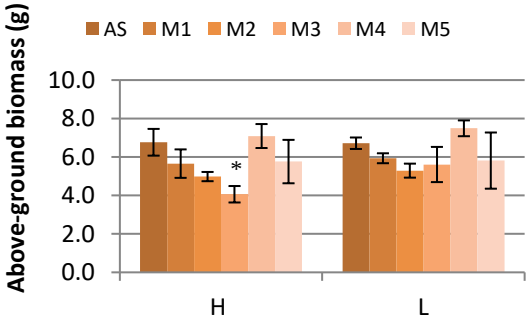
G Seed biomass_Myb67



J Seed P content_Myb67



H Above-ground biomass_Myb67



K Above-ground P content_Myb67

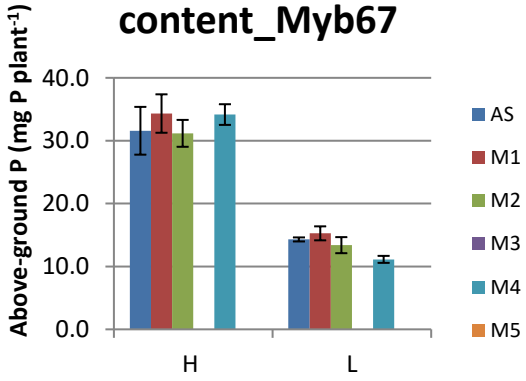


Fig. 64 Phenotypic analysis of *TaMyb67* transgenic and the control lines under high-P and low-P treatments. H: High-P treatment. L: Low-P treatment. AS: the control line (wild-type wheat AS0556). M1–M5: *TaMyb67* transgenic lines. In each line of each treatment, n=3. Differences between the transgenic and control lines under high-P and low-P were analysed by two-way ANOVA respectively. *: A difference significant at $P<0.05$. **: A difference significant at $P<0.01$. Harvest-related variables include A: Main-spike height; B: Main-spike weight; C: Spikelet number of the main spike; D: Grain number; E: Thousand-grain weight; F: Shoot biomass; G: Seed biomass; H: Above-ground biomass. P-related variables include I: Shoot P content; J: Seed P content; K: Above-ground P content**.** Note: “Shoot” represents the non-seed potion after harvest.

C) P-related variables

Only three transgenic lines, M1, M2 and M4, were chosen for P content and P utilisation efficiency analyses due to technical limitations. M3, which showed the highest overexpression among the five *TaMyb67* transgenic lines under low-P, was not analysed due to pathogen infection. In both high-P and low-P, neither the shoot nor seed P led to a significant difference between the transgenic and control lines, though changes were observed (Figs. 64I, 64J and 64K).

For P utilisation efficiency (Figs. 65A, 65B and 65C), M4 seemed to have distinct patterns of change compared with M1 and M2. However, significant differences were only detected in low-P rather than in high-P. Under low-P, the shoot utilisation efficiency of M1 and M2 significantly decreased ($P<0.01$), while M4 tended towards a higher range than the control but this was not significant. Comparatively, the seed utilisation efficiency of M4 generated a distinctively significant increase ($P<0.01$), whereas M1 and M2 only exhibited lower ranges than the control without significance. The above-ground utilisation efficiency under low-P resembled those patterns of the seed utilisation efficiency in these lines. Furthermore, the plant/P soil ratio in M4 was significantly reduced ($P<0.01$), but M1 and M2 were more comparable to the control under low-P (Fig. 65D).

Together, most of the *TaMyb67* transgenic wheat did not significantly differ from the control plants. Although a few statistically significant differences were observed, there seemed to be a lack of support by the other transgenic lines to them, e.g. anthesis date, spikelet number chlorophyll concentration, tiller number and shoot utilisation efficiency. Also, phenotypes of M4 seemed to have strong dissimilarity with other lines in several aspects under both high-P and low-P. These will be discussed in 6.4.3.

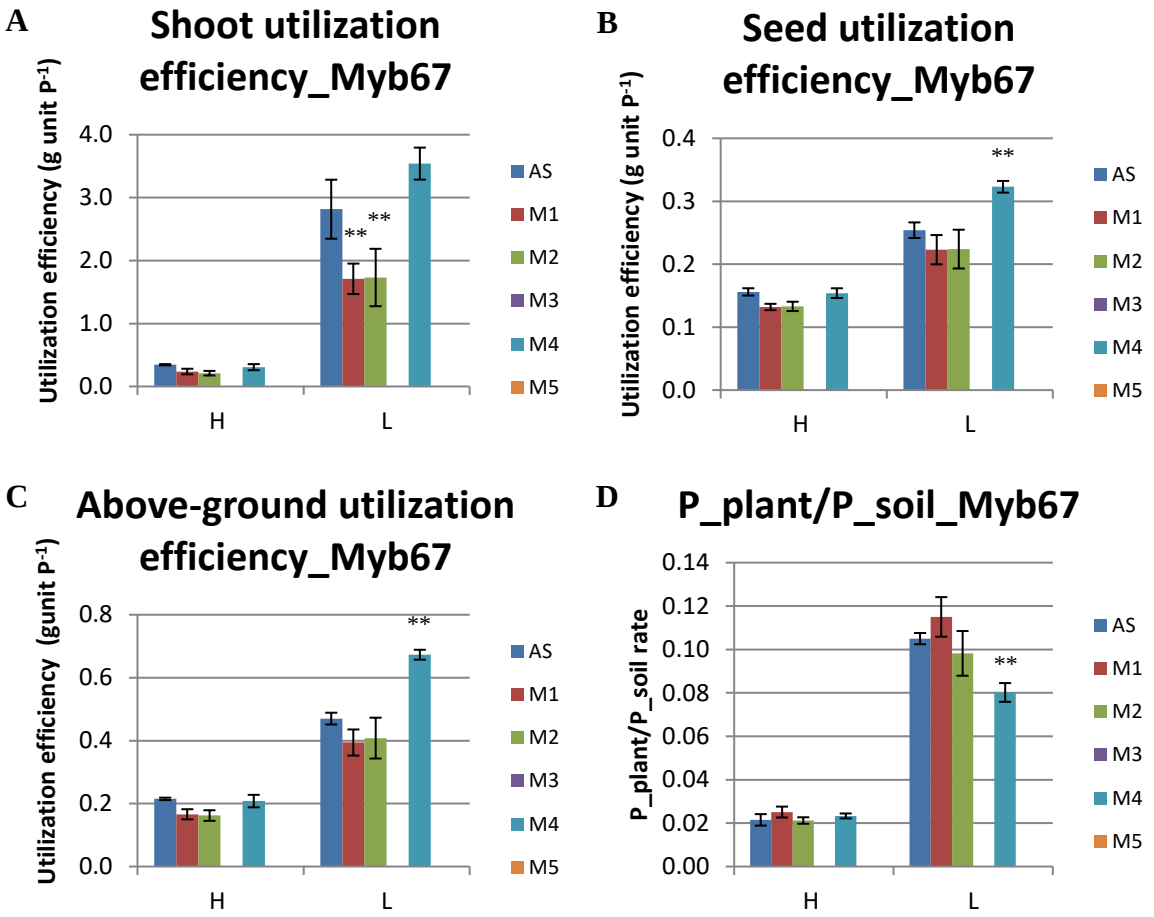


Fig. 65 Phenotypic analysis of *TaMyb67* transgenic and the control lines under high-P and low-P treatments. H: High-P treatment. L: Low-P treatment. AS: the control line (wild-type wheat AS0556). M1–M5: *TaMyb67* transgenic lines. In each line of each treatment, n=3. Differences between the transgenic and control lines under high-P and low-P were analysed by two-way ANOVA respectively. *: A difference significant at P<0.05. **: A difference significant at P<0.01. P-related variables include A: Shoot P utilisation efficiency ("Shoot" represents the non-seed potion after harvest); B: Seed P utilisation efficiency; C: Above-ground P utilisation efficiency; D: P_plant/P_soil ratio.

6.3.2.4 Correlation analysis of *TaMyb67* expression and phenotypes

Phenotypic analysis in previous sections could not imply the transgene effects. To further explore the data, the 20 transgenic plants that were assessed for *TaMyb67* expression from the five *TaMyb67* transgenic lines were investigated by correlation analysis using the method previously described, with results expressed as correlation coefficients in the matrix table.

As shown in Tables 29 and 30, none of the correlation coefficients obtained for phenotypic variables of *TaMyb67* transgenic lines and the expression levels of *TaMyb67* under high-P and low-P were significant and thus neither positively nor negatively correlated. Nevertheless, different phenotypic variables showed partial correlations with each other.

Table 29. Correlation analysis of *TaMyb67* transgenic wheat under high-P

Name of variables	r	A	B	c	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
TaMyb67 expression	A	-																									
Chlorophyll	B	0.195	-																								
Leaf area 1	C	0.440	0.184	-																							
Leaf area 2	D	-0.208	0.211	0.484	-																						
Leaf area 3	E	-0.514	0.082	0.173	*0.7091	-																					
Total leaf area	F	-0.311	0.156	0.487	**0.8749	**0.9309	-																				
Height	G	-0.253	*0.7704	0.308	0.618	*0.6504	*0.6927	-																			
Valid tiller	H	-0.168	-0.127	0.157	-0.256	0.120	0.057	0.002	-																		
Invalid tiller	I	0.301	-0.021	-0.365	-0.021	-0.262	-0.278	-0.313	-0.373	-																	
Total tiller	J	0.242	-0.083	-0.317	-0.145	-0.224	-0.271	-0.336	0.080	**0.8951	-																
Anthesis date	K	-0.202	-0.205	0.245	*0.6641	*0.6686	*0.7023	0.251	-0.289	-0.267	-0.426	-															
Main-spike height	L	-0.380	0.369	0.250	*0.714	**0.9021	**0.8812	**0.8153	-0.021	-0.402	-0.442	0.707	-														
Main-spike weight	M	0.022	*0.7241	0.271	*0.725	0.534	0.629	**0.8287	-0.319	0.124	-0.020	0.329	*0.7034	-													
Spikelet number	N	-0.203	0.298	0.390	**0.9389	*0.708	0.836	**0.6573	-0.314	-0.117	-0.276	0.778	**0.82	*0.7514	-												
Grain number	O	-0.037	0.626	0.529	*0.7333	0.613	0.749	**0.8552	-0.078	-0.167	-0.218	0.274	*0.7221	**0.8816	*0.6787	-											
Thousand-grain weight	P	-0.299	*-0.8401	-0.048	-0.197	-0.180	-0.190	-0.617	0.191	-0.361	-0.296	0.214	-0.308	*-0.7424	-0.213	-0.608	-										
Shoot biomass	Q	-0.297	0.397	0.382	**0.905	**0.7797	**0.8757	*0.7594	-0.090	-0.088	-0.138	0.525	**0.8184	**0.8044	**0.891	0.846	-0.386	-									
Seed biomass	R	-0.170	0.426	0.599	0.775	0.568	*0.7448	*0.7475	-0.050	-0.350	-0.399	0.362	*0.6766	*0.7369	*0.7071	0.927	-0.288	**0.833	-								
Above-ground biomass	S	-0.251	0.428	0.499	**0.8845	**0.716	**0.8536	*0.7873	-0.075	-0.213	-0.265	0.473	**0.7887	**0.8085	**0.845	0.921	-0.358	**0.9667	**0.9469	-							
Shoot P	T	0.630	-0.543	-0.135	-0.250	-0.590	-0.539	-0.725	-0.782	**0.9398	0.720	-0.175	-0.642	-0.192	-0.354	-0.605	-0.191	-0.686	-0.590	-0.668	-						
Seed P	U	-0.283	0.473	0.462	0.505	0.035	0.231	0.448	0.314	-0.367	-0.275	-0.104	0.074	0.422	0.327	0.643	0.734	0.432	*0.8349	0.636	-0.204	-					
Above-ground p	V	0.178	0.050	0.316	0.277	-0.370	-0.162	-0.097	-0.254	0.315	0.246	-0.211	-0.371	0.243	0.049	0.158	0.520	-0.084	0.338	0.108	0.499	0.746	-				
Shoot utilisation efficiency	W	-0.663	0.338	0.286	0.738	*0.8986	*0.9147	**0.9654	0.676	-0.717	-0.492	0.554	0.886	0.640	0.770	*0.9022	-0.044	**0.9713	0.784	**0.9226	*-0.8266	0.319	-0.280	-			
Seed utilisation efficiency	X	-0.722	0.265	0.169	0.749	**0.939	**0.9241	**0.975	0.571	-0.679	-0.517	0.626	0.928	0.682	0.802	*0.8815	-0.068	**0.9699	0.754	*0.908	-0.792	0.268	-0.301	**0.9902	-		
Above-ground utilisation efficiency	Y	-0.686	0.309	0.224	0.721	**0.9191	*0.9141	**0.9631	0.640	-0.711	-0.510	0.585	0.909	0.640	0.772	*0.8776	-0.072	**0.9637	0.750	*0.9026	*-0.8267	0.266	-0.327	**0.9974	**0.996	-	
Plant/soil P ratio	Z	0.179	0.051	0.318	0.277	-0.370	-0.162	-0.097	-0.252	0.313	0.246	-0.212	-0.371	0.243	0.048	0.158	0.521	-0.084	0.338	0.109	0.498	0.747	**1	-0.279	-0.301	-0.327	-

"r" =correlation coefficient. Correlation analysis was carried out by using the expression and phenotypic data of the 20 *TaMyb67* transgenic plants which were analysed by qRT-PCR from the five *TaMyb67* transgenic lines under high-P. The capital letter A indicates the variable of *TaMyb67* expression levels in *TaMyb67* transgenic lines under high-P. The capital letters B to Z indicate other phenotypic variables in *TaMyb67* transgenic lines under high-P, as described in the first column of the table. The number in cell indicates the "r" produced from the correlation analysis for the two variables in such a row and such a column. The column highlighted in yellow shows the "r"s between *TaMyb67* expression (A) and another phenotypic variable (B to Z). The "r" was validated to be true when P<0.05 (shown in red, *significant at P<0.05. **significant at P<0.01.). Otherwise, it was false (as tested by correlated analysis). The "r" in minus indicates a negative linear correlation, otherwise it indicates a positive linear correlation.

Table 30. Correlation analysis of *TaMyb67* transgenic wheat under low-P

Name of variables	r	A	B	c	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
TaMyb67 expression	A	-																									
Chlorophyll	B	0.545	-																								
Leaf area 1	C	-0.207	-0.204	-																							
Leaf area 2	D	-0.617	-0.134	*0.6535	-																						
Leaf area 3	E	-0.266	-0.173	0.470	0.661	-																					
Total leaf area	F	-0.461	-0.189	*0.7647	0.924	**0.8593	-																				
Height	G	-0.391	-0.020	0.628	0.825	**0.8867	**0.9295	-																			
Valid tiller	H	0.058	-0.244	-0.380	-0.517	-0.529	-0.568	-0.557	-																		
Invalid tiller	I	0.105	0.244	-0.162	0.076	0.344	0.142	0.406	0.000	-																	
Total tiller	J	0.098	-0.112	-0.412	-0.431	-0.323	-0.448	-0.322	**0.898	0.440	-																
Anthesis date	K	-0.382	*-0.8523	0.233	0.086	0.237	0.200	0.111	0.499	0.098	0.491	-															
Main-spike height	L	-0.188	-0.042	**0.7757	*0.686	**0.6501	**0.8001	**0.7724	-0.521	0.303	-0.335	0.157	-														
Main-spike weight	M	-0.026	0.411	0.511	*0.6601	*0.7422	*0.7599	**0.8709	*-0.7579	0.424	-0.494	-0.331	**0.74	-													
Spikelet number	N	-0.514	-0.159	0.594	**0.8116	**0.7544	**0.8584	**0.8785	-0.391	0.479	-0.140	0.309	**0.8701	*0.7004	-												
Grain number	O	-0.056	0.597	0.433	0.615	0.468	0.602	*0.729	-0.461	0.457	-0.213	-0.375	0.594	**0.8601	0.637	-											
Thousand-grain weight	P	0.540	0.194	-0.254	-0.287	0.162	-0.133	-0.162	-0.094	-0.206	-0.175	-0.241	-0.419	-0.017	-0.480	-0.175	-										
Shoot biomass	Q	-0.429	-0.169	0.581	*0.7056	*0.8016	**0.8244	**0.8711	-0.174	0.449	0.042	0.426	*0.6549	0.620	**0.8616	*0.6322	-0.187	-									
Seed biomass	R	-0.050	0.492	0.474	0.613	0.617	*0.675	**0.808	-0.432	0.422	-0.202	-0.262	0.548	**0.8715	0.617	**0.9524	0.016	*0.7356	-								
Above-ground biomass	S	-0.262	0.166	0.567	*0.7089	*0.7638	**0.8066	**0.9019	-0.322	0.468	-0.083	0.097	*0.6467	**0.7974	**0.7963	*0.8466	-0.094	**0.9348	**0.9283	-							
Shoot P	T	0.506	0.800	*-0.8595	*-0.8675	-0.713	**-0.9463	-0.794	0.263	0.126	0.389	*-0.8602	-0.607	-0.632	-0.778	-0.472	-0.596	*-0.8974	-0.748	*-0.8886	-						
Seed P	U	0.510	*0.9299	-0.762	-0.806	*-0.8837	**-0.9785	*-0.8766	0.474	0.029	0.503	**-0.9474	-0.751	-0.774	*-0.9094	-0.166	-0.608	*-0.891	-0.543	*-0.8121	**0.9279	-					
Above-ground p	V	0.516	*0.9118	-0.795	*-0.8307	*-0.8555	**-0.9837	*-0.8688	0.431	0.052	0.483	**-0.9394	-0.727	-0.751	*-0.8907	-0.239	-0.613	*-0.9041	-0.598	*-0.8404	**0.9567	**0.9962	-				
Shoot utilisation efficiency	W	-0.620	**-0.9133	0.637	0.789	*0.8824	**0.9388	**0.9466	-0.353	0.163	-0.190	**0.956	0.516	0.774	*0.8445	0.337	0.739	**0.9908	0.769	**0.9613	**-0.9232	**-0.9179	**-0.9311	-			
Seed utilisation efficiency	X	-0.717	**-0.8714	0.648	*0.859	*0.8355	**0.9494	**0.9243	-0.193	0.042	-0.151	*0.9125	0.445	0.692	0.790	0.412	0.772	**0.9814	*0.8396	**0.9795	**-0.942	*-0.9058	**-0.926	**0.981	-		
Above-ground utilisation efficiency	Y	-0.675	**-0.9344	0.649	0.808	*0.8991	**0.9585	**0.9486	-0.314	0.080	-0.234	**0.9646	0.521	0.747	*0.8418	0.288	0.775	**0.9904	0.756	**0.9563	**-0.9296	**-0.9418	**-0.9512	**0.9925	**0.9879	-	
Plant/soil P ratio	Z	0.512	**0.9074	-0.800	*-0.8339	*-0.8502	**-0.9837	*-0.8655	0.429	0.055	0.484	**-0.9362	-0.728	-0.748	*-0.8892	-0.248	-0.608	*-0.9024	-0.600	*-0.8401	**0.9593	**0.9954	**0.9999	**-0.9302	**-0.9258	**-0.9499	-

"r" =correlation coefficient. Correlation analysis was carried out by using the expression and phenotypic data of *TaMyb67* transgenic plants which were analysed by qRT-PCR from the five *TaMyb67* transgenic lines under low-P. The capital letter A indicates the variable of *TaPtf1* expression levels in *TaMyb67* transgenic lines under low-P. The capital letters B to Z indicate other phenotypic variables in *TaMyb67* transgenic lines under low-P, as described in the first column of the table. Each number in a cell indicates the "r" produced from the correlation analysis for the two variables in such a row and such a column. The column highlighted in yellow shows the "r"s between *TaMyb67* expression (A) and another phenotypic variable (B to Z). The "r" was validated to be true when P<0.05 (shown in red, *significant at P<0.05. **significant at P<0.01.). Otherwise, it was false (as tested by correlated analysis). The "r" in minus indicates a negative linear correlation, otherwise it indicates a positive linear correlation.

6.4 Discussion

6.4.1 Alteration of the transgene expression in *TaPtf1*/*TaMyb67* transgenic wheat

Instability of transgene expression has been well documented and frequently reported for plants generated by a transgenic approach (Kohli *et al*., 2010; Shrawat *et al*., 2007). Although the molecular basis for this is not fully understood to date, it has been found a transgene is regulated by multiple factors including chromosomal position effect, copy number and epigenetic or post-transcriptional regulation of gene expression (Charrier *et al*., 2000; Graham *et al*., 2011; Vilperte *et al*., 2016). In this study, the maize ubiquitin promoter employed is a constitutive promoter widely used for monocot transformation, which can enable high levels of transient expression of a transgene *in vivo* (Christensen and Quail, 1996; Cornejo *et al*., 1993; Meng *et al*., 2003). The promoter thus was expected to drive strong overexpression of the *TaPtf1*/*TaMyb67* transgene. However, instability of transgene expression driven by a maize ubiquitin promoter was also previously reported (Rooke *et al*., 2000; Weinhold *et al*., 2013). Characterisation of the transgene expression by qRT-PCR analysis for selected *TaPtf1*/*TaMyb67* transgenic lines in this study revealed that the overexpression was, in fact, only satisfactorily high in few transgenic lines. One possibility is that *TaPtf1*/*TaMyb67* in nature is post-transcriptionally regulated (e.g. by microRNA regulation), which doesn't allow for the gene expression going beyond certain levels. Moreover, the transgene expression also exhibited different patterns between different *TaPtf1*/*TaMyb67* transgenic lines, as well as between high-P and low-P treatments for each transgenic line. Within each transgenic line under each given P treatment, differential expression was also detectable between the two replicate plants. It is likely that the transgene expression in these transgenic lines has resulted from combined effects of different factors.

The differential expression of the transgene between the two replicate plants of each *TaPtf1*/

TaMyb67 transgenic line under each given P treatment might be explained by the influence of segregation in T1 generation (T0: primary transformants). Wheat plants carrying inconsistent copy numbers of the transgene coexisted in the same *TaPtf1*/*TaMyb67* transgenic line, which probably has affected the transgene mRNA levels of different plants in such a line. Knowledge has been informed from early experiments that the *Agrobacterium*-mediated transformation of wheat or barley usually results in low copies of a transgene (less than 5) (Cheng *et al*., 1997; Travella *et al*., 2005). Integration of only one transgene copy would lead to homozygotes and hemizygotes in T1 generation. Integration of more than one transgene copies that intersperse in the genome would lead to independent segregation. However, the presence of multiple transgene copies, especially "head-to-head" or "tail-to-tail" (IR) structure in the same integration site, is inclined to induce gene silencing, which even complicates the scenario of transgene expression (Kohli *et al*., 2010).

The differences in transgene expression observed between the five *TaPtf1*/*TaMyb67* transgenic lines were thought to possibly associate with chromosomal position effect, which refers to the influence of genomic environments at the integration site of a transgene (Matzke and Matzke, 1998). The genomic environments of different transgene loci could sense, respond to and interact with the presence of transgenes, which may vary within populations of plants generated in the same experiment, resulting in the transgene expression being highly variable (Matzke *et al*., 2000). An integration site located at transcriptional inactive regions of the chromatin (e.g. heterochromatin), in most cases, would lead to silencing or repressed expression of transgenes (Matzke *et al*., 2000). In euchromatin regions that are more permissive for transcription, an integration site may lead to specific position effect when it is accidentally located downstream of other regulatory elements, such as a promoter, enhancer, repressor or insulator, which may influence the transgene expression

through combinatorial effects of these elements (Kohli *et al*., 2010). Given that each *TaPtf1*/*TaMyb67* transgenic line has derived from an independent transformation event, the transgene could have randomly integrated into different locations on the host genome and affected by position effect.

Besides copy number and position effect, DNA methylation status is also indicated to significantly affect the expression of a transgene (Morel *et al*., 2000). Both a transgene and its promoter are subject to DNA methylation. In plants, DNA methylation is a biochemical process of the formation of 5-methylcytosine (mC site) from cytosine. This can occur, mediated by DNA methyltransferas, in three DNA sequence contexts: CG, CHG and CHH (H is any nucleotide except guanine) under regulation of different pathways (Secco *et al*., 2017). It has been widely accepted that DNA methylation in the promoter region, histone, or coding region adversely affects the expression of a gene at both transcriptional and post-transcriptional levels (Huettel *et al.,* 2006; Regulski *et al*., 2013), to a large extent depending on the degree of methylation. Interestingly, there have been reports that suggest P deficiency can trigger changes in DNA methylation status. Using a genome-wide study of DNA methylation in rice and *Arabidopsis*, Secco *et al*. (2015) identified the modification of DNA methylation induced by P-starvation in both species. Another study by Young-Villalobos *et al*. (2015) also showed that DNA methylation in *Arabidopsis* plants deprived of P was globally modified. Therefore, changes in DNA methylation status triggered by P deficiency are likely to offer explanations for the inconsistency of the transgene expression patterns between high-P and low-P treatments in each *TaPtf1*/*TaMyb67* transgenic line. Furthermore, DNA methylation is implicated to be a major factor resulting in complete or incomplete transgene silencing, which is defined as transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS), respectively, according to their

different mechanisms studied in plants (Morel *et al*., 2000). Perhaps the unsatisfactory overexpression observed in *TaPtf1*/*TaMyb67* transgenic lines, are also relevant to different mechanisms of transgene silencing. Transgene silencing is a series of "host-defence" responses commonly observed in plants enabled by a variety of aberrant DNA-DNA, DNA-RNA or RNA-RNA interactions and potentially affected by the degree of homology between the transgene and endogenous genes, the complexity of the host genome, the genomic position of two transgenes, etc. (Meng *et al*., 2003; Waterhouse *et al*., 2001). TGS is often correlated with DNA methylation in the promoter of a transgene (and/or homologous endogenous genes) and a compact chromatin structure, while PTGS is often characterised by inabilities to accumulate mRNA in the cytoplasm due to sequence-specific RNA degradation and coupled with DNA methylation in the coding region of a transgene (and/or homologous endogenous genes) (Matzke *et al*., 2007; Meng *et al*., 2003; Pikaart *et al*., 1998; Vaucheret *et al*., 2001).

6.4.2 Phenotypes in *TaPtf1* transgenic wheat

In this study, overexpression of *TaPtf1* was verified in the five independent *TaPtf1* transgenic lines by qRT-PCR analysis (except P3 in high-P which had suppression), though the overexpression levels in several lines appeared to be restricted to various extents, especially in low-P (Fig. 55). Compared to that in high-P, the transgene expression of P2 and P4 in low-P greatly decreased, although P3 and P5 in low-P showed small increases. As discussed in the previous section, the expression of a transgene can be affected by DNA methylation status in either the transgene or its promoter, while P deficiency can trigger changes of DNA methylation status. The alteration, as stated above, of the transgene expression between two P treatments is thus presumably associated with changes in DNA methylation status. Meanwhile, changes in DNA methylation status seem also to provide a basis for understanding the transgene expression and phenotypes in *TaPtf1* transgenic wheat. In their study on an *Arabidopsis* mutant defective of DNA methylation, Young-Villalobos *et al*. (2016) have reported that DNA methylation acts a crucial role in modulating a group of P starvation-responsive genes which are necessary for the establishment of proper P-stress responses. In *TaPtf1* transgenic wheat treated with high-P, although more satisfactory overexpression was achieved (except P3 under high-P), only a few detectable differences were observed in phenotypic analysis and no correlation with *TaPtf1* expression was found for any phenotypic variable. This could be attributed to a lack of changes in DNA methylation status for induction of the downstream P-stress-responsive genes transcriptionally regulated by *TaPtf1* when P is sufficient; unresponsive to high-P. In *TaPtf1* transgenic wheat treated with low-P, the results of correlation analysis were able to indicate several phenotypic variables in association with the transgene effects of *TaPtf1*. This had provided further support for interpreting the transgene effects of *TaPtf1* when only weak phenotypes under low-P were found by phenotypic analysis and only insufficient expression data were collected from qRT-PCR analysis

(due to technical limits). The combined results seem to reasonably reflect that the phenotypic effects of overexpressing *TaPtf1* are subject to different P supply, which corresponds with previous findings of *TaPtf1* in wild-type wheat.

Furthermore, although the weak phenotypes under low-P may partially be explained by the unsatisfactory overexpression, it also appears, interestingly, that globally overexpressing *TaPtf1* may neutralise the phenotypic effects. As discussed in Chapter 3, it has already been shown in wild-type wheat that the functional roles and expression patterns of *TaPtf1* are tissue-specific, which, so far, can be described by three scenarios (Aziz *et al.*, 2014). In leaves, the expression of *TaPtf1* is constitutive and not P-stress responsive. In contrast, *TaPtf1* in both the stem and roots seems to be involved in regulatory activities of PUE in response to P stress (Aziz *et al*., 2014). In the stem, *TaPtf1* is likely to have a conserved function with that of *OsPtf1*/*ZmPtf1* and increase the flux of sugars from shoots to roots under P deficiency/low-P. Sugars (most likely sucrose), already known as a group of signals involved in systemic P-stress responses, can subsequently induce P-stress responses in roots to enhance P uptake and translocation to shoots, including expression of PTs and other PSI genes, root remodelling and other root metabolic changes (Jain *et al*., 2007; Karthikeyan *et al*., 2007). In roots, *TaPtf1* is suggested to be mainly a negative regulator in lateral roots but induced in the early stage of P deficiency/low-P, with the expression being reduced by degrees along with time (Espindula *et al*., 2009; Aziz *et al*., 2014). Therefore, *TaPtf1* seems to have contrasting effects on PUE in the stem and roots in response to P stress. Because of this, it is possible overexpression of *TaPtf1* in the stem and roots can potentially counter each other, resulting in the weak phenotypes or no phenotypic difference in *TaPtf1* transgenic wheat under low-P. On the other hand, while some implications of the association with the transgene effects of *TaPtf1* were found, due to the small sample size of the *TaPtf1* transgenic lines in this study, the findings remain to be further validated using a larger population in the subsequent generation.

6.4.2.1 Plant height and tiller number in *TaPtf1* transgenic lines under low-P

Based on results analysed for plant height, there was a tendency to increased height in *TaPtf1* transgenic lines under low-P, although a statistical significance was only detected for P3 and P5. The unsatisfactory statistical significance could be attributed to the low and inconsistent overexpression between replicates in P1 and P2 and pathogen infection in P4 during plant growth. Further evidence obtained in correlation analysis confirmed that the mRNA expression of *TaPtf1* positively contributed to plant height. Of the five transgenic lines, the highest plant height of P5 coincided with the maximum *TaPtf1* expression levels detected in this line. So far, there is no information describing a regulatory role of *Ptf1* on plant height in the literature, but evidence has been accumulated to suggest that phytohormones are actively involved in manipulation of Pi homeostasis (Franco-Zorrilla *et al*., 2005; Rubio *et al*., 2009). The promotional effects of *TaPtf1* on plant height are likely to be related to changes in phytohormone activities resulting from the combined effects of overexpressing *TaPtf1* under P deficiency/low-P.

Studies on tiller numbers yielded results indicating that the tiller emergence in all *TaPtf1* transgenic lines under low-P significantly declined. However, the control under low-P was slightly higher than that under high-P. A reduction in the control was initially expected because P-deficient conditions can adversely affect P accumulation in shoots, causing reduced tiller emergence in wheat (Rodríguez *et al*., 1999). Meanwhile, a correlation with the mRNA expression of *TaPtf1* was not found. It thus becomes contestable, unless further evidence can be collected, to ascertain whether the statistical significance detected in each line is valid or to conclude a negative transgene effect of *TaPtf1* on the tiller number. On the other hand, there might be a likelihood that the degree of P stress used in the experiment was insufficient to affect the tillering ability in the control. In that case, the transgenic lines appear to be more sensitive to P stress than the control, which may be a consequence of the combined effects of overexpressing

TaPtf1 under P deficiency/low-P. This hypothesis nevertheless is subject to further investigation.

6.4.2.2 Biomass and P accumulation in *TaPtf1* transgenic lines

From correlation analysis, the shoot biomass in *TaPtf1* transgenic lines under low-P was shown to increase with the mRNA expression of *TaPtf1*, which correspondingly supported the indication for the increases of shoot biomass under low-P characterised by phenotypic analysis, though the increases (except P3) were small compared with the control. The increased shoot biomass seems to be largely attributed to the fact of the increased plant height under low-P, which requires improved PUE, either enhanced P uptake and translocation to shoots or more efficient shoot P utilisation (e.g. P recycling/metabolic bypass), to be achieved during vegetative growth for higher biomass accumulation in shoots than the control. As described at the beginning of the section, *TaPtf1* in the stem and roots appears to have opposite regulatory roles in contributing to P uptake (Aziz *et al*., 2014; Espindula *et al*., 2009). Overexpression of *TaPtf1* in the stem under P deficiency/low-P is supposed to further increase sugar flux and signalling, which accordingly can expand systemic P-stress responses of expression of PTs and other PSI genes, root remodelling and other root metabolic changes, thereby leading to more significantly increased P uptake (than the control). Of these, lateral root growth is a primary strategy for root remodelling and thus pivotal to P uptake. However, *TaPtf1* is suggested to be a negative regulator of lateral roots, though with an early induction in roots (Aziz *et al*., 2014). Overexpression of *TaPtf1* in roots under P deficiency/low-P, in a context of global overexpression used in this study, though may be briefly beneficial during the early induction of *TaPtf1*, can considerably impair lateral root growth and thus the positive regulatory effects on P uptake contributed by overexpression of *TaPtf1* in the stem. Hence, the P accumulation in shoots during vegetative growth would be largely determined by a combination of the positive and adverse effects of overexpressing *TaPtf1* in the stem and

roots, whereas it seems obscure which one is more influential than the other overall. Given the effects of the stem over roots, P uptake would be largely weakened by the adverse effect from roots, though still enhanced in *TaPtf1* transgenic wheat compared with the control, resulting in the insufficiently increased shoot biomass as shown in this study. Alternatively, given the effects of roots over the stem, where P uptake in *TaPtf1* transgenic wheat would be lower than the control, the insufficient increases of shoot biomass may have resulted from some other systemic P-stress responses of P-efficient utilisation strengthened by overexpression of *TaPtf1* (e.g. P recycling/bypass). It has been shown that involved in systemic P-stress responses are also those metabolic changes in plant shoots which improve P recycling or bypass (e.g. PAPs, SQD) (Plaxton and Tran, 2011).

While P accumulation in shoots during vegetative growth was not assessed in this study, the shoot P measured for each plant represents the remaining P in the non-seed portions of the above-ground phase at harvest after graining-filling (mainly the leaf and stem of shoots). Wheat is one of those crops that have rapid senescence at post-anthesis, with most grain P deriving from vegetative tissues (leaves, stem, roots) rather than post-anthesis P uptake (Rose *et al*., 2007). Most P accumulated in shoots during vegetative growth is expected to be redistributed to supply grain-filling and stored in the form of phytate. Indeed, reductions of P accumulation in shoots during vegetative growth can consequently bring down grain P at harvest (Hocking, 1994; Wang *et al*., 2016b). Therefore, P accumulation in shoots during vegetative growth is logically inseparable and positively correlated with grain P as well as the remaining P in shoots. Interestingly, the results in *TaPtf1* transgenic lines under low-P seemed not to be consistent with this. The “seed P” showed lower ranges than the control, suggesting similar patterns ought to be seen in the “shoot P” remaining in the non-seed portions. Surprisingly, the shoot P characterised by phenotypic analysis was, in fact, found to be

marginally higher than the control (except P3). Correlation analysis also further revealed that the mRNA expression of *TaPtf1* positively contributed to the shoot P in these lines under low-P. Moreover, reduction potentials of seed biomass were also observed in most *TaPtf1* transgenic lines under high-P and low-P in comparison with the control. These together might imply *TaPtf1* functions as a negative regulator and inhibit P partition to seeds for grain-filling, thereby leading to the shoot P being reversely accumulated.

Meanwhile, it is unclear why significant reductions of the seed biomass were observed to be greater under high-P than under low-P, but perhaps associated with accelerated carbon translocation from shoots. Furthermore, a positive correlation between the mRNA expression of *TaPtf1* and the shoot P under high-P was also not detected like that under low-P, while neither the seed P nor biomass under high-P or in low-P was demonstrated to correlate with the mRNA expression of *TaPtf1*. On the other hand, the shoot utilisation efficiency in *TaPtf1* transgenic lines under low-P was detected to be negatively correlated with the mRNA expression of *TaPtf1*, which might reflect that the negative contribution of *TaPtf1* to grain-filling (reverse accumulation of the shoot P) is over the positive contribution of *TaPtf1* to the shoot biomass. Also, the above-ground P in the transgenic lines under low-P (calculated as shoot P plus seed P) may have been affected by the capacity of P pool in roots in contributing to grain-filling and thus should not be simply explained as the P accumulation in shoots during vegetative growth. All these appear to require further evidence.

In addition, the transgenic line of P3 has given a poor representation of the transgene effects of *TaPtf1* on biomass, P accumulation or utilisation and abnormally displayed strong growth repression, with several extremely low mean values of the shoot, seed and above-ground biomass or P accumulation being observed in phenotypic analysis. The reason was unclear, but

it might associate with some destructive effects resulting from position effect (e.g. insertional inactivation of the endogenous genes related to essential metabolism pathways), or somaclonal variation (see next section) in this line. As such, P3 is unsuitable for the study of progeny generations.

6.4.2.3 Phenotypes independent of P status in *TaPtf1* transgenic lines

Apart from the phenotypic variables described above, statistically significant differences were also partially observed in *TaPtf1* transgenic lines under both P treatments for a few other growth-related variables in phenotypic analysis. However, their causality was poorly explained by the transgene expression patterns and it was also difficult to find further support from correlation analysis.

Significant reductions of chlorophyll were shown by a few and a majority of *TaPtf1* transgenic lines under high-P and low-P respectively. Under high-P, there was no correlation between the mRNA expression of *TaPtf1* and chlorophyll concentrations. Interestingly, however, the chlorophyll concentrations under low-P was found to be positively correlated with the mRNA expression of *TaPtf1*, which is believed to connect with overexpression of *TaPtf1*. This also seems to indicate that other influential factors, rather than the transgene effects of *TaPtf1*, have caused the observed reductions of chlorophyll in these lines. Given the measurement was taken before anthesis, the results were unlikely to be affected by natural leaf senescence. It is possible the results of reduction are associated with a metabolic disorder or other cell abnormalities in consequence of the expression of *bar* gene.

Significant increases of anthesis date were generated by several *TaPtf1* transgenic lines (three under high-P and two low-P), with other lines similarly showing higher ranges than the control. Nevertheless, the delay in anthesis, neither in high-P nor low-P, was revealed to correlate with

the mRNA expression of *TaPtf1*. Perhaps it is rational to consider the small increases without significance as natural variations while those significant increases could possibly be due to somaclonal variations. Somaclonal variation is tissue culture-induced genetic and epigenetic instabilities resulting in phenotypic variation, which may be heritable in the progeny generations (Larkin and Scowcroft, 1981). Due to their different interactions with the transgene expression, the impacts of somaclonal variation could complicate the inconsistency of phenotypes within transgenic populations and even further extend distinctions between independent transgenic lines (Meng *et al*., 2003). Alternatively, the changes may also associate with the expression of *bar* gene as previously discussed.

A surprisingly significant increase of leaf area 3 was detected in a greater proportion of *TaPtf1* transgenic lines (except P4) under high-P and low-P compared with the control, whereas no correlation was obtained. Under high-P, although P3 resulted in a significant increase like other lines, its suppressed expression of *TaPtf1* was not in accordance with the overexpression in other lines, thereby providing no evidence for the transgene effects of *TaPtf1*. Together, these probably imply the transgene effects of *TaPtf1* are unrelated to leaf growth.

Significant increases under low-P and higher ranges than the control under high-P were also seen for the spikelet number in phenotypic analysis, while the results of correlation analysis did not indicate a contribution of the mRNA expression of *TaPtf1* to these changes. It seems uncertain if these increases are linked to the transgene effects of *TaPtf1* or maybe they are merely outcomes of other contributing factors such as the expression of *bar* gene as previously discussed.

6.4.3 Phenotypes in *TaMyb67* transgenic wheat

In this study, overexpression of *TaMyb67* was detected in the five independent *TaMyb67* transgenic lines under high-P excluding M4, whereas in low-P overexpression was only detectable in M2 and M3 but not in other three lines where suppression, which might be co-suppression, seemed to have occurred (Fig. 62). Also, the expression of *TaMyb67* in all transgenic lines except M3 was more restricted in low-P when compared to that in high-P. Apart from the cue of changes in DNA methylation status triggered by P deficiency as previously discussed, the suppression under low-P might also associate with the homology between the transgene and endogenous genes. A transgene partially or entirely homologous to the host genes is likely to cause suppression in a co-ordinated manner (co-suppression) of both the transgene and homologous endogenous genes (Depicker and Van Montagu, 1997). However, co-suppression can be delayed when the endogenous genes are not expressed or not induced (Smith *et al*., 1990; Vaucheret *et al*., 1997). The endogenous gene *TaPhr1*, which is highly similar to *TaMyb67*, is induced under P deficiency/low-P (Wang *et al*., 2013b), while other splice variants derived from *TaPhr1*/ *TaMyb67* homoeoloci, as suggested by the data obtained in Chapter 3, might also be induced under P deficiency/low-P. These together also seemed to have increased the possibilities of the transgene and homologous endogenous genes prone to suppression co-ordinately in P-stressed *TaMyb67* transgenic lines.

The expression of *TaMyb67* characterised for the two replicate plants of each *TaMyb67* transgenic line was not found to correlate with any phenotypic variable of the corresponding transgenic plants as assessed by correlation analysis. Meanwhile, the results of phenotypic analysis, when compared with the expression data of *TaMyb67*, also offered no indication of the transgene effects of *TaMyb67*, despite some statistically significant differences being found for a few

phenotypic variables. Among these, significant increases of anthesis date and spikelet number in partial transgenic lines, with other lines showing higher ranges than the control, were observed under both P treatments but unlikely explained by the inconsistent expression of *TaMyb67* between different lines. Since similar increases also occurred to *TaPtf1* transgenic lines as previously discussed in 6.4.2, it is therefore assumed the increases in *TaMyb67* transgenic lines may likewise be relevant to the expression of *bar* gene or some other unknown factors. Other significant differences detected on even fewer occasions (or even only in individual lines) in *TaMyb67* transgenic lines under high-P or low-P seem not to have sufficient support from the remaining transgenic lines under the same P-treatment. Of these, the significant reductions of harvest-related phenotypic variables in M3 under high-P might have been affected by pathogen infection as observed during plant growth while the rest of significant differences might be random variations or caused by unclear reasons.

Furthermore, M4 appeared to be more dissimilar from other transgenic lines, as well as the control, in phenotypes, relating to greater performance in leaf growth, biomass and P utilisation under both high-P and low-P. This was initially thought to have resulted from the suppression of *TaMyb67* in this line. However, the idea could not be supported by M1 and M5 under low-P, where suppression, though to a lesser extent than that of M4, was also detected but not shown to impact on their phenotypes which were closer to the control. The observed dissimilarity of phenotypes in M4 may otherwise have derived from somaclonal variation in consequence of tissue culture, as previously discussed in 6.4.2. Alternatively, position effect of integration of transgenes may also lead to variation in phenotypes. It has been widely accepted that position effect can disturb the equilibrium of natural gene expression, which may subsequently cause metabolic disorders and ultimately variations of phenotypes between transgenic lines (Kohli *et al*., 2010). Differently,

direct insertional inactivation of an endogenous gene (by the transgene) can cause the loss-of-function of the gene, resulting in disturbance of gene expression and relevant pathways and hence variation in phenotypes (Birch, 2000). In addition, the expression of an endogenous gene could also be inactivated or repressed due to being translocated to a different transcriptional inactive region of the chromatin following heterochromatinization that is frequently caused by DNA methylation of transgenes, which is also a potential explanation for variations in phenotypes in transgenic populations, as with the classical example of the eye colour in drosophila (Eiler and Wakimoto, 1995). Therefore, the transgenic line M4 probably has been affected by these factors.

Except for the above-mentioned phenotypic differences, other aspects of the phenotypes shown by the studied *TaMyb67* transgenic lines nevertheless looked more like the control either under high-P or low-P, although overexpression was detected in four lines under high-P and two lines under low-P. The likely role of *TaMyb67* as an ecotypic variation of *TaPhr1-B1* has been discussed in Chapter 3. Overexpression of *TaMyb67* was expected to confer PUE-improved phenotypes like those previously observed in *TaPhr1-A1* overexpression lines under P deficiency/low-P, such as increases in tiller numbers, biomass and P accumulation (Wang *et al*., 2013b). However, the studied *TaMyb67* transgenic lines did not show these phenotypes. It might be possible that the downstream P-stress-responsive genes regulated by *TaMyb67* were unresponsive to high-P (as previously discussed in 6.4.2 for *TaPtf1* transgenic lines), while the low level of (or no) overexpression in these lines under low-P was ineffective to alter the phenotypes.

Chapter summary

In summary, the *TaPtf1*/*TaMyb67* transgene expression exhibited different patterns. Overexpression *TaPtf1*/*TaMyb67* seemed not to affect the phenotypes under high-P. Under low-P, the combined results of phenotypic and correlation analyses of *TaPtf1* transgenic wheat indicated that the transgene effects of *TaPtf1* is likely to positively associate with chlorophyll, plant height, shoot P and biomass but adversely affect grain-filling. On the other hand, no transgene effects were implied by *TaMyb67* transgenic wheat under low-P, presumably due to the low (or no) overexpression in these plants. In addition, unexpected phenotypes seemed to be obtained as well.

Chapter 7 Studying the sub-cellular localisation of TaPtf1 and TaMyb67 in wheat protoplast (pipeline 3)

7.1 Overview

Transcriptional factors (TFs) primarily have a sub-cellular location in the nucleus due to their essential functions of DNA-binding (Kauffman and O'Shea, 1999). Navigation of a TF to the nucleus relies on the NLS element existing within the TF as a signal (Stewart, 2007). In previous studies on lower ploidy crops species, Ptf1 was reported to have a nucleic location in rice (Yi *et al.,* 2005) and soybean (Li *et al*., 2014). It was thus hypothesised that TaPtf1 would be similarly located in the nucleus. Meanwhile, previous reports of OsPhr2 (Zhou *et al*., 2008a) and its orthologues in *Arabidopsis* (Nilsson *et al*., 2007) and maize (Wang *et al*., 2013a) indicated these TFs are located in the nucleus. Therefore, TaMyb67 might also have a nucleic location. Nevertheless, bread wheat, as a polyploidy crop, has a more complex and highly repetitive genome compared to the lower ploidy species (Glover *et al*., 2015). The gene function and sub-cellular location in wheat may vary or have species-specific features.

With the aim to validate the sub-cellular location of TaPtf1/TaMyb67, this chapter described the work of pipeline 3 (Fig. 8), where two constructs for transition expression of TaPtf1:GFP and TaMyb67:GFP fusions were generated and transformed to wheat protoplast cells, followed by fluorescence analysis under a laser-scanning microscope (LSM). However, reliable results were not able to be obtained in the described work due to errors in the constructs when they were generated, which was discussed in this chapter.

7.2 Materials and methods

7.2.1 Generation of 16318PTF1/16318MYB67

7.2.1.1 Vector for cloning

A sub-cloning vector pZeroback was supplied by TianGen (Beijing, China) and the plasmid vector 16318GFP was provided by Dr Zengyan Zhang (CAAS, China). The pZeroback (Fig. 66) is a small linearised vector (2974bp) that mainly serves for fast and efficient sub-cloning. It allows insertion of a DNA fragment with blunt ends, but also has MCS to enable insertion of a DNA fragment with sticky-ends. It employs an ampicillin-resistant gene (*ampr*/*bla* gene) as the bacteria selection marker. The GFP vector 16318GFP (Fig. 67) is a 4400bp vector featured with a fluorescence *hGFP* gene downstream of MCS. The MCS allows insertion of a target DNA while a doubled-35S promoter can enhance the expression of the target DNA. The reading frame between the doubled-35S promoter upstream of MCS and the CAMV terminator downstream of the *hGFP* gene can enable the generation of GFP fusion. The vector also employs the ampicillin-resistant gene (*ampr*/*bla* gene) as the bacteria selection marker.

7.2.1.2 Cloning method

To generate the 16318PTF1/16318MYB67 construct achieved by using restriction enzyme digestion and ligation, a sub-cloning step was involved. BamHI and SalI were chosen as suitable cloning sites on the 16318GFP vector to allow the insertion of *TaPtf1*/*TaMyb67*. Primers (described as GFP primers) were designed based on the CDS primers previously used to amplify the CDS of *TaPtf1*/*TaMyb67* [PTF(27)/PTF(20) and MYB(23)/MYB(18), as described in Chapter 3] and added with BamHI and SalI onto the 5’ end and 3’ end of the CDS primers respectively.

The CDS of *TaPtf1*/*TaMyb67* added with restriction enzyme sites was amplified from the

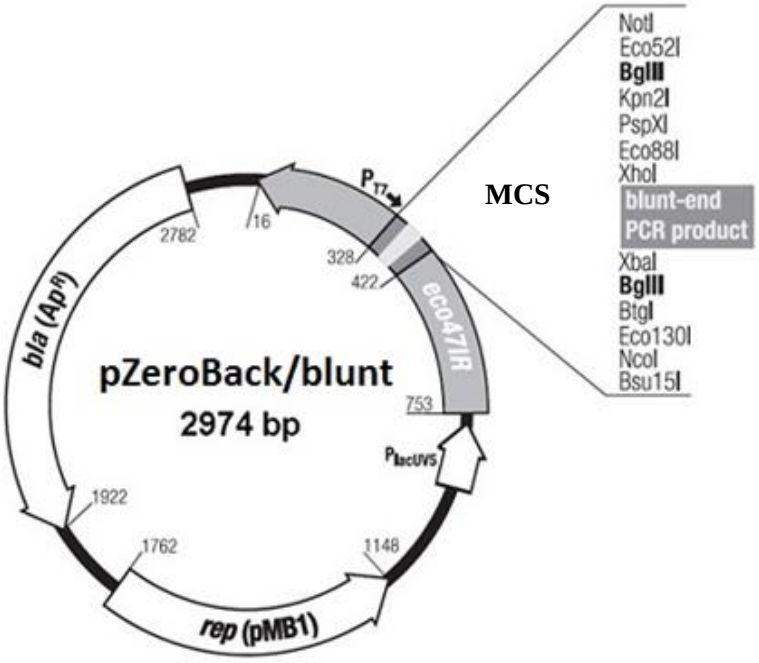


Fig. 66 Vector map of pZeroback and its MCS. Source: reprinted with permission by Tian-Gen (Beijing, China).

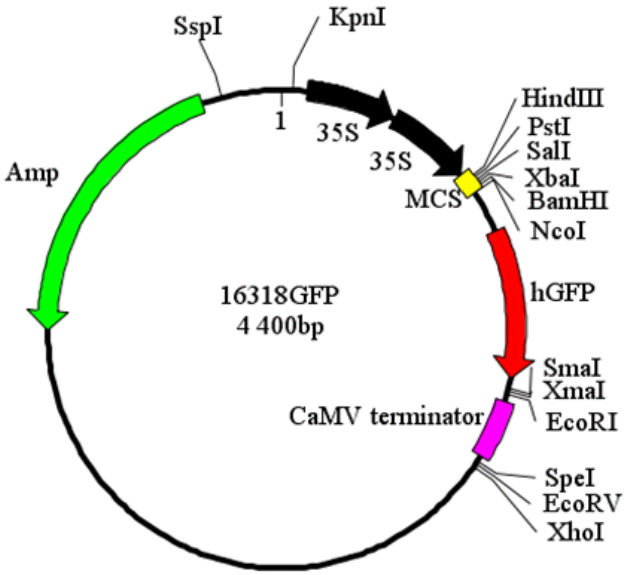


Fig. 67 Vector map of 16318GFP and its MCS. Source: reprinted with permission by Dr Zeng-yan Zhang (CAAS, China).

Table 31. Ligation of pZeroback vector and the CDS of *TaPtf1*/*TaMyb67*

	ZePTF1	ZeMYB67
***TaPtf1* (µL)**	2	-
***TaMyb67* (µL)**	-	2
pZeroback (µL)	0.5	0.5
T4 ligation buffer (µL)	1	1
T4 ligase (µL)	0.5	0.5
PEG4000 (µL)	1	1
ddH20 (µL)	5	5
Total (µL)	10	10

Table 32. Ligation of 16318GFP vector and the CDS of *TaPtf1*/*TaMyb67*

	1638PTF1	1638MYB67
***TaPtf1* (µL)**	1.5	-
***TaMyb67* (µL)**	-	2
Linearised 16318GFP vector fragment (µL)	0.5	0.5
ddH_2O (µL)	6.5	6
T4 Ligation buffer (µL)	1	1
T4 Ligase (µL)	0.5	0.5
Total (µL)	10	10
The reaction was incubated at 22°C for 1 hour		

G1390PTF1/G1390MYB67 constructs (described in Chapter 4) by using the GFP primers, high-fidelity KOD-Plus polymerase (1U μL^{-1}) with buffer (ToYoBo, Shanghai, China) in a 50µL standard PCR reaction system according to Method 2) as described in 2.7. The PCR products were assessed by agarose gel electrophoresis and further purified from the gel according to Method 2) as described in 2.8 and 2.11, followed by being adjusted to appropriate concentrations for the downstream cloning.

Mediated by T4 ligase, the purified PCR products were ligated to pZeroback, as described in Table 31. Following this, the ligation products were used to transform competent *E.coli* TOP10 cells individually and selected on LB+Ampicillin (100mg L^{-1}) plates. Single colonies picked from the plates were assessed by a screening PCR using primers of PTF(27)/PTF(20) and MYB(23)/ MYB (18) and agarose gel electrophoresis according to Method 2) as described in 2.7 and 2.8, to select successful sub-clones ZePTF1/ZeMYB67. Plasmids of ZePTF1/ZeMYB67 were subsequently extracted according to Method 2) as described in 2.5 and assessed by restriction endonuclease analysis with BamHI and SalI (Appendix 18) and agarose gel electrophoresis.

Plasmids of ZePTF1/ZeMYB67 and 16318GFP were prepared to up to 1µg, based on concentrations determined by spectrophotometric analysis, and double digested with BamHI and SalI according to the method described in 2.9, prior to being assessed by agarose gel electrophoresis. Corresponding insert and vector DNA fragments were purified from the gel as previously described and adjusted to appropriate concentrations. The ligation of the purified insert and vector fragments was performed as in Table 32. The ligation products were used to transform competent *E.coli* TOP10 cells individually and selected on LB+Ampicillin (100mg L^{-1}) plates. The following procedures for PCR screening, plasmid extraction and restriction endonuclease analysis were carried out as previously described, with one positive clone of

16318PTF1/16318MYB67 being confirmed. Plasmids of 16318PTF1/16318MYB67 were also assessed by DNA sequencing with primers of PTF(27)/PTF(20) and MYB(23)/MYB(18) according to Method 2) as described in 2.11.

7.2.2 Polyethylene glycol (PEG)-mediated transformation of wheat protoplast and LSM analysis

The method was adapted from a protocol provided by Dr Zengyan Zhang's lab (CAAS, China). The blank GFP vector, 16318GFP, was used as the control. Plasmids of 16318GFP, 16318PTF1 and 16318MYB67 were prepared as previously described to the required concentration of 1μg μL^{-1} and transformed respectively into wheat protoplast cells mediated by PEG, with the results observed under a Laser scanning microscope (LSM).

7.2.2.1 Preparation of buffer solutions

Prior to protoplast isolation, several chemical stock solutions were prepared separately and sterilised by syringe and filter-membrane (0.45μm) strainer, including MES (0.1M, pH=5.7), Mannitol(0.8M), $CaCl_2.2H_2O$(1M), KCl (2M) and $MgCl_2$ (2M), glucose (5mM). Five buffer solutions listed below were further prepared by using the stock solutions:

-Buffer WI: MES (4mM), Mannitol (0.5M), KCl (20mM)

-Buffer MMG: MES (2mM), Mannitol (0.4M), $MgCl_2$ (15mM).

-Buffer PEG: PEG4000 (40%, w/v), Mannitol (0.4M), $CaCl_2.2H_2O$ (10mM)

-Buffer W5: MES (1.5mM), NaCl (1.5M), $CaCl_2.2H_2O$ (1.25M), glucose (5mM) and KCl (5mM). Buffer W5 was prepared and sterilised by autoclave at 121°C for 20 minutes and cooled to room temperature.

-Enzymolysis buffer: Cellulose R10 (1.5%, w/v), Macerozyme R-10 (0.4%, w/v), Mannitol (0.4M), KCl (20mM) and $CaCl_2.2H_2O$ (20mM). To make the buffer, 1mL of MES was added to a 50mL falcon tube

and heated at 70°C water bath for 5 minutes, after which the others four stock solutions were individually added. The mixed solution was incubated at 55°C water bath for 10 minutes and cooled down before mixing with 0.05mL of additional $CaCl_2.2H_2O$. The solution was finalised at the volume of 5mL with ddH_2O and sterilised by syringe and filter-membrane (0.45 µm) strainer.

7.2.2.2 Plant material, protoplast isolation and transformation

Wheat seeds (Var. Chinese spring) were germinated in a polystyrene petri dish (90x15mm, sterile) filled with two layers of moist filter paper (Ø 85-90mm, sterile) at room temperature (25°C) under natural sunlight for 3 days. The seedlings were grown for another 7 days before protoplast isolation. Young leaf tissues (0.5–1g) were taken and carefully cut into 0.5–1mm pieces, which were subsequently digested in 5mL of buffer Enzymolysis in the dark for 3 hours at room temperature. Next, 5mL of buffer W5 was added into the lysis and the 10mL mixture was filtered by a 75µm filter-sieve to elute protoplast cells into a 50mL centrifuge tube (flat bottom), which was centrifuged at 945rpm for 1 minute at room temperature. The supernatant was discarded and 2mL of buffer W5 was gently added to re-suspend the protoplast cells. The resuspension was incubated for 30 minutes on ice, after which the supernatant was discarded without disturbing the bottom phase before 1mL of buffer MMG was added at room temperature. For transformation, 100µL of the protoplast cells were gently mixed with 10µg plasmid DNA in a 2mL microcentrifuge tube prior to the addition of an equal-volume (110µL) of buffer PEG into the tube which was incubated at room temperature for 30 minutes' reaction. The reaction was deactivated by a double-volume (440µL) of buffer W5, after which the tube was centrifuged at 945rpm for 1 minute and the supernatant was further removed. At last, 300µL of buffer WI was used to gently dilute and re-suspend the protoplast cells, followed by the tube being incubated flatwise in the dark for 24–48 hours at room temperature.

7.2.2.3 Laser scanning microscope (LSM)

Following the previous step, 100µL of the incubated protoplast cells was pipetted to spread onto a microscope slide and a cover slice was placed over for microscope analysis. This was then observed on a Zeiss LSM700 confocal laser scanning microscope according to manufacturer's instructions (Zeiss, Carl-Zeiss-Strasse, 73447, Oberkochen, Germany), and the imaging analysis was performed with Zen2009 software (Zeiss, Germany).

7.3 Results

7.3.1 Generation of 16318PTF1/16318MYB67 construct

7.3.1.1 Sub-cloning and cloning of 16318PTF1/16318MYB67

For 16318PTF1 and 16318MYB67, the planned approach to cloning was to add two restriction enzyme sites, BamHI and SalI, to the end of the CDS of *TaPtf1*/*TaMyb67* and to construct the sub-cloning vectors ZePTF1/ZeMYB67 through a blunt-end ligation by using pZeroback. The sub-cloning was employed in that the BamHI and SalI restriction enzyme sites remained uncut after PCR amplification. This was followed by the cleavage of *TaPtf1*/*TaMyb67* from ZePTF1/ZeMYB67 and re-ligation with the linearised 16318GFP vector through the BamHI and SalI restriction enzyme sites of the vector.

The GFP primers, designed based on the CDS primers of *TaPtf1*/*TaMyb67* as described in method section 7.2.1.2, are displayed in Table 33. The forward CDS primer [PTF(27) or MYB(23)] was flanked by SalI outside the 5' end, while the reverse CDS primer [PTF(20) or MYB(18)] was flanked by BamHI outside the 5' end. The PCR amplification, sub-cloning and PCR screening were performed as described in method section 7.2.1.2, with positive clones of ZePTF1/ZeMYB67 being screened out and confirmed. The double digestion of ZePTF1/ZeMYB67 and G16318GFP with BamHI and SalI for subsequent cloning steps was carried out as described in method section 7.2.1.2. The purpose was to allow *TaPtf1*/*TaMyb67* to acquire sticky ends when they were excised from ZePTF1/ZeMYB67 and, meanwhile, to linearise G16318GFP with the same sticky ends being obtained for ligation. As the gel result in Fig. 68, only one DNA band was observed in lanes 1–2 resulting from linearisation of 16318GFP. Moreover, the lower DNA bands of both the digested ZePTF1 (Fig. 68, lanes 3–4) and ZeMYB67 (Fig. 68, lanes 5–6) agreed with the expected sizes at approximately 1443bp and 1326bp respectively, indicating the CDSs of *TaPtf1* and

TaMyb67 were successfully excised from the sub-cloning vectors. Following recycling and purification of the insert and vector DNA fragments, the ligation of *TaPtf1*/*TaMyb67* with 16318GFP was performed and one positive clone of 16318PTF1/16318MYB67 was finally selected out as described in method section 7.2.1.2.

Table 33. Primers with enzyme restriction sites for amplification of *TaPtf1/TaMyb67*

primers	Restriction enzyme site 5' to 3'	CDS primer 5' to 3'
SalI-PTF(27)	5'-G$^{\vee}$T C G A C-3' 3'-C A G C T$_{\wedge}$G-5' →	ATGGACTACTCTAATGGTTCTTTCTTT-3'
SalI-MYB(23)		ATGAGGAGGTGTGATCTGAGACA-3'
BamHI-PTF(20)	5'-G$^{\vee}$G A T C C-3' 3'-C C T A G $_{\wedge}$G-5' →	TCACCTTTCAGGAGGGATTG-3'
BamHI-MYB(18)		TCAGCGCTTCTCTTGCGG-3'

*The stop codon "TGA" on its reverse strand was highlighted in yellow

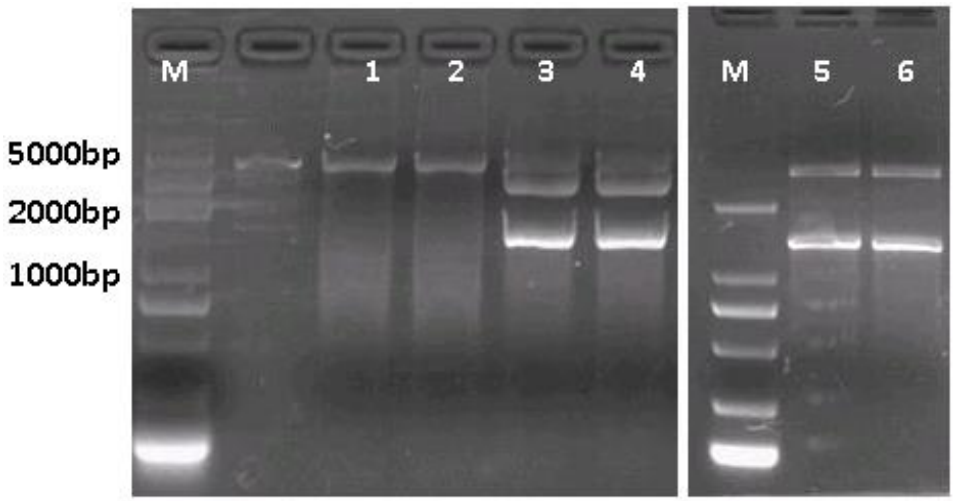


Fig. 68 Restriction enzyme digestion of 16318GFP, ZePTF1 and ZeMYB67 by BamHI and SalI. Lanes 1–2: 16318GFP. Lanes 3–4: ZePTF1. Lanes 5–6: ZeMYB67. M: DNA ladder BM5000 and BM2000.

7.3.1.2 Restriction endonuclease and DNA sequencing analysis for 16318PTF1/ 16318MYB67

Plasmids of 16318PTF1/16318MYB67 were prepared and further validated for successful insertion of *TaPtf1*/*TaMyb67* on the constructs through restriction endonuclease analysis with BamHI and SalI, as described in method section 7.2.1.2. As the gel result shows in Fig. 69, only two DNA bands were obtained from the digestion products in each lane. The upper bands reflected the vector fragments while the lower bands of lane 2 and lane 1 were observed to be consistent with the expected sizes at approximately 1443bp for *TaPtf1* and 1326bp for *TaMyb67* respectively.

Given no sequence information for the 16318GFP vector could be provided by Dr Zengyan Zhang (CAAS, China) except for the vector map, the CDS primers of PTF(27)/PTF(20) and MYB(23)/MYB(18) were used in DNA sequencing as described in the method section. Although the results of DNA sequencing could justify the presence of *TaPtf1*/*TaMyb67* as shown in alignment (Fig. 71 and Fig. 72), the regions of the start and stop codon were not fully detected. Designing primers complementary to regions within the start and stop codon (Fig. 70) for sequencing in the reverse orientation (from *TaPtf1*/*TaMyb67* to the vector) could have helped to confirm the correctness of the restriction enzyme sites near the start and stop codon regions, and also, to check the reading frame for the GFP fusion. However, due to insufficient consideration, this information was not able to be collected at this time.

The 16318GFP/16318MYB67 constructs were expected to lead to the production of transient expression of TaPtf1:GFP/TaMyb67:GFP fusion proteins. This requires the *hGFP* gene to be incorporated downstream of *TaPtf1*/*TaMyb67* into the same reading frame driven by

the 35S promoter on the constructs (Fig. 70) and can be achieved through the removal of the stop codon from the full-length CDS of *TaPtf1*/*TaMyb67*. Hence, the *hGFP* gene would be transcribed together and fused to the C-terminus of the resulting protein of *TaPtf1*/*TaMyb67* when translated (Fig. 70). However, although the 16318GFP/16318MYB67 constructs were successfully established, an error of including the stop codon "TGA" at the 3' end of the CDS primers used to amplify the CDS of *TaPtf1*/*TaMyb67* as highlighted in Table 33, was, in fact, made. Predictions for the errored-constructs would be that *TaPtf1*/*TaMyb67* and the *hGFP* gene still be transcribed into one transcript, whereas the translation stops at "TGA". The outcome of this is the GFP protein not being translated and fused with the resulting protein of *TaPtf1*/*TaMyb67*.

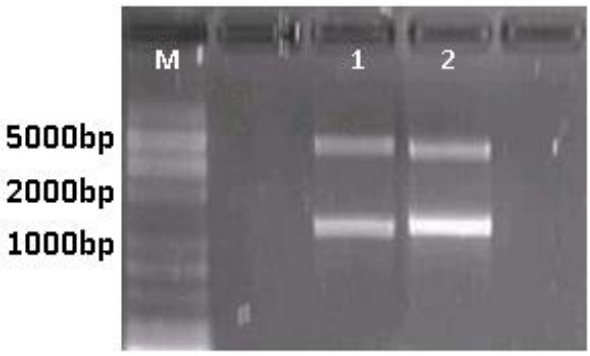


Fig. 69 Double digestion of 16318PTF1 and 16318MYB67 with BamHI and SalI. Lane 1: 16318MYB67. Lane 2: 16318PTF1. M: DNA ladder BM5000.

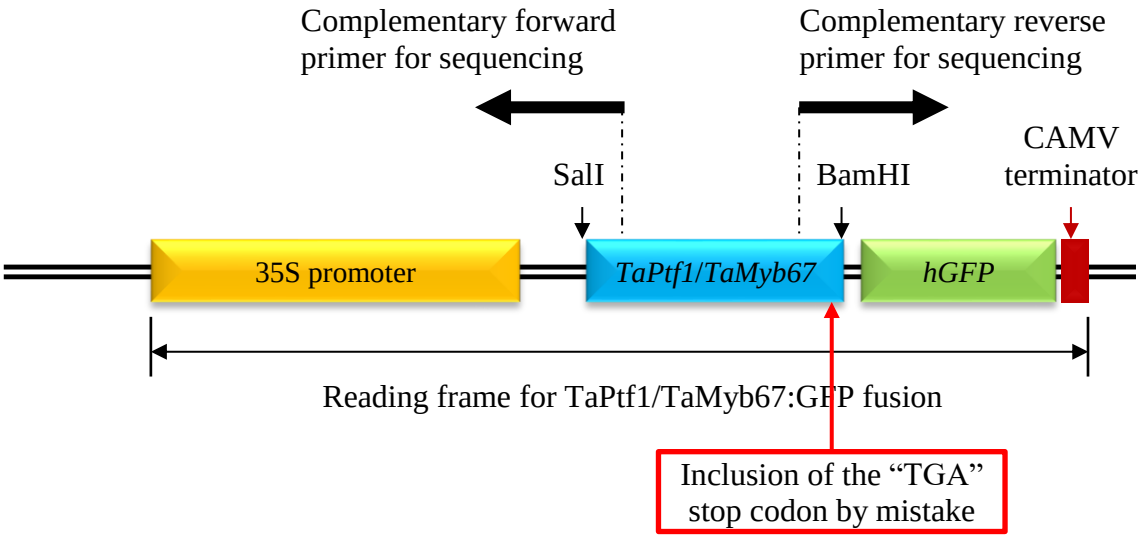


Fig. 70 Diagram of reading frame for the expected TaPtf1/TaMyb67:GFP fusion. The reading frame is displayed between the 35S promoter and CAMV terminator. The red arrow indicates the mistake of including the "TGA" stop codon on the constructs. The two black arrows on the top indicate the priming sites for complementary primers that could be used for sequencing in the reverse orientation.

(I) Sequencing results of 16318PTF1

```
# Length: 1447
# Identity:    1390/1447 (96.1%)
# Similarity:  1390/1447 (96.1%)
# Gaps:          51/1447 (3.5%)
# Score: 6884.5

16318PTF1          1 -------------------------TGCCAGCATTC-TGG-CTGGC-ATT     22
                                               |.||    ||| ||| ||||| |||
G1390PTF1          1 ATGGACTACTCTAATGGTTCTTTCTTTCC----TTCATGGCCTGGCAATT     46

16318PTF1         23 CCGCTTCCGAGAATTATAGCTTTGTTGATGGTTCAGTGGAATCATATGCA     72
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1         47 CCGCTTCCGAGAATTATAGCTTTGTTGATGGTTCAGTGGAATCATATGCA     96

16318PTF1         73 GAAGAAGGAAGTATGCCACCTACAGGCTATTTCAGAGCTAGATCAAATCA    122
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1         97 GAAGAAGGAAGTATGCCACCTACAGGCTATTTCAGAGCTAGATCAAATCA    146

16318PTF1        123 GAATTTAACATTTGATGAGCATGAACAGAACCCTGCTATGCTTGCAAATG    172
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1        147 GAATTTAACATTTGATGAGCATGAACAGAACCCTGCTATGCTTGCAAATG    196

16318PTF1        173 GGTGCTTGCCGTACAACACCCAGACTGATCTATTATCTGGTGAGATTCTG    222
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1        197 GGTGCTTGCCGTACAACACCCAGACTGATCTATTATCTGGTGAGATTCTG    246

16318PTF1        223 TCAGAGGACAAACATTCCAACAGCCTTATGGAGCTTCCACAACTTCAGAA    272
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1        247 TCAGAGGACAAACATTCCAACAGCCTTATGGAGCTTCCACAACTTCAGAA    296

16318PTF1        273 CAATGGCAGTCTGCAAAGTAATTTAATCCCACCAGGGACTCTTCAGTGCA    322
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1        297 CAATGGCAGTCTGCAAAGTAATTTAATCCCACCAGGGACTCTTCAGTGCA    346

16318PTF1        323 CTTCAACACCTGGAACATTTGACCTGCAGTTGGATACCCCTGGCCTTCTA    372
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1        347 CTTCAACACCTGGAACATTTGACCTGCAGTTGGATACCCCTGGCCTTCTA    396

16318PTF1        373 GAACTTCCTCATGCCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGAAGT    422
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1        397 GAACTTCCTCATGCCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGAAGT    446

16318PTF1        423 TTCAGCTTTTCTTGCTGATGTACATGCTGTTTCTTCAGCCTCAACTCTGT    472
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1        447 TTCAGCTTTTCTTGCTGATGTACATGCTGTTTCTTCAGCCTCAACTCTGT    496

16318PTF1        473 GCTCGACATTCCAAAATGTTCCTTCCTACATGGAACCAGTAAGCCTAGAA    522
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1        497 GCTCGACATTCCAAAATGTTCCTTCCTACATGGAACCAGTAAGCCTAGAA    546

16318PTF1        523 GCTTTCAGTTTTCAAGGGATACAAAATGCTGCTATGTTCAACAATACAAG    572
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1        547 GCTTTCAGTTTTCAAGGGATACAAAATGCTGCTATGTTCAACAATACAAG    596

16318PTF1        573 TCATTCAAATGGGAACCTGTCAGTATTTGATGAGGCAACCATGGCATCAC    622
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1        597 TCATTCAAATGGGAACCTGTCAGTATTTGATGAGGCAACCATGGCATCAC    646
```

```
16318PTF1       623 TACATGATAGCAAAGAATTTCTCAGTGGTAGCATCTCATCTTTTGGTACG    672
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1       647 TACATGATAGCAAAGAATTTCTCAGTGGTAGCATCTCATCTTTTGGTACG    696

16318PTF1       673 GCCGAGCAGTCACAACTAGCTGGTAGTGGTTTGAAGGCTGAACAACAGGA    722
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1       697 GCCGAGCAGTCACAACTAGCTGGTAGTGGTTTGAAGGCTGAACAACAGGA    746

16318PTF1       723 ACAAAATGCGATGTGCAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGA    772
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1       747 ACAAAATGCGATGTGCAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGA    796

16318PTF1       773 TGGCAGTGAGTGAAGCACAAGGGGCAATGATTCCTTCGAAGATAAGCTCA    822
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1       797 TGGCAGTGAGTGAAGCACAAGGGGCAATGATTCCTTCGAAGATAAGCTCA    846

16318PTF1       823 ACGATGCATAACAATAAAAGTGAGTACCCTGTCCCTATCAGCCATTCTGC    872
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1       847 ACGATGCATAACAATAAAAGTGAGTACCCTGTCCCTATCAGCCATTCTGC    896

16318PTF1       873 TGATGCGCAGAACAAGGCAAATTCAGCTAATGGAAACAGTGCCAGTGCTA    922
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1       897 TGATGCGCAGAACAAGGCAAATTCAGCTAATGGAAACAGTGCCAGTGCTA    946

16318PTF1       923 AGCCACGAGCAAGGGCTCGTCGTGGACAGGCAACTGACCCTCATAGTATT    972
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1       947 AGCCACGAGCAAGGGCTCGTCGTGGACAGGCAACTGACCCTCATAGTATT    996

16318PTF1       973 GCTGAACGGCTTCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCTCCA   1022
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1       997 GCTGAACGGCTTCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCTCCA   1046

16318PTF1      1023 AGACCTTGTACCAAACTCAAATAAGGCAGATAAATCATCAATGCTCGATG   1072
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1      1047 AGACCTTGTACCAAACTCAAATAAGGCAGATAAATCATCAATGCTCGATG   1096

16318PTF1      1073 AAATAATTGATTATGTGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGC   1122
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1      1097 AAATAATTGATTATGTGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGC   1146

16318PTF1      1123 ATGAGTAGGCTAGGAGCTCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATC   1172
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1      1147 ATGAGTAGGCTAGGAGCTCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATC   1196

16318PTF1      1173 TCAAACTGAGGGCCGTAGCAATTCACCTCTATCATCTCCAACCGCTTCAC   1222
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1      1197 TCAAACTGAGGGCCGTAGCAATTCACCTCTATCATCTCCAACCGCTTCAC   1246

16318PTF1      1223 AAGGGCTTCTGGACGCAGCAGGCCCAGAAGACAGCTTGGTCTTTGAGCAA   1272
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1      1247 AAGGGCTTCTGGACGCAGCAGGCCCAGAAGACAGCTTGGTCTTTGAGCAA   1296

16318PTF1      1273 GAAGTTATAAAGCTGATGGAAACAAGCATCACAAATGCAATGCAGTACCT   1322
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1      1297 GAAGTTATAAAGCTGATGGAAACAAGCATCACAAATGCAATGCAGTACCT   1346

16318PTF1      1323 TCAGAACAAGGGCCTCTGCCTGATGCCTATCGCTCTTGCTTCAGCCATAT   1372
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
G1390PTF1      1347 TCAGAACAAGGGCCTCTGCCTGATGCCTATCGCTCTTGCTTCAGCCATAT   1396
```

```
16318PTF1       1373 CCAACCAGAAAGGCACTTCTGCAGCTGG------------------   1400
                     ||||||||||||||||||||||||||||.
G1390PTF1       1397 CCAACCAGAAAGGCACTTCTGCAGCTGCAATCCCTCCTGAAAGGTGA   1443
```

** 16318PTF1: sequenced *TaPtf1* on 16318PTF1.
G1390PTF1: sequenced *TaPtf1* on G1390PTF1.

** Sequences highlighted in green indicate the areas near to the sequencing primers.

** Excluding the primer areas, the sequenced *TaPtf1* on 16318PTF1 is identical to the *TaPtf1* sequence (1443bp) on G1390PTF1 .

Fig. 71 Sequencing result for *TaPtf1* clone on the 16318PTF1 construct. The sequenced *TaPtf1* clone on 16316PTF1 was compared with that on G1390PTF1 (Chapter 4) by alignment.

(II) Sequencing results of 16318MYB67

```
# Length: 1328
# Identity:    1277/1328 (96.2%)
# Similarity:  1277/1328 (96.2%)
# Gaps:          48/1328 (3.6%)
# Score: 6293.0

16318MYB67         1 --------------------GCAGATCTCACAGCA----GGTTTCTGGAG     26
                                         .||| |||||||.||    ||||||||||
G1390MYB67         1 ATGAGGAGGTGTGATCTGAGACAG-TCTCACAACAGCAGGGTTTCTGGAG     49

16318MYB67        27 G-ATGTCATCCTCTTTACCTATTCTGCCAAATTCTCTGAAAGAAACCTTC     75
                     | ||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67        50 GAATGTCATCCTCTTTACCTATTCTGCCAAATTCTCTGAAAGAAACCTTC     99

16318MYB67        76 CATGGGCCTTACAATCCGCAGCTCACTCCGATGCAAAGGCAACTGACGAG    125
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       100 CATGGGCCTTACAATCCGCAGCTCACTCCGATGCAAAGGCAACTGACGAG    149

16318MYB67       126 TGATCTTGTGCCCTTACATCAGAGTGCACTTCCGTCTGCTACTTTGCACC    175
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       150 TGATCTTGTGCCCTTACATCAGAGTGCACTTCCGTCTGCTACTTTGCACC    199

16318MYB67       176 CAAGAGCTGGGGCTATGAGATCATCATATGCAGCCTCATTAGGATACTCA    225
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       200 CAAGAGCTGGGGCTATGAGATCATCATATGCAGCCTCATTAGGATACTCA    249

16318MYB67       226 CCTAATCCTCTTGATTCTGCGCTTAACCATGAGAGGCAGTCTATGGTTGC    275
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       250 CCTAATCCTCTTGATTCTGCGCTTAACCATGAGAGGCAGTCTATGGTTGC    299

16318MYB67       276 TCCTTTTGCTCCTCAGCCATCAAATATTGAAGTATTTCAGACCTTATCTA    325
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       300 TCCTTTTGCTCCTCAGCCATCAAATATTGAAGTATTTCAGACCTTATCTA    349

16318MYB67       326 ATAATATCCCTGGAGGACACACTGAGGCAACTTGGTTCCCAGGTTCGGCT    375
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       350 ATAATATCCCTGGAGGACACACTGAGGCAACTTGGTTCCCAGGTTCGGCT    399

16318MYB67       376 GATGGTTTATCAGATTACAGGGATAACATCCCTGCTTCTGGTAGTCAGAT    425
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       400 GATGGTTTATCAGATTACAGGGATAACATCCCTGCTTCTGGTAGTCAGAT    449

16318MYB67       426 CCAGAATGGCGGTCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATG    475
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       450 CCAGAATGGCGGTCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATG    499

16318MYB67       476 AATGGTGGGCAGAGATAATGAATGATGATTGGAGAGATATTCTAGATGCA    525
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       500 AATGGTGGGCAGAGATAATGAATGATGATTGGAGAGATATTCTAGATGCA    549

16318MYB67       526 ACGGCTGCTGATCCCCAGTCAAAGCCTTCCAATTCGTCTGCATCACAGCC    575
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       550 ACGGCTGCTGATCCCCAGTCAAAGCCTTCCAATTCGTCTGCATCACAGCC    599

16318MYB67       576 TGCTGTCAACCAGCCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAG    625
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       600 TGCTGTCAACCAGCCAGCTTCATCTCATGGTGGAGAGATTTGCAATGTAG    649
```

```
16318MYB67       626 CTAGTCCTCCCAATAGCAACTCTGCAGCCAAACAACGGATGAGGTGGACT    675
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       650 CTAGTCCTCCCAATAGCAACTCTGCAGCCAAACAACGGATGAGGTGGACT    699

16318MYB67       676 CCAGAACTCCATGAATGCTTCGTAGACTCTGTAAATAAGCTTGGTGGTAG    725
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       700 CCAGAACTCCATGAATGCTTCGTAGACTCTGTAAATAAGCTTGGTGGTAG    749

16318MYB67       726 TGAAAAAGCTACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTTGACGGTT    775
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       750 TGAAAAAGCTACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTTGACGGTT    799

16318MYB67       776 TGACAATATATCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGC    825
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       800 TGACAATATATCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGC    849

16318MYB67       826 TATAAGCCAGACCTAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGA    875
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       850 TATAAGCCAGACCTAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGA    899

16318MYB67       876 AGAGTTGACTCTAGACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGC    925
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       900 AGAGTTGACTCTAGACCTGAAATCGAGCATGGATCTTACTGAAGCGCTGC    949

16318MYB67       926 GTCTTCAGATGGAAGTTCAGAAACGTCTTCATGAACAACTTGAGACCCAG    975
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67       950 GTCTTCAGATGGAAGTTCAGAAACGTCTTCATGAACAACTTGAGACCCAG    999

16318MYB67       976 AGAAAGTTGCAATTGCGAATTGAAGAACAAGGGAAGTATCTTCAGATGAT   1025
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67      1000 AGAAAGTTGCAATTGCGAATTGAAGAACAAGGGAAGTATCTTCAGATGAT   1049

16318MYB67      1026 GTTTGAAAAGCAGTCTAAATCCAATACGGAGAAGGTGCAGGATCTATCCT   1075
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67      1050 GTTTGAAAAGCAGTCTAAATCCAATACGGAGAAGGTGCAGGATCTATCCT   1099

16318MYB67      1076 CGGGAGCTACAACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGA   1125
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67      1100 CGGGAGCTACAACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGA   1149

16318MYB67      1126 AATAGGGGTGATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCC   1175
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67      1150 AATAGGGGTGATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCC   1199

16318MYB67      1176 CGTGAGTGCCGAAATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGG   1225
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
G1390MYB67      1200 CGTGAGTGCCGAAATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGG   1249

16318MYB67      1226 AGATGGCAGAAAGCGAGTCTTCTGAGCCCC-TGCAAATACTAATGAGTG-   1273
                     |||||||||||||||||||||||||||||| ||||||||||||||| ||
G1390MYB67      1250 AGATGGCAGAAAGCGAGTCTTCTGAGCCCTTGCAAATACTAATGA-TGG   1298

16318MYB67      1274 ---CAATGC---------------CTG-   1282
                        |||.||               |||
G1390MYB67      1299 TTCCAAGGCCCCGCAAGAGAAGCGCTGA   1326
```

** 16318MYB67: sequenced TaMyb67 on 16318MYB67.
G1390MYB67: sequenced TaMyb67 on G1390MYB67.

** Sequences highlighted in green indicate the areas near to the sequencing primers.

** Excluding the primer areas, the sequenced *TaMyb67* is identical to the *TaMyb67* sequence (1326bp) on G1390MYB67 .

Fig. 72 Sequencing result for *TaMyb67* clone on the 16318MYB67 construct. The sequenced *TaMyb67* clone on 16318MYB67 was compared with that on G1390MYB67 (Chapter 4) by alignment.

7.3.2 Observation of 16318PTF1/16318MYB67 transformed wheat protoplast cells

Since the inclusion of "TGA" in the constructs was not considered, the experiment still proceeded to the PEG-mediated transformation of 16318PTF1 and 16318MYB67 into wheat protoplast cells and the results were analysed with LSM as described in method section 7.2.2. The outcomes of LSM analysis were presented as confocal images of wheat protoplast cells under three channels, GFP, visible and chlorophyll, as displayed in separate or merged images in Fig. 73. The control (Fig. 73A) demonstrated constitutive expression of GFP green fluorescence within the whole cell. As discussed in the previous section, negative signals of GFP green fluorescence were assumed to be the most likely results for 16318GFP/16318MYB67 due to the inclusion of the stop codon resulting in no translation and fusion of the GFP with TaPtf1/TaMyb67.

It was surprising that the images produced from LSM for 16318PTF1 (TaPtf1) showed positive signals of GFP green fluorescence in the nucleus as well as the extensive cytoplasm areas (Fig. 73B), where the fluorescence was not as strong as the control. This indicates the *hGFP* gene was expressed and translated. Reasons for such a result were unclear, but it might be possible that the translation of GFP was re-activated independently. Therefore, the observed GFP green fluorescence would have derived from GFP only, while the resulting protein of TaPtf1 is not fused. The observation thus cannot reflect the sub-cellular location of TaPtf1. Alternatively, there was a small likelihood of the loss of effectiveness of the stop codon, probably caused by a point mutation in the stop codon during PCR amplification, though the reading frame would not be altered. In that case, the GFP green fluorescence detected may suggest that TaPtf1 has a sub-cellular location not only in the nucleus but also in the cytoplasm. However, the region of the stop codon was not properly assessed by sequencing during the experiment due to insufficient consideration on this occasion. The result thus may not be reliable for the sub-cellular location of TaPtf1 and remains to be further

investigated. Furthermore, using constructs absent of the NLS sequence of *TaPtf1* as the comparison may also be useful in any further investigation. If *TaPtf1* were to be validated to express in the nucleus as well as the cytoplasm in any future results, it might suggest the sub-cellular location of TaPtf1 is species-specific compared with OsPtf1 and GmPtf1 which, as previously studied, have the nucleic location only. It is possible that TaPtf1 participates in more extensive cellular activities with the potential of functional divergence from other lower ploidy species. Meanwhile, whether TaPtf1 is located on the cell membrane remains unknown and may be achieved in any future experiment by using an osmotic treatment with a sucrose solution.

For 16318MYB67 (TaMyb67), on the other hand, red fluorescence signals, theoretically produced by chloroplasts, were found in the images produced from LSM (Fig. 73C), implying no expression of GFP green fluorescence in sub-cellular areas. The tiny spots of green fluorescence in the middle of the red fluorescence might be background signals. The result was in accordance with the predicted no translation of GFP in the presence of the stop codon of *TaMyb67* on the construct. It thus turned out that the sub-cellular location of TaMyb67 remained to be studied in any future experiment.

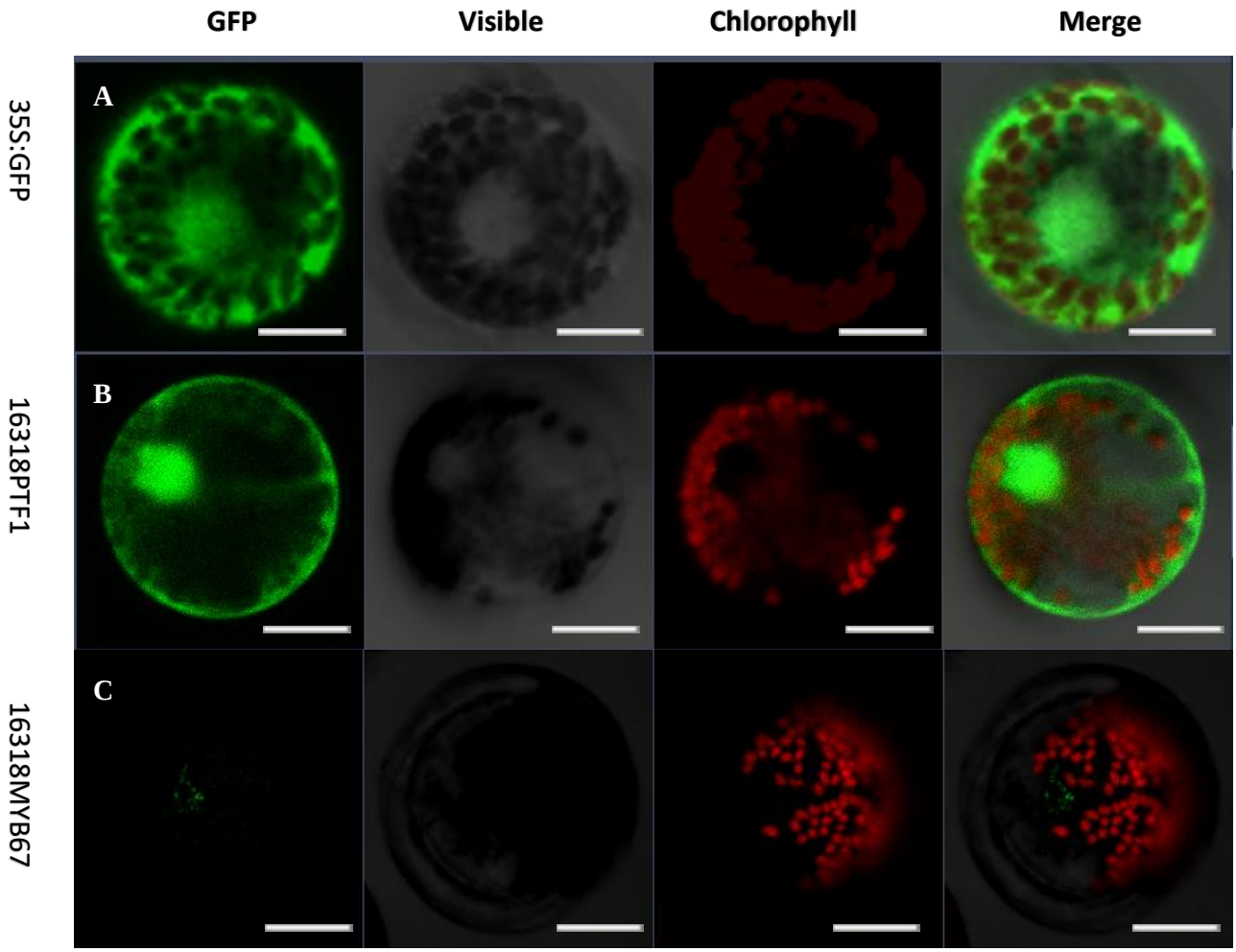


Fig. 73 LSM observation of transformed wheat protoplast cells. Wheat protoplast cells were transformed with plasmids of 16318GFP as the control or with those of 16318PTF1/16318 TaMYB67, mediated by polyethylene glycol (PEG), for the transient expression of (A) 35S:GPF control; (B) 16318PTF1; (C) 16318MYB67. Images were captured using the following wavelengths (522nm; 630nm). Images are shown in separate modes in GFP, visible, chlorophyll and merge. Scale bar: 50μm.

Chapter 8 General discussion and future work

8.1 Overview

P deficiency/low-P is a major nutritional disorder and hence a limiting factor for crop yield in agriculture. This study aimed to initially understand gene functions of *TaPtf1* and *TaMyb67*, which are two transcription factors (TFs) investigated for their roles in the regulatory complexity of PUE in hexaploid wheat. The sequential work presented in this thesis started with a bioinformatics approach by following several steps of sequence analyses, through which the two wheat TFs were selected and their functions were predicted based on their orthologous genes in the model crop rice. The genome location and sequence structure of the two wheat TFs genes were explored and analysed (Chapter 3). Following this, the manipulation of wheat plants was achieved by a transgenic approach, during which the coding sequences of *TaPtf1* and *TaMyb67* were molecularly cloned and expressed in wheat (Chapter 4 and 5). To assess the phenotypic effects of *TaPtf1*/*TaMyb67* on plant growth, yield and PUE, with more attention to the above-ground phase, *TaPtf1*/*TaMyb67* mRNA expression patterns and phenotypes of selected transgenic lines were analysed (Chapter 6). Attempts were also made to study the sub-cellular location of *TaPtf1*/*TaMyb67* (Chapter 7). Finally, in this chapter, the results and future work will be further discussed.

8.2 *TaPtf1*

In previous reports, *Ptf1* has been suggested to maintain plant growth under P deficiency/low-P by regulating carbon allocation and metabolism. Global overexpression of *OsPtf1* was demonstrated to significantly increase tiller numbers, P accumulation and biomass in both shoots and roots in P-deficient rice during vegetative growth (Yi *et al*., 2005). Consistent with that, significantly greater tiller numbers, P accumulation and biomass in both shoots and roots during vegetative growth, as well as the increase of grain yields, in P-deficient maize were described to result from global overexpression of *ZmPtf1* (Li *et al*., 2011). The functional role of *Ptf1* nevertheless is not well-defined in wheat, whereas some functional aspects of *TaPtf1* have been investigated by expression analysis in wild-type wheat suggesting that *TaPtf1* has functionally diverged (Aziz *et al*., 2014). In this study, *TaPtf1* was identified as the wheat homologue of *OsPtf1* and its three hoeomoloci were found in the wheat genome. *TaPtf1* overexpression lines were generated by using the coding sequence of *TaPtf1-D1*, despite the restricted overexpression to various extents in several lines, and phenotypically studied with more attention to the above-ground phase. Through phenotypic analysis, combined with correlation analysis, we were able to obtain a few implications for functional roles of *TaPtf1* under P-deficient conditions. However, we did not observe an increase in tiller numbers or seed P accumulation and biomass, although the shoot P accumulation and biomass at harvest were shown to be slightly higher than the control; plant height and chlorophyll concentration were found to be affected as well. Our results also seemed to support that *TaPtf1* has, to a large extent, functionally diverged from *OsPtf1*/*ZmPtf1*.

8.2.1 Potentially conserved and diverged functional roles of *TaPtf1*

It has been well recognised that P_i homeostasis elaborately co-ordinates with carbon supply and photosynthesis in plants (Wissuwa *et al*., 2005). Evidence built up in rice and maize has

pointed to an important functional role of *Ptf1* in shoot-to-root sugar translocation and sugar signalling in systemic P-stress responses to facilitate PUE. Related observations in the leaf and stem in wild-type wheat appeared to comply with this and to suggest *TaPtf1* is likely to have a conserved role of regulating sugar translocation through the recruitment of H^+-transporting ATPase for phloem loading of photosynthetic sugars; this has been discussed in Chapter 3 and 6. Current results of this study did not provide direct evidence for sugar translocation, whereas the observed increases in shoot biomass and plant height in *TaPtf1* transgenic lines under low-P might indicate the existence of strengthened sugar translocation and sugar signalling (by overexpression of *TaPtf1*) in induction of systemic P-stress responses required for enhanced P uptake and translocation. Sugars are believed to be important systemic signals in systemic P-stress responses, while many PSI genes have been confirmed as sugar-responsive (Jain *et al*., 2007; Karthikeyan *et al*., 2007). Sucrose, the most abundant transportable sugar, has been proposed to be a most likely candidate to mediate sugar signalling in systemic P-stress responses (Chiou and Lin, 2011). Sucrose biosynthesis takes place in the cytoplasm following fixed carbon being exported from the chloroplast by the triose-P/P-translocator (TPT) in photosynthetic leaves (Lloyd *et al*., 2004). Sucrose exported from photosynthetic leaves can be transported through the phloem to roots or other tissues (Braun *et al*., 2014). Exogenous application of sucrose can remarkably upregulate the expression of many PSI genes, including PTs, in both shoots and roots (Karthikeyan *et al*., 2007; Müller *et al*., 2005, 2007), supporting the hypothesis that sucrose acts a systemic signal in P-stress responses. In maize, precursors of sucrose synthesis including fructose-1,6-bisphosphatase and sucrose phosphate synthase were found to be modulated by *ZmPtf1*. Interestingly, Aziz *et al*., (2014) also suggested that in wheat a plastid enzyme TaGPho1, which mediates transient starch degradation in the chloroplast, was likely to be positively regulated by *TaPtf1* in the stem and leaves (Tiwari and Kumar, 2012). Overexpression of *TaPtf1* may thus increase starch

degradation in shoots, which provides more substrates for sucrose synthesis and phloem loading of sucrose. Furthermore, when P is deficient, TPT translocation and cell exporting of sucrose (and other sugars) could be limited, resulting in cytoplasmic accumulation of these molecules (Wang and Ruan, 2013). Meanwhile, sucrose (and other sugars) unloading in companion cell and sieve element (CC/SE), as well as post-phloem delivery, in roots could also be affected. Therefore, apart from the contribution to synthesis and phloem loading of sucrose (and other sugars), *TaPtf1* may in some way also regulate other related processes.

Growing evidence has shown increased lateral root and root hair formation is an important root remodelling strategy as part of systemic P-stress responses mediated by sugar signalling (Hammond and White, 2008; Jain *et al*., 2007; Karthikeyan *et al*., 2007). *OsPtf1* and *ZmPtf1*, both significantly induced in roots, could positively regulate lateral root formation in their overexpression lines under P deficiency/low-P, leading to greater tiller number, P accumulation and biomass in both shoots and roots (Li *et al*., 2011; Yi *et al*., 2005). However, related-observations in wild-type wheat indicated *TaPtf1* is significantly repressed in lateral roots, though with an early induction in roots, under P deficiency/low-P; this has been discussed in Chapter 3 and 6. Global transcriptome profiling in previous studies has revealed that P deficiency/low-P are concerned with expressional changes of genes involved in biosynthesis, metabolism and signalling pathways of different phytohormones (Hammond *et al*., 2004; Misson *et al*., 2005). The early induction of *TaPtf1* is likely to associates with phytohormone activities for the generation of primary signals which are transmitted from roots to shoots in systemic P-stress responses. Strigolactones (SLs), a group of phytohormone mainly generated in roots and indicated to be transportable in long-distance to shoots as initially supported by grafting studies of SLs and SL intermediates (Booker *et al*., 2005), have been proposed to be a candidate of primary signals (Chiou and Lin, 2011). SLs have also been identified to function as

regulators of shoot architectural responses to P stress by suppressing bud outgrowth and shoot branching (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008). Recent work in *Arabidopsis* showed that the presence of SL orobanchol, one type of SLs, in the xylem sap was related to shoot architectural changes under P deficiency/low-P (Kohlen *et al*., 2011), which agreed with previous findings. In rice, P deficiency/low-P resulted in inhibition of bud outgrowth in shoots accompanied with increases of some root SLs (Umehara *et al*., 2010). With these in mind, it is perhaps reasonable that *TaPtf1* regulates the generation of SLs and other primary signals or their delivery from roots to shoots in systemic P-stress responses. On the other hand, the negative effects of *TaPtf1* in lateral roots denoted functional divergence, by which the results of tiller number, P accumulation and biomass in *TaPtf1* transgenic lines under low-P might be largely explained. As demonstrated by the successful lateral root (LR) organogenesis model established in *Arabidopsis*, LRs originate from pericycle founder cells which undergo programmed cell division and expansion to form lateral root primordia (LRP) that emerge from parent root and become functional meristem for LR growth onwards (Benková and Bielach, 2010; Dubrovsky *et al*., 2001, 2008; Malamy and Benfey, 1997). Of several phytohormones found to modulate these processes, auxin is generally accepted to be pivotal in determining LR formation, whereas cytokinin plays an inhibitory role (Franco-Zorrilla *et al*., 2005; Lavenus *et al*., 2013; Nacry *et al*., 2007). The establishment of an auxin gradient is vital for LR formation while cytokinin inhibits auxin transport in the primordia through interfering with the expression of *PIN* (PIN-FORMED) genes that encode auxin efflux carriers (Benková *et al.*, 2003; Geldner *et al.*, 2004; Laplaze *et al*., 2007). In this stage, the molecular basis of the negative regulatory effects of *TaPtf1* on lateral roots under P deficiency/low-P is unclear, but it is probably related to carbon metabolism (sugars) in crosstalk with phytohormone activities. In support of this, initial evidence has been found in P-deficient wild-type wheat indicating that TaPtf1 seems to be co-regulated with a fructose 1,6-bisphosphate aldolase, TaALD, in glycolysis or glycolysis bypass

where sucrose and glucose are involved (Aziz *et al*., 2014). Sucrose and glucose have been previously reported to potentially interact with auxin biosynthesis, signalling and transport in root development (Booker *et al*., 2010; Gibson, 2004; Gupta *et al*., 2014; Jain *et al*., 2007; Mishra *et al*., 2009; Ohto *et al*., 2006; Sairanen *et al*., 2012). By contrast, there is also a likelihood that *TaPtf1* is recruited to positively regulate cytokinin and thus counteracts LR formation under P deficiency/low-P. Furthermore, the involvement of *TaPtf1* in regulating other phytohormones which also affect LR formation, such as gibberellic acids (GA), brassinosteroid (BRs), ethylene (ET), abscisic acid (ABA) and SLs (Chang *et al*., 2013), could also be the case for the negative effects of *TaPtf1* on lateral roots. However, it seems the specificity of the regulation remains to be discovered.

8.2.2 Impacts of overexpressing *TaPtf1* on plant height

Another finding further dissimilar with those in studies on overexpression of *OsPtf1* and *ZmPtf1* is the promotional effects of *TaPtf1* on plant height. Plant height influences shoot architecture, lodging resistance and yield performance and, to a large extent, decides on the growth of the shoot apical meristem (SAM) regulated by multiple phytohormones (Qi *et al*., 2011; Wang *et al*., 2017). GA is known to be a crucially positive regulator but acts synergistically and antagonistically with other phytohormones (e.g. auxin, BRs, ET and SLs, etc.) in controlling plant height (Wang *et al*., 2017). GA mainly works through the GA signalling pathway composed of GA, GA receptor (GID1) and DELLA repressor protein (a GRAS transcription factor) in crosstalk with signalling pathways of other phytohormones (Griffiths *et al*., 2006; Livne *et al*., 2015; Ueguchi-Tanaka *et al*., 2005, 2008). Experiments using exogenous GA or in DELLA-deficient mutants in *Arabidopsis* and tomato showed phenotypes of boosted stem elongation (King *et al*., 2001; Livne *et al*., 2015). Moreover, an SLs receptor, Dwarf14 (D14), has been reported to interact with DELLA repressor protein of GA signalling (Slr1) in rice, indicating the possible crosstalk between SLs

and GA signalling pathways. While the association between *TaPtf1* and SLs still needs to be proven as discussed in the previous section, the promoted plant height in *TaPtf1* transgenic lines under low-P might have been caused by excessive SLs, due to overexpression of *TaPtf1*, being generated and transmitted to shoots and interacting with the GA signalling pathway. Alternatively, overexpression of *TaPtf1* could have intervened in other aspects of GA biosynthesis or interactions of GA with other relevant phytohormone signalling pathways, resulting in elevated GA levels and thus the alteration of plant height in these lines under low-P. Furthermore, numerous studies notably indicate connections between phytohormone and sugars (Moore *et al*., 2003; Rouached *et al*., 2010; Wang and Ruan, 2013). Sugars, as signalling metabolites in addition to the role of energy supply, are also important in manipulating gene expression (Hammond and White, 2008; Jain *et al*., 2007; Karthikeyan *et al*., 2007). *TaPtf1* itself is assumed to regulate sugar translocation and sugar signalling, as previously discussed. Therefore, it is also possible the promotional effects of *TaPtf1* on plant height are derived from changes in GA levels contributed by sugar signalling. Meanwhile, meristematic growth relies on sugars (Van't Hof, 1966), whereas sugar homeostasis needed for meristem growth may be affected by overexpression of *TaPtf1* in shoots under P deficiency/low-P, which would consequently exert direct effects on meristematic growth. One piece of evidence recently emerging from *Arabidopsis* suggested that sugar signalling promotes meristematic proliferation by activating a key cell cycle gene that boosts the G2 phase to M transition during mitosis (Skylar *et al*., 2011).

8.2.3 Chlorophyll concentration as a later result of overexpressing *TaPtf1* in wheat

Plant leaves are primary sites for photosynthetic carbon assimilation where chlorophyll plays a fundamental role in the light capture of photosynthesis (Kim *et al*., 2013). The synthesised free chlorophyll requires to bind with proteins to form the more stable light-harvesting complex II (LHC II)

or antenna complex on the thylakoid; otherwise, the free chlorophyll ought to be quickly degraded (Li *et al*., 2000). Therefore, the chlorophyll concentration is mainly decided by a dynamic balance between biosynthesis and degradation, which are regulated by separate pathways. The chlorophyll biosynthesis pathways, in *Arabidopsis*, for example, have been characterised as tetrapyrrole and methylerythritol phosphate (MEP) pathways respectively (Kim *et al*., 2013). The basic common breakdown pathway of chlorophyll is the pheide and oxygenase (PAO) pathway (Hörtensteiner and Kräutler, 2011). In this study, the upregulated expression of *TaPtf1* was found to correlate with increases of unit chlorophyll concentration in *TaPtf1* transgenic lines under low-P. Meanwhile, the fact of promoted plant height and increased shoot biomass similarly detected in these lines under low-P has implications for improved PUE, either enhanced P uptake and translocation to shoots or shoot P-efficient utilisation (P recycling/bypass). Improvements of PUE can alleviate the inhibition of photosynthesis caused by P deficiency/low-P and often accompanies stimulated response to nitrogen and nitrogen use efficiency, while nitrogen is the major ingredient for chlorophyll biosynthesis and a sensitive indicator of chlorophyll concentration (Marschner and Marschner, 2012). It thus looks more likely that the increases of unit chlorophyll concentration detected in *TaPtf1* transgenic lines under low-P, marked by a state biosynthesis greater than degradation of chlorophyll, have been a later result of improved PUE, rather than the direct participation of *TaPtf1* in chlorophyll biosynthesis/degradation pathways.

8.2.4 *TaPtf1* as a potential negative regulator of grain-filling

TaPtf1 appears to differ from *OsPtf1* and *ZmPtf1* in grain-filling-associated seed P and seed biomass, as shown by *TaPtf1* transgenic lines under low-P. The combined results of phenotypic and correlation analyses for the shoot and seed P in Chapter 6 were indicative of a negative regulatory role of *TaPtf1* in redistributing P from vegetative tissues to seeds for grain-filling.

However, this is not in line with those of global overexpression of *ZmPtf1,* where seed P and biomass increased consistently with shoot P and biomass, while the global overexpression of *OsPtf1* was only studied during seedling stage and thus could not be compared. In plants, P remobilisation is mainly facilitated by PTs (Wang *et al*., 2016b). For instance, OsPht1:8 and HvPht1:6 are both found to undertake P remobilisation from senescing vegetative tissues to reproductive tissues (Jia *et al*., 2011; Rae *et al*., 2003). Probably the poor grain-filling in *TaPtf1* transgenic lines is due to limits of P remobilisation by PTs during senescence, which implies a potential involvement of *TaPtf1* in negative regulation of PTs. Also, P remobilisation during senescence consumes major part of the organic P pool which is composed of nucleic acid-P, phospholipids and other phospho-esters (Veneklaas *et al*., 2012). The downstream targets of *TaPtf1* may have impeded some essential pathways in breakdown processes of these organic compounds. As suggested by Stigter and Plaxton (2015), genes involved in both leaf senescence and P-stress responses may include those encoding PTs, ribonucleases (RNases), phospholipid modelling enzymes and P recycling-associated PAPs. Moreover, the grain-filling in *TaPtf1* transgenic lines could also have been adversely affected by disruption of normal carbon status in shoots upon an excessive source-to-sink supply of carbon from shoots to roots in consequence of the overexpression of *TaPtf1* (Braun *et al*., 2013). This nevertheless remains to be further studied.

Taken together, the results gained in this study seemed to suggest *TaPtf1* only functions partially in a similar manner to *OsPtf1 and ZmPtf1*, possibly the shoot-to-root sugar translocation and signalling. The disparity between this and their studies may indicate *Ptf1* works through an interplay between distinct pathways in these crop species while the variability of the regulatory effects of *TaPtf1* is species-specific. Previously, it has been suggested that the regulatory

system of PUE in higher plants is much more complicated and under polygenic control (Su *et al*., 2009; Yi *et al*., 2005). Different species or even different genotypes within a species thus could have diversification of PUE regulatory systems, accompanied with diversified gene functions. Considering the complex and highly repetitive genome of hexaploid wheat (Glover *et al*., 2015), *TaPtf1* could potentially be even more highly-regulated than those in diploid species, with which the results of this study are consistent. Understandings of *TaPtf1* functions, as well as its interconnection with other regulatory components, can be further exploited to better elucidate the relationship between P deficiency and carbon metabolism and maybe the crosstalk with phytohormone in plants.

8.2.5 Future work

As far as *TaPtf1* is concerned, global overexpression seemed not to be an ideal model for studying P uptake and translocation, owing to the opposite regulatory effects of *TaPtf1* in the stem and lateral roots on these processes. However, it may be still useful to further purify the progeny generations of *TaPtf1* transgenic lines for investigating some functional aspects, especially the elite lines like P4 and P5 that have more satisfactory overexpression under P stress. Following purification, atypical lines can thus be screened out, giving a homogenised genetic background of progeny generations. Several areas of investigation may be considered, for which hydroponics with different P supply may be preferentially used to allow the study of roots.

Given *TaPtf1* has been shown as a negative regulator in lateral roots (Aziz *et al*., 2014), observations can be taken to study lateral root phenotypes in greater details in *TaPtf1* overexpression lines. It will be interesting to investigate the molecular mechanisms by studying the expression of key genes involved in regulating lateral root development, e.g. *Arf19* and *Tlr1* (Pérez-Torres *et al*., 2008). In the stem, positively regulating sugar

translocation from shoots to roots through the recruitment of H^+-transporting ATPase has been hypothesised to be the functional role of *TaPtf1*, whereas there is a lack of experimental support. Therefore, concentrations of key soluble sugars (e.g. sucrose) in shoots and roots in *TaPtf1* overexpression lines can be assessed and compared with those in wild-type. MALDI-Tof spectrometry can be one of the attractive options for analysing the spectrum of carbohydrates (Robinson *et al*., 2006). The role of H^+-transporting ATPase and other relevant genes in regulating carbohydrate metabolism in the stem and/or leaves have previously been described for *ZmPtf1* and *OsPtf1* (Li *et al*., 2011; Yi *et al*., 2005). Similar studies can be performed for *TaPtf1* in wheat.

The promotion of plant height is thought to be a result of altered meristematic activities associated with crosstalk between phytohormone and sugar signalling directly or indirectly regulated by *TaPtf1* under P deficiency/low-P. Hormone profiling has been one of the potent methods to investigate the role of phytohormones in plant growth and development (Müller *et al*., 2017). To carry this out in *TaPtf1* overexpression lines may collect valuable information on hormonal regulation by *TaPtf1*. Moreover, since grain-filling appears to be negatively regulated by *TaPtf1*, studies attending to grain quality by examining important components of grain size and shape, coupled with analysis of tissue-specific expression patterns of related PTs and other relevant genes in a further aspect, may help to obtain evidence about this.

An alternative approach to study the distinction between the regulatory effects of *TaPtf1* in the stem and roots on P uptake and translocation, as well as their different regulatory activities in contributing to root remodelling and PUE, probably is to create separate *TaPtf1* overexpression/knockdown wheat lines using tissue-specific promoters. Once a population, where the transgene expression is more stable and consistent in all plants, is obtained, differences

in the behaviour of P uptake, root growth and biomass accumulation can be analysed in these plants grown under different P supply. Certainly, it would also be interesting to collect data for differential expression of a group of genes potentially involved in these processes, such as PTs or other PSI genes, the results of which may provide additional messages for the regulatory effects of *TaPtf1*. Furthermore, various aspects of photosynthetic activities in plant leaves would be worth studying. A direct way for this may be to use the LICOR portable photosynthesis system (Long *et al*., 1996), which is likely to extend the data about influences on photosynthesis following alternations of P uptake and translocation. In addition, it would also be informative to create reporter lines for *TaPtf1* to study the tissue-specific expression and sub-cellular location or regulation by external (e.g. P supply) and internal (e.g. phytohormone) factors. The outputs of the future work are expected to extend current understandings of this TF and provide more evidence on how the carbohydrate metabolism is regulated by *TaPtf1* in response to P stress in wheat.

8.3 *TaMyb67*

8.3.1 Roles of *TaMyb67*

In this study, *TaMyb67* (CDS) was identified and hypothesised as the wheat orthologue of *OsPhr2.* The central role of *OsPhr2* or its equivalents in other plant/crop species in regulating PUE and promoting plant growth in adaptation to P deficiency/low-P has been well-demonstrated in previous studies (reviewed in section 1.7). In Chapter 3, we already discussed that the CDS of *TaMyb67* is a truncated sequence and most likely to be an ecotypic variation of *TaPhr1-B1* but unlikely to be a splice variant. This may suggest that more than one alleles of *TaPhr1-B1* exist in different wheat cultivars. With the expectation of validating whether *TaMyb67* has a functional role in improving PUE in response to P stress, attempts were made by creating and studying *TaMyb67* overexpression lines in this study. However, due to no clear evidence being obtained, the biological functions of *TaMyb67* could not be ascertained.

According to the sequence analysis in Chapter 3, apart from the missing peptides which are not located in the known functional regions, alterations of amino acids were also observed in the TaMyb67/TaPhr1-B1 protein sequence, when compared with TaPhr1-A and TaPhr1-D1 protein sequences. However, much is unknown for the impacts of these alterations on the functions of the resulting protein. Perhaps they are associated with the selection of, or the binding affinity and stability to, downstream targets of the resulting protein. It has been studied that *Phr1* regulates several different pathways through binding to downstream microRNAs or PSI genes to improve PUE under P deficiency/low-P (reviewed in section 1.7). The *cis*-element P1BS (GNATATNC) in the promoter region of PSI genes is the target of *Phr1* (Bustos *et al*., 2010; Rubio *et al*., 2001; Zhou *et al*., 2008a). It is possible the discrepancy of amino acids in the TaPhr1-B1/TaMyb67 protein sequence can enable different signal sensing and responding

pathways in regulating PUE under P deficiency/low-P. *TaPhr1-A1* works through a group of high-affinity PTs to increase P acquisition and translocation for improved PUE under P deficiency/low-P, remarkably *TaPht1:6* and *TaPht1:2* predominant in shoots and roots, respectively (Davies *et al*., 2002; Miao *et al*., 2009; Wang *et al*., 2013b). Unlike that, *TaPhr1-B1*/*TaMyb67* might employ other high- or low- affinity PTs to assist in P acquisition, translocation and utilisation to improve PUE in resistance to P deficiency/low-P.

Furthermore, data obtained in Chapter 3 also suggested the *TaPhr1-B1*/*TaMyb67* homoeolocus is highly spliced compared with homoeoloci of *TaPhr1-A1* and *TaPhr1-D1*. This may imply the AS in the homoeolocus of *TaPhr1-B1* is more variable, whereas those in homoeoloci of *TaPhr1-A1* and *TaPhr1-D1* seem to be more conserved. AS has been shown as a dynamic and complex process broadly affected by intrinsic and environmental factors (Brett *et al*., 2002; Mazzucotelli *et al*., 2008; Palusa, 2007), with the selection of splice sites regulated spatially and temporally. One good example for this is *AtDCL2*, a dicer gene playing roles in RNA interference pathways, which generates four splicing variants that encode four functionally distinct protein isoforms in different tissues and developmental stages in *Arabidopsis* and rapeseed plants (Yan *et al*., 2009). Notably, AS events that are driven by environmental stress can profoundly change plants' resilience to the stress (Pil *et al*., 2013; Staiger and Brown, 2013). AS events are important for TFs to achieve precise modulation of gene expression, which has been discussed in different studies in plants (Talavera *et al*., 2009; Raddy *et al*., 2013). While most splice variants of the *TaPhr1-B1* homoeolocus are not well-studied, they may have differential expression patterns, distributions and regulation within the plant, which could be correlated with developmental stages, tissue/cell-types and P stress or other abiotic stresses. Perhaps these splice variants express mutually or individually in a tissue/cell-specific manner or even universally within the whole plant depending on

developmental stages. It might also not be improper to infer that a dynamic balance of transcription or co-regulation exists for these splice variants when they express mutually, which is possibly controlled by some unknown feedback loops interacting with AS. In addition, such a unique feature of the *TaPhr1-B1* homoeolocus might be associated with the miniature inverted-repeat transposable elements (MITEs) insertion found in the intron-4 of *TaPhr1-B1* as suggested by Wang *et al*. (2013b); also found in *TaPhr1-B1* in the Ensembl wheat genome in this study. MITEs are one type of transposable elements (TEs) that present extensively in plant genomes (Chen *et al*., 2014b). The insertion of MITEs into the intron of a gene has been indicated to influence the regulatory expression of the gene in various ways, although the mechanisms have not been well-understood. Moreover, MITEs have been recently discovered to also play regulatory roles in plant stress responses (Negi *et al*., 2016).

8.3.2 Future work

There was no evidence derived from the current study to show *TaMyb67*, which has been thought to be an ecotypic variant of *TaPhr1-B1*, has a functional role in PUE as discussed in the previous section. Nevertheless, progeny generations with a homogenised genetic background of more satisfactory overexpression might still be obtained using other *TaMyb67* transformant sublines untested in this study. It will be interesting to study the transcriptional activities of a set of high- or low- affinity PTs or other genes associated with P acquisition and potentially regulated by *TaPhr1* (Nilsson *et al*., 2007; Rubio *et al*., 2001). In the meantime, P uptake or root architecture/morphology under different P supply can also be examined in *TaMyb67* overexpression lines. Furthermore, studies on expression patterns of other PSI genes regulated by *TaPhr1* in different pathways, e.g. PAP, SQD (Zhou *et al*.,2008a), may also shed light on the regulatory effects of *TaPhr1*-B1 in PUE. Network analysis could also be helpful in

exploring how other regulatory components are interacting with or linking to *TaPhr1* pathways, e.g. regulators of phytohormone or sugar signalling pathways. The study can also be extended by further validating whether the feature of highly-splicing at post-transcriptional level of the *TaPhr1-B1* homoeolocus is related to MITEs. For this, to create another mutant line, where the MITEs that *TaPhr1-B1* contains is non-functional, through transgenic approaches (e.g. CRISPR) may be a strategy. There may be advantages of comparing the transcriptional activities of *TaPhr1-B1* and their relevant regulators in the mutant line to be given different P supply. In addition, it may also be useful to re-generate a new GFP reporter construct, using the CDS of *TaMyb67* without a stop codon, for studying the sub-cellular location of TaMyb67 and comparing with the nucleic location of TaPhr1-A1 previously identified (Wang *et al*., 2013b). The outputs of the future work are expected to contribute to the knowledge pool of abiotic stress responses in plants and hopefully to facilitate breeding wheat cultivars with improved PUE.

Reference

Abbo, S., Lev-Yadun, S., Heun, M., Gopher, A. (2013). On the 'lost' crops of the Neolithic Near East. *Journal of Experimental Botany*, 64(4), 815–822.

Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K., Yamaguchi-Shinozaki, K. (2003). *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell*, 15(1), 63–78.

Adesemoye, A.O., Kloepper, J.W. (2009). Plant-microbes interactions in enhanced fertilizer-use efficiency. *Applied Microbiology and Biotechnology*, 85(1), 112.

Agarwal, M., Hao, Y., Kapoor, A., Dong, C.H., Fujii, H., Zheng, X., Zhu, J.K. (2006). A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *The Journal of Biological Chemistry*, 281(49), 37636–37645.

Ahmad, P., and Prasad, M.N.V. (2012). *Abiotic stress responses in plants: metabolism, productivity and sustainability*. New York: Springer, 2012.

Ai, P., Sun, S., Zhao, J., Fan, X., Xin, W., Guo, Q., Yu, L., Shen, Q., Wu, P., Miller, A.J., Xu, G. (2009). Two rice phosphate transporters, OsPht1:2 and OsPht1:6, have different functions and kinetic properties in uptake and translocation. *The Plant Journal*, 57(5), 798–809.

Akhtar, M.S., Oki, Y., Adachi, T. (2008). Intraspecific variations of phosphorus absorption and remobilisation, P forms, and their internal buffering in Brassica cultivars exposed to a P-stressed environment. *Journal of Integrative Plant Biology*, 50(6), 703–716.

Akhtar, M.S., Oki, Y., Adachi, T., Murata, Y., Khan, M.H.R. (2007). Relative phosphorus utilisation efficiency, growth response, and phosphorus uptake kinetics of Brassica cultivars under a phosphorus stress environment. *Communications in Soil Science and Plant Analysis*, 38 (7–8), 1061–1085.

Akhunov, E.D., Sehgal, S., Liang, H., Wang, S., Akhunova, A.R., Kaur, G., Li, W., Forrest, K.L., See, D., Šimková, H., Ma, Y., Hayden, M.J., Luo, M., Faris, J.D., Doležel, J., Gill, K.S. (2013). Comparative analysis of syntenic genes in grass genomes reveals accelerated rates of gene structure and coding sequence evolution in polyploid wheat. *Plant Physiology*, 161(1), 252–265.

Allen, A. M., Winfield, M.O., Burridge, A. J., Downie, R.C., Benbow, H.R., Barker, G.L.A., Wilkinson, P.A., Coghill, J., Waterfall, C., Davassi, A., Scopes, C., Pirani, A., Webster, T., Brew, R., Bloor, C., Griffiths, S., Bentley, A.R., Alda, M., Jack, P., Phillips, A.L., Edwards, K.J. (2017). Characterisation of a wheat breeders' array suitable for high throughput SNP genotyping of global accessions of hexaploid bread wheat (*Triticum aestivium*). *Plant Biotechnology Journal*, 15(3), 390–401.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25(17), 3389–402.

Anghinoni, I. and Barber, S.A. (1980). Phosphorus influx and growth characteristics of corn roots as influenced by phosphorus supply. *Agronomy Journal*, 72(4), 685–688.

Arpat, A.B., Magliano, P., Wege, S., Rouached, H., Stefanovic, A., Poirier, Y. (2012).

Functional expression of PHO1 to the Golgi and *trans*-Golgi network and its role in export of inorganic phosphate. *The Plant Journal*, 71(3), 479–491.

Ashley, K., Cordell, D., and Mavinic. D. (2011). A brief history of phosphorous: From the philosopher's stone to nutrient recovery and reuse. *Chemosphere*, 84(6), 737–746.

Assuero, S.G., Mollier, A., Pellerin, S. (2004). The decrease in growth of phosphorus–deficient maize leaves is related to a lower cell production. *Plant, Cell and Environment*, 27(7), 887–895.

Aukerman, M.J., Hirschfeld, M., Wester, L., Weaver, M., Clack, T., Amasino, R.M., and Sharrock, R.A. (1997). A deletion in the PHYD gene of the *Arabidopsis Wassilewskija* ecotype defines a role for phytochrome D in red/far-red light sensing. *Plant Cell*, 9(8), 1317–1326.

Aung, K., Lin, S.I., Wu, C.C., Huang Y.T., Su, C.l., Chiou, T.J. (2006). pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. *Plant Physiol*ogy, 141(3), 1000–1011.

Aziz, T., Finnegan, P.M., Lambers, H., Jost, R. (2014). Organ-specific phosphorus-allocation patterns and transcript profiles linked to phosphorus efficiency in two contrasting wheat genotypes. Plant, *Cell and Environment*, 37(4), 943–96.

Babitha, K.C., Ramu, S.V., Pruthvi, V., Mahesh, P., Nataraja, K.N., Udayakumar, M. (2013). Co-expression of AtbHLH17 and AtWRKY28 confers resistance to abiotic stress in *Arabidopsis*. *Transgenic Research*, 22(2), 327–341.

Baek, D., Park, H., Kim, M., Yun, D. (2013). The role of *Arabidopsis* MYB2 in miR399f-mediated phosphate-starvation response. *Plant Signaling and Behavior*, 8(3), e23488. doi: org/10.4161/psb.23488.

Baker, A., Ceasar, S.A., Palmer, A.J., Paterson, J.B., Qi, W., Muench, S.P., Baldwin, W.A. (2015). Replace, reuse, recycle: improving the sustainable use of phosphorus by plants. *Journal of Experimental Botany*, 66(12), 3523–3540.

Bakrim, N., Brulferta, J., Vidala, J., Cholletb, R. (2001). Phospho*enol*pyruvate Carboxylase Kinase Is Controlled by a Similar Signaling Cascade in CAM and C4 Plants. *Biochemical and Biophysical Research Communications*, 286 (5), 1158–1162.

Balemi, T., Negisho, K. (2012). Management of soil phosphorus and plant adaptation mechanisms to phosphorus stress for sustainable crop. *Journal of Soil Science and Plant Nutrition*, 12(3), 547–561.

Baligar, V.C., Fageria, N.K., He, Z.L. (2001). Nutrient use efficiency in plants. *Communications in Soil Science and Plant Analysis*, 32(7–8), 921–950.

Barber, S.A. (1995). *Soil Nutrient Bioavailability. A mechanistic approach.* 2nd ed. New York: John Wiley and Sons Inc.

Barber, S.A., Walker, J.M., Vasey, E.H. (1963). Mechanisms for the movement of plant nutrients from the soil and fertilizer to the plant root. *Journal of Agricultural Food Chemistry*, 11, 204–207.

Bari, R., Datt Pant, B., Stitt, M., Scheible, W.R. (2006). PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiology*, 14(3), 1988–1999.

Bates, T.R., Lynch, J.P. (1996). Stimulation of root hair elongation in *Arabidopsis thaliana* by low phosphorus availability. *Plant, Cell and Environment*, 19(5), 529–538.

Bates, T.R., Lynch, J.P. (2000a). Plant growth and phosphorus accumulation of wild-type and two root hair mutants of *Arabidopsis thaliana*. *American Journal of Botany*, 87(7), 958–963.

Bates, T.R., Lynch, J.P. (2000b). The efficiency of *Arabidopsis thaliana* root hairs in phosphorus acquisition. *American Journal of Botany*, 87(7), 964–970.

Batjes, N.H. (1997). A world dataset of derived soil properties by FAO-UNESCO soil unit for global modelling. *Soil Use and Management*, 13(1), 9–16.

Bayuelo-Jiménez, J.S, Gallardo-Valdéz, M., Pérez-Decelis, V.A., Magdaleno-Armas, L., Ochoa, I., Lynch, J.P. (2011). Genotypic variation for root traits of maize (*Zea mays* L.) from the *Purhepecha Plateau* under contrasting phosphorus availability. *Field Crops Research*, 121(3), 350–362.

Benková, E., Bielach, A. (2010). Lateral root organogenesis—from cell to organ. *Current Opinion in Plant Biology*, 13(6), 677–683.

Benková, E., Michniewicz. M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell*, 115(5), 591–602.

Bennett, E.M., Carpenter, S.R., Caraco, N.F. (2001). Human impact on erodable phosphorus and eutrophication: a global perspective. *BioScience*, 51(3), 227–34

Bennett, M.D., Leitch, I.J. (2011). Nuclear DNA amounts in angiosperms: targets, trends and tomorrow. *Annals of Botany*, 107(3), 467–590.

Bernard, P., Couturier, M. (1992). Cell Killing by the F Plasmid *ccd*B Protein Involves Poisoning of DNA-Topoisomerase II Complexes. *Journal of Molecular Biology*, 226(3), 735–745.

Bhalla, P.L. (2006). Genetic engineering of wheat-current challenges and opportunities. *Trends in Biotechnology*, 24(7), 305–311.

Bhat, K.K.S., Nye, P.H. (1973). Diffusion of phosphate to plant roots in soil. I. Quantitative autoradiography of the depletion zone. *Plant and Soil*, 38(1), 161–175.

Bhat, K.K.S., Nye, P.H. (1974). Diffusion of phosphate to plant roots in soil: III. Depletion around onion roots without root hairs. *Plant Soil*, 41(2),383–394.

Bieleski, R.L. (1973). Phosphate pools, phosphate transport, and phosphate availability. *Annual Review of Plant Physiology*, 24, 225–252.

Birch, R.G. (2000). Application of gene transfer to crop improvement. In: L. O'Brien, R.J. Henry ed., *Transgenic cereals*. Minnesota, USA: American Association of Cereal Chemists, p.267–276.

Birnboim, H.C., Doly, J. (1979) A rapid alkaline lysis procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, 7(6), 1513–1522.

Bodelier, P.L.E.; Roslev, P., Henckel, T., Frenzel, P. (1999). "Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots". *Nature*, 403(6768), 421–424.

Bolan, N.S. (1991). A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. *Plant and Soil*, 134(2), 189–207.

Bombarely, A., Rosli, H.G., Vrebalov, J., Moffett, P., Mueller, L.A., Martin, G.B. (2012). A draft genome sequence of *Nicotiana benthamiana* to enhance molecular plant-microbe biology research. *Molecular plant-microbe interactions,* 25(12), 1523–1530.

Booker, J., Sieberer, T., Wright, W., Williamson, L., Willett, B., Stirnberg, P., Turnbull, C., Srinivasan, M., Goddard, P., and Leyser, O. (2005). MAX1 encodes a cytochrome P450 family

member that acts downstream of MAX3/4 to produce a carotenoid-derived branch-inhibiting hormone. *Developmental Cell*, 8(3), 443–449.

Booker, K.S., Schwarz, J., Garrett, M.B., Jones, A.M. (2010). Glucose attenuationof auxin-mediated bimodality in lateral root formation is partly coupled by the heterotrimeric G protein complex. *PLoS ONE*, 5, e12833. doi: 10.1371/journal.pone.0012833

Borrill, P., Ramirez-Gonzalez, R., Uauy, C. (2016). *expVIP: a customisable RNA-seq data analysis and visualisation platform*. *Plant Physiology*, *170 (4), 2172–2186.*

Bouwman, L., Goldewijk, K.K., Van Der Hoek, K.W., Beusen, A.H.W., Van Vuuren, D.P., Willems, J., Rufino, M.C., Stehfest, E. (2011). Exploring global changes in nitrogen and phosphorus cycles in agriculture induced by livestock production over the 1900–2050 period. In: *Proceedings of the National Academy of Sciences.* Washington, DC, USA: *Proceedings of the National Academy of Sciences. USA*, 110 (52), 20882–20887.

Bovill, W.D., Huang, C.Y., McDonald, G.K. (2013). Genetic approaches to enhancing phosphorus-use efficiency (PUE) in crops: challenges and directions. *Crop and Pasture Science*, 64, 179–198.

Brabant, P. (2010). A land degradation assessment and mapping method. A standard guideline proposal. Les dossiers thématiques du CSFD. N°8. France: CSFD/Agropolis International, Montpellier, p.52.

Bradley, S.R., Berry, P., Blake, J., Kindred, D., Spink, J., Bingham, I., McVittie, J., Foulkes, J. (2008). *The wheat growth guide*. London: HGCA, p.30.

Branscheid, A., Sieh, D., Pant, B.D., May, P., Devers, E.A., Elkrog, A., Schauser, L., Scheible, W.R., Krajinski, F. (2010). Expression pattern suggests a role of MiR399 in the regulation of the cellular response to local Pi increase during arbuscular mycorrhizal symbiosis. *Molecular Plant-Microbe Interaction*, 23(7), 915–926.

Braun, H.J., Atlin, G., and Payne, T. (2010). Multi-location testing as a tool to identify plant response to global climate change. In: M.P. Reynolds, ed., *Climate Change and Crop Production*. Surrey: CABI, p.115–138.

Braun, M.D., Wang, L., Ruan, Y. (2014). Understanding and manipulating sucrose phloem loading, unloading, metabolism, and signalling to enhance crop yield and food security. *Journal of Experimental Botany*, 65(7), 1713–1735.

Brenchley, R., Spannagl, M., Pfeifer, M., Barker, G. L. A., D'Amore, R., Allen, A. M., Hall, N. (2012). Analysis of the bread wheat genome using whole-genome shotgun sequencing. *Nature*, 491(7426), 705–710.

Brett, D., Pospisil, H., Valc´arcel, J., Reich, J., Bork, P. (2002). "Alternative splicing and genome complexity," *Nature Genetics*, 30(1), 29–30.

Bridgwater, W. and Aldrich, B. (1966). *Triticum* L. In: *The Columbia-Viking Desk Encyclopedia*. Columbia: Columbia University, p.1959.

Briggle, L.W. (1980). Origin and botany of wheat. In: E. Häfliger, ed., *Wheat Documenta Ciba-Geigy*. Basle, Switzerland. p.6–13.

Brooks, A., Woo, K.C., Wong, S.C. (1988). Effect of phosphorus nutrition on the response of photosynthesis to CO2 and O2, activation of ribulose bisphosphate carboxylase and amount of

ribulose bisphosphate and 3-phosphoglycerate in spinach leaves. *Photosynthesis Research*, 15(2), 133–141.

Brundrett, M.C. (2002). Coevolution of roots and mycorrhizas of land plants. *New Phytologist*, 154(2), 275–304.

Bushman, W., Thompson, J. F., Vargas, L., and Landy, A. (1985). Control of directionality in Lambda site-specific recombination. *Science*, 230(4728), 906–911.

Bustos, R., Castrillo, G., Linhares, F., Puga, M.I., Rubio, V., Pérez-Pérez, J., Solano, R., Leyva, A., Paz-Ares, J. (2010). A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in *Arabidopsis*. *PLoS Genetics*, 6(9), e1001102. doi.org/10.1371/journal.pgen.1001102.

Byrne, P.F., McMullen, M.D. (1996). Defining genes for agricultural traits: QTL analysis and the candidate gene approach. *Probe*, 7, 24–27.

Byrne, S.L., Foito, A., Hedley, P.E., Morris, J.A., Stewart, D., Barth, S. (2011). Early response mechanisms of perennial ryegrass (*Lolium perenne*) to phosphorus deficiency. *Annuals of Botany*, 107(2), 243–254.

Cailloux, M. (1972). Metabolism and the absorption of water by root hairs. *Canadian Journal of Botany*, 50(3), 557–573.

Calderini, D.F., Savin, R., Abeledo, L.G., Reynolds, M.P., Slafer, G.A. (2001). The importance of the period immediately preceding anthesis for grain weight determination in wheat. *Euphytica*, 119(1–2), 199–204.

Cannon, S.B., May, G.D. and Jackson, S.A. (2009). Three sequenced legume genomes and many crop species: rich opportunities for translational genomics. *Plant Physiology*, 151(3), 970–977.

Cao Z. H., Zhang S. Z., Wang R. K., Zhang R. F., Hao Y. J. (2013). Genome-wide analysis of the apple MYB transcription factor family allows the identification of MdoMYB121 gene conferring abiotic stress tolerance in plants. *PLoS ONE*, 8, e69955. doi.org/10.1371/journal. pone.0069955.

Carretero-Paulet, L., Galstyan, A., Roig-Villanova, I., Martı´nez-Garcı´a, J.F., Bilbao-Castro, J.R., Robertson, D.L. (2010). Genome-Wide Classification and Evolutionary Analysis of the bHLH Family of Transcription Factors in *Arabidopsis*, Poplar, Rice, Moss, and Algae. *Plant Physiology*, 153(3), 1398–1412.

Carver, B.F. (2009). *Wheat science and trade*. Danvers: Wiley, Blackwell, Oxford, UK, p.569.

Caswell, K.L., N.L.L., Chibbar, R.N. (2000). An efficient method for in *vitro* regeneration from immature inflorescence explants of Canadian wheat cultivars. *Plant Cell, Tissue and Organ Culture*, 60(1), 69–73.

Chang, L., Ramireddy, E., Schmülling, T. (2013). Lateral root formation and growth of *Arabidopsis* is redundantly regulated by cytokinin metabolism and signalling genes. *Journal of Experimental Biology*, 64(16), 5021-5032.

Charrier, B., Scollan, C., Ross, S., Zubko, E., Meyer, P. (2000). Co-silencing of homologous transgenes in tobacco. *Molecular Breeding*, 6(4), 407–419.

Chen, A., Guo, X., Chen, Z., Chen, W., Liu, D., Zheng, Y., Liu, Y., Wei, Y., Wang, J. (2016). Genome-wide characterization of developmental stage- and tissue-specific transcription factors in wheat.

BMC Genomics, 16, 125. doi: 10.1186/s12864-015-1313-y.

Chen, A., Chen, X., Wang, H., Liao, D., Gu, M., Qu, H., Sun, S., Xu, G. (2014a). Genome-wide investigation and expression analysis suggest diverse roles and genetic redundancy of Pht1 family genes in response to Pi deficiency in tomato. *BMC Plant Biology*, 14, 61. doi: 10.1186/1471-2229-14-61.

Chen, J., Hu, Q., Lu, C., Kuang, H. (2014b). In Evolutionary biology: Genome evolution, speciation, coevolution and origin of life. In: P. Pontarotti, ed., *Evolutionary genomics of miniature inverted-repeat transposable elements (MITEs) in plants*. Berlin: Springer International Publishing, p.157–168.

Chen, J.Y., Liu, Y., Ni, J., Wang, Y.F., Bai, Y.H., Shi, J., Gan, J., Wu, Z.C., Wu, P. (2011). OsPHF1 regulates the plasma membrane localisation of low- and high-affinity inorganic phosphate transporters and determines inorganic phosphate uptake and translocation in rice. *Plant Physiology*, 157(1), 269–278.

Chen, Z., Nimmo, G.A., Jenkins, G.I., Nimmo, H.G. (2007). BHLH32 modulates several biochemical and morphological processes that respond to Pi starvation in *Arabidopsis. Biochemical Journal*, 405(1), 191–198.

Cheng, M., Fry, J.E., Pang, S.Z., Zhou, H.P., Hironaka, C.M., Duncan, D.R., Conner, T.W., Wan, Y.C. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiology*, 115(3), 971–980.

Cheng, Y., Kim, M., Deng, X., Kwak, S., Chen, W. (2013). Enhanced Salt Stress Tolerance in Transgenic Potato Plants Expressing IbMYB1, a Sweet Potato Transcription Factor. *Journal of Microbiology and Biotechnology*, 23(12), 1737–1746.

Chiera, J., Thomas, J., Rufty, T. (2002). Leaf initiation and development in soybean under phosphorus stress. *Journal of Experimental Botany*, 53(368), 473–481.

Chin, J.C., Scott, K.J. (1977). Studies on the formation of roots and shoots in wheat callus cultures. *Annals of Botany*, 41(3), 473–481.

Chiou, T.J., and Lin, S.I. (2011). Signalling Network in Sensing Phosphate Availability in Plants. *Annual Review of Plant Biology*, 62, 185–206.

Chomczynski, P. (1993). A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*, 15(3), 532–537.

Christensen, A.H., Quail, P.H. (1996). Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research*, 5(3), 213–218.

Clarkson, D.T. (1981). Nutrient interception and transport by root systems. In: C.B. Johnson, ed., *Physiological processes limiting plant productivity*. London: Butterworths, p.307–314.

Cominelli, E., Galbiati, M., Vavasseur, A., Conti, L., Sala, T., Vuylsteke, M., Leonhardt, N., Dellaporta, S.L., Tonelli, C. (2005). A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. *Current Biology*, 15(13), 1196–1200.

Cordell, D., Drangert, J.O., White, S. (2009). The story of phosphorus: global food security and food for thought. *Glob Environ Change*, 19(2), 292–305.

Cordell, D., Rosemarin, A., Schröder, J.J., Smit, A.L., (2011). Towards global phosphorus security: a systems framework for phosphorus recovery and reuse options. *Chemosphere,* 84(6), 747–758.

Cordell, D., White, S., (2010). Securing a sustainable phosphorus future for Australia. *Farm Policy Journal*, 7(3), 1–17.

Cornejo, M.J., Luth, D., Blankenship, K.M., Anderson, O.D., Blechl, A.E (1993). Activity of a maize ubiquitin promoter in transgenic rice. *Plant Molecular Biology*, 23(3), 567–581.

Corrales, I., Amenos, M., Poschenrieder, C., Barcelo, J. (2007). Phosphorus efficiency and root exudates in two contrasting tropical maize varieties. *Journal of Plant Nutrition*, 30(6), 887–900.

Correll, D.L., (1998). The role of phosphorus in eutrophication of receiving waters: a review. *Journal of Environmental Quality*, 27(2), 261–266.

Dai, X., Wang, Y., Yang, A., Zhang, W.H. (2012). OsMYB2P-1, an R2R3 MYB transcription factor, is involved in the regulation of phosphate-starvation responses and root architecture in rice. *Plant Physiology*, 159(1), 169–183.

Dalmay, T., Horsefield, R., Brautenstein, T.H., Baulcombe, D.C. (2001). SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in *Arabidopsis*. *EMBO Journal*, 20(8), 2067–2077.

Damian, C. (2013). NFU claims extreme weather poses biggest threat to British farming. *The Guardian*, [online] p. A1. Available at: https://www.theguardian.com/environment /2013/jul/28/weather-heatwaves-climate-change-uk-farming. [Accessed 28/09/2015]

Daniel, P. S., Robert J. R., Ayling, S.M. (1998). Phosphorus uptake by plants: from soil to cell. *Plant Physiology,* 116 (2), 447–453.

Daram, P., Brunner, S., Persson, B. L., Amrhein, L. and Bucher, M. (1998). Functional analysis and cell-specific expression of a phosphate transporter from tomato. *Planta*, 206(2), 225–233.

Davies, T., Ying, J., Xu, Q., Li, Z., Li, J., Gordon-Weeks, R. (2002). Expression analysis of putative high-affinity phosphate transporters in Chinese winter wheat. *Plant, Cell and Environment*, 25(10), 1325–1339.

De Groot, C. C., Marcelis, L. F. M., Van den Boogaard, R., Lambers, H. (2001). Growth and dry-mass partitioning in tomato as affected by phosphorus nutrition and light. *Plant Cell &Environment,* 24(12), 1309–1317.

De Ridder, M., De Jong, S., Polchar, J., Lingemann, S. (2012) Risks and opportunities in the global phosphate rock market-Robust strategies in times of uncertainty. [online] Hague: The Hague Centre for Strategic Studies (HCSS), p.2 Available at: http://www.snb.nl/ files/Nieuws/Internet/Rapport_Marjolein-de-Ridder_Fosfaat.pdf. [Accessed 15/05/2015].

Delhaize, E., Randall, P. (1995). Characterization of a phosphate-accumulator mutant of *Arabidopsis thaliana*. *Plant Physiology*, 107(1), 207–213.

Delhaize, E., Taylor, P., Hocking, P.J., Simpson, R.J., Ryan, P.R., Richardson, A.E. (2009). Transgenic barley (*Hordeum vulgare* L.) expressing the wheat aluminium resistance gene (TaALMT1) shows enhanced phosphorus nutrition and grain production when grown on an acid soil. *Plant Biotechnology Journal*, 7(5), 391–400.

Delporte, F., Mostade, O., Jacquemin, J.M. (2001). Plant regeneration through callus initiation from thin mature embryo fragments of wheat. *Plant Cell, Tissue and Organ Culture*, 67(1), 73–80.

Depicker, A., Van Montagu, M. (1997). Post-transcriptional gene silencing in plants. *Current Opinion in Cell Biology*, 9(3), 373–382.

Devaiah, B.N., Madhuvanthi, R., Karthikeyan, A.S., Raghothama, K.G. (2009). Phosphate starvation responses and gibberellic acid biosynthesis are regulated by the MYB62 transcription factor in *Arabidopsis*. *Molecular Plant*, 2(1), 43–58.

Ding, W., Yu, Z., Tong, Y., Huang, W., Chen, H., Wu, P. (2009). A transcription factor with a bHLH domain regulates root hair development in rice. *Cell Research*, 19(11), 1309–1311.

Dittmer, H.J. (1937). A quantitative study of the roots and root hairs of a winter rye plant (*Secale cereale*). *American Journal of Biotechnology*, 24(7),417–420.

Dolan, L., Costa, S. (2001). Evolution and genetics of root hair stripes in the root epidermis. *Journal of Experimental Biotechnology*, 52(S1), S413–S417.

Dolan, L., Roberts, K. (1995). The development of cell pattern in the root epidermis. Philosophical Transactions of the Royal Society B, 350(1331), 95–99.

Dong, B., Rengel, Z., Delhaize, E. (1998). Uptake and translocation of phosphate by pho2 mutant and wild-type seedlings of *Arabidopsis thaliana*. *Plant Physiol*ogy, 205(2), 251–256.

Dong, B., Ryan, P.R., Rengel, Z., Delhaize, E. (1999). Phosphate uptake in *Arabidopsis* thaliana: dependence of uptake on the expression of transporter gene and internal phosphate concentrations Plant. *Cell Environment*, 22(11), 455–146.

Dong, D., Peng, X., Yan, X. (2004). Organic acid exudation induced by phosphorus deficiency and/ or aluminium toxicity in two contrasting soybean genotypes. *Physiologia Plantarum*, 122(2), 190–199.

Driessen, P., Deckers, S., Spaargaren, O., Nachtergaele, F., (2001). *Lecture notes on the major soils of the world. World Soil Resources Reports 94*. [online] Rome, Italy: FAO, p.1–327. Available at: http://www.fao.org/3/a-y1899e.pdf. [Accessed 02/02/2016].

Du, H., Feng, B.R., Yang, S. S., Huang, Y.B., Tang, Y.X. (2012b). The R2R3- MYB transcription factor gene family in maize. *PLoS ONE*, 7, e37463. doi: 10.1371/journal.pone.0037463.

Du, H., Yang, S.S., Liang, Z., Feng, B.R., Liu, L., Huang, Y.B., Tang, Y.X. (2012a). Genome-wide analysis of the MYB transcription factor superfamily in soybean. *BMC Plant Biology*, 12, 106. doi: 10.1186/1471-2229-12-106.

Du, H., Zhang, L., Liu, L., Tang, X.F., Yang, W.J., Wu, Y.M., Huang, Y.B., Tang, Y.X. (2009). Biochemical and molecular characterization of plant MYB transcription factor family. *Biochemistry (Mosc)*, 74(1), 1–11.

Dubrovsky, J., Rost, T., Colón-Carmona, A., Doerner, P. (2001). Early primordium morphogenesis during lateral root initiation in *Arabidopsis thaliana*. *Planta*, 214(1), 30–36.

Dubrovsky, J.G., Sauer, M., Napsucialy-Mendivil, S., Ivanchenko, M.G., Friml, J., Shishkova, S., Celenza, J., Benková, E. (2008). Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. *Proceedings of the National Academy of Sciences. USA*, 105(25), 8790–8794.

Duff, S.M.G., Moorhead, G.B.G., Lefebvre, D.D., Plaxton, W.C. (1989). Phosphate starvation inducible bypasses of adenylate and phosphate dependent glycolytic enzymes in Brassica nigra suspension cells. *Plant Physiology*, 90(4), 1275–1278.

Duff, S.M.G., Sarath, G., Plaxton, W.C. (1994). The role of acid phosphatases in plant phosphorus metabolism. *Physiologia Plantarum,* 90(4), 791–800.

Dupont, F.M., Vensel, W.H., Tanaka, C.K., Hurkman, W.J., Altenbach, S.B. (2011). Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases and tandem mass spectrometry. *Proteome Science*, 9, 10. doi: 10.1186/1477-5956-9-10.

Dvorak, J., Luo, M.C., Akhunov, E.D. (2011). NI Vavilov's theory of centres of diversity in the light of current understanding of wheat diversity, domestication and evolution. *Czech Journal of Genetic Plant Breeding,* 47, S20–S27.

Dvorak,J., Deal, K.R., Luo, M.C., You, F.M., Von Borstel, K. (2012). The origin of spelt and free-threshing hexaploid wheat. *Journal of Heredity*, 103(3), 426–441.

Elser, J., Bennett, E., (2011). Phosphorus cycle: a broken biogeochemical cycle. *Nature,* 478(7367), 29–31.

Erisman, J.W. (2009). Integrating nitrogen use and food production with environmental expectations. In: *No. 664 Proceedings of the International Fertilizer Society*. York, UK: International Fertilizer Society, p.1–24.

Erisman, J.W., Bleeker, A., Van Jaarsveld, J.A., (1998). Evaluation of ammonia emission abatement on the basis of measurements and model calculations. *Environmental Pollution*, 102(1), S269–S274.

Espindula, L. F., Minella, E., Delatorre, C.A. (2009). Low-P tolerance mechanisms and differential gene expression in contrasting wheat genotypes. *Pesquisa Agropecuária Brasileira*, 44 (9), 1100–1105.

Eswaran, H., Reich, P., and Beinroth F (1997). Global distribution of soils with acidity. In: A.C. Moniz, R.E. Furlani, N.K. Schaffert, C.A. Fageria, C.A. Rosolem, H. Canttarella, ed., *Plant-Soil Interactions at Low pH: sustainable agriculture and forestry production*. Campinas: Brazilian Society of Soil Science, p.159–164.

Fageria, N.K., V.C. Baligar, and Y.C. Li. (2008). The role of nutrient efficient plants in improving crop yields in the 21st century. *Journal of Plant Nutrition*, 31(6), 1121–1157.

Fairhust, T., Lefroy, E., Mutert, E., Batjes, N.H., (1999). The importance, distribution and causes of phosphorus deficiency as a constraint to crop production in the tropics. *Agroforestry Forum*, 9, 2–8.

Fang, W., Ding, W., Zhang, F., Gao, S., Li, X., Xiao, K. (2016). Expression profile and function characterization of the MYB type transcription factor genes in wheat (*Triticum aestivum* L.) under phosphorus deprivation. *Acta Physiologiae Plantarum*, 38, 5. doi:10. 1007/s11738-015-1997-2.

FAO. (2002). *World Agriculture Towards 2015/2030, Summary Report*. Rome: FAO - Food and Agriculture Organisation of the United Nations, p.97.

FAO. (2008). *The State of Food and Agriculture 2008: Biofuels: Prospects, Risks and Opportunities.* Rome: FAO - Food and Agriculture Organisation of the United Nations, p.138.

FAO. (2012). *World Agriculture Towards 2030/2050: the 2012 revision*. *ESA Working paper No. 12-03*. Rome: FAO - Food and Agriculture Organisation of the United Nations, p.154.

FAO. (2014). *FAOSTAT*. [online] Available at: http://faostat.fao.org/. [Accessed 10/02/ 2016].

FAO. (2015). *World Fertilizer Trends and Outlook to 2018*. Rome: FAO - Food and Agriculture Organisation of the United Nations, p.66.

Ferré-D'Amaré, A.R., Prendergast, G.C., Ziff, E.B., Burley, S.K. (1993). Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. *Nature*, 363(6424), 38–45.

Feuillet, C. and Muehlbauer, G.J. (2009) Genetics of the Triticeae. In: G.J. Muehlbauer, C. Feuillet, ed., *Plant Genetics and Genomics of the Triticeae*. Berlin: Springer, p.3–30.

Filichkin, S., Priest, H.D., Megraw, M., Mockler, T.C. (2015). Alternative splicing in plants: directing traffic at the crossroads of adaptation and environmental stress. *Current Opinion in Plant Biology*, 24, 125–135.

Filichkin, S.A., Priest, H.D., Givan, S.A., Shen, R., Bryant, D.W. Fox, S.E., Wong, W.K., Mockler, T. C. (2010). Genome-wide mapping of alternative splicing in *Arabidopsis thaliana*. *Genome Research*, 20(1), 45–58.

Flint-Garcia, S.A., Thornsberry, J.M., Buckler, E.S. (2003). Structure of linkage disequilibrium in plants. *Annual Reviews of Plant Biology*, 54, 357–374.

Flügge, U.I., Freisl, M., Heldt, H.W. (1980). Balance between metabolite accumulation and transport in relation to photosynthesis by spinach chloroplasts. *Plant Physiology*, 65(4), 574–577.

Föhse, D., Claassen, N., Jungk, A. (1988). Phosphorus efficiency of plants. I. External and internal P requirement and P uptake efficiency of different plant species. *Plant and Soil*, 110(1), 101–109.

Föhse, D., Jungk, A. (1983). Influence of phosphate and nitrate supply on root hair formation of rape, spinach and tomato plants. *Plant and Soil*, 74(3), 359–368.

Folling, L., Olesen, A. (2001). Transformation of wheat (*Triticum aestivum* L.) microspore-derived callus and microspores by particle bombardment. *Plant Cell Report*, 20(7), 629–636.

Fowler, S., Thomashow, M.F. (2002). *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell,* 14(8), 1675–1690.

Franco-Zorrilla, J.M., Martin, A.C., Leyva, A. and Paz-Ares, J. (2005). Interaction between phosphate-starvation, sugar, and cytokinin signalling in *Arabidopsis* and the roles of cytokinin receptors CRE1/AHK4 and AHK3. *Plant Physiology*, 138(2), 847–857.

Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., García, J.A., Paz-Ares, J. (2007). Target mimicry provides a new mechanism for regulation of microRNA activity. *Nature Genetics*, 39(8), 1033–1037.

Fricke, W. (2002). Biophysical limitation of cell elongation in cereal leaves. *Annals of Botany*, 90(2), 157–167.

Fujii, H., Chiou, T.J., Lin, S.I., Aung, K., Zhu, J.K. (2005). A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Current Biology*, 15(22), 2038–2043.

Gahoonia, T.S, Nielsen, N.E. (1997). Variation in root hairs of barley cultivars doubled soil phosphorus uptake. *Plant Soil*, 98(3), 177–182.

Gahoonia, T.S. Nielsen, N.E. (2004). Root traits as tools for creating phosphorus efficient varieties. *Plant and Soil*, 260(1–2), 47–57.

Gahoonia, T.S., Nielsen, N.E. (1996). Variation in acquisition of soil phosphorus among wheat and barley genotypes. *Plant and Soil*, 178(2), 223–230.

Gahoonia, T.S., Nielsen, N.E. (1998). Direct evidence on participation of root hairs in Phosphorus (32^P) uptake from soil. *Plant and Soil*, 198(2), 147–152.

Gahoonia, T.S., Nielsen, N.E. (1998). Direct evidence on participation of root hairs in phosphorus (32P) uptake from soil. *Plant and Soil*, 198(2), 147–152.

Gahoonia, T.S., Nielsen, N.E. (2003). Phosphorus (P) uptake and growth of root hairless barley mutant (bald root barley, brb) and wild-type in low- and high-P soils. *Plant Cell and Environment*, 26(10), 1759–1766.

Gahoonia, T.S., Nielsen, N.E. (2004). Root traits as tools for creating phosphorus efficient crop varieties. *Plant Soil*, 260(1), 47–57.

Gahoonia, T.S., Nielsen, N.E., (1997). Variation in root hairs of barley cultivars doubled phosphorus uptake from soil. *Euphytica*, 98(3), 177–182.

Gahoonia, T.S., Nielsen, N.E., Lyshede, O.B. (1999). Phosphorus (P) acquisition of cereal cultivars in the field at three levels of P fertilisation. *Plant Soil*, 211(2), 269–281.

Geer, L.Y., Marchler-Bauer, A., Geer, R.C., Han, L., He, J., He, S., Liu, C., Shi, W., Bryant, S.H. (2010). The NCBI BioSystems database. *Nucleic Acids Research*, 38, (D1), D492–D496.

Geldner, N., Richter, S., Vieten, A., Marquardt, S., Torres-Ruiz, R.A., Mayer, U., Jürgens, G. (2004). Partial loss-of-function alleles reveal a role for GNOM in auxin transport-related, post-embryonic development of *Arabidopsis*. *Development*, 131(2), 389–400.

Gelvin, S. B. (2003). *Agrobacterium*-Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool. *Microbiology and Molecular Biology Reviews*, 67(1), 16–37.

Gibson, S.I. (2004). Sugar and phytohormone response pathways: navigating a signalling network. *Journal of Experimental Biotechnology*, 55(395), 253–264.

Gilbert, N. (2009). Environment: the disappearing nutrient. *Nature*, 461(7265), 716–718.

Glass, A.D.M. and Siddiqi, M.Y. (1984). The control of nutrient uptake rates in relation to the inorganic composition of plants. In: P.B. Tinker, A. Lauchli, ed., *Advances in plant nutrition*. New York: Praeger, p.103–147.

Glover, N.M., Daron, M.N.J., Pingault, L., Vandepoele, K., Paux, E., Feuillet, C.C., Choulet, R. (2015). Small-scale gene duplications played a major role in the recent evolution of wheat chromosome 3B. *Genome Biology*, 16, 188. doi.org/10.1186/s13059-015-0754-6.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., Pretty, J., Robinson, S., Thomas, S.M., Toulmin, C. (2010). Food Security: the challenge of feeding 9 billion people. *Science,* 327(5967), 812–818.

Goff, S.A., Ricke, D., Lan, T.H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., Hadley, D., Hutchison, D., Martin, C., Katagiri, F., Lange, B. M., Moughamer, T., Xia, Y., Budworth, P., Zhong, J., Miguel, T., Paszkowski, U., Zhang, S., Colbert, M., Sun, W. L., Chen, L., Cooper, B., Park, S., Wood, T. C., Mao, L., Quail, P., Wing, R., Dean, R., Yu, Y., Zharkikh, A., Shen, R., Sahasrabudhe, S., Thomas, A., Cannings, R., Gutin, A., Pruss, D., Reid, J., Tavtigian, S., Mitchell, J., Eldredge, G., Scholl, T., Miller, R. M., Bhatnagar, S., Adey, N., Rubano, T., Tusneem,

N., Robinson, R., Feldhaus, J., Macalma, T., Oliphant, A., Briggs, S. (2002). A draft sequence of the rice genome (*Oryza sativa* L. ssp. Japonica). *Science,* 296(5565), 92–100.

Gomez-Roldan, V., Fermas, S., Brewer, P.B., Puech-Page`s, V., Dun, E.A., Pillot, J.P., Letisse, F., Matusova, R., Danoun, S., Portais, J.C., Bouwmeester, H., Bécard, G., Beveridge, C.A., Rameau, C., Rochange, S.F. (2008). Strigolactone inhibition of shoot branching. *Nature*, 455(7210), 189–194.

González, E., Solano, R., Rubio, V., Leyva, A., Paz-Ares, J. (2005). Phosphate transporter traffic facilitator 1 is a plant-specific SEC12-related protein that enables the endoplasmic reticulum exit of a high-affinity phosphate transporter in *Arabidopsis*. *Plant Cell*, 17(12), 3500–3512.

González, F.G., Miralles, D.J., Slafer, G.A. (2011). Wheat floret survival as related to pre-anthesis spike growth. *Journal of Experimental Botany*, 62(14), 4889–4901.

Graham, M.W., Mudge, S.R., Sternes, P.R., Birch, R.G. (2011). "Understanding and avoiding transgene silencing". In: C.N. Stewart, A. Touraev, V. Citopvsky, T. Tzfira, ed., *Plant Transformation Technologies*. West Sussex: Blackwell Publishing, p.169–196.

Gregory, A.L., Hurley, B.A., Tran, H.T., Valentine, A.J., She, Y.M., Knowles, V.L., Plaxton, W.C. (2009). *In vivo* regulatory phosphorylation of the phosphoenolpyruvate carboxylase AtPPC1 in phosphate-starved *Arabidopsis thaliana*. *Biochemical Journal*, 420(1), 57–65.

Griffiths, J., Murase, K., Rieu, I., Zentella, R., Zhang, Z.L., Powers, S.J., Gong, F., Phillips, A.L., Hedden, P., Sun, T.P., Thomas, S.G. (2006). Genetic characterization and functional analysis of the GID1 gibberellin receptors in *Arabidopsis*. *Plant Cell*, 18(12), 3399–414.

Gunes, A., Inal, A., Aplaslan, M., Cakmak, I. (2006). Genotypic variation in phosphorus efficiency between wheat cultivars grown under greenhouse and field conditions. *Soil Science and Plant Nutrition*, 52(4), 470–478.

Guo, B., Jin, Y.H., Wussler, C., Blancaflor, E.B., Motes, C.M., Versaw, W.K. (2008). Functional analysis of the *Arabidopsis* PHT4 family of intracellular phosphate transporter. *New Phytologist*, 177(4), 889–898.

Guo, M., Ruan, W., Li, C., Huang, F., Zeng, M., Liu, Y., Yu, Y., Din, X., Wu, Y., Wu, Z., Mao, C., Yi, K., Wu, P., Mo, X. (2015). Integrative Comparison of the role of the phosphate response 1 subfamily in phosphate signalling and homeostasis in rice. *Plant Physiology*, 168 (4), 1762–1776.

Gupta, A., Singh, M., Jones, A.M., Laxmi, A. (2012). Hypocotyl directional growth in Arabidopsis: a complex trait. *Plant Physiology*, 159(4), 1463–1476.

Hamburger, D., Rezzonico, E., Petetot, J.M., Somerville, C., Poirier, Y. (2002). Identification and characterization of the *Arabidopsis* PHO1 gene involved in phosphate loading to the xylem. *Plant Cell*, 14(4), 889–902.

Hammond, J.P., Bennett, M.J., Bowen, H.C., Broadley, M.R., Eastwood, D.C., May, S.T., Rahn, C., Swarup, R., Woolaway, K.E., and White, P.J. (2004). Changes in gene expression in *Arabidopsis* shoots during phosphate starvation and the potential for developing smart plants. *Plant Physiology,* 132(2), 578–596.

Hammond, J.P., White, P.J. (2008). Sucrose transport in the phloem: integrating root responses to phosphorus starvation. *Journal of Experimental Botany*, 59(1), 93–109.

Hancock, J.F. (2004). *Plant Evolution and the Origin of Crop Species*. 2nd ed. Cambridge: CABI Publishing.

Harris, R., Wright, M., Byrne, M. (1988). Callus formation and plantlet regeneration from protoplast derived from suspension cultures of wheat (*Triticum aestivum)*. *Plant Cell Reports*, 7(5), 337–340.

Hartwell, J., Gill, A., Nimmo, G.A., Wilkins, M.B., Jenkins, G.I., Nimmo, H.G. (1999). Phosphoenolpyruvate carboxylase kinase is a novel protein kinase regulated at the level of expression. *The Plant Journal*, 20(3), 333–342.

Harvey, A., Moisan, L., Suzanne, L., Lonsdale, D. (1999). Wheat regenerated from scutellum callus as a source of material for transformation. *Plant Cell, Tissue and Organ Culture*, 57(2), 153–156.

Harwood, W.A. (2012). Advances and remaining challenges in the transformation of barley and wheat. *Journal of Experimental Botany*, 63(5), 1791–1798.

Heffer, P., Prud'homme, M. (2008). *Medium-Term Outlook for Global Fertilizer Demand, Supply and Trade 2008 – 2012. 76th IFA Annual Conference Vienna (Austria), 19-21 May 2008.* [online] France, Paris: IFA, p.11. Available at: http://www.fertilizer.org/Search?SearchTerms=78th%20IFA.

Heffer, P., Prud'homme, M. (2010). *Fertilizer outlook 2010–2014. 78th IFA Annual Conference, 31 May–2 June 2010.* [online] France: Paris IFA, p.11. Available at: http://www.fertilizer.org/Search?SearchTerms=78th%20IFA.

Heid, C. A., Stevens, J., Livak, K. J., Williams, P. M. (1996). Real-time quantitative PCR. Genome Research, 6(10), 986–994.

Heim, M.A., Jakoby, M., Werber, M., Martin, C., Weisshaar, B., Bailey, P.C. (2003). The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. *Molecular Biology and Evolution*, 20(5), 735–747.

Helyar, K.R. (1998). Efficiency of nutrient utilisation and sustaining soil fertility with particular reference to phosphorus. *Field Crop Research*, 56(1–2), 187–95.

Hermans, C., Hammond, J. P., Verbruggen, N. (2006). How do plants respond to nutrient storage by biomass allocation? *Trends Plant Science*, 11(12), 610–617.

Hocking, P. (1994). Dry matter production, mineral nutrient concentrations, and nutrient distribution and redistribution in irrigated spring wheat. *Journal of Plant Nutrition*, 17(8), 1289–1308.

Holford, I.C.R. (1997). Soil phosphorus: its measurement, and its uptake by plants. *Australian Journal of Soil Research*, 35(2), 227–239.

Hörtensteiner, S., Kräutler, B. (2011). Chlorophyll breakdown in higher plants. *Biochimica et Biophysica Acta (BBA)-Bioenergetic*, 1807(8), 977–988.

Hsieh, L.C., Lin, S.I., Shih, A.C.C., Chen, J.W., Lin, W.Y., Tseng, C.Y., Li, W.H., Chiou, T.J. (2009). Uncovering small RNA-mediated responses to phosphate deficiency in *Arabidopsis* by deep sequencing. *Plant Physiology*, 151(4), 2120–2132.

Huang, T.K., Han, C.L., Lin, S.I., Chen, Y.J., Tsai, Y.C., Chen, Y.R., Chen, W.Y., Lin, W.Y., Chen, P.M., Liu, T.Y., Chen, Y.S., Sun, C.M., Chiou, T.J. (2013). Identification of downstream components of ubiquitin-conjugating enzyme PHOSPHATE2 by quantitative membrane proteomics in *Arabidopsis* roots. *The Plant Cell*, 25(10), 4044–4060.

Huettel, B., Kanno, T., Daxinger, L., Aufsatz, W., Matzke, A.J.M., Matzke, M. (2006). Endogenous targets of RNA-directed DNA methylation and PolIV in *Arabidopsis*. *The EMBO Journal*, 25(12), 2828–2836.

Hyun, Y., Lee, I. (2006). KIDARI, encoding a non-DNA Binding bHLH protein, represses light signal transduction in *Arabidopsis thaliana*. *Plant Molecular Biology*, 61(1–2), 283–296.

Jaillon, K.O., Bouhouche, J.F. Gout, J.M. Aury, B. Noel, B. Saudemont, M. Nowacki, V. Serrano, B.M. Porcel, B. Ségurens, Le Mouël A, Lepère G, Schächter V, Bétermier M, Cohen J, Wincker P, Sperling L, Duret L, Meyer E. (2008). Translational control of intron splicing in eukaryotes. *Nature*, 451(7176), 359–362.

Jain, A., Poling, M.D., Karthikeyan, A.S., Blakeslee, J.J., Peer, W.A., Titapiwatanakun, B., Murphy, A.S., Raghothama, KG. (2007). Differential effects of sucrose and auxin on localised phosphate deficiency-induced modulation of different traits of root system architecture in *Arabidopsis*. *Plant Physiology*, 144(1), 232–247.

Jain, A., Nagarajan, V.K., Raghothama, K.G. (2012). Transcriptional regulation of phosphate acquisition by higher plants. *Cell and Molecular Life Sciences*, 69(19), 3207–3224.

Jasinski, S.M., (2010). *Phosphate Rock, Mineral Commodity Summaries*. Reston, Virginia USA: U.S. Department of the Interior, US Geological Survey-USGS. Available at: http:// minerals.usgs.gov/minerals/pubs/commodity/phosphate_rock.

Jia, H.F., Ren, H.Y., Gu, M., Zhao, J.N., Sun, S.B., Zhang, X., Chen, J.Y., Wu, P., Xu, G.H. (2011). The phosphate transporter gene OsPht1; 8 is involved in phosphate homeostasis in rice. *Plant Physiology*, 156(3), 1164–1175.

Jin, H., Cominelli, E., Bailey, P., Parr, A., Mehrtens, F., Jones, J., Tonelli, C., Weisshaar, B., Martin, C. (2000). Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in *Arabidopsis*. *EMBO Journal*, 19(22), 6150–6161.

Jin, J.P., Zhang, H., Kong, L., Gao, G., Luo, J.C. (2014). PlantTFDB 3.0: a portal for the functional and evolutionary study of plant transcription factors. *Nucleic Acids Research*, 42(D1), D1182–D1187.

Jin, Y., Bian, T. (2004). Isolation and partial characterization of a novel pollen-specific cDNA with multiple polyadenylation sites from wheat. *Acta Biochimica Biophysica Sinica* (Shanghai), 36(7), 467–476.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., Madden, T.L. (2008). "NCBI BLAST: a better web interface" *Nucleic Acids Research*, 36(W1), W5-W9.

Jones, H.D., Doherty, A., Wu, H. (2005). Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. *Plant Methods*, 1(1), 5–14.

Joseph M. A. (2011). *Major Cereal Grains Production and Use around the World*. ACS Symposium Series, American Chemical Society: Washington, DC, p.1–8.

Jung C., Seo J. S., Han S. W., Koo Y. J., Kim C. H., Song S. I., Nahm, B.H., Choi, Y.D., Cheong, J.J. (2008). Overexpression of AtMYB44 enhances stomatal closure to confer abiotic stress tolerance in transgenic *Arabidopsis*. *Plant Physiology*, 146(2), 623–635.

Jungk, A. (1996). Dynamics of nutrient movement at the soil-root interface in Plant roots. In: Y. Waisel, A. Eshel, U. Kafkafi, ed., *The hidden half*. New York, USA: Marcel Dekker, Inc., p.259–272.

Jungk, A. (2001). Root hairs and acquisition of plant nutrients from soil. *Journal of Plant Nutrition and Soil Science*, 164(2), 121–129.

Kaeppler, S.M., Kaeppler, H.F., Rhee, Y. (2000). Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology*, 43(2), 179–188.

Kang, Y.J., Lee, T., Lee, J., Shim, S., Jeong, H., Satyawan, D., Kim, M.Y., Lee, S. (2016). Translational genomics for plant breeding with the genome sequence explosion. *Plant Biotechnology Journal*, 14(4), 1057–1069.

Kant, S., Peng, M., Rothstein, S.J. (2011). Genetic regulation by NLA and microRNA827 for maintaining nitrate-dependent phosphate homeostasis in *Arabidopsis*. *PLoS Genetics,* 7(3), e1002021. doi: 10.1371/journal.pgen.1002021.

Karas, B., Amyot, L., Johansen, C., Sato, S., Tabata, S., Kawaguchi, M., Szczyglowski, K. (2009). Conservation of lotus and *Arabidopsis* basic helix-loop-helix proteins reveals new players in root hair development. *Plant Physiology*, 151(3), 1175–1185.

Karthikeyan, A.S., Varadarajan, D.K., Jain, A., Held, M.A., Carpita, N.C., and Raghothama, K.G. (2007). Phosphate starvation responses are mediated by sugar signalling in *Arabidopsis*. *Planta*, 225(4), 907–918.

Karthikeyan, A.S., Varadarajan, D.K., Mukatira, U.T., D'Urzo, M.P., Damsz, B., Raghothama, K.G. (2002). Regulated expression of *Arabidopsis* phosphate transporters. *Plant Physiol*ogy, 130(1), 221–233.

Katiyar, A., Smita, S., Lenka, S.K., Rajwanshi, R., Chinnusamy, V., Bansal, K.C. (2012). Genome-wide classification and expression analysis of MYB transcription factor families in rice and *Arabidopsis*. *BMC Genomics*, 13, 544. doi.org/10.1186/1471-2164-13-544.

Kato, K., Kidou, S., Miura, H., Sawada, S. (2002). Molecular cloning of the wheat CK2a gene and detection of its linkage with Vrn-A1 on chromosome 5A. *Theoretical and Applied Genetic*s, 104(6/7), 1071–1077.

Katzen, F. (2007). Gateway recombinational cloning: a biological operating system. *Expert opinion and Drug Discovery*, 2(4), 571–589.

Kauffman, A. and O'Shea, E. K. (1999). Regulation of nuclear localisation: a key to a door. *Annual Reviews of Cell Development Biology*, 15, 291–339.

Kaur, G., Kumar, S., Nayyar, H., Upadhyaya, H.D. (2008). Cold stress injury during the pod-filling phase in chickpea (*Cicer arietinum* L.): effects on quantitative and qualitative components of seeds. *Journal of Agronomy and Crop Science*, 194(6), 457–464.

Kauwenbergh, S.J.V., (2010). *World phosphate rock reserves and resources.* Muscle Shoals, Alabama, USA: IFDC Technical Bulletin No. 75, p.58.

Kavanova, M., Lattanzi, F.A., Grimoldi, A.A., Schnyder, H. (2006). Phosphorus deficiency decreases cell division and elongation in grass. *Plant Physiology*. 141(2), 766–775.

Keller-Grein, G., B.L. Maass, and J. Hanson (1996). Phosphorus deficiency decreases cell division and elongation in grass leaves. *Plant Physiology*, 141(2), 766–775.

Kellogg, E.A. (2001). Evolutionary history of the grasses. *Plant Physiology*, 125(3), 1198–1205.

Kempken, F., and Jung, C. (2010). *Genetic Modification of Plants - Agriculture, Horticulture and Forestry*. Berlin, Heidelberg: Springer, p.113.

Kim, S., Schlicke,H., Van Ree, K., Karvonen,K., Subramaniam,A., Richter,A., Grimm, B., Braam, J. (2013). *Arabidopsis* Chlorophyll Biosynthesis: An essential balance between the methylerythritol phosphate and tetrapyrrole pathways. *The Plant Cell*, 25(12), 4984–4993.

Klug, W. S., Cummings, M. R., Spencer, C. A., Palladino, M. A. (2014). *Concepts of Genetics*. 10th ed. San Francisco: Person Education Inc., p.896.

Kochian, L.V. (2012). Rooting for more phosphorus. *Nature*, 488 (7412), 466–467.

Kohlen, W., Charnikhova, T., Liu, Q., Bours, R., Domagalska, M.A., Beguerie, S., Verstappen, F., Leyser, O., Bouwmeester, H., Ruyter-Spira, C. (2011). Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in nonarbuscular mycorrhizal host *Arabidopsis*. *Plant Physiology*, 155(2), 974–987.

Kohli, A., Miro, B., Twyman, R.M. (2010). "Transgene integration, expression and stability in plants: strategies for improvements". In: C. Kole, C.H. Michler, A.G. Abbott, T.C. Hall, ed., *Transgenic Crop Plants: Principles and Development*. Berlin: Springer-Verlag, p.201–237.

Konvalina, P., Capouchova, I., Stehno, Z., Moudry, J. (2010). Agronomic characteristics of the spring forms of the wheat landraces (einkorn, emmer, spelt, intermediate bread wheat) grown in organic farming. *Journal of Agrobiology*, 27(1), 9–17.

Kotewicz, M.L., D'Alessio, J.M., Driftmier, K.M., Blodgett, K.P., Gerard, G.F. Cloning and overexpression of Moloney murine leukemia virus reverse transcriptase in *Escherichia coli*. *Gene*, 35(3), 249–258.

Kouas, S., Debez, A., Slatni, T., Labidi, N., Drevon, J.J., Abdelly, C. (2009). Root proliferation, proton efflux, and acid phosphatase activity in common bean (*Phaseolus vulgaris*) under phosphorus shortage. *Journal of Plant Biology,* 52(5), 395–402.

Kraus, M., Fusseder, A., Beck, E. (1987). In situ-determination of the phosphate-gradient around a root by radioautography of frozen soil sections. *Plant Soil*, 97(3), 407–418.

Kriventseva, E.V., Koch, I., Apweiler, R., Vingron, M., Bork, P., Gelfand, M.S., Sunyaev, S. (2003). Increase of functional diversity by alternative splicing. *Trends in Genetics*, 19(3), 124–128.

Krutovsky, K.V., Troggio, M., Brown, G.R., Jermstad, K.D., Neale, D.B. (2004). Comparative mapping in the Pinaceae. *Genetics*, 168(1), 447–461.

Kunitz, M. (1950). Crystalline Desoxyribonuclease II. Digestion of thymus nucleic acid (Deoxyribonucleic acid) the kinetics of the reaction. *Journal of General Physiology*, 33(4), 363–377.

Kurata, N., Moore, G., Nagamura, Y., Foote, T., Yano, M., Minobe, Y., Gale, M. (1994). "Conservation of genome structure between rice and wheat". *Nature Biotechnology*, 12 (3), 276–278.

Lai, J., Ma, J., Swigonova, Z., Ramakrishna, W., Linton, E., Llaca, V., Tanyolac, B., Park, Y.J., Jeong, O.Y., Bennetzen, J.L., Messing, J. (2006). Gene loss and movement in the maize genome. *Genome Research,* 14(10A), 1924–1931.

Lambers, H., Shan, M.W., Cramer, M.D., Pearse, S.J. and Veneklaas, E.J. (2006). Root structure and functioning for efficient acquisition of phosphorus: matching morphological and physiological traits. *Annual of Botany*, 98(4), 693–713.

Landy, A. (1989). Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. *Annual Review of Biochemistry*, 58, 913–949.

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, L., Weijers, D., Calvo, V., Parizot. B., Herrera-Rodriguez, M.B., Offringa, R., Graham,N., Doumas, P., Friml, J., Bogusz,D., Beeckman, T., Bennett, M. (2007). Cytokinins act directly on lateral root founder cells to inhibit root initiation. *The Plant Cell*, 19(12), 3889–3900.

Lareau, L.F., Green, R.E., Bhatnagar, R.S., Brenner, S.E. (2004). The evolving roles of alternative splicing. *Current Opinion in Structural Biology*, 14(3), 273–282.

Latchman, D.S. (1997). "Transcription factors: an overview". *International Journal of Biochemistry and Cell Biology*, 29(12), 1305–1312.

Lauer, M.J., Blevins, D.G., Sierputowska-Gracz, H. (1989a). ^{31}P-nuclear magnetic resonance determination of phosphate compartmentation in leaves of reproductive soybeans (*Glycine max* L.) as affected by phosphate nutrition. *Plant Physiology*, 89(4), 1331–1336.

Lauer, M.J., Pallardy, G.S., Blevins, D.G., Randall, D.D. (1989b). Whole leaf carbon exchange characteristic of phosphate-deficient soybeans (*Glycine max* L.). *Plant Physiology*, 91(3), 848–854.

Laurie, D.A., Griffiths, S., Dunford, R.P., Christodoulou, V., Taylor, S.A., Cockram, J., Beales, J., Turner, A. (2004). Comparative genetic approaches to the identification of flowering time genes in temperate cereals. *Field Crops Research*, 90(1), 87–99.

Lee, T.I., Young, R.A. (2000). Transcription of eukaryotic protein-coding genes. *Annual Review of Genetics*, 34(1), 77–137.

Lemoine, R., La Camera, S., Atanassova, R., Dédaldéchamp, F., Allario, T., Pourtau, N., Bonnemain, J.L., Laloi, M., Coutos-Thévenot, P., Maurousset, L., Faucher, M., Girousse, C., Lemonnier, P., Parrilla, J., Durand, M. (2013). Source to sink transport and regulation by environmental factors. *Frontiers in Plant Science*, 4(272). doi: 10.3389/fpls.2013.00272.

Lev-Yadun, S., Gopher, A., Abbo, S. (2000). The cradle of agriculture. *Science*, 288(5471), 1602–1603.

Li, J., Li, X., Guo, L., Lu F, Feng, X., He, K., Wei, L., Chen, Z., Qu, L.J., Gu, H. (2006). A subgroup of MYB transcription factor genes undergoes highly conserved alternative splicing in *Arabidopsis* and rice. *Journal of Experimental Biotechnology*, 57(6), 1263–1273.

Li, S., Yamada, M., Han, X., Ohler, U., and Benfey, P.N. (2016). High-resolution expression map of the *Arabidopsis* root reveals alternative splicing and lincRNA regulation. *Developmental Cell*, 39(4), 508–522.

Li, X., Björkman, O., Shih, C., Grossman, A.R., Rosenquist, M., Jansson, S., Niyogi, K.K. (2000). A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature*, 403(6768), 391–395.

Li, X., Duan, X., Jiang, H., Sun, Y., Tang, Y., Yuan, Z., Guo, J., Liang, W., Chen, L., Yin, J., M, H., Wang, J., Zhang, D. (2006). Genome-wide analysis of basic/helix-loop-helix transcription factor family in rice and *Arabidopsis*. *Plant Physiology*, 141(4), 1167–1184.

Li, X., Wang, Y., Wu, B., Kong, Y., Li, W., Chang, W., Zhang, C. (2017). GmPHR1, a novel homolog of the AtPHR1 transcription factor, plays a role in plant tolerance to phosphate starvation. *Journal of Integrative Agriculture*, 13(12), 2584–2593.

Li, X.H., Wu, B., Kong, Y.B., Zhang, C.Y. (2014). GmPTF1, a novel transcription factor gene, is involved in conferring soybean tolerance to phosphate starvation. *Genetics and Molecular Research*, 13(1), 926–937.

Li, Y., Luo, A., Hassan, M.J., Wei, X. (2006). Effect of phosphorus deficiency on leaf photosynthesis and carbohydrates partitioning in two rice genotypes with contrasting low P susceptibility. *Rice Science*, 13(4), 283–290.

Li, Y.D., Wang, Y.J, Tong, Y.P., Gao, J.G., Zhang, J.S., Chen, S.Y. (2005). QTL Mapping of phosphorus deficiency tolerance in soybean (*Glycine max* L. Merr.). *Euphytica*, 142(1–2), 137–142.

Li, Y.J., Liu, J.Z., Li, B., Li, J.Y., Li, Z.S. (1999). Chromosomal control of the tolerance to soil phosphorus deficiency in genome of common wheat. *Chinese Journal of Genetics*, 26(2), 529–538.

Li, Z., Gao, Q., Liu, Y., He, C., Zhang, X., Zhang, J. (2011). Overexpression of transcription factor ZmPTF1 improves low phosphate tolerance of maize by regulating carbon metabolism and root growth. *Planta*, 233(6), 1129–1143.

Liang Y. K., Dubos C., Dodd I. C., Holroyd G. H., Hetherington A. M., Campbell M. M. (2005). AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in *Arabidopsis thaliana*. *Current Biology*, 15(13), 1201–1206.

Liao, H., Yan, X.L., Rubio, G., Beebe, S.E., Blair, M. W., Lynch, J.P. (2004). Genetic mapping of basal root gravitropism and phosphorus acquisition efficiency in common bean. *Functional Plant Biology*, 31(10), 959–970.

Liao, Y., Zou, H.F., Wang, H.W., Zhang, W.K., Ma, B., Zhang, J.S. (2008). Soybean GmMYB76, GmMYB92, and GmMYB177 genes confer stress tolerance in transgenic *Arabidopsis* plants. *Cell Research*, 18(10), 1047–1060.

Lin, W. Y., Lin, S. I., Chiou, T. J. (2009). Molecular regulators of phosphate homeostasis in plants. *Journal of Experimental Botany*, 60(5), 1427–1438.

Lin, W.Y., Huang, T.K., Chiou, T.J. (2013). Nitrogen limitation adaptation, a target of microRNA827, mediates degradation of plasma membrane localised phosphate transporters to maintain phosphate homeostasis in *Arabidopsis*. *Plant Cell*, 25(10), 4061–4074.

Lippold, F., Sanchez, D. H., Musialak, M., Schlereth, A., Scheible, W. R., Hincha, D. K., Udvardi, M.K. (2009). AtMyb41 regulates transcriptional and metabolic signalling responses to osmotic stress in *Arabidopsis*. *Plant Physiology*, 149(4), 1761–1772.

Liu, C., Muchhal, U.S., Uthappa, M., Kononowicz, A.K., Raghothama, K.G. (1998). Tomato phosphate transporter genes are differently regulated in plant tissues by phosphorus. *Plant Physiology*, 116(1), 91–99.

Liu, F., Wang, Z., Ren, H., Shen, C., Li, Y., Ling, H.Q., Wu, C., Lian, X., Wu, P. (2010b). OsSPX1 suppresses the function of OsPHR2 in the regulation of expression of OsPT2 and phosphate homeostasis in shoots of rice. *Plant Journal*, 62(3), 508–517.

Liu, J., Samac, D.A., Bucciarelli, B., Allan, D.L., and Vance, C.P. (2005). Signalling of phosphorus deficiency-induced gene expression in white lupin requires sugar and phloem transport. *The Plant Journal*, 41(2), 257–268.

Liu, J.Q., Allan, D.L., Vance, C.P. (2010a). Systemic signaling and local sensing of phosphate in common bean: cross-talk between photosynthate and microRNA399. *Molecular Plant*, 3(2), 428–

437.

Liu, L., White, M.J., MacRae, T.H. (1999). Transcription factors and their genes in higher plants: functional domains, evolution and regulation. *European Journal of Biochemistry*, 262(2), 247–257.

Liu, T.Y., Huang, T.K., Tseng, C.Y., Lai, Y.S., Lin, S.I., Lin, W.Y., Chen, J.W., Chiou, T.J. (2012). PHO2-dependent degradation of PHO1 modulates phosphate homeostasis in *Arabidopsis*. *The Plant Cell*, 24(5), 2168–2183.

Liu, W., Tai, H, Li S, Gao, W., Zhao, M., Xie, C., Li, W.X. (2014). bHLH122 is important for drought and osmotic stress resistance in *Arabidopsis* and in the repression of ABA catabolism. *New Phytologist*, 201(4), 1192–1204.

Liu, X., Zhao, X., Zhang, L., Lu, W., Li, X., Xiao, K. (2013). TaPht1:4, a high-affinity phosphate transporter gene in wheat (*Triticum aestivum*), plays an important role in plant phosphate acquisition under phosphorus deprivation. *Functional Plant Biology*, 40(4), 329–341.

Livak, K.J., Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25(), 402–408.

Livne, S., Lor, V.S., Nir, I., Eliaz, N., Aharoni, A., Olszewski, N. E., Eshed, Y., Weissa, D. (2015). Uncovering DELLA-independent Gibberellin responses by characterizing new tomato procera mutants. *The Plant Cell*, 27(6), 1579–1594.

Lizana X.C., Calderini D.F. (2013). Yield and grain quality of wheat in response to increased temperatures at key periods for grain number and grain weight determination: considerations for the climatic change scenarios of Chile. *Journal of Agricultural Science*, 151(2), 209–221.

Lloyd, J.C., Zakhleniuk, O.V. (2004). Responses of primary and secondary metabolism to sugar accumulation revealed by microarray expression analysis of the *Arabidopsis* mutant, *pho3*. *Journal of Experimental Botany*, 55(400), 1221–1230.

Long, S.P., Farage P.K., Garcia, R.L. (1996). Measurement of leaf and canopy photosynthetic CO_2 exchange in the field. *Journal of Experimental Botany*, 47(304), 1629–1642.

Long, T.A., Tsukagoshi, H., Busch, W., Lahner, B., Salt, D.E., Benfey, P.N. (2010). The bHLH transcription factor POPEYE regulates response to iron deficiency in *Arabidopsis* roots. *Plant Cell*, 22(7), 2219–2236.

Lynch, J., Läuchli, A., Epstein, E. (1991). Vegetative growth of common bean in response to phosphorus nutrition. *Crop Science*, 31(2), 380–387.

Lynch, J.P., Brown, K.M. (2001). Topsoil foraging–an architectural adaptation of plants to low phosphorus. *Plant and Soil*, 237(2), 225–237.

Lynch, J.P. (2007). Roots of the second green revolution. *Australian Journal of Botany*, 55(5), 493–512.

López-Arredondo, D.L., Leyva-Gonza´lez, M.A., Gonza´lez-Morales, S.I., Lo´pez-Bucio, J., Herrera-Estrella, L. (2014). Phosphate nutrition: improving low-phosphate tolerance in crops. *Annual Review of Plant Biology*, 65, 95–123.

Malamy, J.E., Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development*, 124(1), 33–44.

Manske, G.G.B., Ortiz-Monasterio, J.I., Ginklel, M.V., González, R.M. Rajaram, S., Molina, E., Vlek, P.L.G. (2000). Traits associated with improved P-uptake efficiency in CIMMYT's semidwarf spring bread wheat grown on an acid Andisol in Mexico. *Plant and Soil*, 221(2), 189–204.

Manske, G.G.B., Ortiz-Monasterio, J.I., van Ginkel, M., González, R.M., Fischer, R.A., Rajaram, S., Vlek, P.L.G. (2001). Importance of P uptake efficiency versus P utilisation for wheat yield in acid and calcareous soils in Mexico. *European Journal of Agronomy*, 14(4), 261–274.

Marschner, H. (1995). *Mineral nutrition of higher plants*. London: Academic, p.889.

Marschner, H. and Marschner, P. (2012). *Marschner's mineral nutrition of higher plants*. 3rd ed. London, Waltham, MA: Elsevier Academic Press, p.672

Marschner, H., Dell, B. (1994). Nutrient uptake in mycorrhizal symbiosis. *Plant and Soil*, 159(1), 89–102.

Martin, A.C., del Pozo, J.C., Iglesias, J., Rubio, V., Solano, R., de la Pena, A., Leyva, A., and Paz-Ares, J. (2000). Influence of cytokinins on the expression of phosphate starvation-responsive genes in *Arabidopsis*. *Plant Journal*, 24(5), 559–567.

Massari, M.E., Murre, C. (2000). Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Molecular Cell Biology*, 20(2), 429–440.

Masucci, J.D., Schiefelbein, J.W. (1994). The rhd6 Mutation of *Arabidopsis thaliana* Alters Root-Hair Initiation through an Auxin- and Ethylene-Associated Process. *Plant Physiology*, 106(4), 1335-1346.

Matzke, A.J., Matzke, M.A. (1998). Position effects and epigenetic silencing of plant transgenes. *Current Opinion in Plant Biology*, 1(2), 142–148.

Matzke, M., Kanno, T., Huettel, B., Daxinger, L., Matzke, A.J. (2007). Targets of RNA directed DNA methylation. *Current Opinion in Plant Biology*, 10(5), 512–519.

Matzke, M.A., Mette, M.F., Matzke, A.J. (2000). Transgene silencing by the host genome defence: implications for the evolution of epigenetic control mechanisms in plants and vertebrates. *Plant Molecular Biology*, 43(2/3), 401–415.

Mayer, K. F. X., *et al*., (2014). A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. *Science*, 345 (6194), 1251788.

Mazzucotelli, E., Mastrangelo, A.M., Crosatti, C., Guerra, D., Stanca, A.M., Cattivelli, L. (2008). Abiotic stress response in plants: When post-transcriptional and post-translational regulations control transcription. *Plant Science*, 174(4), 420–431.

McLaughlin, K.A., Hatzenbuehler, M.L., Mennin, D.S., Nolen-Hoeksema, S. (2011). Emotion dysregulation and adolescent psychopathology: A prospective study. *Behaviour Research and Therapy*, 49(9), 544–554.

McLaughlin, M.J., Fillery, I.R. and Till, A.R. (1992). Operation of the phosphorus, sulphur and nitrogen cycles. In: R. Gifford and M. Barson, ed., *Australia's renewable sources: sustainability and global change*. Parkes, A.C.T.: Bureau of Rural resources and CSIRO division of plant industry, p.67–116.

Meissner, R., Michael, A.J. (1997). Isolation and characterization of a diverse family of *Arabidopsis* two- and three-fingered C2H2 zinc-finger protein genes and cDNAs. *Plant Molecular Biology*, 33(4), 615–624.

Menand, B., Yi, K., Jouannic, S., Hoffmann, L., Ryan, E., Linstead, P., Linstead, P., Schaefer, D.G., Dolan, L. (2007). An ancient mechanism controls the development of cells with a rooting function in land plants. *Science*, 316(5830), 1477–1480.

Meng, L., Bregitzer, P., Zhang, S., Lemaux, P.G. (2003). Methylation of the exon/intron region in the Ubi1 promoter complex correlates with transgene silencing in barley. *Plant*

Molecular Biology, 53(3), 327–340.

Meyer, S. R., Purugganan, D. M., (2013). Evolution of crop species: genetics of domestication and diversification. *Nature Reviews Genetics*, 14(12), 840–852.

Meyers, B.C., Galbraith, D.W., Nelson, T., Agrawal, V. (2004). Methods for transcriptional profiling in plants. Be fruitful and replicate. *Plant Physiology*, 135(2), 637–652.

Miao, J., Sun, J., Liu, D., Li, B., Zhang, A., Li, Z, Tong, Y. (2009). Characterization of the promoter of phosphate transporter TaPHT1.2 differentially expressed in wheat varieties. *Journal of Genetics and Genomics,* 36(8), 455–466.

Miller, M.J., Barrett-Wilt, G.A., Hua, Z., Vierstra, R.D. (2010). Proteomic analyses identify a diverse array of nuclear processes affected by small ubiquitin-like modifier conjugation in *Arabidopsis*. *Proceedings of the National Academy of Sciences. USA*, 107(38), 16512–16517.

Mimura, T., Sakano, K., Shimmen, T. (1996). Studies on the distribution, re-translocation and homeostasis of inorganic phosphate in barley leaves. *Plant, Cell and Environment*, 19(3), 311–320.

Minami, M., Huh, G.-H., Yang, P. Iwabuchi, M. (1993). Coordinate gene expression of five subclass histones and the putative transcription actors, HBP-1a and HBP-1b, of histone genes in wheat. *Plant Molecular Biology*, 23(2), 429–434.

Ming, F., Zheng, X.W., Mi, G.H., He, P., Zhu, L.H., Zhang, F.S. (2000). Identification of quantitative trait loci affecting tolerance to low phosphorus in rice (*Oryza Sativa* L.). Chinese. *Science Bullitin*, 45, 520. doi.org/10.1007/BF02887097.

Mishra, B., Singh, M., Aggrawal, P., Laxmi, A. (2009). Glucose and auxin signalling interaction in controlling *Arabidopsis thaliana* seedlings root growth and development. *PLoS ONE*, 4: e4502. doi: 10.1371/journal.pone.0004502.

Misson, J., Raghothama, K.G., Jain, A., Jouhet, J., Block, M.A., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland,N., Doumas, P., Nacry,P., Herrerra-Estrella, L., Nussaume, L., and Thibaud, M. (2005). A genome-wide transcriptional analysis using *Arabidopsis* thaliana Affymetrix gene chips determined plant responses to phosphate deprivation. *Proceedings of the National Academy of Sciences. USA,* 102(33), 11934–11939.

Mitsuda, N., Ohme-Takagi, M. (2009). Functional Analysis of Transcription Factors in *Arabidopsis*. *Plant Cell Physiol*ogy, 50(7), 1232–1248.

Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A.S., Raghothama, K.G., Baek, D.W., Yoon, D.K., Jin, J.B., Bressan, R.A.,Yun, D.J., Hasegawa, P.M. (2005). The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. *Proceedings of the National Academy of Sciences. USA*, 102(21), 7760–7765.

Monna, L., Kitazawa, N., Yoshino, R., Suzuki, J., Masuda, H., Maehara, Y., Tanji, M., Sato, M., Nasu, S., Minobe, Y. (2002). Positional cloning of rice semi-dwarfing gene, sd-1: rice “green

revolution gene" encodes a mutant enzyme involved in gibberellin synthesis. *DNA Research*, 9(1), 11–17.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Liu, Y.X. (2003). Roles of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signalling. *Science,* 300(5617), 332–336.

Morel, J., Mourrain, P., Béclin, C., Vaucheret, H. (2000). DNA methylation and chromatinstructure affect transcriptional and post-transcriptional transgene silencing in *Arabidopsis*. *Current Biology*, 10(24), 1591–1594.

Muchhal U.S., Pardo J.M., Raghothama K.G. (1996). Phosphate transporters from the higher plant *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences. USA*, 93(19), 10519–10523.

Mudge, S.R., Rae, A.L., Diatloff, E., Smith, F.W. (2002). Expression analysis suggests novel roles for members of the Pht1 family of phosphate transporters in *Arabidopsis*. *The Plant Journal*, 31(3), 341–353.

Müller, M., Munné-Bosch, S. (2017). Hormone Profiling in Plant Tissues. *Methods in Molecular Biology*, 1497, 249–258.

Müller, R., Morant, M., Jarmer, H., Nilsson, L., and Hamborg Nielsen, T.H. (2007). Genome-wide analysis of the *Arabidopsis* leaf transcriptome reveals interaction of phosphate and sugar metabolism. *Plant Physiol*ogy, 143(1), 156–171.

Müller, R., Nilsson, L., Nielsen, L.K., and Nielsen, T.H. (2005). Interaction between phosphate starvation signalling and hexokinase independent sugar sensing in *Arabidopsis* leaves. *Plant Physiology*, 124(1), 81–90.

Muthamilarasan, M., Khandelwal R., Yadav C. B., Bonthala V. S., Khan Y., Prasad M. (2014). Identification and molecular characterization of MYB Transcription Factor Superfamily in C4 model plant foxtail millet (*Setaria italica* L.). *PLoS ONE*, 9, e109920. doi: 10.1371/journal.pone.0109920.

Nacry, P., Canivenc, G., Müller, B., Azmi, A., Onckelen, H., Rossignol, M., Doumas, P. (2005). A Role for Auxin Redistribution in the Responses of the Root System Architecture to Phosphate Starvation in *Arabidopsis*. *Plant Physiology*, 138 (2005), 2061–2074.

Nagarajan, V.K., Jain, A., Poling, M.D., Lewis, A.J., Raghothama, K.G., Smith, A.P. (2011). *Arabidopsis* Pht1:5 mobilizes phosphate between source and sink organs and influences the interaction between phosphate homeostasis and ethylene signalling. *Plant Physiol*ogy, 156(3), 1149–1163.

Natr, L. (1992). Mineral nutrients - a ubiquitous stress factor for photosynthesis. *Photosynthetica*, 27, 271–294.

Negi, P., Archana, N.R., Penna, S. (2016). Moving through the stressed genome: emerging regulatory roles for transposons in plant stress response. *Frontiers in Plant Science*, 7(1448). doi: 10.3389/fpls.2016.01448.

Nestler, J., Keyes, S.D., Wissuwa, M. (2016). Root hair formation in rice (*Oryza sativa* L.) differs between root types and is altered in artificial growth conditions. *Journal of Experimental Botany***,** 67(12)**,** 3699–3708.

Neumann, G., Römheld, V. (1999). Root excretion of carboxylic acids and protons in phosphorus-deficient plants. *Plant and Soil*, 211(1), 121–130.

Ni, J.J., Wu, P., Senadhira, D., Huang, N. (1998). Mapping QTLs for phosphorus deficiency tolerance in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, 97(8), 1361–1369.

Nilsson, L., Müller, R., Nielsen, T.H. (2007). Increased expression of the MYB-related transcription factor, PHR1, leads to enhanced phosphate uptake in *Arabidopsis thaliana*. *Plant, Cell and Environment*, 30(12), 1499–1512.

Nilsson, L., Müller, R., Nielsen, T.H. (2010). Dissecting the plant transcriptome and the regulatory responses to phosphate deprivation. *Physiology Plant*, 139(2), 129–143.

Nimwegen, V.E. (2003). "Scaling laws in the functional content of genomes". *Trends in Genetics*, 19 (9), 479–484.

Niu, Y., Chai, R., Jin, G., Wang, H., Tang, C., Zhang, Y. (2013). Responses of root architecture development to low phosphorus availability: a review. *Annuals of Botany*, 112(2), 391–408.

Ohto, M.A., Hayashi, S., Sawa, S., Hashimoto-Ohta, A., Nakamura, K. (2006). Involvement of HLS1 in sugar and auxin signaling in Arabidopsis leaves. *Plant Cell Physiology*, 47(12), 1603–1611.

O'Malley, R.C.., Huang, S.S., Song, L., Lewsey, M.G., Bartlett, A., Nery, J.R., Galli, M., Gallavotti, A., Ecker, J.R. (2016). Cistrome and epicistrome features shape the regulatory DNA landscape. *Cell*, 165(5), 1280–1292.

O'Rourke, J.P., Ness, S.A. (2008). Alternative RNA splicing produces multiple forms of c-Myb with unique transcriptional activities. *Molecular and Cellular Biology*, 28(6), 2091–2101.

Obasi, G.O.P., and Dowdeswell, E. (1998). *Climate Change 1995: IPCC Second Assessment: A Report of the Intergovernmental Panel on Climate Change*. [online] Darby, PA: Diane Publishing Co., p.73 Available at: https://www.ipcc.ch/publications_and_data/ publications_and_data_reports.shtml#1 [Accessed 10/12/2015].

Okumura, S., Mitsukawa, N., Shirano, Y. and Shibata, D. (1998). Phosphate transporter gene family of *Arabidopsis thaliana*. *DNA Research*, 5(5), 261–269.

Ortiz, J.P.A., Reggiardo, M.I., Ravizzini, R.A., Altabe, F., Cervigni, G.D.L., Spitteler, M.A., Morata, M.M., Elias, F.E., Vallejos, R.H. (1996). Hygromycin resistance as an efficient selectable marker for wheat stable transformation. *Plant Cell Reports*,15(12), 877–881.

Osborne, L. D., Rengel, Z. (2002). Screening cereals for genotypic variation in efficiency of phosphorus uptake and utilisation. *Australian Journal of Agricultural Research*, 53, 295–303.

Owen, D., Williams, A.P., Griffith, G.W., Withers, P.J.A. (2014) Use of commercial bio-inoculants to increase agricultural production through improved phosphorus acquisition. *Applied Soil Ecology*, 86, 41–54.

Ozturk, L., Eker, S., Torun, B. Cakmak, I. (2005). Variation in phosphorus efficiency among 73 bread and durum wheat genotypes grown in a phosphorus-deficient calcareous soil. *Plant Soil*, 269(1), 69–80.

Palusa, S.G., Ali, G.S., Reddy, A.S.N. (2007). Alternative splicing of pre-mRNAs of *Arabidopsis* serine/arginine-rich proteins: regulation by hormones and stresses. *Plant Journal*, 49(6), 1091–1107.

Parentoni, S.N., Júnior, C.L.S. (2008). Phosphorus acquisition and internal utilisation efficiency in tropical maize genotypes. *Pesquisa Agropecuária Brasileira*, 43(7), 893–901.

Park, B.S., Seo, J.S., Chua, N.H. (2014). Nitrogen limitation adaptation recruits to target the phosphate transporter PT2 for degradation during the regulation of *Arabidopsis* phosphate homeostasis. *Plant Cell*, 26(1), 454–464.

Parker, J.S., Cavell, A.C., Dolan, L., Roberts, K., Grierson, C.S. (2000). Genetic interactions during root hair morphogenesis in *Arabidopsis*. *Plant Cell*, 12(10), 1961–1974.

Pattanaik, S., Kong, Q., Zaitlin, D., Werkman, J. R., Xie, C. H., Patra, B., Yuan, L. (2010). Isolation and functional characterization of a floral tissue-specific R2R3 MYB regulator from tobacco. *Planta*, 231(5), 1061–1076.

Peng, J., Richards, D.E., Hartley, N.M., Murphy, G.P., Devos, K.M., Flintham, J.E., Beales, J., Fish, L.J., Worland, A.J., Pelica, F., Sudhakar, D., Christou, P., Snape, J.W., Gale, M.D., Harberd, N.P. (1999). "Green revolution" genes encode mutant gibberellin response modulators. *Nature*, 400(6741), 256–261.

Peng, J.H., Sun, D., Nevo, E. (2011). Domestication evolution, genetics and genomics in wheat. *Molecular Breeding*, 28(3), 281–301.

Pérez-Torres, C. A., López-Bucio, J., Cruz-Ramırez, A., Ibarra-Laclette, E., Dharmasiri, S., Estelle, M., Herrera-Estrella, L. (2008). Phosphate availability alters lateral root development in *Arabidopsis* by modulating auxin sensitivity via a mechanism involving the TIR1 auxin receptor. *Plant Cell*, 20(12), 3258–3272.

Pestsova, E., Ganal, M.W., Röder, M.S. (2000). Isolation and mapping of microsatellite markers specific for the D genome of bread wheat. *Genome*. 43(4), 689-697.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29(9), e45.

Pierzynski, G. M., Sims, J. T., Vance G. F. (1994) *Soils and Environmental Quality*. Boca Raton, F.L.: Lewis Publishing Inc., p.1–17.

Pikaart, M.J., Recillas-Targa, F., Felsenfeld, G. (1998). Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators. *Genes and Development*, 12(18), 2852–2862.

Pil, J. S., Park, M., Park, C. (2013). Alternative splicing of transcription factors in plant responses to low-temperature stress: mechanisms and functions. *Planta*, 237(6), 1415–1424.

Plaxton W.C., Tran H.T. (2011) Metabolic adaptations of phosphate-starved plants. *Plant Physiology*, 156(3), 1006–1015.

Plaxton, C.W. and Lambers, H. (2015). Phosphorus: Back to the roots. In: C.W. Plaxton, H. Lambers, ed., *Phosphorus metabolism in plants*. Chichester, United Kingdom: Wiley Blackwell, p.480.

Plaxton, W.C. (2004). *Plant response to stress: Biochemical adaptations to P deficiency*. In: R. Goodman, ed., *Encyclopedia of Plant and Crop Science*. New York: Marcel Dekker, Inc., p.976–980.

Plaxton, W.C. and Carswell, M.C. (1999). Metabolic aspects of the phosphate starvation response

in plants. In: H.R. Lerner, ed., *Plant Responses to Environmental Stresses: From Phytohormones to Genome Reorganization*. New York: Marcel Dekker, Inc., p.349–372.

Poirier, Y. and Bucher, M. (2002). Phosphate transport and homeostasis in *Arabidopsis*. In: C.R. Somerville, E.M. Meyerowitz, ed., *The Arabidopsis Book*. Rockville, MD: American Society of Plant Biologists, p.1–35.

Poirier, Y., Thoma, S., Somerville, C., Schiefelbein, J. (1991). A mutant of *Arabidopsis* deficient in xylem loading of phosphate. P*lant Physiology,* 97(3), 1087–1093.

Pop, M. (2009). "Genome assembly reborn: recent computational challenges". *Briefings in Bioinformatics*, 10(4), 354–366.

Prabu, G., Prasad, D.T. (2012). Functional characterization of sugarcane MYB transcription factor gene promoter (PScMYBAS1) in response to abiotic stresses and hormones. *Plant Cell Reports*, 31(4), 661–669.

Prelich, G. (2012) Gene overexpression: uses, mechanisms, and interpretation. *Genetics*, 190(3), 841–854.

Preuss, C.P., Huang, C.Y., Gilliham, M., Tyerman, S.D. (2010). Channel-like characteristics of the low-affinity barley phosphate transporter PHT1:6 when expressed in *Xenopus oocytes*. *Plant Physiology*, 152(3), 1431–1441.

Preuss, C.P., Huang, C.Y., Tyerman, S.D. (2011). Proton-coupled high-affinity phosphate transport revealed from heterologous characterization in Xenopus of barley-root plasma membrane transporter, HvPHT1:1. *Plant Cell and Environ*ment, 34(4), 681–689.

Prud'homme, M. (2010). World Phosphate Rock Flows, Losses and Uses. In: *Proceedings of Phosphates International 2010 Conference*. Brussels, Belgium: International Fertiliser Industry Association, p.22–24.

Qi, W., Sun, F., Wang, Q., Chen, M., Huang, Y., Feng, Y.Q., Luo, X., Yang, J. (2011). Rice ethylene-response AP2/ERF factor OsEATB restricts internode elongation by down-regulating a gibberellin biosynthetic gene. *Plant Physiology*, 157(1), 216–228.

Rae, A., Cybinski, D.H., Jarmey, J.M., Smith, F.W. (2003). Characterization of two phosphate transporters from barley; evidence for diverse function and kinetic properties among members of the Pht1 family. *Plant Molecular Biology*, 53(1–2), 27–36.

Raghothama, K.G. (1999). Phosphate acquisition. *Annual Review of Plant Physiology and Plant Molecular Biology*, 50(1), 665–693.

Raghothama, K.G. (2000a). Phosphate transport and signalling. *Current Opinion Plant Biology*, 3(3), 182–187.

Raghothama, K.G. (2000b). Phosphorus acquisition: plant in the driver's seat. *Trends in Plant Science*, 5(10), 412–413.

Raghothama, K.G., Karthikeyan, A.S. (2005). Phosphate acquisition. *Plant and Soil*, 274(1–2), 37–49.

Rajamanickam, E., Krishnapriya, V., Pandey, R., Rao, A.S., Abrol, Y.P. (2015). Physiological and molecular approaches for improving phosphorus uptake efficiency of crops. *Current Science*, 108 (7), 1271–1279.

Rajyalakshmi, K., Grover, A., Maheshwari, N. (1991). High-frequency regeneration of plantlets from the leaf-bases via somatic embryogenesis and comparison of polypeptide profiles from morphogenic and nonmorphogenic calli in wheat (*Triticum aestivum*). *Physiologia Plantarum*, 82(4), 617–623.

Ramaekers, L., Remans, R., Rao, I.M., Blair, M.W., Vanderleyden, J. (2010). Strategies for improving phosphorus acquisition efficiency of crop plants. *Field Crops Research*, 117(2/3), 169–176.

Rao, I.M., Arulanantham, A.R., Terry, N. (1989). Leaf phosphate status, photosynthesis, and carbon partitioning in sugar beet. II. Diurnal changes in sugar phosphates, adenylates, and nicotinamide nucleotides. *Plant Physiol*ogy, 9(3), 820–826.

Rao, I.M., Friesen, D.K., and Osaki, M., (1999). Plant adaptation to phosphorus-limited tropical soils. In: Pessarakli, M. (Ed.), *Handbook of Plant and Crop Stress*. New York: Marcel Dekker, Inc., p.61–96.

Rao, I.M., Terry, N. (1995). Leaf phosphate status, photosynthesis, and carbon partitioning in sugar beet. IV. Changes with time following increased supply of phosphate to low phosphate plants. *Plant Physiol*ogy, 107(4), 1313–1321.

Rath, M., Salas, J., Parhy, B., Norton, R., Menakuru, H., Sommerhalter, M., Hatlstad, G., Kwon, J.Y., Allan, D.L., Vance, C.P., Uhde-Stone, C. (2010). Identification of genes induced in proteoid roots of white lupin under nitrogen and phosphorus deprivation, with functional characterization of a formamidase. *Plant Soil*, 334(1), 137–150.

Reddy, A.S.N., Marquez, Y., Kalyna, M., Barta, A. (2013). Complexity of the alternative splicing landscape in plants. *Plant Cell*, 25(10), 3657–3683.

Regulski, M., Lu, Z., Kendall, J., Donoghue, M.T., Reinders, J., Llaca, V., Deschamps, S., Smith, A., Levy, D., McCombie, W.R., Tingey, S., Rafalski. A., Hicks, J., Ware, D., Martienssen, R.A. (2013). The maize methylome influences mRNA splice sites and reveals widespread paramutation-like switches guided by small RNA. *Genome Research*, 23(10), 1651–1662.

Remy, E., Cabrito, T. R., Batista, R. A., Teixeira, M. C., Sá-Correia, I. and Duque, P. (2012). The Pht1:9 and Pht1:8 transporters mediate inorganic phosphate acquisition by the *Arabidopsis thaliana* root during phosphorus starvation. *New Phytologist*, 195(2), 356–371.

Ren, F., Guo, Q., Chang, L., Chen, L., Zhao, C., Zhong, H., Li, X. (2012). *Brassica napus* PHR1 Gene Encoding a MYB-Like Protein Functions in Response to Phosphate Starvation. *PLoS One,* 7(8), e44005. doi: 10.1371/journal.pone.0044005.

Rennie, E.A., Turgeon, R. (2009). A comprehensive picture of phloem loading strategies. *Proceedings of the National Academy of Sciences. USA,* 106(33), 14162–14167.

Reynolds, M., Foulkes, J., Furbank, R., Griffiths. S., King, J., Murchie, E., Parry, M., Slafer, G. (2012). Achieving yield gains in wheat*. Plant Cell and Environment*, 35(10), 1799–1823.

Richardson, A. E. (1994). Soil microorganisms and phosphorus availability. In: C. E. Pankhurst, B. M. Doube, V. V. S. R. Grupta, and P. R. Grace, ed., *Soil Biota, Management in Sustainable Farming Systems*. Melbourne, Australia: CSIRO, p.50–62.

Richardson, A.E., Lynch, J.P., Ryan, P.R., Delhaize, E., Smith, F.A., Smith, S.E., Harvey, P.R., Ryan, M.H., Veneklaas, E.J., Lambers, H., Oberson, A., Culvenor, R.A., Simpson, R.J. (2011). Plant and

microbial strategies to improve the phosphorus efficiency of agriculture. *Plant Soil,* 349(1), 121–156.

Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K., Yu, G. (2000). *Arabidopsis* transcription factor: genome-wide comparative analysis among eukaryotes. *Science*, 290(5499), 2105–2110.

Robinson, S., Warburton, K., Seymour, M., Clench, M., Thomas-Oates, J. Localisation of water-soluble carbohydrates in wheat stems using imaging matrix-assisted laser desorption ionization mass spectrometry. *New Phytologist*, 173(2), 438–444.

Rodríguez, D., Andrade, F.H., Goudriaan, J. (1999). Effects of phosphorus nutrition on tiller emergence in wheat. *Plant and Soil*, 209(2), 283–295.

Roig-Villanova, I., Bou-Torrent, J., Galstyan, A., Carretero-Paulet, L., Portoles, S., Rodriguez-Concepcion, M., Martinez-Garcia, J.F. (2007). Interaction of shade avoidance and auxin responses: a role for two novel atypical bHLH proteins. *EMBO Journal*, 26, 4756–4767.

Rooke, L., Byrne, D., Salgueir, S. (2000). Marker gene expression driven by the maize ubiquitin promoter in transgenic wheat. *Annuals of applied Biology*, 136(2), 167–172.

Rose, T.J., Rengel, Z., Ma, Q., Bowden, J.W. (2007). Differential accumulation patterns of phosphorus and potassium by canola cultivars compared to wheat. *Journal of Plant Nutrition and Soil Science*, 170(3), 404–411.

Rosegrant M.W., Agcaoili M. (2010). *Global food demand, supply, and price prospects to 2010*. Washington D.C.: International Food Policy Research Institute, p.1.

Rosinski, J.A., Atchley, W.R. (1998). Molecular evolution of the Myb family of transcription factors: evidence for polyphyletic origin. *Journal of Molecular Evolution*, 46(1), 74–83.

Rouached, H., Arpat, A.B., Poirier, Y. (2010). Regulation of phosphate starvation responses in plants: signaling players and crosstalks. *MolecularPlant*, 3(2), 288–299.

Roy, S. (2016). Function of MYB domain transcription factors in abiotic stress and epigenetic control of stress response in plant genome. *Plant Signal Behaviour*, 11(1), e1117723. doi: 10.1080/15592324.2015.1117723.

Rubio, V., Bustos, R., Irigoyen, M. L., Cardona-Lopez, X., Rojas-Triana, M., and Paz-Ares, J. (2009). Plant hormones and nutrient signaling. *Plant Molecular Biology*, 69(4), 361–373.

Rubio, V., Linhares, F., Solano, R., Martin, A.C., Iglesias, J., Leyva, A., Paz-Ares, J. (2001). A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Gene and Development*, 15(6), 2122–2133.

Rushton, P.J. Bokowiec, M.T., Han, S., Zhang, H., Brannock, J.F., Chen, X., Laudeman, T.W., Timko, M.P. (2008). Tobacco transcription factors: novel insights into transcriptional regulation in the *Solanaceae*. *Plant Physiology*, 147(1), 280–295.

Róder, M.S., Korzun, V., Wendehake, K., Plaschke, J., Tixier, M.H., Leroy, P., Ganal, M.W. (1998). A microsatellite map of wheat. *Genetics*, 149(4), 2007–2023.

Sah, S.K., Reddy, K.R., Li, J. (2016). Abscisic acid and abiotic stress tolerance in crop plants. *Frontiers in Plant Science*, 7(571). doi.org/10.3389/fpls.2016.00571.

Sairanen, I., Novák, O., Pěnčík, A., Ikeda, Y., Jones, B., Sandberg, G., Ljung, K. (2012) Soluble carbohydrates regulate auxin biosynthesis via PIF proteins in Arabidopsis. *Plant Cell*, 24(12), 4907–4916.

Salamini, F., Özkan, H., Brandolini, A., Schäfer-Pregl, R., Martin, W. (2002). Genetics and geography of wild cereal domestication in the Near East. *Nature Reviews Genetics*, 3(6), 429–441.

Salentijn, E.M.J., Pereira, A., Angenent, G. C., van der Linden, G., Krens, F., Smulders, M.J. M., Vosman, B (2007). Plant translational genomics: from model species to crops. *Molecular Breeding*, 20(1), 1–13.

Salentijn, E.M.J., Pereira, A., Angenent, G.C., van der Linden, C.G., Krens, F., Smulders, M.J.M., Vosman, B. (2007). Plant translational genome: from model species to crops. *Molecular Breeding*, 20(1), 1–13.

Sanchez, P.A., (1976). *Properties and Management of Soils in the Tropics*. New York: John Wiley, p.618.

Sander, C., Schneider, R. (1991). Database of Homology-Derived Protein Structures and the Structural Meaning of Sequence Alignment. *Proteins: Structure, Function and Genetics*, 9(1), 56–68.

Sano, T., Kuraya, Y., Amino, S., Toshiyuki, N. (1999). Phosphate as a limiting factor for cell division of tobacco BY-2 Cells. *Plant Cell Physiology*, 40(1), 1–8.

Sattari, S.Z., Bouwman, A.F., Giller, K.E., van Ittersum, M.K., (2012). Residual soil phosphorus as the missing piece in the global phosphorus crisis puzzle. *Proceedings of the National Academy of Sciences. USA*, 109(16), 6348–6353.

Sattari, S.Z., Van Ittersum, M.K., Giller, K.E., Zhang, F., Bouwman, A.F. (2014) Key role of China and its agriculture in global sustainable phosphorus management. *Environmental Research Letters*, 9(5), 4003–4008.

Schenk, M.K. (2006). Nutrient efficiency of vegetable crops. *Acta Horticulture (ISHS)*, 700, 21–34.

Schjorring, J.K., Nielsen, N.E. (1987). Root length and phosphorus uptake by four barley cultivars grown under moderate deficiency of phosphorus in field experiments. *Journal of Plant Nutrition*, 10(9), 1289–1295.

Schnable, J.C., Freeling, M., Lyons, E. (2002) Genome-Wide Analysis of Syntenic Gene Deletion in the Grasses. *Genome Biology Evolution*, 4(3), 265–277.

Schneider, K., Mathur, J., Boudonck, K., Wells, B., Dolan, L., Roberts, K. (1998). The ROOT HAIRLESS 1 gene encodes a nuclear protein required for root hair initiation in *Arabidopsis*. *Genes and Development*, 12(13), 2013–2021.

Schröder, J.J., Smita, A.L., Cordell, D., Rosemarin A. (2011). Improved phosphorus use efficiency in agriculture: A key requirement for its sustainable use. *Chemosphere*, 84(6), 822–831.

Schünmann, P. H. D., Richardson, A. E., Smith, F. W., and Delhaize, E. (2004). Characterization of promoter expression patterns derived from the Pht1 phosphate transporter genes of barley (*Hordeum vulgare* L.). *Journal of Experimental Botany*, 55(398), 855–865.

Secco, D., Wang, C., Shou, H., Schultz, M.D., Chiarenza, S., Nussaume, L., Ecker, J.R., Whelan, J., Lister, R. (2015). Stress induced gene expression drives transient DNA methylation changes at adjacent repetitive elements. *Elife*, 4, e09343. doi:10.7554/ eLife.09343.

Secco, D., Whelan, J., Rouached, H., Lister, R. (2017). Nutrient stress-induced chromatin changes in plants. *Current Opinion in Plant Biology*, 39, 1–7.

Semenov, M.A., Stratonovitch, P., Alghabari, F., Gooding, M.J. (2014). Adapting wheat in Europe for climate change. *Journal of Cereal Science*, 59(3), 245–256.

Seo, P.J., Park, C.M. (2009). Auxin homeostasis during lateral root development under drought condition. *Plant Signal Behaviour,* 4(10), 1002–1004.

Shane, M.W., Lambers, H. (2005). Cluster roots: a curiosity in context. *Plant Soil*, 274(1), 101–125.

Shenoy, V.V., Kalagudi, G.M. (2005). Enhancing plant phosphorus use efficiency for sustainable cropping. *Biotechnology Advances*, 23(7–8), 501–513.

Shewry P.R., Jones H.D. (2005). Transgenic wheat: where do we stand after the first 12 years? *Annals of Applied Biology*, 147(1), 1–14.

Shimada, T. (1978). Plant regeneration from callus induced from wheat embryo. *The Japanese Journal of Genetics*, 53(6), 374–381.

Shin, H., Shin, H.S., Dewbre, G.R., Harrison, M.J. (2004) Phosphate transport in *Arabidopsis*: Pht1:1 and Pht1:4 play a major role in phosphate acquisition from both low- and high-phosphate environments. *The Plant Journal*, 39(4), 629–642.

Shrawat, A.K., Becker, D., Lorz,H. (2007). *Agrobacterium tumefaciens*- mediated genetic transformation of barley (*Hordeum vulgare* L.). *Plant Science*, 172(2), 281–290.

Skylar, A., Sung, F., Hong, F., Chory, J., Wu X. (2011). Metabolic sugar signal promotes *Arabidopsis* meristematic proliferation via G2. *Developmental Biology*, 351 (1), 82–89.

Slafer G.A. and Whitechurch E.M. (2001). Manipulating wheat development to improve adaptation. In: Reynolds MP, Ortiz-Monasterio JI, McNab A, ed., *Application of Physiology in Wheat Breeding.* Mexico: CIMMYT, p.160–170.

Slewinski, T.L., Braun, D.M. (2010). Current perspectives on the regulation of whole–plant carbohydrate partitioning. *Plant Science,* 178(4), 341–349.

Smith, S.E., Read, D.J. (1997). *Mycorrhizal symbiosis*. San Diego, California*:* Academic Press Inc., p.605.

Smith, S.E., Smith, F.A. (2011). Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystems scales. *Annual Review of Plant Biol*ogy, 63, 227–250.

Soltan, S., Römer, W., Adgo, E., Gerke, J., Schilling, G. (1993). Phosphorus sorption by Egyptian, Ethiopian and German soils and P uptake by rye (*Secale cereale* L.) seedlings. *Journal of Plant Nutrition and Soil Science (Z Pflanzenernähr Bodenkd)*, 156(6), 501–506.

Staiger, D., Brown, J.W.S. (2013). Alternative splicing at the intersection of biological timing, development, and stress responses. *Plant Cell*, 25(10), 3640–3656.

Stamm, S., Ben-Ari, S., Rafalskam, I., Tang, Y., Zhang, Z., Toiber, D., Thanaraj, T.A., Soreq, H. (2005). Function of alternative splicing. *Gene*, 344, 1–20.

Stefanović, S., Kuzmina, M., Costea, M. (2007). Delimitation of major lineages within *Cuscuta* subgenus *Grammica* (Convolvulaceae) using plastid and nuclear DNA sequences. *American Journal of Botany,* 94(4), 568–589.

Stewart, M. (2007). Molecular mechanism of the nuclear protein import cycle. *Nature Reviews Molecular Cell Biology*, 8(3), 195-208.

Stigter, K.A., Plaxton, W.C. (2015). Molecular Mechanisms of Phosphorus Metabolism and Transport during Leaf Senescence. *Plants (Basel)*, 4(4), 773–798.

Stracke, R., Werber, M., Weisshaar, B. (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology*, 4(5), 447–456.

Su, J, Xiao, Y., Li, M., Liu, Q., Li, B., Tong, Y., Jia, J., Li, Z. (2006). Mapping QTLs for phosphorus-deficiency tolerance at wheat seedling stage. *Plant Soil*, 281(1), 25–36.

Su, J., Zheng, Q., Li, H., Li, B., Jing, R., Tong, Yi, Li, Z. (2009). Detection of QTLs for phosphorus use efficiency in relation to agronomic performance of wheat grown under phosphorus sufficient and limited conditions. *Plant Science, 176(6), 824–836.*

Sulpice, R., Ishihara, H., Schlereth, A., Cawthray, G.R., Encke, B., Giavalisco, P., Ivakov, A., Arrivault, S., Jost, R., Krohn, N., Kuo, J., Laliberte´, E., Pearse, S.J., Raven, J.A., Scheible, W.R., Teste, F., Veneklaas, E.J., Stitt, M., Lambers, H. (2014). Low levels of ribosomal RNA partly account for the very high photosynthetic phosphorus-use efficiency of Proteaceae species. *Plant, Cell and Environment*, 37(6), 1276–1298.

Sultana, S., Ho, C., Namasivayam, P., Napis, S. (2014). Genotypic Differences in Response 38 Genotypic Differences in Response to Hygromycin Effect on Untransformed Calli Death and Rice Germination, *Bangladesh Rice Journal*, 18 (1–2), 38–43.

Svistoonoff, S., Creff, A., Reymond, M., Sigoillot-Claude, C., Ricaud, L., Blanchet, A., Nussaume, L., Desnos, T. (2007). Root tip contact with low-phosphate media reprograms plant root architecture. *Nature Genetics*, 39(6), 792–796.

Swarup, R. Crespi, M., Bennett, M.J. (2016). One gene, many proteins: mapping cell-specific alternative splicing in plants. *Developmental Cell*, 39(4), 383–384.

Syers, J.K., Johnston, A.E., Curtin, D. (2008). *Efficiency of soil and fertilizer phosphorus use: reconciling changing concepts of soil phosphorus behaviour with agronomic information. FAO Fertilizer and Plant Nutrition Bulletin; 2008 18*. Rome, Italy: Food and Agriculture Organization of the United Nations - FAO, p.123.

Talavera, D., Orozco, M., de la Cruz, X. (2009). Alternative splicing of transcription factors' genes: beyond the increase of proteome diversity. *Comparative and Functional Genomics*, e9058594. doi: 10.1155/2009/905894.

Tang, H., Li, X., Zu, C., Zhang, F., Shen, J. (2013). Spatial distribution and expression of intracellular and extracellular acid phosphatases of cluster roots at different developmental stages in white lupin. *Plant Physiology*, 170(4), 1243–1250.

Thakur, P., Kumar, S., Malik, J.A., Berger, J.D., Nayyar, H. (2010). Cold stress effects on reproductive development in grain crops: an overview. *Environmental and Experimental Botany*, 67(3), 429–443.

The *Arabidopsis* genome initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, 408(6814), 796–815.

Theodorou, M.E., Plaxton, W.C. (1993). Metabolic adaptation of plant respiration to nutritional phosphate deprivation. *Plant Physiology*, 101(2), 339–344.

Thibaud, M.C., Arrighi, J.F., Bayle, V., Chiarenza, S., Creff, A., Bustos, R., Paz-Ares, J., Nussaume, L. (2010). Dissection of local and systemic transcriptional responses to phosphate starvation in *Arabidopsis*. *The Plant Journal*, 64(5), 775–789.

Thingstrup, I., Kahiluoto, H., Jackobsen, I. (2000). Phosphate transport by hyphae of field communities of arbuscular mycorrhizal fungi at two levels of P fertilisation. *Plant Soil*, 221(2), 181–187.

Thomashow, M.F., Panagopoulos, C.G., Gordon, M.P., Nester, E.W. (1980). Host range of *Agrobacterium tumefaciens* is determined by the Ti plasmid. *Nature*, 283, 794–796.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R., Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature*, 418(6898), 671–677.

Tinker, P.B., Nye, P.H. (2000). *Solute movement in the rhizosphere*. Oxford, UK: Oxford University Press, Inc., p.464.

Tiwari, R., Kumar, A. (2012). Starch phosphorylase: biochemical and biotechnological perspectives. *Biotechnology and Molecular Biology Reviews*, 7(3), 69–83.

Tran, H.T., Hurley, B.A., Plaxton, W.C. (2010a). Feeding hungry plants: the role of purple acid phosphatases in phosphate nutrition. *Plant Science*, 179(1–2), 14–27.

Tran, H.T., Qian, W., Hurley, B.A., She, Y.M., Wang, D., Plaxton, W.C. (2010b). Biochemical and molecular characterization of AtPAP12 and AtPAP26: the predominant purple acid phosphatase isozymes secreted by phosphate-starved *Arabidopsis thaliana*. *Plant Cell Environment,* 33(11), 1789–1803.

Travella, S., Ross, S.M., Harden, J., Everett, C., Snape, J.W., Harwood, W.A. (2005). A comparison of transgenic barley lines produced by particle bombardment and *Agrobacterium*-mediated techniques. *Plant Cell Reports*, 23(12), 780–789.

Trione, E.J., Jones, L.E., Metzgerr, R.G. (1968). In vitro culture of somatic wheat callus tissue. *American Journal of Bot*any, 55(5), 529–531.

Trolove, S.N., Hedley, M.J., Caradus, J.R., Mackay, A.D. (1996). Uptake of phosphorus from different sources by Lotus pedunculatus and three genotypes of *Trifolium repens*. 2. Forms of phosphate utilised and acidification of the rhizosphere. *Australian Journal Soil Res*earch, 34(6), 1015–1026.

Tu, S.I., Cananaugh, J.R., Boswell, R. T. (1990). Phosphate uptake by excised maize root tips studied by *in vivo* ^{31}P nuclear magnetic resonance spectroscopy. *Plant Physiology*, 93(2), 778–784.

Tubiello, F. N., Amthor, J.S., Boote, K.J., Donatelli, M., Easterling, W., Fischerf, G., Gifford, R.M., Howden, M, Reilly, J., Rosenzweig, C. (2007). "Crop Response to Elevated CO2 and World Food Supply—A Comment on 'Food for Thought' by Long et al., (2006). *Science*, 312, 1918–1921, 2006." *European Journal of Agronomy*, 26(3), 215–223.

Udvardi, M.K., Kakar, K., Wandrey, M., Montanari, O., Murray, J., Andriankaja, A., Zhang, J.Y., Benedito, V., Hofer, J.M., Chueng, F., Town, C.D. (2007). Legume transcription factors: global regulators of plant development and response to the environment. *Plant Physiology*, 144(2), 538–549.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T.Y., Hsing, Y.I., Kitano, H., Yamaguchi, I., Matsuoka, M. **(**2005). *GIBBERELLIN INSENSITIVE DWARF1* encodes a soluble receptor for gibberellin. *Nature*, 437(7059), 693–698.

Ueguchi-Tanaka, M., Hirano, K., Hasegawa, Y., Kitano, H., and Matsuoka, M. (2008). Release of the repressive activity of rice DELLA protein SLR1 by gibberellin does not require SLR1 degradation in the gid2 mutant. *Plant Cell*, 20(9), 2437–2446.

Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., Arite, T., Takeda-Kamiya, N., Magome, H., Kamiya, Y., Shirasu, K., Yoneyama, K., Kyozuka, J., Yamaguchi, S. (2008). Inhibition of shoot branching by new terpenoid plant hormones. *Nature*, 455(7210), 195–200.

Umehara, M., Hanada, A., Magome, H., Takeda-Kamiya, N., Yamaguchi, S. (2010). Contribution of strigolactones to the inhibition of tiller bud outgrowth under phosphate deficiency in rice. *Plant Cell Physiology*, 51(7), 1118–1126.

United Nations. (2007). *World Population Prospects: the 2006 Revision, Highlights.* New York: United Nations, p.96.

United Nations. (2013). *World Population Prospects: the 2012 Revision.* New York: United Nations, p.94.

USDA. (2015). USDA *Wheat Baseline 2015-24*. [online] Available at: http://www.ers. usda. gov/topics/crops/wheat/usda-wheat-baseline-2015-24.aspx. [Accessed 24/02/ 2016].

Vaccari, D.A. (2009). Phosphorus: a looming crisis. *Scientific American,* 300, 54–59.

Valdés-López, O., Arenas-Huertero, C., Ram´ırez, M., Girard, L., S´anchez, F., Vance, C.P., Luis Reyes, J., Hernández, G. (2008). Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus-deficiency signalling in common bean roots. *Plant, Cell and Environment*, 31(12), 1834–1843.

Van de Wiel, C. C. M., Van der Linden, C. G., Scholten, O.E. (2016). Improving phosphorus use efficiency in agriculture: opportunities for breeding. Review. *Euphytica*, 207(1), 1–22.

Van Slageren, M.W. (1994). *Wild Wheats: A Monograph of Aegilops L. and Amblyopyrum (Jaub and Spach) Eig (Poaceae).* Aleppo: ICARDA, Agricultural University, Wageningen, p.513.

Vance, C.P., Uhde-Stone, C., Allan, D. (2003). Phosphorus acquisition and use: critical adaptation by plants for securing non-renewable resources. *New Phytologist*, 15(3), 423–447.

Van't Hof, J., (1966). Experimental control of DNA synthesizing and dividing cells in excised root tips of pisum. *American Journal of Botany*, 53(10), 970–976.

Varshney, R.K., Hoisington, D.A., Nayak, S.N. and Graner, A. (2009). Molecular plant breeding: methodology and achievements. *Methods in Molecular Biology*, 513, 283–304.

Vasil, I.K. (2007). Molecular genetic improvement of cereals: transgenic wheat (*Triticum aestivum* L.). *Plant Cell Report*, 26(8), 1133–1154.

Vaucheret, H., Beclin, C., Fagard, M. (2001). Post-transcriptional gene silencing in plants. *Journal of Cell Science*, 114, 3083–3091.

Veneklaas, E.J., Lambers, H., Bragg, J., Finnegan, P.M., Lovelock, C.E., Plaxton, W.C., Price, C.A., Scheible, W.R., Shane, M.W., White, P.J., Raven, J.A. (2012). Opportunities for improving phosphorus-use efficiency in crop plants. *New Phytologist*, 195(2), 306–320.

Viertel, K., Hess, D. (1996). Shoot tips of wheat as an alternative source for regenerable embryogenic callus cultures. *Plant Cell, Tissue and Organ Culture*, 1996, 44(3), 183–188.

Vilperte, V., Agapito-Tenfen, S.Z., Wikmark, O., Nodari, R.O. (2016). Levels of DNA methylation and transcript accumulation in leaves of transgenic maize varieties. *Environ Science Europe*, 28, 29–43.

Vlek, P.L.G., Koch, H. (1992). The soil resource base and food production in the developing world: special focus on Africa. *Gättinger Beitrage zur Landund Forstwirschaft in den Tropen und Subtropen*, 71, 139–160.

Vorasoot, N., Songsri, P., Akkasaeng, C., Jogloy, S., Patanothai, A. (2003). Effect of water stress on yield and agronomic characters of peanut. *Songklanakarin Journal of Science and Technology (SJST),* 25(3), 283–288.

Walk, T.C., Jaramillo, R., Lynch, J.P. (2006). Architectural tradeoffs between adventitious and basal roots for phosphorus acquisition. *Plant Soil*, 279(1), 347–366.

Wang, C., Huang, W., Ying, Y., Li, S., Secco, D., Tyerman, S., Whelan, J., Shou, H. (2012a). Functional characterization of the rice SPX-MFS family reveals a key role of OsSPX-MFS1 in controlling phosphate homeostasis in leaves. *New Phytologist*, 196(1), 139–148.

Wang, F., Rose, T., Jeong, K., Kretzschmar, T., Wissuwa, M (2016b). The knowns and unknowns of phosphorus loading into grains, and implications for phosphorus efficiency in cropping systems. *Journal of Experimental Botany*, 67(5), 1221–1229.

Wang, H., Wang, H., Shao, H., Tang, X. (2016a). Recent Advances in Utilizing Transcription Factors to Improve Plant Abiotic Stress Tolerance by Transgenic Technology. *Frontiers in Plant Science*, 7(67). doi: 10.3389/fpls.2016.00067.

Wang, J., Sun, J., Miao, J., Guo, J., Shi, Z., He, M., Chen, Y., Zhao, X., Li, B., Han, F., Tong, Y., Li, Z. (2013b). A phosphate starvation response regulator Ta-PHR1 is involved in phosphate signalling and increases grain yield in wheat. *Annals of Botany*, 111(6), 1139–1153.

Wang, L., Ruan, Y.L. (2013). Regulation of cell division and expansion by sugar and auxin signaling. *Frontiers in Plant Science*, 4(163). doi.org/10.3389/fpls.2013.00163.

Wang, X., Bai, J., Liu, H., Sun, Y., Shi, X., Ren, Z. (2013a). Overexpression of a maize transcription factor ZmPHR1 improves shoot inorganic phosphate content and growth of *Arabidopsis* under low-phosphate conditions. *Plant Molecular Biology Reporter*, 31(3), 665–677.

Wang, X.R., She, J.B., Liao, H. (2010). Acquisition or utilisation, which is more critical for enhancing phosphorus efficiency in modern crops? *Plant Science*, 179(4), 302–306.

Wang, Y., Zhao, J., Lu, W., Deng, D. (2017). Gibberellin in plant height control: old player, new story. *Plant Cell Reports*, 36(3), 391–398.

Waterhouse, P.M., Wang, M.-B. and Lough, T. (2001). Gene silencing as an adaptive defence against viruses. *Nature*, 411(6839), 834–842.

Weaver, D.M., Wong, M.T.F. (2011). Scope to improve phosphorus (P) management and balance efficiency of crop and pasture soils with contrasting P status and buffering indices. *Plant and Soil*, 349(1–2), 37–54.

Weiler K, Wakimoto B (1995). "Heterochromatin and gene expression in *Drosophila*". *Annual Review of Genetics*. 29(1), 577–605.

Weinhold, A., Kallenbach, M., Ian Thomas Baldwin, T.I. (2013). Progressive 35S promoter methylation increases rapidly during vegetative development in transgenic *Nicotiana attenuata* plants. *BMC Plant Biology*, 13, 99. doi.org/10.1186/1471-2229-13-99.

Weisberg, R. A. and Landy, A. (1983). Site-Specific Recombination in Phage Lambda. In: R. A. Weisberg, ed., *Lambda II. Cold Spring Harbor*, New York: Cold Spring Harbor Press, p.211–250.

White, J., (2000). *Introduction to Biogeochemical Cycles.* Boulder: Department of Geological Sciences. University of Colorado.

Wilkinson, P.A., Winfield, M.O., Barker, G.L., Tyrrell. S., Bian, X., Allen, A.M., Burridge, A., Coghill. J.A., Waterfall, C., Caccamo, M., Davey, R.P., Edwards, K.J. (2016). CerealsDB 3.0: expansion of resources and data integration. *BMC Bioinformatics*, 17, 256. doi: 10.1186 /s12859-016-1139-x.

Wilkinson, P.A., Winfield, M.O., Barker, G.L.A., Allen, A.M., Burridge, A, Coghill, J.A., Burridge, A. and Edwards, K.J. (2012). CerealsDB 2.0: an integrated resource for plant breeders and scientists. *BMC Bioinformatics*, 13, 219. doi.org/10.1186/1471-2105-13-219.

Wissuwa, M., Gamat, G., and Ismail, A.M. (2005). Is root growth under phosphorus deficiency affected by source or sink limitations? *Journal of Experimental Botany*, 56(417), 1943–1950.

Wissuwa, M., Yano, M., Ae, N. (1998). Mapping of Q TLs for phosphorus-deficiency tolerance in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics*, 97(5–6), 777–783.

World Resources Institute - WRI. (2000). *World Resources 2000–2001: People and Ecosystems*. Washington, DC: United Nations Development Programme, United Nations Environment Programme, World Bank, and World Resources Institute.

Wu, C., Wang, Z., Fan, Z., Sun, H.L. (2003). Effects of different concentrations and form ratios of nitrogen on chlorophyll biosynthesis, photosynthesis, and biomass partitioning in Fraxinus mandshurica seedlings. Chinese Journal of Plant Ecology, 2003, 27(6), 771-780.

Wu, P., Shou, H., Xu, G., Lian, X. (2013). Improvement of phosphorus efficiency in rice on the basis of understanding phosphate signaling and homeostasis. *Current Opinion in Plant Biology*, 16, 205–212.

Yan, F., Peng, J., Lu, Y., Lin, L., Zheng, H., Chen, H., Chen, J., Adams, M.J. (2009). Identification of novel splice variants of the *Arabidopsis* DCL2 gene. *Plant Cell Reports*, 28(2), 241–246.

Yan, X., Liao, H., Beebe, S.E., Blair, M.W., Lynch, J.P. (2004). QTL mapping of root hair and acid exudation traits and their relationship to phosphorus uptake in common bean. *Plant Soil*, 265(1), 17–29.

Yan, X.L., Liao, H., Beebe, S.E., Blair, M. W., Lynch, J.P. (2004). QTL mapping of root hair and acid exudation traits and their relationship to phosphorus uptake in common bean. *Plant Soil*, 265(1–2), 17–29.

Yanagisawa, S., Akiyama, A., Kisaka, H., Uchimiya, H., Miwa, T. (2004). Metabolic engineering with Dof1 transcription factor in plants: improved nitrogen assimilation and growth under low nitrogen conditions. *Proceedings of the National Academy of Sciences. USA*, 101(20), 7833–7838.

Yang, T., Hao, L., Yao, S., Zhao, Y., Lu, W., Xiao, K. (2016). TabHLH1, a bHLH-type transcription factor gene in wheat, improves plant tolerance to Pi and N deprivation via regulation of nutrient transporter gene transcription and ROS homeostasis. *Plant Physiology and Biochemistry*, 104, 99–113.

Yanofsky, M.F., Lowe, B., Montoya, A., Rubin, R., Krul, W., Gordon, M.P., Nester, E.W. (1985). Molecular and genetic analysis of factors controlling host range in *Agrobacterium tumefaciens*. *Molecular and General Genetics*, 201(2), 237–246.

Yi, K., Menand, B., Bell, E., Dolan, L. (2010). A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. *Nature Genetics*, 42(3), 264-267.

Yi, K., Wu, Z., Zhou, J., Du, L., Guo, L., Wu, Y., Wu, P. (2005). OsPTF1, a novel transcription factor involved in tolerance to phosphate starvation in rice. *Plant Physiology*, 138(4), 2087–2896.

Yong-Villalobos, L., Cervantes-Pérez, S.A., Gutiérrez-Alanis, D., Gonzáles-Morales, as., Martínez, O., Herrera-Estrella, L. (2016). Phosphate starvation induces DNA methylation in the vicinity of cis-acting elements known to regulate the expression of phosphate-responsive genes. *Plant Signal Behaviour*, 11(5), e1173300.

Yong-Villalobosa, L., González-Moralesa, S.I., Wrobelb, K., Dolores Gutiérrez-Alanis, D., Cervantes-Peréza, S.A., Hayano-Kanashiroa, C., Oropeza-Aburtoa, A., Cruz-Ramíreza, A., Martíneza, O., Herrera-Estrellaa, L. (2015). Methylome analysis reveals an important role for epigenetic changes in the regulation of the *Arabidopsis* response to phosphate starvation. *Proceeding of National Academy of Sciences*. USA, 112(52), E7293-302.

Yu, B., Xu, C., Benning, C. (2002) *Arabidopsis* disrupted in SQD2 encoding sulfolipid synthase is impaired in phosphate-limited growth. *Proceedings of National Academy of Science. USA*, 99(8), 5732–5737.

Yuan, Y., Gao, M., Zhang, M., Zheng, H., Zhou, X., Guo, Y., Zhao, Y., Kong, F., Li, S. (2017). QTL mapping for phosphorus efficiency and morphological traits at seedling and maturity stages in wheat. *Frontiers in Plant Science*, 8(614). doi.org/10.3389/fpls.2017.00614.

Yun, K.Y., Park, M.R., Mohanty, B., Herath, V., Xu, F., Mauleon, R., Wijaya, E., Bajic, V.B., Bruskiewich, R., Reyes B. G. (2010). Transcriptional regulatory network triggered by oxidative signals configures the early response mechanisms of japonica rice to chilling stress. *BMC Plant Biology*, 10, 16. doi:10.1186/1471-2229-10-16.

Zaharieva, M., Ayana, N., Hakimi, A., Misra, S., Monneveux, P. (2010). Cultivated emmer wheat (*Triticum* dicoccon Schrank), an old crop with promising future: a review. *Genetic Resources and Crop Evolution*, 57(6), 937–962.

Zambryski, P., Joos H., Genetello, H., Leemans, J., van Montagu, M., Schell, J. (1983). Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO Journal*, 2(12), 2143–2150.

Zang, Q., Wang, C., Li, Y., Guo, Z., Jing, R., Zhao, J., Chang, X. (2010). Isolation and characterization of a gene encoding a polyethylene glycol-induced cysteine protease in common wheat. *Journal of Bioscience*, 35(3), 379–388.

Zhang K.P., Wang J.J., Zhang L.Y., Rong C.W., Zhao F.W., Peng, T., Li. H., Cheng, D., Liu, X., Qin, H., Zhang, A., Tong, Y., Wang, D. (2013). Association analysis of genomic loci important for grain weight control in elite common wheat varieties cultivated with variable water and fertiliser supply. *PLoS ONE*, 8, e57853. doi.org/10.1371/journal.pone. 0057853.

Zhang, H., Wang, H. (2015). QTL mapping for traits related to P-deficient tolerance using three related RIL populations in wheat. *Euphytica*, 203(3), 505–520.

Zhang, L., Zhao, G., Jia, J., Liu, X., Kong, X. (2012). Molecular characterization of 60 isolated wheat MYB genes and analysis of their expression during abiotic stress. *Journal of Experimental Botany*, 63(1), 203–214.

Zhao, J., Fu, J., Liao, H., He, Y., Nian, H., Hu, Y., Qiu, L., Dong, Y., Yan, X. (2004). Characterization of root architecture in an applied core collection for phosphorus efficiency of soybean germplasm. *Chinese Science Bulletin,* 49 (15), 1611–1620.

Zhou, J., Jiao, F., Wu, Z., Li, Y., Wang, X., He, X., Zhong, W., Wu, P. (2008a). OsPHR2 is involved in phosphate-starvation signalling and excessive phosphate accumulation in shoots of plants. *Plant Physiol*ogy, 146(4), 1673–1686.

Zhou, J., Li, F., Wang, J., Ma, Y., Chong, K., Xu, Y. (2009). Basic helix-loop-helix transcription factor from wild rice (OrbHLH2) improves tolerance to salt and osmotic stress in *Arabidopsis*. *Journal of Plant Physiology*, 166, 1296–1306.

Zhou, K., Yamagishi, M., Osaki, M., Masuda, K. (2008b). Sugar signalling mediates cluster root formation and phosphorus starvation-induced gene expression in white lupin. *Journal of Experimental Botany*, 59, 2749–2756.

Zhou, Y., Zhou, C., Ye, L., Dong, J., Xu, H., Cai, L., Zhang, L., Wei, L. (2003). Database and analyses of known alternatively spliced genes in plants. *Genomics*, 82(6), 584–595.

Zhou, Z., Jiang, Y., Wang, Z., Gou, Z., Lyu, J., Li, W., Yu, Y., Shu, L., Zhao, Y., Ma, Y., Fang, C., Shen, Y., Liu, T., Li, C., Li, Q., Wu, M., Wang, M., Wu, Y., Dong, Y., Wan, W., Wang, X., Ding, Z., Gao, Y., Xiang, H., Zhu, B., Lee, S.H., Wang, W. and Tian, Z. (2015). Resequencing 302 wild and cultivated accessions identifies genes related to domestication and improvement in soybean. *Nature Biotechnology*, 33(4), 408–414.

Zhu, J., Brown, K.M., Lynch, J.P. (2010). Root cortical aerenchyma improves the drought tolerance of maize (*Zea mays* L.). *Plant, Cell and Environment*, 33(5), 740–749.

Zhu, J.M., Kaeppler, S.M., Lynch, J.P. (2005). Mapping of Q TL controlling root hair length in maize (*Zea mays* L.) under phosphorus deficiency. *Plant Soil*, 270(1), 299–310.

Appendices

Appendix 1 *E. coli* plasmid extraction

Plasmids of transformed *E.coli* TOP10 were prepared by using PurePlasmid Mini Kit (CWBio, Beijing, China) following the method adapted from manufacturer's protocol. A volume of 5mL transformed *E.coli* culture was pelleted by spinning at 8,000rpm for 5 minutes in a 15mL falcon tube and re-suspended with up to 2mL ddH_2O (DNase-free) before transferring to a 2mL microcentrifuge tube. The culture was re-pelleted by spinning at 13,000rpm for 1 minute, after which the supernatant was discarded. The tube was added and mixed with 250µL of buffer P1 (RNase A contained), followed by adding 250µL of buffer P2 and inverting the tube gently for 6–8 times. Next, the mixture was added with 250µL of buffer N3 and inverted immediately and gently for 6–8 times before spinning at 13,000rpm for 5 minutes. The supernatant was removed to a Spin Column DM (placed in a 2mL collection tube), which was then centrifuged at 13,000rpm for 30 seconds, with the flow-through discarded. The column was added with 150µL of buffer PB and centrifuged at 13,000rpm for 30 seconds, with the flow-through discarded. Subsequently, the column was washed twice by adding 400µL of buffer PW (ethanol contained) and spinning at 13,000rpm for 30 seconds with the flow-through discarded each time. After replacing the collection tube with a new 1.5mL microcentrifuge tube, the DNA was eluted by adding 50µL ddH_2O (DNase-free) and standing for 2 minutes at room temperature, before spinning at 13,000rpm for 1 minute. This was repeated twice when necessary by using the same elution. The *E. coli* plasmid sample was stored at -20°C for later use.

Appendix 2 Purification of DNA

A2.1 DNA purification using PEG

A volume of 50µL DNA was diluted 4-fold with 150µL of TE buffer (10mM Tris-HCl with pH=7.5, 1mM EDTA) in a 1.5mL microcentrifuge tube and thoroughly mixed with 100µL of 30% PEG8000/30mM $MgCl_2$ by vortex. The mixture was subsequently centrifuged at 9,500rpm for 15 minutes at room temperature, followed by the supernatant carefully removed without interrupting the DNA pellet. The pelleted DNA was re-suspended in 20µL of TE buffer prior to the downstream experiments.

A2.2 DNA purification from agarose gel using Gel Extraction Kit

DNA was purified from agarose gel by using Gel Extraction Kit (CWBio, Beijing, China) following the method adapted from manufacturer's protocol. The gel slices collected in a 1.5mL microcentrifuge tube were thawed at room temperature from -20°C. They were measured for net weight to be converted to volume (e.g. 100mg equals to 100µL). An equivalent volume of buffer PG was added to the gel slices, followed by heating at 50°C and occasionally inverting the tube until the gel was fully dissolved. Next, the dissolution was cooled to room temperature and transferred to a Spin Column DM (750µL each time), before which the column was normalised by using 200µL of buffer PS. The column was incubated at room temperature for 2 minutes and centrifuged at 13,000rpm for 1 minute, with the flow-through discarded. The column was then washed twice by adding 450µL of buffer PW (ethanol contained) and spinning at 13,000rpm for 1 minute, with the flow-through discarded each time. Subsequently, the column was centrifuged at 13,000rpm for 1 minute without any buffer added. After replacing the collection tube with a new 1.5mL microcentrifuge tube, the DNA was eluted by adding 50µL ddH_2O (DNase-free) and

standing for 2 minutes at room temperature, before spinning at 13,000rpm for 1 minute. This was repeated twice when necessary by using the same elution. The DNA sample was then stored at -20°C for later use.

Appendix 3 Extraction of genomic DNA

A3.1 Extraction of genomic DNA by DNeasy Plant Mini kit

Genomic DNA was extracted by using DNeasy Plant Mini kit (QIAGEN, UK). Each sample, comprised of no more than 100mg fresh leaf tissues, was collected in a 1.5mL microcentrifuge tube and frozen in liquid nitrogen upon collection. The sample was disrupted by mortar and pestle before mixing with 400µL of buffer AP1 and 4µL RNase by vortex. The tube was incubated at 65°C for 10 minutes occasionally inverted during incubation. This was followed by adding and mixing with 130µL of buffer P3 into the tube, which was incubated on ice for 5 minutes and centrifuged at 14,000rpm for 5 minutes at room temperature. The upper phase of the lysate was removed to a QIAshredder spin column placed in a 2mL collection tube, which was then centrifuged at 14,000rpm for 2 minutes. A volume of 350µL of the flow-through was pipetted into a new 2mL microcentrifuge tube with the pellet left out, after which 525µL of buffer AW1 was added and mixed by pipetting. Following this, the 650µL mixture was transferred to a DNeasy Mini spin column placed in a 2mL collection tube, which was centrifuged for 1 minute at 8,000rpm, with the flow-through discarded. This was repeated with the remaining mixture. Subsequently, the spin column was moved to a new 2mL collection tube before adding 500µL of buffer AW2 and spinning at 8,000rpm for 1 minute, with the flow-through discarded. This was repeated a second time. Next, the tube was centrifuged at 14,000rpm for an extended time of 2 minutes. The genomic DNA was eluted from the spin column into a new 1.5mL microcentrifuge tube by adding 50µL ddH_2O (DNase-free) directly onto the spin column membrane, which was incubated at room temperature for 5 minutes and centrifuged at 10,000rpm for 1 minute. The genomic DNA sample was stored at 4°C for later use.

A3.2 Extraction of genomic DNA by Nuclean PlantGen DNA Kit

Genomic DNA was extracted by using Nuclean PlantGen DNA Kit (CWBio, Beijing, China). For each sample, no more than 100mg fresh leaf tissues were collected into a 2mL microcentrifuge tube (contained with 3x2mm mill balls and placed on ice after collection). The sample was frozen in liquid nitrogen and immediately ground by Bullet blender (Next Advance, Inc., 1548, Burden Lake Road, Averill Park, NY 12018-2818) before mixing with 400µL of buffer LP1 and 6µL RNase by vortex. The tube was incubated at room temperature for 10 minutes and occasionally inverted during incubation. This was followed by adding and mixing with 130µL of buffer LP2 into the tube, which was centrifuged at 12,000rpm for 5 minutes. A volume of 300µL supernatant was removed into a 1.5mL microcentrifuge tube and added with 450µL of buffer LP3, followed by inverting the tube to mix well. Following this, the 750µL mixture was transferred to a spin column DM placed in a 2mL collection tube, which was centrifuged at 12,000rpm for 1 minute, with the flow-through discarded. This was repeated with the remaining mixture. Subsequently, the spin column was moved to a new 2mL collection tube before adding 500µL of buffer GW2 and spinning at 12,000rpm for 1 minute, with the flow-through discarded. This was repeated with an extended time of 2 minutes. The genomic DNA was eluted from the spin column into a new 1.5mL microcentrifuge tube by adding 100µL ddH_2O (DNase-free) directly onto the spin column membrane, which was incubated at room temperature for 5 minutes and finally centrifuged at 12,000rpm for 1 minute. The genomic DNA sample stored at -20°C for later use.

Appendix 4 Long Ashton solution

100x stock macronutrients

NaH_2PO_4 20.8g L^{-1}

$CaCl_2$ 50.0g L^{-1}

$MgSO_4 \cdot 7H_2O$ 36.9g L^{-1}

K_2SO_4 21.75g L^{-1}

100x stock micronutrients

$MnSO_4 \cdot H_2O$ 0.223 g L^{-1}

Fe EDTA 3.0g L^{-1}

$CuSO_4 \cdot 5H_2O$ 0.024g L^{-1}

$ZnSO_4 \cdot 7H_2O$ 0.029g L^{-1}

H_3BO_3 0.186 g L^{-1}

$(NH4)_6Mo_7O_{24}\cdot 4H_2O$ 0.004g L^{-1}

$CoSO_4 \cdot 7H_2O$ 0.003g L^{-1}

NaCl 0.585g L^{-1}

Stock solutions (100x) of macro- and micro-nutrients were prepared individually. The hydroponic solution was prepared by using appropriate volumes of stock solutions which were diluted to working concentration (1x).

Appendix 5 Concentrations of total RNA assessed by Nanodrop

Readings	Concentration (ng μL^{-1})	260/230	260/280	Average Concentration
Root 1-a	49.3	2.00	2.05	
1-b	48.8	2.26	2.01	245ng μL^{-1}
1-c	49.1	2.04	2.07	
Root 2-a	70.1	2.17	2.07	
2-b	69.7	2.04	2.07	349.8ng μL^{-1}
2-c	70.1	2.11	2.13	

Root 1 and Root 2: RNA sample 1 and sample 2 extracted from wheat roots respectively.

The concentration of each RNA sample was calculated by averaging three readings. The purity of each RNA sample was roughly reckoned based on readings of 260/230 and 260/280 ratios, suggesting these samples were satisfactory to carry on RT-PCR (cDNA synthesis).

Appendix 6 Primers for confirmation of the transgenes

A6.1 Primers for confirmation of the *TaPtf1* transgene

ATGGACTACTCTAATGGTTCTTTCTTTCCTTCATGGCCTGGCAATTCCGCTTCCGAGAA
TTATAGCTTTGTTGATGGTTCAGTGGAATCATATGCAGAAGAAGGAAGTATGCCACCT
ACAGGCTATTTCAGAGCTAGATCAAATCAGAATTTAACATTTGATGAGCATGAACAGA
ACCCTGCTATGCTTGCAAATGGGTGCTTGCCGTACAACACCCAGACTGATCTATTATCT
GGTGAGATTCTGTCAGAGGACAAACATTCCAACAGCCTTATGGAGCTTCCACAACTTC
AGAACAATGGCAGTCTGCAAAGTAATTTAATCCCACCAGGGACTCTTCAGTGCACTTC
AACACCTGGAACATTTGACCTGCAGTTGGATACCCCTGGCCTTCTAGAACTTCCTCATG
CCTTGTCCAGTTCAATTGAAAGCAATGGTAGTGAAGTTTCAGCTTTTCTTGCTGATGTA
CATGCTGTTTCTTCAGCCTCAACTCTGTGCTCGACATTCCAAAATGTTCCTTCCTACAT
GGAACCAGTAAGCCTAGAAGCTTTCAGTTTTCAAGGGATACAAAATGCTGCTATGTTC
AACAATACAAGTCATTCAA(P2)ATGGGAACCTGTCAGTATTTGATGAGGCAACCATGG
CATCACTACATGATAGCAAAGAATTTCTCAGTGGTAGCATCTCATCTTTTGGTACGGCC
GAGCAGTCACAACTAGCTGGTAGTGGTTTGAAGGCTGAACAACAGGAACAAAATGCG
ATGTGCAATATTCCACTCCCTTTCGCTTCTGGTAGTCAGATGGCAGTGAGTGAAGCAC
AAGGGGCAATGATTCCTTCGAAGATAAGCTCAACGATGCATAACAATAAAAGTGAGT
ACCCTGTCCCTATCAGCCATTCTGCTGATGCGCAGAACAAGGCAAATTCAGCTAATGG
AAACAGTGCCAGTGCTAAGCCACGAGCAAGGGCTCGTCGTGGACAGGCAACTGACCC
TCATAGTATTGCTGAACGGCTTCGCAGAGAGAAGATCTCAGAGAGGATGAAAAATCT
CCAAGACCTTGTACCAAACTCAAATAAGGCAGATAAATCATCAATGCTCGATGAAATA
ATTGATTATGTGAAATTTCTTCAGCTTCAGGTGAAGGTCTTAAGCATGAGTAGGCTAG
GAGCTCCCGGGGCAGTTCTTCCCCTCCTTGCAGAATCTCAAACTGAGGGCCGTAGCAA
TTCACCTCTATCATCTCCAACCGCTTCACAAGGGCTTCTGGACGCAGCAGGCCCAGAA
GACAGCTTGGTCTTTGAGCAAGAAGTTATAAAGCTGATGGAAACAAGCATCACAAAT
GCAATGCAGTACCTTCAGAACAAGGGCCTCTGCCTGATGCCTATCGCTCTTGCTTCAG
CCATATCCAACCAGAAAGGCACTTCTGCAGCTGCAATCCCTCCTGAAAGGTGA======
====TTTCCCCGATCGTTCAAACATTTGGCAA(Tnos)TAAAGTTTCTTAAGATTGAATCCT
GTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTTGAATTACGTTAAGCATGTAAT
AATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGC
AATTATACA(NosR2)TTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGA
TAAATTATCGCGCGCGGTGTCATCTATGTTACTAGATCGGGAATTCAAGCTT

** Sequence underlined: primers for PCR screening; P2: forward primer; NosR2: reverse primer
** Sequence highlighted in yellow: fragment of expected PCR products
** Sequence in red: full-length CDS of *TaPtf1*; ** Sequence in black: Nos-polyA
** Black frame: Tnos reverse primer for sequencing
** ===== vector fragment between *TaPtf1* and Nos-polyA

A6.2 Primers for confirmation of the *TaMyb67* transgene

GTTTTATAATTATTTTGATCTTGATATACTTGGATGATGGCATATGCAGCAGCTATATG
TGGATTTTTTTA(Ubi1922)GCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCT
TTTGTCGATGCTCACCCTG(Pubi)TTGTTTGGTGTTACTTCTGCAGGTCGACTCTAGA===
=======ATGAGGAGGTGTGATCTGAGACAGTCTCACAACAGCAGGGTTTCTGGAGGAA
TGTCATCCTCTTTACCTATTCTGCCAAATTCTCTGAAAGAAACCTTCCATGGGCCTTAC
AATCCGCAGCTCACTCCGATGCAAAGGCAACTGACGAGTGATCTTGTGCCCTTACATC
AGAGTGCACTTCCGTCTGCTACTTTGCACCCAAGAGCTGGGGCTATGAGATCATCATA
TGCAGCCTCATTAGGATACTCACCTAATCCTCTTGATTCTGCGCTTAACCATGAGAGGC
AGTCTATGGTTGCTCCTTTTGCTCCTCAGCCATCAAATATTGAAGTATTTCAGACCTTA
TCTAATAATATCCCTGGAGGACACACTGAGGCAACTTGGTTCCCAGGTTCGGCTGATG
GTTTATCAGATTACAGGGATAACATCCCTGCTTCTGGTAGTCAGATCCAGAATGGCGG
TCCTGCTGTGACATCTGATGTGGTTGCTAAACAAAATGAATGGTGGGCAGAGATAATG
AATGATGATTGGAGAGATATTCTAGATGCAACGGCTGCTGATCCCCAGTCAAAGCCTT
CCAATTCGTCTGCATCACAGCCTGCTGTCAACCAGCCAGCTTCATCTCATGGTGGAGA
GATTTGCAATGTAGCTAGTCCTCCCAATAGCAACTCTGCAGCCAAACAACGGATGAGG
TGGACTCCA(M2718)GAACTCCATGAATGCTTCGTAGACTCTGTAAATAAGCTTGGTGG
TAGTGAAAAAGCTACTCCCAAGGGTGTGCTGAAGCTTATGAAAGTTGACGGTTTGACA
ATATATCATGTGAAAAGCCATCTGCAGAAGTACCGAACAGCTCGCTATAAGCCAGACC
TAACGGAAGGTACAGCAGAAAAAAGGACTACCACCGAAGAGTTGACTCTAGACCTGA
AATCGAGCATGGATCTTACTGAAGCGCTGCGTCTTCAGATGGAAGTTCAGAAACGTCT
TCATGAACAACTTGAGACCCAGAGAAAGTTGCAATTGCGAATTGAAGAACAAGGGAA
GTATCTTCAGATGATGTTTGAAAAGCAGTCTAAATCCAATACGGAGAAGGTGCAGGAT
CTATCCTCGGGAGCTACAACAACCCTATCATCTGAACCAAGCCATCCTGCAACCAGAA
ATAGGGGTGATGATGCAGCTGATGACCTAAATAGAACAGGAGAGAACCCCGTGAGTG
CCGAAATAGGAGAAACTTTGATGCATGCAGGTGGCAACCAGGAGATGGCAGAAAGCG
AGTCTTCTGAGCCCCTTGCAAATACTAATGATGGTTCCAAGGCCCCGCAAGAGAAGCG
CTGA

** Sequence underlined: primers for PCR screening; Ubi1922: forward primer; M2718: reverse primer
** Sequence highlighted in yellow: fragment of expected PCR products
** Sequence in red: full-length CDS of *TaMyb67*; ** Sequence in black: ubiquitin promoter
** Black frame: Pubi forward primer for sequencing
** ===== vector fragment between ubiquitin promoter and *TaMyb67*

Appendix 7 Restriction endonuclease analysis of G1390PTF1 and G1390MYB67

A7.1 The formula used to calculate PCR products in Gateway BP reactions

$$\mathrm{ng} = (100\mathrm{fmol})(\mathrm{N})(\frac{660\mathrm{fg}}{\mathrm{fmol}})\left(\frac{1\mathrm{ng}}{10^{6}{}_{\mathrm{fg}}}\right)$$

*N refers to the size of DNA in base pair

* fmol refers to the femtomoles of DNA

A7.2 Restriction endonuclease analysis of G1390PTF1 and G1390MYB67

A double digestion reaction was prepared in a 30μL reaction system, as in the table below, and incubated at 37°C overnight. The reaction was inactivated by incubating at 65°C for 20 minutes. The reaction products were assessed by agarose gel electrophoresis.

	G1390PTF1	G1390MYB67
Reaction 1		
Plasmid (μL)	26	26
XhoI (μL)	0.5	0.5
KpnI (μL)	0.5	0.5
10x buffer M (μL)	3	3
Total (μL)	30	30
Reaction 2		
Plasmid (μL)	27.5	26
KpnI (μL)	0.5	0.5
HindIII (μL)	-	0.5
SphI (μL)	0.5	-
10x buffer M (μL)	-	3
10x buffer K (μL)	1.5	-
Total (μL)	30	30

Appendix 8 Tri-parental mating

For each prep, *E.coli* DH5a that contains the target construct and *E.coli* PRK2013 were revived from glycerol stocks on LB medium+Kana (50mg L^{-1}) plates (37°C, 16 hours) separately. Single colonies were then used to individually inoculate 10mL of LB medium plus Kana (in a working concentration of 50mg L^{-1}) in 15mL falcon tubes, which were then incubated at 37°C+180rpm in a shaking incubator overnight (12–16 hours). *Agrobacterium* C58C1 was also revived from glycerol stocks on a YEP+ Rif (50mg L^{-1})+Gent (100mg L^{-1}) plate (28°C, 48–72 hours). Single colonies were used to individually inoculate 10mL of YEP medium plus Rif (50mg L^{-1}) and Gent (100mg L^{-1}) in a 15mL falcon tube and incubated at 28°C+180rpm in a shaking incubator overnight (12–16 hours). A volume of 100µL culture from each strain was pipetted and mixed into a 1.5mL (autoclaved) microcentrifuge tube. The tube was briefly centrifuged at 5,000rpm for 2 minutes, with the supernatant discarded. The pelleted culture was re-suspended in 100µL of LB medium and then dropped onto the middle of a LB+non-antibiotics plate for co-cultivation at 28°C for 24 hours. An appropriate amount of the incubation products was carefully scraped off by a transferring loop

and re-suspended in 100µL of LB medium. This was used to streak a new YEP+Rif (50mg L^{-1})+Gent (100mg L^{-1})+Kana (50mg L^{-1}) plate, followed by incubation at 28°C for 48–72 hours. Single colonies of *Agrobacterium* were selected from the YEP plate and used to individually inoculate YEP medium plus appropriate antibiotics in separate 15mL falcon tubes, which were then incubated at 28°C+180rpm in a shaking incubator for 12–16 hours before expanded culture with 25–30mL more YEP in 100mL flasks (autoclaved). During incubation, screening PCR was carried out by using compatible primers and was assessed

by agarose gel electrophoresis. Subsequently, positive clones were used for extraction of plasmids, which were assessed by restriction endonuclease analysis and agarose gel electrophoresis. *Agrobacterium* strains of positive clones were also individually incorporated into *Agrobacterium*-glycerol stock by mixing 1mL of the culture with 1mL of 50% glycerol (autoclaved) by vortex in a 2mL microcentrifuge tube, after which the stock was stored at -80°C.

Appendix 9 *Agrobacterium* plasmid extraction

Plasmids of transformed *Agrobacterium* were extracted by using EndoFree Plasmid Mini Kit (CWBio, Beijing, China). A volume of 5mL transformed *Agrobacterium* culture was precipitated by spinning at 8,000rpm for 5 minutes in a 15mL falcon tube and re-suspended with up to 2mL ddH_2O (DNase-free) before transferring into a 2mL microcentrifuge tube. The culture was re-precipitated by spinning at 13,000rpm for 1 minute, after which the supernatant was discarded. The tube was added and mixed with 250µL of buffer P1 (RNase A contained), followed by adding 250µL of buffer P2 and inverting the tube gently for 8–10 times. The mixture was incubated at room temperature for 3 minutes. Next, the tube was added with 250µL of buffer N3 and inverted immediately and gently for 8–10 times, after which the mixture was incubated at room temperature for 5 minutes before spinning at 13,000rpm for 5 minutes. The supernatant was removed to an EndoRemover FM column (placed in a 2mL microcentrifuge tube), which was then centrifuged at 13,000rpm for 1 minute. Following this, the flow-through was added and mixed with 225µL of isopropanol. The mixture was then transferred to a Spin Column DM, before which the column was normalised by using 200µL of buffer PS. The column was centrifuged at 13,000rpm for 1 minute, with the flow-through discarded. Subsequently, the column was added with 750µL of buffer PW and centrifuged at 13,000rpm for 1 minute, with the flow-through discarded. The column was placed back to the collection tube and centrifuged at 13,000rpm for another 1 minute. After replacing the collection tube with a new 1.5mL microcentrifuge tube, the DNA was eluted by adding 50µL ddH_2O (DNase-free) and standing for 2 minutes at room temperature, before spinning at 13,000rpm for 2 minutes. This step was repeated when necessary using the same elution. The *Agrobacterium* plasmid sample was stored at -20°C.

Appendix 10 *Agrobacterium*-mediated transformation, selection and regeneration of transformants - Method I

A10.1 Isolation of immature embryos and pre-incubation

All procedures, if not specified, were carried out in a sterile Lamina flow hood which was sterilised by ultraviolet for 30 minutes before use.

After sterilisation, immature embryos were manually and carefully isolated from the germ area of wheat seeds using sterile forceps and knife (surgical grade). Approximately 60–70 embryos were placed in an array and scattered in a polystyrene petri dish (90x20mm, sterile) containing pre-incubation medium (medium made of 4.3g L^{-1} MS basal salt mix + 30g L^{-1} sucrose + 2.0mg L^{-1} Dicamba + 2.4g L^{-1} phytagel, PH=5.8), with the scutellum oriented upward and the radicle (embryo axis) oriented downward in contact with the medium. The plates were sealed with Parafilm (BioDee, Beijing, China) and incubated at 25°C in the dark for 4 days prior to inoculation (Shaoguan Taihong Medical Apparatus and Instruments Co., Ltd., Guangdong, China).

A10.2 Inoculation and co-cultivation

Agrobacterium C58C1 culture was transferred into a 15mL/50mL falcon tube according to the volume required for inoculation and centrifuged at 3,500rpm for 10 minutes, with the supernatant discarded. The C58C1 culture was re-suspended with the 1/10WCC inoculation buffer (1/10WCC buffer made of 0.43g L^{-1} MS basal salt mix + 6.4mg L^{-1} MS vitamins + 1.95g L^{-1} MES + 0.75g L^{-1} $MgCl_2$ + 78mg L^{-1} acetosyringone + 20 g L^{-1} glucose + 40 g L^{-1} maltose + 2.0mg L^{-1} Dicamba + 4.4mg L^{-1} Picloram + 1.0g L^{-1} glutamine + 200mg L^{-1} Vitamin C + 200mg L^{-1} casein hydrolysate + 100mg L^{-1} AGPs + 0.01% Silwet-77, pH=5.4) in a ratio of 1:1 to make the C58C1 suspension. Approximately 30–40 pre-cultured embryos

were gathered into a 2mL microcentrifuge tube or a polystyrene petri dish (30x15mm, sterile), which was then quickly added with 4mL C58C1 suspension and stood for 30 minutes with the tube/dish occasionally swirled. When the inoculation finished, the fluid phase was pipetted out and discarded, with the embryos left moist but not too wet. The embryos were transferred into the central area of a polystyrene petri dish (90x20mm, sterile) filled with two layers of filter paper (Ø 85-90mm, sterile), following which the plate was sealed with Parafilm and incubated at 25°C in the dark for co-cultivation for 3 days.

A10.3 Callus induction

The isolated embryos were inoculated and co-cultivated with *Agrobacterium* C58C1 containing G1390PTF1/G1390MYB67 as described in the previous section. Subsequently, the embryos were transferred into another polystyrene petri dish (90x20mm, sterile) containing the callus induction medium (medium made of 4.43g L^{-1} MS+Vitamins mix + 30g L^{-1} sucrose + 2.0mg L^{-1} Dicamba + 100mg L^{-1} AGPs + 200mg L^{-1} Carb + 100mg L^{-1} Cef + 2.4g L^{-1} phytagel, PH=5.8). Approximately 30–50 embryos were placed and scattered in one plate to leave space for growth. The plates were sealed with Parafilm and incubated at 25°C in the dark for 5 days to grow calli.

A10.4 Selection and regeneration of transformants

Following callus induction, induced calli were transferred into a new polystyrene petri dish (90x20mm, sterile) containing selection medium (medium made of 4.43g L^{-1} MS+Vitamins mix + 30g L^{-1} sucrose + 2.0mg L^{-1} Dicamba + 100mg L^{-1} AGPs + 200mg L^{-1} Carb + 100mg L^{-1} Cef +2.4g L^{-1} phytagel, PH=5.8 supplement with 10mg L^{-1} Hyg). The plates were sealed with Parafilm and incubated at 25°C in the dark for 21 days to grow and screen calli. After 3 weeks, any callus bearing somatic embryos was transferred to new plates containing

differentiation medium (medium made of 4.43g L^{-1} MS+Vitamins mix + 20g L^{-1} sucrose + 2.0mg L^{-1} Dicamba + 200mg L^{-1} Carb + 100mg L^{-1} Cef + 2.4g L^{-1} phytagel, PH=5.8 supplement with 20mg L^{-1} Hyg). The plates were sealed with Parafilm and incubated at 24°C in the light (45µmol $m^{-2}s^{-1}$, 16hours day^{-1}) for 14 days in a growth room in CAAS. After this, any surviving callus/sprout was removed to plates containing new differentiation medium and similarly incubated at 24°C in the light. The calli were continued to be screened on Hyg, where two selection levels were used, at 35mg L^{-1} and 50mg L^{-1}. Once sprouting shoots were clearly defined and could be separated easily from the calli, they were transferred to new differentiation medium (supplement with Hyg at the same level, either 35mg L^{-1} or 50mg L^{-1}) and then incubated at 24°C in the light. Following differentiation, the seedlings were carefully separated and transferred to glass tubes containing rooting medium (capped and autoclaved, medium made from 4.43g L^{-1} MS+Vitamins mix + 20g L^{-1} sucrose + 2.0mg L^{-1} Dicamba + 200mg L^{-1} Carb + 100mg L^{-1} Cef + 2.4g L^{-1} phytagel, PH=5.8).

A10.5 Histochemical staining analysis

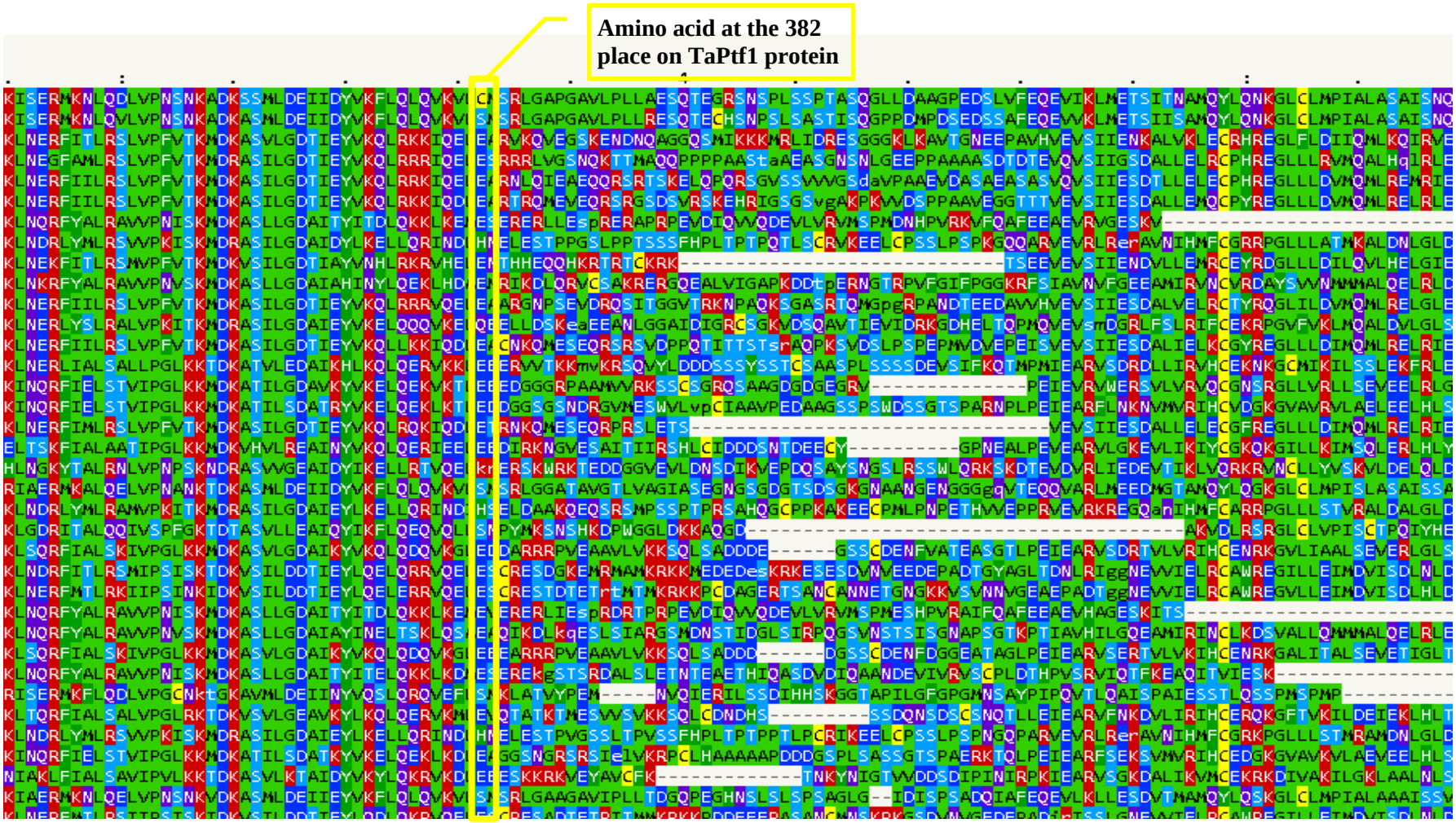
Immature embryos were isolated, pre-incubated, inoculated and co-cultivated with GUS290-contained *Agrobacterium* C58C1 using the method as described above, after which two to three embryos were randomly selected from each plate to be examined by histochemical staining using X-Gluc. Randomly selected embryos were placed into a 2mL microcentrifuge tube and added with 1mL of X-Gluc buffer. The tube was incubated at 37°C overnight (12–16 hours). The buffer was replaced with 70% (v/v) ethanol to stand for another 48 hours before the assay result was recorded.

Appendix 11 Concentrations of purified PCR products amplified by *att*B primers

Sample/Measurement	*att*B-*TaPtf1*	*att*B-*TaMyb67*
Measurement 1 (ng µL^{-1})	23.6	62.9
Measurement 2 (ng µL^{-1})	22.5	62.2
Average (ng µL^{-1})	23.05	62.55

The concentration of each *TaPtf1* or *TaMyb67* (added with *att*B adaptors) DNA sample was calculated by averaging two readings. In addition, the purity of each DNA sample was roughly reckoned based on readings of 260/230 and 260/280 ratios, suggesting these samples were satisfactory to carry out the downstream Gateway cloning.

Appendix 12 Protein alignment analysis of TaPtf1 by PHYRE2.0



***The results of Position-Specific Iterated (PSI) BLAST produced from PHYRE2.0 by using TaPtf1 (GenBank) sequence as a query. C: Cys. (Cysteine); S: Ser. (Serine); other letters in the figure stand for their corresponding amino acids.**

Appendix 13 Restriction endonuclease analysis of 3301PTF1 and 3301MYB67 by XhoI

A single digestion reaction using XhoI was prepared in a 25μL reaction system, as in the table below, and incubated at 37°C overnight. The reaction was inactivated by incubating at 65°C for 20 minutes. The reaction products were assessed by agarose gel electrophoresis.

	3301PTF1	3301MYB67
Plasmid(μL)	22	22
XhoI (μL)	0.5	0.5
10x buffer H (μL)	2.5	2.5
Total (μL)	25	25

Appendix 14 Transformation of *Agrobacterium* competent cells

Each stock of 50µL competent cells of a disarmed-T_i *Agrobacterium* strain EHA105 was thawed on ice from -80°C and added with 3µL (<1ng) of the target plasmid DNA. This was followed by incubation on ice for 30 minutes before the tube was frozen in liquid nitrogen for 2 minutes. Immediately after this, the tube was placed back in a 37°C water bath for 3 minutes and on ice for 2 minutes. Subsequently, 450µL of YEP medium was mixed to the competent cells in the tube and incubated in a 28°C+150rpm shaker-incubator for 3 hours. The tube was centrifuged at 5,000rpm for 2 minutes, with the supernatant discarded. The pelleted culture was re-suspended to 100µL, which was then spread onto a YEP Rif (50mg L^{-1})+ Kana (50mg L^{-1}) plate with a sterilised "hockey puck" spreader and incubated at 28°C for 48–72 hours. Single colonies of *Agrobacterium* were selected from the YEP plate and used to inoculate YEP medium plus appropriate antibiotics in separate 15mL falcon tubes, which were incubated at 28°C+180rpm in a shaking incubator for 16-18 hours before expanded culture with 25–30mL more YEP in 100mL flasks (autoclaved). During incubation, screening PCR was carried out by using compatible primers and was assessed by agarose gel electrophoresis. Subsequently, positive clones were used for extraction of plasmids, which were assessed by restriction endonuclease analysis and agarose gel electrophoresis. *Agrobacterium* strains of positive clones were also individually incorporated to *Agrobacterium*-glycerol stock by mixing 1mL of the culture with 1mL of 50% glycerol (autoclaved) by vortex in a 2mL microcentrifuge tube, after which the stock was stored at -80°C.

Appendix 15 *Agrobacterium*-mediated transformation, selection and regeneration of transformants - Method II

A15.1 Isolation of immature embryos and pre-incubation

All procedures, if not specified, were carried out in a sterile Lamina flow hood which was sterilised by ultraviolet for 30 minutes before use.

After sterilisation, immature embryos were microscopically and carefully removed from the germ area of wheat seeds using a pair of sterile forceps (surgical grade). Approximately 50–60 embryos were placed one by one into a 2mL microcentrifuge tube containing the modified-1/10WCC inoculation buffer (to prevent dehydration of embryos).

A15.2 Inoculation and co-cultivation

Agrobacterium EHA105 culture was transferred into a 15mL/50mL falcon tube according to the volume required for inoculation and centrifuged at 3,500rpm for 10 minutes, with the supernatant discarded. The EHA105 culture was re-suspended with the modified-1/10WCC inoculation buffer (modified-1/10WCC buffer: modified from 1/10WCC inoculation buffer as previously described, unpublished by CAAS) in a ratio of 1:1 to make the EHA105 suspension. The 2mL microcentrifuge tube carrying the immature embryos in the buffer was centrifuged at 7,500rpm for 10 minutes at 4°C, followed by the buffer pipetted and discarded. The tube was added with 1.5–2mL EHA105 suspension and stood for 5–10 minutes with the tube occasionally and gently inverted for 3–5 times. When the inoculation finished, the fluid phase was pipetted out and discarded, with the embryos left moist but not too wet. Approximately 30–40 embryos were scattered into a polystyrene petri dish (90x20mm, sterile) containing co-cultivation medium (medium: modified-1/10WCC + 1.95g L^{-1} MES + 15g L^{-1} agarose, PH=5.8, unpublished by CAAS), with the scutellum

oriented upward and the radicle (embryo axis) removed to prevent precocious germination. The plates were sealed with Parafilm and incubated at 25°C in the dark for co-cultivation for 2 days.

A15.3 Callus induction

The isolated embryos were inoculated and co-cultivated with *Agrobacterium* EHA105 containing 3301PTF1/3301MYB67 as described in the previous section. Subsequently, the embryos were transferred into another polystyrene petri dish (90x20mm, sterile) containing modified-callus induction medium (medium: modified from callus induction medium as previously described + 1.95g L^{-1} MES + 15g L^{-1} agarose, PH=5.8, unpublished by CAAS). Approximately 30–50 embryos were placed and scattered in one plate to leave space for growth. The plates were sealed and incubated at 25°C in the dark for 5 days to grow calli.

A15.4 Selection and regeneration of transformants

Following callus induction, induced calli were transferred into a new polystyrene petri dish (90x20mm, sterile) containing modified-selection medium (medium: modified from selection medium as previously described + 1.95g L^{-1} MES + 15g L^{-1} agarose, PH=5.8, supplement with 5mg L^{-1} PPT, unpublished by CAAS). The plates were sealed and incubated in the dark at 25°C for 14 days to grow and screen calli. After 2 weeks, any callus bearing somatic embryos was transferred to new plates containing modified-differentiation medium (medium: modified from differentiation medium as previously described + 1.95g L^{-1} MES + 15g L^{-1} agarose, PH=5.8, supplement with 10mg L^{-1} PPT, unpublished by CAAS). Approximately 20–30 embryos were placed and scattered in one plate to leave space for growth. The plates were sealed and incubated at 25°C in the dark for another 14 days to screen and grow sprouts. After this, any surviving callus with

sprouts was cautiously and partially separated (in order to allow more surface to be exposed to light), which was placed into plates containing new modified-differentiation medium (medium: modified from differentiation medium as previously described + 1.95g L^{-1} MES + 15g L^{-1} agarose, PH=5.8, supplement with 5mg L^{-1} PPT, unpublished by CAAS) and incubated at 25°C in the light (90μmol $m^{-2}s^{-1}$, 16hours day^{-1}) for another 14 days (depending on growth rate and size) in a growth room in CAAS. When the sprouts grew into bigger plantlets, they were carefully removed from the remaining parts, without any injury to the bottom, and transferred to a polycarbonate tissue culture box (78x78x95mm, 36cm^2, filter membrane) containing modified-rooting medium (medium: modified from rooting medium as previously described + 1.95g L^{-1} MES + 15g L^{-1} agarose, PH=5.8, supplement with 5mg L^{-1} PPT, unpublished by CAAS).

A15.5 GFP fluorescence imaging analysis for transformation efficiency

Immature embryos were isolated, inoculated and co-cultivated with 16318hGFP-contained *Agrobacterium* EHA105 using the method as described above and then cultured on modified-callus induction medium for 5 days at 25°C in the dark. Induced calli were selectively used to prepare samples for fluorescence imaging analysis, for which the calli were cut into thin pieces and placed onto a slice glass under a cover slice, which was then observed on a Zeiss LSM700 confocal laser scanning microscope according to manufacturer's instructions.

Appendix 16 Bar assay by using LibertyLink (bar) QuickStix Kit

For each sample, a piece of 3cm wheat young leaf was collected into a 1.5mL microcentrifuge tube (extraction tube) 1) not containing mill balls; 2) containing mill balls (3x2mm). The leaf was then 1) manually ground by pushing the leaf down to the bottom of the tube with a disposable plastic pestle (provided in the kit) and twisting the pestle around against the sides of the tube (a few more times of grinding were repeated until the leaf tissue was well ground); or 2) frozen in liquid nitrogen and well ground by Bullet blender. The tube was then added with 0.5mL of extraction buffer (warmed to room temperature before use) and mixed well. A QuickStix strip was removed from canisters (warmed to room temperature before use) and placed into the extraction tube to allow the sample to travel up the strip. The extraction was stood for 10 minutes' reaction-time and the assay result was recorded. The strip was retained and labelled after the bottom section being cut off and discarded.

Appendix 17 Hot acid digestion

Seed and non-seed-portion samples were digested separately in two experiments. Approximately 200mg samples of finely ground seed or non-seed-portion samples were individually weighted into vessels (with smart vent), followed by carefully and sequentially adding 6mL of Conc. (seeds: 3mL of Conc. HNO3, 3mL Milli-Q H_2O and 2mL of 30%; 100 Vols H_2O_2) in a fume cupboard. The closed vessel microwave digestion for the 42 samples plus two blanks was completed with a 24-place Multiwave PRO microwave (ANTON PAAR Ltd., Unit F, The Courtyard, St Albans, UK). Once the digestion was complete, the digestion vessels were individually opened in the fume cupboard to exhaust gas slowly and safely. Following this, 4mL Milli-Q H_2O was pipetted into each digestion vessel. The mixture of each vessel was then transferred into a 25mL clean universal tube. Each vessel was rinsed by 5mL (seeds: 3mL) Milli-Q H_2O, after which the fluid was transferred to the corresponding universal tube to make up a volume of 20mL (seeds: 15mL) for each sample. The samples were diluted 5 times prior to ICP-MS analysis.

Appendix 18 Restriction endonuclease analysis of ZePTF1/ZeMYB67 and 16318PTF1/ 16318MYB67

A double digestion reaction was prepared in a 25µL reaction system, as the table below, and incubated at 37°C overnight. The reaction was inactivated by incubating at 65°C for 20 minutes. The reaction products were assessed by agarose gel electrophoresis.

	ZePTF1	ZeMYB67
	16318PTF1	16318MYB67
Plasmid (µL)	24.5	24.5
BamHI (µL)	0.5	0.5
SalI (µL)	0.5	0.5
10x buffer T (µL)	4.5	4.5
Total (µl)	30	30