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Influence of drought stress on pollen viability and fertility

Amira Elsaid Mohammed Hassan

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Declaration

I hereby declare that all the studies in this thesis is my own work, except were acknowledgement is made by reference. The work described here has not been submitted anywhere for any other degree of qualification. All assistance given to me during the preparation of this thesis is also acknowledged.

Amira E. Hassan

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I am extremely grateful to my father Elsaid who always telling me "a journey of a thousand miles begins with a single step", my lovely mother Amany for her love and support and my two brothers Mohamed and Ahmed and my sister Asma for their continuous support.

Abstract

Pollen viability is important in fertilization. Drought is one of the greatest stresses that affects food security (Wilhite *et al*, 1985). Yield loss vulnerability is closely correlated to pollen viability, with a significant reduction in pollen viability having a major impact on seed set. Genes involved in this process still are largely unknown. In *Arabidopsis thaliana* the expression of thirty genes, before and after pollen mitosis I associated with drought stress were examined using qRT-PCR under severe drought stress. Some genes showed specific expression patterns and regulation to drought at specific stage of pollen development. A subset of seven genes that showed altered expression in the anther during drought stress were studied by modifying their expression by knock out and overexpression lines, the candidate genes have the following locus identifiers: AT1G52570, AT4G36600, AT3G28980, AT2G24450, AT3G20220, AT3G23770 and AT1G63060. The overexpression lines of AT3G20220 displayed pleiotropic phenotype such as extreme dwarf, short stamen filament, low number of pollen grains, stem architecture and internode defects, changes in the inflorescence branch angles. AT1G52570 displayed an early flowering phenotype. AT3G23770 has a role in callose wall degradation and exhibited pleiotropic phenotype such as early anther dehiscence, internode defects, siliques and inflorescence pattern defect. Other lines showed

impacts on flowering time and male fertility. Selected T-DNA insertion/knock out lines exhibited short siliques and inviable pollen (AT3G20220, AT2G24450 and AT4G36600).

Fluorescent protein reporter lines that monitor Ca^{+2}, pH and auxin facilitating their subcellular localisation and signalling were studied during drought stress.

In summary, the candidate genes play a role in pollen maturation and anther dehiscence. However, their role at the cellular level and regulatory pathway associated with drought requires further investigation.

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Abbreviations

bp: base pair
BC: Bicellular
TC: Tricellular
CaMV: Cauliflower Mosaic Virus
cDNA: complementary Deoxyribonucleic Acid
DNase: deoxyribonuclease
dNTP: Deoxynucleotide triphosphate
dUTP: Deoxyuridine triphosphate
EDTA: Ethylenediaminetetraacetic acid
FRET: Förster resonance energy transfer
G: gram
GFP: Green Fluorescent Protein
HCl: Hydrochloric acid
HM: Homozygous
HT: Heterozygous
Kb: kilobase pair
LB: Luria Broth
min: minute
ml: millilitre
mM: milimolar
mRNA: messenger Ribonucleic Acid
MS: Murashige and Skoog Basal Medium
MS35: MALE STERILE35
NASC: Nottingham Arabidopsis Stock Centre
PBS: Phosphate buffered saline
PCR: Polymerase Chain Reaction

PMI: Pollen Mitosis I

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

RT: Room Temprature

TAIR: The Arabidopsis Information Resource

T-DNA: Transferred-Deoxyribonucleic Acid

TF: Transcription Factor

Wt: Wild type

YFP: Yellow Fluorescent Protein

µg/ml: microgram per millilitre

µl: microlitre

µM: micromolar

Callase: Glucan endo-1,3-b-D-glucosidase

Callose: b-1,3-glucan

mm: millimetre

Kan: Kanamycin

PCD: Programmed Cell Death

LEA: late embryogenesis abundant

RPKM: Reads Per Kilobase of transcript per Million

Chapter 1 Introduction

Seventy-five years ago, the German scientist Laibach first suggested the weed, *Arabidopsis thaliana* as a model plant. His collection of seeds in 1960s become a source for many worldwide *Arabidopsis* centers including The Nottingham (NASC) (Laibach., 1965; Kranz, 1978 Cited in Somssich, 2018). *Arabidopsis* is a small, rosette plant (Figure1.1) that belongs to the taxonomic family of the Brassicaceae in the eudicotyledonous group. It is distinguished by a short life cycle (6 weeks) from seed germination until seed formation, self-pollination, with high amount of seeds and a genome which was sequenced in 2000 (The *Arabidopsis* Genome Initiative 2000). It has been used extensively to help understand plant development and molecular biology including signalling mechanisms that occur during flowering which are very well known. Although much is known about floral development in a broad molecular context, there is still much to learn about the molecular genetics and the biochemical details of floral organ development.

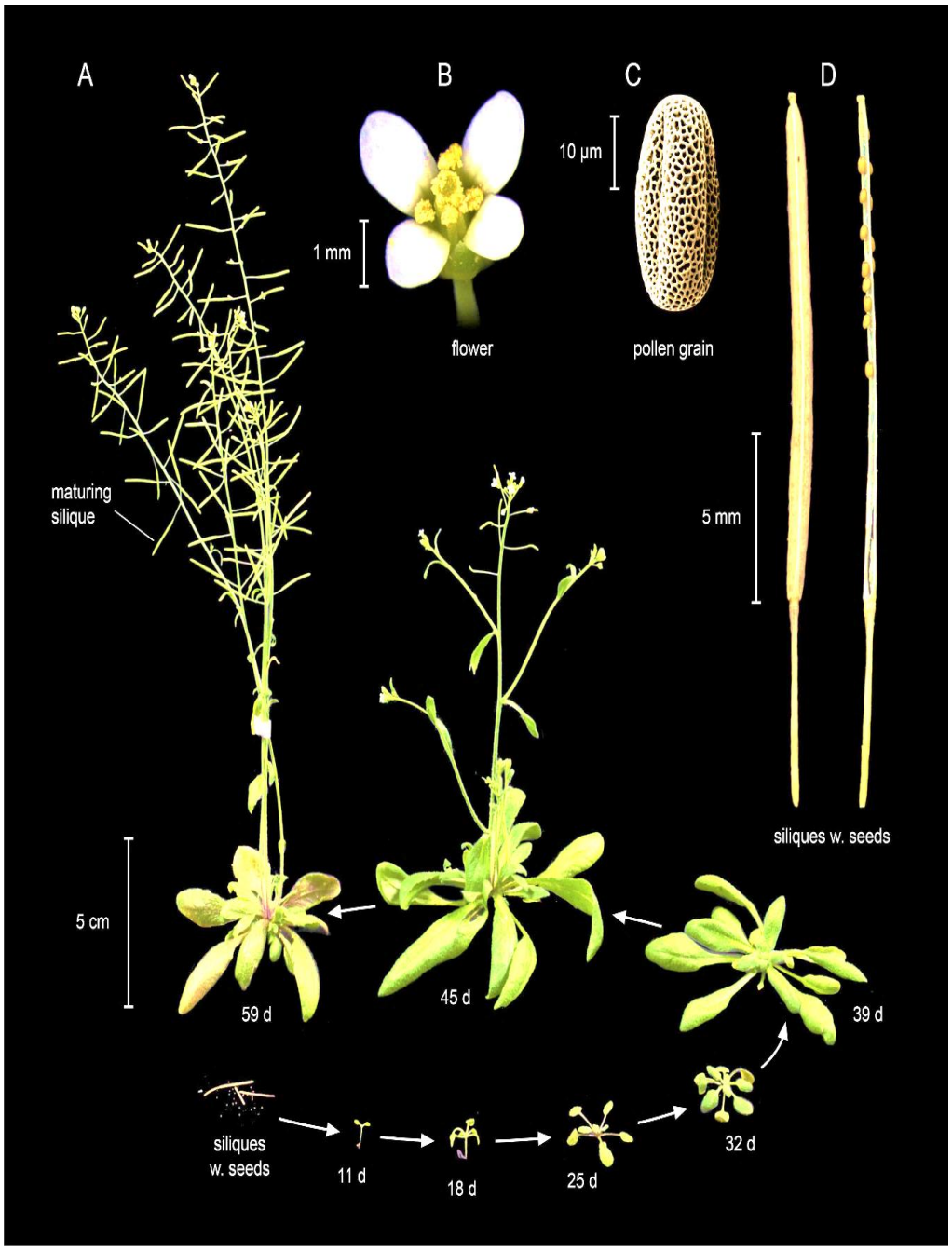


Figure 1.1 Life cycle of *Arabidopsis thaliana* Columbia. (A) Mature *Arabidopsis thaliana* with siliques on, (B) a flower, (C) a pollen grain (scanning electron micrograph), and (D) mature siliques. Image credits: B and C, Maria Bernal and Peter Huijser; other photographs, Ines Kubigsteltig and Klaus Hagemann, Image taken from (Krämer, 2015).

1.1 Flower development

Arabidopsis flowers are acropetal, meaning that the oldest flowers are at the base of the inflorescence, and the new flowers are at the tip. Sepals, petals, stamens and carpels are the four whorls of *Arabidopsis* flower. Stamen considered as the third whorls, which are responsible for pollen production, while carpels produce ovules and are in the fourth whorl. A complex network and overlapped signalling control the process of flower development. The developmental stages from floral initiation until bud opening, it takes twelve stages of development while plant continues growth until the silique dehiscence (Smyth et al., 1990; Alvarez-Buylla., 2010). The transition from inflorescence meristem into floral meristem announces the beginning of the floral development which depends on several induction signals such as temperature and photoperiod (Williams, 1959c).

1.2 Male gametophyte development

Flower and anther development are divided into 14 stages (Sanders et al., 1999). The anther is composed of four layers, the epidermis, endothecium, middle layer and the innermost tapetum layer, surrounding the central region where pollen grain development take place (Figure 1.2). At flower stage 6 each microspore mother cell in the anther undergoes a meiotic division and produces a tetrad surrounded by a callose wall with four haploid microspores. Callase enzyme secreted by the tapetum separates the tetrads to form free microspores (Scott et al., 2004). From flower stage 9 uni-nucleate

microspores develop into meiocytes inside the loculus, which is surrounded by the tapetum providing nutrition for pollen development. Meiocytes undergo a mitotic division named as Pollen Mitosis I(PMI) at this stage the pollen coat is synthesized. These polarize, with a large vacuole at one end and the nucleus at the other, preparing the microspore for an asymmetric cell division. The cell plate formed during pollen mitosis I has a distinctive hemispherical shape, and after division is completed the smaller generative cell detaches from the cell wall and is enveloped within the vegetative cell (Paupiere et al., 2014). The generative cell undergoes Pollen Mitosis II (PMII) of cell division to form the two sperm cells of the mature pollen grain (Figure 1.3). Pollen grains start to accumulate starch during their final stages of the development (Koonjul *et al.,* 2004). Mature pollen is then released into environment (dehiscence) the movement of pollen grains onto the stigma usually occurs by wind, water or insects. Germination then occurs by the formation of a pollen tube through a pore in the pollen wall until it reaches the embryo sac. One special feature of pollen grains is their wall which is different from any other plant cell. The pollen wall is multi-layered and consists of materials that are highly resistant to degradation, as important determine for pollen viability.

On the surface of the pollen wall is a layer called the pollen coat (Taylor and Helper, 1997). This layer is sticky and contains lipids, proteins and phenolic compounds. It is believed to be important for pollen transfer and as well as protection against UV- radiation, and

the lipids and proteins are thought to be important in pollen stigma interaction (Dickinson *et al*., 2000). After dehiscence, the pollen is dehydrated and become inactive however some pollen grains are still partially hydrated and metabolically active (Nepi *et al*., 2001).

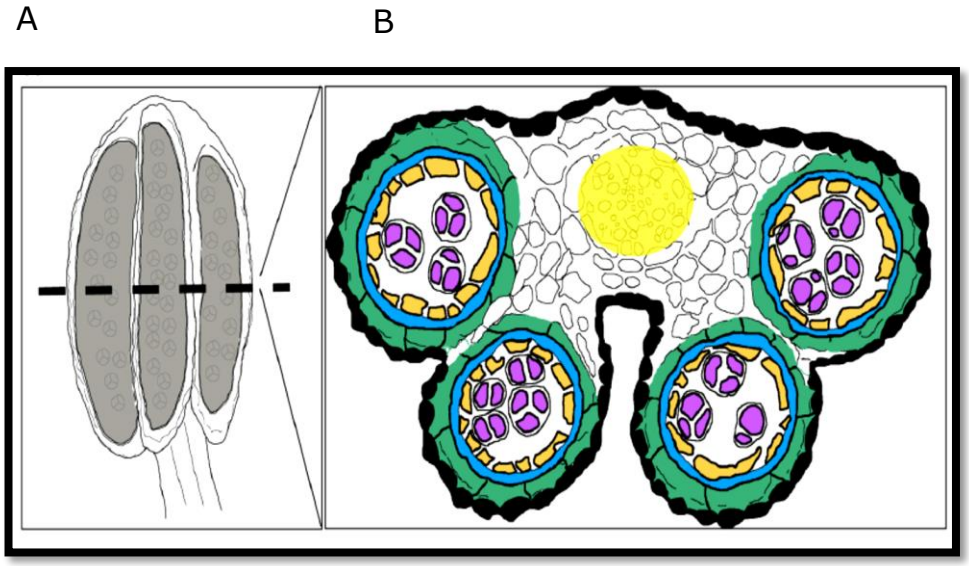


Figure 1.2 Overview of a stage 7 stamen and its internal structure. (A) Section of the stamen (B) Close look up to anther section showing cell layers highlighted in different colors. Black: Epidermis; Green: Endothecium; Blue: Middle layer; Orange: Tapetum; Purple: Gametophyte; White: Connective tissue; Yellow: Procambium, (Image taken and adapted from Estornell et al., 2018)

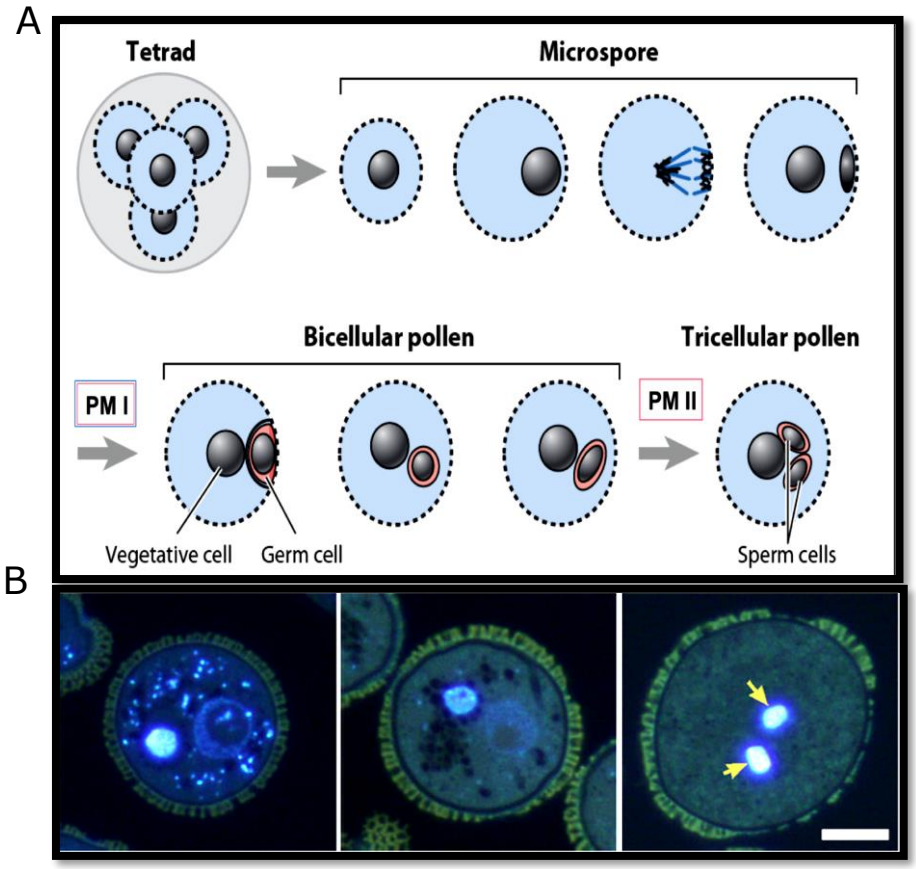


Figure 1.3 Male gametophyte development. A) Pollen meiosis to produce a tetrad of haploid microspores. Microspore stage, where nuclear migrate and the asymmetric spindle formed followed by pollen mitosis I (PMI) to produce bicellular pollen, contains a small germ cell and large vegetative cell. Pollen mitosis II (PMII) occurred to produce twin sperm cells. (Image adapted from Berger and Twell, 2011). **B**) DAPI-stain of bicellular and tricellular pollens. Strong signals are indicated by arrows in tricellular pollen and sperm nuclei, Bars = 5 mm. (Image taken and adapted from Matsushima, et al, 2011)

1.3 The impact of global warming on crop yield

Drought and heat waves threaten crop yield (Lesk et al 2016, Schaubergeret al 2017). Naturally occurring droughts are becoming more frequently and intense due to climate change and global warming (Trenberth et al., 2014). It has been recognized that drought affects up to a third of all arable land and it is one of the largest threats to food security (Wang et al., 2003). Billions of people rely on cereals crops such as rice, wheat and maize which are impacted by drought; under drought stress wheat (*Triticum aestivum* L.) showed a yield decrease less than of 25%, while Rice (*Oryza sativa* L.) was reduced by 50% (Bakul et al., 2009; Shamsi and Kobraee.,2011; Daryanto et al.,2017). The past two decades have witnessed increasing global concern with the need for sustainable water, water security and land management. Water stress is not only due to water loss but also salinity, high temperature and severe cold are the main factors that affect plant water absorption. Each stress acts through a complex cellular and molecular stress response system to minimize cell damage. 'Agricultural drought' can be defined as moisture deficits in the root zone (Solomon et al., 2007). Approximately 70 % of all available fresh water resources are used to irrigate crops, this is expected to increase over the next twenty years due to global

population expansion, changes in climatic conditions and increased requirements for water resources (Rost et al., 2008 cited in Bonsch Markus et al., 2015). Food production rates by 2050 are predicted to leave from 2 to 3 billion people hungry (Wheeler and Von Braun, 2013). Drought deficit has a great impact on plant growth and development reducing growth and cell division rate, leaf size, stem and root elongation, disturbance of stomatal closure and leaf growth (Li et al., 2009; Dubois et al., 2017). Reproductive development is especially sensitive to drought (Zinselmeier et al., 1995; Samarah et al., 2009), and changes in the reproductive programme, alerted flower development and flower number, panicle extension, anther dehiscence and pollen abortion all impact on seed set and yield (Prasad et al., 2008; Al-Ghzawi et al., 2009). Floral meristem development in rice, wheat and barley were shown to be affected by water desiccation, resulting in a reduction of spikelet initiation and floral abortion (Dolferus et al., 2011). A reduction of pollen grain viability in *Zea mays* L. and *Trigonella moabitica*_ (Schoper 1986; Al-Ghzawi et al. 2009), pollen germination, pollen tube growth (Lee., 1988) and reduced maize mega-gametophyte fertility (Young et al., 2004) have also been observed.

Drought before and after anthesis is particularly important as it affects grain numbers and grain size which are major determinants of yield productivity (Sanjari Pireivatlou and Yazdansepas., 2010; Liu et al., 2015). Generally, stress affects carbohydrate metabolism in the plant by decreasing the photosynthesis rate (Efeoğlu et al., 2009). Drought reduces anther sink strength and inhibits pollen grain development (Ji et al., 2010). Because of disturbtion sugar accumulation in pollen during drought (Lalonde et al., 1997, Koonjul et al., 2004).

1.4 Plant responses to drought

Understanding plant responses to drought stress is therefore of crucial importance for improving plant breeding and management techniques in agriculture. In broad plant physiological terms, plant responses to water stress have been defined as escape, avoidance, and tolerance mechanisms (Levitt, 1980; Ludlow, 1989). In" Escape mechanisms" plants complete their life cycle by speeding up flowering and setting seeds before drought become critical. "Avoidance mechanisms" aim to limit transpiration and enhance water uptake to avoid dehydration, adaptations for this strategy include longer roots, a waxy cuticle, sunken stomatal or early flowering (Taiz and Zeiger, 1991; Bray *et al.*, 2000).

One example was *DEEPER ROOTING 1* (*DRO1*) in rice and its paralog in *Arabidopsis* was found to be beneficial for enhancing root system architecture and in turn increase drought tolerance the harvest rate, the deeper penetration into the soil layers the more moisture the plant could find (Uga et al., 2013; Guseman et al., 2017). Abiotic plant stress responses have been described in four phases (1) alarm phase which starts with the stress reaction leading to functional decline and prepares the plant for the resistance phase;(2) resistance phase;(3) exhaustion;(4) regeneration phase in which plant regenerate its physiological function for survival after stress removal before dying (Lichtenthaler, 1988). Drought affects different aspects of plant physiology, from cell membrane modification to cell wall architecture, cell cycle and cell division changes (Krasensky & Jonak, 2011). These occur alongside a complex series of signals and pathways involved in alerting gene expression to cope with the coming stress, for example activation of ion channels, kinase cascades, reactive oxygen species, hormones accumulation like salicylic acid, ethylene, jasmonic acid and abscisic acid (Velázquez, 2013).

1.5 Plant developmental process

Photosynthesis in leaves is arrested by water desiccation, although respiration continues. During periods of photosynthetic activity, the plant utilizes stored carbohydrate reserved until resumption of photosynthesis (Ruan et al., 2010). Drought can reduce leaf area development, leaf size and leaf dry matter accumulation; under mild water stress, shoot growth is restricted while root growth continues (Burke., 2007). However, drought potential and duration affect the progression of developmental phases for example the length of period from floral initiation to anthesis is decreased by moderate drought and increased by severe stress, in severe drought stress panicle development can be stopped at any stage until flowering (Prasad et al., 2008). Moreover, pollen grains fail to accumulate starch and lose viability with ovule malfunction, thus causing sterility of the plant. Drought also shortens the time required for siliques growth and grain filling, so consequently seed numbers decrease (Prasad et al., 2008). A series of gene signals are triggered as a response to water stress, however the physiological mechanisms of failure of fertility under drought is still not well understood. It is important to know whether the drought stress is controlled by changes in carbon/nitrogen flux, or it is related to the whole plant signalling system involving hormones like abscisic acid (ABA) (Prasad et al., 2008).

1.6 Pollen development during drought

Water balance has a strong influence on the viability of mature pollen. The period from tetrad release to the first pollen mitosis is the formation period of the two microspore walls exine and intine. Pollen size was observed to be decreased by drought as a conseques of water loss (Pacini., 2000), to cause early anther dehiscence and to trigger pollen wall folding in to prevent from further desiccation (Pacini.,1990). A possible explanation for this is that the wall, which is known for its role in the uptake and loss of water, is making changes to pollen volume (Franchi et al., 2011). Other reasons could be related to pollen vacuoles that are thought to play an important role during drought stress (Li et al., 2008). Vacuoles that appear throughout pollen development help pollen to increase in volume due to water storage and osmolite accumulation during pollen wall formation and allow a high rate of water and solute movement across its membrane (Kaldenhoff and Fischer. 2006). The presence of locular fluid, which separate the microspores from the maternal anther cells may also contribute to the high sensitivity of the developing pollen grains to drought; locular fluid was shown to store sucrose, glucose and fructose during pollen development (Castro and Cle´ment., 2007). Pollen grains may differ in their water and carbohydrate contents, depending on pollen position inside the locule and the effects of different environmental changes (Firon, et al, 2012). The nature of damage and the impact on yield depends on degree, duration and the

particular stages that stress occurs (O'Toole & Moya, 1981). The most vulnerable stage to water deficit is during the meiosis of pollen mother cells (PMCs) causing a decline in grain set and a number of engorged pollen (Dolferus et al., 2011; Liu et al., 2015), and/or reduce grain size (Sanjari Pireivatlou and Yazdansepas, 2010), While drought during anthesis inhibits anther dehiscence and represses anther development (Saini & Westgate, 1999). Despite the changes occurring to anthers during drought stress such as presence of starch in the connective tissue where it normally not exists. More than 70% of the stressed anthers still contained microspores (Lalonde *et al*., 1997). A review by Storme and Geelen (2014) suggests that abnormal tapetum development caused by drought is a primary reason for pollen sterility. In wheat plants, under water deficiency pollen microspores developed normally until the first pollen mitosis then lose contact with the tapetum (Ji et al., 2010; Saini et al., 1984). Wheat (Triticum aestivum L.) showed sensitivity to drought (Ji et al. 2010), as well as various abiotic stresses such as heat and oxidative stress (Chen et al., 1999; Dumont et al., 1999). The tapetum is a layer of cells surrounding anther gametophytes (Wilson and Zhang, 2009), which is important to support of pollen development (Pacini et al., 1985). Moreover, it is thought to play a vital role in providing pollen with carbohydrates, and in the signalling, structure involved in the sugar pathway (Golberg et al., 1993). Several scientists describe the importance and the sensitivity of tapetum to abiotic stress; for

example, wheat (Lalonde et al., 1997; Saini et al., 1984) and rice (Ji et al., 2010). With any interruption to tapetum function causing inviable pollen (Mamun et al. 2006; Parish and Li., 2010). In some pollen grains were sterile due to a failure to accumulate starch during the final stage of development (Figure 1.4 D, E) (Saini et al. 1984). For cereal crops, the extent to which drought stress reduces grain yield largely depends on the developmental stages during which stress is experienced (Dolferus et al., 2011). In conclusion, flower development during drought stress before /at anthesis caused early embryo abortion, by disruption of the following processes; microspores, pollen viability, stigmatic receptivity, anthesis, pollination, pollen tube growth and fertilization, which are highly susceptible to both drought and heat stress (Prasad et al., 2008). This leads to a lower number of seeds and reduced crop productivity. Nevertheless, a few pollen grains are still able to divide and continue growing to mature pollen. Seed number and size are the main determinants of yield potential (Reynolds et al., 2009). Great interest has been given to improve yield potential under drought by increase the partitioning of assimilates to the developing grain during grain fill, which stabilizes grain size (Xue et al., 2008).

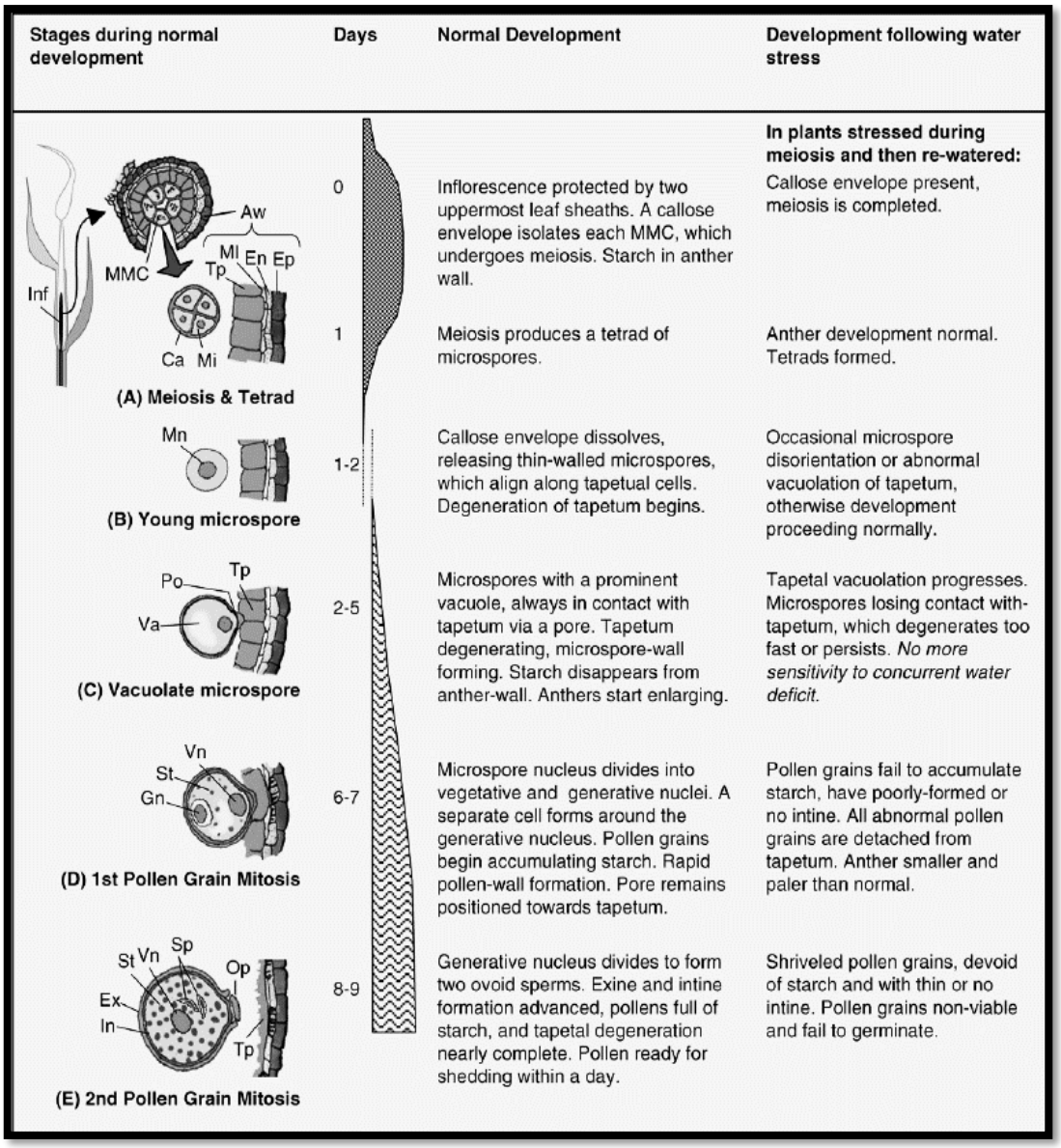


Figure 1.4 Summary of pollen development in normal wheat anthers, and under water deficit. Abbreviations denote: Aw, anther wall; Ca, callose; En, endothecium; Ep, epidermis; Ex, exine; Gn, generative nucleus; In, intine; Inf, inflorescence; Mi, microspore; Ml, middle layer; MMC, microspore mother cell; Mn, microspore nucleus; Op, operculum; Po, pore; Sp, sperm; St, starch; Tp, tapetum; Va, vacuole; Vn, vegetative nucleus. The information is adapted from Saini et al. (1984) and Lalonde et al. (1997) cited in (koonjul et al ,2005).

1.7 Molecular mechanisms activated during drought

When a plant is hunting for water due to environmental changes, a cascade of signalling between two pathways, growth and defence, exist. Thus, roots change their direction and architecture in a process called tropisms to stabilize their water content or to give signals to reduce growth rate (Dietrich.,2018). Root cortex has been identified as a sensor water zone (Dietrich et al., 2017). Genes involved in this process could be important targets for drought tolerance. However, increasing crop productivity has always been challenging as stress-tolerant plants often show low growth rates (Chapin, 1991). Hormones such as auxin, ABA and cytokinin (Moriwaki et al., 2012; Saucedo et al., 2012), jasmonic acid (JA), and ethylene (ET) and secondary messengers like calcium and transcription factors are known to play vital roles in the regulation of drought response (Nakashima, et al.,2013). ABA plays a key role in stress responses, while auxin plays a major role in promoting plant growth as well as brassinosteroids by activation of the transcription factors BRASSINAZOLE RESISTANT1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1) (Yang et al., 2016). First sign of plant defence against drought occurs when ABA signals translocated from the roots to the rest of the plant to close stomata, during this process a huge number of stress-related genes are switched off/on (Harb, A., et al., 2010; Philippe et

al., 2010). Plant responses to drought stress depend on the species and its developmental stage alongside with the degree of the drought (Gall et al., 2015). The timing of stress at flowering stage has a tremendous effect on productivity. Genes encoding late embryogenesis abundant (LEA) proteins function in protecting proteins and membrane from dehydration (Ingram and Bartels, 1996). Heat shock proteins believed to improve drought tolerance, HSFA1b is considered as a master regulatory in response to abiotic stress and (O'Toole, 1994; Albihlal et al., 2018). Aquaporins play an important role for water distribution among different tissues (Almeida-Rodriguez, et al.,2010).

1.8 Phytohormones Abscisic acid (ABA)

The hormone abscisic acid (ABA) is one of the key growth hormones controlling the pathways involved in dormancy and stress responses (Panjabi et al., 2010). ABA accumulates in plant tissues during drought controlling stomatal closure, enhancing plant transpiration, maintaining water balance and osmotic stress tolerance (Fraire-Velázquez., 2013). Rook et al., (2006) indicate that ABA and sugar signalling overlap and that ABA is involved in controlling sink-source relationships and sugar metabolism. It has been reported that increasing ABA levels by zeaxanthin epoxidase and 9-cis-epoxycarotenoid dioxygenase (NCED) overexpression enhanced drought tolerance (Nambara & Marion-poll., 2005).

Low levels of ABA are important for stress tolerance and pollen fertility, while high levels of ABA repress sugar transport and affect many genes that are required for anther sink strength. For example, anther ABA levels in wheat drought-tolerant lines are lower than those in sensitive lines which indicates the importance of ABA for abiotic stress tolerance and pollen fertility (Ji et al., 2011). ABA responsive genes considered as good candidates for drought tolerance (Cutler et al.,2010). Jasmonate (JA) and gibberellin (GA) both are mediated by ABA for plant defence responses and seed dormancy (Kerchev et al., 2011; Shu et al., 2013, 2016).

1.9 ABA-dependent signalling pathway

Three proteins: Pyrabactin Resistance/Pyrabactin Resistance-Like/Regulatory (PYR/PYL/RCARs) are considered as ABA Receptors that bind to Protein Phosphatases 2Cs (PP2Cs) and inhibit its enzymatic activity and phosphorylate SNF1-Related Protein Kinase 2s (SnRK2s) (Park *et al.*, 2009; Hubbard et al., 2010; Cutler et al., 2010). PP2Cs therefore act as negative regulators of the pathway, while SnRKs act as positive regulators of downstream signalling (Ma et al., 2009), which then allow for the phosphorylation of ABA-responsive element binding factors (ABFs) leading to gene expression (Figure.1.5). Several transcription factors (TFs) are mediated by ABA to regulate gene expression. For example, *ATMYB2*, *ATMYC2* and *RESPONSIVE TO DEHYDRATION 26* (*RD26*) (Abe et al., 1997; Abe et al., 2003; Yoshida et al., 2010; Yoshida *et al.*, 2015). The *CALCIUM-*

DEPENDENT PROTEIN KINASES (CDPKs) has been reported as positive regulator in ABA signalling controlling stomatal movement (Mori et al., 2006).

1.10 ABA-independent signalling pathway

The APETELLA2/ETHYLENE RESPONSE FACTOR AP2/ERF subgroup dehydration responsive element (*DRE*) as well as *C-repeat binding factor* were considered as cis elements that interact with a signalling cascade and work independently of ABA for regulation of the drought induced genes (Shen et al., 1996; Nakashima et al., 2009; Huang *et al.*, 2012).

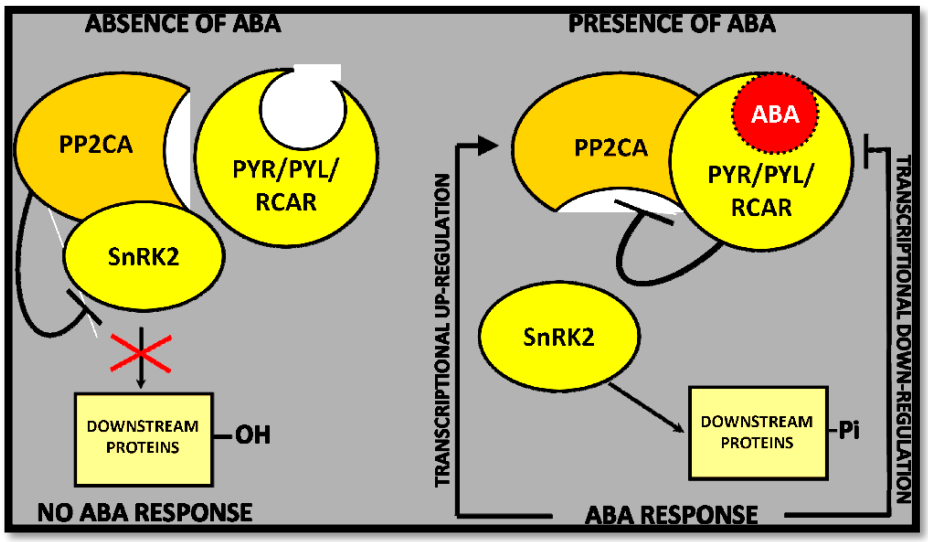


Figure 1.5. ABA perception signalling pathway. A) In absence of ABA failed to bind to ABA receptors PYR/PYL/RCARs (PYLs), preventing the activation of SnRK2s. B) In the presence of ABA showed binding to PYR/PYL/RCARs (PYLs) to inhibit PP2CA and phosphorylate SnRK2s and activated ABA-responsive element binding factors to regulate gene expression (Gascón, 2012).

1.11 Role of PCD during tapetum development

The tapetum has a complex regulatory gene network to facilitate pollen development and to communicate with the developing pollen. The tapetum serves as a regulatory cell layer but also as a factory for pollen wall materials. The tapetum undergoes developmental programmed cell death (PCD) to facilitate pollen wall deposition and viable pollen formation. The timing of tapetum PCD is critical for pollen development, the inhibiting of PCD results in abnormal tapetal expansion due to vacuolization and hypertrophy (Kawanabe et al.2006; Parish and Li 2010). PCD is critical in plant responses to environmental stresses such as cold and drought, causing tapetum pre-mature cell death (Ji et al., 2010).

1.12 Aims of study

Many studies have focused on plant responses to drought during vegetative development rather than reproductive development. Currently there is limited understanding of the genetic factors, regulatory networks and the physiological mechanisms of reproductive failure under water deficit. Understanding stamen and pollen development under drought stress at different stages of development and investigating gene expression under dry condition is important to help address the challenges of climate change on crop fertility. The aims of this study were to help address some of these issues, as follows: -

- To identify and characterize genes that show differential expression during drought stress during reproduction.
- To identify the impact of selected genes on development and stress response using overexpression study.
- To determine the impact of drought on pollen development in *Arabidopsis*.

Chapter 2 Bioinformatics

2.1 Selection of candidate genes

To investigate drought related genes critical for anther and pollen development, three publicly available microarray databases have been used:

- "Moderate drought causes dramatic floral transcriptomic reprogramming to ensure successful reproductive development in Arabidopsis" (Ma et al., 2014)
- "Arabidopsis transcriptome analysis reveals key roles of melatonin in plant defence systems." (Weeda et al., 2014)
- "Flower development under drought stress: morphological and transcriptomic analyses reveal acute responses and long-term acclimation in Arabidopsis." (Su, et al., 2013)

2.2 Gene expression profile Analysis

Co-expression networks were generated from the three microarrays data sets to give a network with nodes (genes) and links (correlations) as the basic elements (Pearce et al., 2015). Genes and correlations were explored in Cytoscape 3.4.0 (Shannon et al., 2003). The generated list of genes showing alerted expression under drought conditions were then compared to gene expressed in male sterile mutants and floral tissues using The University of Nottingham Flower Net tool (Pearce et al., 2015). Genes with high expression in young buds, which reduced in anther development mutants were considered of particular importance (Pearce et al., 2015).

These analyses resulted in 6 clusters 2, 7, 21, 37,110 and 116 (Figure 2.1 red square); thirty genes were chosen as highly expressed in different stages of pollen development (Figure 2.2) these were selected for further analysis (Table 2.1) based upon them correlated gene expression profiles and expression in the floral tissues and alerted expression in the male sterile mutant. For example, AT1G52570 *PLDALPHA2* gene plot reveals that this gene is highly expressed in the stamen and pollen with significant difference between the wild type and the mutants *ms1* and *ams* , as shown in (Figure 2.2) each of the 7 (highlighted in red square) clusters for the candidate genes are divided into 3 highlighted panels as shown Figure 2.2. The 1st subpanel shows *PLDALPHA2* expressed in pollen uninucleate microspores, BCP, TCP and mature pollen, as well as in different mutants such as auxin response factors (*arf6arf8*) ,*MYB* transcription factors, *dyt1*, *myb80* and *myb26*, and JA biosynthesis pathway. The 2nd panel shows gene expression in the *ms1* mutant at flower stage from 1-13 with wild-type and *ms1* buds, in sequential non-open buds.

The 3rd panel shows gene expression in Wild-type (Col-0) and *ams* buds collected at Meiosis (Mei), Pollen Mitosis I (PMI), Bicellular Pollen (BCP), Pollen Mitosis II (PMII) stages. The expression patterns for all the candidate genes were assessed by looking at available microarray data, using the *Arabidopsis* eFP browser (Winter *et al*., 2007) (Appendix II). Both the relative and absolute expression levels

were assessed. For the absolute expression level all genes that had either universal expression or specific expression in bud stages 9/10, 12/13 and mature pollen were listed.

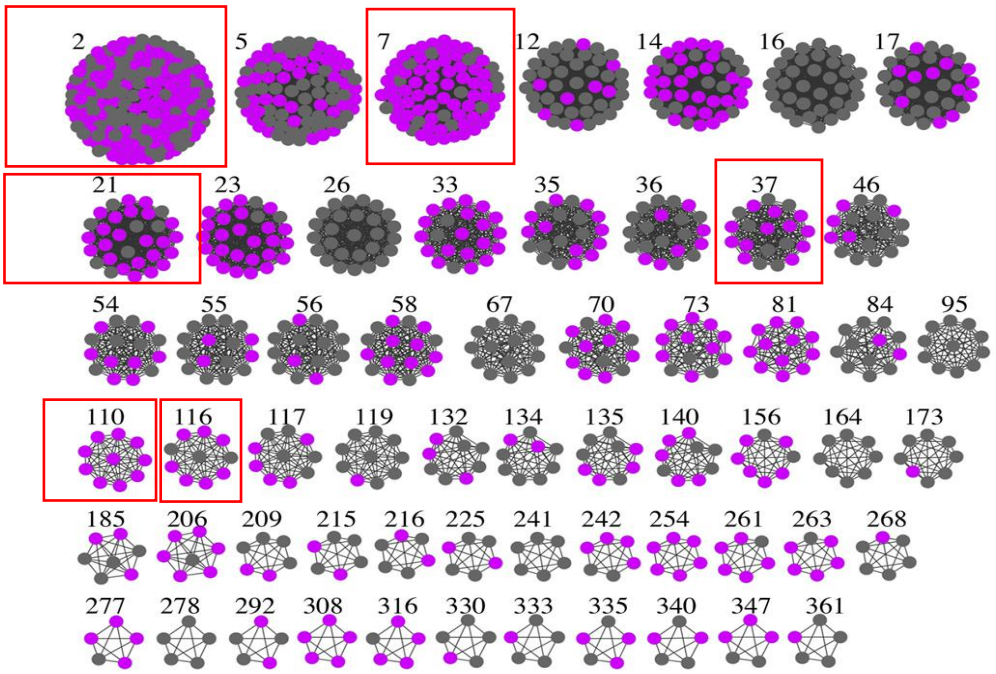
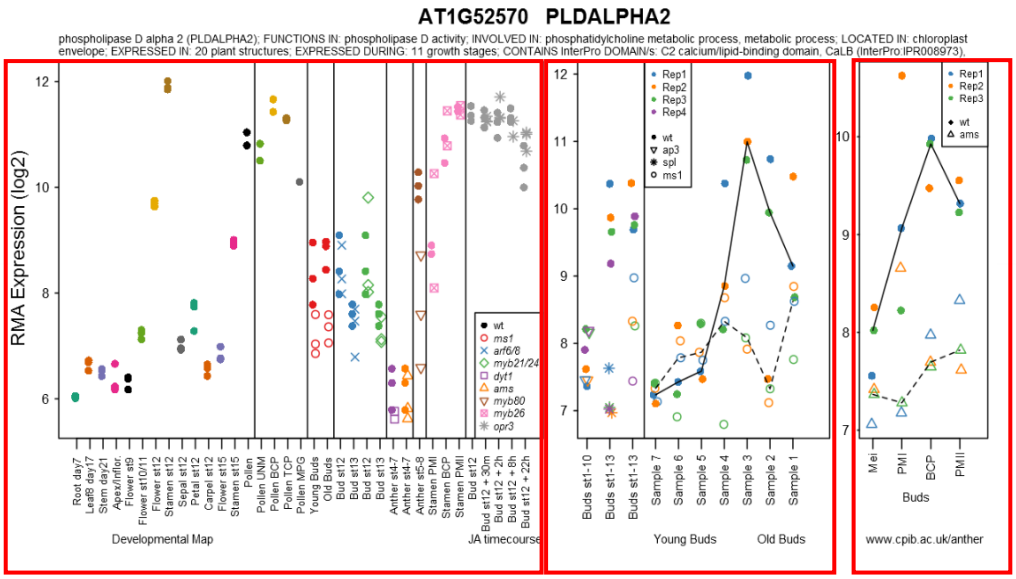


Figure 2.1. Selection of FlowerNet clusters, known for their anther-related genes and male sterile mutant expression. Red squares are indicated those clusters of genes of interest. Highlighted in pink are those genes found to be stamen specific (Wellmer et al., 2004 cited in Pearce et al., 2015).



First panel Second panel Third panel

Figure 2.2. *PLDALPHA2* Expression Plot. In various anther and floral microarrays (First panel) with differences between wild type and mutant in *ms1* (second panel) and *ams* (Third panel) gene mutants (http://www.cpib. ac.uk/anther) (Pearce *et al*., 2015).

Table 2.1. List of drought associated candidate genes expressed preferentially or specifically during pollen development. Brief gene description obtained from the TAIR website https://www.arabidopsis.org/.[1]Cluster information is available from Flower Net, https://www.cpib.ac.uk/anther/ (Pearce et al. 2015), values = correlation. Co-expression database obtained from ATTD-II version 9.2 (http://attend.jp).

AGI	Other name	Function Annotation	[1]Custer	Co-expression/RNAseq
AT3G17630	CHX19	Member of Putative Na+/H+ antiporter family/Regulate pH	21	At1g52570 (PLDALPHA2) At4g00040 At3g56960 (PIP5K4)
AT4G37840	HKL3	Encodes a putative hexokinase. Function in ATP binding	21	N/A
AT2G19770	PRF5	Encodes profilin 5, regulates the organization of actin cytoskeleton.	110	At3g01250 At5g52360 (ADF10) At3g62170 (VGDH2) At5g39330 (DUF1163)
AT3G26110	NIL	Anther-specific protein agp1-like protein	7	At5g28690 (DUF1685) At1g05580 (CHX23) At2g47030 (VGDH1)
AT1G24520	BCP1	Pollen sperm cell differentiation	7	At1g05580 (CHX23) At1g02790 (PGA4) At1g35490 (bZIP family transcription factor)
AT4G27580		Family of unknown molecular function	7	At5g49920 At3g17980 (AtC2) At5g12000
AT3G09530	ATEXO70 H3	Exocytosis, vesicle docking involved in exocytosis	2	At1g05580 (CHX23) At5g28680 (ANX2) At3g62710
AT1G68610	PCR11	Involved in pollen sperm differentiation	2	At4g24450 At1g05580 (CHX23) At5g15500 (ATGWD2)

AT1G18370	HINKEL/ ATNACK1	Involved in: response to cyclopentenone, cellularization of the embryo sac, gametophyte development, pollen development, cytokinesis. Function in: ATP binding	37	At2g28620 At5g55520 At3g23890 (ATTOPII) At4g26660 At3g15550 At5g48310 At2g22610 At1g49870 At5g17160 At3g05330 (ATTAN) At5g51600 (ATMAP65-3) At1g26760 (ATXR1) At4g35730 At4g17000 At5g56580 (ATMKK6)
AT5G57560	TCH4/ATX TH22	Involved in: cell wall biogenesis, plant-type cell wall organization, rapidly upregulated in response to environmental stimuli Has hydrolase activity	21	At1g35140 (PHI-1) At3g62720 (ATXT1) At4g08950 (EXO) At3g50060 (MYB77) At5g46710 (PLATZ transcription factor) At2g01300
AT2G28190	CSD2	Encodes a chloroplastic copper/zinc superoxide dismutase CSD2 that can detoxify superoxide radicals. Its expression is affected by miR398-directed mRNA cleavage.	37	AT1G12520 (SOD1) AT1G08830 (CSD1) AT3G15640
AT1G06410	ATTPS7	Encodes an enzyme putatively involved in trehalose biosynthesis in response to stress.	37	At1g61100 At3g62010 At3g13530 (MAP3K) At4g01210 At1g68580 At2g42030 AT3G51550 (FER)

AT4G12430	TPPF	Involved in: trehalose biosynthetic process	37	N/A
AT4G35700	DAZ3	Target promoter of the male germline-specific transcription factor DUO1.	37	At1g34095 At3g43120 (SAUR-like auxin- protein family) At3g05725 (DUF3511) At2g20430 (RIC6) At4g15650
AT2G26150	ATHSFA2	Member of Heat Stress Transcription Factor (Hsf) family. Involved in response to misfolded protein accumulation in the cytosol. Regulated by alternative splicing and non-sense-mediated decay.	37	At1g74310 (HSP101) At5g52640 (HSP90.1) At3g12580 (HSP70) At3g14200 (Chaperone DnaJ-domain superfamily protein) At1g59860 (HSP20-like chaperones superfamily protein) At1g71000 (Chaperone DnaJ-domain superfamily protein) At5g64401 At1g59865
AT5G03720	ATHSFA3	Member of Heat Stress Transcription Factor (Hsf) family. Expression is regulated by DREB2A and in turn HSFA3 regulates the expression of hsps Hsp18.1-CI and Hsp26.5-MII35S. Involved in establishing thermotolerence.	37	At4g32060 (calcium-binding EF hand family protein) At2g46610 (RNA-binding (RRM/RBD/RNP motifs) family protein) At3g15530 (S-adenosyl-L-methionine-dependent methyltransferases superfamily protein)

AT2G32460	ATMYB101	Member of the R2R3 factor gene family.	37	At3g49540 At1g23510 At2g03850 (LEA family protein) At3g46520 (ACT12) At5g57380 (VIN3) At1g29090 (Cysteine proteinases superfamily protein)
AT3G27810	ATMYB21	Encodes a member of the R2R3-MYB transcription factor gene family. Induced by jasmonate. Involved in jasmonate response during stamen development.	37	At5g40350 (MYB24) At3g27809 At1g44542 (Cyclase family Protein)
AT1G35490	bZIP	bZIP family transcription factor	37	At2g47030 (VGDH1) At3g43120 (SAUR-like auxin-responsive protein family) At3g01240 At3g26860 (Plant self-incompatibility protein S1 family) At5g56640 (MIOX5) At3g62180 (Plant invertase/pectin methylesterase inhibitor superfamily protein) At5g50830 At3g54800 (Pleckstrin homology (PH) and lipid-binding START domains-containing protein) At1g24520 (BCP1) At5g16500 (Protein kinase superfamily protein) At5g62750 At5g47000 (Peroxidase superfamily protein) At2g27180 At1g23350 (Plant invertase/pectin methylesterase inhibitor superfamily protein) At3g61230 (PLIM2c)

AT2G38090		Duplicated homeodomain-like superfamily protein DNA binding. Has DNA binding transcription factor activity	37	At3g06590 (basic helix-loop-helix (bHLH) DNA-binding superfamily protein At5g38970 (ATBR6OX) At3g56480 (myosin heavy chain-related)
AT4G38960	BBX19	Involved in circadian rhythm Has DNA binding transcription factor activity	37	At4g15430 (early-responsive to dehydration stress) family protein At2g47490 (ATNDT1) At1g01060 (Homeodomain-like superfamily protein) At2g34720 (nuclear factor Y, subunit A4) At3g21390 (Mitochondrial substrate carrier family protein) At3g08730 (ATPK1) At5g49230 (Drought-responsive family protein)
AT5G01200		Duplicated homeodomain-like superfamily protein. Has DNA binding transcription factor activity	37	At4g27435 (DUF1218) At1g75800 (Pathogenesis-related thaumatin superfamily protein) At5g42200 (RING/U-box superfamily protein)
AT3G02120		Hydroxyproline-rich glycoprotein family protein	37	At3g02640 At5g16250 At4g31840 **(**early nodulin-like protein 15) At3g42725 (Putative membrane lipoprotein At1g31335 At3g13175 At2g26520

Continued from previous

AT1G52570	**PLDALPHA2**	**Abscisic acid-activated signalling pathway, ethylene-activated signalling pathway, lipid catabolic process, phosphatidylcholine metabolic process**	**21**	**At3g17630 At1g06250 At3g05270 (DUF869)**
AT4G36600		Embryo development ending in seed dormancy with unknown function	21	At1g54870 At4g39130 At4g21020 (LEA) At1g23070 (DUF300) At3g15280
AT1G63060		Biological process with unknown function	110	At5g07410 At2g28355 At4g11485
AT3G28980	**DUF1216**	Biological process with unknown function	110	At3g14040 At3g62170 At3g28790 (DUF1216) At3g01250 At4g00350
AT3G23770		Carbohydrate metabolic process with unknown molecular function	37	At1g06280 At4g12920 At1g01280 At4g14080 At3g50580
AT2G24450	**FLA3**	Anchored component of membrane, plasma membrane	7	At3g62710 At5g39880 At1g05580

		with unknown molecular function		
AT3G20220	**SAUR47**	Response to auxin with unknown molecular function	7	At1g51250 At2g26850 At3g26860

2.3 Conclusion

Genes selected based on their expression level during pollen development and alerted expression in drought conditions (section 2.1). Candidate genes will be examined during drought by quantitative analysis, T-DNA insertion and overexpression lines (for seven genes) which will be discussed through the thesis.

Chapter 3 Materials and Methods

3.1 General growth conditions under drought stress.

Arabidopsis thaliana ecotype *Landsberg erecta* (L*er*-0) was obtained from The Nottingham Arabidopsis Stock Center (NASC). Five seeds were sown in 13 cm pots containing equal weight of Levington M3 compost (The Scotts Company Ltd, UK), which was soaked in water until saturated. Seeds were germinated and plants grown in a growth room at 23/18℃ (day/night) and 16 hrs photoperiod at a light intensity (measured as photon intensity) of 180±20 µmol/s/m2 once flowering had commenced and two siliques had formed the drought treatment was started and plants were moved into the glasshouse (23°C and 27% humidity/22h day light) and water was withheld for 12 days. At the end of the drought treatment plants were watered daily until all the siliques were mature and ready for harvesting. Control plants (watered) were watered every day until seed harvesting. Buds were collected at the beginning of the drought (day 2) and at the end of the drought treatment (day 12) and after 48hr

of re-watering from both drought and well-watered plants. Buds were selected based on size and shape (Xu et al.,2010 and Pearce et al.,2015) to obtain anthers at the correct developmental stages. *of* pollen mother cell meiosis, pollen mitosis I, bicellular pollen, and pollen mitosis II.

3.2 Expression Analysis

3.2.1 RNA extractions

Total RNA, from frozen buds at different stages (Tetrad, Microspore, Bicellular and Tricellular microspores) of both treated and control plants, was extracted using an RNeasy Plant Mini kit (Qiagen,UK) following the manufacturer's protocol. Briefly, anthers were ground to a fine powder in liquid nitrogen. Approximately 10-15 mg of this anther powder was transferred to a microcentrifuge tube containing 450 µL of RLT buffer with 1% (v/v) β-mercaptoethanol followed by 9 mins incubation at 65℃. The lysate was centrifuged for 2 min at 13,000 rpm in a microcentrifuge. The clear flow-through lysate was then transferred to a new collection tube and mixed with 250 µL of 96% (v/v) ethanol. This mixture was transferred to a RNeasy column in a 2 mL centrifuge tube and centrifuged for 15 seconds at 11,000 rpm. The RNeasy column was subsequently washed with 700 µL of RW1 buffer and centrifuged for 15 seconds at 11,000 rpm. Potential contaminant genomic DNA was digested on-column with RNase-free DNase (Qiagen, UK). A DNase I mix containing 10 µL DNase I stock solution [(1500 Kunitz

units) in 550 ul of RNase-free water)] and 70 µL buffer RDD was pipetted directly onto the RNeasy spin column membrane and incubated for 20 min at room temperature. After incubation, the spin column was washed with 500 µL of RW1 buffer and centrifuged for 15 seconds at 11,000 rpm. RPE twice before finally eluted in 30 µl of RNAse-free water. Total RNA was quantified using NanoDrop fluorospectrometer (ND-1000 Spectrophotometer, NanoDrop Technologies Inc., USA). Total RNA was diluted with RNase-free PCR grade water to desired concentrations for RT-PCR as required. The RNA was stored at -80°C.

3.2.2 cDNA preparation

Transcripts of the specific genes were reverse transcribed into corresponding cDNA using SuperscriptTM III One-Step RT-PCR Platinum® Taq (Invitrogen, UK). Briefly, 1 µL total RNA 2.5 µg was mixed with 1µl oligo (dT) 12-18 (500µg/ml), 1µl 10 mM dNTP Mix and sterile distilled water to a total volume of 13µl. The mixture was incubated in a PCR machine at 65℃ for 5 min then cooled on ice for at least 1 min before adding 4µl 5X First-Strand Buffer, 1µl DTT (0.1M), 1µl RNaseOUT Recombinant RNase Inhibitor and 1µl SuperScript III Reverse Transcriptase (Invitrogen, UK). The total 13 µl reaction was incubated at 50℃ for 60 minutes, then the reaction was inactivated by heating to 70℃ for 15 min. The cDNA samples were then used as template for PCR or stored at -20℃.

3.2.3 RT-PCR

Gene specific primers were designed based on sequence information (http://www.ncbi.nlm.nih.gov), using Primer3 (http://primer3.sourceforge.net) Primers were designed (Appendix II primers). Initial RT-PCR tests were applied to all genes under investigation. Mixing 0.1-1µl of cDNA template, 5µl REDTaq 2x Master Mix (VWR, UK), 0.3µl primer pairs (10pmol/µl), and molecular grade distilled water (SIGMA, UK) to a total volume of 10 µl. The cycling profile of the standard RT-PCR was initiated with a reverse transcription step depending on the melting temperature (Tm) of the different primers; the annealing temperature usually ranged from 52℃ to 60℃. The initial denaturation at 95°C for 3 min followed by 30-35 cycles of 95°C for 30 seconds denaturing, 52-65°C for 30 seconds annealing, 62-72°C for 1 min elongation depending on gene target see (Table 3.1).

Table.3.1 PCR Reaction for Red Taq® Ready Mix® PCR Reaction Mix

Reagent	Volume
2xRed Taq® Ready Mix®	5 µl
10p.M Forward Primer	0.3 µl
10p.M Reverse Primer	0.3 µl
0.2µl cDNA template	1 µl
ddH2O	Up to 10 µl

3.2.4 Agarose gel electrophoresis

Amplified products were electrophoresed in a 0.8-1.5% (w/v) agarose gel (Melford, UK), 0.5x TBE buffer (45 mM Tris-borate, 1 mM EDTA) with 0.025µg/ml ethidium bromide (Sigma, UK). RedTaq PCR product (loading dye included) was loaded onto the gel and electrophoresed in 0.5x TBE buffer at an electric field strength of 100 V/45 minutes until DNA fragments separation. Hyper Ladder™ 1kb (Bioline, UK) was run in parallel with the samples. The DNA bands on the gel were then visualized under UV light. The gel images were captured using SnapGene Software (GSL Biotech LLC).

3.2.5 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Real-time qRT-PCR reaction was carried out using Maxima SYBR Green QPCR 2x Master Mix (Thermo Fisher Scientific, UK) to detect and quantify gene expression (Table 3.2). The fluorescence from SYBR Green I is detectable when bound non-specifically to double strand DNA and increases as PCR products are amplified, making it possible for the detection of PCR product in real-time. QPCR was performed and analysed using the LightCycler® 480 System (Roche, UK). The experimental reaction comprised 4.5µl Maxima SYBR Green QPCR 2x Master Mix, 0.2µl cDNA template, 0.2µl primer pairs (10pmol/µl) and 4µl nuclease-free water. Initial denaturation at 95^{o}C for 10 min, following 35-40 cycles of 95^{o}C for 30 seconds denaturing,

57-60°C for 30 seconds annealing, 72°C for 1 min/Kb elongation. Specificity of amplified products and primer dimer formation were monitored by the dissociation programme (1°C per cycle from 55°C to 95°C) for 30 seconds during cycles, and a melt curve at the end of the qRT-PCR reaction (Table 2.3). To calculate primer efficiency, wild-type cDNA series (undiluted pool, 1:5, 1:25, 1:125; dilution) was used as templates; the house-keeping PP2A3 was used to normalize amplification between experimental samples. Relative expression levels were determined in comparison to PP2A3 expression using the 2-ΔΔCT analysis method (Livak and Schmittgen, 2001). Samples were analysed using separate wells of a QPCR Light Cycler 480 multi-well plate 384 (Roche, UK). All samples were run at least in duplicate. The plate was centrifuged at 2000g for 2 minutes before loading to the LightCycler 480 QPCR machine.

Table 3.2 Real-Time Quantitative PCR Reaction

Reagent	Volume
2xSYBR® Green Master Mix	4.5µl
10µM Forward Primer	2.2 µl
10µM Reverse Primer	2.2 µl
ddH2O	Up to 10µl

3.3 Genotyping

3.3.1 Identification of knockout mutants from T-DNA insertional lines

Arabidopsis thaliana T-DNA insertion "knockout" (KO) lines were obtained from Nottingham *Arabidopsis* Stock Centre (NASC) in *Arabidopsis* ecotype Columbia (C*ol*) background. Gene information for these lines along with insertion positions was obtained from SIGnAL T-DNA-Express facility (Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/cgi-bin/tdnaexpress) (Appendix II figures). SALK (Alonso *et al.*, 2003), SAIL (Sessions *et al.*, 2002) and GABI-Kat (Rosso *et al.*, 2003) lines in a Col-0 background were used in this thesis, and a list of all T-DNA insertional mutants screened are given in (Appendix II primers).

3.3.2 DNA Extraction

Genomic DNA from T-DNA KO lines and wild type (ecotype Columbia) control plants were extracted using the sucrose method (Hosseinpour *et al*, 2013). 2-3mm diameter of leaf tissues were cut from the plant and immediately transferred into a tube on ice with 100µl of the Sucrose Buffer (50mM Tris-HCl, 300mM NaCl, 300mM sucrose). These were roughly crushed with a pipette tip in the tube, each sample was then boiled for 10 mins at 95°C prior to briefly spun at 2000–6000g for 5 seconds. 0.5-1µl of the extracted DNA was immediately used for PCR amplification.

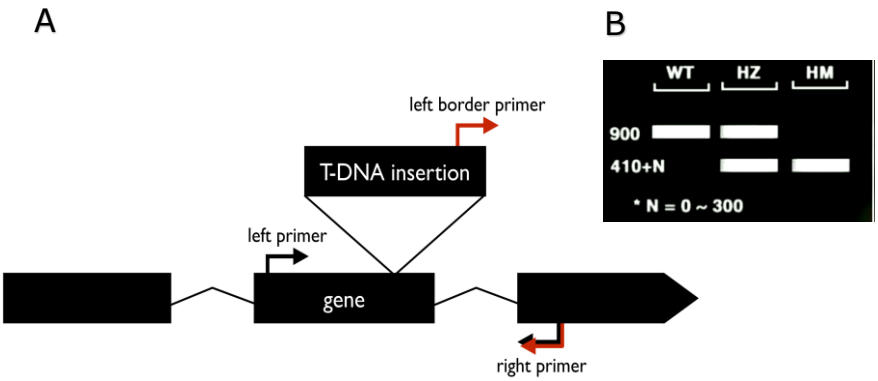


Figure 3.1. A) T-DNA insertion Strategy used for genotyping. Two pairs of primers were used to verify the presence of the T-DNA insertion. The left border in red primer specific to the T-DNA sequence pairs with the right gene-specific primer. **B)** Guide for the PCR genotyping and gel analysis of the insertion lines in the heterozygous (HZ) and homozygous (HM) lines as compared to their respective wild type (WT), provided by the SALK Institute, Genomic Analysis Laboratory (SIGnAL) (http://signal.salk.edu/tdnaprimers.2.html). N: difference of the actual insertion site and the flanking sequence position, usually 0-300 bases.

3.3.3 PCR to Identify T-DNA Insertional Mutants

PCR amplification from genomic DNA was carried out using REDTaq 2x Master Mix (VWR, UK), in a volume of 10 µl (Table 3.1). Salk-lines were analysed for mRNA expression to see if gene expression was completely knocked out. Genotyping was performed using forward and reverse gene-specific primers and SALK-LBb1 and LB3 primers and T-DNA insertion "knockout" (KO) primer (Appendix III primers).

Table 3.3 PCR information of T-DNA Insertional Mutants.

Reaction	Volume µl
Distilled water dH2O	1.5
2xRed Taq® Ready Mix®	5.0
10µM LBb1 primer 10µM Lb3 primer	0.5
10µM Forward Primer	0.5
10µM Reverse Primer	0.5
0.2 µl cDNA template	2.0
Total volume	10.0

3.4 Molecular Cloning Methods

3.4.1 Production of Lines Overexpressing the Gene of Interest

To construct overexpression vectors for the genes of interest, the Gateway® Technology (Invitrogen™ from ThermoFisher Scientific) was used. according to the manufacturer's instructions using the minimum suggested reaction composition. All BP and LR reactions were incubated at 25℃ overnight and the reactions were terminated by adding 1µl proteinase K and incubating for 10 mins at 37℃ before transforming into *E. coli* competent cells.

3.4.2 Amplification of the Gene of Interest for Gateway® Cloning

The full-length protein-coding sequence of the genes of interest was amplified from wild-type *Ler* cDNA by PCR. Q5 High-fidelity DNA polymerase (NEB, UK) according to the manufacturer's instructions, for a 20 µl reaction and using the recommended PCR conditions. The forward primer contained the CACC sequence at the 5' end to ensure directional cloning into the TOPO® vector. The sequences of the forward and reverse primers used in the PCR are given in (Appendix III). 100-200 ng of cDNA was added to each reaction and an annealing temperature of 60 °C was used. The PCR products were run on a 1% (w/v) agarose TBE gel and purified by gel extraction as described in (section 3.2.4).

3.4.3 TOPO® Clone and Gateway® Technology

3.4.3.1 TOPO clone

Topoisomerase based cloning technology allows ligation of DNA with compatible ends. PCR products were amplified using Phusion. The core of TOPO cloning is the enzyme DNA topoisomerase I, which functions both as a restriction enzyme and as a ligase. The topoisomerase I specifically recognises the 5'-(C/T) CCTT-3' sequence and forms a covalent bond with the phosphate group attached to the 3' thymidine and cleaves one DNA strand to unwind the DNA and then re-ligate the ends before releasing from the DNA. The TOPO® vector is Gateway®-adapted and is linearized with topoisomerase I covalently bound to the 3' phosphate on each end. This enables the vector to ligate the insertion DNA with compatible ends to produce entry clone. pCR™8/GW/TOPO® TA Cloning Kit (Invitrogen, UK) was used to generate the Entry Clones with inserted CDS of AT3G20220, AT2G24450, AT3G23770, AT3G28980, AT1G63060, AT4G36600 and AT1G52570 individually. According to the manufacturer's instructions, 2µl of PCR products (with 3' adenine) was mixed with 0.5µl salt solution (200 mM NaCl; 10 mM $MgCl_2$), 0.5µl TOPO® vector and incubated overnight at room temperature. This 3µl TOPO cloning reaction was transformed into One Shot Mach1™ competent cells (Invitrogen, UK), after 30 min of incubation on ice, the cells were heat shocked at 42°C for 1 min and 30 seconds, immediately transferred to ice and 250 µl of LB medium

added. The samples were incubated for 1 hour at 37°C then spread onto LB + Kanamycin plates (50 µl /ml Kanamycin) and incubated overnight at 37°C. Colonies were screened using vector specific sequences (M13F/M13R) flanking the cloning sites (Appendix III primers). Colony PCR and sequencing were performed to ensure the inserted DNA had the correct orientation and sequence.

3.4.3.2 LR Reaction

Gateway cloning was performed using Gateway™ BP/LR Clonase™ II Enzyme Mix (Invitrogen, UK) according to the manufacturer's instructions. All BP and LR reactions were incubated at 25℃ overnight and the reactions were terminated by adding 1µl proteinase K and incubating for 10 mins at 37℃ before transforming into DH5α competent cells.

3.4.3.3 Plasmid DNA extraction and sequencing

Plasmid DNA was extracted using the GenElute™Plasmid Miniprep Kit (Sigma, UK). 10 µl plasmid DNA with a concentration 100ng/µl DNA concentration was sent for sequencing. During the cloning procedure, plasmid DNA was extracted from both entry clones and expression clones and sequenced with primers from both ends to ensure the insertion DNA was correct. Sequencing was performed using the Sanger sequencing services provided by Source Bioscience (Nottingham). The sequencing data was analysed using SnapGene Software (GSL Biotech LLC, US).

3.4.3.4 Storage of bacteria as glycerol stocks

A single colony was transferred to 1 ml of LB liquid media and incubated overnight (*E. coli* at 37°C, 180 rpm; *Agrobacterium* at 28 °C, 200 rpm). The culture was then mixed to a final concentration of 20% (v/v) glycerol and flash frozen in liquid nitrogen. The glycerol stock was kept at -80°C.

3.4.3.5 Transformation of *Agrobacterium tumefaciens*

The pGWB5-GFP vector expression constructs were transformed into electro-competent *Agrobacterium tumefaciens*, (strain GV3101, Genomics facility, University of Nottingham) (Koncz and Schell, 1986) by electroporation. 1 µl of 200 ng plasmid was gently mixed with a 50 µl aliquot of the cells and transferred to ice-cold 2 mm electroporation cuvettes. Electroporation was of 1.25 kV/cm was then applied to the cells. 1 ml of ice-cold LB medium was added to the cells and the samples were shaken at 28 °C for 3 hours. 200 µl of the cells were then plated on LB Agar plates containing 50 µg/ml of Kanamycin. The plates were incubated at 28 °C for 2 days and positive transformants were verified by colony PCR using the same primers used to screen *E. coli* colonies after LR cloning.

3.4.3.6 Transformation of Arabidopsis by *Agrobacterium*-mediated transformation

Positive colonies (Section 3.4.3.5) were grown overnight at 28 °C in 10 ml LB medium 50 µg/ml of Kanamycin. These were used to inoculate 500 ml LB cultures and wild-type *Ler* Arabidopsis plants were transformed with the overexpression vectors using the floral-dip method described by Clough and Bent (1998). The seeds (T1) of the transformed plants were screened on MS medium (Appendix I) with kanamycin (50 mg/ml) antibiotic then after 5 weeks transferred to soil. Positive transformants were confirmed by PCR (Section 3.2.3) and then analysed by qPCR (section 3.2.5), to verify overexpression of the gene of interest. Homozygous and heterozygous lines were obtained by screening selected T2 lines to confirm that there was no segregation in the lines.

3.5 Microscopy and Staining Methods

3.5.1 Alexander Staining

Anthers were dissected from flowers and mature buds under a stereomicroscope and were placed onto a clean slide. 20-40 µl of Alexander staining solution (Alexander 1969) was dropped onto the sample. The cover slide was gently pressed to facilitate pollen release. The samples were observed under a light microscope after staining 30 min. Viable pollen stained dark red while non-viable pollen stained green.

3.5.2 Fluorescein Diacetate (FDA) Staining

Mature microspores were stained with fluorescein diacetate (FDA) (Heslop Harrison et al. 1970) (Appendix I). Viability assessment is based on the uptake of non-fluorescing FDA by the viable pollen grain and subsequent hydrolysis by esterases to release fluorescein under UV, while nonviable cells are incapable of hydrolyzing FDA and no fluoresce. Open flowers buds (at anthesis) were used to determine pollen viability. 2 mg of fluorescein diacetate was dissolved in 1 ml acetone to prepare FDA stock solution. Freshly collected pollen added by 1 or 2 drops of FDA buffer on a clean slide. Observation was taken using a fluorescence microscope (Leica DM5000) and ten view areas were captured for further analysis. Viable and non-viable young microspores were immediately counted.

3.6 Gene Network Construction.

The STRING network tool was used for network analysis. STRING is a database of known and predicted protein interaction data from multiple sources including experiments, co-expression, and text mining. In total, STRING covers nearly 10,000,000 proteins from over 2,000 organisms (http://string-db.org).

Chapter 4 Expression Analysis of Genes Involved in Drought Stress during Pollen Development

4.1 Introduction

Water provides the basis of all known forms of life. Drought stress is a major problem to plant growth and crop development, which impacts upon various stages of development with varying degrees of severity, including the rapid production of flowers and seed set (Krasensky and Jonak., 2012). Male gametophyte development plays an important role in plant fertility and is very sensitive to abiotic stress. The development of mature pollen grains is a very complex process that is regulated by specific gene expression patterns. Plants respond to drought by the induction of both regulatory and functional sets of genes (Bartels and Sunkar., 2005). Stress-inducible transcription factors (TFs), for example *NAM* (*no apical meristem*), *ATAF* (*Arabidopsis transcription activation factor*), *CUC* (*cup-shaped cotyledon*) (Souer et al., 1996; Aida et al., 1997), *MYB/MYC* (Abe et al., 1997), *WRKY* (Banerjee and Roychoudhury., 2015) play important roles in regulating stress responses and tolerance. *Nuclear factor-y*(*NF-Y*) and dehydration-responsive element binding protein (*DREB*) all are involved in drought responses through the ABA-independent pathways genes and ABA-responsive element (*ABRE*) binding protein (Yamaguchi-Shinozaki and Shinozaki.,2006, Nakashima et al., 2014).

In this chapter, the impact of drought stress on fertility was assessed and genes associated with drought stress investigated. The identification of subnetworks of genes expressed at different stages of pollen development were identified linked to drought stress using two databases, a microarray co-expression network (drought related) followed by a FlowerNet network, this identified thirty genes based upon their significant alerted expression under drought, and their preferential or specific expression during pollen development (Chapter2 Table 2.1). Many of these genes were associated with regulatory proteins such as chaperones, osmotin, LEA (late embryogenesis abundant) proteins, transporters (sugar and proline), detoxification enzymes, hormonal signalling pathways such as Jasmonate, auxin, gibberellin, and stress response transcription factors involved in metabolism and protein processing, cell wall metabolism, cytoskeleton, transport and trafficking. These were analysed using RT-PCR expression analysis and reverse genetics, to determine their potential involvement in pollen development during drought treatments and to provide insight into the possible gene regulatory mechanisms underlying male fertility.

4.2 Methods

4.2.1Drought Stress Treatment

Plant growth conditions were as described previously (Chapter3 section 3.1). Plants were well-watered until two siliques had formed. The soil moisture for well-watered plants was maintained at 90% (90 g water/100 g soils) whereas the soil moisture content of drought-treated plants was gradually decreased to about 35% of the soil water-holding capacity.The humidity was set to the normal growth condition (60%) when the soil moisture of drought plants reached 35%. Post-drought stresses the soil water condition of well-watered and drought plants was maintained by daily watering until the fruits (siliques) matured.

4.2.2 Relative Water Content (RWC) Measurement

A leaf from a droughted plant were cut and the fresh weight was determined. Then, the immersed in water overnight and blotted-dry with paper towel. The leaves were then weighed to obtain turgid weight. The same leaf was then dried in an oven overnight to acquire

dry weight (Figure 4.2 A). Relative water content (RWC) was determined by using the formula below (Smart and Bingham, 1974).

$$\mathbf{RWC\ (\%) = [(W\text{-}DW) / (TW\text{-}DW)] \times 100}$$

W – Sample fresh weight
TW – Sample turgid weight
DW – Sample dry weight

4.3 Results

4.3.1 Effect of Drought on Plant Fertility

To determine the impact of drought on plant growth, particularly pollen development and viability, plants were exposed to a period of drought, bud samples were collected from primary and secondary flowering stems from well-watered and drought plants according to (Chapter3 section 3.1). The buds were collected based upon size corresponding to developmental stage and were labelled as Bud Stage 1(SI), Bud Stage 2(SII), Bud Stage 3(SIII) and Bud Stage 4(SIV) (Figure 4.1A). Drought affected seed number and reduced siliques length accordingly (Figure 4.2C) and reduced the number of viable pollen during the drought treatment (Figure 4.2D). Under severe drought stress, flowers were abnormal and mostly non-fertile. Flowers frequently exhibited stunted stamens, immature anthers, with distorted sepals and petals were separated from each other (Figure 4.1C). Early anther dehiscence was observed and collapsed non-viable pollen grains were seen compared to the plants with a continuous water regime (Figure 4.3). Drought had a significant detrimental effect on flower development and seed set, analysis of gene expression during drought was therefore investigated to aid understanding of the impact of drought stress on flower development at the cellular and molecular level.

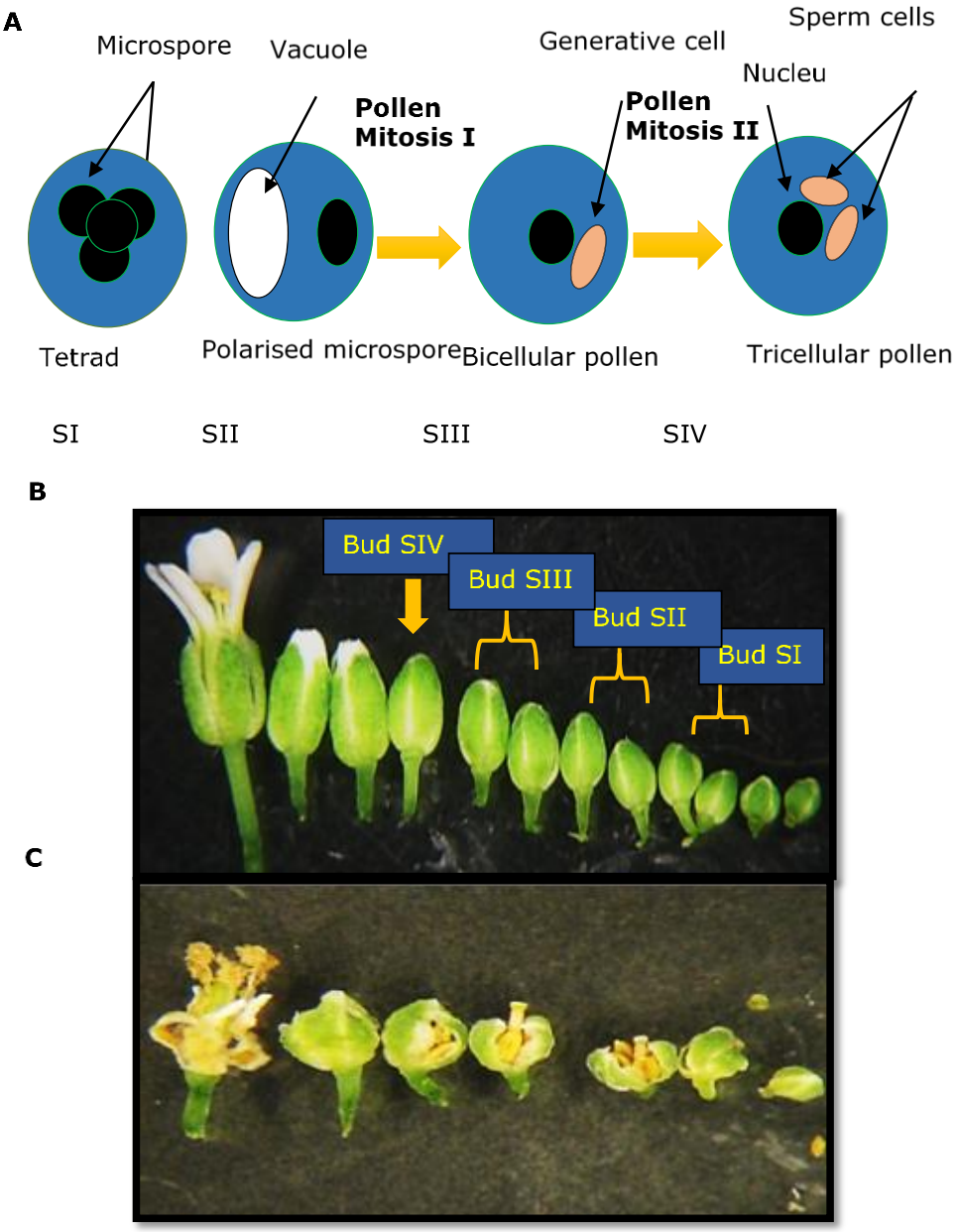


Figure 4.1. *Arabidopsis* ler dissected flowers under drought. A) Pollen development stages tetrad (SI), microspore (SII), bicellular pollen (SIII), tricellular pollen (SIV). **B)** Separated buds from well-watered illustrating staging process. **C)** Separated buds from plant after 12 days of continuous drought.

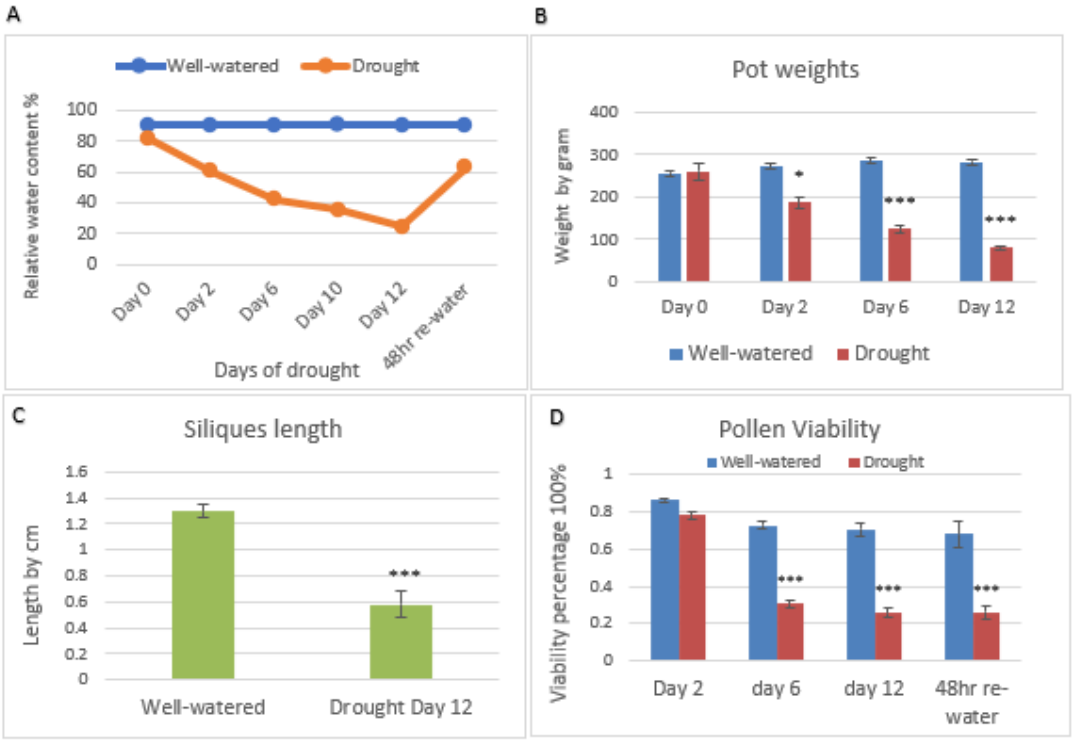


Figure 4.2. Drought impact on *Arabidopsis ler* growth. **A)** Leaf relative water content (RWC %), leaves were immersed in water overnight then weighed to obtain turgid weight then dried to acquire dry weight**. B)** Pot weights to indicate water loss during drought days for both well-watered and drought materials. Asterisks indicate values that are significantly different with $P < 0.001$ by a Student's *t* test. The data and error bars represent the mean and se ($n = 3$). **C)** Total fruit number and the sterile silique number on the main stem under the Well-watered and drought condition. Asterisks indicate values that are significantly different ($P < 0.001$) by a Student's *t* test. The data and error bars represent the mean and se ($n = 4$). **D)** The percentage of viable pollen grains. Asterisks indicate values that are significantly different with $P < 0.001$ by a Student's *t* test. The data and error bars represent the mean and se ($n = 10$).

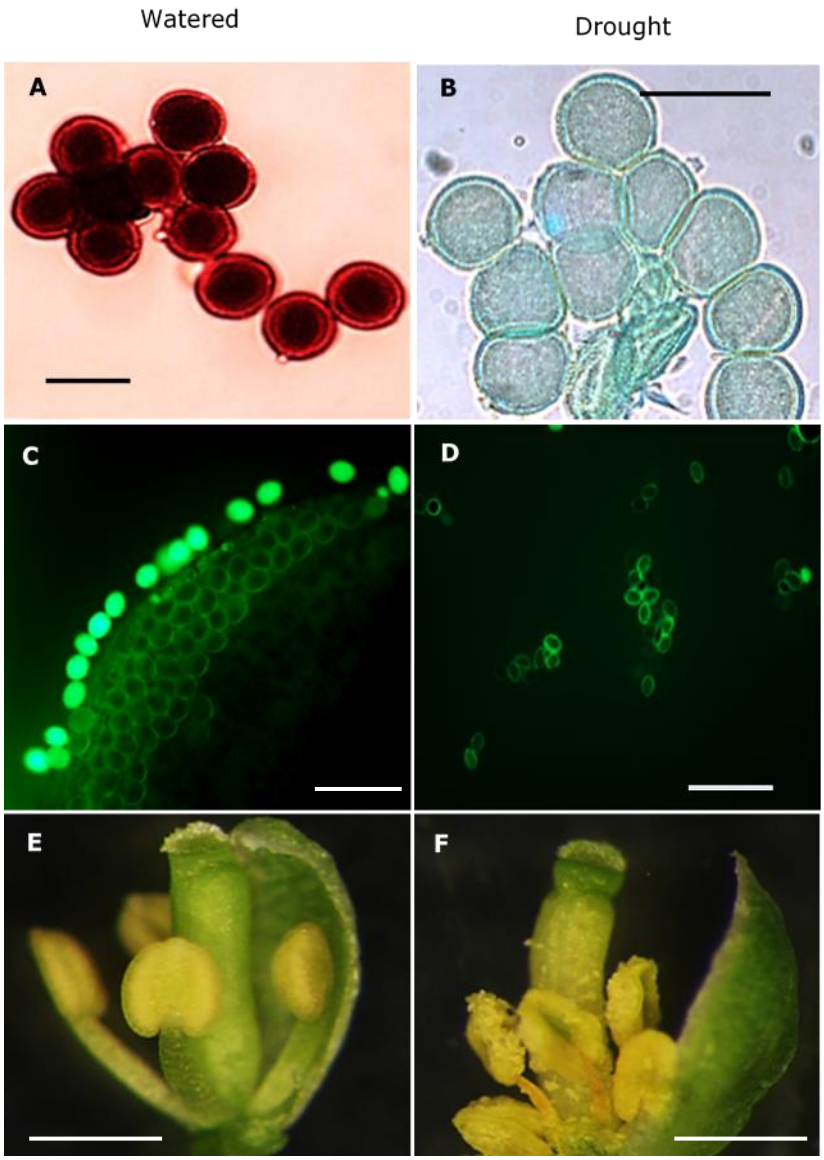


Figure 4.3. Anther and pollen images under normal and drought condition. **A)** Viable pollen stained red and **B)** Unviable pollen stained green, Pollen viability test by Alexander staining (Alexander, 1969). Distinction between viable and nonviable grains is based on the differential staining of the cytoplasm and the cellulose-containing wall. Viable pollen stains red, and non-viable cells are stained green. Stained samples were visualized by bright-field microscopy. Scale bar 10 and 20 µm for viable and non-viable respectively. **C)** and **D)** Pollen viability was also evaluated with cell-permeable esterase substrate fluorescein diacetate (FDA) Viable cells stained bright green. Scale bars, 10 µm. **E)** Wild-type *Arabidopsis* L*er* open bud from well-watered samples displaying normal stamen. **F)** wild-type in severe drought indicates shrunk anther and filaments**.** Scale bar 200 µm

4.3.2 Validation of Selected Genes via Quantitative Real-Time PCR in Wild Type Buds under Drought Treatment

Buds samples were collected from different stages of pollen development (Figure 4.1b). To determine the expression of the drought associated genes, semi-quantitative RT-PCR was conducted on the staged bud samples from well-watered and water stressed plants. RNA and cDNA were prepared (Chapter3 Section 3.2.1 & 3.2.2) and qRT-PCR conducted as described (Chapter 3 Section 3.3.5), expression was normalised to the reference gene PP2A3.

4.3.3 Expression Analysis of Drought Responsive Genes Using Quantitative RT-PCR.

Drought is the most prevalent environmental stress that impacts upon the agricultural economy. To improve production under drought stress, plant breeders and scientists have worked for decades to improve varieties undertaken using recombinant inbred lines (RIL), molecular markers, and quantitative trait locus (QTL) mapping (Cattivelli et al., 2008; Yu et al., 2008; McMullen et al., 2009). Investigating the role of drought-responsive genes in genetic regulation will be of value for understanding drought and minimising yield losses, particularly given the great importance of functioning pollen for the plant life cycle and for agricultural production. Gene expression was investigated by qRT-PCR during early and late stages of pollen formation under different days of drought.

Details of the qRT-PCR expression analysis of each gene are in Appendix I and summarised in Figure 4.4 to 4.7. Expression was similar to that represented by the eFP browser (Figure 4.8). In the control (well-watered) samples, several interesting findings were found with gene showing stage specific expression. For example, a group of genes were expressed in buds specifically and predominantly in BCP and TCP stages: (*BCP1, ATCHX19, PRF5, ATEXO70H3*, *SFH5LP*) (Figure 4.6&4.7), *BCP1* was known to be active in microspores and tapetum and is essential to pollen viability (Xu et al., 1995), however Li et al (2017) illustrated that *BCP1* was not expressed in the tapetum during anther stage 6 to anther stage 10, which is consistent with the qRT-PCR data which presented expression of *BCP1* at both anther stage 11(BCP) and stage 12 (TCP).

ATCHX19 has expression reported in sperm cells and pollen tubes, but the mutant of *ATCHX19* shows abnormalities in exine wall and reduced seeds numbers (Padmanaban et al., 2017), suggesting gene functioning during late anther and pollen development, consistent with my data where *ATCHX19* shows high expression in BCP and TCP stages (Figure 4.7). *PRF5* shows high expression at BCP and TCP and is induced in apical actin polymerise in pollen tubes (Liu et al.,2015). *PRF5* has shown high expression in mature pollen and is predominantly in the polysomal mRNA fraction (Hafidh et al., 2018). *ATEXO70H3* gene is involved in cytokinesis and cell plate maturation

(Fendrych et al., 2010), which has a role in vesicle trafficking processes and is considered as pollen specific gene (Chong et al., 2010); this expression profile was confirmed by qRT-PCR analysis of TCP specific expression (Figure 4.6). Another interesting finding was genes that are expressed predominantly at TCP such as *FLA3*. *ALP*, PCR*11*, *TPPF*, *DAZ3*, *ATMYB21*, *bZIP*, SAUR*47* (Figure 4.4 to 4.7) ALP shows highest expression in pollen and siliques and affect male sterility (We et al., 2016). My data showed maximum expression of the *ALP* transcript at TCP stage. *bZIP*, showed strong expression in pollen (Alves-Ferreira et al., 2007), which is consistent with my finding with maximum expression at TCP stage. Many of the MYB genes has been reported for their expression at high level in sperm cells. *ATMYB21* is stamen gene that affects male sterility and flower regulation (Shim et al., 2002), similar expression at TCP was shown in my data. Both *bZIP* and *ATMYB21* were downregulated in *myb80* mutant and are involved in PCD at tetrad stage (Phan et al., 2011; Yi et al.,2016). *PCR11* and *DAZ3* are sperm specific genes targeted by the male germline-specific R2R3 MYB transcription factor DUO1 POLLEN1 (*DUO1*) (Borg et al., 2011). Thus, explain their highest expression at TCP stage. *SAUR47* is induced by auxin and promotes cell expansion (Spartz *et al.*, 2012) also shows high expression at TCP. *FLA3*, is involved in pollen intine and is highly expressed at TCP (Li et al.,2010; Li et al., 2017), which was confirmed by the qRT-PCR expression at BCP and TCP (Figure 4.4).

In chapter 5 overexpression lines have been generated showing complete male sterility for both *SAUR47 and FLA3* that will be discussed in more details.

DUF1216 show highest expression at Microspore stage (II) and TCP in my study. It has peak expression pattern at uninucleate microspores and TCP with high expression levels at early tapetum with high RPKM values 76 reported by (Li et al., 2017). Suggesting *DUF1216* unique role in early and late tapetum. *DUF1216* generated overexpression lines indicate male sterility as presented by my data in chapter 4. *ATHSFA3* and *MYB101* show high expression at both tetrad and TCP stages. Boavida., et al (2005) has reported *ATMYB101* expression in mature pollen and microsporogenesis, *ATMYB101* which is regulated by JA and is required for anther dehiscence and is believed to trigger programmed cell death PCD (Phan et al., 2011). Intriguing, *Beta-1,3-glucanase* show specific Tetrad expression as presented by my data and reported by (Kang et al., 2008; Li et al., 2017) with RNA-seq, analysis Reads Per Kilobase per Million mapped reads (RPKM) value 33. However, the rest of the candidate genes were expressed in all stages of pollen developmental such as; *HKL3*, *ATNACK1, TCH4/ATXTH22, CDS2, ATTPS7, ATHSFA2, AtDIV3, BBX19, AtDIV4, HRGPs*, *PLDALPHA2*, *NEP1LP* a ribosome biogenesis and *LEA* gene that prevent cell from dehydration and are downregulated by *myb80* mutant (Phan et al., 2011). *AtNACK1* has shown expression in all the four stages of buds

analysed, suggesting a role to maintain pollen cell walls during and after every cell division. *ATXTH22* is involved in cell wall strengthening and increasing cell wall biogenesis, *CDS2* exhibits localization of Cu in all stages of pollen. Trehalose-phosphatase gene *ATTPS7* which are believed to stabilize proteins and membranes structures during sugar metabolism. Genes with *BBOX* domain has been illustrated as tapetum specific as well as *HSF* in *Medicago truncatula* (Shao, et al., 2016). This suggested function for *BBX19* and *ATHSFA2* in tapetum development through all the developmental stages is supported by the expression data shown (Figure 4.5). *HKL3* which is essential for somatic cell cytokinesis, especially for the cell- plate formation, reported in my work as mostly expressed in BCP and TCP has been reported by (Li et al., 2017) as a late tapetum gene which is down regulated by MS188. Despite, that Hexokinase has a role in inhibition of PCD as reported by Kim et al., (2006). This suggests that *HKL3* may function in tapetum degradation.

Figure 4.4 Quantitative RT-PCR of *LEA*, *DUFF8*, *PLDALPHA2*, *FLA3* and *SAUR47* expression analysis in different buds of the wild-type (Wt) and drought stress. (pollen tetrad) stage (I), (microspore MS) stage (II), (bicellular pollen) BCP stage (III) and (tricellular Pollen) TCP stage (IV). Data was normalised to the reference gene PP2A3 Error bar, standard error. Statistical difference represented by Student's T-Test; an asterisk (*) $P \leq 0.05$ (**) indicate $P \leq 0.01$, (***) $P \leq 0.001$.

Figure 4.5 Quantitative RT-PCR of *ATDIV3*, *ATDIV4*, *ATHSFA2*, *CSD2*, *HRGPS*, *ATMYB101*, *BBX19*, *ATHSFA3* expression analysis in different buds of the wild-type (Wt) and drought stress. (pollen tetrad) stage (I), (microspore MS) stage (II), (bicellular pollen) BCP stage (III) and (tricellular Pollen) TCP stage (IV). Data was normalised to the reference gene PP2A3 Error bar, standard error. Statistical difference represented by Student's T-Test; an asterisk (*) P ≤ 0.05 (**) indicate P ≤ 0.01, (***) P ≤ 0.001.

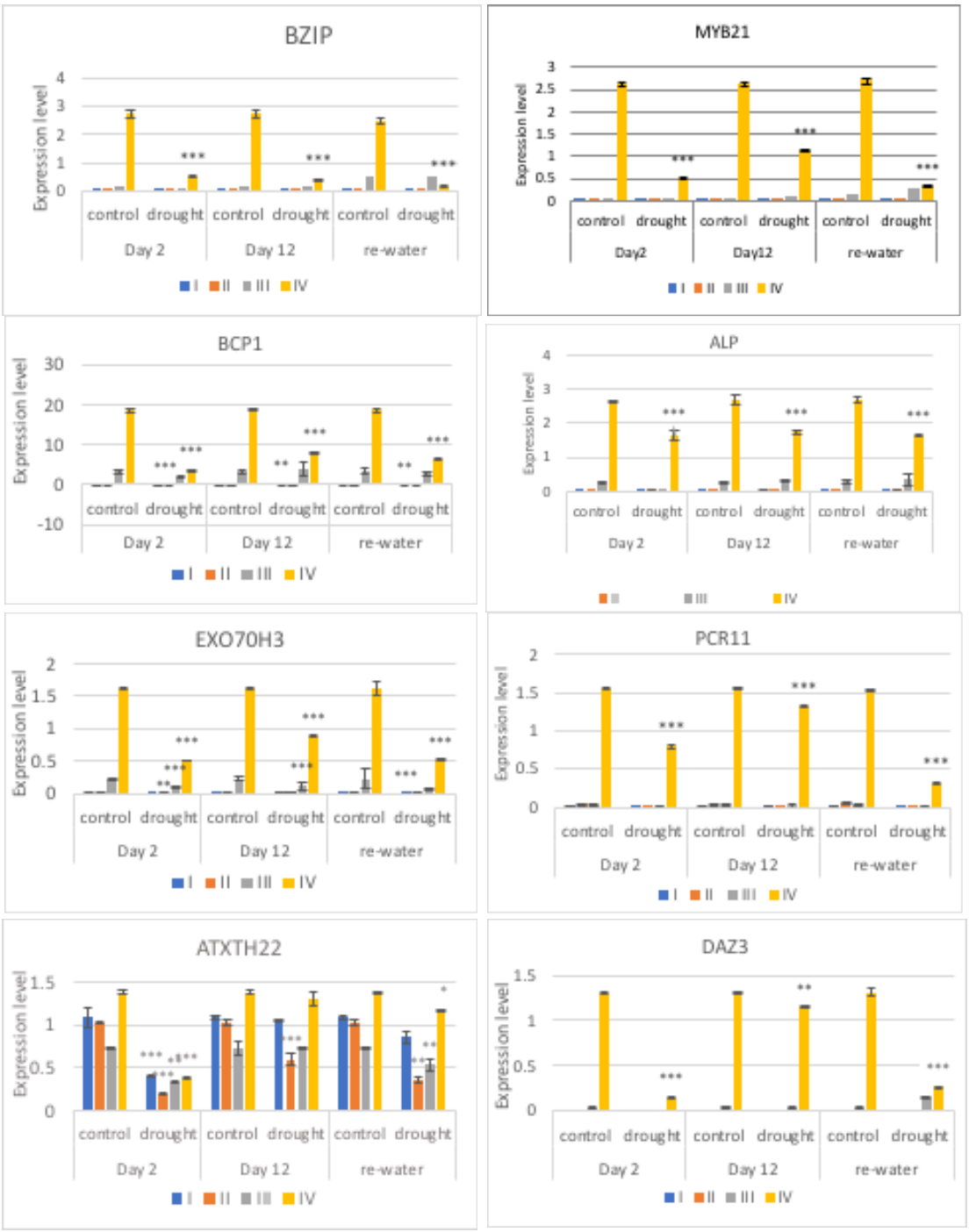


Figure 4.6 Quantitative RT-PCR of *BZIP, MAY21*, *BCP1*, *ALP*, *EXO70H3*, *PCR11*, *DAZ3* and *ATXTH22*, expression analysis in different buds of the wild-type (Wt) and drought stress. (pollen tetrad) stage (I), (microspore MS) stage (II), (bicellular pollen) BCP stage (III) and (tricellular Pollen) TCP stage (IV). Data was normalised to the reference gene PP2A3 Error bar, standard error. Statistical difference represented by Student's T-Test; an asterisk (*) $P \leq 0.05$ (**) indicate $P \leq 0.01$, (***) $P \leq 0.001$.

Figure 4.7 Quantitative RT-PCR of *HKL3*, *ATTPS7*, *ATCHX19*, *NEP1LP*, *ATNACK1*, *SFH5LP*, *TPPF* and *PRF5* expression analysis in different buds of the wild-type (Wt) and drought stress. (pollen tetrad) stage (I), (microspore MS) stage (II), (bicellular pollen) BCP stage (III) and (tricellular Pollen) TCP stage (IV). Data was normalised to the reference gene PP2A3 Error bar, standard error. Statistical difference represented by Student's T-Test; an asterisk (*) $P \leq 0.05$ (**) indicate $P \leq 0.01$, (***) $P \leq 0.001$.

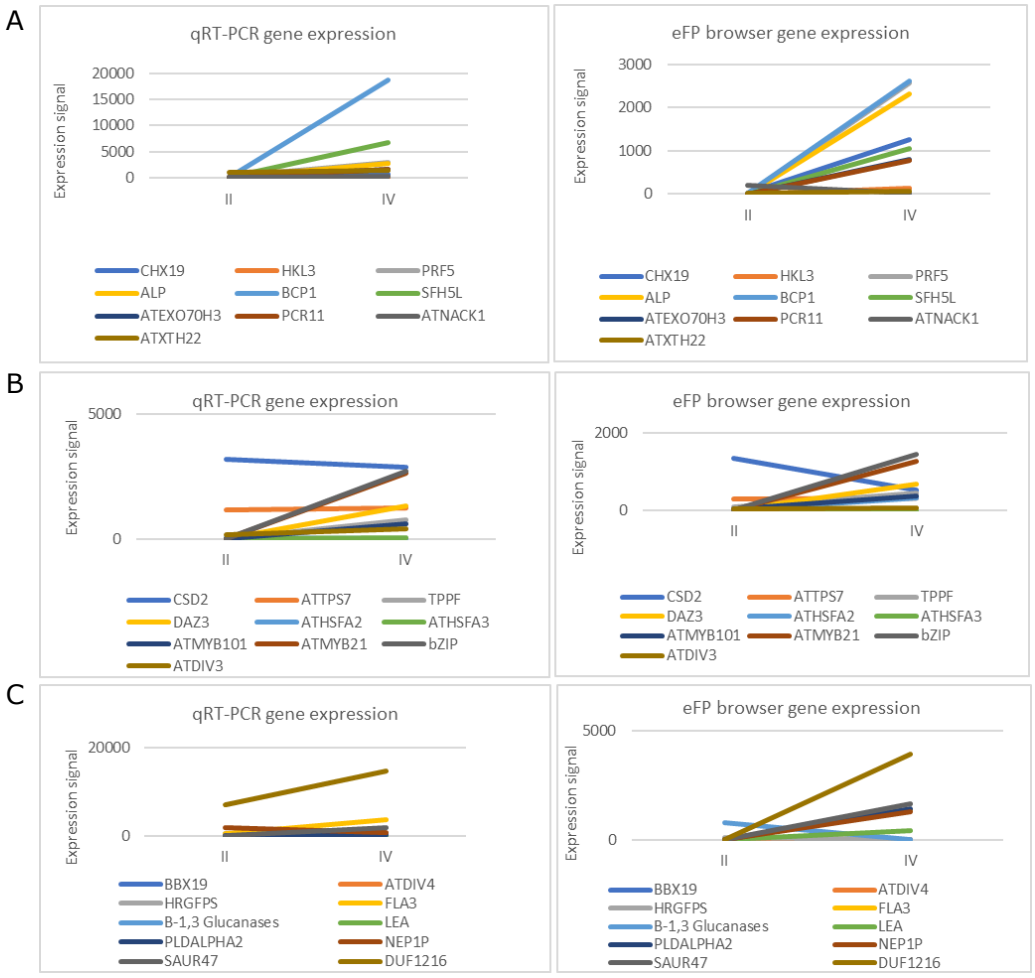


Figure 4.8 Graphs show comparison between qPCR expression analysis of the 30 genes with the data provided from the eFP browser in both anther stage 9 (stage II) and stage 12 (stage IV). A-C)30 genes were divided by 10 genes in each sun panel, all qRT-PCR expression exhibited similar expression as presented from eFP browser except for B-1,3 Glucanases that showed highest expression in pollen stage I by the qPCR while the highest expression existed at pollen Stage II by the eFP browser, the NEP1P showed opposite expression as in the eFP browser.

4.3.4 Genes Expression during Drought

A survey of the expression changes seen for the drought and watering measurements are shown in table 4.2 and 4.3 and in a heat map (Figure 4.9)

4.3.4.1 Pollen Tetrad (Anther Stage 7-8)

Callose beta 1,3-glucan cell wall physiologically isolates the microspores from the surrounding sporophytic tissue. Some genes were up-regulated by the drought at this stage, such as *CSD2*, *ATHSFA3*, *BBX19*, *HRGPs* and *B-1,3-Glucanases*) (Figure 4.4 &4.5). This implies that these genes have distinct roles in maintaining cell tolerance and degrading the callose wall, may have the exact localization of these genes may be by release from the tapetum, as shown for (Tratt., 2016) however *B-1,3-Glucanases* gene and this hypothesis needs further investigation. The down-regulated genes at this stage are the heat stress gene *ATHSFA2* as well as *ATMYB101* and its two paralogs (*AtDIV3, AtDIV4*), which has been reported as a specific tapetum genes (Li et al., 2017). HSPs are considered to prevent tapetum differentiation (Xing et al., 2018). This suggest changes *ATHSFA2* and *ATHSFA3* in response to drought at tetrad, and all of them might have a role in during tapetum PCD.

4.3.4.2 Early Microspore (Anther Stage 9-10)

The nucleus is pushed towards the cell wall to allow rapid vacuole biogenesis. The large vacuole is important to prepare pollen cells for haploid mitosis producing a large vegetative and smaller generative cell. The cellular trafficking gene *DUF1216* and *LEA* were up-regulated during drought during the asymmetric cell division phase. However, Hexokinase gene *HKL3*, *ATXTH22, CSD2, CHX19, NACK1, ATTPS7, PLDALPHA2, HKL3,* and *ATHSFA2, ATDIV3*, *BBX19, AtDIV4, HRGPs NEP1-like gene* were down-regulated in response to drought (Figure 4.4 to 4.7).

4.3.4.3 Bicellular Pollen

In which the pollen generative cell is prepared for mitosis II division. Genes like *HKL3*, *ATHSFA2* and *LEA* were up-regulated by the drought. Although, those genes *CHX19*, *PRF5, ALP,* SFH5LP, ATXTH22, *ATEXO70H3, ATNACK1, SFH5L,* CSD2, ATTPS7, AtDIV3, BBX19, PLDALPHA2, *MYB21* were down-regulated in response to drought (Figure 4.4 to 4.7).

4.3.4.4 Tricellular Stage

After mitosis II the cell produces two sperm cells which is the mature pollen grain stage, the following genes were important to the development for example, *FLA3* and *LEA* were the only genes that up-regulated by the drought, except for all the rest of the twenty-eight genes were they down regulated by drought including DAZ3. Honys and Twell (2004) indicated

that gene expressed during later pollen development may be correlated to desiccation and other specific pollen function.

Table 4.2 List of genes up and down regulated at the end of the drought day12.
In buds at different pollen developmental stages. Symbol (-) means no change in the expression.

AGI	Other names	Tetrad	Microspore	Bicellular	Tricellular
AT3G17630	CHX19	-	-	-	Down
AT5G57560	ATXTH22	-	Down	-	-
AT3G02120	HRGFPS	Up	-	-	Down
AT2G24450	FLA3	-	-		Up
AT3G23770	B-1,3 Glucanases	Up	-	-	-
AT2G19770	PRF5	-	-	Down	Down
AT1G52570	PLDALPHA2	Up	Down	Down	Down
AT3G09530	ATEXO70H3	-	-	Down	Down
AT1G18370	HINKEL/ATNACK1	-	Down	Down	Down
AT4G27580	SFH5L	-	-	-	Down
AT1G06410	ATTPS7	-	Down	-	-
AT4G12430	TPPF	-	-	-	Down
AT4G37840	HKL3	-	-	Up	Down
AT4G35700	DAZ3	-	-	-	Down
AT2G26150	ATHSFA2	Down	Down	Up	Down
AT5G03720	ATHSFA3	Up	-	-	Down
AT2G32460	ATMYB101	Down	-	-	Down
AT3G27810	ATMYB21	-	-	-	Down
AT1G35490	bZIP	-	-	-	Down
AT2G38090	ATDIV3	Down	Down	Down	Down
AT4G38960	BBX19	Up	Down	Down	Down
AT5G01200	ATDIV4	Down	Down	-	Down
AT3G28980	DUF1216	-	Up	-	Down
AT3G20220	SAUR47	-	-	-	Down
AT4G36600	LEA	-	Up	Up	Down
AT1G68610	PCR11	-	-	-	Down
AT1G24520	BCP1	-	-	-	Down
AT2G28190	CSD2	Up	Down	Down	Down
AT1G63060	NEP1P	-	-	Up	Up
AT3G26110	ALP	-	-	-	Down

Table 4.3 List of genes up and down regulated after 48hr of re-watering.
In buds at different pollen developmental stages. Symbol (-) means no change in the expression.

AGI	Other names	Tetrad	Microspore	Bicellular	Tricellular
AT3G17630	CHX19	-	Up	Down	Down
AT5G57560	ATXTH22	-	Down	Down	Down
AT3G02120	HRGFPS	Down	-	-	Down
AT2G24450	FLA3	-	-		Down
AT3G23770	B-1,3 Glucanases	-	-	-	-
AT2G19770	PRF5	-	-	Down	Down
AT1G52570	PLDALPHA2	-	Down	-	Down
AT3G09530	ATEXO70H3	-	-	Down	Down
AT1G18370	HINKEL/ATNACK1	-	Down	Down	Down
AT4G27580	SFH5L	-	-	Down	Down
AT1G06410	ATTPS7	Down	Down	Down	Up
AT4G12430	TPPF	-	-	-	Down
AT4G37840	HKL3	-	Down	Down	Down
AT4G35700	DAZ3	-	-	-	Down
AT2G26150	ATHSFA2	Down	Down	Up	Down
AT5G03720	ATHSFA3	-	-	-	Down
AT2G32460	ATMYB101	Down	-	-	Down
AT3G27810	ATMYB21	-	-	-	Down
AT1G35490	bZIP	-	-	-	Down
AT2G38090	ATDIV3	Down	Down	Down	Down
AT4G38960	BBX19	Down	Down	Down	Down
AT5G01200	ATDIV4	Down	Down	-	Down
AT3G28980	DUF1216	-	Down	-	Down
AT3G20220	SAUR47	-	-	-	Up
AT4G36600	LEA	-	-	Up	Down
AT1G68610	PCR11	-	-	-	Down
AT1G24520	BCP1	-	-	-	Down
AT2G28190	CSD2	Down	Down	Down	Down
AT1G63060	NEP1P	-	Down	Down	Down
AT3G26110	ALP	-	-	-	Down

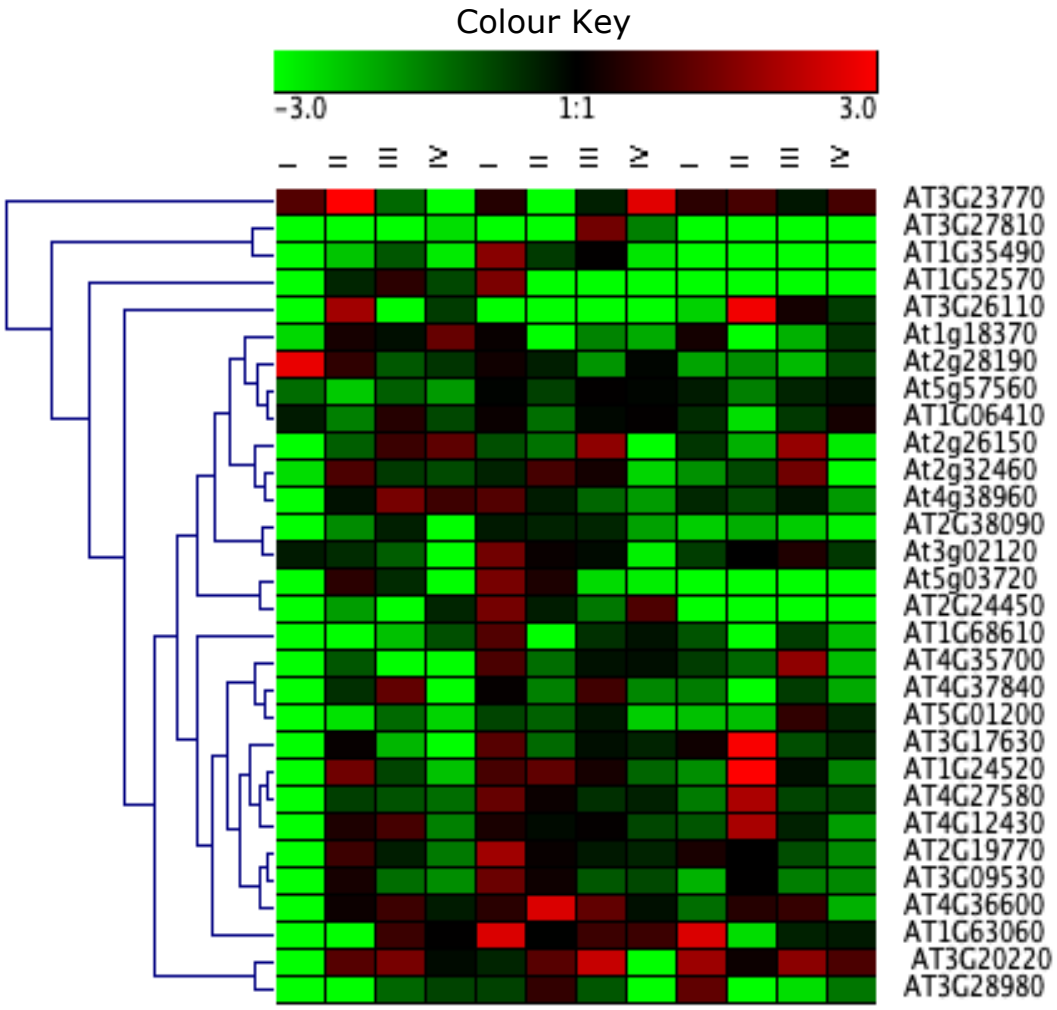


Figure 4.9 Heatmap showing relative expression of four stages of pollen development compared drought to control of 30 genes. (pollen tetrad) stage (I), (microspore MS) stage (II), (bicellular pollen) BCP stage (III) and (tricellular Pollen) TCP stage (IV). Heatmap showing hierarchical clustering of 30. Dark shades indicate higher expression and light shades indicate lower expression. Colour key indicates the intensity associated with normalized expression set to 3.0 and -3.0 respectively.

4.4 Discussion

Plants respond to drought stress differently in different tissues and at different developmental stages. To validate gene expression analysis obtained from the qRT-PCR all the presented data were compared with the data provided from the eFP browser (online *Arabidopsis* microarray data allows visualization of gene expression data) that showed consistency with the eFP browser except for B-1,3 Glucanase that showed highest expression in pollen stage I by the qRT-PCR while the highest expression occurred at pollen Stage II by the eFP browser and NEP1P which showed opposite expression to the eFP browser. To develop immediate visualisation of a summary of the qRT-PCR information a heat map, which allows a better understanding of the complex data sets were generated based upon expression under drought stress of the relative expression to control (watered) of the 30 genes as shown in (Figure 4.9) in the four different stages of pollen development.

Some genes showed specific expression pattern and regulation to drought at only TCP or tetrad while others have changes at microspore, BCP and TCP (Table 4.2). Significant expression changes have been seen as a consequence of drought stress and these may have a general impact on fertility causing impaired pollen development.

DYSFUNCTIONAL TAPETUM1 (DYT1), DEFECTIVE in TAPETAL DEVELOPMENT FUNCTION1 (TDF1), A BORTED MICROSPORES (AMS), AtMYB103/MS188 form a genetic pathway for tapetum development and pollen wall formation (Zhu et al., 2011). A new study by Li et al., (2017) indicates that *ALP*, *BCP1*, *HKL3*, *SFH5LP*, *AtMYB101*, *bZIP*, *PLDALPHA2*, *NEP1P*, *LEA*, *DUF1216*, *b-1,3-Glucanases, FLA3*, *SAUR47, CHX19 and DUO1* directs of target *DAZ3* and *PCR11* were down-regulated in all the four mutants *dyt1*, *tdf1*, *ams*, and *ms188*. Interestingly, *HKL3*, *ALP*, *BCP1*, *SFH5LP*, *FLA3*, *SAUR47* were not expressed during early tapetum. However, MYB*21*, *ATDIV3*, *ATDIV4* and *B-1,3- GLUCANASES* were defined as tapetum specific genes. Furthermore Xing et al., (2018) implies that abnormal tapetum thickening during early stage of the development causes male sterility. All of these are supporting evidence for the importance of the candidate genes in my study and explaining that the role of target gene in tapetum development is critical for male fertility.

4.4.1 Pollen target Genes for drought tolerance

The plant hormone abscisic acid (ABA) is a master regulator of plant development and water loss responses, regulates embryo and seed development, seed dormancy, germination, seedling establishment, vegetative development, general growth and, during dehydration, accumulation of osmocompatible solutes and synthesis of dehydrins and *LEA* proteins (Cutler et al., 2010). ABA-induced genes encode for proteins

such as dehydrins, enzymes that detoxify reactive oxygen species, enzymes of compatible solute metabolism, a variety of transporters, transcription factors, protein kinases and phosphatases, and enzymes involved in phospholipid signalling (Cutler et al., 2010). Increasing the knowledge of ABA-dependent drought responses and its core signalling components offers possibilities for the development of drought tolerant crops. Since Parish et al (2012) explained that during drought ABA signalling downregulates tapetum cell wall invertase which blocks sucrose production allowing hexokinase for feeding the pollen. Trehalose synthesis genes considered as a reserve carbohydrate and stress protectant, reported as up-regulated genes in Saccharomyces cerevisiae drought (Taymaz-Nikerel et al., 2016) such as *HKL3* degrade tapetum cells and this may explain gene (*TPPF, TPS2, TPS3, TPS4, TPS7*) downregulation by the drought. This suggests that disruption of sucrose metabolism and signalling is causing reproductive failure under abiotic stress. Transcription factors like *ATMYB101* which are reported as ABA mediated by miR159 to drought up-regulation (Reyes and Chua 2007). *bZIP*, *MYB*, and genes with zinc-finger domain such as *BBX19* are stress modulators (Dubouzet et al., 2003; Yang et al., 2012; Mukhopadhyay et al., 2004) and are highly expressed under drought. *HSPS* is known as a molecular *chaperone* to prevent protein folding are mostly induced by the drought (Campalans et al., 2001). Cell wall related genes such as, *PLD* which is down-regulator to

ABA mediated by miR167 to drought (Morad et al.,2016). *SFH5LP* induced in response to drought by ABA (El-Maarouf *et al*., 2001). *CHX19* was reported as regulated by Jasmonate (Mandaokar et al., 2006) and is down regulated by drought as well as *FLA3* and *ATXTH22*.

BCP1 a pollen sperm gene also regulated by ABA targeted by miR169 in response to drought in *Medicago truncatula* plant (Wang et al., 2011). Hormonal related genes like *SAUR* genes response to different plant stresses, including drought (Du et al., 2013). Many miRNAS has been reported expressed in drought stress (Kantar et al., 2011; Frazier et al., 2011). ABA mediated miR394 was found to regulate *CU/ZN CDS* genes in response to drought (Sunkar et al., 2006).

4.4.2 Analysis of gene network

Given the importance of the analysed genes in pollen development and stress responses, their interaction as parts of a functional protein network were investigated. Genes were evaluated using the STRING network tool (http://string-db.org). Interestingly, two main groups were observed; First showed 11 out of the 30 gene networks *(PRF5, SAUR47, DAZ3, PCR11, SFH5LP, FLA3, BCP1, ALP, MYB101, BZIP, EXO70H3*) , second group showed 5 out 30 (*CSD2, TPS7, BBX19,HKL3,TPPF*) , moreover 4 separate genes with each 2 were connected (*HSFA2, HSFA3*) and (*HIK, HRGPs*) and two triple connection mediated by *PLDALPHA2* (*CHX19, HKL3*) and *NEP1LP* (*PRF5, DUF1216*) (Figure 4.10) the enrichment p-value= (< 1.0e-16). This

finding support that those genes may act in common networks and pathways and may affect the same biological mechanisms. As in each stage of pollen development there was a group of genes (chapter4 section 4.3.4) that could contribute to the same process and response in different ways to drought.

To summarise, the qRT-PCR analysis provides clues for the most highly expressed pollen genes in *Arabidopsis* during water desiccation, which suggests a new avenue for further investigations of its role in pollen development. Further analysis of these targets and altered expression may provide mechanisms for developing resilience and may assist in developing better breeding strategies for improving drought tolerance. A next step in such analysis is altering their expression possibly by mutagenesis by CRISPR-Cas9 system (Zhang et al., 2014), or generating over expression lines. Based on the transcriptomic data from the four pollen development stages a group of seven genes (*LEA*, *DUF1216*, *FLA3*, *SAUR47*, *NEP1LP*, *PLDALPHA2*, *B-1,3- GLUCANASES*) were selected for over expression lines which will be discussed in more details in the chapter 5.

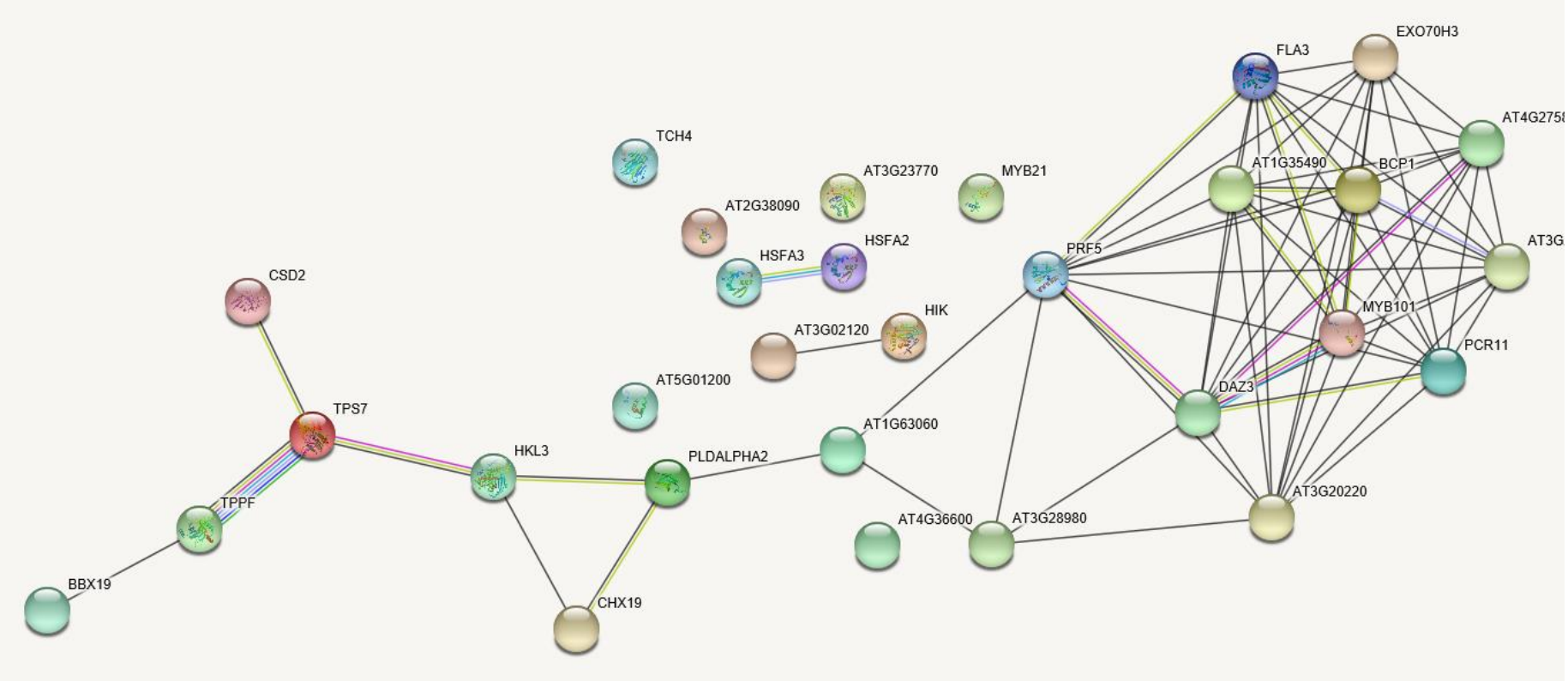


Figure 4.10. Protein interaction network of the 30 genes. The network was constructed using the STRING tool (http://string-db.org) and the 30 candidate genes as input. *PRF5, SAUR47, DAZ3, PCR11, SFH5LP, FLA3, BCP1, ALP, MYB101, BZIP,* EXO70H3 interacted to maintain pollen gametophyte, cell differentiation, pollen wall and pollen tube germination, while *CSD2, TPS7, BBX19, HKL3,TPPF* has a role in sugar metabolism during pollen development. The width of the interactions depends on the confidence score to each association in STRING.

Chapter 5 Characterization of Drought Candidate Genes

5.1 Introduction

Several transcription factors have been reported to be important for anther and pollen development such as *TAPETUM DETERMINANT1* (*tpd1*) (Yang et al., 2005), *MALE STERILITY1* (*MS1*) (Wilson et al., 2001), *ABORTED MICROSPORES* (*AMS*) (Sorensen et al., 2003), and *DYSFUNCTIONAL TAPETUM1* (*DYT1*) (Zhang et al., 2006; Feng et al., 2012). For better understanding for gene regulatory network controlling pollen development and based on gene expression analysis obtained from the previous chapter, six genes were selected *LEA*, *DUF1216*, *FLA3*, *SAUR47*, *PLDALPHA2*, *NEP1LP* based on changes in their expression after re-watering where they considered as drought responsive genes, while *B-1,3- GLUCANASES* showed up regulation by drought, and has been proposed as having a role in callose accumulation where Callose to help absorb water molecules (Li et al., 2010). The candidate genes are highly expressed at different stages of pollen development (Chapter 4) and were chosen as good candidate for drought stress studies. Genes were studied in this chapter by T-DNA knock out lines and overexpression lines.

5.2 Knockout Mutant Lines Confirmation

Based on the *Arabidopsis* Gene Mapping Tool of the Salk Institute (http://signal.salk.edu/cgi-bin/tdnaexpress), potential T-DNA insertion lines were identified for the candidate genes (Appendix II) and ordered from the Nottingham Arabidopsis Stock Centre (NASC). Insertion lines were identified for five of the selected genes [AT1G52570 *(PLDALPHA2)*, AT4G36600 (*LEA)*, AT2G24450 (*FLA3)*, AT3G20220 (*SAUR47),* AT3G23770 (*B-1, 3- GLUCANASES)*], these contained T-DNA insertions in the protein coding region; however, no T-DNA insertion lines were found for both AT1G63060 and AT3G28980 genes. Figure 5.1 shows a map for each gene with the T-DNA insertion. Genotyping was conducted by PCR using gene-specific primers combined with LBb1 and LB3 primers. Plants amplifying a PCR product for the gene-specific primer combination only were considered homozygous wild type, plants with only products for the T-DNA insertion primers homozygous mutant, and plants displaying bands for both PCR products were considered heterozygous. PCR genotyping for PLDALPHA2 (AT1G52570) gene SALK-lines represented by the (GABI-Kat) GK-212E06 showed that two of six plants were identified as homozygous (Figure 5.2a). as well as SALK_098375C (Figure 5.2c4). However, the other 4 plants of GK-212E06 (Figure 5.2a) and SALK_053957 were Wild type, (Figure 5.2d3) and showed the expected

WT gene size (1210 bp). For the AT3G20220 (*SAUR47*) gene insertion, two Salk lines were identified GK-171H05 (Figure 5.2b) and SAIL_84_D03 which hits AT3G20230 (Ribosomal L18p/L5e family protein) and ends at AT3G20220 gene; eight out of the ten of GK-171H05 plant were homozygous insertions, the SAIL_84_D03 was also homozygous (Figure 5.2e4). The Late embryogenesis abundant (*LEA*) AT4G36600 was studied using two T-DNA insertion lines SALK_131759C (Figure 5.2c2) and SALK_046270 (Figure 5.2d5) which were identified as homozygous mutants. AT3G23770 was analysed using four insertion lines Salk_033100, SAIL_885_D07 (Figure 5.2c), SALK_138003 and SAIL_883_D01 (Figure 5.2d) the first two were homozygous while the last two were wild-type. SAIL_1233_G05 (Figure 5.2c10) and SALK_016582 (Figure 5.2e2) were homozygous lines for FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 3 *(FLA3*) AT2G24450 gene.

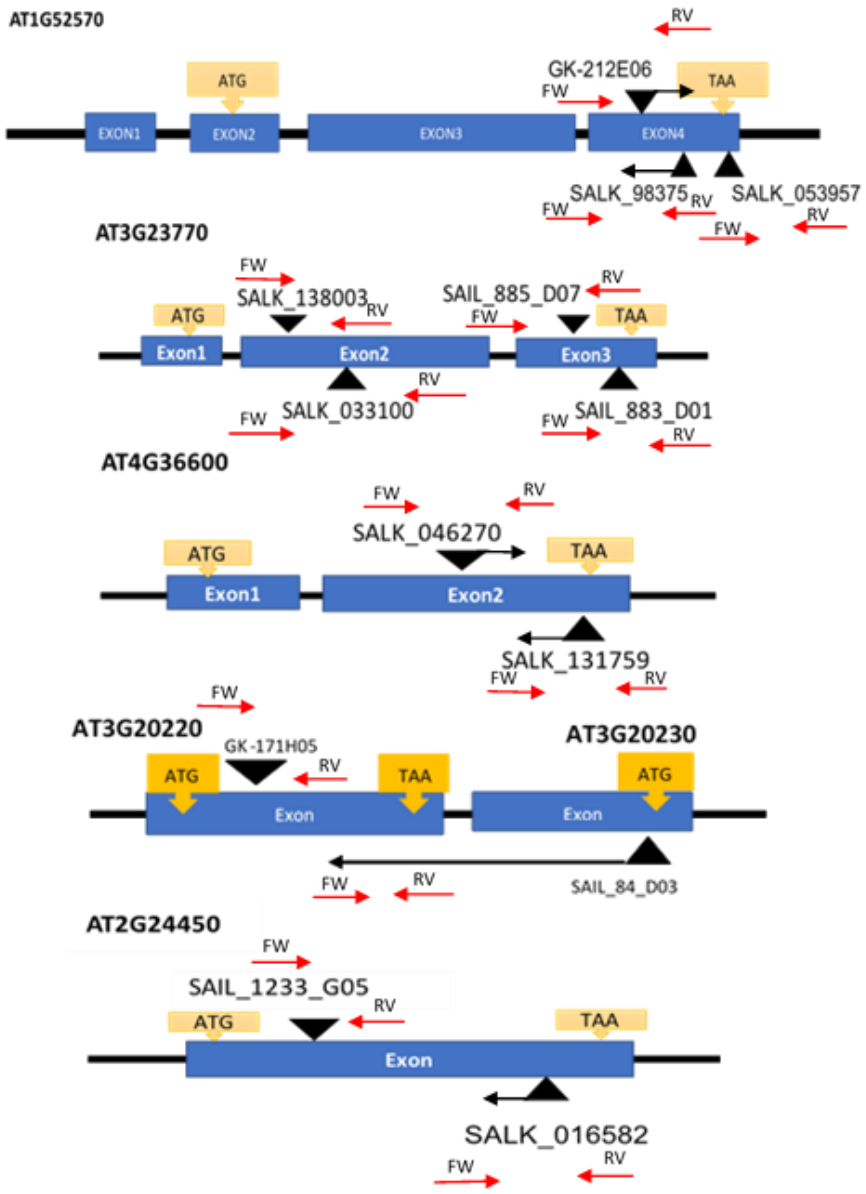


Figure 5.1. Schematic represent of the target genes with T-DNA insertion information. The diagram shows the SALK T-DNA insertion sites for the AT1G52570, AT3G23770, AT4G36600, AT3G20220 and AT2G24450 genes. Red arrow indicates both forward and reverse primer used. T-DNA insertion sites were obtained from the SIGnAL website (http://signal.salk.edu) whilst gene models were based on genomic DNA sequence information from the NCBI database.

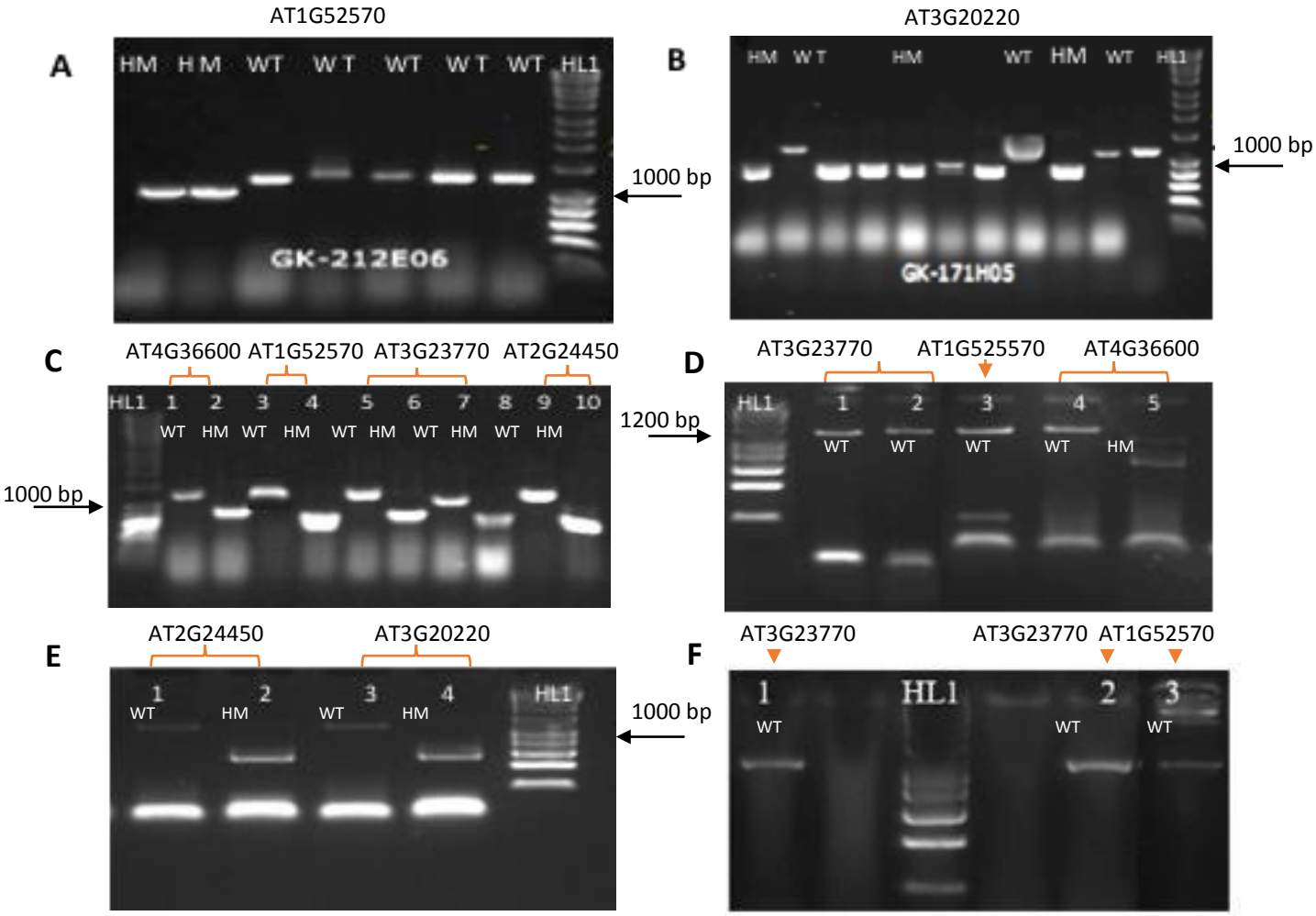


Figure 5.2. Genotyping of T-DNA insertions lines.

(A) GK-212E06 expected band size for WT ~1210 bp and ~1000 bp for the homozygous T-DNA insertion.**(B)** GK-171H05 expected band size for WT ~ 1081 bp and ~800 bp for the homozygous T-DNA insertion.**(C)** 1-SALK_131759C expected band size for WT ~1136, 2- SALK_131759C band size ~800 bp for the homozygous T-DNA insertion, 3- SALK_098375C expected band size for WT~1000 bp, 4-SALK_098375C band size ~600 bp for the homozygous T-DNA insertion, 5- SAIL_885_D07 expected band size for WT ~1190 bp, 6- SAIL_885_D07 band size ~800 bp for the homozygous T-DNA insertion, 7- SALK_033100 expected band size for WT ~1068 bp, 8- SALK_033100 band size ~600 bp for the homozygous T-DNA insertion. 9- SAIL_1233_G05 expected band size for WT ~1191 bp, 10-SAIL_1233_G05 band size ~600 bp for the homozygous T-DNA insertion.**(D)** 1- SALK_138003 expected band size for WT~ 1200, 2- SAIL_883_D01 expected band size for WT~ 1200, 3- SALK_053957 expected band size for WT~ 1200, 4- SALK_046270 expected band size for WT~ 1200 , 5- SALK_046270 ~600 bp for the homozygous T-DNA insertion.**(E)** 1- SALK_016582 expected band size for WT~1200 bp, 2-SALK_016582band size ~800 bp for the homozygous T-DNA insertion, 3- SAIL_84_D03 expected band size for WT~1200 bp, 4- SAIL_84_D03 band size ~883 bp for the homozygous T-DNA insertion. **(F)** 1-SAIL_883_D01 expected band size for WT ~1093 bp, (2) SALK_138003 expected band size for WT ~ 1051 bp , 3- SALK_053957 expected band size for WT ~ 1080 bp.

5.3 Expression Analyses of The Salk Knockout Lines

RT-PCR expression analysis for SALK-lines was conducted using gene-specific primers on the homozygous insertion lines (Appendix II) PP2A3 primers were used to check the integrity of the cDNA and as housekeeping genes for normalization, wild type Col cDNA was used as the positive control. The results of the target gene expression analysis showed amplification in the homozygous mutant lines (Figure 5.3). Suggesting changes in gene expression which need to be tested by qRT-PCR.

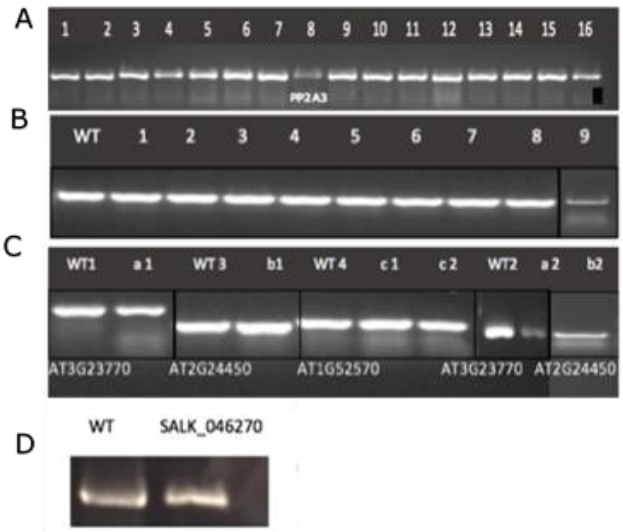


Figure 5.3. RT-PCR Expression analysis of T-DNA insertional homozygous lines: A) PP2A3 was used as a control to check the integrity of the cDNAs and consistory between samples. **B)** Gene specific expression of AT3G20220 in GK-171H05 (1) GK-171H05-1, (2) GK-171H05-11, (3) GK-171H05-5, (4) GK-171H05-10, (5) GK-171H05-9, (6) GK-171H05-12, (7) GK-171H05-4, (8) GK-171H05-5 (~187bp), (9) SAIL-84D03 (400bp). **C)** Gene specific expression of AT3G23770 in SALK_033100 (~395bp), (a1) SALK_033100 (~395bp), WT2 SAIL_885_D07(~ 139bp), (a2) SAIL_885_D07 (~ 139bp). Gene specific expression of AT2G24450 in for SAIL_1233_G05 (~ 167bp), (b1) SAIL_1233_G05 (~ 167bp), (b2) SALK-016582 band size (~354bp). Gene specific expression of AT1G52570 in GK-212E06 expected band size (~273bp), (C1) GK-212E06-7, (C2) GK-212E06-11 band size (~ 187 bp). **D)** Gene specific expression of AT4G36600 for SALK_046270 band size (~ 330bp).

5.4 Phenotypic Analysis Of T-DNA Insertion Lines

All the homozygous T-DNA lines were assessed for changes in phenotype compared to the wild-type, and by light microscopy for any changes to pollen development. All lines for genes *SAUR47*, *PLDALPHA2*, *LEA*, *B-1,3-Glucanases* and *FLA3* (GK-171H05, GK-212E06, SALK_131759C, SALK_033100, SALK_098375C, SAIL_885_D07 and SAIL_1233_G05) exhibited a reduction in the main stem height that varied between SALK-lines, although the vegetative growth was normal compared to wild type, particularly SALK_098375C for *PLDALPHA2* showed 29 mm in length compared to the wild type (Figure 5.4a&5.5b). The insertion seems to reduce number of auxiliary branches approximately by half as observed in GABI-Kat collection GK-171H05-1, GK-171H05-5 for *SAUR47* gene, SALK_131759C for *LEA* and by a quarter in GK-171H05-12 for *SAUR47* gene. However, number of branches is increased in GK-212E06-11 (Figure 5.4a&5.5b). Since mutation of *atmbd9* transcription factor increases the axillary branches in (Yaish et al. 2009). Therefore, *SAUR47*, *PLDALPHA2* and *LEA* show possible roles in regulation of axillary branching and stem length; these genes could be regulated by *ATMBD9* under ABA and auxin signalling this hypothesis needs further investigation. Brewer et al., 2009 reported the role of auxin in the control of shoot branching. While ABA

modulates auxin to represses axillary bud growth (Chatfield et al. 2000). No differences were observed in germination, the time of flowering or general plant or flower morphology. The siliques length measurements such as SALK_131759C, GABI-Kat collection and SAIL_885_D07 also did not show any significant differences compared to the wild type suggesting no impact on fertility (*F16, 59*= 0.82, *P*=0.65) P > 0.05 not significant (Figure 5.5a). However, SALK 046270, SALK_016582 and SAIL_84_D03 in gene *LEA*, *FLA3* and *SAUR47* respectively show a strong phenotype of reduced fertility based upon reduced siliques length compared to the wild type which is statistically significant P < 0.001 (Figure 5.6a). Plants were partially fertile with shorter siliques, indicating reduced fertility (Figure 5.6b).

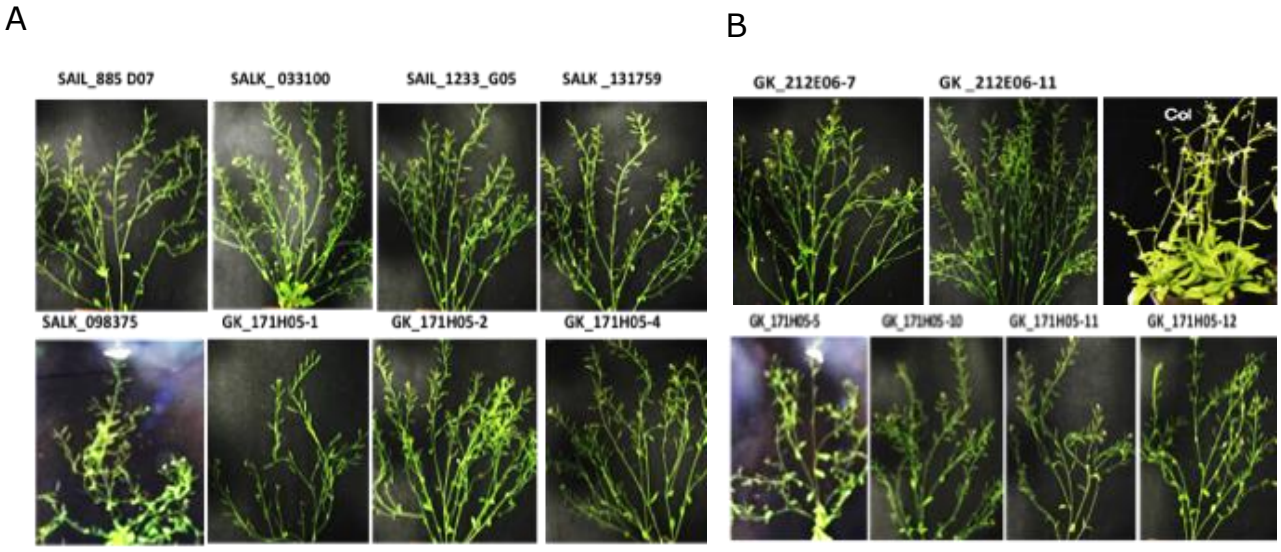
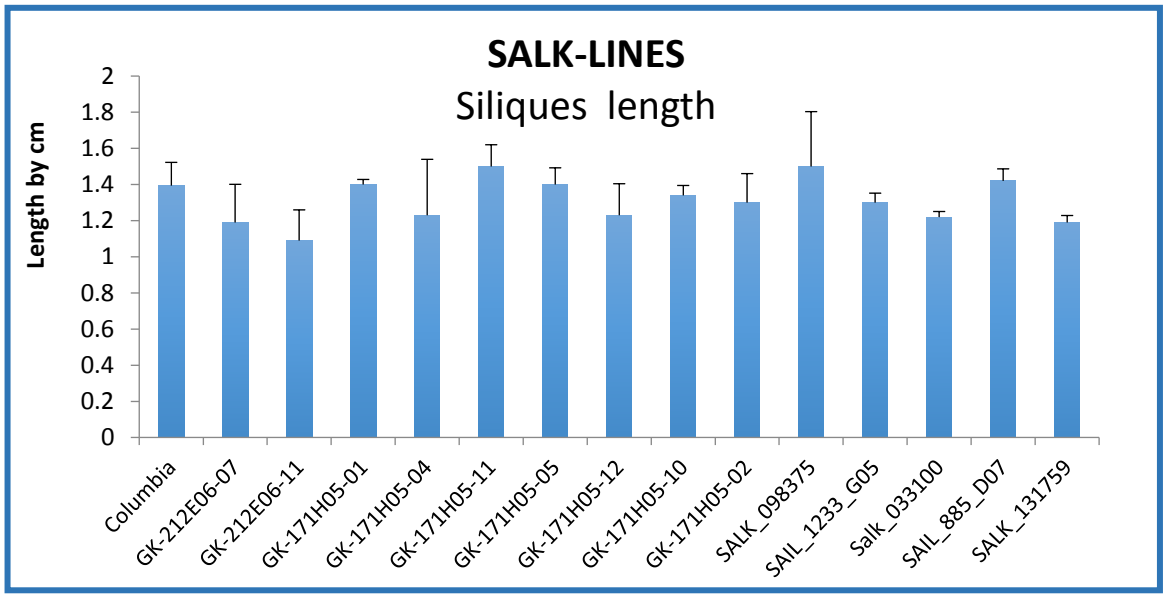


Figure 5.4. Phenotyping analysis of the T-DNA insertional lines. A -B) General plant morphology of the homozygous T-DNA insertional lines

A



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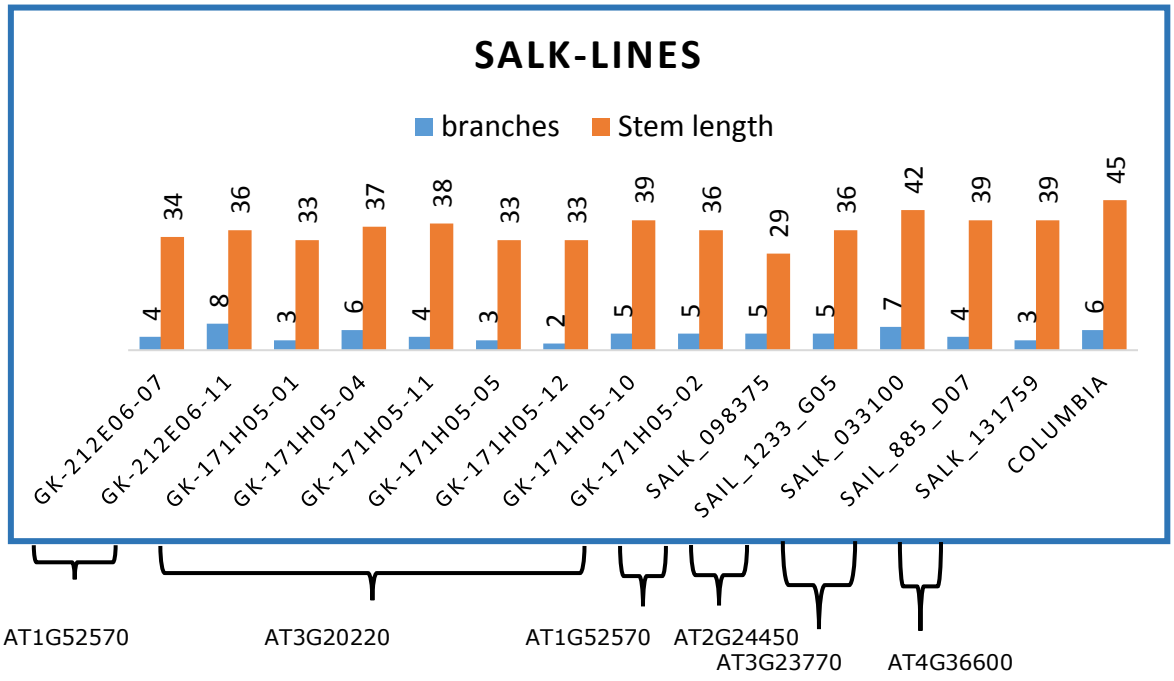


Figure 5.5. Phenotyping analysis of the T-DNA insertional lines. **A)** Siliques length measurement by (cm) for insertion -lines compared to wildtype Col. **B)** SALK-lines stem length and number of branches

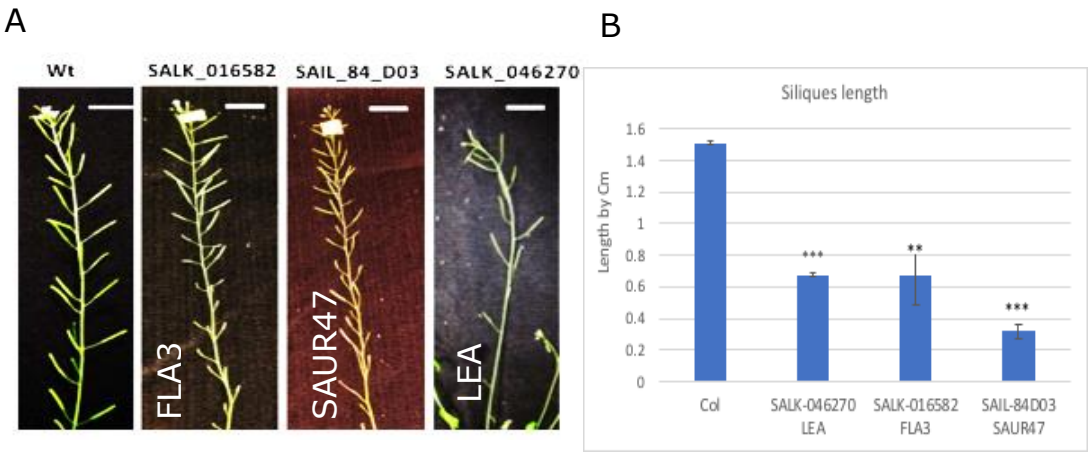


Figure 5.6 Phenotyping analysis of the T-DNA insertional lines. A-B) Morphological growth and siliques length of homozygous lines. Scale bar 3cm.

T-DNA lines were further studied for their impact on gene expression and possible phenotype in response to water stress and fertility. When drought was imposed by withholding water for ten days, homozygous mutant lines lost 19-35% more water than wild type (Figure 5.7a). Inflorescences were collected at drought day 10 for gene expression analysis. In the well water (WW) condition qRT-PCR data of *SAUR47* gene (SAIL_84_D03) showed strong expression ~ 33-fold in the wildtype which was not statistically affected by the drought (Figure 5.8c), *FLA3* (SALK_016582) revealed expression reduction which reduced by drought stress with significant reduction in gene expression ($P \leq 0.01$). While, SALK_046270 showed very low expression of *LEA* gene ~0.005 fold in the WW condition which reduced under drought ($P \leq 0.003$) (Figure 5.8c). The three T-DNA insertion lines had much greater reduction in pollen viability ($P \leq 0.001$) (Figure 5.8a). Alexander staining showed that pollen viability

was reduced in SALK_046270 and SALK_016582 under drought compared to the WW controls (Figure 5.8b). In the well-watered plants SALK_046270 and SALK_016582 siliques length was also significantly shorter than the wild type col $P \leq 0.01$ while SAIL_84_D03 siliques showed a greater length reduction ($P \leq 0.001$) under drought stress (Figure 5.7b). However, drought in SAIL_84_D03 did not reduce pollen viability or siliques length.

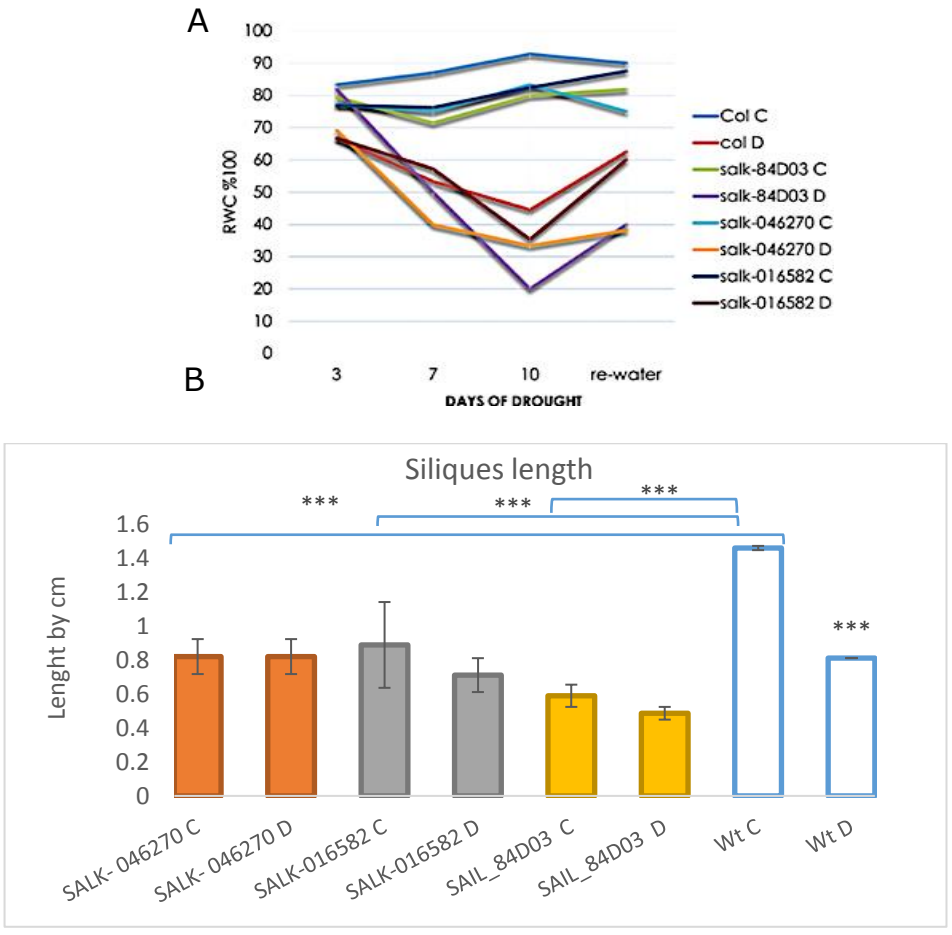


Figure 5.7. A) Relative water content of wild type Arabidopsis Columbia in well-watered and drought, SAIL_84_D03 C and SAIL_84_D03 D, SALK_046270 C and SALK_046270 D, SALK_016582 C and SALK_016582 D under drought, C control, D drought. **B)** Analysis of silique length under drought stress.

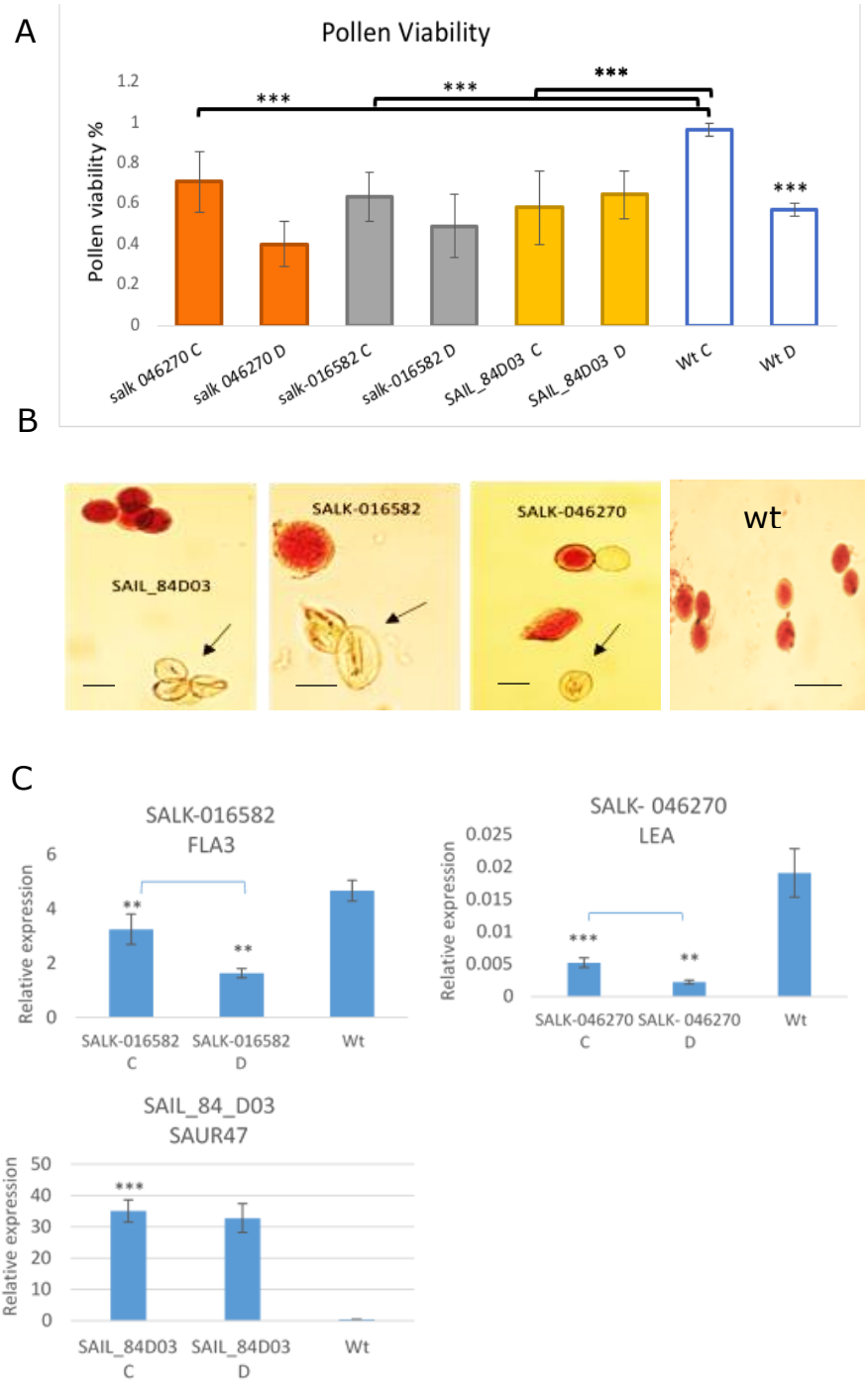


Figure 5.8. **A)** Pollen viability percentage. **B)** Pollen viability using Alexander's stain under light microscope. **C)** qRT-PCR of *SAUR47* gene in WT, SAIL_84_D03Cand SAIL_84_D03D, *LEA* gene in WT, SALK_046270C and SALK_046270D, *FLA3* gene in WT, SALK_016582C and SALK_016582D under drought stress. Scale bar 25 µm

5.5 Preparation of Over-Expression Constructs

Given the preliminary results from the analysis of the T-DNA insertion lines overexpression lines were generated for genes of interest. The appropriate cDNAs, were cloned into the pGWB5-GFP vector to generate overexpression (OEx) lines driven by the CaMV 35S promoter. Constructs were introduced into *Arabidopsis Ler* plants by Agrobacterium–mediated transformation (Chapter3 section 3.4.3.5). Seeds were selected in 50 mg/L kanamycin-containing medium and the positive transformants were transferred to soil (Chapter3 section 3.4.3.6), T1 generation seeds were collected and the presence of the antibiotic-resistance gene was tested in selective medium and the segregation was scored. Seven independent overexpression lines were generated for the following genes AT1G52570 *PLDALPHA2*, AT4G36600 *LEA*, AT3G28980 *DUF1216*, AT2G24450 *FLA3*, AT3G20220 *SAUR47*, AT3G23770 *B-1,3-Glucanases* and AT1G63060 to be phenotypically analysed under optimal growth conditions for quantitative RT-PCR was performed to confirm the expression level. AT1G63060 T1 seeds failed to be obtained on the selective media because of the contamination, future analysis of this gene is therefore needed.

5.5.1 SAUR47 Overexpression in Arabidopsis

Six independent (T1) transgenic lines survived after kanamycin selection, half of T1 plants (3 lines) exhibited alterations in flower development while the other 3 lines had a wild-type appearance. Constitutive overexpression of *SAUR47* in *Arabidopsis* showed pleiotropic growth-related phenotypes observed in OEx1, OEx5 and OEx6 lines, such as curled leaves with early leaf death phenotype, wide branch angle as well as abnormal cauline leaves in the inflorescence area indicated by arrow different from wild type (Figure 5.9A lower panel). A minor defect in petal development with narrow petals compare to the wild type observed in OEx5 (Figure 5.9a &b middle panel). Furthermore, distinct differences in siliques architecture were seen (Figure 5.10.a) (OEx2, OEx3, OEx4, OEx5). The siliques length of each individual line was significantly reduced (OEx1, OEx5, OEx6); t-test $P \leq 0.01$, $P \leq 0.05$ and $P \leq 0.001$ respectively to the wild type (Figure 5.10b). While OEx2, OEx3, OEx4 showed normal growth and silique lengths (Figure 5.10.a). Overexpression of *SAUR47* alerted flower development by changes in male fertility and plant morphology. Quantitative RT-PCR confirmed high expression of *SAUR47* in OEx1, OEx5 and OEx6 lines, this ranged from 1.8 to 9-fold greater than the wild type (Figure 5.10 c). Dwarf OEx1 line had a final stem length of~ 15 cm after 34 days, compared to the wild type main stem height which reached

45 cm. This suggested a role for *SAUR47* in cell expansion and growth. Dissected flowers from OEx1, OEx5, OEx6 lines revealed abnormal and mostly infertile short anthers filaments, pollen rarely reached the stigma, fertility was reduced by a defect in stamen development while the wild type showed stamen that reaches the stigma normally (Figure 5.9b). The T2 generation has been generated confirming the existing phenotype. On a kanamycin selective media, seven transgenic lines were obtained that showed a strong dwarf phenotype. After 5 weeks, plants with smaller shoots were also observed which lacked in the internode elongation compared to the wild type, while three lines did not grow as indicated by arrows (Figure 5.11 a). qRT-PCR revealed different expression of the gene where some transgene was overexpressed such as *SAUR47* OEx3 while others showed lower expression compared to the wildtype (*SAUR47* OEx1) (Figure 5.11a,b&5.12I). It appears that increasing *SAUR47* expression impacted significantly on growth depending on *SAUR47* expression level Plants that failed to grow had very low gene expression with a major influence on plant development. Cauline leaves devoid of coflorescence (secondary branch) development or empty axil phenotype, (red arrow), flowers showing carpelloid sepals in one transgenic line were seen (Figure 5.11 e&d). In addition, the overexpression of *SAUR47* caused defects in flower development as anther filaments at anthesis where short

with a reduced number of released pollen grains, thus cause sterile plants (Figure 5.12a,c). However, pollen grains were still viable using Alexander stain (Figure 5.12e).

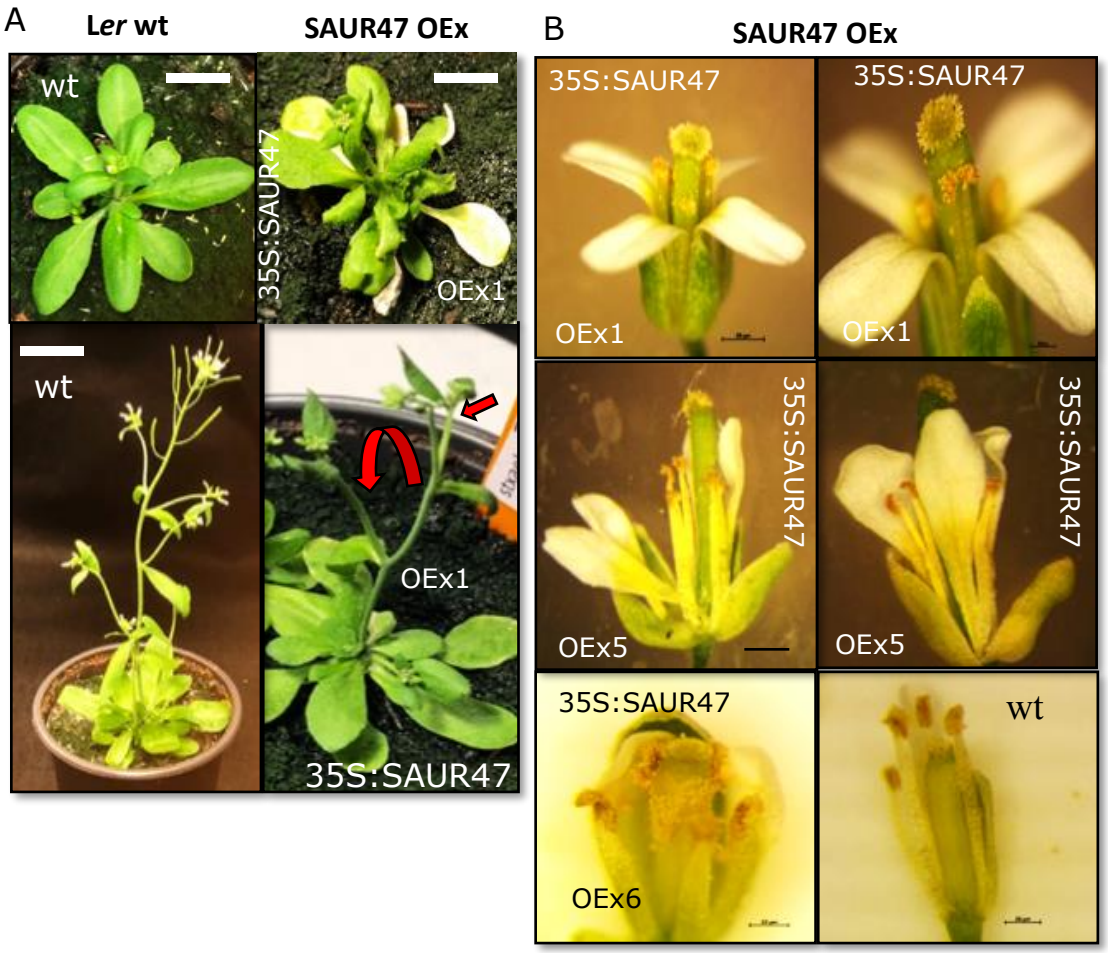


Figure 5.9. Overexpression of SAUR47 in L*er* (T1). A) Upper panel show rosettes leaves of 3 weeks L*er* and SAUR47 (35S: cds) OEx line, lower panel show SAUR47 (35S: cds) OEx 5 weeks old plants with wild-type L*er*. Scale bar 1cm. **B)** Upper panel of 5 weeks *ler* show short filaments of the OEx1 line with low number of pollens on the stigma, middle OEx5 show very short filaments and petals defect, while the lower panel represent OEx6 that show short filaments were pollen does not reach the stigma compare to the wildtype

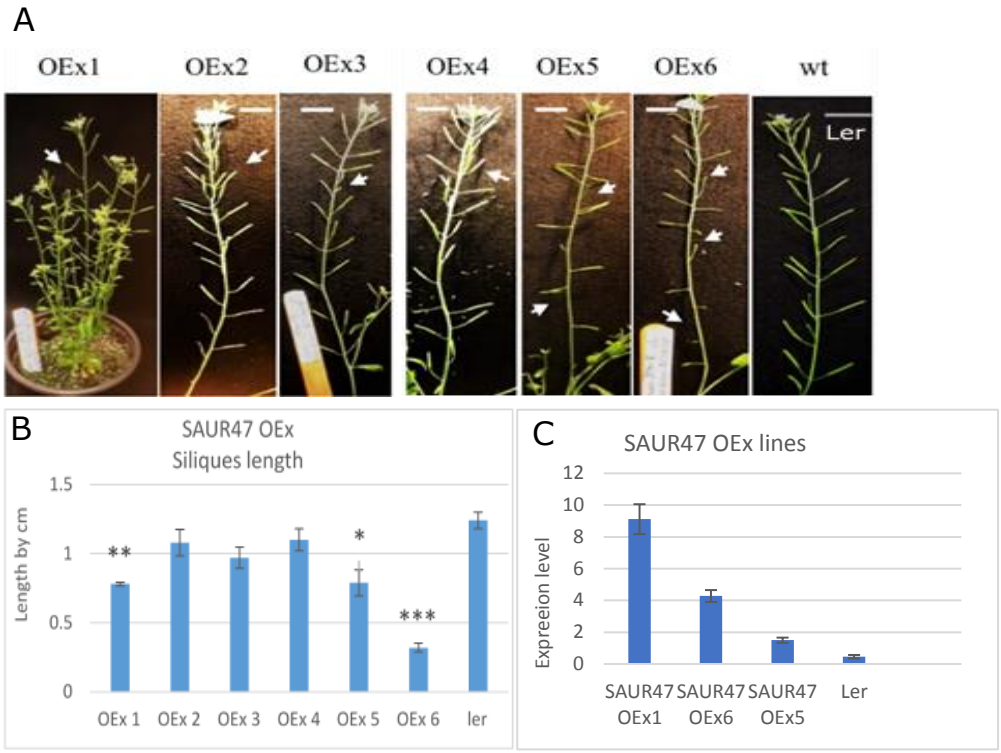


Figure 5.10. Overexpression of *SAUR47* in L*er* (T1). **A)** Main stem of SAUR47 (35S: cds) OEx lines showing siliques. Scale bar 3cm. **B)** Siliques length summary of the SAUR47 (35S: cds) OEx lines Error bar and standard error. **C)** qPCR showing expression level of different SAUR47 (35S: cds) OEx lines with the wild-type.

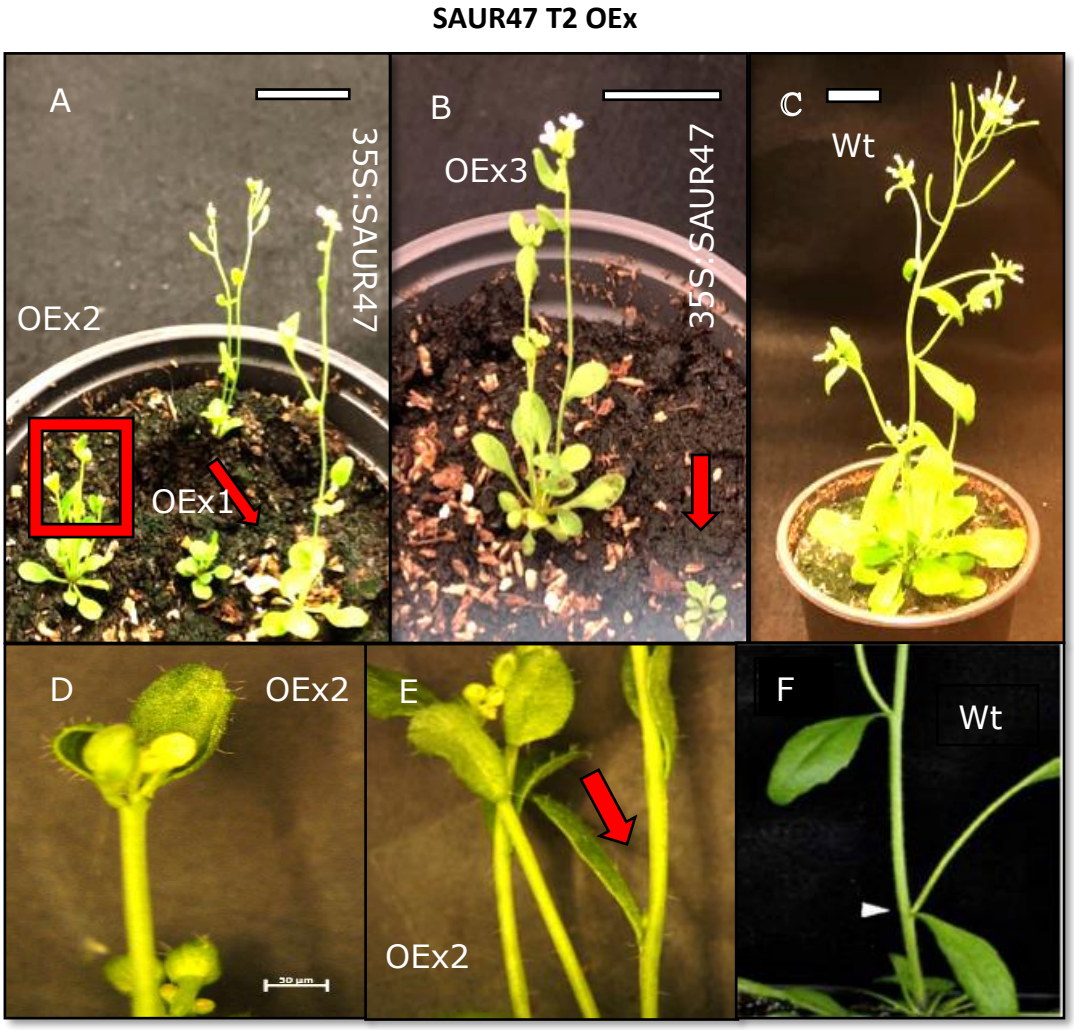
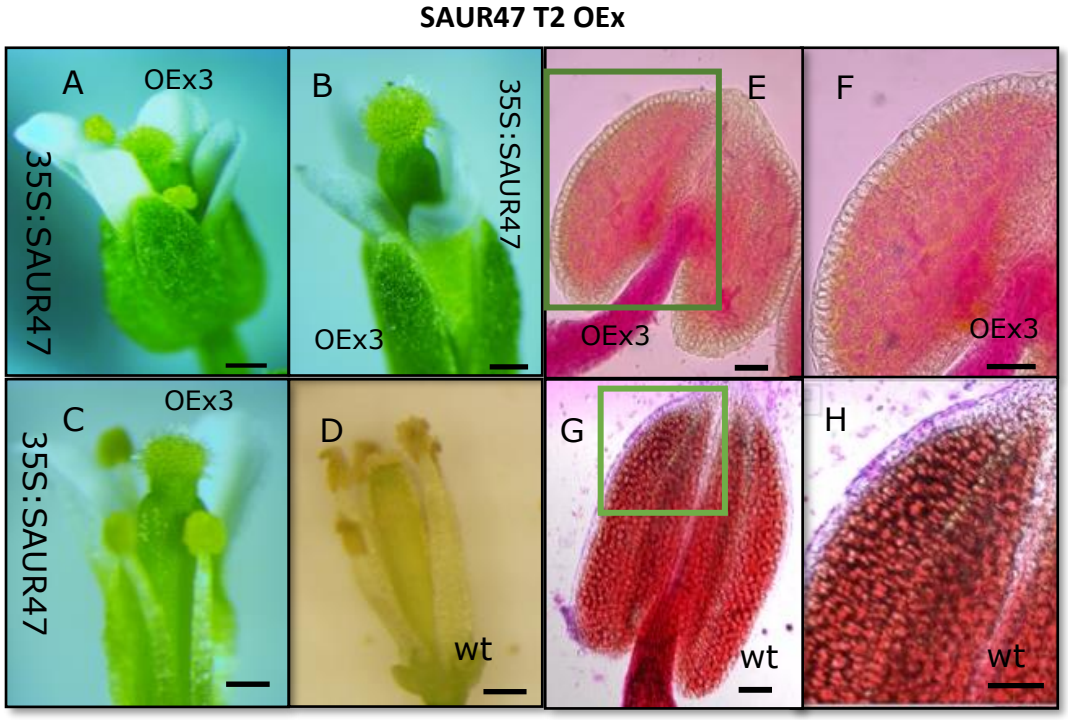


Figure 5.11. Overexpression of SAUR47 in Ler (T2). Segregating population showing **A-B)** 5 weeks old SAUR47 OEx plants. Scale bar 1 cm. **C)** Wild-type 5 weeks old. **D-E)** close up of OEx2 plant (red square) showing 35s: SAUR47cauline leaf/stem boundary. **F)** Wild-type Ler.



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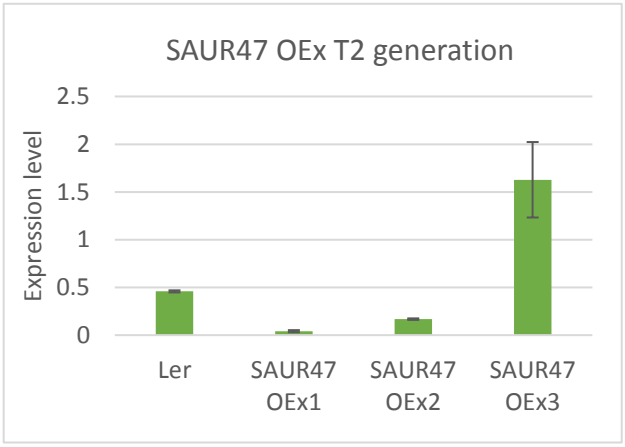

Figure 5.12. Overexpression of *SAUR47* in OEx3 line *Ler* (T2). A-D) 35s: SAUR47 line flowers showing short filaments as well as L*er* wild-type. **E&G)** 35S: SAUR47 and wild-type anthers stained with Alexander stain for viability test, (green square) show close up look on anthers in the right **(F&H)**. **I)** qRT-PCR represent gene expression level in the T2 segregation lines. Scale bar 25 µm

5.5.2 *PLDALPHA2* Overexpression in Arabidopsis

Three independent transgenic lines were generated for overexpression of (*PLDa2*) in the *Ler* background. The transcript level of T1 generation of the transgene was checked by RT-PCR two lines were positively expressed compared to wild-type, *PLDa2* OEx1 and *PLDa2* OEx2. These lines showed early flowering at approximately 9 days (Figure 5.13b Upper panel) compared to the wildtype plant which flowered after 25 days, at the same time an early leaf death senescence phenotype was seen (Figure 5.13a). Both of the transgenic lines showed short silique length (Figure 5.13c) compare to the wildtype. Average length of the first ten siliques indicate significant reduction in length $P<0.001$ and thus seed set (Figure 5.13d). Early dehiscence was observed in several flowers over-expressing *PLDa2* OEx1 and OEx2 line (Figure 5.13b lower panel). However, this phenotype needs to be confirmed in the next generation.

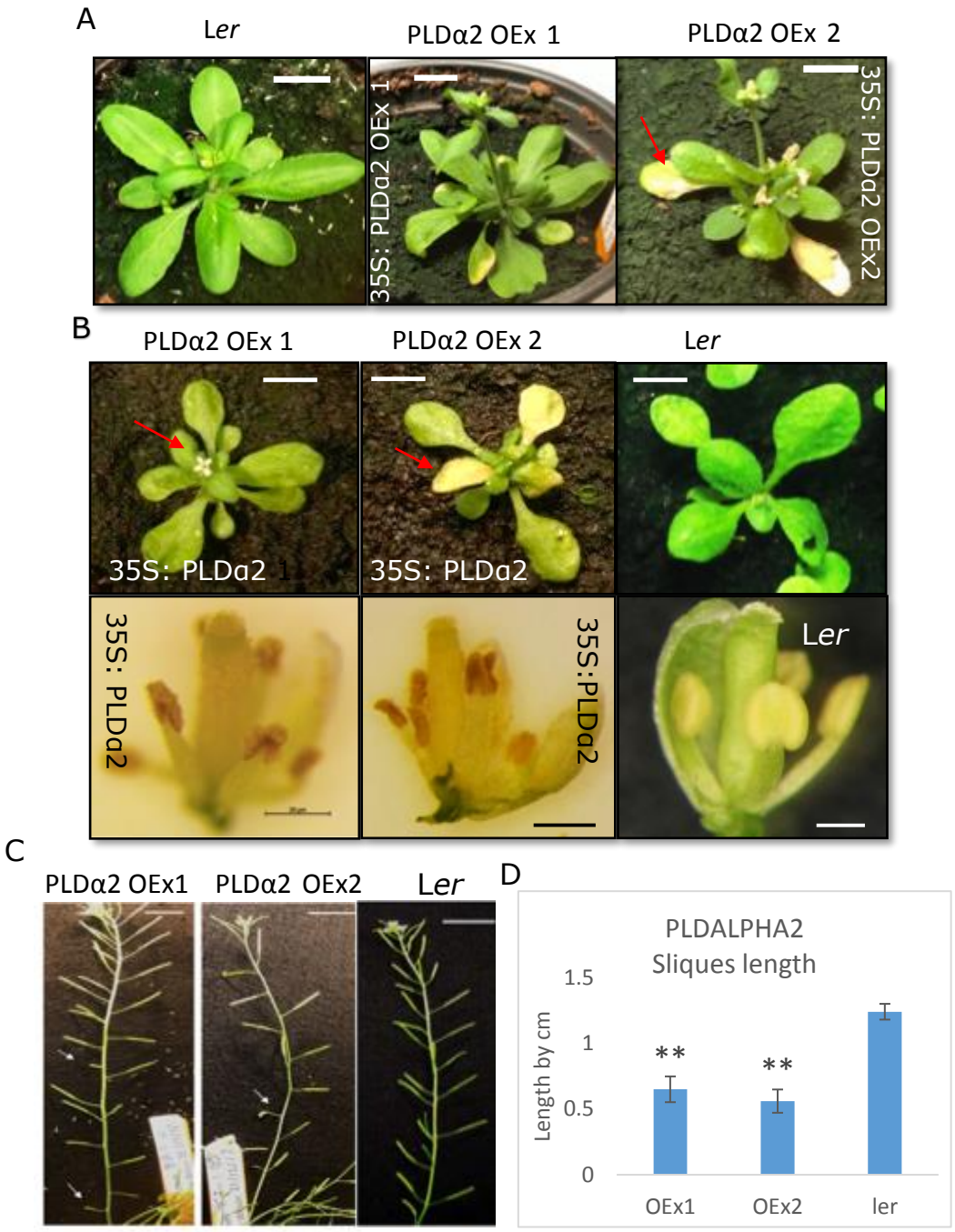

Figure 5.13. **Overexpression of *PLDα2* in Ler**. **A)** Rosettes leaves of wild-type L*er* and *PLDα2* OEx2 exhibited early flowering phenotype. Scale bar 1 cm. **B)** Upper panel 2 weeks old plant show very early flowering of OEx1 and early leaf senescence compare to wildtype respectively. Scale bar 1cm. Lower panel show early anther dehiscence compared to wild type. Scale bar 20μm. **C)** Main stem of PLDα2 OEx1 and OEx2 shows silique size, length and arrangement with wild type L*er*. Scale bar 3cm. **D)** Siliques length summary of the *PLDα2* OEx lines Error bar and standard error

5.5.3 *B-1,3-Glucanases* Overexpression in Arabidopsis

Overexpression of *B-1,3-Glucanases* was seen in three independent lines (T1 generation) these showed an early leaf death phenotype, abnormal inflorescence meristem, and increasing number of nodes with secondary inflorescences and wide branch angle (Figure 5.14a). The overexpression of *B-1,3-Glucanases* affects plant sterility by significantly reducing siliques length in all of the three lines (*B-1,3-Glucanases* OEx 1 $P<0.001$, *B-1,3-Glucanases* OEx 2 $P<0.05$ as well as *B-1,3-Glucanases* OEx3) (Figure 5.14b). Transcript expression has been confirmed as increased by qRT-PCR (Figure 5.14d). OEx2 line showed altered siliques structure with empty pedicels (empty places of siliques) while OEx3 exhibited clusters of siliques that indicate an internode defect, the main florescence Flowers were also transformed into secondary inflorescence-like structures (Figure 5.14c). Stamens did not show any changes compared to the wild type plant (Figure 5.14c lower panel). Which indicates gene effects on pollen development with normal anther filaments elongation. *B-1,3-Glucanases* shows an expression peak at bud S1 pollen tetrad stage as confirmed by the qRT-PCR (Chapter4 Figure4.4) and is believed to be a tapetum specific gene with a role in callose degradation (Hird et al, 1993). Generating and analysis the second generation (T2) of the observed phenotype for further investigation.

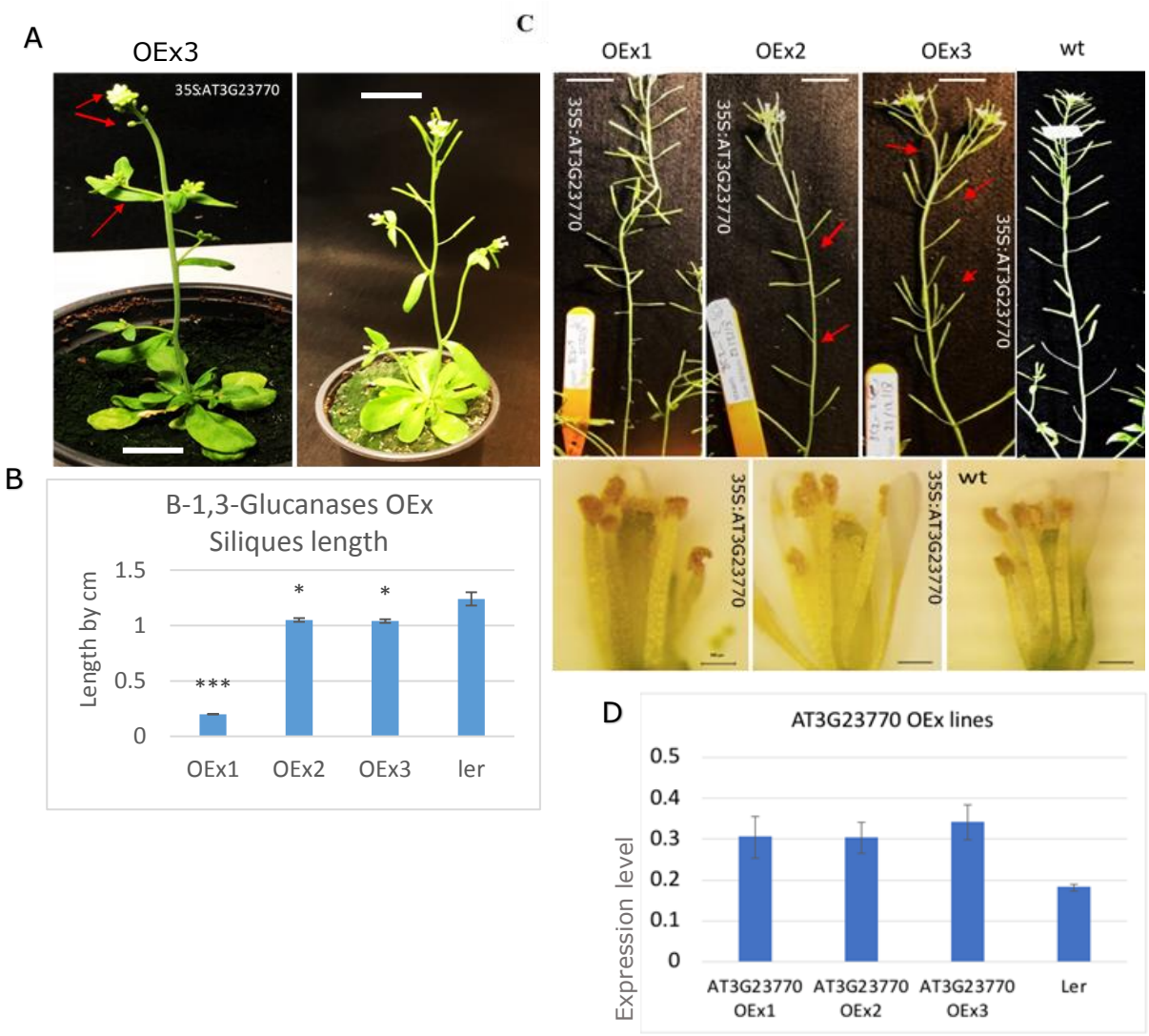

Figure 5.14. Overexpression of *B-1,3-Glucanases* in L*er.* A) 4 weeks old *B-1,3-Glucanases* OEx and wildtype plant. Scale bar 3cm. **B)** Siliques length by mm of the three OEx lines Error bar and standard error. **C)** Upper panel show main stem of the three OEx lines with presenting siliques length and arrangement. Scale bar 3cm, lower panel Open flowers of OEx1 and OEx2 show anther filaments and Stamens. Scale bar 20µm. **D)** Expression level of *B-1,3-Glucanases* OEx lines by QRT-PCR.

5.5.4 *LEA* Overexpression in *Arabidopsis*

T1 seeds of *LEA* overexpression lines were screened on kanamycin. Five independent transgenic lines were screened for phenotype, early leaf senescence and curled leaves were seen, with flowers transformed into secondary inflorescence-like structures (Figure 5.15.a), arrowheads indicate the first flower formed after the transition from secondary inflorescences to flowers (Figure 5.15.a). Independent *LEA* (OEx1, OEx2, OEx3, OEx4, OEx5) lines displayed similar inflorescence architecture defects, with different orientated siliques and clustered inflorescences as in OEx5 (Figure 5.15.b) compared to wild-type plants. Silique lengths showed significant reductions in the three lines OEx2, OEx3, OEx4 (t-test value $P<0.05$) ;(Figure 5.15.c). Expression pattern of two lines OEx1 and OEx2 showed increased expression compared to wild-type (Figure5.15.d). Twenty transgenic T2 from both homozygous and heterozygous lines were screened. Curiously, late flowering phenotype was only observed in ten of the transgenic lines. However, the majority of the flowers failed to open with small size which was correlated with high expression levels (Figure 5.16.d). No siliques were produced over the duration of the experiment. Furthermore, inflorescence meristem co-initiates cauline leaves and co-florescences in positions where flowers would normally developed in the axils of some cauline leaves (Figure 5.16.a) (blue arrows). Interesting, inflorescences produced cauline leaves (Leafy shoots) (Figure 5.16.a). Manually opened buds

revealed anthers that did not open causing severe male sterility, shorter curved filaments were seen also as well as dehydrated anthers that dehiscenced early (Figure 5.16.b). While the other ten transgenes showed early flowering that to produce a few flowers with siliques length did not exceed 2mm in length; defects in branching with changes in branch angle and orientation were observed in most of the lines, one of the lines show areal rosettes and others lacked branches (Figure 5.17.a). Short stamen filaments and shrunken stamen that did not reach pistil were observed, anthers retained a small, immature heart shape and make a small number of mature pollen grains (Figure 5.16.b). Alexander's stain was used to indicated unviable pollen (Figure 5.18.a lower panel).

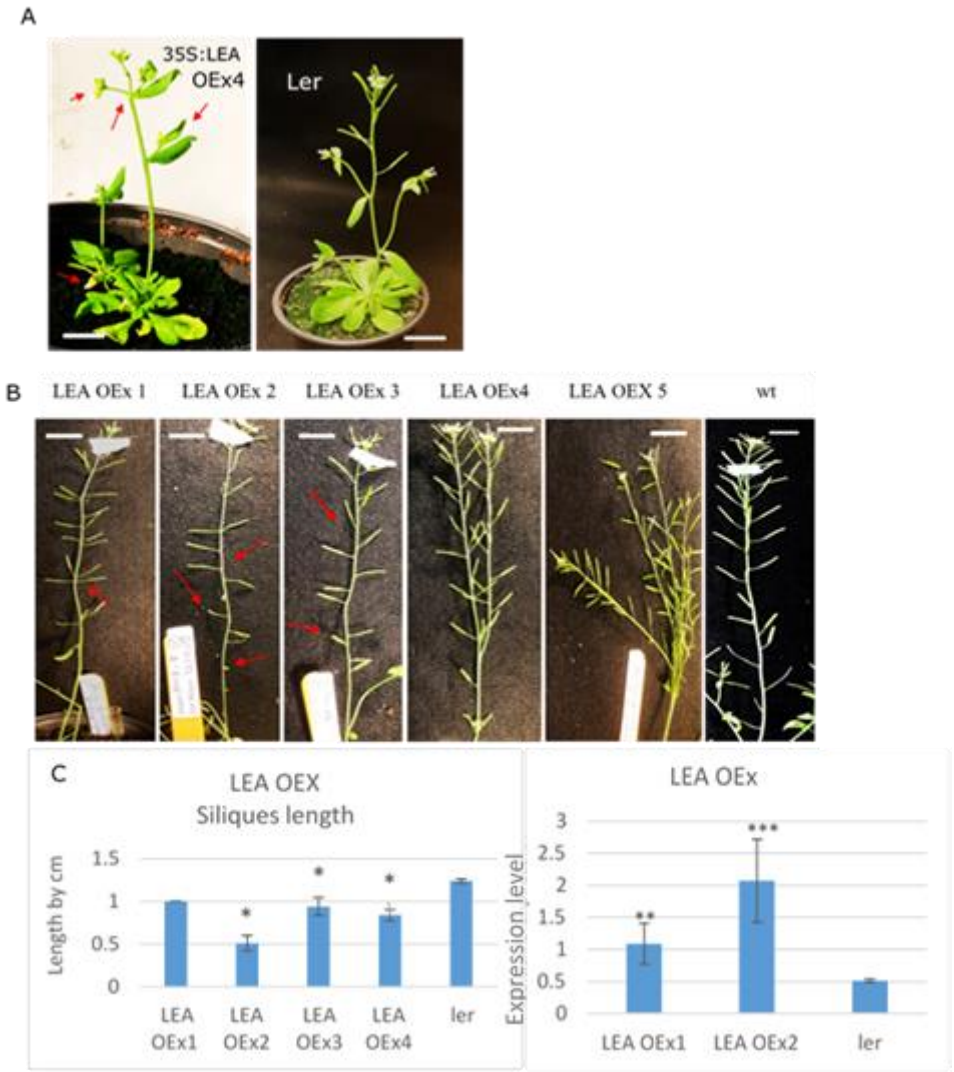

Figure 5.15. Overexpression of *LEA* in Ler (T1). A) Left panel show 5 weeks old Arabidopsis overexpressing *LEA* , right panel show wild-type l*er*. Scale bar 1cm**. B)** Images of *LEA* OEx lines showing Stem length and siliques length and inflorescences pattern. Scale bar 3cm. **C)** Siliques Length of *LEA* OEx lines by cm Error bar and standard error. **D)** Expression of LEA OEx lines by QRT-PCR.

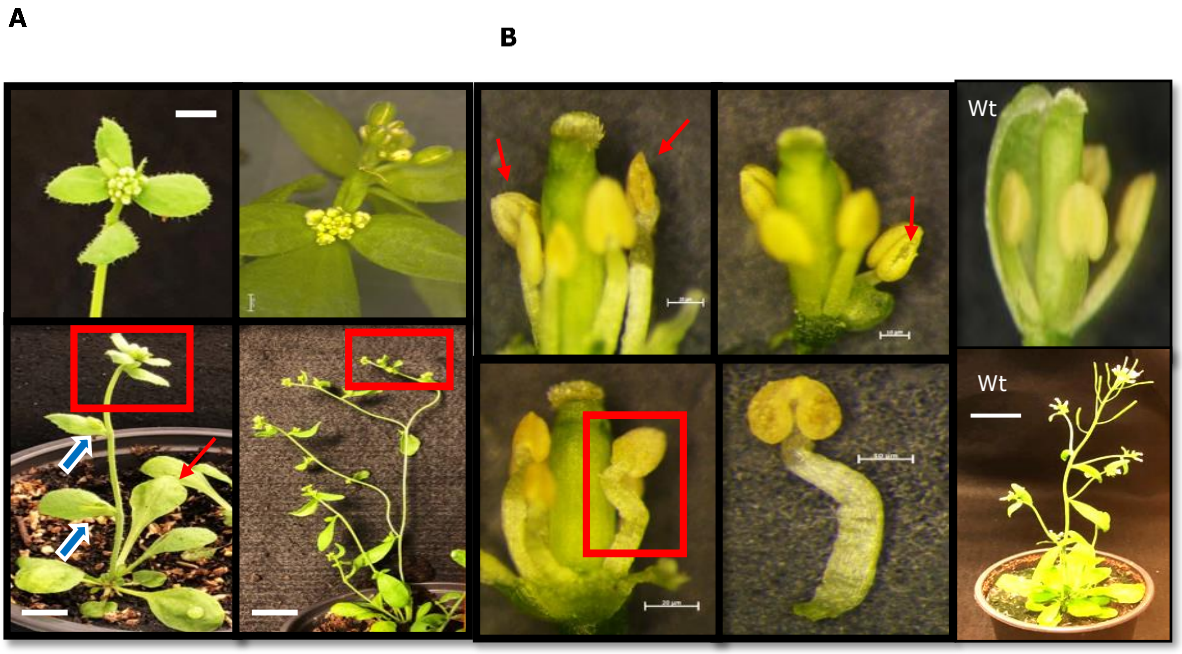

Figure 5.16. Overexpression of *LEA* in Ler (T2) OEx1 line. A) Upper panel (close up of lower panel) leafy like -structure (mixed floral and shoot character) in the Florescence 35S: LEA, lower panel present 5 weeks old 35S: LEA plants on the left, 7 weeks old 35S: LEA plants on the right. Scale bar 1cm. **B)** Upper panel flower stage12 shows early dehiscent anthers of 35S: LEA plants, lower panel early dehiscent anthers close up on the left distorted filaments with dehiscent anther and small number of pollen. 5 weeks old Arabidopsis l*er*. Scale bar 1cm

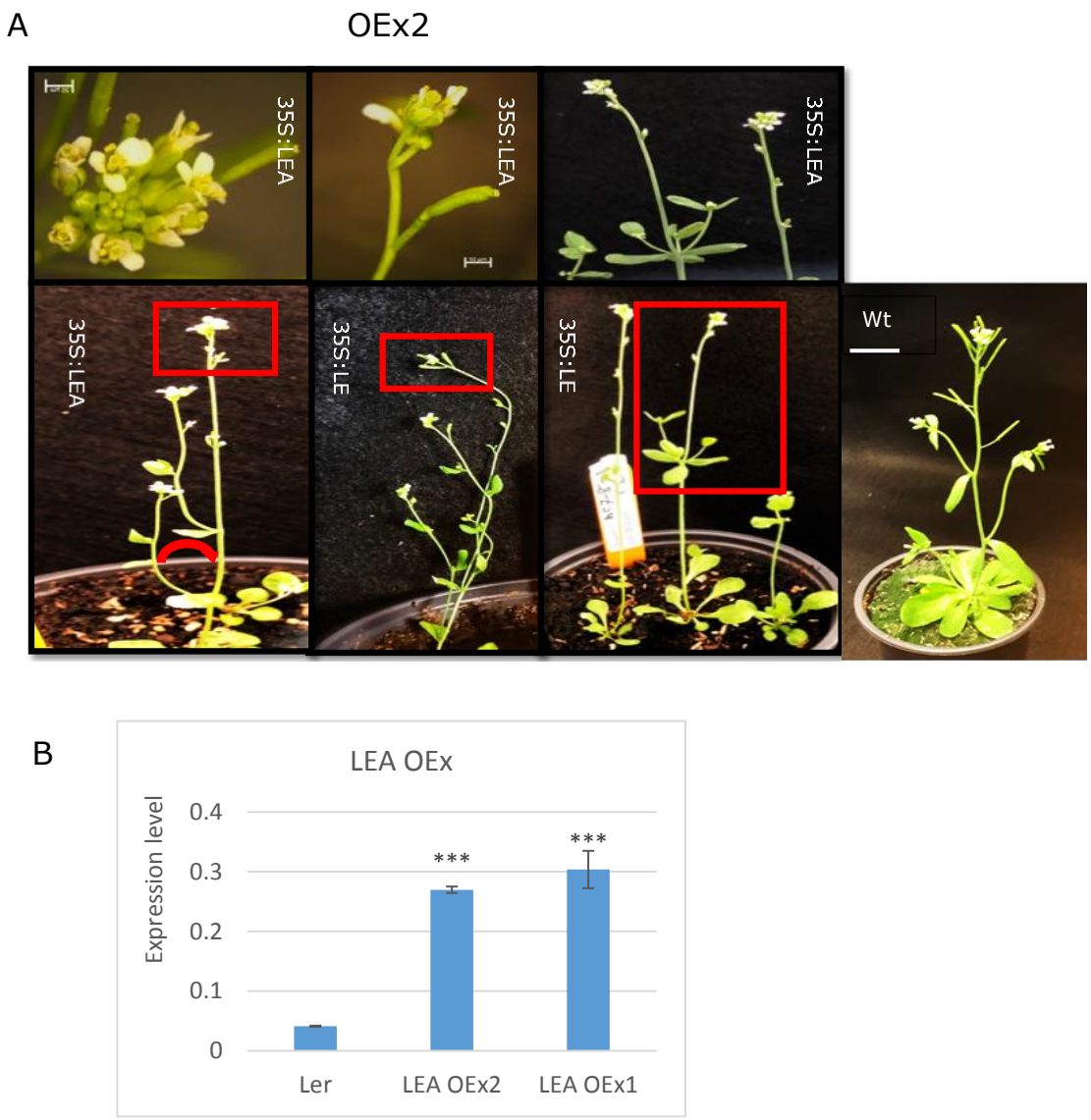

Figure 5.17. Overexpression of *LEA* in Ler (T2) OEx2. A) Left-upper panel close up to the main florescence of 35S: LEA, middle flower with anther and petal defect, right panel shows areal rosette leaves with tiny siliques. Lower panel 5 weeks old 35S: LEA shows changes in the branching angle (left), areal rosette leaves (right). Scale bar 1cm. 5 weeks old Arabidopsis l*er*. Scale bar 1cm. **B)** The graph show gene expression of the transgene by qRT-PCR.

Figure 5.18. Overexpression of *LEA* in *Ler* (T2) OEx2 line. **A)** Flower at anthesis of 35S: LEA plants had short anthers with clear stigma in the three images, the left panel shows defects in petal size, the middle panel shows retarded development of flower organs, the right panel present swelling stigma, the left image shows slight change to female part, lower panel pollen viability with alexander stain, middle and left panel LEA OEx. Scale bar 50 µm.

5.5.5 DUF1216 Overexpression in Arabidopsis

Overexpression of *DUF1216* was seen in two independent lines (T1 generation). *DUF1216* OEx1 and OEx2 lines indicated by qRT-PCR data analysis were gene expression increased in both lines as compared to the wildtype as shown in (Figure 5.19.d). T1 generation of *DUF1216* OEx lines showed male sterility, displaying shorter filaments (Figure 5.19.b left panel) relative to wildtype, small siliques with significant length reduction (t-test value $P<0.05$ in *DUF1216* OEx1 and $P< 0.01$ in *DUF1216* OEx2; Figure 5.19.c). Pedicels were observed with empty siliques as in *DUF1216* OEx2, less number rosette leaves were seen compared to wild-type, the secondary inflorescence lacked a leaf axil in contrast the inflorescence did not grow from the leaf axil (indicated by arrow Figure 5.19.a). *DUF1216* OEx1 showed second inflorescence like structures and swollen stigma (Figure 5.19.b left panel), flowers formed from subtended angle indicated by the arrows. Random defect in the internode area were also detected in OEx1 and OEx2 lines (blue bars). Plant height was also shorter than wild type, (19 and 17 cm respectively). Further analysis is needed to confirm the phenotype in the second generation (T2).

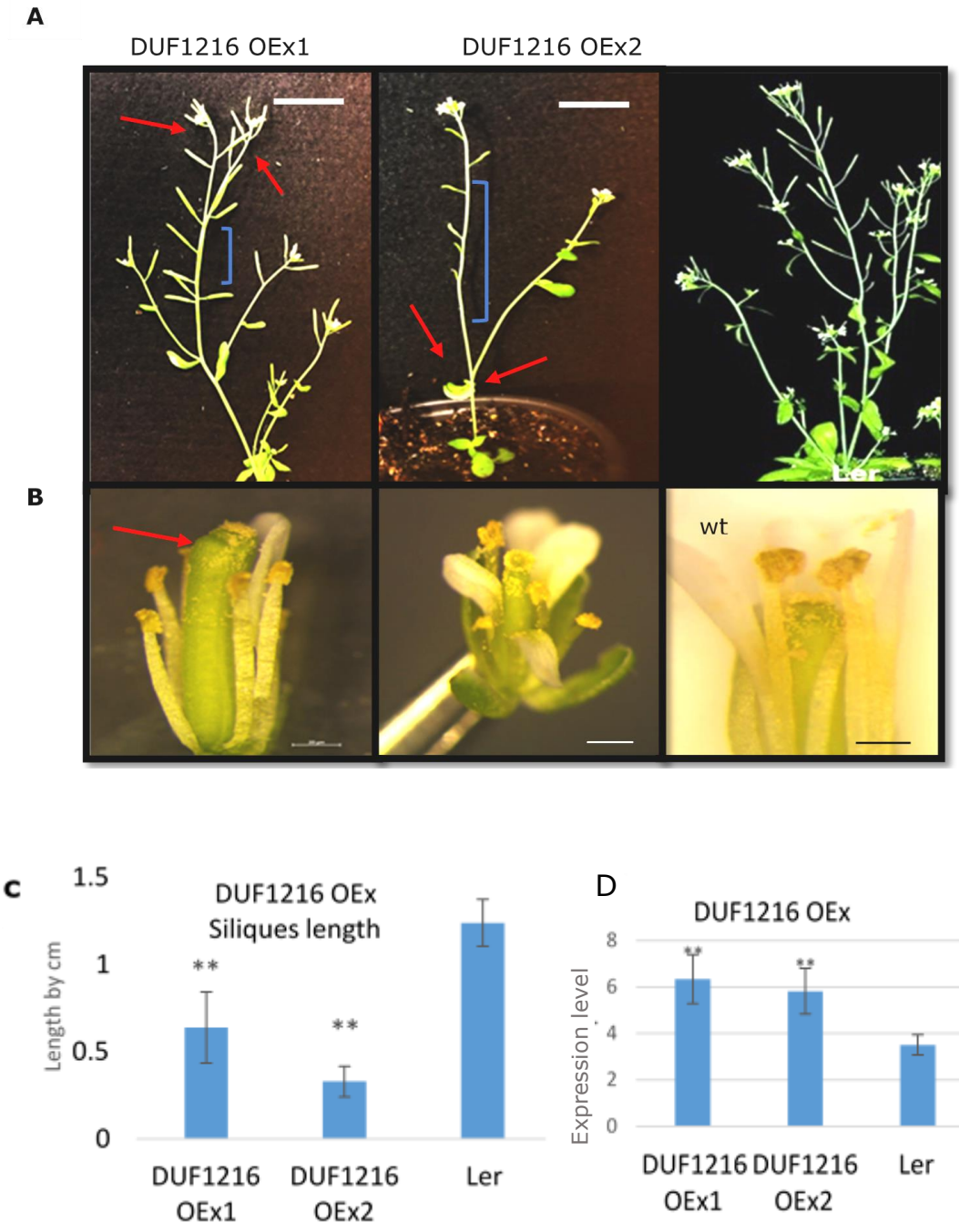

Figure 5.19. Overexpression of *DUF1216* in *Ler*. A) DUF1216 OEx plants and wild-type 5 weeks old as. Scale bar 3cm. **B)** DUF1216 OEx flowers stage 13 at anthesis shows short stamen, the left panel show short petal and swelling stigma, narrow petals in the middle image. Scale bar 100 μm. **C)** Means of DUF1216 OEx plants siliques length Error bar and standard error. **D)** Gene expression analysis by qRT-PCR.

5.5.6 FLA3 Overexpression in Arabidopsis

Abnormal phenotype was observed in *FLA3* overexpression lines. One transgenic line has been generated for the overexpression of *FLA3* (Figure 5.20d). Severe sterility with shorter siliques with an average length of mature siliques (0.5 cm) that was much shorter than in WT plants (1.3 cm) was seen (Figure 5.20 a,c), with defects in the internode area (bracts) ad empty siliques near the main florescence (Figure 5.20a). In addition, anthers of OEx plants were short and did not reach the stigmatic surface at anthesis (Figure 5.20b), some anthers also did not dehiscence. However, the role of *FLA3* OEx in anther dehiscence, short stamen needs further molecular investigation and the phenotype of the T2 generation needs to be confirmed. This result is consistent with the previous study by Li et al. 2010 that reported the role of *FLA3* in microspore development, pollen intine formation as the overexpression of *FLA3* caused defective elongation of stamen filaments and reduced female fertility.

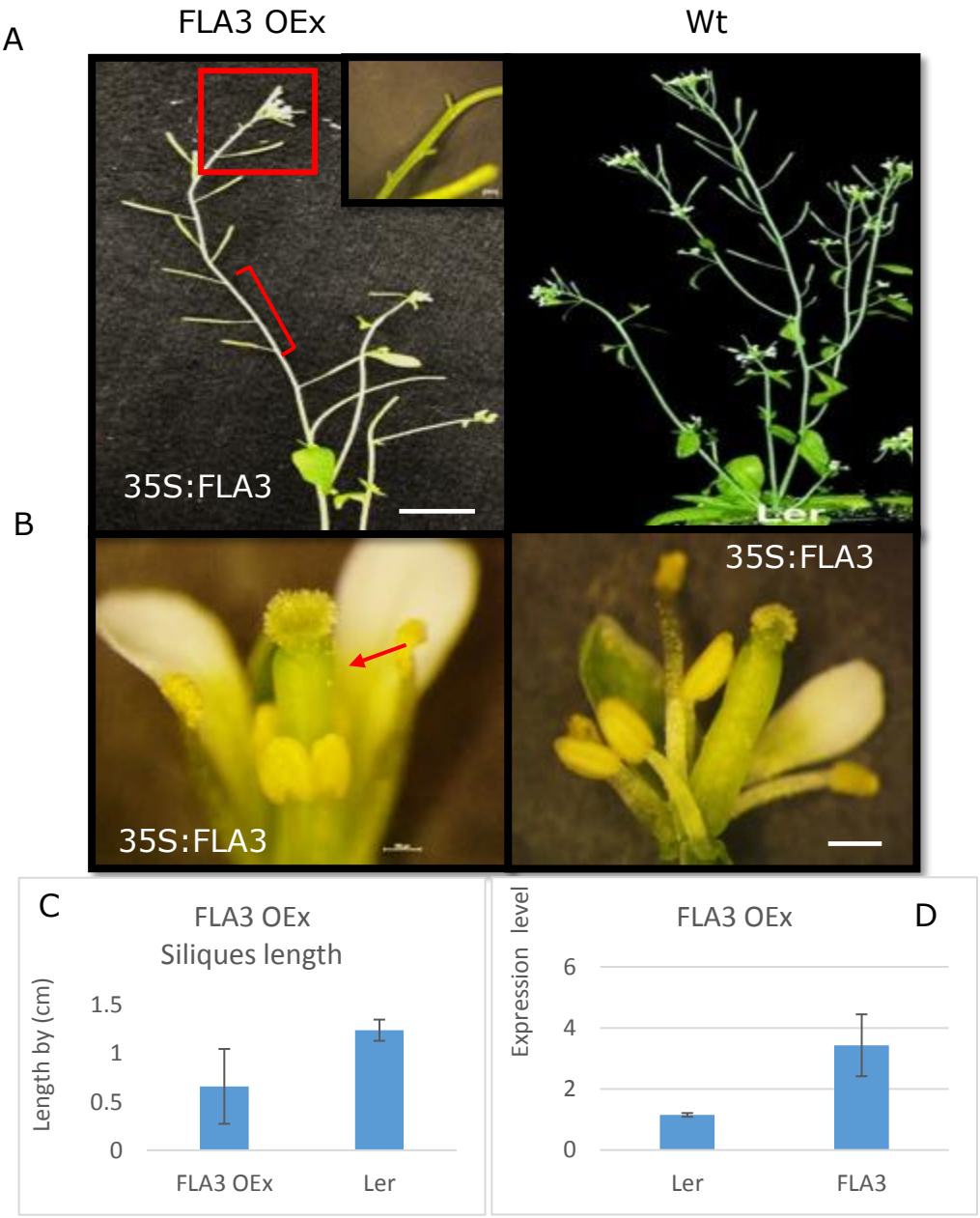

Figure 5.20. Overexpression of *FLA3* in *Ler*. A) *FLA3* OEx plant 5 weeks old with wilt-type on the right. Scale bar 3cm. **B)** *FLA3* OEx flowers at anthesis, short filaments and indehiscent anther **C)** Siliques length of the main stem error bar and standard error. **D)** Gene expression analysis by qRT-PCR.

5.6 Discussion

5.6.1 SAUR47 affects male fertility and plant growth through hormonal cross talk.

Seven genes were characterized for their impact when expressed specifically. Auxin is well-known for controlling plant development and growth by regulating cell division and expansion (Ren and Gray., 2015) The SMALL AUXIN UPREGULATED RNA (*SAUR*) genes are part of a large family of auxin regulated genes (Hagen and Guilfoyle., 2002). *SAUR47* overexpression lines exhibited short stamens filament and lower amounts of pollen grains causing severe sterility. The expression analysis of *SAUR47* in wild type showed high expression of the transcript in the tricellular pollen stage (Chapter4 Figure 4.4). Mutant deficient GA RGA (repressor of *ga1-3*) and JA (12-Oxophytodienoate reductase 3 *opr.3*) have been known to impact on anther filament length (Stintzi., 2000; Koornneef and Van der Veen.,1980a). This suggests a possible regulatory role of *SAUR47* under the regulation of GA and JA hormone in controlling filament elongation. JA regulates stamen elongation through *MYB21* and *MYB24* and may therefore be that *SAUR47* is induced by GA under the regulation of *MYB21-MYB24* and thus disrupts Jasmonate production (Reeves et al., 2012).

Several *SAUR* overexpression studies have been reported as positively regulating cell expansion and causing a long hypocotyls phenotype for example *SAUR19* (Spartz et al., 2014; Spartz et al., 2017 Tomato). *SAUR36* (Stamm and Kumar, 2013), *SAUR41* (Kong et al., 2013), *SAUR63* (Chae et al., 2012), *SAUR76-78* (Li, et al, 2015), *SAUR10* (Ren and Gray, 2015). These data are not consistent with my findings, where the overexpression of *SAUR47* showed an extreme dwarf phenotype in the OExT2 segregation lines, suggesting that *SAUR47,* directly or indirectly, negatively regulates cell expansion or cell division. The low expression of *SAUR47* also negatively regulates plant growth, which suggests that auxin level in the cell is required to maintain normal growth condition. Since the gibberellin mutant *ga1-3* displayed a dwarf phenotype, this implies the possible regulation of *SAUR47* by GA. Auxin and BR have been reported as promoting cell expansion (Nemhauser et al.,2004). BR signalling is believed to control cell elongation through the regulation of auxin induced (*SAUR19*) expression (Favero et al., 2017). Suggesting the possible link between BR and *SAUR47* associated with the dwarf phenotype (Figure 5.11.a). Moreover, the pleiotropic phenotype which is observed in *SAUR47* OEx lines such as cauline leaf shape, stem architecture, inflorescence branch angles, and shorter internode could be correlated with the MADS-domain factor FRUITFULL (*FUL*) which believed to regulate target genes in the network of several hormonal signalling to determines both fruit

development and patterning (Gu et al., 1998; Ferrándiz et al., 2000b). *FUL* exhibited a similar pleiotropic phenotype (Ren and Gray., 2015), suggesting that *SAUR47* may work under the regulation of *FUL*. This explanation is based on the evidence that (*FUL*) repress *SAUR10* cell elongation (Bemer et al., 2017).

To summarise, *SAUR47* may work under the cross-signalling pathway between JA, GA, Auxin, BR and FUL to regulate general plant growth, cell expansion and male fertility. Future work is required to determine gene subcellular localization in pollen and anthers as well as stem tissues. How *SAUR47* gene interacts with different mutant lines targeting each of the hormone network pathways is also of interest. Furthermore, *SAUR* proteins negatively regulate D-clade PP2C (PP2C.D) family phosphatases in the plasma membrane to control cell expansion (Appendix I). It has been recently reported that PP2C.D5 overexpression exhibits a dwarf phenotype and short stamen filament, interestingly PP2C.5 as one of the primary plasma members factor to bind with *SAUR* to promote cell expansion (Ren et al.,2018). Investigating whether *SAUR47* belongs to PP2C.5 phosphatase member could help understanding gene regulation. Since *SAUR47* was down-regulated by the drought (Chapter4 Figure 4.4) and the T-DNA mutant line that show severe male sterility by reducing siliques length and pollen viability was up-regulated by drought. Therefore, this suggests that *SAUR47* may improve pollen development during drought condition. Future

generation of overexpression *SAUR47* lines under drought stress will help clarifying this.

5.6.2 PLDALPHA2 promotes early flowering and early anther dehiscence

Overexpression of a family member (*PLDALPHA2*) of phospholipases enzyme showed an early flowering phenotype. Suggesting *PLDa2* may altered flowering time and plant life cycle. Since *PLDa2* OEx lines produces siliques earlier than wild-type. However, the endogenous signals of *PLDa2* is still unclear. *PLDa3* overexpression has been shown to induce early flowering under drought conditions (Hong et al., 2008). Since the FLOWERING LOCUS T (*FT*) gene is a key integrator of signals that influence *Arabidopsis* flowering time (Corbesier et al., 2007; Mathieu et al., 2007), further investigation of the expression patterns of FT in *PLDa2* OEx is a key question for future work. The phospholipase D family contribute to the hydrolysis of different membrane phospholipids, producing phosphatidic acid PA, induced by different hormonal signalling and affect PCD (Chapter4 Figure 4.4). Hence, *PLDa2* has strong expression in microspore, BCP and TCP during pollen development. This could explain the early anther dehiscence by inducing tapetum PCD. Anther dehiscence was induced by JA biosynthesis through the regulation of DEFECTIVE IN ANTHER DEHISCENCE1 (*DAD1*) by catalysing the initial step of JA biosynthesis using a phospholipase A1 (PLA1) enzyme. This suggest that *PLDa2* may be regulated by JA

biosynthesis through DAD1 influencing early anther dehiscence (Inoue et al., 2006). Future work to investigate *PLDα2* subcellular localisation and the relationship to *PLA1* in pollen development in required. The gene mode of action under drought stress is also very important since many *PLDs* showed drought tolerance Appendix I.

5.6.3 *Beta-1,3-glucanase* a pollen wall gene regulated by Brassinosteroids

Beta-1,3-glucanase has a role in pollen callose wall degradation (Levy et al., 2007; De Storme and Geelen, 2014). The T1 generation of *Beta-1,3-glucanase* OEX lines showed normal filaments and pollen covered the stigma. Siliques length was reduced but varied between the OEx lines, however this work is lacking pollen viability testing. A study by Ye, et al (2010) implies that *Beta-1,3-glucanase* might control male sterility by affecting pollen wall formation and is regulated by BR, mediated by MALE STERILITY1 (MS1). Indicating that *Beta-1,3-glucanase* is an important gene for male fertility. However, Tratt, (2016) stated that *Beta-1,3-glucanase* may not be involved in callose wall degradation during tetrad stage based on an absence expression in the anther locule at the microspore release stage. They concluded that *Beta-1,3-glucanase* may play a role in regulating callose accumulation around the tapetal layer or may regulate callose degradation of the generative cell wall later in pollen development. Both hypotheses need further investigation, *Beta-1,3-glucanase* is highly expressed in pollen tetrads and showed up-

regulation response to drought stress (Chapter4 Figure 4.4). This suggests a regulatory role for *Beta-1,3-glucanase* in drought stress associated the pollen cell wall development. On the other hand, *Beta-1,3-glucanase* overexpression lines revealed similar phenotypes as observed in the double mutant of *PENNYWISE* (*pny*) and BREVIPEDICELLUS (*bp*) an *Arabidopsis* BELL gene, such as internode defects, siliques like-clusters and inflorescence patterning defects which may be regulated by auxin and GA signalling (Smith and Hake., 2003). However, the mechanism of hormonal control in *pny* and *bp* are still unknown. Suggesting that *Beta-1,3-glucanase* might work under the regulatory pathway of both *pny* and *bp* in the inflorescence's meristem controlling patterning. Analysis of the T2 generation is required to explore the analysis at different stages of pollen development particularly tetrad stage where *Beta-1,3-glucanase* was highly expressed.

5.6.4 LEA A Flower Time Regulatory Gene Targeted by LEAFY Transcription Factor

The LEA OEx lines showed two phenotypes in the T1 generation suggesting either multiple inserts or some additional factor achieving 50% of the T2 generation of *LEA* OEx lines showed late flowering phenotype while most of the flowers failed to open and were replaced by mixed floral and cauline leaf clusters. Moreover, rosettes carried upward-curled leaves (Figure 5.16.a). However, the other half of the OEx lines showed an early flowering phenotype producing very short

siliques length and some changes in branch angle and orientation. However, the LFY mutant showed a similar phenotype with flowers replaced by leafy shoots under the activation of APETALA1 (*AP1*) expression (Weigel et al., 1992) and reduced male fertility by affecting number of stamens (Dornelas and Rodriguez., 2005). This suggests that *LEA* may work under the regulatory network of (*LFY*) TF to control floral identity. The late flowering plants, which produced only a few flowers appeared after 16 days of moving the plants from the selective media to the soil. However, the other half of the population showed early flowering only after 2 days of moving the plants to the soil. *Arabidopsis* flowering time is determined by long-day (LD) (16 h light:8 h dark) and short-day (SD) (8 h light:16 h dark) (Andrés and Coupland, 2012). However, all the transgenic lines were conducted under the same conditions. This implies a role for *LEA* in inducing flowering time, since there is cross-talk between *LFY* and FLOWERING LOCUS T (*FT*) transcription factor (Ruiz-Garcia et al., 1997; Nilsson et al., 1998), suggesting that *LEA* may be regulated by the LFY/FT pathway. GA is induced in both LD and SD conditions to regulate male fertility while *bHLH* transcription which is known to be regulated under (SD) also have a role in male fertility (Porri et al., 2012; Sharma et al., 2016). Therefore, the severe male sterility phenotype in both lines could be linked to GA level by LFY/FT pathway. This hypothesis needs further investigation. The T-DNA mutant lines exhibited male sterility by reducing siliques length and

pollen viability and were down regulated by the drought. In the wild-type plant LEA show expression at microspore, PCB and TCP and is up-regulated in all stage of pollen development by the drought (Chapter4 Figure 4.4).

5.6.5 DUF1216 Pollen Specific Gene Induce Male Sterility

A pollen specific *DUF1216* gene family (*DUFF8*) were expressed in BCP and TCP and showed strong expression in the TCP, several mutants targeting different *DUF1216* members including *DUFF8* caused pollen abortion, where *DUFF8* is highly expressed in TCP and microspore stage (Nestorova., 2016). This is consistent with my finding that *DUFF8* also showed high expression in microspores and TCP (Chapter4 Figure 4.4). Interestingly, a distinct phenotype has been observed in the overexpression lines of *DUFF8* that influence male sterility by affecting siliques and stamen length, minor defect in the female organ, petal size and inflorescence structure. However, this work is lacking pollen study which is necessary to help us understand gene function. The *HUA ENHANCER 1 (HEN1-1)* is regulated under the *AGAMOUS* (AG) network and has the same function as *APETALA2 (AP2)* (Chen et al., 2002a), and exhibited a similar pleiotropic phenotype as observed in *DUFF8* OEx lines. Nestorova., (2016) reported that *DUFF8* is down regulated in the *hen1-1* mutant, suggesting that *DUFF8* may work under AG to control male fertility. While *AP1* works under *LFY* to control meristematic floral identity, therefore *DUF88* may work under *LFY* as

defects in the inflorescence and secondary inflorescences subtended by cauline leaves have been observed. This hypothesis needs further investigation by analysis of the T2 lines.

5.6.6 FLA3 Induced Male Sterility

FLA3 has been studied by two transgenic lines OEx and T-DNA mutant both have shown severe male sterility. The OEx lines reduced stamen filament length and affect anther dehiscence. However, The T-DNA mutant lines exhibited reduction in silique lengths and pollen viability (section 5.4 above), This is consistent with findings reported that *FLA3* OEx lines exhibited short stamen and siliques (Li et al., 2010). RNAi lines have shown previously pollen intine layer defects possibly by affecting cell wall cellulose deposition (Li et al., 2010). The qRT PCR expression analysis revealed high expression of *FLA3* at the TCP stage which is up-regulated by drought stress (Chapter4 Figure 4.4). Future work is required to test the hormone network with *FLA3* such as auxin and JA which is associated with cell expansion and anther dehiscence. T2 lines must be generated to confirm the phenotype and test gene subcellular localisation.

Chapter 6 Monitoring Ca^{+2}, pH and auxin changes in anther

6.1 Introduction

In response to drought stress a cascade of physiological responses to enhances plant adaptation such as, water regulation, photosynthate transport and sugar metabolism (Chapter1 section1.8). ABA dependant transcription factors have been well studied however little is known about ABA calcium dependent proteins, although Ca^{2+} signalling has been associated with abiotic stress responses (Dodd et al., 2010). The plant hormone auxin has a key role in regulating growth responses to environmental cues. However, we are still lacking a cellular and molecular understanding of how ABA dependent signalling is associated with auxin and pH changes and Ca^{2+} signalling during male reproduction. The expression of Ca^{+2}, pH and auxin during different stages of pollen development from tetrad to tricellular pollen, were investigated using reporter constructs to determine how drought will affect each of them.

6.2 Ca^{2+} response in anther

Ca^{2+} is an essential element to regulate several biological processes such as cell wall structure, cell division, differentiation, and PCD (Rizzuto et al., 2008). *Arabidopsis* root cells transport Ca^{2+} to the plasma membrane and endomembrane; Ca^{2+} is stored in the endoplasmic reticulum (ER), space within the lumen and vacuoles (Hedrich et al., 2012). Ca^{2+} deficiency affects root development, leaf

and apical meristem cells (White and Broadley, 2003). Various biotic and abiotic stresses triggers Ca^{2+} signalling such as temperature, salt and drought changes in hormonal signalling (Sanders et al., 1999), which are likely to increase cytoplasmic Ca^{2+} concentration $[Ca^{2+}]$ cyt (Dodd et al., 2010). Ca^{2+} permeable channels, Ca^{2+} pumps and Ca^{2+}/H^{+} exchangers (Kudla et al., 2010), control Ca^{2+} gradients across the plasma membrane and the inner membrane. Several on/off regulatory mechanisms work to control cell Ca^{2+} excess. Ca^{2+} signalling has been reported to exist in the nucleus, chloroplasts and mitochondria (Kudla et al., 2010). The nucleus $[Ca^{2+}]$ nuc contributes to several signal-transduction pathways such as DNA replication and degradation (Reddy et al., 2011). Ca^{2+} homeostasis plays a role in pollen tube growth and egg fertilization (Li, et al., 2018), forming a concentration gradient across the length of the pollen tube that specifically increases at the tip (Hepler et al., 2001). The initial step of a calcium signaling occurs when Ca^{2+} binds to Ca^{2+} sensors, which divide into two types (sensor relays and sensor responders) such as Calmodulin (CaM) (sensor relay) contains Ca^{2+}-binding motif and Ca^{2+}dependent protein kinases (sensor responders) with EF-hand motif, CDPKs) (Cheng et al., 2002). For example, Calmodulin Ca^{2+} signalling targeting *ATMYB2* in responsive to drought (Yoo et al., 2005); *ATWRKY7* (Kim et al., 2006) and SAUR (Yang and Poovaiah, 2000). Crosstalk between Ca^{2+} and pH changes has been demonstrated in response to salt stress

and the control of Na^+/K^+ exchange rate (Yamaguchi et al., 2005). The Ca^{2+} binding protein OsDEX1 (Defective in Exine Formation1 in Rice) has been shown to regulate tapetum degeneration (Yu, et al., 2016). For our understanding of tapetum PCD most of our knowledge comes from studying transcription factors and their regulatory mechanisms. Hence, calcium imaging produces a powerful technique to study the changes of Ca^{2+} in plant cells. The tagged Cameleon YC3.6 (Nagai et al., 2004) which targeted the cytoplasm, with a nuclear export sequence (NES-YC) (Krebs et al.,2012), and to mitochondria, 4mt-YC3.6 $[Ca^{2+}]$m (Loro et al.,2012), which have been measured by aequorin and fluorescence resonance energy transfer (FRET)-based yellow cameleon indicators (Miyawaki et al., 1997).

6.3 pH changes during anther development

The stability of plant cell is pH dependant especially when plants perceive environmental changes such as osmotic or drought stress. Every protein requires an optimal pH to function, the cytosol and the nucleus exhibited almost similar pH value 7.3 and 7.2 respectively (Shen, et al., 2013).

Increasing cell alkalinity helps regulate actin cytoskeleton during pollen tube growth in the *Lilium longiflorum* (Lovy-Wheeler et al., 2006). Cytosolic acidification has been associated by cell death and activation of the programmed cell death (Wilkins, et al., 2015). Furthermore, several genes have been activated during drought by

cell acidification. Drought stress enhanced pH expression in *Commelina communis* xylem (Wilkinson., 1997). Understanding changes in the cytoplasmic pH signals during drought is limited, Here I present a study about pH signals at different stages of pollen development from tetrad until TCP in normal watered plants and under drought.

6.4 Role of auxin in anther development

Auxin plays an essential role in regulating plant growth and development, for example, embryogenesis, cell division and elongation, vascular tissue differentiation, root patterning, shoot elongation, and embryonic patterning (Leyser, 2006). Auxin controls multiple genes and changes their expression in response to exogenous auxin supply (Paponov et al., 2008). The disruption of auxin biosynthesis leads to the failure of flower formation (Cheng et al, 2007) the auxin polar transport system plays an important role in an early stage of *Arabidopsis* floral bud formation (Okada *et al*, 1991). Auxin plays a role in pollen development and anther dehiscence (Wilson et al., 2011). Several auxin genes have a role in male sterility and pollen viability including *YUC2* and *YUC6* a flavin monooxygenase of tryptophan-dependent auxin biosynthetic pathway (Cheng et al., 2006). Double mutant in *yuc2yuc6* arrests pollen development causing defects in first mitotic cell division (PMI) and microspores stages, which implies the role in auxin in early stage of pollen development (Yao et al., 2018). Therefore, studying auxin

signalling is crucial for understating anther and pollen development in normal watered condition and after drought stress.

6.5 Materials and Methods

6.5.1 Reporter Gene Constructs

Calcium Cameleon NES-YC3.6 that has cytoplasmic localization nuclear export signal (NES) (Krebs et al., 2012). The Yellow cameleons, are composed of a donor chromophore (CFP), calmodulin (CaM), a glycylglycine linker, the CaM-binding peptide of myosin light-chain kinase (M13), and an acceptor chromophore (YFP). When Ca2+ binds to CaM form interaction between CaM and M13 resulting in an increased FRET-efficiency between CFP and YFP (Miyawaki et al., 1997). NES-YC3.6 is a pTkan-based vector with a UBQ10 promoter (Krebs et al.,2012). For fluorescence mitochondria Ca^{2+} sensor the mitochondrial targeting sequence from subunit VIII of human cytochrome *c* oxidase fused to the N-terminal end of the probe. This sequence is very efficient especially when repeated four times (4mt) under the control of a single CaMV 35S promoter (Loro et al.,2012). pH was monitored using *seapen Ptilosarcus gurneyi* (*Pt*-GFP) vector was expressed in ecotype Columbia-0 of *Arabidopsis thaliana* under the control of the CaMV 35S promoter (Alexander et al., 2006). Auxin has been studied by *DR5-GFP* reporter which has been previously detected in anther (Feng et al., 2006). The DR5GUS construct contains seven copies of DR5 cloned up- stream of a -46 CaMV 35s promoter with a TMV 5' leader (Ulmasov et al., 1997).

6.5.2 Growth Conditions

Four *Arabidopsis thaliana* (col) background seeds were grown in 13 cm pots filled with Levington M3 compost (The Scotts Company Ltd, UK) at 16 h light (120 µmol m^{-2} s^{-1})/8 h dark at 22°C and exposed to drought stress for ten days. Images were taken at the beginning of the drought treatment at day2 and at the end of the drought, day10 and13 days drought treatment for the auxin signalling study.

6.6 Results

6.6.1 Monitoring Ca^{2+} changes in anther

In this chapter two Ca^{2+} signalling probes have been studied to determine Ca^{2+} concentration in the anther and tapetum layers at different pollen developmental stages from tetrad stage (I), microspore stage (II) bicellular pollen BCP stage (III) and tricellular Pollen TCP stage (IV). Mitochondrial based Ca^{2+} the 4mt-YC3.6 $[Ca^{2+}]_m$, at drought day2 the transgenic plants showed expression of the 4mt-YC3.6 probe clearly in anthers (Figure.6.1), in the epidermis and endothecium as well as plasma membrane of the tapetum cells from pollen tetrad until TCP, as presented by the laser Confocal scanning microscopy (Figure.6.2).However the expression remained high in the drought samples and similar to control anthers. While at drought, day10 Ca^{2+} expression decreased in comparison to the control, suggesting that mitochondrial calcium is impacted in by drought stress. The NES-YC3.6 prop showed Ca^{2+} concentration in anther epidermis, endothecium, tapetum epidermis and cytoplasm

in the watered samples (Figure.6.1). While samples under severe drought stress showed induced Ca^{2+} spikes in the drought samples (Figure.6.5). The cytoplasmic and nucleus Ca^{2+} have been enhanced in response to different stresses as reported by several authors (Rodrigues et al., 2007; Huang et al.,2017).

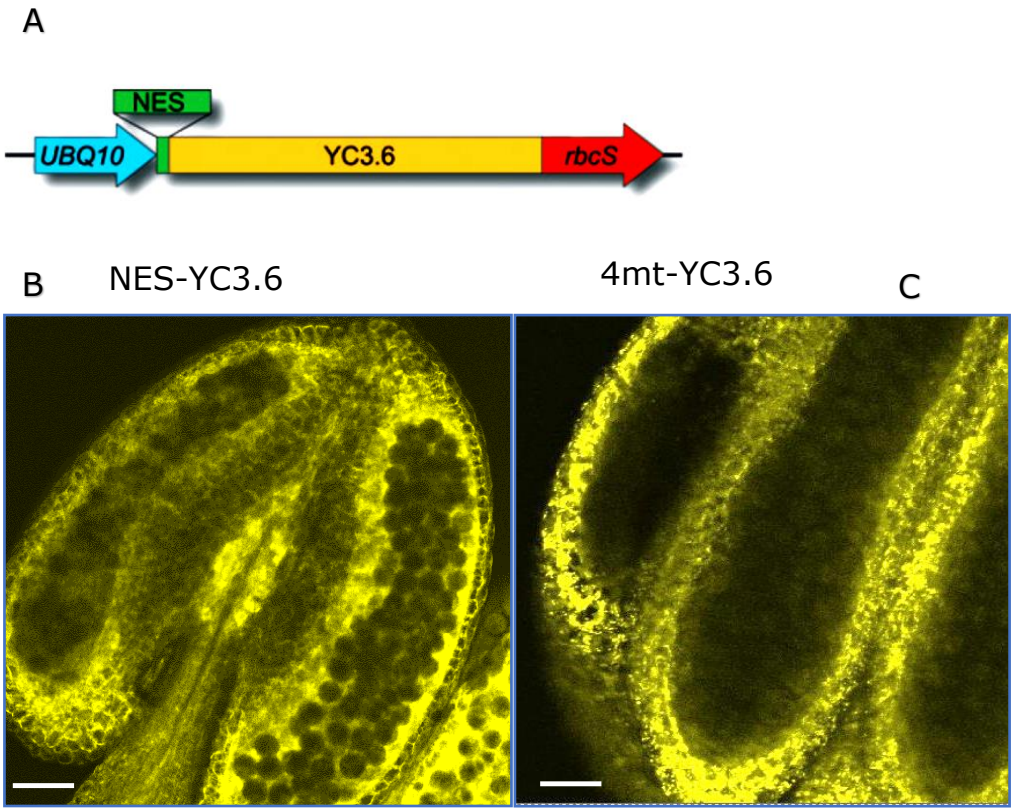

Figure 6.1 *Arabidopsis* anther expressing 4mt-YC3.6 and NES-YC3.6 Probe in the well- watered anther. **A)** Schematic representation of the pGPTVII-based cameleon expression vectors, expression is controlled by the UBIQUITIN10 (UBQ10) promoter and nopaline synthase (nos) promoter conferring resistance to BASTA. **B)** Presenting strong nucleus Ca signal in anther loculus and plasma membrane, as well as tapetum membrane and cytoplasm, the 4mt-YC3.6 construct used is 35s promoter. **C)** Showing Mitochondria localisation of strong Ca signals exist in anther epidermis, endothecium and tapetum plasma membrane. Images taken by TCS SP2 confocal laser scanning microscope captured with the CFP (438Ex/483Em), CpVenus (500Ex/542Em). Scale bars, 100 µm

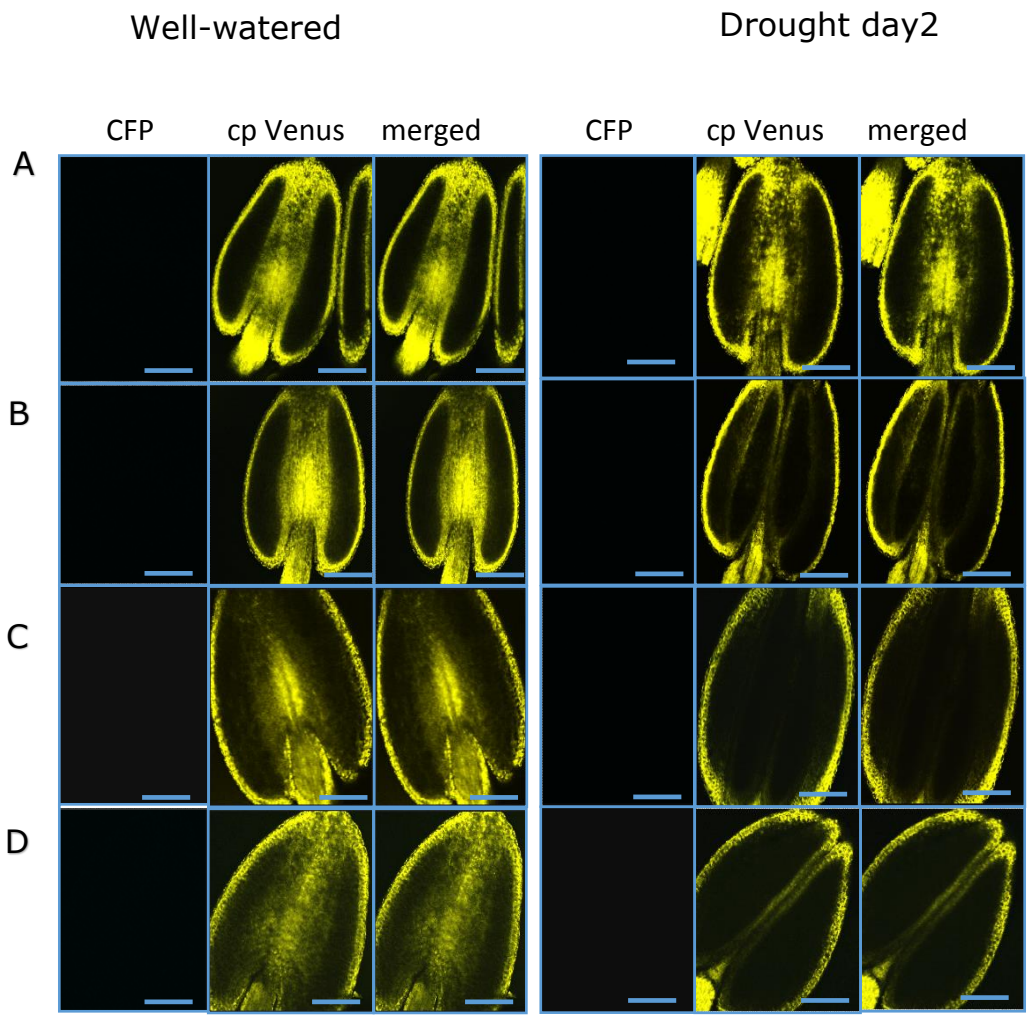

Figure 6.2 Arabidopsis anthers expressing 4mt-YC3.6 probe in well-watered and drought stressed plant based on YFP emission. Confocal images of anthers at the following stages **A)** Pollen tetrad**, B)** Microspore pollen, **D)** Bicellular pollen **E)** and Tricellular pollen image. The left panel is for the well-watered tissues while the right panel is for the drought stressed tissues. Confocal images of Ca^{2+} expression in Mitochondria are taken using a confocal microscope. Images taken by TCS SP2 confocal laser scanning microscope captured with the CFP (438Ex/483Em), CpVenus (500Ex/542Em). Scale bars, 100 µm

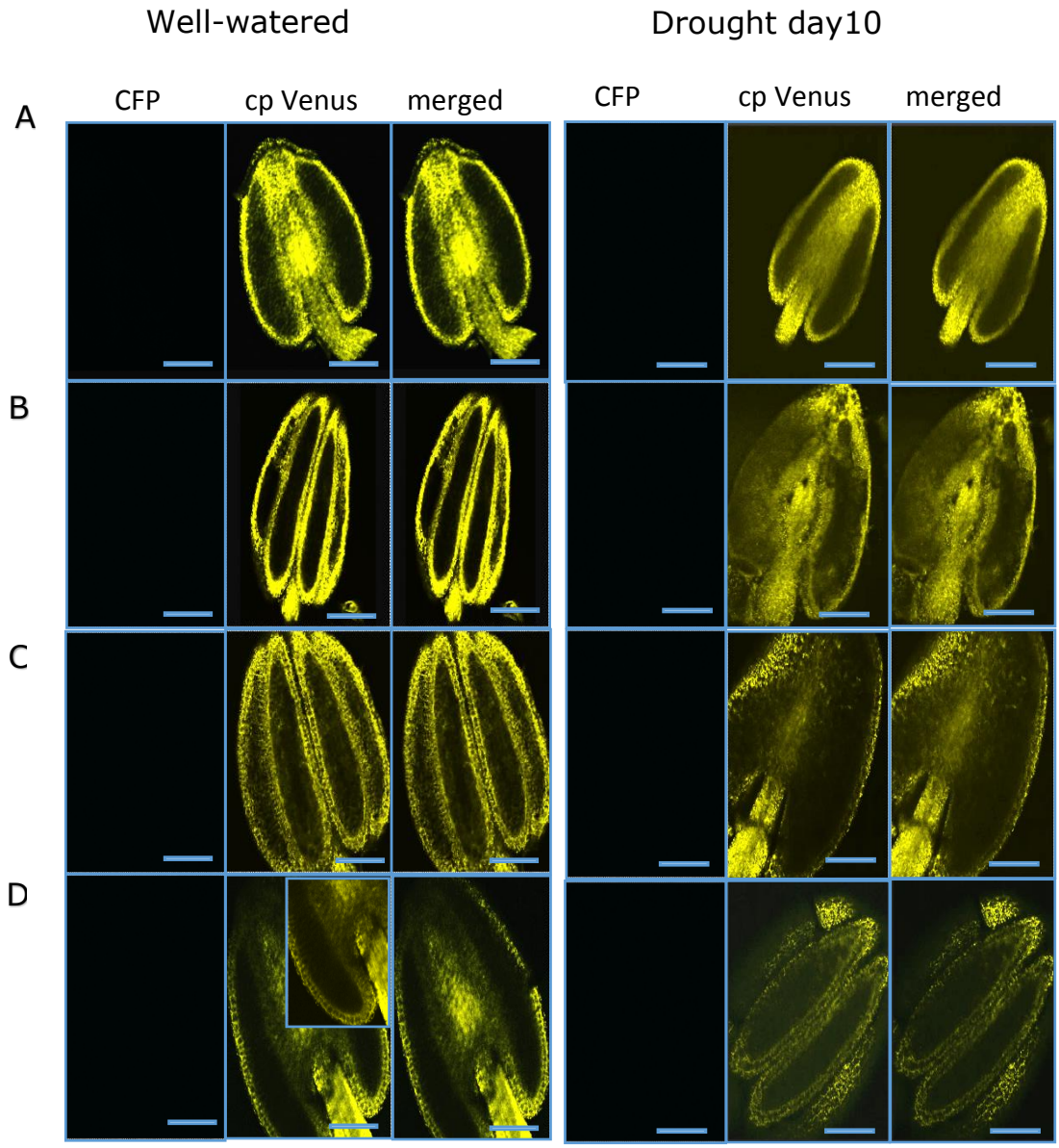

Figure 6.3. *Arabidopsis* anther expressing 4mt-YC3.6 probe in well-watered and drought stressed plant based on YFP emission. Confocal images of anthers at the following stages. **A)** Pollen tetrad, **B)** Microspore pollen, **D)** Bicellular pollen **E)** and Tricellular pollen image. The left panel is for the well-watered tissues while the right panel is for the drought stressed tissues. Wild type confocal images of Ca^{2+} expression in mitochondria taken by sp2 confocal microscope. Images taken by TCS SP2 confocal laser scanning microscope captured with the CFP (438Ex/483Em), CpVenus (500Ex/542Em). Scale bars, 100 µm

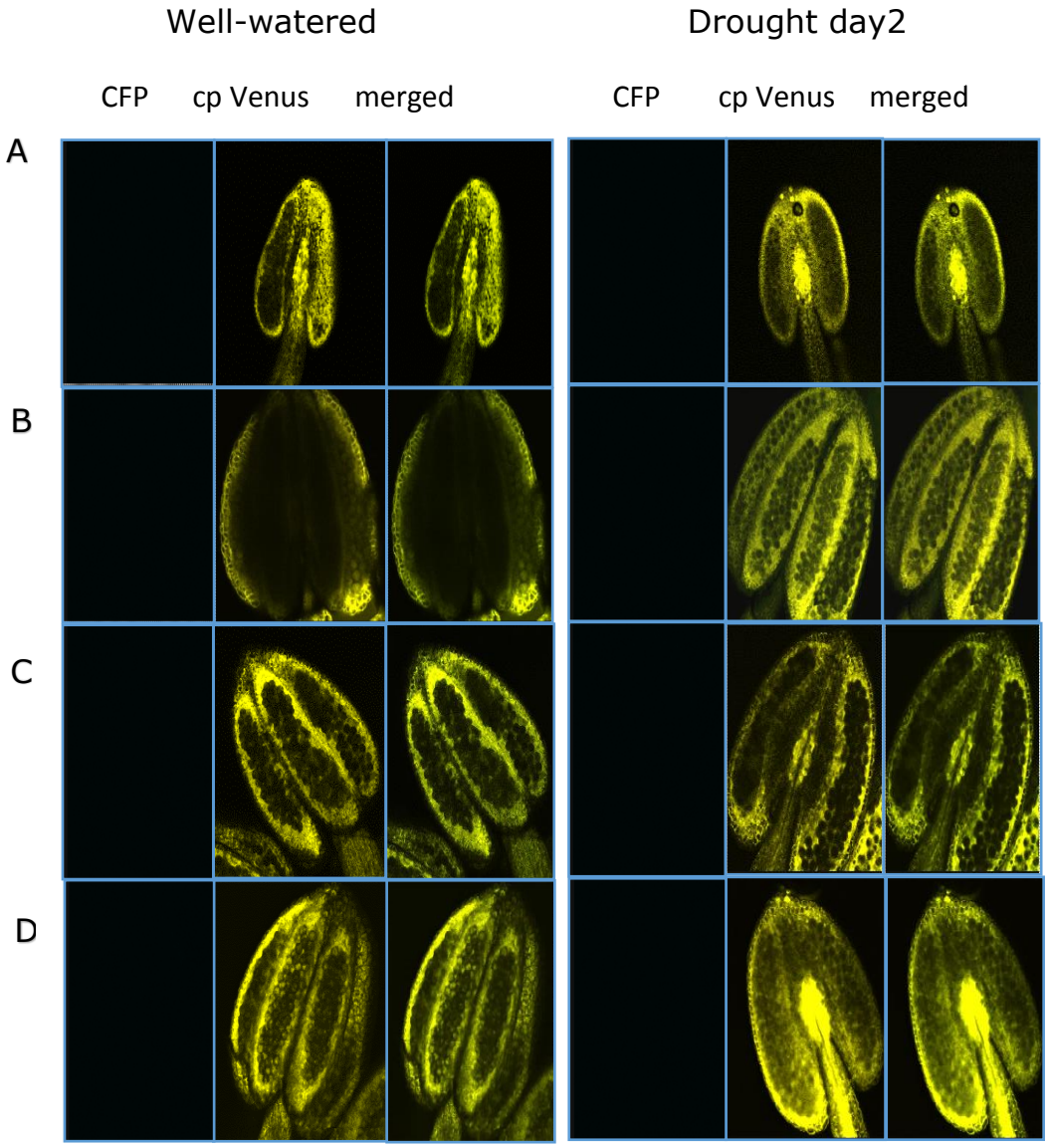

Figure 6.4 *Arabidopsis* anther expressing NES-YC3.6 probe in well-watered and drought stressed plant based on YFP emission. Confocal images of **A)** Pollen tetrad, **B)** Microspore pollen, **D)** Bicellular pollen **E)** and Tricellular pollen image. The left panel is for the well-watered tissues while the right panel is for the drought stressed tissues. Wild type confocal images of Ca^{2+} expression in mitochondria taken by sp2 confocal microscope. Images taken by TCS SP2 confocal laser scanning microscope captured with the CFP (438Ex/483Em), CpVenus (500Ex/542Em). Scale bars, 100 µm

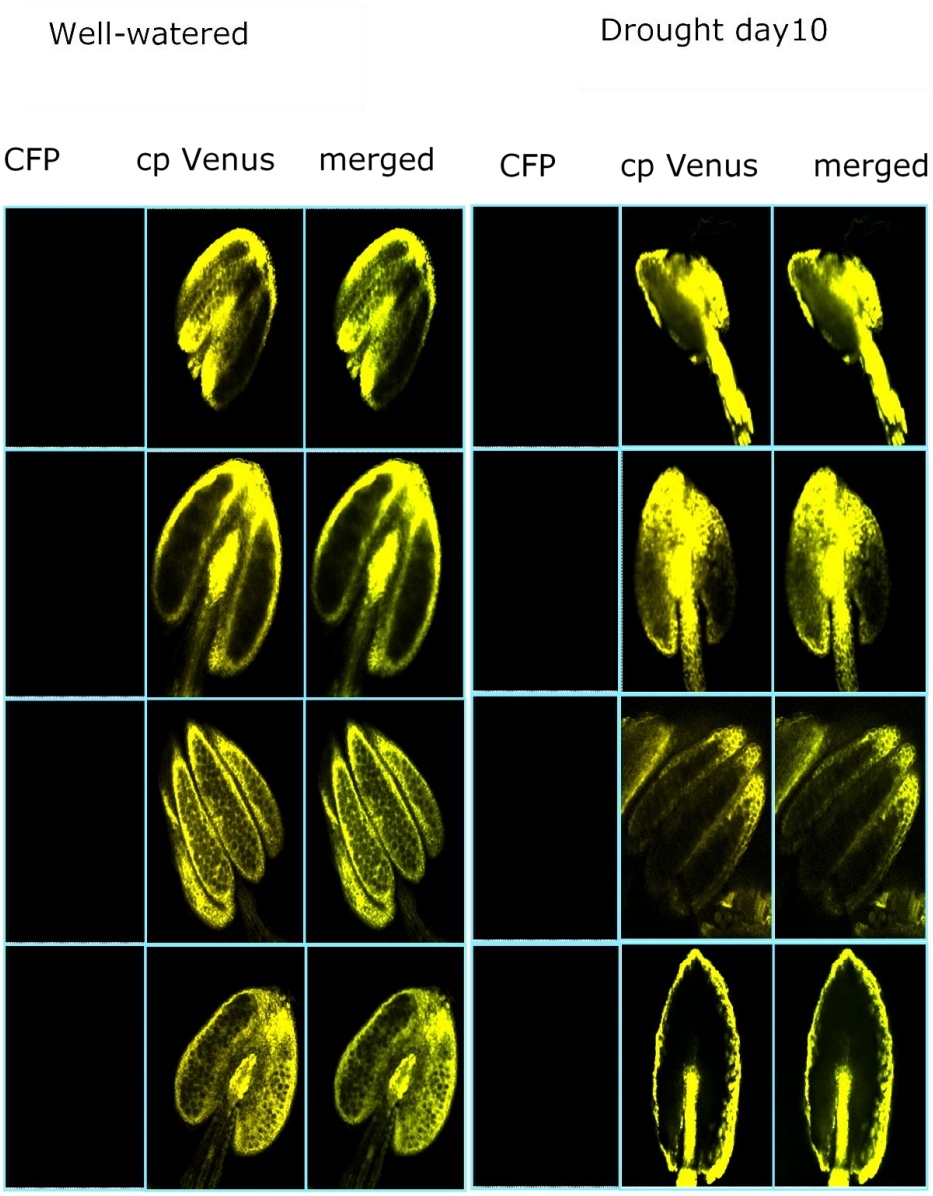

Figure 6.5. *Arabidopsis* anther expressing NES-YC3.6 probe in well-watered and drought stressed plant based on YFP emission. Confocal images of **A)** Pollen tetrad, **B)** Microspore pollen, **D)** Bicellular pollen **E)** and Tricellular pollen image. The left panel is for the well-watered tissues while the right panel is for the drought stressed tissues. Wild type confocal images of Ca^{2+} expression in mitochondria taken by sp2 confocal microscope. Images taken by TCS SP2 confocal laser scanning microscope captured with the CFP (438Ex/483Em), CpVenus (500Ex/542Em). Scale bars, 100 µm

6.6.2 Monitoring pH changes in anther

pH signal emission in well-watered anther tissues showed peak expression in the anther epidermis, endothecium and tapetum plasma membrane and cytoplasm as seen in Figure.6.6. At day2 drought strong pH signals was observed in anthers at pollen tetrad, microspore, BCP and TCP, stages; signalling remained similar in the drought treatments. After 10 days of withholding water the pH signalling was observed at epidermis, and endothecium in the control samples. However, the signal disappeared from tapetum cytoplasm in all stages (Figure.6.7). The emission spectrum at 470 represent pH value at 7.4 in a radiometric fluorescent measurement under different pH concentration as reported by (Alexander et al., 2006). Suggesting normal viable cells as discussed earlier, reducing the pH signals by the drought stress is an indicator of cell acidification and reduced the pH value.

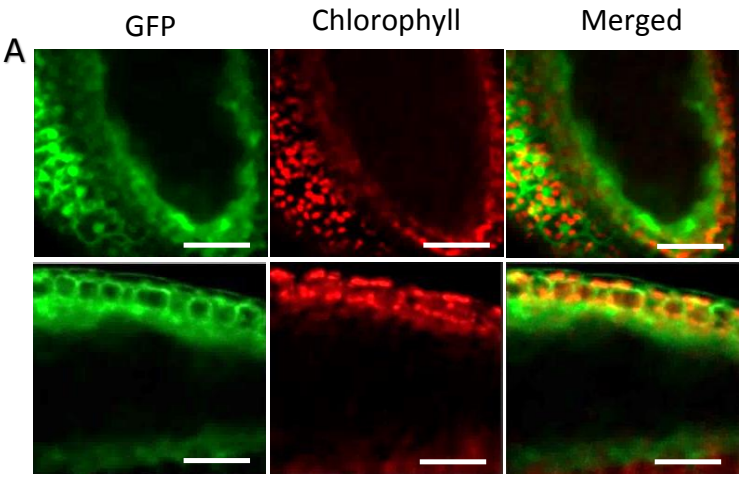

Figure 6.6. Pt-pH-GFP in wild type *Arabidopsis* anther. **A)** Close up confocal image of *Arabidopsis* anthers BCP stage expressing *Pt*-GFP. Show expression in anther epidermis, endothecium, tapetum plasma membrane and cytoplasm. The red fluorescence is chlorophyll auto fluorescence from chloroplasts. Scale bars, 100 µm

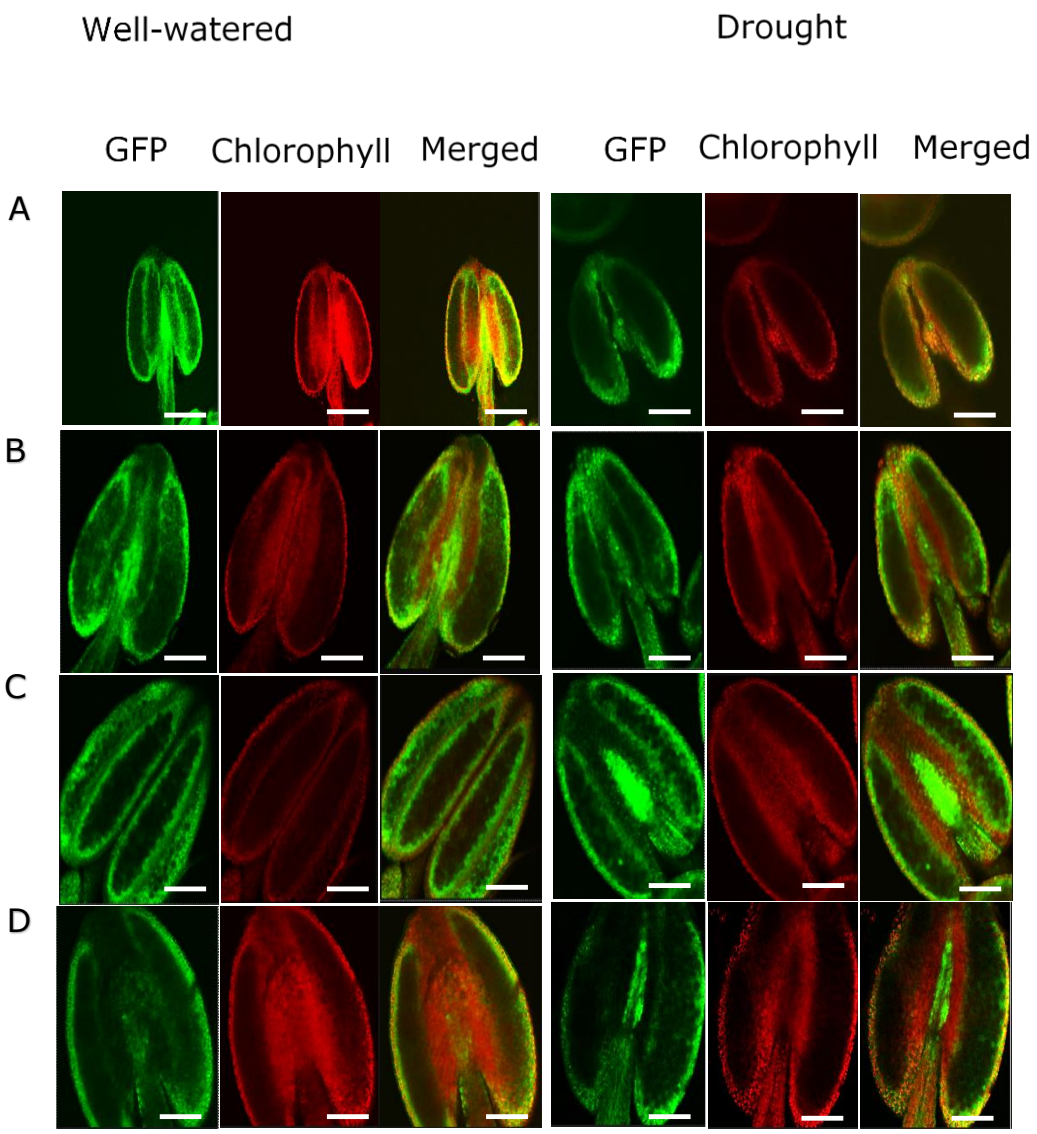

Figure 6.7 *Pt*-pH-GFP in *Arabidopsis* anther drought day2 from withholding water. **A)** Confocal images of pollen tetrad, **B)** Microspore pollen, **C)** Bicellular pollen **D)** and Tricellular pollen image. The left panel is for the well-watered tissues while the right panel is for the drought stressed tissues. 488 nm beamline of the Argon laser was chosen for GFP fluorescence and 600–660 nm (red channel) for chlorophyll auto fluorescence. Images taken by SP2 confocal microscope Scale bars, 100 µm

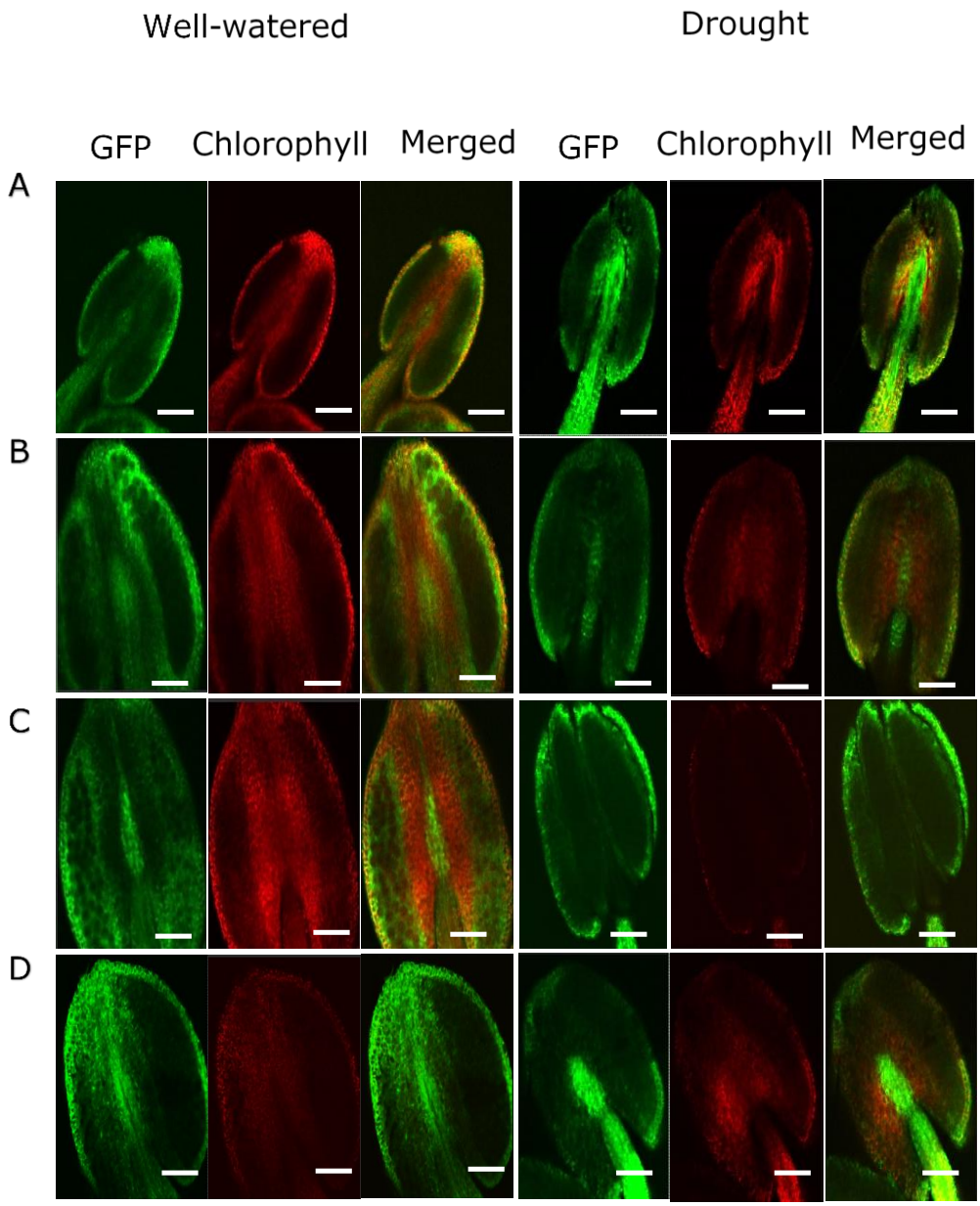

Figure 6.8 *Pt*-pH-GFP in *Arabidopsis* anther drought day10 from withholding water. **A)** Pollen tetrad confocal image expressing *Pt*-GFP. The red fluorescence is chlorophyll auto fluorescence from chloroplasts. **B)** Microspore pollen, **D)** Bicellular pollen **E)** and Tricellular pollen image. The left panel is for the well-watered tissues while the right panel is for the drought stressed tissues where pH signal disappeared from tapetum cytoplasm in all stages while remained in both epidermis and endothecium. 488 nm beamline of the Argon laser was chosen for GFP fluorescence and 600–660 nm (red channel) for chlorophyll auto fluorescence. Images taken by SP2 confocal microscope Scale bars, 100 µm.

6.6.3 Monitoring auxin response in anther

Auxin is known to trigger plasma membrane (PM) proton pumps (PM H^+-ATPases), resulting in cell acidification and regulate cell expansion (Spartz et al., 2014). Auxin regulates anther dehiscence, pollen development, and filament elongation (Cecchetti et al.,2008). The auxin response reporter DR5: GFP was used to determine auxin signals in the anther during drought. Day2 showed strong expression in tapetum cell layer and cytoplasm in the microspore, BCP and TCP stages, while auxin signals in the tetrad were not detected. The signals remain the same and did not change at drought day2, however at day13, in the well-watered samples auxin signals were not seen in mature pollen anther stage13 but remained in all anther stages (Figure 6.9). In response to drought at drought day13 auxin signals completely disappeared from all the stages from tetrad to TCP stage (anther stages 9 to 12) in comparison to the control watered plants (Figure 6.9).

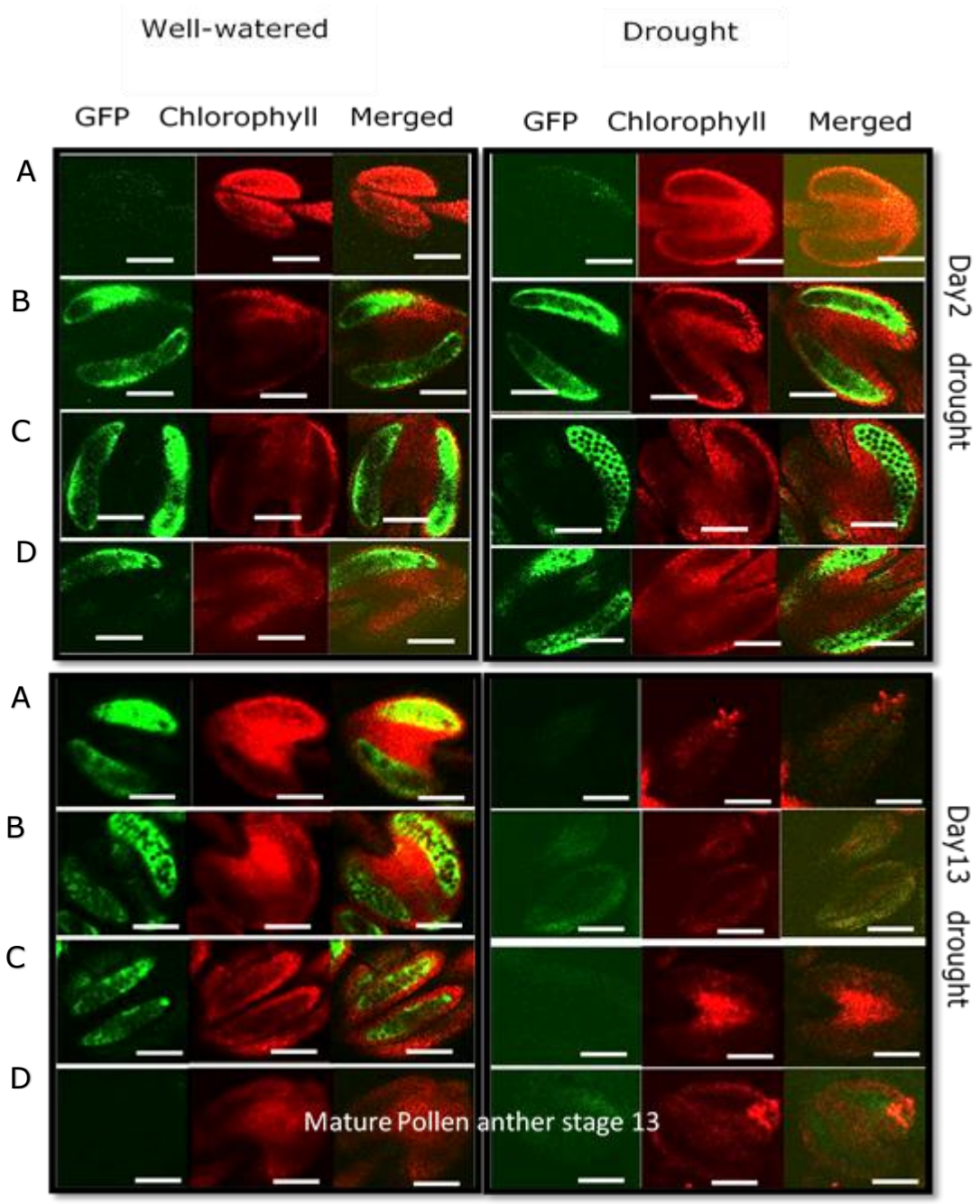

Figure 6.9. Expression of the *DR5*: *GFP* auxin-responsive reporter in the anthers of *Arabidopsis thaliana*. A) Distribution of auxin in anther development during bud stage I(tetrad), **B)** stage II **(**Microspore), **C)** stage III (BCP), **D)** stage IV (TCP) under normal and drought treatment at day3 in well-watered and drought treatment. Auxin expression level was decreased in days13 of withholding water in bud stage II microspore, stage III (BCP)and stage IV (TCP), while **D)** Drought day 13 show mature pollen in both control and drought where no auxin expressed. Scale bar 100 µm

6.7 Discussion

Drought stress before anthesis causes pollen abortion and reduces seeds number (Dolferus et al., 2011), in part due to reduced starch accumulation in pollen grains and disrupted tapetum function (Ji et al., 2010), which implies a role of sugar signalling in maintaining pollen viability. Sugar has been reported to induce increased cytosolic Ca^{2+} concentrations and therefore induce expression of biosynthesis and stress responses genes (Furuichi, et al., 2001). In well-watered tissues, Ca^{2+} signals appeared in anther loculus, plasma membrane, tapetum membrane and cytoplasm (Figure 6.5), while severe drought has induced Ca^{2+} signals in all the four stages of pollen development. Ca^{2+} is known to regulate a wide range of biological processes, including PCD and cell death, (Lopez-fernandez et al., 2015) by triggering increase of cytosolic Ca^{2+}. Suggesting that drought may triggers Ca^{2+} signalling events and regulates cell stress perception at the anther membrane level with a cellular response in tapetum layers. Changes in cytosolic Ca^{2+} concentration are also associated with hormone signalling (Bush, 1995). For example, AtCPK1 leads to salicylic acid accumulation and plant immune system (Coca et al., 2010), while GA which induces $[Ca^{2+}]$ cyt expression through DELLA-independent pathway (Okada et al.,2017).

Mitochondrial Ca^{2+} is crucial for oxidative phosphorylation and the control of metabolic activity, it also plays an important role in ROS production and allows mitochondrial Ca^{2+} increases that leads to PCD and cell death (Rizzuto et al.,2012). Stored mitochondrial Ca^{2+} entered mitochondria either from the plasma membrane or the endoplasmic reticulum (ER) through Ca^{2+}-permeable channels (Kim et al.,2016). Salt stress induced high level of mitochondrial Ca^{2+} concentration and increased ROS generation (Mallilankaraman et al 2012). Drought stress also has been reported to induce mitochondrial ROS and participate in plant leaves PCD (Ruberti, et al.,2014). A 4mt-YC3.6 signal revealed normal mitochondrial Ca^{2+} concentration equilibrium between the influx and efflux rates in the well-watered and during drought day2 in anthers tissues (Figure 6.2). The signals at drought day10 appeared to be reduced in compared to the well-watered anthers. Suggesting mitochondrial Ca^{2+} efflux may rapidly disappear by diffusion to the cytoplasm and reduces mitochondrial Ca^{2+} uptake (Figure 6.3).

The cytosolic pH is coordinated with cytosolic Ca^{2+} concentration with a wide range of cellular functions and in defence responses against stresses (Kader et al., 2010). pH-GFP signal appeared in anther epidermis, endothecium and tapetum plasma membrane and cytoplasm as seen in (Figure 6.6) in watered conditions. However, pH-GFP signal disappeared from tapetum cytoplasm by the drought, which corresponded to

cytoplasmic acidification. Islam et al, (2010) reported that Ca^{2+} induced cytosolic alkalization in guard cells to prevent stomatal closure. While Poburko et al., (2012) reported that mitochondrial pH decreased during cytosolic Ca^{2+} elevations and causes cytosolic acidification.

Auxin concentration reduced under drought (Figure 6.9) illustrating the importance of auxin in early and late stage of pollen development where tapetum layers not only provides pollen with nutrients and supplements but also auxin hormone. Cell wall extension and relaxation triggers calcium accumulation at the plasma membrane level in an alkaline environment and inhibits the H^{+}-ATPase proton pumps. Since auxin has a role in cell acidification (section 6.6.3). This suggest the activation of several enzymes and cell wall related genes to regulate cell growth, cell responded to drought by decreasing auxin expression level which might increases cell alkalinity and inhibits growth.

Chapter 7 General Discussion

Water deficit during reproductive development affects male fertility and grain set (Boyer & Westgate, 2004). This work investigated male fertility reduction under water deficit stress by investigations of drought gene expression at the molecular level. In addition, by studying gene function by overexpression and knockout lines, and by studying protein localization and expression patterns of Ca^{+2}, pH and auxin in response to drought.

The plant hormone abscisic acid (ABA) is a master regulator of plant development and water loss responses, regulates embryo, seed development, seed dormancy, germination, vegetative development, general growth and plant defence system by producing a massive network of osmocompatible solutes and dehydrins, enzymes that detoxify reactive oxygen species, enzymes of compatible solute metabolism, a variety of transporters, transcription factors, protein kinases and phosphatases, enzymes involved in phospholipid signalling and LEA proteins during drought to prevent cell damage (Cutler et al., 2010). Increasing the knowledge of ABA-dependent drought responses and its core signalling components offers possibilities for the development of drought tolerant crops.

During normal pollen development, the microspore mother cells divide by meiosis to form tetrads of haploid microspores, before undergoing two rounds of cell division to develop into mature pollen. Initial work in this study involved an analysis of the expression patterns of genes in different stages of pollen development (tetrads, microspores, polarized microspores and mature pollen) under drought conditions; selected genes were shown to be enriched for different processes in specifying stamen identity, regulating anther cell division and differentiation, controlling male meiosis, supporting pollen development, and promoting anther dehiscence For example, cell wall metabolism and cytoskeleton, transport and transcription factors. When *A. thaliana* plants were exposed to drought, cell wall related genes, sugar metabolism, late embryogenesis abundant (LEA), and heat shock (HSP) proteins genes were upregulated by drought during early and late pollen development. However, selected transcription factors (that work as master switches of the genetic reprogramming) and selected pollen specific genes were down-regulated in response to drought (Chapter 4). The ABA dependent genes as reported from other authors were *PLDALPHA2, HSP*, *bZIb*, *BBX19*, *ATMYB101*, *HKL3*, *SFH5LP, BCP1 and CU/ZN CDS* (Chapter 4) and these were down-regulated by the effect of drought. However, the rest of the candidate genes which were induced by the drought their interaction with ABA is still unknown and requires further investigation.

The up-regulated and down-regulated genes, for example *HRGPs, FLA3, PLDALPHA2, BBX19, DUF1216, SAUR47, LEA* and *CSD2,* exhibited opposite responses when plants were rehydrated. This indicates that these genes are responding to the drought stress conditions and may be involved in either the manifestation of the drought stress, or potential avoidance mechanism(s) to minimise the impact of the drought stress. Their specific expression in the floral tissues during pollen development also supports a role for these genes in pollen development gene networks.

Drought stress has a tremendous effect on flowering and impacts on male fertility and seed set, as indicated by a lack of silique elongation, pollen viability defect, short stamen length, early anther dehiscence, abnormalities to general floral shape, sepals and petals. To further explore possible causes of male sterility, a significant part of this work was devoted to understanding the role of those genes in anthers under normal (watered) and drought conditions. The transgenic *Arabidopsis* overexpressing the following genes *FLA3*, *PLDALPHA2*, *DUF1216*, *SAUR47*, *LEA,* showed a distinct impact on male fertility including alterations in stamen filament length, anther dehiscence, pollen number, pollen viability and silique length. Further a proposed model for genes mechanise and pathways in (Figure 7.1) as stated in chapter 5 (section 5.6).

In addition, plants were affected in their general growth, such as main stem & internode length, stem and siliques architecture and flowering time. The observed impact of overexpression in *FLA3, SAUR47, LEA,* were investigated further by T-DNA mutant lines. Each of *LEA*, *SAUR47* and *FLA3* were studied by two T-DNA insertion lines, however gene expression analysis that they show maintained same expression levels (Chapter5 section 5.4). *LEA*, and *FLA3* mutant lines showed a reduction in gene expression while *SAUR47* insertion line enhanced gene expression and all of the three lines revealed severe male sterility (Chapter5 Figure 5.6a&b) by reducing siliques length and when exposed to a period of drought *FLA3* and *LEA* lines were negatively affected by the drought, however *SAUR47* was positively regulated by drought. The two lines for (*FLA3, LEA)* SALK 046270, SALK_016582 showed a reduction in pollen viability in response to drought. However, the siliques length did not change significantly. According to the viability assay this suggests that drought may impact pollen number or size, or female fertility, which has not been tested in this work. The T-DNA insertion of AT3G20230 and *SAUR47* by SAIL_84_D03 seems to enhance plant reproductive development during drought by maintains siliques length and pollen viability as the control (watered). Hence the FLA3 and LEA affects pollen viability which reduced yield, this implies a possible role of *SAUR47*, *FLA3* and *LEA* as drought resilience

genes for future work. Both GK-171H05 for *SAUR47* and SALK_131759C and *LEA* exhibited reductions in secondary branch numbers however, GK-212E06 for *PLDALPHA2* showed increased number of the secondary branches. *PLDALPHA2* insertion line SALK 098375c showed the most reduce stem length (Chapter5 Figure 5.5b). Increasing auxiliary branching means potentially increasing yield biomass. Since *PLDALPHA2* showed a phenotype of increased number of branches, Implying that this gene may be a candidate to increase yield biomass.

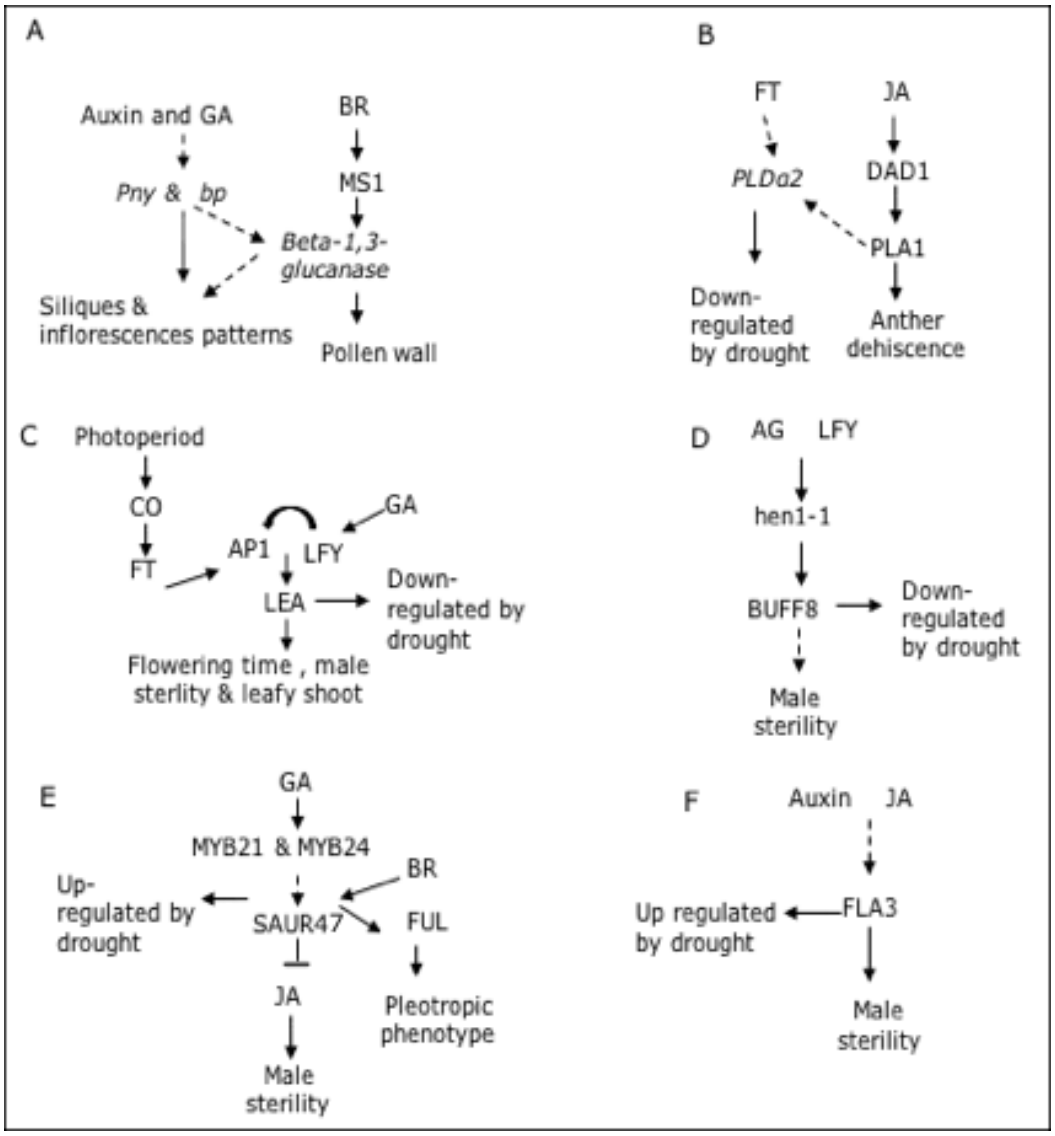

Figure 7.1 A proposed model and regulatory mechanism for each of the six genes . A) *Beta-1,3-glucanase* is down regulated by *MS1* and may work under auxin and GA to control siliques and inflorescences architecture, Beta-1,3-glucanase is not affected by drought stress. *B) PLDα2* may works under FT and PLA1 to control male fertility. C) *LEA* works under the regulatory mechanism of *LFY* and FT to control flowering time and male fertility . D) *BUFF8* is down regulated by hen1-1 and affects male fertility. E) *SAUR47* works under MYB21 and *MYB24* to inhibits JA to cause male fertility , *SAUR47* also works under BR to control various phenotype such as dwarfism . F) *FLA3* may works under JA and auxin to control male fertility.

The role of Ca^{+2} is critical in pollen tube development (Ge et al., 2007), however little is known regarding calcium distribution and function in anther development. The nature of changes in Ca^{+2} (nucleus & mitochondria) and pH_{cyt} in drought responsive plants may increase our ability to improve stress resistance in crops. When *Arabidopsis* is exposed to ten days of drought stress Ca^{+2} signalling increases in all stages of pollen development as well as anther filaments. Suggesting a role Ca^{+2} role in regulating antioxidant metabolism to avoid dehydration stress. However, pH signals decreased in all stages from tetrad to TCP and disappeared from tapetum cell membrane and cytoplasm indicates changes in membranes, cytoplasmic buffering by inducing cell acidification, while strong pH signals exist in anther filaments. This implies some mediation of pH to physiological responses to stress and may affect tapetum PCD. Finally during drought mitochondrial $[Ca^{2+}]_m$ decreased by releasing and increasing cytoplasmic calcium $[Ca^{2+}]_{cyt}$ which causes cell acidification by decreasing pH concentration and affects tapetum function and pollen abortion. Consistent with finding from Felle., (1988) when cytoplasmic pH decreased the in $[Ca^{2+}]_{cyt}$ increased.

Auxin application has been reported to control cytosolic acidification of epidermal cells in corn coleoptiles (Felle et al., 1986). Auxin hormones is essential for normal plant development. Therefore, studying auxin signalling produced during pollen development is required for better

understanding. This study illustrated that tapetum may provide pollen with nutrition and auxin which required for both sporophytic and gametophytic development. Yao et al., (2018) reported that auxin in diploid microsporocytes is sufficient for early stages of pollen development through YUC2 and YUC6 enzymes. Severe drought affects auxin levels by a loss of DR5 reporter signal in anthers from microspore to tricellular pollen stage. It has been reported that ABA promotes auxin biosynthesis and polar auxin transport in rice root tip (Wang et al., 2017). Auxin can also reverse the inhibitory effect of ABA on stomatal opening (Gehring et sl., 1990). However, the role of ABA controlling male fertility through auxin in tapetum cell layer before and after miosis still unknown. Drought may be speeding up development and hence auxin is lost earlier.

Future perspectives

- Generating second generation which both homozygous and heterozygous (T2) of overexpression lines for the following genes *FLA3*, *PLDALPHA2*, *DUF1216, B-1,3-Glucanases*
- All the over expression lines need to be analysed under drought stress
- Investigating possible hormone signalling network connected to each gene particularly *SAUR47* and *LEA* where supposed to be integrated in different hormonal pathway
- Subcellular localization for each gene in the anthers during tetrad, microspore, bicellular and tricellular pollen stages.

The overexpression lines using 35S CaMV promoter increased the expression level of the gene in all organs of plants, as observed in all of the overexpression lines in this study and affected general plant growth. Therefore, using a specific drought promoter could be beneficial for engineering plants. As reported by Ge et al., (2018) the synthetic promoters Ap (containing four tandem repeats of ABRE), Dp (containing two tandem repeats of DRE), and ANDp (containing two tandem repeats of DRE and four tandem repeats of ABRE) showed strong tolerance to drought in addition to keeping normal plant growth in the watered plants.

Future work could include specific drought promoters for studying drought stress tolerance.

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APPENDICES

APPENDIX I

Expression of Cell Wall-Related Genes

ATCHX19 (AT3G17630)

ATCHX19 is a membrane transporter that is localised in the dynamic endomembrane system, which modulates K^+ and pH homeostasis. It belongs to the 28-member *Arabidopsis CHX* protein family, 18 members of which are expressed exclusively or predominantly in the pollen; cell wall and plasma membrane remodelling appear to be influenced by *CHX* transporters. It is expressed in bicellular and tricellular stages, but expression was decreased in mature pollen, which suggests a role in cation/proton exchange in early male gametogenesis, possibly associated with vacuole morphogenesis and osmotic adjustment and K^+ homeostasis as mature pollen desiccates and then rehydrates at germination (Sze et al., 2004). Recent reports suggest a role for *ATCHX19*, alongside *ATCHX17* and *ATCHX 18*, in pollen development, wall formation and pollen tube sensing, resulting in male fertility defects (Padmanaban et al, 2017). Several vacuole ion channels, such as H^+-ATPase or calcium channels appear to be expressed at all stages of pollen development (Bock et al., 2006). *ATCHX19* is up regulated in the *MALE GAMETOGENESIS IMPAIRED ANTHERS* (*mia*) (subfamily of P-type ATPases) mutant, which negatively affects cation homeostasis (Jakobsen et al., 2005). It has been suggested

that the fertilization failure is mainly due to pollen tube defects or reduced sperm fertility. Loss of function of PM-localized *CHX19* may affect tube growth and rupture perhaps through ANX1/ANX2 receptor-like kinases (Boisson-Dernier et al., 2009). The qRT-PCR analysis indicated expression of *ATCHX19* at the tetrad stage (I) and microspore stage (II) but did not show any obvious changes during drought compared to the control samples where the expression was low. Higher expression was observed at BCP (III) and TCP (IV) in the control samples that showed a very significant reduction ($P \leq 0.001$) during early drought and after re-watering. Expression was reduced by drought and although it was rescued to some extent by re watering expression it was still reduced compared to the control (Chapter4. Figure 4.7).

ATCHX19 expressed at late pollen development BCP and TCP. Has a role in pH and cation homeostasis that may act a membrane trafficking and is involved in pollen development and pollen tube growth as mentioned previously. *ATCHX19* is down regulated by the drought. However, gene function and regulatory network under drought still unknown.

ATXTH22 **(AT5G57560)**

ATXTH22 is a cell wall-modifying enzyme xyloglucan endotrans-glucosylase/hydrolase (*XTH*) enzyme, which is rapidly upregulated in response to environmental stimulation and has a unique activity to split and reconnect xyloglucan chains without the requirement of Uridine

diphosphate glucose (UDP-glucose). *ATXTH22* exclusively transfers xyloglucans to xyloglucan oligosaccharides and is able to form cellulose-like insoluble material from a soluble cello-oligosaccharide (Shinohara, et al 2017). *AtXTH22* also known as *TCH4* has been identified as one of the touch-inducible genes regulated by auxin and Brassinosteroids by *AtMYB30* that contribute to cell elongation and cell wall modification (Xu et al.,1995). The e-FP browser shows predominant expression of *ATXTH22* in rosette leaves (Appendix I), cotyledon, flower stage 12 carpels and petals. The qRT-PCR data indicates that *ATXTH22* is contributing to the development of pollen by different expression levels in all the 4 stages in control samples. At tetrad stage, gene expression was reduced significantly by two days of drought ($P \leq 0.001$). Which enhanced by day twelve of drought and did not show any changes to the control even after plant recovery, expression at microspore stage shows significant reduction from early drought till the end of the treatment. At microspore stage, expression decreased from day2 until re-watering ($P \leq 0.001$). Gene expression at BCP appeared at very low expression compare to the control samples at the beginning of the drought, the expression enhanced by the drought at day 12 however after 12 days the expression reduced again ($P \leq 0.001$). TCP expression is the highest and sensitive to water-holding continue after plant recovery (Chapter4. Figure.4.6). Different expression pattern of *ATXTH22* in different stages of pollen development indicates its

importance in several biological processes. *ATXTH22* is down-regulated by the drought. A similar study by Seki et al., (2002) indicated the down regulation of several *XTH* gene family in response to drought. *Capsicum XTH* transgenic lines showed increased salt tolerance that enhance root length (Choi et al., 2011). Suggesting that *ATXTH22* may have a role in stress responses.

HRGPs **(AT3G02120)**

The Hydroxyproline-rich glycoprotein family protein (*HRGPs*) family is subdivided into three groups, arabinogalactan-proteins (*AGPs*), extensins (*EXTs*), and proline-rich proteins (*PRPs*) (Showalter, 1993; Cassab, 1998; Nothnagel, 1997; Kieliszewski and Lamport, 1994; Seifert and Roberts, 2007). *HRGPs* have multiple roles in cell wall formation and modifying including the early construction of walls surrounding a vascular element (Stiefel V et al., 1990), AT3G02120 plays a role in plant defence to *Botrytis cinerea* defence in *Arabidopsis* down-regulated by *WRKY33* overexpression and mutant lines (Sham et al., 2017). The e-FP browser shows expression in almost every part of the plant with low levels in mature pollen (Appendix I). The control samples showed high expression at tetrad stage which was enhanced by drought, but this was less evident at the later developmental stages (Chapter4. Figure.4.5). However, after plant recovery the expression decreased, expression at microspore stage reduced by drought at day12 extremely significantly $P \leq 0.001$ and recovered by adding water.

While expression at BCP showed low expression at day2 therefore remining at a constant level in both treatments. Expression at TCP stage was reduced by the drought and could not be recovered as expression decreased extremely significantly $P \leq 0.001$ (Chapter4. Figure.4.5).

HRGPs are believed to have functions in plant growth and development (Showalter, 2001). AT3G02120 expression in tetrad were up-regulated by the drought, suggesting their function in the earlier stage of pollen development.

FLA3 (AT2G24450)

A member of *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 3* precursor (*FLA3*) in Arabidopsis, which is involved in microspore development and response to abiotic stress, *FLA3* is distributed at the plasma membrane with a glycosylphosphatidylinositol anchor, it thought to modulated cellulose deposition during intine formation (Li et al. 2010). *Arabinogalactan* proteins are abundant in reproductive tissues, and may function in gametophytic cell differentiation, pollination and pollen tube growth (Cheung and Wu, 1999; Coimbra et al., 2007), egg cell fertilization, and zygote division (Qin and Zhao, 2006). *FLA3* expression was low level in young floral buds at floral stage 10 however had strong expression at TCP which appeared lowest expression at early drought then the expression was highly increased to 7- fold by drought day 12, after 24

hours of adding water gene expression was severely reduced (Chapter4.Figure.4.4). *FASCICLIN-LIKE ARABINOGALACTAN PROTEINS* (*FLAs*) are a secondary cell wall-related, subclass of *AGPs*, which have glycosylated regions and cell adhesion domains (Gaspar et al., 2001). *FLA3* is up-regulated by the drought at later stage of pollen development (TCP) but significantly down-regulated after re-watering. Suggesting *FLA3* role in secondary thickening to protect cell wall and avoid dehydration, that may happen by activating another gene family network. Since *FLA3* was up-regulated in hypocotyl development in response to salt stress and is involved in fibre development to protect cell wall during the stress (Guerriero et al., 2017).

B-1,3-GLUCANASES (AT3G23770)

B-1,3-Glucanases are a diverse family of hydrolytic enzymes that are classified as endoglucanases or exoglucanases according to the nature of their enzymatic action. Endoglucanases cleave *b-1,3-glucans* into short-chain reducing sugars, whereas exoglucanase hydrolysis releases single glucose units from the reducing ends of the substrate. In Lilies, endo-b-1,3-glucanase was shown to be responsible for callose wall degradation (Stieglitz and Stern.,1973). Most of the endoglucanase activity occurs in the tapetum, immediately surrounding the meiocytes, whereas the majority of exoglucanase activity occurs in the outer somatic layers of the anther (Steiglitz., 1977., Steiglitz and Stern, 1973). However, callose also

surrounds the gap between generative and vegetative cell after mitosis I (Mogami et al., 2002). AT3G23770 has high expression at tetrad stage during the formation of free microspore (Smyth et al. 1990). AT3G23770 show similar expression pattern to *A6* a tetrad callose wall degradation, related gene (Hird et al.,1993). The e-FP browser showed distinct expression at flower stage 9/10 (Appendix I) this is similar to the qRT-PCR data that showed tetrad specific expression which was enhanced by the drought; expression was not recovered by re-watering (Chapter4.Figure 4.4). Callose which is deposited between the primary cell wall and the plasma membrane during meiosis (McCormick, 2004), it is considered as temporary and is degraded to maintain cell wall structure and differentiation (Chen and Kim., 2009). AT3G23770 is up-regulated by the drought implies gene deposition as a defence mechanism.

PRF5 **(AT2G19770)**

AT2G19770 encodes a *profilin 5* protein, an actin monomer-binding protein that regulates actin cytoskeleton organization. *Arabidopsis* contains five genes encoding three vegetative (*PRF1*, *PRF2*, and *PRF3*) and two reproductive (*PRF4* and *PRF5*) profilins. AT2G19770 (*PRF5*) expression is limited to pollen (Christensen et al., 1996; Huang et al., 1996; Ma et al., 2005). All five members are very similar in amino acid sequences and genomic structure. *PRF5* is expressed in mature pollen and growing pollen tubes (Wang et al., 2008). *Profilin* plays a role in cell elongation, cell shape,

and flowering (Ramachandran et al., 2000). *PRF5* is down-regulated in *siago1b* mutant which is affected in pollen tube elongation (Liu X et al,2016). Plant cells respond to different environmental stimulation by regulating the actin cytoskeleton (Staiger, 2000; Pleskot et al., 2013), however actin and interactions with environment changes remains unclear (Henty-Ridilla et al., 2013; Li et al., 2014a., 2015). *PRF5* is highly expressed in stamens and mature pollen e-FP browser (Appendix I). Expression analysis by qRT-PCR illustrated expression at Tetrad stage (I) and a very low expression at pollen microspore stage (II) that did not change by drought. *PRF5* gene is highly expressed at BCP stage (III) of pollen development, which decreases by the drought significantly. A higher expression was also observed in pollen tricellular stage (IV) in the control samples which reduced by the drought very significantly during the drought days and after plant recover $P \leq 0.001$ (Chapter4.Figure.4.7).

Proline has been reported as up-regulated by the drought in maize leaves (Cao et al., 2018); proline accumulation is associated with the plant response to environmental stress (Matysik et al., 2002). *PRF5* was down-regulated at BCP and TCP stage where proline accumulation is important for pollen germination and tube growth, which is consistent with down-regulation of proline during stress (Yang et al., 2011).

PLDALPHA2 (AT1G52570)

PLDALPHA2 is a member of the phospholipase D (*PLD*) family of phospholipases enzyme first identified in plants by Hanahan and Chaikoff in 1947. They contribute to hydrolyses of different membrane phospholipids, producing phosphatidic acid PA, choline and ethanolamine (Munnik and Musgrave, 2001). They are involved in hormone signalling including abscisic acid regulation of stomatal movement (Peters et al., 2010), salicylic acid (Janda et al., 2015), jasmonic acid (Zhao et al., 2013), and auxin (Li and Xue., 2007) ; reactive oxygen generation (Wu et al., 2010); and gibberellic acid as well as programmed cell death, root hair patterning and root growth moreover in response to different environmental stresses such as drought, freezing, wounding, heavy metal toxicity, (Wang et al., 2006, Peng et al, 2010; Lu et al., 2012). Phosphatidic acid (*PA*) is believed to be a target of different genes through different pathways (Fu et al., 2014; Henty-Ridilla et al., 2014). In plants, *PLDs* comprise a large and diverse family of enzymes with over 80 described genes. In *Arabidopsis thaliana*, twelve isoforms of *PLDs* have been identified (Bargmann & Munnik, 2006), which are classified into six major categories (with 2 member), α(with 3 members), γ (with 3 members) δ-,ε- and ζ- with 2 members (Qin and Wang, 2002, Wang, 2005, Hong et al., 2016), based on their protein domain structure and

biochemical properties (Li et al., 2009). All family members contain a C2 (Ca^{2+}/phospholipid-binding) domain near the N-terminus Except PLDζ (Elias et al., 2002, Qin & Wang, 2002). *PLDALPHA2* activity is influenced by various factors such as pH, $Ca^{2+,}$ SDS, PIP2, oleic acid and PA production in Arabidopsis (Wang et al., 2006). *PLDa1* knockout affect stomatal closure (Guo et al., 2012; Jiang et al., 2014). Indicating role of *PLDa1* in balancing plant water through ABA signalling (Mishra et al., 2006). Tang and Liu, (2017) reported that *PLDALPHA2* may act in vesicle trafficking in cotton (*Gossypium hirsutum*) with a role in secondary cell wall thickening. The knockout of *PLDa1* disrupts cortical microtubules (Zhang et al., 2017a) under salt stress conditions (Zhang et al., 2012), and heat shock (Zhang et al., 2017). Microtubules are important for mitosis, cytokinesis, cell elongation, and signal transduction (Wymer and Lloyd, 1996; Hashimoto and Kato, 2006; Jiang et al., 2014). *PLDALPHA1* is expressed during microtubule mitotic progression in root apical meristem and leaf petioles (Novák D et al., 2018). Consistent with this is the finding of Potocký et al. (2014) who reporting PA localization in the plasma membrane of tip growing pollen tubes. *PLDALPHA1* and *PLDALPHA2* are very similar in terms of gene structure and sequence similarity, sharing about 92% amino acid similarity (Qin and Wang., 2002). Expression of *PLD* has been identified in vegetative tissues in highly activate and rapidly growing regions, such as shoot apexes and the

secondary meristem producing axillary buds and vascular tissues of young leaves and stems (Wang et al, 2002). PLD-mediated lipid degradation has been proposed to play a role in membrane degradation in tissue senescence, suppression of *PLDα* in Arabidopsis increased the period during which ABA and ethylene promote senescence (Ryu et al, 1996). *PLD* expression and activity are intimately linked to ripening and senescence, application of hexanal inactivate *PLD* enhances membrane stability and longevity of raspberry fruit (El Kayal et al., 2017). *PLDALPHA2* expression by qRT-PCR during drought that revealed strong expression at microspore stage (II), BCP stage (III) and TCP stage (IV) in the hydrated sample enhanced expression by drought day2 at tetrad and BCP, that decreased significantly in all stages except for pollen tetrad day twelve and again enhanced when applying water. Gene expression at TCP decreased till the end of the treatment compare to the control and enhanced by water, however the expression remains low compare to the control in microspore and TCP (Chapter4.Figure.4.4). Consistent with the absolute expression of *PLDALPHA2* in stamen and mature pollen by the e-FP browser (Appendix I). *PLDALPHA2* is important for all stages of pollen development and is down-regulated by the severe drought. Phospholipid metabolism enzymes are important for ABA signalling pathways. Since *PLDALPHA1* Knockdown enhance drought resistance by controlling transpiration water loss in the whole plant (Sang et al. 2001; Zhang et al.

2004). Therefore, *PLDALPHA2* is a good candidate to study drought tolerance.

EXO70H3 (AT3G09530)

EXO70H3 is one of 23 exocytosis (*EXO70*) Arabidopsis gene family, associated with pollen development and pollen tube growth (Synek et al., 2006; Chong et al., 2010). Exocytosis is the process of expelling substances from cells through the fusion of vesicles with the cell membrane (Boyd et al., 2004). *EXO70* genes are believed to contribute to many exocytosis activities, such as cytokinesis, cell expansion, cell wall thickening, tip growth, and intercellular signalling during cell differentiation (Bloch D et al., 2016). *EXO70* has a role in pollen tube growth (Li et al., 2010; Sekereš et al., 2017). The e-FP browser shows that *EXO70H3* gene is expressed in flowers stage 12 especially stamens and mature pollen (Appendix I). The qRT-PCR data show higher expression of *EXO70H3* at TCP in the control samples that significantly decreased by drought Days and after watering as well as in BCP ($P \leq 0.001$) (Chapter4. Figure.4.6).

Exocysts are involved in the regulation of secondary cell wall deposition in tracheary elements by facilitates moving of lipids and proteins and water to the cell membrane (Lucas et al.,2013). Thus, may explain reduced *EXO70H3* expression by decreased water level in the cell where *EXO70H3* is down-regulated in tricellular pollen. Since role of *EXO70H3* contribute to

pollen tube growth. However, *EXO70H3* interaction during drought still unknown.

ATNACK1 (AT1G18370)

ATNACK1 encodes for a kinesin *HINKEL*, which is required for cytokinesis in pollen. Cytokinesis in plants involves the formation of unique cellular structures such as the phragmoplast as well as the formation of the cell plate to separate the forming daughter cells; the *Arabidopsis* genome contains 61 kinesins. Mutations in *ATNACK1*, results embryos defect (Strompen et al., 2002). *ATNACK1* is required for cellularization of the female gametophyte and post-meiotic cytokinesis in the male gametophyte (Tanaka et al.,2004). *ATNACK1* was predominantly expressed in flower stage 9 from the e-FP browser (Appendix I). Day2 drought in the control samples, a consistent expression of *ATNACK1* gene was seen in all the four stages of pollen development. While drought enhance *ATNACK1* expression at TCP stage (IV) very significantly $P \leq 0.001$. Then reduced at the end of the treatment and after adding water. Expression was reduced in both microspore and BCP stage by the drought and could not be recovered by adding water. However, *ATNACK1* expression in tetrad stage show almost equal expression level in the drought compare to the control (Chapter4. Figure.4.7). ATNACK1 is down-regulated by drought in different stage of pollen development except for the tetrad stage.

SFH5LP (AT4G27580)

AT4G27580 encodes a Phosphatidylinositol transfer SFH5-like protein. *SFH5LP* is one of the genes that is down-regulated in the *ms1* mutant in the anther Flower Net. *SFH5LP* is one of 171 genes strongly up-regulated by salt stress in roots, some of these genes show a moderate induction in drought stresses (Ma et al.,2006). The e-FP browser shows *SFH5LP* expressed in stamens and mature pollen (Appendix I). In the control samples low expression levels of *SFH5LP* occurred in early stages of pollen development. While, the expression in BCP varied up to ~1-fold change that did not affect by the drought. However, the highest expression of *SFH5LP* gene occurred in TCP in the well-watered plant while significantly reduced by drought $P \leq 0.001$. (Chapter4. Figure.4.7). *FH5LP* regulate actin polarization and protein trafficking at the PM (Yakir-Tamang and Gerst., 2009). *FH5LP* is highly expressed in TCP stage and is down-regulated by the drought, this suggests that gene may play a role in polarized cell and pollen tube growth, where cytoskeleton is crucial for pollen tube growth (Staiger et al., 2010).

Trehalose Biosynthesis Related Genes.

***ATTPS7* (AT1G06410)**

ATTPS7 TREHALOSE-PHOSPHATE SYNTHASE 7 Trehalose, a disaccharide limited to organisms adapted to situations of extreme desiccation, protects proteins and membranes together with the precursor trehalose-6-phosphate, and plays an important role in sugar metabolism and plant development (Iordachescu and Imai, 2008). The overexpression of *ATTPS1* gene in Arabidopsis seedlings confers drought tolerance and delayed flowering time (Avonce et al., 2004). Phosphorylation of *TPS7*, was increased following oxidative stress treatment (Chen and Hoehenwarter., 2015). *TPS7* is involved in starch metabolism during pollen development (Hedhly et al., 2016). The e-FP browser implied that *ATTPS7* is expressed in different part of the plant including leaves, seeds, sepals, petals and stamen (Appendix I). Gene expression showed minor changes in expression over the drought stress, it decreased at microspore and TCP stage after three days of withholding water, whilst was enhanced at the BCP stage, while after the twelve days expression decreased in microspore and bicellular stage while enhanced in tetrad and TCP stage as seen in (Chapter4.Figure.4.7). However, after plant recovery the reduction occurred at tetrad, microspore and BCP while increased at TCP significantly compared to wild-type $P \leq 0.001$.

Trehalose expression level altered in response to environmental stresses by accumulating in tissue to prevent cell membrane dehydration (Drennan et al., 1993; Crowe et al, 1984). Pollen grain water content has been shown to vary depending on its phase since water content increased at early stage pollen and decreased in mature pollen (Firon et al., 2012). This suggests changes in *ATTPS7* expression at different stage in response to drought.

TPPF (AT4G12430)

TPPF Trehalose is known to protect membranes, proteins, and whole cells against dehydration stress. Its function as a reserve carbohydrate and is considered as an important stress-protecting molecule (Elbein et al., 2003). Trehalose- related genes are highly expressed in guard cells, *TPPF* enzyme is involved in the conversion of Tre6p into trehalose, which induces stomatal closure (Daloso et al.,2016). The data presented by e-FP browser showed the absolute expression of the gene *TPPF* at mature pollen (Appendix I). *TPPF was* mainly expressed in the TCP stage in the well-watered plants affected by drought consequences day 12 and after plant rehydration data presented by qRT-PCR see (Chapter4.Figure.4.7). Since high levels of trehalose accumulates in lepidophylla plant as a desiccation tolerant conferring drought stress (Pampurova et al., 2014) by preventing the lipid phase transition and fusion of drying vesicles (Crowe., 2007). *TPPF* appears important to pollen viability and the gene is down regulated

in the *male gametogenesis impaired anthers* (mia) mutant which influence pollen grains with tetrads failing to dissociate (Jakobsen et al.,2005). This may explain *TPPF* down regulation by the drought in tricellular pollen.

4.2.3.3 *HKL3* (AT4G37840)

The Hexokinase-like (*HXK*) gene family is highly conserved in plants and plays an important role in plant development in the first step of the glycolytic pathway for sugar accumulation and metabolism (Jang at al.,1997) *HXK* can phosphorylate several hexoses including d-glucose, d-fructose, d-mannose and glucosamine (Schnarrenberger, 1990). The enzyme hexokinase (*HXK*) catalyses ATP dependant hexose to hexose-6-phosphate in the glycolysis process. *Glucose 6-phosphate* (*GLc6p*) acts as a precursor for cell wall biosynthesis and secondary metabolism, it provides the building blocks for starch biosynthesis in the reproductive tissues, as well as energy source for stomata opening (Daloso et al., 2015). Glucose is crucial for the controlling of plant growth, development, metabolism, and stress resistance (Ramon et al., 2008). In rice the *oshxk10* mutant affects anther dehiscence and pollen germination (Xu et al., 2008). *Arabidopsis thaliana* has six *HXK* genes, three encode catalytically active proteins localised to the mitochondria while the other three encode non-catalytic proteins, which have been recently renamed as *hexokinase-like* (*HKL*) proteins (Karve et al., 2008). Each individual isoform has a physiological role within the plant. Expression of *HKL3* in

citrus guard cells is thought regulate sugar-sensing functions during fruit development and maturation stimulating stomatal opening and closing (Lugassi et al., 2015).

The microarray database from e-FP browser has shown absolute expression of gene *HKL3* at flower stage 12 (stamen) and predominantly at mature pollen stage (Appendix I). Gene expression at early pollen development tetrad stage (I) through all the treatment was at low levels and did not show any changes to the drought. However, pollen microspore stage (II) showed low expression level in the control samples and reduced significantly after re-watering compared to the well-watered (Chapter4.Figure.4.7). Gene expression in BCP and TCP were higher that early stages of development in the control samples, which up-regulated in BCP by day two and day twelve (severe drought) by ($P \leq 0.001$ and $P \leq 0.01$) respectively. However, after 24-hours of watering the plant gene expression reduced very significantly $P \leq 0.001$. While the expression at tricellular Pollen stage (IV) showed consistent reduction by the drought in day 2, Day 12 until plant recovery ($P \leq 0.001$), (Chapter4.Figure.4.7).

Starch accumulates and increased gradually in pollen from tetrad to tricellular then completely disappear from the stamen and accumulates in filaments (Hedhly et al., 2016). Starch content was down regulated by drought in potato cultivars (Rudack et al., 2017). Since *HKL3* expression in fruits contributes to starch accumulation, freezing tolerance and seed

longevity (Zhang et al., 2016; Dyson et al., 2014; Lugassi et al., 2015). This suggests the down regulation of *HKL3* in response to drought during pollen development by inducing starch reduction in the cell.

Expression of Transcription Factors

***DAZ3* (AT4G35700)**

DAZ3 is a sperm cell-specific transcription factor identified only in the sperm cell transcriptome, which belongs to the C2H2 zinc finger family (Borg et al., 2011). *DAZ3* have been shown to be a *DUO1*-regulated target (Englbrecht et al., 2004). It is believed that *DUO1* (TF) regulates sperm cell specification (Durbarry et al., 2005). In addition to identifying several pollen specific genes such as *BCP1* and *PCR11*, which down- regulated in the suppression of *Arabidopsis Shaggy-like protein Kinases* (*ASKß*) a putative male fertility related gene (Dong et al.,2015; Borg et al., 2011). *DAZ3* expression is predominantly localized in the sperm cell cytoplasm and the expression pattern persists in the developing pollen tubes, expression is seen late after germ cell division, specifically at tricellular stage of pollen development (Gillmor et al., 2016). The e-FP browser clarified *DAZ3* as a pollen specific gene (Appendix I). Similar to the data obtained from the qRT-PCR, high expression was seen at TCP stage in the well-watered samples. However, the expression decreased significantly through all the drought treatment. Although the expression in the other

three stages of pollen development was very low (Chapter4.Figure.4.6). *DAZ3* (TF) is down regulated by the drought.

ATHSFA2 **(AT2G26150)**

ATHSFA2, is a *HEAT SHOCK TRANSCRIPTION FACTOR A2* its overexpression in Arabidopsis results in the up-regulation of heat shock protein (*HSPs*), leading to enhancement of heat tolerance (Nishizawa A et al., 2006). *ATHSFA2* is expressed in petals and stamen as shown by the e-FP browser tool (Appendix I). Gene expression in tetrad stage show significant reduction during day2, day12 and after re-watering $P \leq 0.01$, $P \leq 0.001$ and $P \leq 0.05$ respectively. Similar to the expression at bicellular pollen where expression reduction happened at day 2, day 12 and after re-watering $P \leq 0.05$, $P \leq 0.001$ and $P \leq 0.01$ respectively. Transcript levels at BCP seems to be enhanced by the drought during the drought treatment. While opposite expression occurred at TCP since the gene enhanced by the drought at day2 then dramatically decreased till the end of the treatment $P \leq 0.001$ (Chapter4.Figure.4.5). *ATHSFA2* suppressed *ATHSPS* expression and improved heat tolerance in Arabidopsis mediated by ABA signalling (Wang et al., 2017) and implies a role for *ATHSFA2* in pollen where its strongly expressed in TCP stage and down-regulated by the drought.

ATHSFA3 (AT5G03720)

A member of Heat Stress Transcription Factor (*HSF*) family. *HSFA3* Expression is regulated by DREB2A during heat stress (Sakuma et al., 2006). *HSFA3* is involved in establishing thermotolerance Stress granules (SGs), a transient dynamic particle for mRNA storage that are formed in the plant cytoplasm under stress conditions, SGs are actively involved in protecting mRNAs from degradation. (Lambermon et al., 2000; Weber et al., 2008). *HSFA3* is expressed in plant tissues by e-FP browser the expression of *HSFA3* is mainly expressed at the tetrad stage (I) and TCP stage (IV) in the normal condition. Gene expression at the tetrad stage was increased by the drought at day 12 at and did not show any significant changes during plant rehydration period. Expression at BCP show significant reduction during day 2 drought, then continue without any changes in the drought compare to the control samples. Although gene expression at TCP significantly decreased by severe drought and after plant recovery (Chapter4.Figure.4.5). Since overexpression of *HSFA3* results in the induction of many heat-inducible genes and increases thermotolerance, while *hsfa3* knockout mutants exhibit reduced thermotolerance (Yoshida et al., 2008). Suggesting drought role in controlling *HSFA3* which is up regulated in tetrad stage and down regulated in TCP stage.

MYB101 (AT2G32460)

ATMYB101 is a member of *MYB* transcription factors identified as *GAMYB-like* genes that have been implicated in GA signalling in anthers and germinating seeds, *GAMYBs* are essential for GA-mediated programmed cell death during seed germination and in tapetum development (Millar and Gubler.,2005), As well as being involved in Arabidopsis flowering time regulation (Blazquez et al. 1998, Blazquez and Weigel.,1999). Overexpression of *ATMYB101* increases plant sensitivity to ABA during germination and miR159 (Reyes and Chua.,2007), causing abnormal leaf phenotypes (An et al., 2014). The e-FP browser showed gene expression in mature pollen (Appendix I). Expression analysis indicated that *MYB101* transcript was highly expressed at both tetrad (I) and TCP stage (IV) in the control samples. However, during the drought treatment significantly reduce expression at tetrad and BCP stage (III), even after plant rehydrated (Chapter4.Figure.4.5). *ATMYB101* is considered as a drought responsive gene (Baldoni et al., 2015) and is highly expressed in mature pollen grains with a role in pollen tube reception (Liang et al., 2013). *ATMYB101* showed higher expression at early and late pollen stages and is down-regulated by the drought.

MYB21 (AT3G27810)

MYB21 and *MYB24* transcription factors regulated by jasmonate JA, which influence flower development, pollen germination, anther dehiscence, filament elongation and male fertility (Mandaokar et al., 2006; Song et al., 2011; Reeves et al., 2012). *JASMONATE ZIM-DOMAIN* (*JAZ*) proteins act as repressors of target transcription factors to modulate JA in response to environmental stress (Chini et al., 2016; Gimenez- Ibanez et al., 2017; Major et al., 2017). *JAZs* proteins interact with *MYB21/24* to regulate stamen development and fertility in response to environmental stress. A proper overexpression of *MYB24* partially restores male fertility of opr3 (Qi et al., 2015, Huang et al.,2017). The e-FP browser showed *ATMYB21* expression at flower stage 12 (Appendix I). qRT-PCR analysis explained specific gene expression at pollen (TCP) bud stage (IV) ranged from ~ 2.5-fold, while other stages remain at a very low level. Expression at TCP stage reduced by the drought $P \leq 0.001$. However, expression at BCP enhanced by the drought after plant rehydration $P \leq 0.001$ (Chapter4.Figure.4.6). *MYB21* is highly expressed at TCP and is down-regulated by drought stress. Since *ATMYB21* reported as a drought-responsive gene plays a role in the maintenance of fertility (Baldoni et al., 2015). And a crosstalk of the role of *MYB21* in the pathway of JA, GA and ABA in drought response (Su et al., 2013).

BZIP (AT1G35490)

BZIP encodes a basic Leucine Zipper Domain (*bZIP*) transcription factor, which has strong expression in the tapetum and microspores at late floral stage 9. However, gene expression is being restricted to microspores at floral stage 10 (Alves-Ferreira et al.,2007). *bZIP* gene family are a well-known ABA-dependent signal transduction factor involved in abiotic stress tolerance as well as gibberellic acid (GA) signalling and sucrose signalling (Dubos, et al.,2010). *BZIP* transcript is highly expressed in mature pollen. The e-FP browser qRT-PCR analysis indicated strong expression in stage (IV) TCP in the control which significantly ($P \leq 0.001$) decreased during the drought treatment and after re-watering the plant (Chapter4.Figure.4.6). Gene expression at BCP was at low levels in the control and the expression remain consistence in the drought with no changes till plant recovery. Since At1g35490 has been reported as a stress responsive gene that is down regulated by *NAC019* under drought stress (Sukiran et al.,2013). Indicated the role of *BZIP* in pollen development that show expression peak at TCP and is down-regulated by the drought.

ATDIV3 (AT2G38090)

ATDIV3 is a paralog of the DIV lineage transcription factors from the MYB gene family DIVIRICATA (DIV) (Gao et al.,2017), which interact antagonistically to regulate floral dorsoventral asymmetry (Almeida et al.,1997). The e-FP browser showed *ATDIV3* expression in petals, flower

stage 15 and seed stage3. qRT-PCR analysis showed expression of the gene in buds at all the 4 stages of pollen development. Expression of the gene at tetrad stage (I), BCP stage (III) and TCP (IV) reduced significantly through all the treatment in drought as compared to the well-watered as seen in (Chapter4.Figure.4.5). *Plantago lanceolate* (*PlDIV*) has been reported to contribute to cell proliferating and undergoing mitosis during anther development since high expression level was seen in tapetal cells which then decreased and increased until the microspores were released, Moreover *PlDIV* expression was downregulated in *ms1* mutant (Reardon et al.,2014). Suggesting gene role in pollen viability and sensitivity to drought were its down-regulated by drought.

ATDIV4 **(AT5G01200)**

ATDIV4 is a paralog of the DIV lineage transcription factors from the MYB gene family DIVIRICATA (*DIV*) (Gao et al.,2017), which interact antagonistically to regulate floral dorsoventral asymmetry (Almeida et al.,1997). The e-FP browser showed *ATDIV4* expression in sepals and seeds stage 3, 4 and 5. Where qRT-PCR analysis showed high expression of the gene allocated in tetrad, microspore and TCP of pollen development which decreased from day two of the treatment until day twelve (Chapter4.Figure.4.5).

BBX19 (AT4G38960)

B-BOX DOMAIN PROTEIN 19 (*BBX19*) is a class of zinc-finger transcription factors containing a B-box domain with one or two B-box motif which believed to play a role in plant stress responses (Wang et al., 2013). *BBX19* is a negative regulator of flowering time under the inductive photoperiod and its overexpression causes delayed flowering (Wang et al.,2014). *BBX19* is a potential target of mediated mRNA decay (*NMD*) that regulate flowering time through the FLC-independent pathway (Nasim et al ,2017). *BBX19* expression from microarray database e-FP browser showed strong levels of the transcript in tissues. qRT-PCR showed expression in all four stages of pollen development, where expression reduced significantly at day 2 at tetrad stage while increased in BCP and TCP stage, at day 12 very significant increases expression occurred at tetrad and a very significant reduction at microspore pollen, BCP and TCP. After plant watering significant reduction occurred at all the stages. During tetrad stage (I) expression of the gene appeared reduced by day 2 drought while enhanced at day 12 drought, therefore after adding water decreased again. Expression in microspore, BCP and TCP stage reduced by severe drought as compared to the well-watered samples as seen in (Chapter4.Figure.4.5). BBX19 may contribute to pollen viability particularly in tetrad as this is up-regulated by drought while the rest of pollen stages were down-regulated.

DUFF8 (AT3G28980)

AT3G28980 is a member of *DUF1216* family identified as *DUFF8* (Nestorova., 2016). DUF1216 family proteins are part of the cellular trafficking machinery (Grobei et al., 2009). Ten of the *DUF1216* family members have been found as mRNA in earlier stages of pollen development and mature pollen (Honys and Twell, 2004). The mutant lines of these genes displayed pollen degeneration and reduced male fertility. The exact molecular role of this novel group of proteins is unclear. The qRT- PCR indicate strong expression at both microspore and TCP stage in hydrated tissues. Microspore gene expression was dramatically reduced by day two drought and enhanced by day twelve. Then the expression was low by drought effect. However, severe drought significantly reduced the expression at TCP which show higher expression during early days (Chapter4.Figure.4.4). The e-FP browser Show absolute gene expression at mature pollen.

DUFF8 may has a role in the early proliferative stages and later differentiation phase in pollen. Suggest expression peak in microspore and TCP stage, which showed sensitivity to drought by enhancing the expression in microspore while reduced it at TCP stage.

Expression of Hormone-Related Genes

SAUR47 (AT3G20220)

SAUR47 belongs to a large family of small auxin-up regulated RNAs (*SAUR*), that are rapidly induced by auxin and encode highly unstable mRNAs. *SAUR* genes are involved in various developmental and physiological processes (McClure and Guilfoyle, 1987). The *Arabidopsis* genome contains 79 SAUR genes (Ren and Gray, 2015), however, the functions of these proteins are largely unknown. Different factors have been reported recently as targeting *SAUR*, rather than auxin, including different hormones, abscisic acid (ABA), ethylene, gibberellic acid (GA) and brassinosteroids (BR) (Kodaira et al., 2011; Walcher and Nemhauser, 2012; Stamm and Kumar, 2013; Oh et al., 2014; Li et al., 2015; David, et al 2017), light response factors and transcription factors including *MADS* domain transcription factors *SEPALLATA3* (*SEP3*), *APETALA1* (*AP1*), *TCP* (TEOSINTE *BRANCHED1/CYCLOIDEA / PROLIFERATING CELL FACTOR1*) family protein *TCP20* (Kaufmann et al., 2009; Kaufmann et al., 2010b; Danisman et al., 2012) and TF *FRUITFULL* (*FUL*) (Bemer et al ., 2017), BR response factor *BRASSINAZOLE RESISTANT 1* (*BZR1*) (Oh et al., 2014) . Two-third of the *SAURs* can respond to auxin downstream of the Auxin Response Factors *ARF5*, *ARF6*, *ARF7*, *ARF8* or *ARF19* (Goda, et al,2004; Paponov et al.,2008; Bargmann et al., 2013). Arabidopsis *SAURs* show general capacity to promote cell elongation (Chae et al., 2012; Spartz

et al., 2012; Stamm and Kumar, 2013; Ren and Gray, 2015; Sun et al., 2016). They interact with protein phosphatases of the PP2C-D family to inhibit their function, preventing dephosphorylation of plasma membrane H^+-ATPases, resulting in membrane acidification, which enables cell elongation (Spartz et al., 2014; Sun et al., 2016). GA and BR are well known for their role in plant dwarfism, reduced seed germination, delayed flowering and reduced male fertility (Clouse et al., 1996; Steber et al.,2001; Jaillais et al 2010; Ye et al., 2010, Oh et al., 2011). *SAUR* is regulated by auxin and BR (Goda et al. 2004). The e-FP browser showed mature pollen high expression (Appendix I). The data from qRT-PCR show specific high expression at tricellular pollen (TCP) stage at day 2 in both control and drought samples, which reduced by drought day 12 and after plant rehydration expression increased again (Chapter4.Figure.4.4). *SAUR47* has been studied by T-DNA insertion mutant lines and overexpression lines in Chapter 5. *SAUR47* is down-regulated by drought at TCP stage which implies gene role in drought response.

LEA (AT4G36600)

LEA (*late embryogenesis abundant*) is one of a large group of hydrophilic proteins with major role in drought and other abiotic stresses tolerance in plants. The physiological and biochemical functions of *LEA* genes in desiccation are largely unknown (Wise et al, 2003). *LEA* proteins have several possible functions, including roles as antioxidants, membrane and

protein stabilisers during water stress and "space fillers" to prevent cellular collapse at low water activities (Tunnacliffe and Wise, 2007). Maize overexpressing Rab28 *LEA* exhibited sustained growth under osmotic stress (Amara et al., 2013). ABA also induces the expression of genes encoding *LEA* proteins during seed development and abiotic stress (Zhao et al., 2011). The microarray database e-FP browser showed expression of the gene at mature pollen (Appendix I). qRT-PCR showed gradually expression of the gene in the four stages, low levels at tetrad stage and microspore stage which increased by drought day 12 drought and enhanced significantly. Expression in BCP was also increased by drought day twelve significantly and recovered by water. The highest expression was seen in TCP in the control samples which reduced through all the drought treatment (Chapter4.Figure.4.4). LEA expression is up-regulated by drought in microspore and BCP stage, However expression in down-regulated in TCP.

Pollen Specific Related Genes

***ALP* (AT3G26110)**

Arabidopsis AGP1-LIKE PROTEIN (ALP) is an anther specific gene shows 47% homology at the amino acid level to At1g24520.1 *HOMOLOG OF BRASSICA CAMPESTRIS POLLEN PROTEIN 1*(*BCP1*). is highly expressed in tapetum and microspores cells (Wu et al., 2016). Arabinogalactan proteins

(*AGPs*) are cell wall proteoglycans important for pollen development and pollen tube germination, there are 47 genes encoding for *AGP* polypeptide in *Arabidopsis* (Pereira et al.,2014). *AGPs* are believed to play a role as calcium reservoirs to deliver calcium for pollen tube growth (Lamport and Várnai., 2012); calcium is involved in pollen tube cell wall growth as well as fertilization (Schiøtt M., et al.,2004). The e-FP browser indicated that *ALP* was not highly expressed in any tissues other than those of mature pollen (Appendix I). In the control sample *ALP* is expressed at very low level in pollen tetrad and microspore but was expressed higher in later stages of the development, at tricellular pollen expression was significantly reduced from the beginning of the drought until the end of the treatment $P \leq 0.001$(Chapter4.Figure.4.6). In conclusion *ALP* is expressed at TCP stage and is down regulated by the drought treatment.

BCP1 (AT1G24520)

BCP1, HOMOLOG OF BRASSICA CAMPESTRIS POLLEN PROTEIN 1 is an anther-specific gene expressed in both tapetum and microspores, which is essential for pollen development, the Arabidopsis *bcp1* mutant affects anther and pollen abortion, resulting in pollen grains lacking cytoplasmic contents with flattened exine shells, (Xu et al., 1995). *BCP1* is downregulated by the *TDF1* transcription factor and regulated by *ASKβ* a pollen regulatory gene (Dong et al., 2015). *BCP1* has been reported to be involved in two pathways; firstly, in 60S biogenesis by binding with

chaperones in the nucleus to block interactions between Ribosomal protein L23 (Rpl23) allow the chaperones to bind with Rpl23 instead, the Loss of Bcp1 function causes instability of Rpl23 and 60S subunits. Secondly it functions in the synthesis of phosphatidylinositol (PI) by exporting the Mss4 (PI4P 5-kinase) gene in the plasma membrane (Ting et al.,2017; Audhya and Emr.,2003; Homma et al., 1998). The level of cross talk between the two pathways is currently unknown. Mss4 regulates different biological function such as sporulation, endocytosis, membrane trafficking, normal organization of the actin cytoskeleton and cell viability (Audhya and Emr.,2002). The e-FP browser showed that *BCP1* was not highly expressed in any tissues other than mature pollen. In the watered tissues the gene *BCP1* was strongly expressed in TCP stage (IV) and was significantly reduces by drought from day 2 until the end of the drought $P \leq 0.001$, higher expression of the gene was seen in BCP stage (III) which did not change compare to drought (Chapter4.Figure.4.6). *BCP1* is late development pollen gene and is down-regulated by the drought.

PCR11 **(AT1G68610)**

PCR11 belongs to the PLANT CADMIUM RESISTANCE family of genes, they are membrane proteins involved in the efflux of heavy metals in Arabidopsis (Song et al., 2004., 2010). *PCR11* is sperm cell specific gene and is down-regulated by different transcriptional factors; **A)** male germline-specific transcription POLLEN1 (*DUO1*), that's plays a critical role

in regulating sperm cell production and cell fertilization (Borg M et al., 2011), **B)** Arabinogalactan proteins (*AGPs*) double mutant in *agp6 agp11* reduced pollen tube growth and aborted pollen grains (Costa M. et al.,2013). **C)** Tapetal development and function 1 (*TDF1*) throught *ASKß* (At1g68610) (Dong et al., 2015). Since the over-expression of *ASKß* affects *DAF1*, *PCR11* in tetrad stage, causes abnormal microgametogenesis (Borg et al., 2011). *PCR11* is a pollen specific gene as reported in the e-FP browser, in the watered samples our data showed high expression of *PCR11* in pollen stage (IV) TCB that reduced by the severe drought extremely significant $P \leq 0.001$ compared to the control (Chapter4.Figure.4.6). However, very low expression of the gene was observed in the tetrad, microspore and BCP stages. *PCR11* was reported as a pollen sperm specific gene with important to pollen viability. Suggesting high expression in the TCP as seen from the expression analysis. *PCR11* is down-regulated by drought stress.

Cu/ZnSOD (AT2G28190)

CSD2 a conserved protein which exist in the chloroplasts (Kliebenstein et al., 1998); *Cu/Zn SOD* that is down-regulated under mild Zn deficiency (Ibarra-Laclette et al., 2013). It is involved in detoxification of superoxide radicals and is important for photosynthesis (Randall and Bouma, 1973; Li et al., 2013). *Arabidopsis CSD2* is; **A)** upregulated by down regulation of miR398 in response to heat stress (Guan et al., 2013). **B)** activated by

AP2/EREBP transcription factor through the interaction with RCD1 (RADICAL-INDUCED CELL DEATH 1), in young leaves and protects mesophyll cells from early cell death (Heiber et al., 2017). **C)** Overexpression of AP2/EREBP regulates TRANSLUCENT GREEN (TG) for drought tolerance through aquaporin genes (Zhu et al., 2014). During Cu deficiency, *CDS2* is downregulated by JA by the activation of SPL7 which affects plant fertility, however the role of Cu in anther and pollen development still unknown (Jiapei Yan et al., 2017). **D)** it also reduced ROS accumulation regulated by *AST1* in response to drought (Xu et al.,2017). The e-FP browser showed *CSD2* was predominantly expressed in vegetative rosette and leaves (Appendix I). *CSD2* is expressed in buds at the pollen microspore, BCP, TCP stages with higher expression occurring at tetrad stage in the normal watered condition. Day 2 drought present higher expression in microspore stage (II) compared to the well-watered samples and an opposite lower expression in tetrad, BCP and TCP. Expression at tetrad stage appeared higher during day 12, while expression at microspore and TCP did not change statistically, although expression reduced at BCP. After plant recovery *CDS2* reduced significantly in all stages (Chapter4.Figure.4.5). *CSD2* is important for pollen viability and is down-regulated by the drought.

NEP1LP (AT1G63060)

Ribosome biogenesis takes place in the nucleolus. The nucleolus is a specialized sub-compartment in the nucleus of eukaryotic cells. Ribosome biogenesis is a complex process that begins with the transcription of ribosomal RNA (rRNA) and continues to process ribosomal proteins are assembled (Henras et al., 2008). The nucleolar protein *NEP1LP* (also named *EMG1P*) has been shown to be an essential factor for 18S rRNA maturation which is a highly conserved protein required for ribosome biogenesis and found in organisms from archaea to humans (Liu & Thiele, 2001; Eschrich et al., 2002). The qRT-PCR expression at microspore, BCP and TCP stage in the well-watered condition, *NEP1LP* expression in the microspore stage reduced at the early days of drought, enhanced by day 12 drought and reduced again significantly after re-watering the plant compare to control plant. *NEP1LP* expression in BCP stage enhanced by the drought significantly which then decreased after adding water. While *NEP1LP* expression in TCP stage remained in a constant level on both samples at early drought, which enhance at the end of the treatment and decreased after adding water gene significantly reduced $P \leq 0.05$ (Chapter4.Figure.4.7). The e-FP browser showed high expression in stamen and mature pollen.

APPENDIX II
CHEMICALS

10 X TBE BUFFER	Concentrations
NaCl	0.89 M
Tris-HCl (PH 7.5)	10mM
Na2 –EDTA	1mM
Dilute to 0.5 x to use in electrophoresis	

Murashige and Skoog Basal (MS) medium	Concentrations
MS powder	2.15 g
Agar	9 g
ddH2O	Add up to 1L
Total volume	1L
Adjust pH to 5.2-5.7 and autoclave to sterilize; antibiotics added	
when sterilized medium cools to 50tC.	
Luria-Bertani	

Fluorescein diacetate (FDA)staining

BK buffer S15 MOPS	**concentrations**
Ca(NO3)2·4H2O (MW 236)	30 mg/L (0.127 mM)
MgSO4·7H2O (MW 246.5)	20 mg/L (0.081 mM)
KNO3 (MW 101)	10 mg/L (0.1 mM)
Sucrose	15%
MOPS (MW209)	10 mM (pH 7.5)
Stored at -20 °C in an Eppendorf tube	

BK buffer S15	**(50 ml)**
100 mM MOPS (pH 7.5)	5 ml
Sucrose	7.5 g
Ca(NO3)2 (1 M)	6.35 µl
MgSO4 (1 M)	4.05 µl
KNO3 (1 M)	5 µl
Luria-Bertani (LB) medium	**Concentrations**
Bacto-Typtone	20g
Bacto-yeast extract	10g
NaCl 10g	15g
Agar (if solid medium)	
dH2O Add up to	1L
Adjust PH to 7.0 and autoclave to sterilize: antibiotic added	
when sterilized medium cools to≈50˚C	

Alexander Staining	**Concentrations**
Ethanol 1%	20ml
Malachite green in ethanol	95%
dH2O	50ml
Glycerol	40ml
Acid fuchsin 1% in dH2 O	10ml
Phenol	5g
Lactic acid	1-6ml
Pollen stained can be used immediately or stored for later	
use. Staining is hastened by lightly flaming the slides or by	
storing at 55±2 C for 24 hr.	

GENE EXPRESSION AND GENE PLOTS

Expression of each of the candidate genes from microarrays in *A thaliana*. Data adopted from the Arabidopsis eFP browser (Winter *et al*., 2007).

AGI	Expression highest	Floral stage9	Floral stage 12	Mature pollen	Highest expression tissue
AT3G17630	1251.93	12.65	1251.93	365.7	Flower Stage 12, Stamens
AT4G37840	136.83	0.58	136.83	75.45	Flower Stage 12, Stamens
AT2G19770	4991.93	2.96	2574.54	4991.93	Mature pollen
AT3G26110	14106.9	3.2	2308.4	14106.9	Mature pollen
AT1G24520	14492.63	12.03	2609.36	14492.63	Mature pollen
AT4G27580	11457.33	4.51	1042.93	11457.33	Mature pollen
AT3G09530	8594.5	1.76	804.15	8594.5	Mature pollen
AT1G68610	5634.85	6.71	778.41	5634.85	Mature pollen
AT1G18370	325.16	206.43	7.86	21.63	Shoot Apex, Inflorescence
AT5G57560		28.45	57.18	35.9	Rosette Leaf 4
AT1G06410	593.66	288.38	307.2	176.69	Seeds Stage 8 w/o Siliques
AT4G12430		90.7	431.96	1461.81	Mature Pollen
AT4G35700	3636.41	2.54	674.26	3636.41	Mature pollen
AT2G26150	946.56	0.51	320.05	2.78	Flower Stage 12, Petals
AT5G03720	43.01	9.33	5.9	8.38	Leaf 7, Distal Half
AT2G32460	3013.1	25.05	356.23	3013.1	Mature pollen
AT3G27810	1062.13	0.54	1271.1	8.75	Flower Stage 15
AT1G35490	9276.53	4.11	1447.45	9276.53	Mature pollen
AT2G38090	269.08	34.56	65.25	5.09	Stem, 2nd Internode
AT4G38960	140.91	68.05	29.11	59.48	Flower Stage 15, Stamen
AT5G01200	270.88	56.4	22.5	32.44	Seeds Stage 4 w/ Siliques

AT1G52570		24.11	1438.08	1187.1	Flower Stage 12, Stamens
AT4G36600	2260.85	2.96	398.48	179.51	Dry seed
AT1G63060	1285.86	0.41	1285.86	1008.8	Flower Stage 12, Stamens
AT3G28980	7666.68	2.86	3897.28	7666.68	Mature pollen
AT3G23770	795.11	795.11	5.03	9.65	Flower Stage 9
AT2G24450	11395.8	3.16	1363.66	11395.8	Mature pollen
AT3G20220	10340.66	2.95	1642.31	10340.66	Mature pollen
AT2G28190	3855.11	1351.45	528.26	110.66	Rosette Leaf 8
AT3G02120	238.85	123.08	9.71	12.88	Shoot Apex, Vegetative
AT3G20230	1323	1203	1218	1284	Flower

APPENDIX III
PRIMERS

RT-PCR gene expression primers

AGI	Forward primer	Reverse primer
AT3G17630	AGAGAAGCCGTGACACTTGG	TTCATCCCGTATGCGAGAGC
AT4G37840	GAGCGGCGGTTGAAACATAC	TTCCGCGTCAAGAGAAGCAT
AT2G19770	ATGATGGTAGCGTTTGGGCT	GCTCGCCTTGGATGACCATA
AT3G26110	ATGATGAAGTGGCAGTCGCC	AAGAAGAGCGATCCAGCAACT
AT1G24520	ATCATGTCGAGGCTCCAGTC	GGATGTAACACCAACGACGA
AT4G27580	GAGTCCAAGAATGCCGCGA	TGGTGGCTCAGTCTCAGAAGA
AT3G09530	CGTTCAGCTGGTGACTCCAT	CGAACCACTGTCTTGGACGA
AT1G68610	TGACTTGTTGGTGCCCTTGT	AGTCGTGCATGTCGTTCCAT
AT1G18370	GTGGACTTAGCAGGAAGCGA	AGGCGTTACGGCAACAGAAT
AT5G57560	AGCTTGTCCCTGGAAACTCC	CACCTCTCGTTGCCCAATCA
AT2G28190	CGTCGAAAGCGTTGACAGTT	TCGTGAACCACAAAGGCTCT
AT1G06410	TGGGTCGGATTCAATCGGTG	AGCTTCAACGTTCCATGGGT
AT1G35910	CATCAGCATTCCCCGGAAGA	AGGCAAAAACTCATTCGCCG
AT4G12430	ATCCTTCGGCGATTAGCTCG	CTCGACCGCTCTTCCTTTGT
AT1G74930	TTTCGACGCCGCTCAATTTT	ATCCACCGTCGATGTAGCAA
AT4G35700	GCAAGGCCCTCCTATGTGTA	ACCAACGGAAATAGCAGTGG
AT2G26150	TAACGAAACAGGGCCACCAC	ACCGTATTGCCCAACCTCAA
AT5G03720	TACGTCGACACTGACATGGG	ACCCTTGGCTTTGGCTAGTG
AT2G32460	CTCCGGTGTGGCAAAAGTTG	ACTGAACGAATGGGTCGGTC
AT3G06490	ATGGAGAAGGTCGCTGGAAC	GGGTTTGGAGCGCTGTAGTA
AT3G27810	ACGTACGACGGGGAAACATC	TGCTTGCAGCTTGATCGTTG
AT5G40350	GTGGCTGAACTACCTCCGAC	GAACCCTCTTTGGACCGTAA
AT1G35490	CGTCTCCTAATCTCGTACGTCTTC	GCAAATTGGGCATCGGGTTC
AT2G38090	AGCAAGCGGTAACAATGGGT	GGAGAGTCGGGGATGTTGAC
AT3G10470	AAGCCGCTATGGGTCTTCAC	CCTGAATTGGACCCTCCGAC
AT4G38960	ATTTTGTGCGATGCTTGCGA	AAGCTCGGTTACACCAGCAA
AT5G01200	GCTTTGTGACGACTCGAACG	TCCACTGCGGAAACGCATTA
AT3G02120	AGGTACAGGAGCCCAACTGA	CCGATTCCGGTTTGCGTAGT

Primers used for qRT-PCR

Gene ID	Fw primers (5' to 3')	Rv primers (5' to 3')
AT2G24450	CAAGACCGAGCTCACACCAA	AATCCCCTTCATCAGCAGGA
AT1G52570	ACCTGAACCGGACACTGACT	CAAGACAACCAGCTCGAGGT
AT1G63060	TGCACCGGCAATCAGTACAA	GAATGGGTTAGGTCGAGTGGG
AT3G20220	TCAAGACACCATTCCGCTTG	CCCAAACTCGTCCGCTACTT
AT3G28980	ACGATGATTGCTCCCCGAAA	CGACTCACTCTGAGCTGCAA
AT3G23770	AAGATGACTGCTAACCCGCC	TCCGCAAGCAAAGTTAAGCG
AT4G36600	AAGACCGGTCAAGCCAAGTA	GCCTTCTCAGATGCTGATCC

Primers used for RT-PCR Gateway cloning primers

AGI code	Forward sequence	Reverse sequence
AT3G20220	ATGATTTCGCTAAGTTTCATCG	TTATTTCTTCTTGTCACAAGACAT
AT3G23770	TCACAATGTAACGCTAGGAAACTTG	ATGACTCCTTTTGCTCTGTTCC
AT1G52570	ATCAGATTTGACACCGAGGATAC	ATGGAAGAGTGTTTGTTACATGGA
AT4G36600	TCAGCGCTACGGTCTCTCAT	ATGGATGGGGAACGGAGACA
AT1G63060	AGATTCCCTTTGGAGCCAC	ATGGCTTTCAGAATCACCAGC
AT3G28980	TTACTTTGCTTGCATCTTGC	ATGGCAAGAGTTCAACTATTG
AT2G24450	TTAGAACCCAACAAAGCTAGCC	ATGGGTCTCAAGGTCTCCTC

Primer	Sequence (5'-3')	Tm°C
M13F	TGTAAAACGACGGCCAG	55
M13R	CAGGAAACAGCTATGAC	

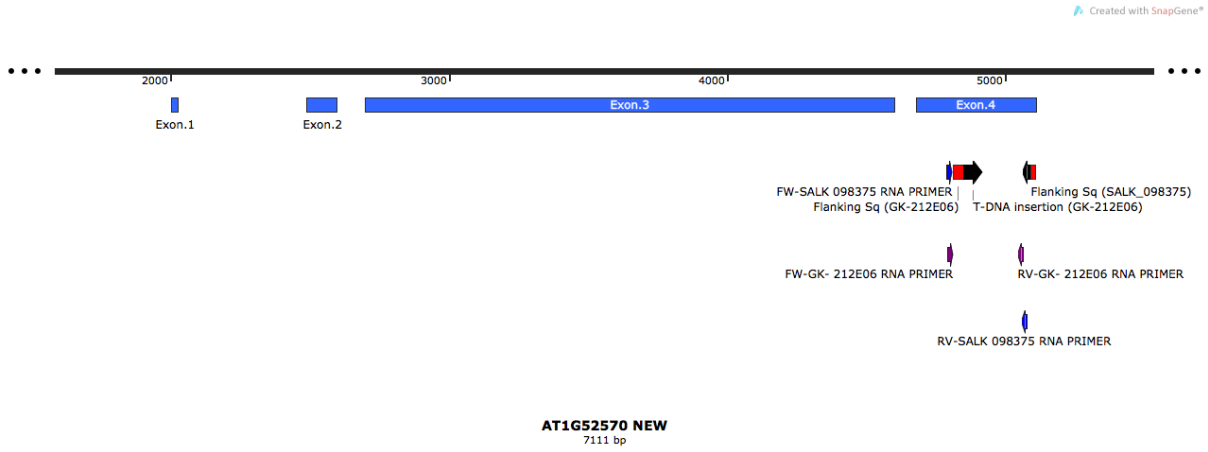
Description of T-DNA insertion lines and their gene-specific primers used for genotyping and RT-PCR analysis

AGI Code	SALK name	NASC ID	(5' to 3') Forward	(5' to 3') Reverse
AT1G52570	GK-212E06	N420310	F-GATTTCCTGACTCCCCTGAAG	R-AGCCGAATGAAAAACCAAAAC
	SALK_098375C	N673018	F-ATACCTCGTGGAACCAAATCC	R-TCGATGAAACATTCCTCGATC
	SALK_053957	N553957	F-GTACCCATCTTACCGGAAACG	R-TCGTATCCTCGGTGTCAAATC
AT4G36600	SALK_131759C	N673498	F-TCTTGTGGGGTGAAAGTGATC	R-ATGCTAAAGACATTGCATCGG
	SALK_046270	N546270	F-ATGACACAAAGGACAAAACCG	R-ATCAGAAGCCTTCTCCTTTGC
AT3G23770	Salk_033100	N859965	F- ACATGTTGCATTGCTTCTTCC	R-AAAACCATTAAAGCAGGCCAC
	SAIL_885_D07	N839833	F-TCCTCAGACCATGTGGTTAGG	R-CAGGAGGAGAATGCTATGCAC
	SALK_138003	N638003	F-TGTTGGGGAAACAAAGAATTG	R-AGGAAAACTCGAACGAAGAGC
	SAIL_883_D01	N839780	F- CTACTTCAATGGTTTGGCTCG	R-TCCTCAGACCATGTGGTTAGG
AT2G24450	SAIL_1233_G05	N878717	F-CCTAGTTGGGCTTCGAAAATC	R-GGGTCTACCAGAGATGGAACC
	SALK_016582	N516582	F-AACAAACGTCAGACCATCACC	R-AGTCTTTCGTTTCCTCCTTGG
AT3G20220	GK-171H05	N416409	F-CGTACCAACTGTACGGTACGG	R-TCGGTAAGCTCATAGCTGAGC
	GK-171H05	N416409	F-CTCGAAGATCACGAGGTGAAG	R-TGAACTTTTCTCAAGACACCATTC
	SAIL_84_D03	N803973	F-GACTCGAATCTGACTCGTTCG	R-GTATTCTTTCTCCCCATTGGC

RT-PCR gene knockout test primers

Gene ID	Primers FW	Primers RV	Target gene	Expected size bp
SALK 033100	GTCAAGCTCTATGACGCCGA	AACATCACCGGAACAGCGA	AT3G23770	395
SAIL_883_D01	GACGGTTACTTGGCATGCAA	AGATCTTGCACAACAATGG	AT3G23770	425
SALK-138003	GACGGTTACTTGGCATGCAA	AGATCTTGCACAACAATGG	AT3G23770	251
SAIL_885-D07	TTTGTTCCTCATCCCTTCTCA	TGAATCAAAAACTTCATATT	AT3G23770	139
SAIL_84_D03	CCATTCCGCTTGATTTTTTT	GCCAATGGGGAGAAAGAA	AT3G20220	400
GK-171H05	AGAAACAAGAACAACTAGG	CAAGCGGAATGGTGTCTTG	AT3G20220	187
GK- 212E06	AGACAACCAGCTCGAGGTCA	ACGAGCTTTTGTGTCGGGA	AT1G52570	273
SALK 098375	CAAGACAACCAGCTCGAGGT	TTGACACCGAGGATACGAG	AT1G52570	289
SALK_016582	CAGCGAGTCGAGGATAAATT	CGGGTCTCCGGTTAAGGTT	AT2G24450	354
SAIL 1233G05	TGCAAACTCAGGCTTTGAGG	TTGGCAAGAAGCTCGGTCA		167
SALK-046270	CAAAGCCGGATCTGCTTACG	TATGACAAGGCAGCTCACG	AT4G36600	330
SALK_131759	CACAAAAGAAGCTTATGAGA	AACTCCACCATTCTTACATA	AT4G36600	226

FIGURES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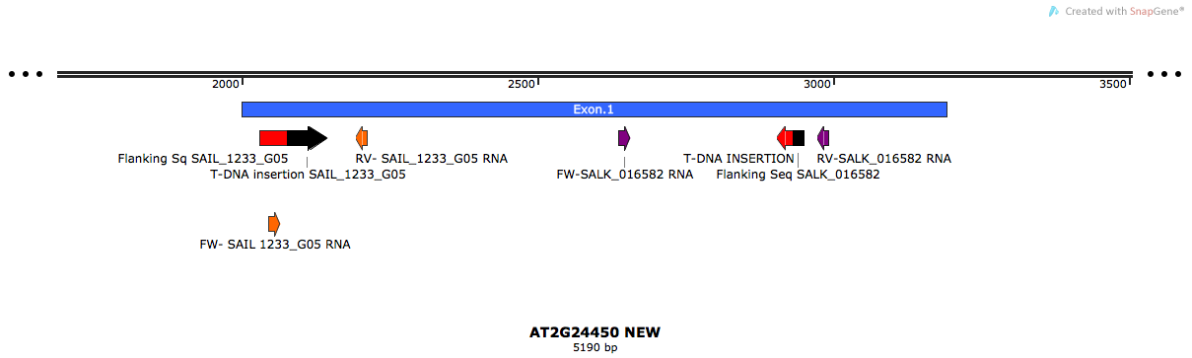
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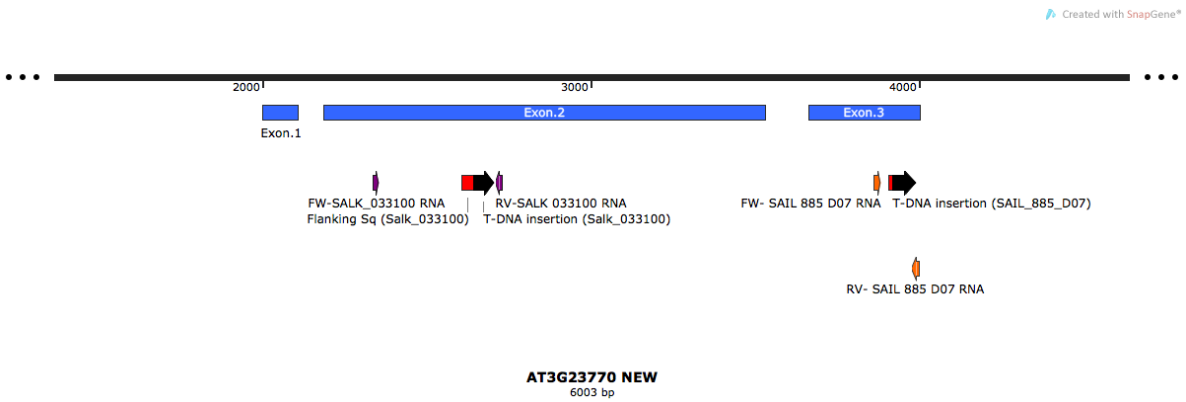

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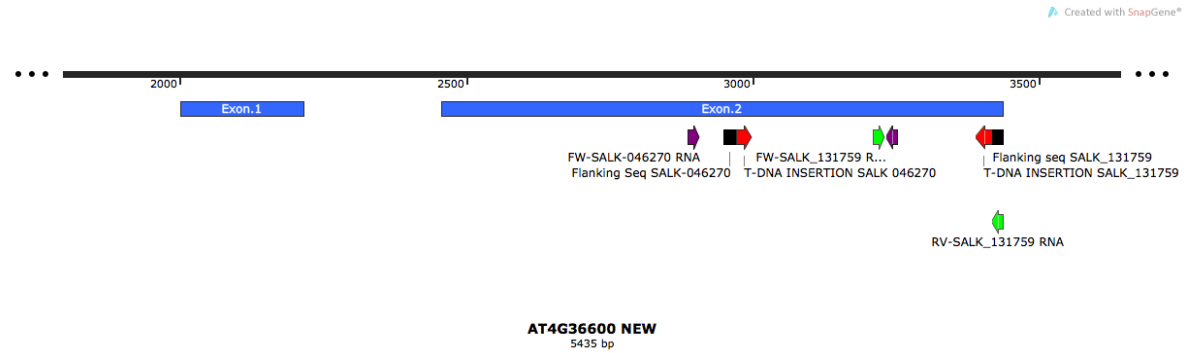

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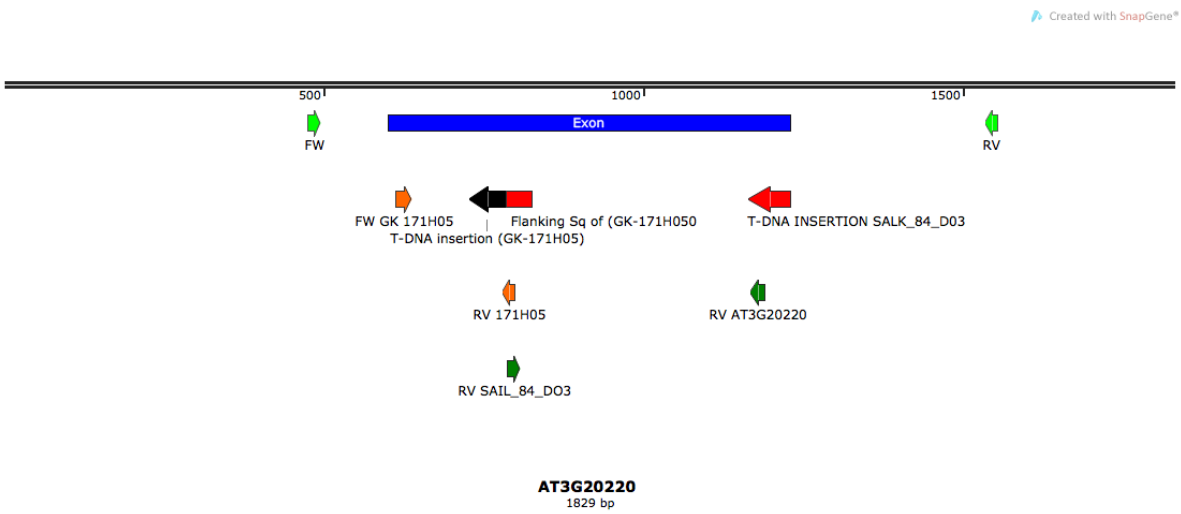
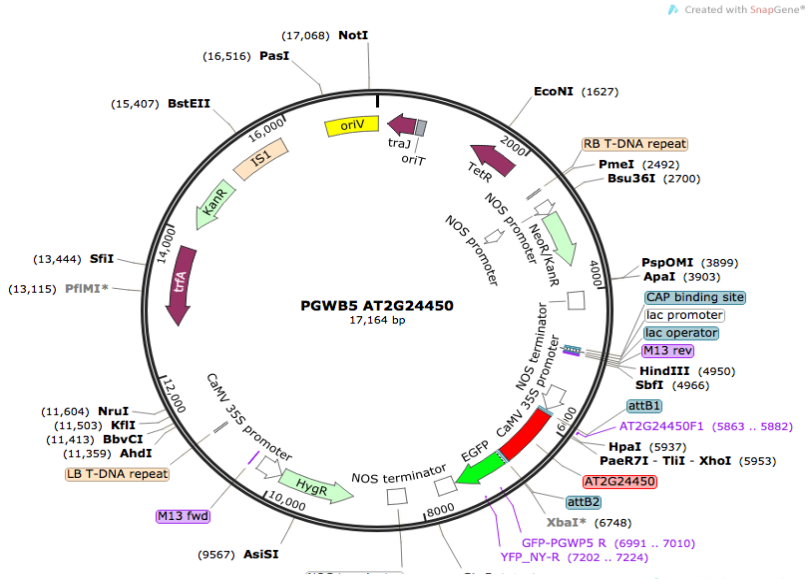


APPENDIX IIV
VECTORS

pGWB5(Nakagawa et al. 2007)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PspOMI (3899)
ApaI (3903)
CAP binding site
lac promoter
lac operator
M13 rev
HindIII (4950)
SbfI (4966)
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HpaI (5937)
PaeR7I - TliI - XhoI (5953)
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attB2
XbaI* (6748)
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YFP_NY-R (7202 .. 7224)
(13,444) SfiI
(13,115) PflMI*
(11,604) NruI
(11,503) KflI
(11,413) BbvCI
(11,359) AhdI
LB T-DNA repeat
M13 fwd
(9567) AsiSI
oriV
traJ
oriT
TetR
NOS promoter
NeoR/KanR
NOS terminator
CaMV 35S promoter
EGFP
NOS terminator
HygR
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IS1
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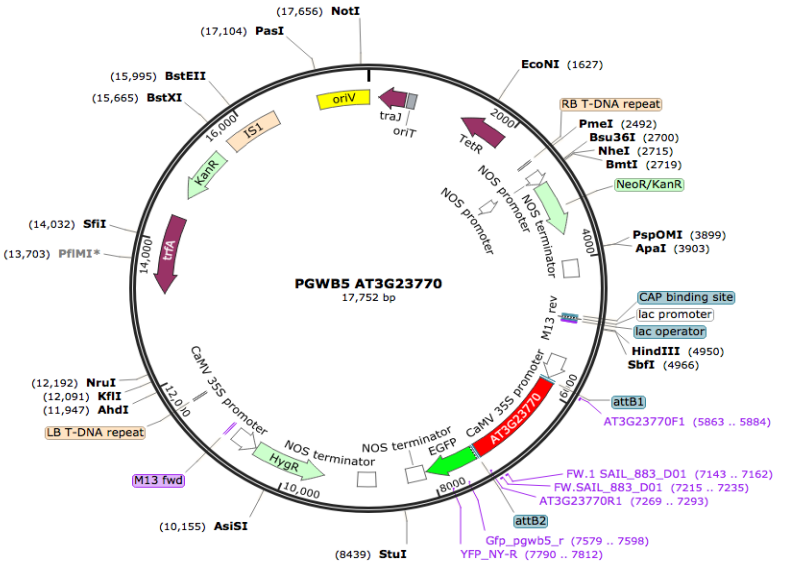
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BmtI (2719)
NeoR/KanR
PspOMI (3899)
ApaI (3903)
CAP binding site
lac promoter
lac operator
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traJ
oriT
TetR
NOS promoter
NOS terminator
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CaMV 35S promoter
EGFP
NOS terminator
HygR
KanR
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trfA
PGWB5 AT3G23770
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6000
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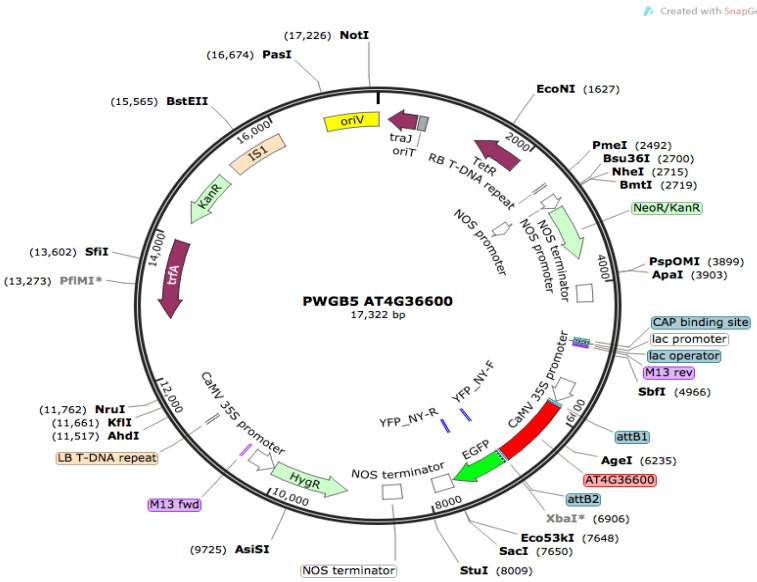
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Bsu36I (2700)
NheI (2715)
BmtI (2719)
NeoR/KanR
IS1
KanR
NOS promoter
NOS terminator
PspOMI (3899)
ApaI (3903)
(13,602) SfiI
(13,273) PflMI*
trfA
PWGB5 AT4G36600
17,322 bp
CAP binding site
lac promoter
lac operator
M13 rev
SbfI (4966)
CaMV 35S promoter
YFP_NY-F
YFP_NY-R
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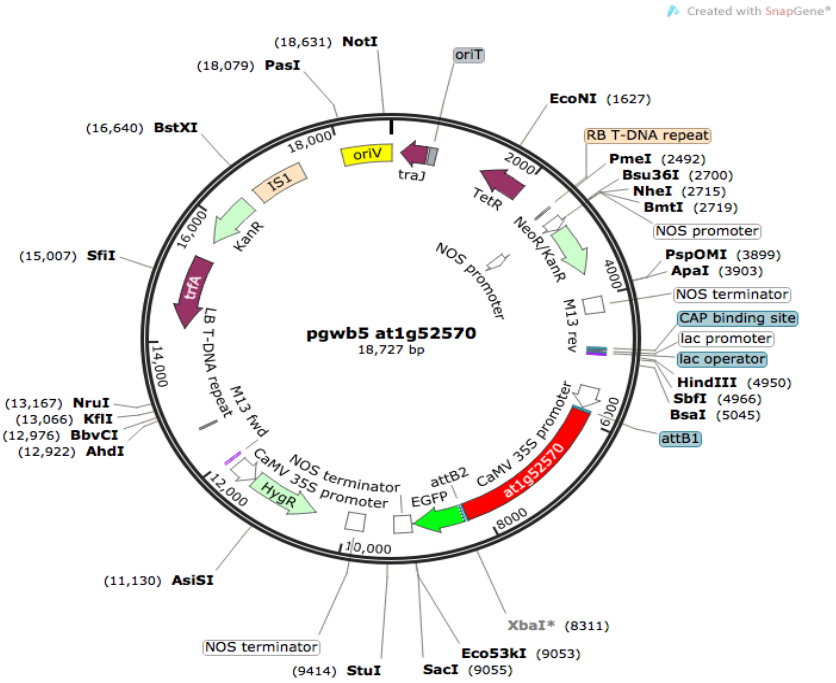
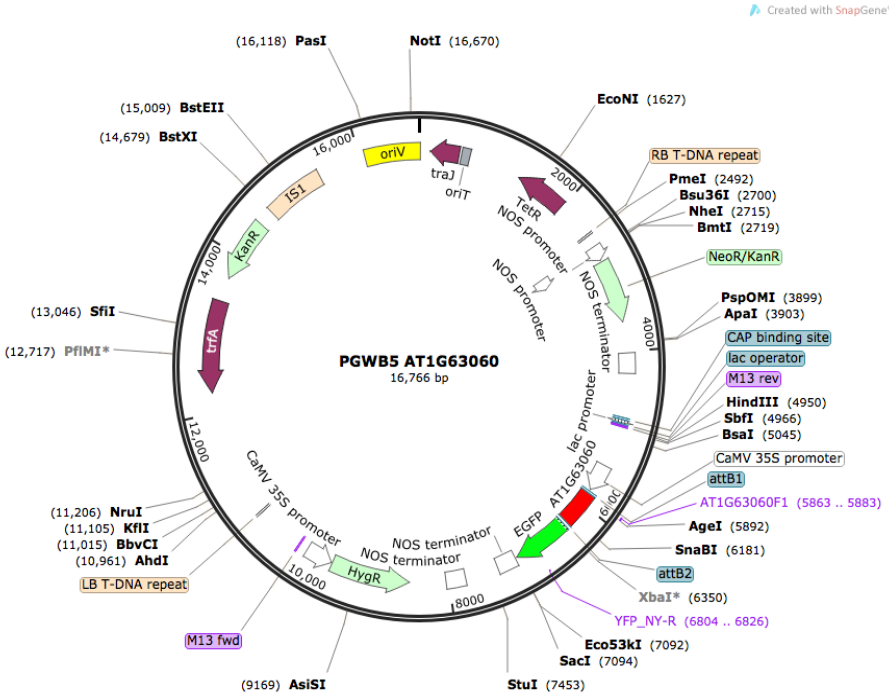
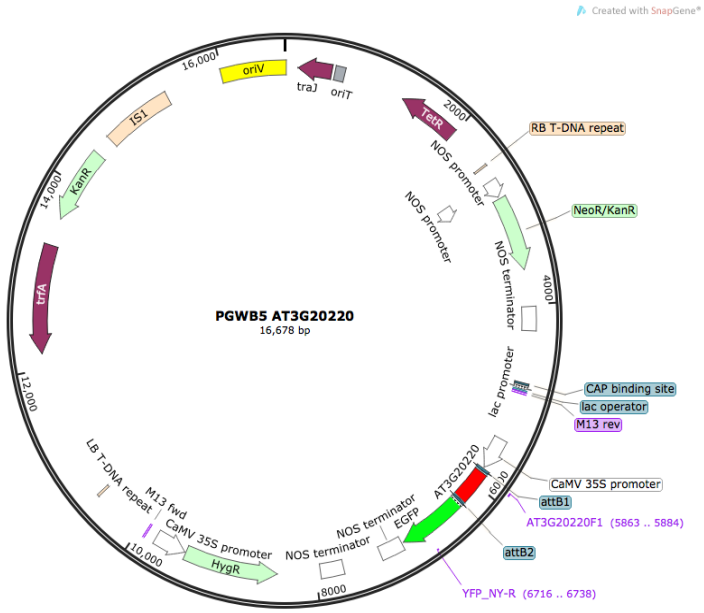
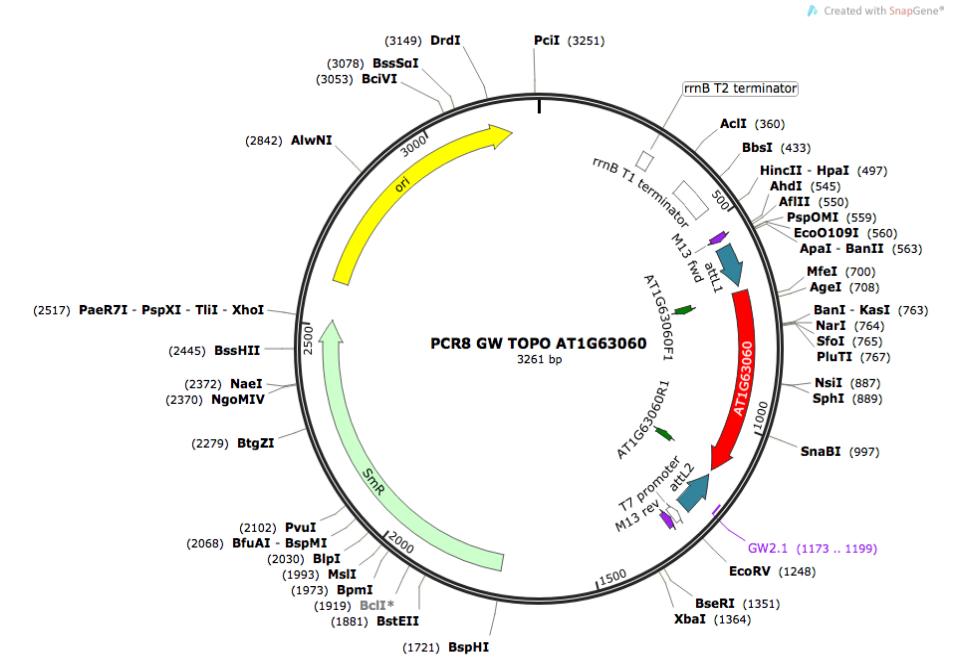
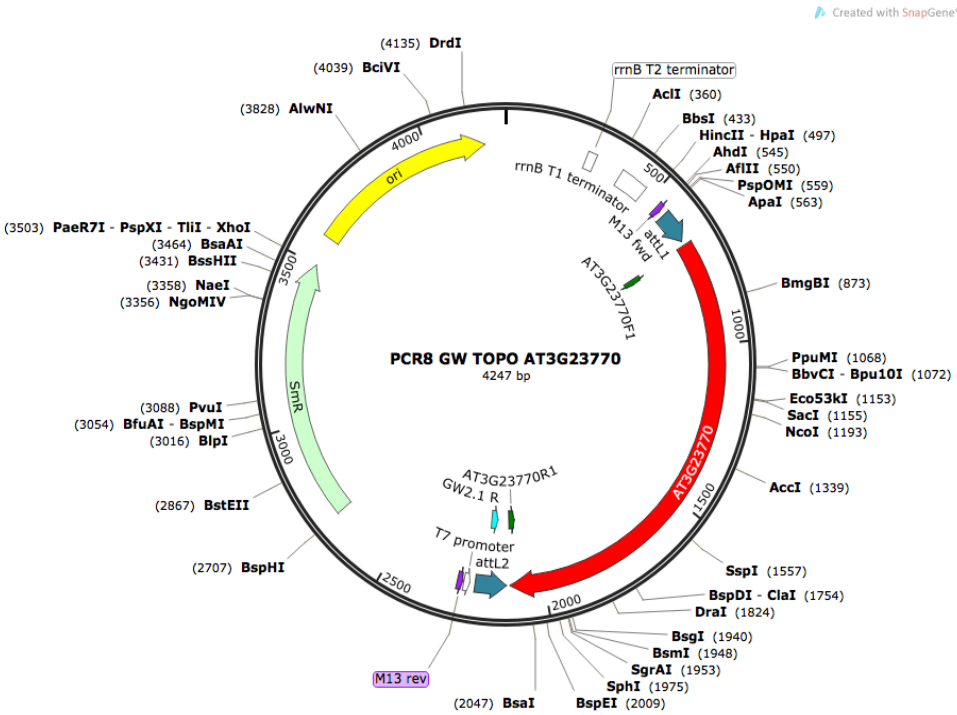
AT4G36600
attB2
XbaI* (6906)
Eco53kI (7648)
SacI (7650)
StuI (8009)
EGFP
NOS terminator
HygR
(11,762) NruI
(11,661) KflI
(11,517) AhdI
LB T-DNA repeat
M13 fwd
(9725) AsiSI
NOS terminator


Created with SnapGene®
(18,631) NotI
(18,079) PasI
oriT
EcoNI (1627)
(16,640) BstXI
RB T-DNA repeat
oriV
traJ
TetR
PmeI (2492)
Bsu36I (2700)
NheI (2715)
BmtI (2719)
IS1
KanR
NeoR/KanR
NOS promoter
NOS terminator
PspOMI (3899)
ApaI (3903)
(15,007) SfiI
trfA
LB T-DNA repeat
M13 rev
CAP binding site
lac promoter
lac operator
pgwb5 at1g52570
18,727 bp
HindIII (4950)
SbfI (4966)
BsaI (5045)
attB1
(13,167) NruI
(13,066) KflI
(12,976) BbvCI
(12,922) AhdI
M13 fwd
CaMV 35S promoter
at1g52570
attB2
EGFP
HygR
(11,130) AsiSI
XbaI* (8311)
NOS terminator
Eco53kI (9053)
(9414) StuI
SacI (9055)

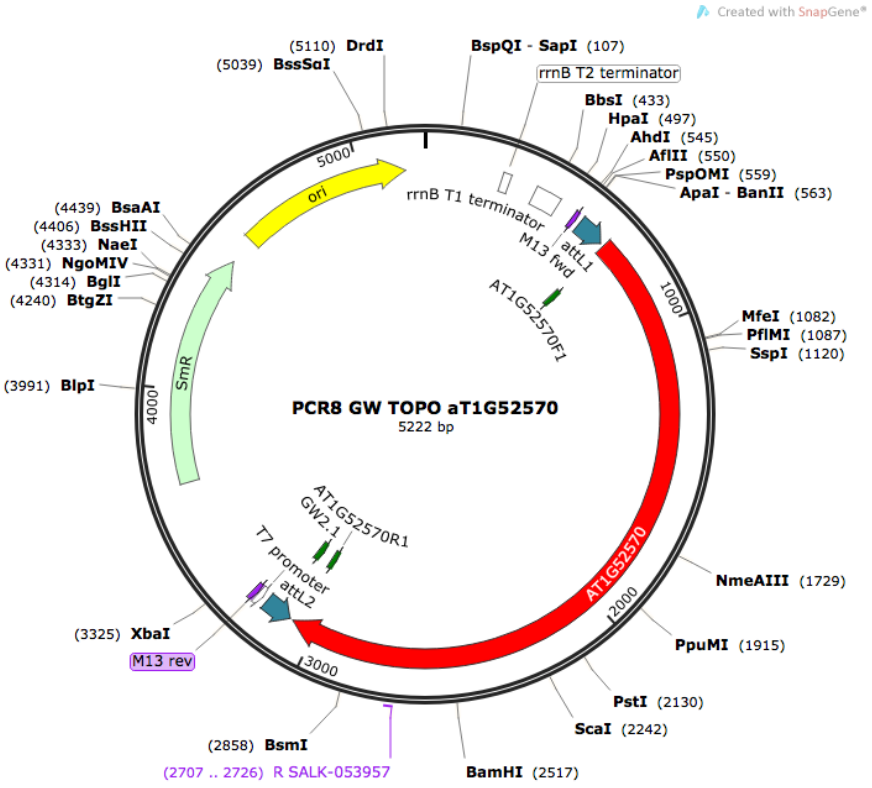

Created with SnapGene®
(16,118) PasI
NotI (16,670)
EcoNI (1627)
(15,009) BstEII
(14,679) BstXI
RB T-DNA repeat
PmeI (2492)
Bsu36I (2700)
NheI (2715)
BmtI (2719)
NeoR/KanR
PspOMI (3899)
ApaI (3903)
CAP binding site
lac operator
M13 rev
HindIII (4950)
SbfI (4966)
BsaI (5045)
CaMV 35S promoter
attB1
AT1G63060F1 (5863 .. 5883)
AgeI (5892)
SnaBI (6181)
attB2
XbaI* (6350)
YFP_NY-R (6804 .. 6826)
Eco53kI (7092)
SacI (7094)
StuI (7453)
(9169) AsiSI
M13 fwd
LB T-DNA repeat
(10,961) AhdI
(11,015) BbvCI
(11,105) KflI
(11,206) NruI
(13,046) SfiI
(12,717) PflMI*
oriV
traJ
oriT
TetR
NOS promoter
NOS promoter
NOS terminator
lac promoter
AT1G63060
EGFP
NOS terminator
NOS terminator
HygR
CaMV 35S promoter
trfA
KanR
IS1
16,000
2000
4000
6000
8000
10,000
12,000
14,000
PGWB5 AT1G63060
16,766 bp

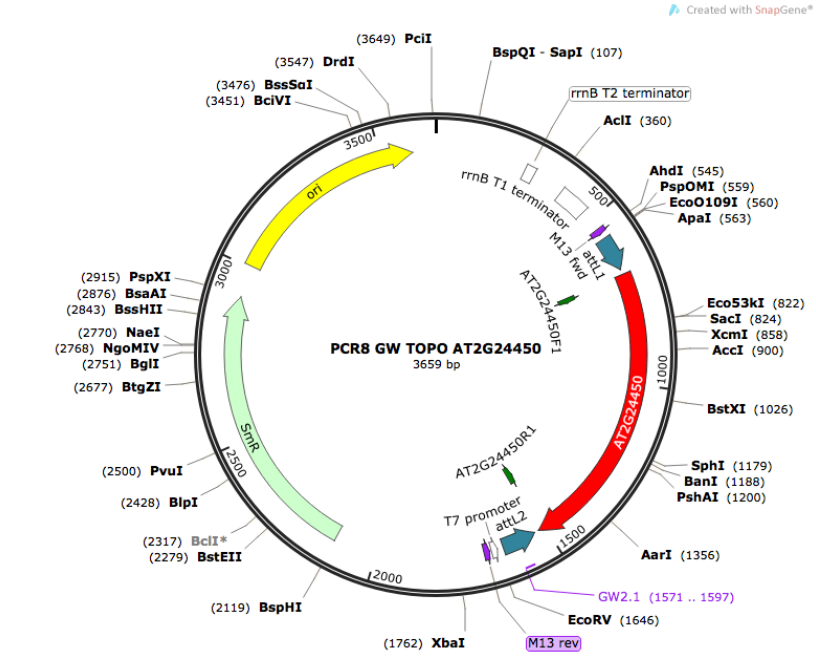

Created with SnapGene®
RB T-DNA repeat
NeoR/KanR
CAP binding site
lac operator
M13 rev
CaMV 35S promoter
attB1
AT3G20220F1 (5863 .. 5884)
attB2
YFP_NY-R (6716 .. 6738)
oriV
traJ
oriT
TetR
NOS promoter
NOS promoter
NOS terminator
lac promoter
AT3G20220
EGFP
NOS terminator
NOS terminator
HygR
CaMV 35S promoter
M13 fwd
LB T-DNA repeat
trfA
KanR
IS1
16,000
2000
4000
6000
8000
10,000
12,000
14,000
PGWB5 AT3G20220
16,678 bp

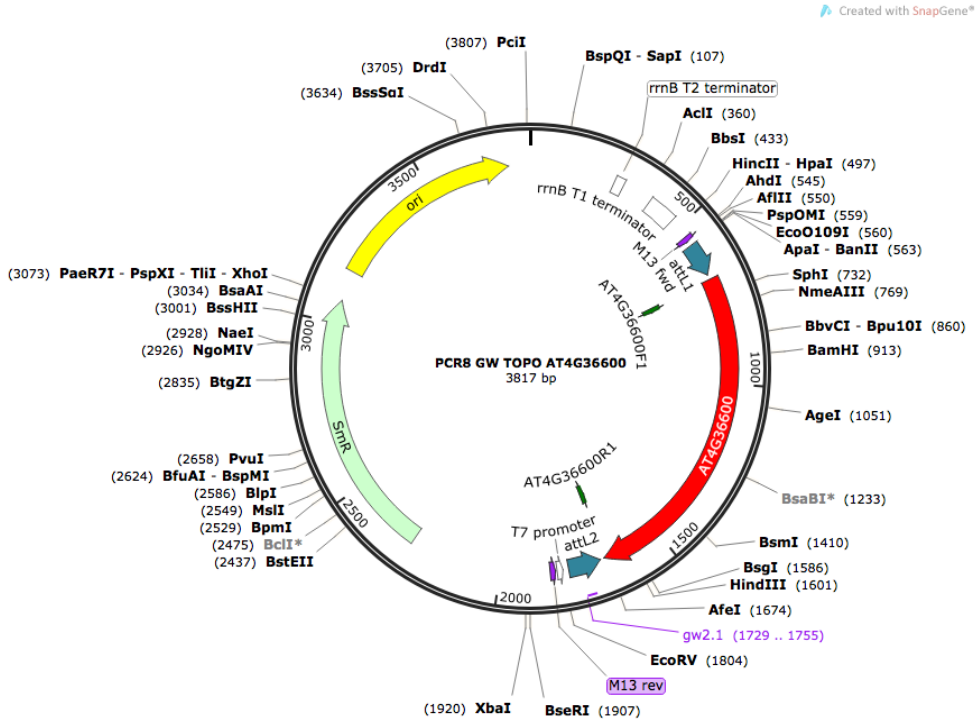

Created with SnapGene®
PCR8 GW TOPO AT1G63060
3261 bp
(3149) DrdI
PciI (3251)
(3078) BssSaI
(3053) BciVI
rrnB T2 terminator
(2842) AlwNI
AclI (360)
BbsI (433)
HincII - HpaI (497)
AhdI (545)
AflII (550)
PspOMI (559)
EcoO109I (560)
ApaI - BanII (563)
MfeI (700)
AgeI (708)
(2517) PaeR7I - PspXI - TliI - XhoI
BanI - KasI (763)
NarI (764)
SfoI (765)
PluTI (767)
(2445) BssHII
NsiI (887)
SphI (889)
(2372) NaeI
(2370) NgoMIV
SnaBI (997)
(2279) BtgZI
(2102) PvuI
(2068) BfuAI - BspMI
(2030) BlpI
(1993) MslI
(1973) BpmI
(1919) BclI*
(1881) BstEII
(1721) BspHI
EcoRV (1248)
BseRI (1351)
XbaI (1364)
rrnB T1 terminator
M13 fwd
attL1
AT1G63060F1
AT1G63060R1
AT1G63060
T7 promoter
M13 rev
attL2
GW2.1 (1173 .. 1199)
ori
SmR
3000
500
1000
1500
2000
2500

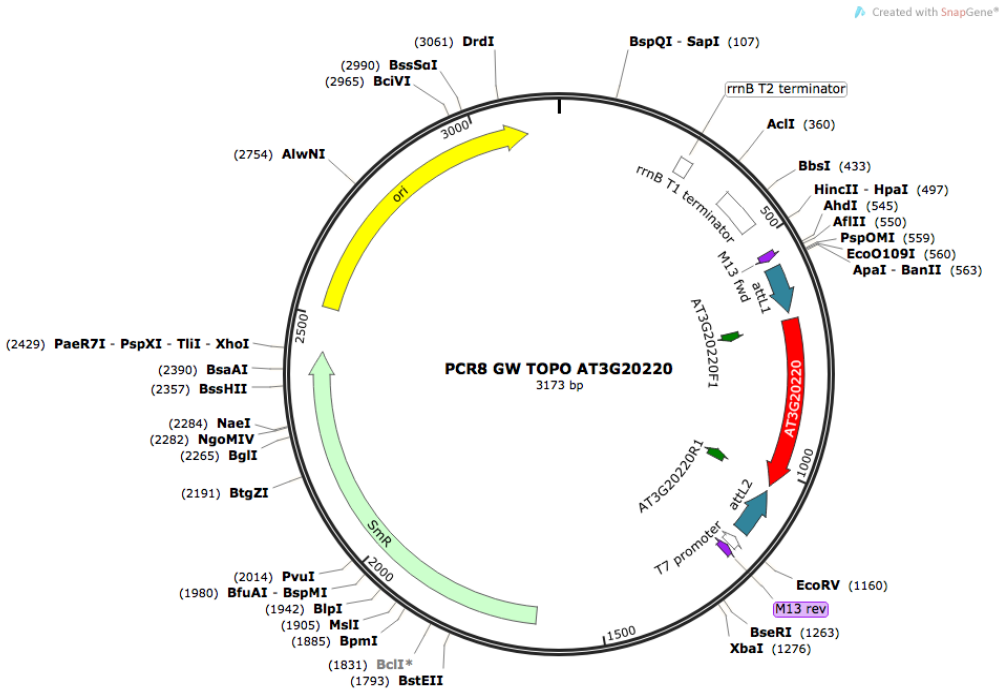

Created with SnapGene®
PCR8 GW TOPO AT3G23770
4247 bp
(4135) DrdI
(4039) BciVI
rrnB T2 terminator
(3828) AlwNI
AclI (360)
BbsI (433)
HincII - HpaI (497)
AhdI (545)
AflII (550)
PspOMI (559)
ApaI (563)
rrnB T1 terminator
M13 fwd
attL1
AT3G23770F1
BmgBI (873)
(3503) PaeR7I - PspXI - TliI - XhoI
(3464) BsaAI
(3431) BssHII
(3358) NaeI
(3356) NgoMIV
PpuMI (1068)
BbvCI - Bpu10I (1072)
Eco53kI (1153)
SacI (1155)
NcoI (1193)
(3088) PvuI
(3054) BfuAI - BspMI
(3016) BlpI
AccI (1339)
AT3G23770R1
GW2.1 R
(2867) BstEII
T7 promoter
attL2
SspI (1557)
(2707) BspHI
BspDI - ClaI (1754)
DraI (1824)
BsgI (1940)
BsmI (1948)
SgrAI (1953)
SphI (1975)
M13 rev
(2047) BsaI
BspEI (2009)
ori
SmR
3500
4000
500
1000
1500
2000
2500
3000

pCR™8/GW/TOPO Vector (Invitrogen™)




Created with SnapGene®
PCR8 GW TOPO AT2G24450
3659 bp
(3649) PciI
BspQI - SapI (107)
(3547) DrdI
(3476) BssSαI
(3451) BciVI
rrnB T2 terminator
AclI (360)
AhdI (545)
PspOMI (559)
EcoO109I (560)
ApaI (563)
rrnB T1 terminator
M13 fwd
attL1
AT2G24450F1
AT2G24450
Eco53kI (822)
SacI (824)
XcmI (858)
AccI (900)
BstXI (1026)
(2915) PspXI
(2876) BsaAI
(2843) BssHII
(2770) NaeI
(2768) NgoMIV
(2751) BglI
(2677) BtgZI
ori
SmR
AT2G24450R1
T7 promoter
attL2
SphI (1179)
BanI (1188)
PshAI (1200)
(2500) PvuI
(2428) BlpI
(2317) BclI*
(2279) BstEII
(2119) BspHI
(1762) XbaI
AarI (1356)
GW2.1 (1571 .. 1597)
EcoRV (1646)
M13 rev
Created with SnapGene®
PCR8 GW TOPO AT3G28980
4154 bp
(4144) PciI
BspQI - SapI (107)
(4042) DrdI
(3971) BssSαI
(3946) BciVI
(3735) AlwNI
rrnB T2 terminator
AclI (360)
HincII - HpaI (497)
AhdI (545)
PspOMI (559)
EcoO109I (560)
ApaI - BanII (563)
rrnB T1 terminator
M13 fwd
attL1
AT3G28980F1
AT3G28980
EcoNI (859)
StuI (892)
MscI (950)
(3410) PspXI
(3371) BsaAI
(3338) BssHII
(3265) NaeI
(3263) NgoMIV
(3172) BtgZI
ori
SmR
AT3G28980R1
T7 promoter
attL2
(2995) PvuI
(2961) BfuAI - BspMI
(2923) BlpI
(2886) MslI
(2866) BpmI
(2812) BclI*
(2774) BstEII
BsmBI (1522)
ZraI (1532)
AatII (1534)
BanI (1696)
HindIII (1760)
(2257) XbaI
M13 rev
(2141) EcoRV
gw2.1 (2066 .. 2092)


Created with SnapGene®
(3807) PciI
BspQI - SapI (107)
(3705) DrdI
(3634) BssSαI
rrnB T2 terminator
AclI (360)
BbsI (433)
HincII - HpaI (497)
AhdI (545)
AflII (550)
PspOMI (559)
EcoO109I (560)
ApaI - BanII (563)
SphI (732)
NmeAIII (769)
BbvCI - Bpu10I (860)
BamHI (913)
AgeI (1051)
BsaBI* (1233)
BsmI (1410)
BsgI (1586)
HindIII (1601)
AfeI (1674)
gw2.1 (1729 .. 1755)
EcoRV (1804)
M13 rev
BseRI (1907)
(1920) XbaI
(2437) BstEII
(2475) BclI*
(2529) BpmI
(2549) MslI
(2586) BlpI
(2624) BfuAI - BspMI
(2658) PvuI
(3034) BsaAI
(3001) BssHII
(3073) PaeR7I - PspXI - TliI - XhoI
(2928) NaeI
(2926) NgoMIV
(2835) BtgZI
rrnB T1 terminator
M13 fwd
attL1
AT4G36600F1
AT4G36600
AT4G36600R1
T7 promoter
attL2
ori
SmR
500
1000
1500
2000
2500
3000
3500
PCR8 GW TOPO AT4G36600
3817 bp


Created with SnapGene®
(3061) DrdI
BspQI - SapI (107)
(2990) BssSαI
(2965) BciVI
rrnB T2 terminator
AclI (360)
BbsI (433)
HincII - HpaI (497)
AhdI (545)
AflII (550)
PspOMI (559)
EcoO109I (560)
ApaI - BanII (563)
EcoRV (1160)
M13 rev
BseRI (1263)
XbaI (1276)
(1793) BstEII
(1831) BclI*
(1885) BpmI
(1905) MslI
(1942) BlpI
(1980) BfuAI - BspMI
(2014) PvuI
(2390) BsaAI
(2357) BssHII
(2429) PaeR7I - PspXI - TliI - XhoI
(2284) NaeI
(2282) NgoMIV
(2265) BglI
(2191) BtgZI
(2754) AlwNI
rrnB T1 terminator
M13 fwd
attL1
AT3G20220F1
AT3G20220
AT3G20220R1
attL2
T7 promoter
ori
SmR
500
1000
1500
2000
2500
3000
PCR8 GW TOPO AT3G20220
3173 bp