HORMONAL REGULATION OF CROP ARCHITECTURE IN RESPONSE TO STRONG SOIL

DAVID PAUL ALEX LLOYD, BSc (Hons)

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Abstract

The architecture of crop plants is determined by adaptation to environmental conditions within genetic constraints and has considerable influence on the ability of a crop to produce high yields. The abiotic stress of strong soil reduces growth of roots and shoots, limiting the yields of wheat and rice plants. This project investigates the hormonal signals mediating architectural responses to strong soil, aiming to improve the understanding of this plant-environment interaction.

Strigolactones are plant hormones known to inhibit shoot branching (tillering in grasses) in a range of plant species. This project demonstrates that strigolactones are, at least in part, responsible for mediating the reduction in tillering in rice plants in response to strong soil. This is demonstrated by a diminished tillering response to strong soil in rice mutants defective in biosynthesis or perception of strigolactones. Additionally, roots of rice plants grown in strong soil contained higher levels of endogenous strigolactone than roots of rice plants grown in weak soil. The identification of wheat orthologues of strigolactone biosynthesis genes is conducted and transgenic lines with reduced expression of these genes are produced, enabling the dissection of the role of strigolactones in wheat in response to strong soil to be explored.

Gibberellin is known to be responsible for promoting leaf elongation in plants. This project demonstrates that gibberellin is responsible for the reductions in leaf elongation in wheat in response to strong soil. Increased levels of bioactive gibberellin are shown to be required for the response to occur and exogenous application of bioactive gibberellin is shown to recover leaf elongation in strong soil conditions.

Finally, this project implicates a number of other hormones as being involved in responding to strong soil in wheat through global hormone profiling.

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Table of Contents

List of Figures and Tables

List of Abbreviations

ABA	Abscisic acid
ANOVA	Analysis of Variance
bp	Base pair
cDNA	Complimentary DNA
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
gDNA	Genomic DNA
$GA_{(x)}$	Gibberellin (species x)
GA2ox	GA 2-oxidase
GFP	Green fluorescent protein
GID1	GIBBERELLIN INSENSITIVE DWARF 1
GC-MS	Combined gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
IAA	3-Indole acetic acid
iP	Isopentenyladenine
JA	Jasmonic acid
JA-Ile	Jasmonate isoleucine conjugate
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LSD	Least Significant difference
ml	Millilitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
PCR	Polymerase chain reaction
PIN	PIN-FORMED
pg	Picogram
qRT-PCR	Quantitative real time PCR
RNA	Ribonucleic acid
RNAi	RNA interference
SA	Salicylic acid
SE	Standard Error

SL	Strigolactone
tZ	Trans-zeatin
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar

Chapter 1: Soil Strength and Plant Hormones

1.1 Food security

According to the World Food Summit of 1996, food security will have been achieved “when all people at all times have access to sufficient, safe, nutritious food to maintain a healthy and active life” (WHO, 2015). Over the coming decades, the global food system will face immense pressures. The global population is set to increase from 7.3 billion today to 9.7 billion in 2050 and will demand more food (UN, 2015). Much of the increases in both people and demand for food will be in emerging economies which are likely to be wealthier and demand more varied and higher-quality diets (Foresight, 2011). At the same time, increased competition for land, water and energy will require great increases in food production without a concomitant increase in the amount of land used or the quantities of inputs into the system. These challenges need to be met with the spectre of climate change ever-present. There are vast discrepancies between food supply and demand around the world and many types of food production are considered unsustainable (Foresight, 2011). The food availability crises and associated global commodity price spikes in 2008 highlighted many weaknesses in the current food production system (Gregory and George, 2011). The latest Food and Agriculture Organization of the United Nations (FAO) figures showed that the number of people undernourished in the world reduced to 795 million people (11% of the global population) compared to over 1 billion (19% of the global population) in 1990-1992 (FAO, 2015b). Continued decline of global food insecurity will require sustained efforts in many areas of research and policy. The sustainability of current and future levels of food production is of integral importance and will require a “redesign of the whole food system” to be achieved (Foresight, 2011). Improving yield is one fundamental factor of a food system rejuvenation that is essential to achieving sustainable food security for all. Indeed, Grierson et al. (2011) state that it is “essential that we address the yield gap; the difference between future yield requirements and yields available with current technologies, management and gene pools”. Globally, it is estimated that around 21% of productivity gains by 2030 will have come from increases in cultivated land area, while 67% will be from increased crop yields with 12% from higher cropping intensity (Gregory and George, 2011).

In the mid-1960s scientists developed High Yielding Varieties or Modern Varieties of crops that were to play such an important role in the Green Revolution (Evenson and Gollin, 2003). The incorporation of semidwarfing genes enabled rice and wheat plants with shorter, stiffer stems to be developed that could respond to high inputs of fertilizer and water with large increases in grain production without the lodging that was associated with tall varieties (Evenson and Gollin, 2003; Pearce et al., 2011). The reduced height in wheat was largely due to the *Reduced height* (*Rht*) alleles *Rht-B1b* and *Rht-D1b* that showed reduced (although not abolished) responsiveness to gibberellin (GA) (Pearce et al., 2011). Although the term Green Revolution often refers to the initial wave of release and adoption of high yielding varieties during the 1960s and 1970s, increases in food production during that time were also due to increases in area of land under cultivation and inputs other than the varieties; while the contribution to yield of high yielding varieties was actually higher in the 1980s and 1990s, when the adoption of these varieties was more widespread (Evenson and Gollin, 2003). It is noteworthy that discrepancies in the adoption of High Yielding Varieties of crops existed, with widespread adoption in areas to which the backgrounds were adapted (Asia and Latin America) and later and smaller adoption in areas such as sub-Saharan Africa (Evenson and Gollin, 2003). In Asian countries 82% of land under production used High Yielding Varieties by 1998, while only 27% of land under production in African countries used High Yielding Varieties (Pingali, 2012).

Wheat is an integral part of the global diet, providing around 20% of humanity's calories and protein (Shiferaw et al., 2013). Over 1 million tonnes of wheat was used for animal feed in Great Britain during the crop year July to September 2015 (DEFRA, 2015). In 2009, wheat was cultivated on more than 200 million hectares (ha) worldwide and production reached almost 687 million tonnes (FAOSTAT, 2012). In 2014 global wheat production reached 733 million tonnes and, as of November 2015, was expected to reach an all-time high of 736 million tonnes in 2015 (FAO, 2015a). As can be seen in Figure 1.1, global average yield per hectare has increased greatly since FAO records began. However, a large amount of this increase is likely to be due to application of fertilizers containing nitrogen, phosphorus and potassium and

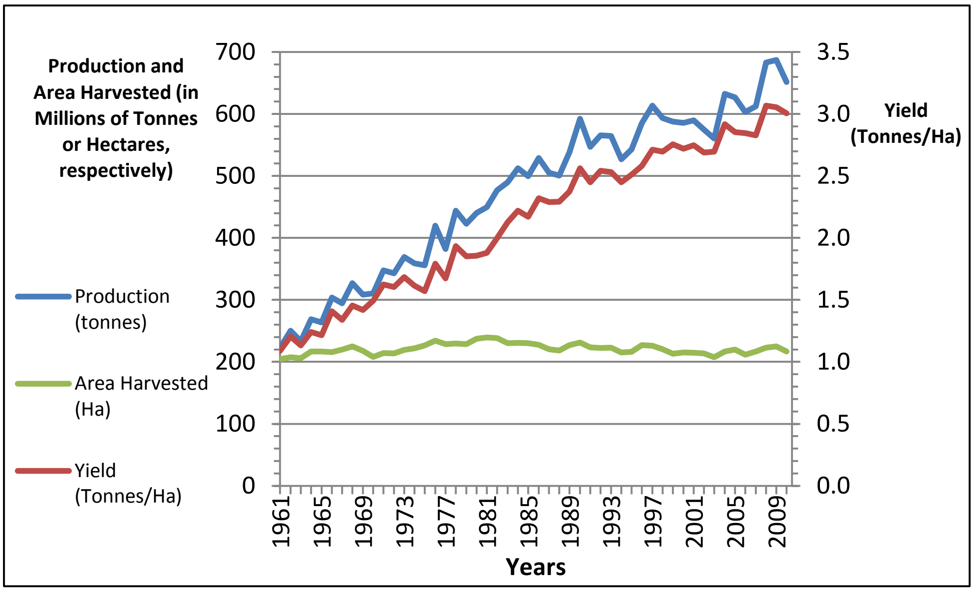


Figure 1.1: Annual global wheat production, area harvested and yield. Data from FAOSTAT, accessed January, 2012.

to the use of synthetic fungicides and pesticides. This means that these gains are likely to be unsustainable in the long term, especially with regard to phosphorus. Phosphorus is an essential element for growth and cannot be substituted or manufactured (Neset and Cordell, 2012). The vast majority of agricultural phosphorus is obtained from a finite resource (phosphate rock) that is found in very few locations (with Morocco/Western Sahara containing 77-85% of global reserves) and peak production is likely to occur in this century (Neset and Cordell, 2012). Similarly, the production of nitrate fertilizer through the Haber-Bosch process is currently dependent on fossil fuels which may also be running out as well as contributing to climate change (although the rise of shale gas may have delayed the decline of fossil fuel utilisation).

Crops obtain their nutrients and water from the soil via the root system. A vigorous root system can improve yield with increased root biomass and deeper roots providing improved water and nitrogen uptake, although the risk of depleting water resources is increased in environments dependent on stored soil water (Palta et al., 2011). Shallow root growth and wide spread of roots provide the best phosphate acquisition (Lynch and Brown, 2012). The ability of a wheat plant to access maximal nutrients, fix large quantities of carbon and remobilise these resources to grain without lodging or succumbing to drought or disease requires a range of processes. Better understanding of root growth and architectural traits and their interaction with the environment will also be necessary for developing new ideotypes of crops that can adapt (or be adapted) to a changing climate (Haling et al., 2011). Increased efficiency of root uptake of applied nutrients will contribute to greater sustainability of the food system. The focus of this thesis will be improved understanding of crop (wheat and also rice) architectural responses to the environmental stress of strong soil, which can limit root growth and result in reduced yields (Whalley et al., 2008). Bread wheat (*Triticum aestivum*) is an allopolyploid (containing chromosomes derived from different species) hexaploid plant that emerged around 8000 years ago in the Fertile Crescent and had “profound impact on the development of human societies”(Matsuoka, 2011). In the coming decades of substantial global population growth, wheat will continue to play an integral role in avoiding human hunger.

1.2 Plant architecture

The geometric and topological organization of plant components, in three-dimensions (3D) makes up the architecture of a plant (Evers et al., 2005). Jungk (1991) refers to root architecture in terms of branching pattern and how roots are distributed in the soil profile. Lynch (1995) defines root architecture as the spatial configuration of the root system. Root topology must be known for root architecture to be understood (Lynch, 1995). However, root topology (how axes are connected via the branching pattern and distribution of roots) does not provide information on orientation. Morphology refers to the form or structure of anatomical features, such as root hairs. According to Lynch (1995), "anatomical features of a root related to cell and tissue organization are not usually part of architectural considerations" . However, a consideration of cellular arrangements is necessary to understand how and where lateral roots (and shoots) are formed. Indeed, the localisation and frequency of lateral shoots and roots and the direction and extent of root tip growth determine plant architecture (Laskowski et al., 2008; Jones and Ljung, 2012). Hence, how lateral shoots and roots form is of fundamental interest to a plant architecture researcher.

The root system of wheat is comprised of seminal roots (that develop in the embryo or from the embryonic nodes) and adventitious roots (that emanate from shoot nodes) (Waisel and Eshel, 1991). In the developmental approach to describing root systems, the primary axes are given an order of magnitude of 1 and subsequent branches (i.e. secondary or tertiary) are given ascending orders of magnitude (Fitter, 1991). The major problem with this developmental approach is that arbitrary decisions are often made as to which of two roots at a junction are of higher order when an existing root system is examined (Fitter, 1991). Branching patterns in root systems can be represented as topological diagrams (Figure 1.2) (Fitter, 1991), with links numbered based on their order in the exterior path length. The root branching patterns in Figure 1.2 represent extremes of a spectrum. A dichotomous topology is costs fewer resources to construct and maintain, more efficient at transporting material but less efficient at acquisition of mobile resources than a herringbone topology (Fitter, 1991).

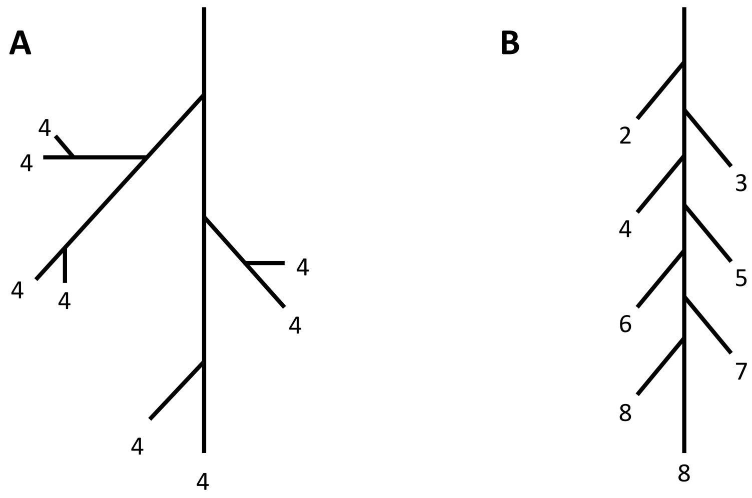


Figure 1.2: Diagrams of two extremes of root topology.
A) Diagram of dichotomous root topology. B) Diagram of herring bone root topology. Numerals represent the exterior path length (the number of links from an exterior link to the base link). Images recreated from images by Fitter (1991).

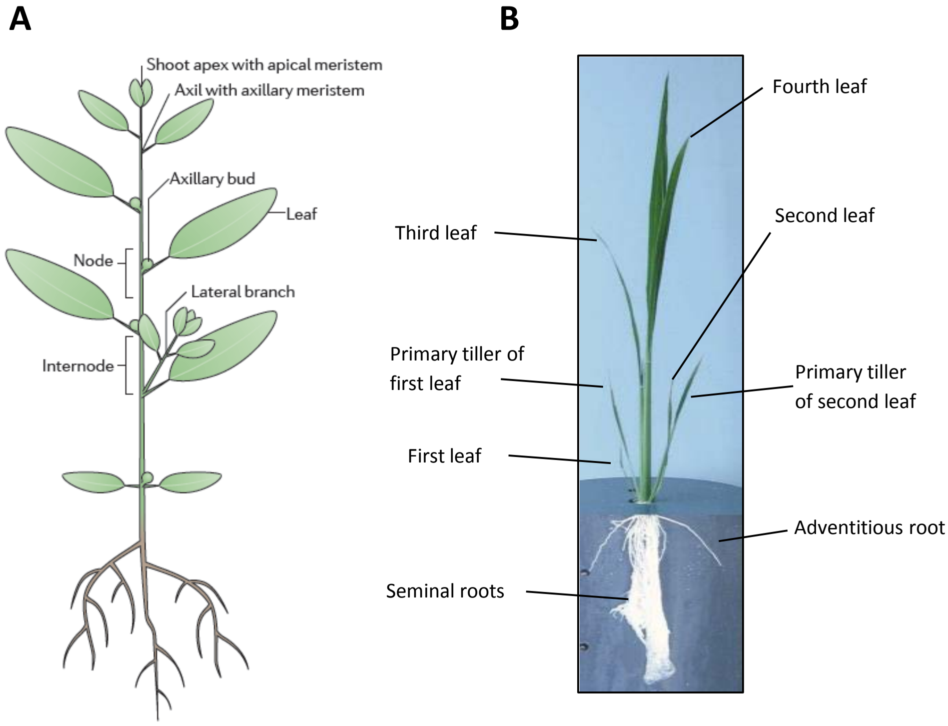


Figure 1.3: Morphology of dicots and monocots
A) Diagram of plant architecture (based on a dicot species) (Domagalska and Leyser, 2011).
B) Diagram of grass morphology (with rice used as a model monocot)

The herringbone topology would be favoured by slow-growing perennial plants in resource-poor soils. The morphology of the aerial parts of dicotyledonous (dicot) plants and monocotyledonous (monocot) plants display certain differences and similarities, as shown in Figure 1.3. Axillary buds form in the axils of the leaves of dicots and lateral branches form at these axillary buds all the way up the stem. By contrast, axillary buds of monocots (including wheat) are formed during the vegetative phase of growth and shoot branches originate from the basal nodes, elongate while under the leaf sheaths and emerge from the leaf axil. The diversity of aerial architectures seen in plants is based largely on branching patterns (Aguilar-Martinez et al., 2007). Shoot branching involves two steps: initiation of lateral (axillary) meristems in the axils of leaves to form axillary buds and outgrowth of these axillary buds to form branches (Durbak et al., 2012). In wheat (as in other grasses) shoot branches are termed tillers and result from the outgrowth of axillary buds in the basal nodes of the stem during the vegetative phase of plant growth (McSteen, 2009). During reproductive development axillary meristems give rise to flowering branches or flowers (McSteen, 2009).

The ability of a plant to grow new organs throughout its lifecycle relies on meristems. The primary shoot apical meristem is responsible for all above-ground organs, the root apical meristem is responsible for all below-ground organs and the axillary meristems are responsible for all “secondary axes of growth” (Durbak et al., 2012). The shoot apical meristem is a region of active cell division which initiates primordia (Kirby and Appleyard, 1984). In wheat primordia develop into distinct plant organs including leaves, tillers and ears with the fate of the primordia being dependent on when and where the primordia are formed (Kirby and Appleyard, 1984). Secondary (axillary) meristems to the shoot apical meristem and root apical meristems can have the same potential as the primary meristem (Domagalska and Leyser, 2011). After initiation, axillary meristems develop into axillary buds (Aguilar-Martinez et al., 2007). The axillary buds have their own apical meristem and can develop in the same way as the main shoot. In the mature embryo of wheat and barley, an axillary tiller bud is already present in the respective axils of the coleoptile and leaf 1 (Kirby and Appleyard, 1984). The vegetative phase of wheat development lasts from germination until there are between four and eight emerged leaves corresponding to nodes the main shoot (Kirby and Appleyard, 1984). The first tiller bud to form in the vegetative phase of a wheat seedling is that in the axil of leaf 2 (Kirby and Appleyard, 1984). Initiation and outgrowth of axillary meristems are under the control of hormonal regulation.

The outgrowth of available tiller buds can be assessed non-destructively on the basis of the number of leaves produced. Site filling can be calculated as the natural logarithm of emerged tillers per unit leaf appearance interval (Assuero et al., 2012). However, the site filling value used in this thesis is the number primary tillers divided by the number of leaves on the main tiller.

1.3 Roles of hormones in development of plant architecture and responses to environmental stimuli

Plant hormones are small molecules, often present at very low concentrations, that derive from a range of metabolic pathways which regulate plant growth and mediate responses to biotic and abiotic stresses (Santner et al., 2009). An ever-increasing number of plant hormones have been identified including auxins, cytokinins, gibberellins, abscisic acid, brassinosteroids, ethylene, jasmonic acid, salicylic acid and, most recently, strigolactones. The following section is intended to give a sense of the involvement of some of the plant hormones in developmental processes of vegetative architecture and responses to environmental stimuli. Additional emphasis is placed on those hormones of more interest to the subsequent investigations described in this thesis.

1.3.1 Auxins

Auxin is involved in cell division, shoot branching, root formation and fruit production, although the last will not be discussed here (Perrot-Rechenmann and Napier, 2005). Auxin molecules are transported throughout the plant by efflux and influx carrier proteins causing auxin gradients and maxima that control production of meristems. Auxin molecules bind to TIR1 F-box proteins as part of a complex that leads to the degradation of transcriptional repressors called AUX/IAAs (Parry et al., 2009). When auxin maxima form around the shoot apical meristem they promote the formation of leaf primordia which then act as auxin sources allowing the next auxin maximum (and next leaf primordium) to form at a determined minimum distance from the existing leaf primordium, thereby directing the arrangement of leaves around a plant's stem (phyllotaxis) (Reinhardt et al., 2003). Auxin is involved in the initiation of all organ primordia and axillary meristems are no different. Auxin biosynthesis, PIN1 (auxin efflux carriers) and PID (a Ser/Thr protein kinase that phosphorylates PIN1 proteins and regulates their localization), or orthologous genes, have all been implicated in axillary meristem initiation in Arabidopsis, maize and rice (McSteen, 2009). Overexpression of *OsPID1* in rice gives a similar phenotype to that arising from application of an inhibiter of auxin transporters. BA1 is a transcription factor that is bound by the maize co-orthologue of PID and has been shown to be required for axillary meristem initiation (McSteen, 2009). Lateral root primordia development is driven by auxin transport into distinct root tissues (via polar auxin transport, or PAT) stimulating the initial establishment of a group of dividing pericycle cells and the subsequent formation of a meristem that is hormone-autonomous (Casimiro et al., 2001). Wisniewska et al. (2006) were able to show, with the use of green fluorescent protein (GFP)-tagged PIN genes, that the polarity of PIN proteins PIN1 and PIN2 determines the direction of auxin flow. A line of cells with a high level of auxin flux is formed and becomes a canal. These auxin flux canals can later develop into vascular strands and a polar auxin transport (PAT) stream (Domagalska and Leyser, 2011). It has been suggested that buds must export auxin in order to become active. Buds act as auxin sources and the stem acts as an auxin sink, with buds competing for access to the polar auxin transport system. If a bud has a strong flux of auxin into the stem PAT stream it will continue to have one and prevent other buds from initiating an auxin flux and, thus, restrict the activation of other buds (hence the phenomenon of apical dominance) (Domagalska and Leyser, 2011).

Auxin is also involved in growth tropisms – growth responses to directional stimuli – including phototropism (in response to directional light) and gravitropism (in response to gravity) (Taiz and Zeiger, 1991). Phototropism occurs by movement of auxin away from light at the coleoptile tip, where it promotes growth of the shaded side (Taiz and Zeiger, 1991). Shoot gravitropism involves the redistribution of auxin to the lower side of a horizontal shoot, increasing the growth of the lower side and resulting in curvature in the opposite direction to gravity (Taiz and Zeiger, 1991). This response can be dampened by increasing PAT which prevents the necessary differential auxin distribution, as occurs in the *la1-ZF802* rice mutant which has a more obtuse arrangement of tillers (Li et al., 2007). Gravitropism in roots involves redistribution of PIN efflux proteins, lateral redistribution of auxin and a reduced growth rate on the lower-side of a horizontal root relative to the upper-side results in a downward curvature (Swarup et al., 2005). The major natural auxin is indole-3-acetic acid (IAA) (Santner et al., 2009).

1.3.2 Strigolactones

Strigolactones (SLs) are carotenoid-derived terpenoid lactones that were initially identified as promoters of parasitic seed germination and hyphal branching of arbuscular mychorrhizae (Koltai, 2011). Subsequently, they were shown to inhibit shoot branching and to affect primary root length and lateral rooting density (Umehara et al., 2010; Ruyter-Spira et al., 2011) and are thus fundamentally important in determining plant architecture.

SLs are a group of plant hormones consisting of a tricyclic lactone (ABC-rings) joined to a methylbutenolide (D-ring) by an enol ether bond (Umehara et al., 2015) (see Figure 1.4). The biological activity of all strigolactones is thought to require the C-D ring moiety, at least in the case of stimulating germination of parasitic seeds (Yoneyama et al., 2009). Akiyama et al. (2010) determined the minimum concentration required to stimulate branching of an arbuscular mycorrhizal fungus for 44 SLs and SL-like compounds and concluded that the coupling of the C-D rings was essential but that the enol ether bond could be swapped for either alkoxy or imino ethers. In contrast, Umehara et al. (2015) showed replacing the enol ether bond for either alkoxy or imino ether bonds dramatically reduced the biological activity of shoot branching inhibition. Furthermore, the *R* configuration at C-2’ of the D-ring (where the enol ether bond occurs) and the resulting steric position of the D-ring is essential for biological activity of a SL in rice (Umehara et al., 2015).

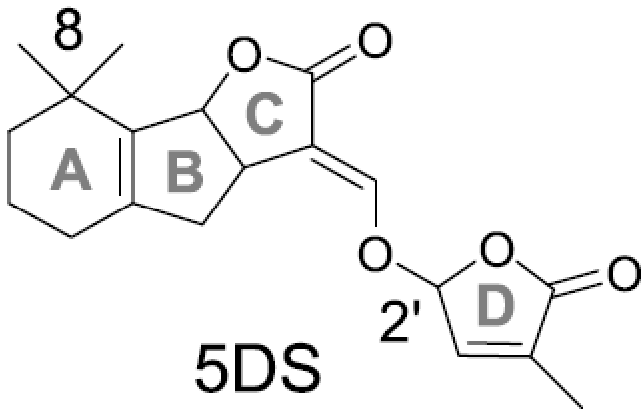


Figure 1.4: Structure of the strigolactone 5-deoxystrigol (5DS)
Image shows the structure of a strigolactone known to be biologically active in rice (5DS). The ABC rings represent the tricyclic lactone and the D ring is a methylbutenolide, both of which are typical structural characteristics of many strigolactones.
Image has been modified from Seto et al. (2014).

Strigolactones can be shoot- or root-derived (predominantly the latter) and move upwards to inhibit shoot branching (Dun et al., 2012). Strigolactones were found in the xylem tissue of Arabidopsis plants, suggesting that the xylem is the transport route to the shoot (Kohlen et al., 2011). Kretzschmar et al. (2012) showed that the transporter PDR1 exports strigolactones from cells that are subsequently colonised by arbuscular mycorrhizae. Furthermore, *PDR1* expression was high in the vascular and nodal regions of petunia stems (Kretzschmar et al., 2012). Kretzschmar et al. (2012) also showed that expression of *PDR1* increased in response to increased application of GR24 (a commonly used synthetic SL analogue), in accordance with the negative feedback regulation of strigolactone biosynthesis shown by Mashiguchi et al. (2009).

The currently known enzymes for SL biosynthesis are an iron-containing isomerization catalyser (D27), two carotenoid cleavage dioxygenases (or CCDs, D17 and D10) and a cytochrome P450 (MAX1) (see Table 1.1). The terms for SL pathway components used in this document are consistent with those used for rice plants, however, Table 1.1 outlines alternative nomenclature used in other species.

Table 1.1 Strigolactone biosynthesis and signalling components

Interspecific differences in gene name prefixes exist. *Dwarf (D)* or *High Tillering Dwarf (HTD)* are used for rice, *More Axillary Growth (MAX)* is used for Arabidopsis, *Ramosus (RMS)* is used for pea and *Decreased Apical Dominance (DAD)* is used for petunia. Information from this table comes from Goulet and Klee (2010), Umehara et al. (2010), Domagalska and Leyser (2011), Zhou et al. (2013) and Zhang et al. (2014).

Strigolactone pathway gene name(s) in rice	Orthologue names in other species	Protein encoded	Location of protein	Protein function
D27		iron-containing protein	Plastid, mainly of vascular cells	Carotenoid cleavage/ strigolactone biosynthesis
D17 **(or *HTD1*)**	MAX3, RMS5, DAD3	CCD7	Plastid	Carotenoid cleavage/ strigolactone biosynthesis
D10	MAX4, RMS1, DAD1	CCD8	Plastid	Carotenoid cleavage/ strigolactone biosynthesis
MAX1 (Os900)	MAX1	cytochrome P450 (Carlactone oxidase)	Vasculature -related tissue	Modification of carlactone into strigolactones
MAX1 (Os1400)		*ent*-2'-*epi*-5DS-4-hydroxylase		Modification of *ent*-2'-*epi*-5DS into orobanchol
D14 **(or *D88* or *HTD2*)**		α/β hydrolase	Vasculature -related tissue	Binds strigolactones and D53 as part of the ubiquitination process
D3	MAX2, RMS4	F-box protein	Nucleus	Incorporates an SCF complex to D53-D14 in the presence of strigolactone
D53		class I ClpATPase protein	Nucleus	SL signalling repressor. Degraded by the proteasome-ubiquitin pathway in a D3 and D14 dependent manner.

D27, D17 (CCD7) and D10 (CCD8) catalyse consecutive steps of conversion of all-*trans*-β-carotene to carlactone, within the plastid (Sorefan et al., 2003; Booker et al., 2004; Snowden et al., 2005; Gomez-Roldan et al., 2008; Umehara et al., 2008; Lin et al., 2009; Alder et al., 2012). MAX1 is involved in SL biosynthesis downstream of D10 and MAX1 mutants behave as expected for plants lacking SL biosynthesis, showing increased shoot branching (Gomez-Roldan et al., 2008; Crawford et al., 2010). In Arabidopsis, MAX1 represents a single member family, while there are five MAX1 paralogues in rice (Booker et al., 2005). Scaffidi et al. (2013) showed that MAX1 functions in the conversion of carlactone to strigolactones. This is followed by signalling specifically through D14, while GR24 functions through both D14 and the paralogue KAI2 (which is part of an alternative plant hormone pathway, the karrikins) (Scaffidi et al., 2013). Seto et al. (2014) successfully proved that carlactone is produced *in planta* and specified that only the (11*R*)-isomer of carlactone is converted to (−)-*ent*-2’-*epi*-5-deoxystrigol (hereinafter referred to as (−)-*ent*-2’-*epi*-5DS), which is both a bioactive SL and a precursor for other SLs. Indeed, Umehara et al. (2015) showed that (−)-*ent*-2’-*epi*-5DS and (+)-5DS (both of which have *R* configuration at C-2’ of the D-ring) function in inhibiting tiller bud outgrowth in rice, while (-)-*ent*-5DS and (+)-2’-*epi*-5DS (which have *S* configuration at C-2’ of the D-ring) have little if any effect on tiller bud outgrowth. Zhang et al. (2014) explored the functionality of the five rice paralogues of MAX1. Os900 was found to be the main paralogue that oxidises carlactone to (−)-*ent*-2’-*epi*-5DS and, hence, termed carlactone oxidase (Zhang et al., 2014). Os1400, Os1900 and Os5100 were found to have much lower activity levels of carlactone oxidation, while Os1500 showed no activity (Zhang et al., 2014). However, the main function of Os1400 seemed to be the conversion of (−)-*ent*-2'-*epi*-5DS to orobanchol, leading to the assigning of the name *ent*-2'-*epi*-5DS-4-hydroxylase (Zhang et al., 2014). Interestingly, the single MAX1 homologue in Arabidopsis was not shown to be able to convert carlactone all the way to orobanchol (Zhang et al., 2014). Figure 1.5 presents a diagrammatic overview of the strigolactone signalling pathway which cross-refers to the list of strigolactone signalling components in Table 1.1.

The known signalling mechanisms for SLs include an α/β hydrolase (D14), an F-box protein (D3) and a SL signalling inhibitor (D53). Through grafting studies, assessment of mutant and overexpression lines, SL application experiments, binding assays, crystallization experiments, and *in-silico* structural analyses, an impressive model for the signalling mechanisms has been built up (Stirnberg et al., 2002; Brewer et al., 2009; Jiang et al., 2013; Nakamura et al., 2013; Zhou et al., 2013; Chevalier et al., 2014; Zhao et al., 2014).

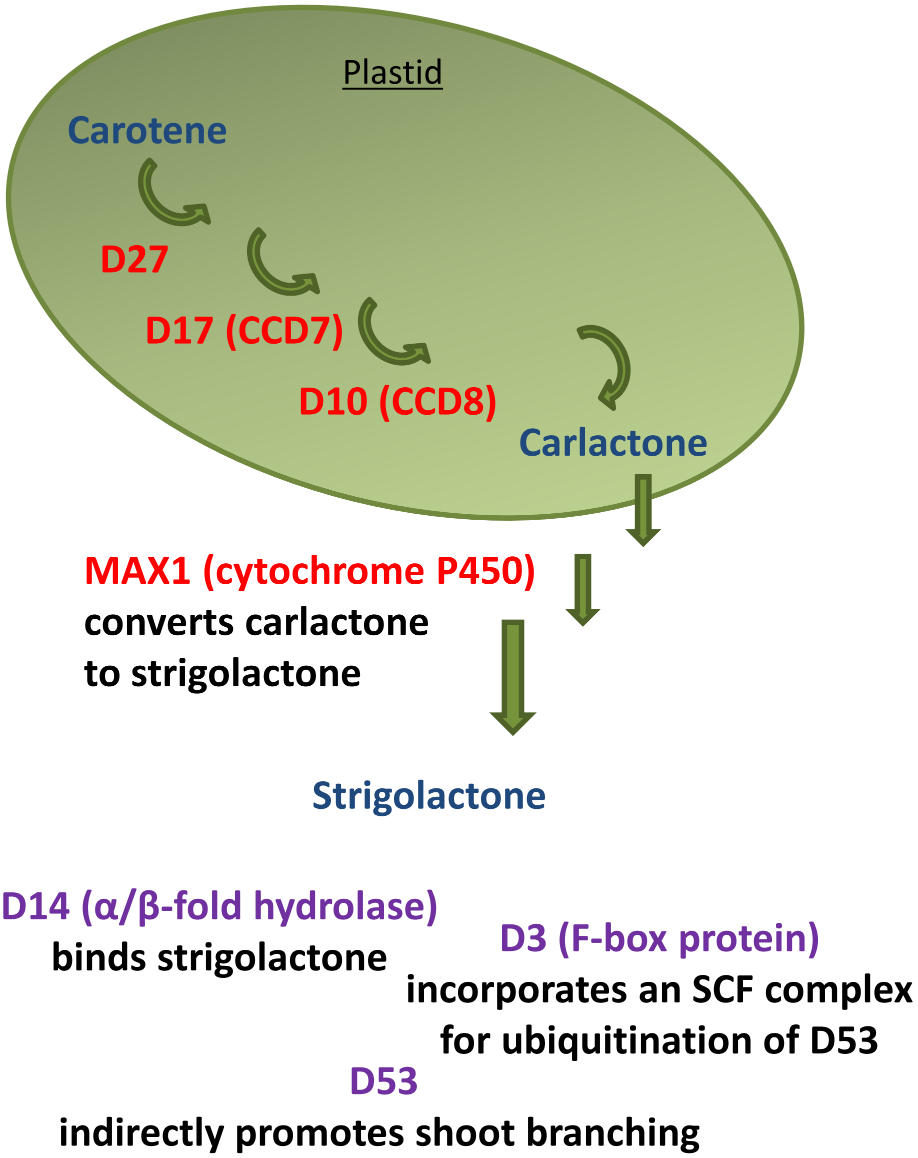


Figure 1.5: Schematic of strigolactone biosynthesis and signalling components
Strigolactone biosynthesis components in red, signalling components in purple and chemicals in blue.
Information from Umehara et al. (2010), Domagalska et al. (2011), Zhou et al. (2013) and Seto et al. (2014).

The current understanding of the roles of D14, D3 and D53 in SL signalling is presented in Figure 1.6. In the absence of SL, D53 is free to inhibit downstream targets (Jiang et al., 2013; Zhou et al., 2013) (Figure 1.6A). D14 (the SL receptor) binds to SL, enabling binding to D53 and D3 (Jiang et al., 2013; Zhou et al., 2013) (Figure 1.6B). D3 becomes part of an SCF complex that results in ubiquitination of D53, thereby targeting D53 for degradation via the proteasome (Jiang et al., 2013; Zhou et al., 2013) (Figure 1.6B and C). Transcription factors are thought to be key downstream targets of D53. Jiang et al. (2013) showed that D53 binds to a transcriptional co-repressor similar to TOPLESS (TPL) that they term TPR2 (a TPL-related protein, also known as ASP1 amongst other names) (Yoshida et al., 2012). ASP1 (or TPR2) was shown to repress axillary bud outgrowth and maintain phyllotaxy in the vegetative tissue of rice plants (as well as proper branching of the spikelet) (Yoshida et al., 2012). The *asp1-2* mutant had fewer crown roots (adventitious, post-embryonic shoot-borne, roots) and shorter seminal roots in rice (Yoshida et al., 2012). Yoshida et al. (2012) showed that ASP1 (or TPR2) is likely to function via a histone deacetylase as a co-repressor, which would negatively regulate transcription via chromatin modification. Another transcription factor family that could be downstream targets of D53 are those of the TCP family. FINE CULM 1 (FC1) is a TCP transcription factor in rice that represses outgrowth of axillary meristems and seems to integrate multiple signalling pathways (including strigolactone signalling) to fine-tune shoot branching, similarly to orthologues in maize (TEOSINTE BRANCHED 1 – TB1) and

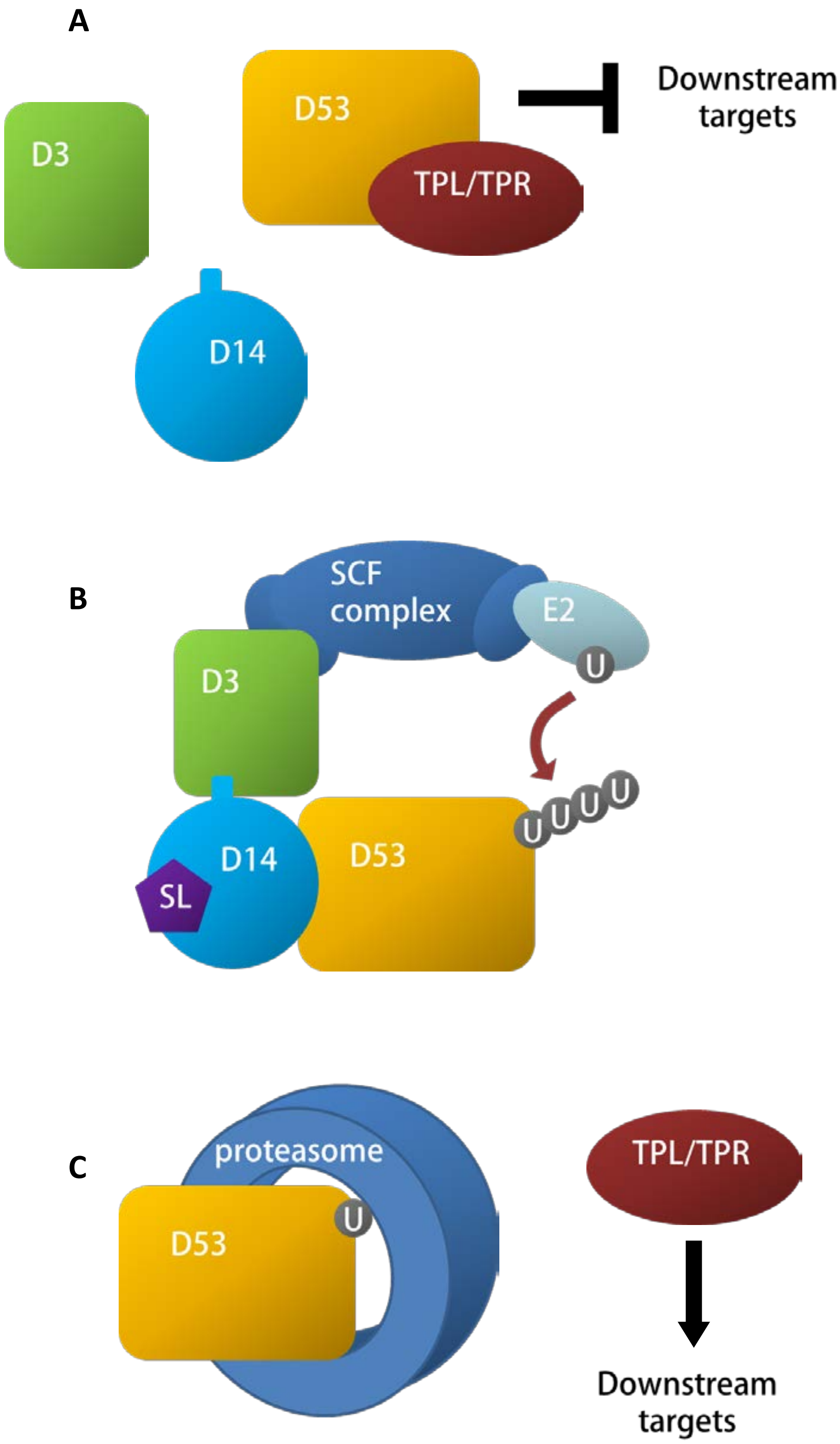


Figure 1.6: Cartoon of strigolactone signalling mechanism
A) D53 inhibits downstream targets by binding TOPLESS (TPL). B) D14 binds strigolactone, D53 and D3 while D3 incorporates the SCF complex required for ubiquitination. C) When D53 has been ubiquitinated, downstream targets can be influenced (such as promotion of FC1 production) causing inhibition of tillering. Information from Jiang et al. (2013) and Zhou et al. (2013).

Arabidopsis (BRANCHED 1 – BRC1) (Doebley et al., 1997; Takeda et al., 2003; Aguilar-Martinez et al., 2007; Minakuchi et al., 2010; Domagalska and Leyser, 2011; Zhou et al., 2013). TCP transcription factors bind DNA as homodimers to perform their function (Aggarwal et al., 2010).

While the TPR and TCP transcription factors are interesting points for SL signalling, it is important to recognise that SL signalling also functions via modifying auxin flux and bud access to the auxin transport stream (Domagalska and Leyser, 2011; Shinohara et al., 2013; Koltai, 2014).

SLs inhibit tiller outgrowth in rice and shoot branching in Arabidopsis, pea and rice (Gomez-Roldan et al., 2008; Umehara et al., 2008). Umehara et al. (2010) showed that decreasing phosphate concentrations lead to tiller inhibition in rice seedlings via increased levels of SL. Expression of SL biosynthesis genes *D10*, *D17* and *D27* was examined by qRT-PCR and all three genes showed increased expression in the roots of rice in response to P deficiency but reduced expression in response to excess P (Umehara et al., 2010). Differences in tiller outgrowth could be seen in the 3rd and 4th leaves between nutrient solutions of different P availability (Umehara et al., 2010).

SLs have been shown to affect root architecture, but the effects can vary between species and with nutrient status. SL-deficient mutants (defective in the *d10* orthologue *max4* or *max1*) had more lateral roots than WT Arabidopsis plants while SL-perceptive plants treated with GR24 (a bioactive synthetic SL analogue) showed reduced lateral root outgrowth and fewer lateral root primordia, with concomitantly reduced auxin levels (Kapulnik et al., 2011; Ruyter-Spira et al., 2011). Lateral root formation was repressed by SL in Arabidopsis, pea and rice plants in a *MAX2*- or *D3*-dependent manner (Gomez-Roldan et al., 2008; Umehara et al., 2008). Endoderm-specific expression of the *D3* orthologue *MAX2* in a *max2* background restored responsiveness to GR24 for lateral root formation and the number of cells in the primary root meristem (Koren et al., 2013). Adventitious root formation (roots derived from non-root tissues such as stems or leaves) was repressed in Arabidopsis and pea plants by GR24 application in a *MAX2* (*D3* orthologue)-dependent manner (Rasmussen et al., 2012). In contrast to inhibiting adventitious root formation and lateral root formation, applied GR24 promoted root hair elongation in Arabidopsis seedlings, in a *MAX2* (*D3* orthologue)-dependent manner (Kapulnik et al., 2011). Endoderm-specific expression of the *D3* orthologue *MAX2* in a *max2* background also restored responsiveness to GR24 for root hair elongation (Koren et al., 2013). Treatment with moderate amounts of GR24 caused significant increases in primary root length of *max4* (*d10*) and WT Arabidopsis plants (Ruyter-Spira et al., 2011). GR24 application also increased seminal root length in rice plants in a *D3*-dependent manner (Sun et al., 2014). Interestingly, SLs promote crown root elongation in rice in a *D3*-dependent manner (Arite et al., 2012), in contrast to the aforementioned inhibition of adventitious roots of Arabidopsis and pea plants by SL shown by Rasmussen et al. (2012).

Both phosphate- and nitrogen-deficiency have been shown to increase the production of SLs, thereby influencing root architecture (Yoneyama et al., 2007; Ruyter-Spira et al., 2011; Yoneyama et al., 2012; Sun et al., 2014). Lopez-Raez et al. (2008) showed that phosphate (P) starvation in tomato plants caused increased production and exudation of SLs by the roots. Phosphate- or nitrogen-deficient conditions increased seminal root length and reduced lateral root density in rice plants in a *D3*-dependent manner (Sun et al., 2014). Under phosphate deficiency, the inhibitory effect of SL on lateral root formation in Arabidopsis is reversed and GR24 application enhanced lateral root formation (Ruyter-Spira et al., 2011). Many of the effects of SL in controlling root architecture seem to occur via modulation of auxin levels and auxin transport (Kapulnik et al., 2011; Ruyter-Spira et al., 2011; Sun et al., 2014).

The roles of SLs in determining shoot and root architecture in response to phosphate and nitrogen deficiencies highlighted the potential of this hormone to be involved in the abiotic stress of strong soil, especially when inhibitory effects of strong soil on tillering (as described in Section 1.4) are taken into consideration.

1.3.3 Cytokinins

Cytokinins are N6-substituted purine derived molecules with involvement in a broad range of developmental processes (Werner et al., 2001). The bioactive molecules are the free bases and their ribosides (Werner et al., 2001). Cytokinin-deficient tobacco plants display stunted shoots, smaller shoot apical meristems (SAM), reduced leaf cell production, faster growing roots and more lateral roots (Werner et al., 2001). Hence, cytokinins promote shoot growth and inhibit root growth and lateral root formation. In the root, cytokinins act directly on xylem-pole pericycle cells (lateral root founder cells) and disrupt the expression of PIN genes, thereby preventing the formation of an auxin gradient that is required for appropriate configuration of lateral root primordia (Laplaze et al., 2007). Cytokinins promote proliferation of undifferentiated cells in the shoot apical meristem (SAM) and suppress meristem cell differentiation in the root apical meristem (Kyozuka, 2007). The LONELY GUY (LOG) gene encodes a cytokinin-activating enzyme and is localised to shoot meristem tips, thereby controlling the spatial distribution of bioactive cytokinin (Kurakawa et al., 2007). Cytokinins may prolong the identity of the panicle branch meristems, causing more lateral spikelets and more secondary panicle branches to be formed (Kyozuka, 2007). Cytokinins act directly in axillary buds to promote bud outgrowth, seemingly through supressing the expression of *PsBRC1* (responsible for a TCP transcription factor – with homologs in maize, Arabidopsis and rice – that inhibits outgrowth of axillary buds), with action antagonistic to that of strigolactones (Dun et al., 2012).

1.3.4 Gibberellins

Gibberellins (GAs) are involved in promotion of seed germination, stem elongation, leaf elongation and seed development as well as the control of flower development (Yamaguchi, 2008). GA_4 and GA_1 (13-hydroxy-GA_4) are the major bioactive GAs endogenous to plants (Thomas et al., 2005). GA_4 is more abundant in Arabidopsis shoot material than GA_1 (Talon et al., 1990). In contrast, GA_4 is of low abundance in vegetative tissues of rice, with GA_1 being the predominant bioactive GA (Magome et al., 2013). It has been shown in rice that 13-hydroxylation of GAs results in partial loss of biological actvity (Magome et al. 2013), but these diffeences between species remain unexplained. GAs promote shoot growth; their discovery was in the context of the excessive stem elongation of rice plants infected with *Gibberella fujikuroi*, while GA signalling is disrupted in semi-dwarf crop varieties widely adopted during the 'Green Revolution' of the 1960s and 1970s (Santner et al., 2009). Rht-A1, Rht-B1 and Rht-D1 are DELLA proteins in wheat and mutations in the *Rht-B1* and *Rht-D1* resulted in the semi-dominant GA-insensitive semi-dwarf varieties of the 'Green Revolution' (Thomas et al., 2005). A simplified depiction of the later stages of GA biosynthesis and DELLA-mediated effects on growth of GA (via nuclear transcription regulation) is shown in Figure 1.7.

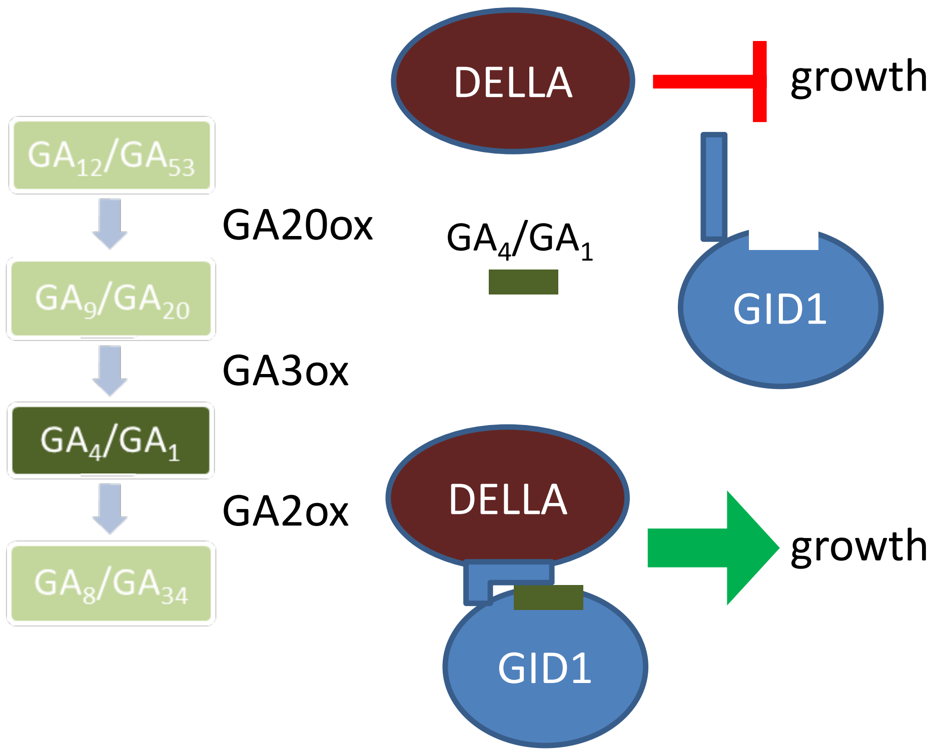


Figure 1.7: GA biosynthesis and perception & DELLA regulated growth
GA20ox and GA3ox convert non-biologically-active GA intermediates into bioactive GA such as GA4 and GA1. GA2ox is involved in inactivation of bioactive GAs. DELLA proteins, such as those encoded by *Rht* in wheat, repress growth in the absence of GA. Bioactive GA binds GID1 and causes a conformational change that allows DELLA proteins to be poly-ubiquitinated and targeted for degradation (not shown here), thus removing growth repression.
Green arrow indicates promotion of growth. Red line indicates repression of growth.
Information from Sun (2010)

Perception and signalling of GAs is similar to that of strigolactones. GA binds to the GA receptor GID1 (GA INSENSITIVE DWARF1), allowing the formation of a GA-GID1-DELLA complex. This creates interaction with GID2, an F-box component that is part of an SCF complex and results in subsequent DELLA degradation via the ubiquitin-proteasome pathway (Sun, 2010). This process is comparable to the targetting of D53 for ubiquitination in the SL pathway. The GA 2-oxidases (GA2oxs) are a multigene family responsible for 2β-hydroxylation of bioactive GAs which results in inactivation (Thomas et al., 2005). The first *GA2ox* gene (*PcGA2ox1*) was identified in runner bean (Thomas et al., 1999). Expression of *PcGA2ox1* in transgenic wheat resulted in dwarf phenotypes including decreased shoot height, dark-green leaves, prostrate growth and increased tillering; these phenotypes could be simulated by application of the GA biosynthesis inhibitor paclobutrazol (Appleford et al., 2007). However, Assuero et al. (2012) suggest that the effect of paclobutrazol on tillering is associated with increased leaf appearance rather than promotion of axillary bud development. Abiotic stress has been shown to induce *GA2ox* expression. At least six genes in the Arabidopsis *GA2ox* family demonstrate upregulation in response to high salinity (Magome et al., 2008). Achard et al. (2008) showed that induced expression of *GA2ox* genes, subsequent reduction in bioactive GA and enhanced DELLA accumulation are part of the dwarfing and tolerance response to freezing.

GA is involved in lateral root formation. GA is at higher levels on the lower side of a horizontal root (during gravitropic response) than on the upper side (Lofke et al., 2013). The higher levels of GA stabilise PIN proteins (auxin transporters) by inhibiting their trafficking to the lytic vacuole, with GA seeming to maintain the gravitropic curving response by stabilising the PIN-dependent auxin stream (Lofke et al., 2013). Total root length of GA-insensitive *Rht-B1c* and *Rht-D1c* wheat plants (*T. aestivum* cv. Mercia) was reduced compared to the Mercia background, but only when plants were grown in soil or the field and not when grown in gel chambers (Wojciechowski et al., 2009).

1.3.5 Abscisic acid

The major roles of ABA are promotion of seed dormancy and improved response to environmental stress conditions including drought. One class of transcription factors that seem to be essential for mediating ABA effects is the ABI3/VP1 transcription factor protein family, with abi3-1 and abi3-5 mutants in Arabidopsis exhibiting ABA insensitivity and reduced seed dormancy (Ooms et al., 1993; Kermode, 2005). The ABI3/VP1 orthologues promote storage reserve accumulation and synthesis of desiccation/stress protectants, repress expression of genes involved in post-germination processes, VP1 inhibits induction of α-amylase gene expression in the aleurone layer of seeds and the ABI3/VP1 orthologues play a role in inception and maintenance of seed and embryo dormancy (reviewed by Kermode (2005)). ABA is involved in inhibiting bud outgrowth and seed germination (the latter in part due to inhibition of α-amylase mRNA transcription) (Taiz and Zeiger, 1991). ABA blocks H+ secretion, inhibiting growth by preventing cell wall acidification and cell elongation (Taiz and Zeiger, 1991). ABA inhibits the expression of several genes encoding hydrolytic enzymes in unfavourable conditions and in dormant seeds (Yazaki and Kikuchi, 2005). In response to drought stress intra-leaf redistribution of ABA, increased ABA concentrations in leaves and root-sourced ABA result in closure of stomata and limit water loss via transpiration (Taiz and Zeiger, 1991). ABA also increases water flow into roots (enhanced ion uptake and hydraulic conductivity), promotes lateral root emergence and suppresses leaf growth; enabling a droughted plant to limit above-ground growth while increasing the size of the root system to cope with the environmental conditions (Taiz and Zeiger, 1991). NCED (9-*cis*-epoxycarotenoid dioxygenase) is an enzyme involved in ABA biosysnthesis and gene expression of NCED precedes ABA accumulation in response to drought stress (reviewed by Nambara and Marion-Poll (2005)). ABA is known to increase under water stress and result in reduced mean cell size, fewer stomata per leaf and greater production of trichomes in wheat (Salisbury and Marinos, 1985). Hypersensitivity to ABA in a mutant wheat line provided improved tolerance to freezing in both non- and cold-acclimated seedlings (Kobayashi et al., 2008). Barley has more tolerance to soil compaction than tomato and the continued growth of barley in moderately compacted soil was attributed to wild-type ABA levels (Mulholland, 1994). High substrate strength stimulates ABA formation in roots and translocation to the leaves via the xylem (Davies et al., 2005).

1.3.6 Ethylene

Ethylene is a gaseous hormone involved in seed germination, root hair development, seedling growth, leaf and petal abscission, fruit ripening and organ senescence as well as response to pathogen attack, wounding, hypoxia and cold (De Paepe and Van Der Straeten, 2005). Hussain et al. (1999) showed that ethylene plays an important role in mediating the growth response of tomato plants to soil compaction. When transgenic tomato plants with greatly reduced synthesis of ACC oxidase (and concomitant reduction in conversion of ACC to ethylene) were grown under split-pot soil compaction conditions (with half of the root system in compacted soil and half in uncompacted soil), the plants were able to grow as if all roots were in uncompacted soil, unlike WT plants that showed reduced leaf area and shoot dry weight (Hussain et al., 1999). Application of the ethylene precursor ACC resulted in reduced leaf elongation and internode length but had no clear effect on axillary buds in maize (Pinthus and Jackson, 1994). Moss et al. (1988) concluded that ethylene did not mediate the growth responses of roots to mechanical impedance, although their use of ballotini (small glass beads which may have caused an additional wounding response) may have introduced an unforeseen confounding variable. DELLA proteins are integrators of growth regulatory signals from ethylene, gibberellin and auxin (Achard et al., 2003).

1.4 Effects of strong soil on plant architecture

Soil may be defined as the upper layer of earth in which land plants grow, consisting of varying proportions of organic materials, clay, sand and rock particles. As a function of being a partly solid structure that can support a plant, a soil provides mechanical impedance to root growth. While some strength is desirable, a soil that is too hard for optimal root growth can prevent adequate acquisition of water and nutrients and preclude yield optimisation. The strength of soil (the ability of soil to resist force) can vary between soil type, soil structure and moisture content, with a dense dry soil being harder than a loosely packed moist soil. Soil compaction (as part of soil structure degradation) is a serious environmental problem caused by modern agriculture, with heavy machinery, short crop rotations, poor soil management and intensive cropping and grazing potentially leading to increasingly deteriorated soil (Hamza and Anderson, 2005). Compaction can be determined by calculating bulk density (mass of a given volume of dry soil) of a given soil type and monitored by measuring soil strength (as measured by resistance to a penetrometer) and soil water content (Hamza and Anderson, 2005). Hamza and Anderson (2005) described how drier soils are stronger (supporting heavier loads), while compaction increases with soil moisture and the number of machine passes. Compaction of soil can result in hypoxia, water deficit and stronger soil (Tracy et al., 2011). Drought can cause both water deficit and stronger soils, and it has been suggested that restriction of plant growth by the increase in soil strength due to drought occurs before water availability becomes limiting (Whitmore and Whalley, 2009).

A range of techniques have been used by researchers to investigate the abiotic stresses of compaction and drought, linked by increased soil strength (or mechanical impedance) to root growth. Some researchers used pressure chambers to increase mechanical impedance (with air or glass beads as the substrate) and others used compacted soils (Goss, 1977; Masle and Passioura, 1987; Moss et al., 1988; Hussain et al., 1999). Each of these methods has limitations when investigating the role of soil strength, with the glass beads (ballotini) in pressure chambers potentially causing wounding and the reduction in pore size in more compacted soils reducing availability of water, oxygen and nutrients to a plant. To avoid those limitations, other authors have used sand columns in tanks of nutrient solution to explore the role of soil strength in impacting on plants architecture (Clark et al., 2000; Clark et al., 2002; Whalley et al., 2006). The work in this thesis uses the latter method, sand columns with different weights applied to the surface to investigate soil strength. It is acknowledged that sand is a greatly simplified, homogenous, version of soil but the terms soil strength, weak soil and strong soil will be used to refer to this sand-based technique. The effects of soil strength on root and shoot architecture of plants are described below, including results from the full range of techniques outlined above.

Researchers using different methods to force roots to work harder, to overcome physically stronger growing media, have shown similar reductions in root elongation and overall size of root systems. Root architecture adapts to the level of resistance to growth (determined by the strength of the medium roots are growing in). To test the effects of mechanical impedance on plant growth Goss (1977) used a pressure chamber with ballotini (glass balls) of a range of sizes, allowing different pore sizes to be investigated. Increased mechanical impedance reduced the growth rate of seminal roots of barley and increased the lateral root density (although the total number of laterals per axis was reduced) (Goss, 1977). In wheat, an applied mechanical impedance of 25 kPa via increased air pressure caused root lengths to be between 51% and 79% of plants grown in an impedance of 0 kPa (Goss, 1977). Increased soil strength (brought about by increased bulk density) also resulted in reduction of root dry weight in wheat plants (Masle and Passioura, 1987). High impedance (strong soil) conditions in a controlled environment reduced the maximum root depth and root dry weight of wheat plants (Whalley et al., 2006).

Shoot architecture is also affected by strong soil. Shoot dry weight and grain yield of field-grown wheat plants were reduced by strong soil in the absence of water deficit (Whalley et al., 2006). Furthermore, high impedance conditions in a controlled environment reduced the number of tillers per plant and shoot dry weight of wheat plants (Whalley et al., 2006). Leaf growth in wheat has also been shown to be affected by soil mechanical impedance with an extended expansion time and shorter leaves, due to shorter cells, arising in response to this stress (Beemster et al., 1996). Increased soil strength brought about by modified bulk density or water content caused a reduction of leaf area and shoot dry weight, while the response could not be rescued with ventilation (implying that hypoxia was not the stimulus) (Masle and Passioura, 1987).

Strong soil effects on crop plant architecture can clearly reduce yield and resource acquisition by crops, affecting food security and profit. Ways of reducing strong soil include soil management techniques and modifying machinery. This includes modifying the timing and depth of ploughing, reducing axle load and ground pressure of machinery and biological loosening of soil with selected rotation crops (Håkansson and Petelkau, 1994; Vermuelen and Perdok, 1994; Hamza and Anderson, 2005). Another option is to understand and adapt the signalling mechanisms of plant responses to strong soil and grow crops that respond more desirably; perhaps plants that are less responsive to temporary increases in strong soil in well irrigated land or rain-fed plants developing plentiful shallow and deep roots (allowing for phosphate uptake and water uptake). The restrictions to shoot growth could be directly due to restricted root growth or be mediated by hormonal signals. It is plausible that a restricted root system will exhaust the localised supply of nutrients and water, leading to nutrient deficiency and subsequent limitation of shoot growth. Indeed, a strong soil had a bigger impact on leaf area for plants subjected to low phosphorus conditions compared to those plants grown in high levels of phosphorus (Masle and Passioura, 1987). However, during early stages of response to strong soil shoot concentrations of phosphorus and nitrogen remained similar to levels measured in plants grown in control strength soil, despite the accompanying reduction of shoot growth (Masle and Passioura, 1987). As suggested by Masle and Passioura (1987), it seems likely that hormonal signalling rather than nutrient deprivation or water deficit triggers the initial responses of plants to strong soil. Indeed, if nutrient and water availability are maintained, the primary mediator(s) of plant architectural response to strong soil could remain hormonal.

Ethylene has been suggested to be responsible for mediating some plant architecture responses to strong soil and plant architecture responses to mechanical impedance (reduced root elongation, increased root diameter and increased lateral root formation) are similar to the effects observed in response to application of ethylene (Kays et al., 1974; Moss et al., 1988; Young et al., 1997; Hussain et al., 1999). Kays et al. (1974) showed that bean roots respond to coming into contact with a physical barrier with a six-fold increase in ethylene production, resulting in increased root diameter. If the roots were able to bend instead of being forced to continue to grow in the direction of an impenetrable barrier, there was no detectable increase in ethylene production (Kays et al 1974). Moss et al. (1988) found a more than two-fold increase in ethylene, reduced root elongation and increased root diameter in maize seedlings in response to mechanical impedance in the form of ballotini. Hussain et al. (1999) used a split-pot experiment where growing some of the roots of tomato plants in more compacted soil caused increased ethylene production and reduced leaf area and root dry weight. Maize seedlings grown in pressure cells responded to application of mechanical impedance with reduced root elongation and increased radial expansion (Sarquis et al., 1991). Ethylene production increased as early as one hour after the mechanical impedance stimulus but growth responses had not occurred within two hours, first being detected at the four-hour time point (Sarquis et al., 1991). Releasing the pressure resulted in reduction of ethylene production within one hour and return to basal levels two hours after removing the stress, while ethylene production could be increased again by turning the pressure back on (Sarquis et al., 1991).

However, these observations are correlative and do not demonstrate that the responses do not occur in the absence of ethylene. To rectify this, Moss et al. (1988) examined the effect of applying the ethylene production inhibitor aminoethoxyvinylglycine (AVG) and found that the root responses still occurred despite a lack of detectable ethylene production. Moss et al. (1988) concluded that wounding of the root rather than response to mechanical impedance was the stimulus for ethylene production. Hussain et al. (1999) found that tomato plants defective in ethylene production still responded with reduced leaf area and root dry weight when the entire root system was grown in high bulk density soil, suggesting that another signal is involved (but not separating the possible stimuli of hypoxia, lack of nutrient availability and soil strength). Sarquis et al. (1991) examined ethylene inhibition further and applied AVG prior to application of mechanical impedance. For Sarquis et al. (1991), treating maize seedlings with ethylene inhibitors meant that only minimal increases in ethylene production occurred in response to mechanical impedance and root and shoot growth recovered to near-control levels. Moss et al. (1988) were not able to measure ethylene production prior to application of the impedance stress so it is plausible that these authors were correct that eventual production of ethylene in their experiment was due to wounding and that (Sarquis et al., 1991) were correct that the early stimulation of ethylene production seen in their work was in response to mechanical impedance. It is perhaps noteworthy that Sarquis et al. (1991) simultaneously applied mechanical impedance to both shoots and roots while Moss et al. (1988) only directly impeded the growth of roots. However, Goss (1977) stated that the effect of mechanical impedance on root elongation was the same whether or not the shoot is also impeded. Unlike other studies, Young et al. (1997) were able to detect reduced leaf elongation only 10 minutes after the initiation of the mechanical impedance stimulus. As the ethylene production response seen by Sarquis et al. (1991) was only known to occur one hour after mechanical impedance, it is arguable that a different signal is responsible for the effects on leaf elongation; potentially linked to transpiration (Young et al., 1997). If ethylene is a signal for response to mechanical impedance, it could be the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) that acts as the root-to-shoot signal, enabling the reduction in shoot growth (Hussain et al., 1999).

Another hormone that has been linked to a role in mediating strong soil is ABA. Many species show increased levels of ABA in response to soil drying and subsequent reduction of stomatal conductance, although the ABA in the xylem of wheat plants does not seem to be the main signal for reduced stomatal conductance in response to soil drying; reviewed by (Dodd, 2005) and references therein. Furthermore, reduced leaf elongation in wheat and barley plants grown in drying soil was not mediated by ABA (Dodd, 2005).

The role of ethylene in mediating root growth in response to strong soil has been quite extensively studied and ABA does not seem to be involved in controlling architectural responses to this abiotic stress. In the work described in this thesis, new lines of investigation are opened. The roles of SLs in inhibiting tillering and in rice in response to nutrient deficiencies and inhibiting the number of lateral roots imply that SLs could be involved in inhibiting tillering in response to strong soil but would be unlikely to be involved in the increase in lateral roots seen in plants grown in strong soil. The importance of GA in promotion of leaf elongation suggests that this hormone could play a role in the reduction of leaf length that has been shown to be a response of plants grown in strong soil conditions. Additionally, further architectural responses of crop shoots and roots may involve the biosynthesis and signalling of other hormones.

Aims of this thesis:

The following chapters explore these possibilities, using molecular biology, hormone quantification and utilisation of available genetic resources to elucidate some of the hormonal signalling in response to strong soil that occurs in rice and wheat plants. The hypotheses investigated are:

- SLs mediate the reduction of tillering in response to strong soil in rice
- SLs mediate the reduction of tillering in response to strong soil in wheat
- GAs mediate the reduction of leaf elongation in response to strong soil and other hormones regulate other shoot and root architectural responses.

Chapter 2: Materials and Methods

2.1 Plant material and growth conditions

2.1.1 Wheat and Rice cultivars and genotypes

Wild-type (WT) rice plants (*Oryza sativa* L. cv. Shiokari) and two high-tillering dwarf SL mutants (*d3* and *d10*) in the Shiokari background were used in Experiment 3.1 and Experiment 3.2. These plants have been described in Ishikawa et al. (2005) and Umehara et al. (2008). Insertion of a transposon into the cds of *D3* causes a lack of SL perception and an amino acid change in the second exon of *D10* dramatically reduces SL biosynthesis (Umehara et al., 2008).

Hexaploid bread wheat (*Triticum aestivum* L. cvs. Mercia and April Bearded) near isogenic lines (NILs) containing a range of *Reduce height* (*Rht*) alleles were used to investigate the role of gibberellin (GA) in strong soil.

Wild-type (*Rht-B1a*) wheat plants (*Triticum aestivum* L. cv. Mercia) were used for analysing gene expression differences between weak and strong soil conditions.

Wild-type (*Rht-B1a*) wheat plants (*Triticum aestivum* L. cv. April Bearded) were used for analysing GA, abscisic acid (ABA) and SL concentrations. GA and ABA levels were measured in leaf samples.

Bread wheat (*Triticum aestivum* L.) cv. Cadenza was used as the basis for production of transgenic lines which were generated by the Rothamsted Cereal Transformation Unit. Wild-type (WT) cv. Additionally, architectural responses to strong soil were assessed in WT Cadenza plants and DNA of WT Cadenza plants was used as template material for cloning SL genes.

2.1.2 Germination of Rice

Threshed rice grains were removed from the glumes and germinated anaerobically by imbibing in the dark in sterile distilled water at 28°C for three days. For soil strength experiments, individual seedlings were then placed into a 3 cm deep hole (made with a pencil) in each sand column (section 2.1.2). A small amount of additional dry sand was used to fill the hole and 1x nutrient solution was added to the top of the sand to moisten.

2.1.3 Germination of Wheat

All cultivars of wheat were germinated in the same way. Clean threshed grains were germinated in Petri dishes under a 14/10hr day/night with temperatures of 22°C /18°C, respectively. However, the petri dishes were covered to maintain darkness during germination. Individual two-day-old seedlings were either transplanted to compost and grown in the glasshouse (the conditions described in Section 2.1.4) or for soil strength experiments transplanted into individual sand columns as described in section 2.1.2, but with a 2 cm deep hole.

2.1.4 Growth conditions

T0 and T1 generations of transgenic wheat lines and rice plants grown for bulking up seed were grown under glasshouse conditions at glasshouse conditions 18-20^{o}C day/14-16^{o}C night temperatures with a 16 hour photoperiod provided by natural light supplemented with banks of SonT 400W sodium lamps (Osram Ltd., UK) generating a light intensity of 400-1,000 μmol/m^2/s photosynthetically active radiation (PAR).

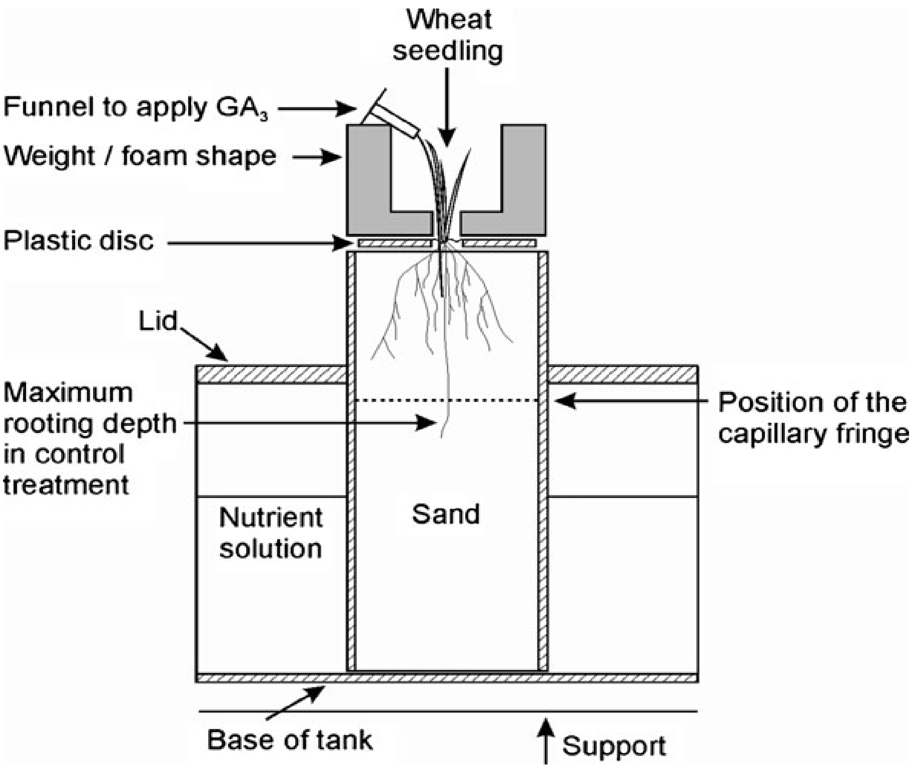
During soil strength experiments, rice and wheat plants were grown in a controlled environment room with day/night temperatures of 22°C /18°C, 14 hour days, day/night humidities of 70%/80% and 450 μmol m^{-2}s^{-1} of light. Because *Oryza sativa* cv. Shiokari (the background for *d10* and *d3* mutants) was a temperate rice, it was possible to grow the rice plants in the same conditions used for wheat plants.

2.1.5 Soil strength apparatus

The investigation into soil strength effects on plant growth utilised the sand culture system described in Coelho-Filho et al. (2013) and shown in Figure 2.1. Rigid plastic tubes of 45cm in length and 15cm in diameter were placed on a base with a mesh lining that allows transfer of nutrient solution into the tubes. These tubes were filled with sand (Redhill T grade silica sand; Sibelco UK, Sandbach, UK) in such a way as to ensure sand was poured gradually and evenly into the nutrient solution, avoiding the formation of air pockets. To complete each sand column sand was poured onto the column above the top of the tube, nutrient solution was slowly poured onto the mound of sand to saturation and a plastic disc (that would be between the weight and the sand surface) was instantly pushed onto the sand column to ensure no air pockets formed in the sand above the surface of the tube. The finished sand surface was protruded 1.5cm above the plastic tube. The plastic disc on the surface of the sand enabled even distribution of weight applied from above and contained a hole in the middle to allow shoots to reach the surface.

Application of a foam weight (0.06kg) constituted the 'weak soil' treatment while application of a 17kg weight constituted the 'strong soil' treatment. Penetrometer measurements were not measured but are assumed to match those measured by Whalley et al. (1999) of 0.19MPa for weak treatment and 0.75MPa for strong. See Figure 2.1 for a visual representation of the strong soil apparatus.

A



B

Figure 2.1 Soil strength apparatus using sand columns in tanks of nutrient solution A) Schematic of apparatus used to grow plants in different soil strengths (image by Dr Richard Whalley), funnel for GA_3 application was an optional extra. B) Photograph of a newly planted soil strength experiment in a controlled environment growth room (with complete randomised block design).

2.1.6 Nutrient solutions

A final volume of approximately 70L of 1x nutrient solution was supplied in the reservoir of each tank (as seen in Figure 2.1B) for each substrate strength experiment. An initial 20L of nutrient solution was added to each tank and then nutrient solutions was added to individual plastic tubes to facilitate the building of the sand columns under nutrient solution (as the tubes are higher than the tanks). The final (1x) component concentrations for each nutrient solution were:

For wheat experiments:

2.0 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 4.0 mM KCl, 2.0 mM $MgSO_4$, 4.0 mM $CaCl_2.2H_2O$, 0.06 mM Si, 0.05 mM B, 0.05 mM Fe, 0.015 mM Mn, 0.0008 mM Zn, 0.003 mM Cu and 0.001 mM Mo. As used by Coelho-Filho et al. (2013).

For rice experiments:

1.5 mM $Ca(NO_3)_2.4H_2O$, 0.15 mM $CaH_4(PO_4)_2.H_2O$, 1mM KCl, 0.3 mM $MgSO_4.7H_2O$, 0.05 mM $C_{10}H_{12}FeN_2NaO_8{\cdot}3H_2O$, 0.05 mM H_3BO_3, 0.01 mM $MnCl_2.4H_2O$, 0.0001 mM $ZnSO_4.7H_2O$, 0.001 mM $CuSO_4.5H_2O$ and 0.0005 mM $H_2MoO_4.H_2O$. As used by Clark et al. (2000).

2.1.7 Plant measurements

Several physiological and developmental measurements were taken from plants growing under different soil strength regimes and for transgenic material under glasshouse conditions.

Tillering data were collected in the sub-categories of primary and secondary tillers. Primary tillers were defined as those tillers emerging from the axils of leaves on the main tiller/culm, secondary tillers emerged from the leaves of primary tillers. Counted tillers were at least 1mm above the ligule. Leaf blade lengths and widths were measured with a Perspex ruler.

Maximum root depth was assessed by gradually removing sand from the base of the sand column and noting the position of the deepest root tip (measured with a Perspex ruler).

Root/shoot fresh weight was determined by removing excess moisture with paper towels, placing the root or shoot of one plant in a pre-weighed metal tray, determining the weight with a digital balance (CP 4202 S; Sartorius, Göttingen, Germany) and subsequently subtracting the weight of the tray from the weight recorded. Root/shoot dry weight was determined by drying the material (in a pre-weighed metal tray) in an oven at 80°C for 48 or 72 hours and subsequently determining the weight with a digital balance. For non-GM material the drying oven used was a Hotbox Oven with fan – Size 3 (Gallenkamp). GM material was dried in a Unitherm Drying Oven (Russel-Lindsey Engineering Limited, Birmingham, England).

2.2 Molecular biology

2.2.1 DNA cloning and manipulation

2.2.1.1 Polymerase Chain Reaction (PCR)

PCR was used to amplify target DNA from template DNA using target-specific primers. Primers were designed to bind to specific DNA sequences, using the Primer3plus software (available at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and Geneious. PCR cycles typically consisted of 3 steps: denaturation (at 94°C), annealing (e.g. at 55°C) and extension (72°C) with an initial (2 min) denaturation step followed by 30-34 cycles of a denaturation (30 sec), annealing (30 sec) and extension (60 sec) steps (thermocycling), before a final extension (72°C) step of 7 minutes. Template DNA was purified gDNA (genomic DNA), cDNA (complimentary DNA) or pDNA (plasmid DNA) or pDNA amplified directly from plasmid in a bacterial colony. The DNA polymerase used depended on the purpose. When initially obtaining a product from gDNA or cDNA it was important to use a proofreading DNA polymerase. For more routine analytical PCR it was desirable to use a more economical/robust DNA polymerase with a slightly reduced proofreading capability. The optimal annealing temperature for a given reaction depends on a multitude of factors including primer size and characteristics, target GC content and $MgCl^{2+}$ concentration of the reaction. The duration of the extension step during thermocycling depended on the size of the amplification product and the capacity of the DNA polymerase used.

The proofreading DNA polymerase used was Phusion (New England Biolabs, Ipswich, U.S.A.). A typical 25µl reaction consisted of 5µl Phusion GC buffer (5x), 0.5µl dNTPs (10mM), 1.25µl forward primer (10µM), 1.25µl reverse primer (10µM), 2µl template gDNA (75ng/µl), 0.75µl DMSO (100%), 0.25µl Phusion DNA polymerase (2units/µl) and sterile distilled water up to the final volume. Phusion GC buffer contains $MgCl^{2+}$ at 7.5mM, giving a final concentration of 1.5mM but more can be added if needed. Reactions were set up on ice. Extension time was 15 seconds per kb of target product. Denaturation temperature for Phusion was 98°C.

The standard DNA polymerases used were GoTaq Flexi DNA polymerase, GoTaq G2 DNA polymerase and GoTaq Hot Start DNA polymerase (Promega, Madison, U.S.A.). The latter differs to the first in that it will not function until an initial 2 minute 94-95°C denaturation step (which inactivates an antibody that prevents bioactivity) has been performed. A typical 25µl reaction for GoTaq Flexi or GoTaq Hot Start DNA polymerase consisted of 5µl Green GoTaq Flexi buffer (5x), 0.5µl dNTPs (10mM), 1.5µl $MgCl^{2+}$ (25mM), 0.5µl forward primer (10µM), 0.5µl reverse primer (10µM), 2µl template gDNA (75ng/µl), 0.16µl GoTaq DNA polymerase (5units/µl). The series of GoTaq G2 uses the GoTaq Reaction buffer, which already contains $MgCl^{2+}$ at 7.5mM so that the final concentration of $MgCl^{2+}$ in a 1x reaction is 1.5mM (equal to the final concentration in the typical reaction for GoTaq Flexi or GoTaq Hot Start DNA polymerases). Reactions were set up on ice. Extension time was 60 seconds per kb of target product.

2.2.1.2 Agarose gel electrophoresis

Agarose gels were made by addition of agarose to 1xTBE (89mM Tris base, 89mM boric acid, 2mM EDTA (pH 8.3)) or 1xTAE (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA (pH 7.6)) prior to melting in a microwave oven. Standard agarose concentrations ranged from 1-2% w/v. Standard gel volumes ranged from 70-260ml. Ethidium bromide (at 10mg/ml in H_2O) (Sigma-Aldrich Co., St Louis, U.S.A.) was added at a concentration of 1µl per

100ml of gel (just before the gel is poured but after heating). Samples either had loading dye (15% w/v ficoll, 0.25% w/v bromophenol blue and 0.25% w/v xylene cyanol FF in sterile distilled water) added to one-sixth the final volume or were loaded directly from PCR reactions run in Green GoTaq Flexi buffer (Promega, Madison, U.S.A.) (which already contains blue and yellow dyes) to allow migration of nucleic acids through a gel to be monitored. Agarose gels were run at 75-150v for 30-120 minutes or until adequate separation of fragments had been achieved. Nucleic acids (bound to ethidium bromide) were visualised under UV fluorescence and an image was taken with the Syngene GelDoc imaging system (Synoptics Ltd., U.K.). Band sizes were determined against DNA ladders added to the gel (GeneRuler 100bp DNA Ladder or GeneRuler 1kb DNA Ladder) (Thermo Fisher Scientific, Waltham, U.S.A.).

2.2.1.3 Gel extraction

Gel fragments containing DNA of expected sizes were excised from agarose gels with a clean razor blade and transferred to a clean Eppendorf tube. DNA was extracted using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions.

2.2.1.4 Restriction digests

Restriction endonucleases were routinely used during the subcloning process to cut both strands of dsDNA at specific sites of recognition sequence and to isolate specific sequences for further manipulation. Restriction enzymes were obtained from either Promega (Madison, U.S.A.) or New England Biolabs (Ipswich, U.S.A.), with enzymes working in appropriate buffers from either company. Single enzyme restriction digests were conducted in either 20µl or 50µl reactions. An example single digest 20µl reaction contained 2µl Promega Buffer D (10x), 0.2µl acetylated BSA (10µg/µl), 5µg DNA (e.g. 3.5µl of 1.5µg/µl DNA), 3µl of *Bgl*II (10units/µl) and was made up to final volume with sterile distilled water. A typical 20µl double digest contained 2µl Promega Buffer C (10x), 0.2µl acetylated BSA (10µg/µl), 1µg DNA, 0.5µl *Bam*HI (10units/µl), 0.5µl *Bgl*II (10units/µl) and was made up to final volume with sterile distilled water. Reactions were left at the required temperature (usually 37°C) for a suitable period of time (typically overnight).

2.2.1.5 Dephosphorylation and recovery

To prevent recircularization of linearized plasmids, 5' phosphate groups were removed from DNA termini. This dephosphorylation was achieved with calf intestinal alkaline phosphatase (CIAP) (Promega, Madison, U.S.A.). Next, 10µl of 10x CIAP buffer was added to a 50µl restriction digest reaction, in addition to 39µl of sterile distilled water and 1µl (1unit/µl) CIAP. This was incubated at 37°C for 30 minutes, a further 1µl of CIAP (1unit/µl) was added to the reaction and the reaction was again incubated at 37°C for 30 minutes. The DNA was recovered using a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions (except that the starting solution was not from a PCR reaction).

2.2.1.6 Ligation

The joining of DNA termini was achieved with T4 DNA ligase, used according to manufacturer's instructions (New England Biolabs, Ipswich, U.S.A.). T4 DNA ligase catalyses the formation of phosphodiester bonds beween 5' phosphate and 3' hydroxyl DNA termini. The ligation reaction mix consisted of 1.5 µl (approx. 100-500ng) linearized (and dephosphorylated) construct/plasmid, 1µl of 10x T4 DNA ligase buffer, 1-3µl (approx. 100-500ng) insert DNA (non-dephosphorylated), 1 µl T4 DNA ligase and sterile distilled water to a final volume of 10 µl. Multiple ratios of plasmid to insert were tested for each ligation step. Control ligation reactions lacking insert DNA were also set up. Ligation reaction conditions were at least one cycle of 18°C for 30 minutes, 22°C for 30 minutes and 20°C for 30 minutes.

2.2.1.7 Plasmid DNA purification

Plasmid DNA was purified from overnight liquid *E. coli* cultures using the QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany), according to manufacturer's instructions.

2.2.2 Bacterial transformation and culturing

2.2.2.1 Bacterial culture media

Sterile 2xYT (16g L^{-1} bacto-tryptone, 10g L^{-1} bacto-yeast extract, 5g NaCl L^{-1}) liquid was used as the standard bacterial growth medium for *E. coli* harbouring plasmids used in this study, with appropriate addition of required antibiotic(s) to maintain selection pressure. For semi-solid media, 2xYT agar, 15g L^{-1} bacto-agar was added prior to autoclaving and poured onto sterile petri dishes following addition of appropriate antibiotics as necessary. Further media were required for more specialist procedures: sterile SOB (20g bacto-tryptone L^{-1} , 5g bacto-yeast extract L^{-1}, 0.5g NaCl L^{-1} pH 7.0) , made up in deionized water and autoclaved; Sterile SOC medium (as SOB with the addition of 20ml of sterile 1M glucose solution L^{-1} following autoclaving the SOB); sterile TB (Terrific Broth) (12g bacto-tryptone L^{-1}, 24g bacto-yeast extract L^{-1}, 4ml glycerol L^{-1}) and was autoclaved. After autoclaving and having cooled to approx. 60°C, 100ml sterile solution of 0.17M KH_2PO_4, 0.72M K_2HPO was added to 900ml of TB solution. Media recipes are as described in Sambrook et al. (1989). Basic media were prepared by members of the prep kitchen and additions of antibiotics and other chemicals were added by the author as required.

2.2.2.2 Bacterial culture on plates and colony screening

Agar plates were made by melting 2xYT agar in a microwave oven, cooling to 55°C in a water bath and pouring into 9cm Petri dishes in a sterile flow hood. Solidified plates were placed in a sterile plastic bag while within the sterile flow hood and the bag was sealed. Inoculum was in the form of liquid culture or re-struck colonies from previous agar plates. Plates were stored lid down at 4°C. Standard antibiotic concentrations: 100µg/ml ampicillin; 17µg/ml chloramphenicol. 5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase (X-gal) was added to the medium at a standard concentration of 40µg/ml when blue/white colony selection was required. When using the strain provided by in the Strata Clone Blunt End cloning kit (containing the *lacZΔM15* mutation) it was not necessary to add IPTG to induce expression of *lacZ'*. Addition of antibiotics or X-gal was conducted in the sterile flow hood immediately after removal of the molten agar from the 55°C water bath. Plates were either inoculated with liquid-cultured bacteria (50-150 µl spread over the surface) or re-struck surviving white colonies (and some light blue colonies) of transformation events. Growth of *E.coli* was conducted overnight at 37°C.

2.2.2.3 Production of chemically competent cells

E. coli cells of strain DH5α were cultured on 2xYT agar plates at 37°C overnight. 10-12 large colonies were picked with sterile wooden sticks and used as inoculum for 250ml SOB in a 1L flask. The liquid culture was then incubated at 19°C for approximately 40 hours until the OD_{600} = 0.5-0.6, with a WPA lightwave spectrophotometer (WPA, Cambridge, UK) used to check OD. The flask was placed on ice and the content was equally distributed between six centrifuge tubes and cells were pelleted by centrifugation at 2500g for 10 minutes at 4°C (cells were kept on ice as much as possible and the centrifuge was pre-chilled). The supernatant was discarded and the pellets were resuspended in 80ml (in total) of ice-cold TB and stored on ice for 10 minutes. Cells were again pelleted by centrifugation at 2500g at 4°C. Pellets were resuspended in 20ml ice-cold TB and 1.4ml DMSO. 50µl aliquots were prepared in pre-chilled 1.5ml microcentrifuge tubes (using pre-chilled 200 µl pipette tips that had been cut with a razor blade and autoclaved).

2.2.2.4 Transformation of *E. coli*

To transform plasmids into *E. coli* hosts, 50µl aliquots of chemically competent *E. coli* cells (strain *DH5a*) were thawed on ice; 2µl of ligation mix (post ligation reaction) added to the competent cells (with a 200µl cut pipette tip) and the reaction left on ice for 45-60 minutes. The reaction was then heat shocked at 42°C for 45 seconds to encourage the uptake of exogenous plasmid. A 2 minute recovery time on ice was followed by addition of 500µl of room temperature SOC medium and incubation at 37°C for 60 minutes with rotation at 180rpm in an incubator/shaker. Finally, the transformation mix was used to inoculate agar plates (2xYT) at 150µl, 100µl and 50µl plate^{-1} which were then incubated, inverted, overnight at 37°C.

Transformation of competent *E.coli* cells of strain ER2925 was carried out in the same way as above, except the initial cell aliquots were of 20µl. Competent ER2925 aliquots were kindly supplied by Dr Pippa Madgwick.

2.2.3 DNA extraction from plants

DNA was extracted from plant samples in a 96 well format using a PVP extraction method. The method was developed by Dr Simon Vaughan and Mrs Aakriti Wanchoo-Kohli. Up to 5cm of leaf tissue was collected in deep-well 96-well blocks (on ice). The samples were freeze dried for 24-48 hours. Two 3mm-ball bearings were added to each sample well. Samples were mechanically ground twice for two minutes using either a TissueLyser (QIAGEN GmbH, Hilden, Germany) at 30 rev per second or a 2010 Geno/Grinder (SPEX SamplePrep, Stanmore, UK) at 1500 rpm. 600µl DNA extraction buffer was added to each sample (using a Multidrop reagent dispenser; Thermo Fisher Scientific, Waltham, U.S.A.) and samples were mixed mechanically for 20 seconds before incubation in a heated shaker at 65°C and 500 rpm. DNA extraction buffer contained 100mM Tizma base, 1M KCl and 10mM EDTA, 7.5g/L PVP-40 and 3.6g/L sodium bisulphite (all chemicals from Sigma-Aldrich, St Louis, U.S.A.). The PVP-40 and sodium bisulphite were added on the day of use. After incubation, the plates were spun for 10 seconds at 2,500 rpm in a Sigma 4-15C centrifuge (QIAGEN GmbH, Hilden, Germany). 200µl 5M potassium acetate at pH 5.8 (as 294g/L potassium acetate and 115ml/L glacial acetic acid) was added to each sample with a Multidrop reagent dispenser, mixed mechanically for twenty seconds and then centrifuged in a Sigma 4-15C centrifuge for 10 minutes at 2,500rpm. 300µl supernatant was transferred to 330µl isopropanol (pre-chilled to -20°C), inverted and incubated at room temperature for 10 minutes. DNA was pelleted by centrifuging in a Sigma 4-15C centrifuge for 10 minutes at 2,500rpm. Supernatant was discarded and 500µl 70% ethanol was added to each well with a Multidrop reagent dispenser. Plates were centrifuged in a Sigma 4-15C centrifuge for 5 minutes at 2,500rpm. Supernatant was discarded and DNA pellets were dried at 40°C for 30 minutes. Samples were resuspended in 200µl TER (TE buffer containing RNAse A at 50µg/ml) added with a Multidrop reagent dispenser, followed by incubation at 50°C for one hour, with mixing every 15 minutes. DNA was stored overnight at 4°C and then at -20C.

2.2.4 RNA methods

RNA was extracted from plant material using one of two methods. The first RNA extraction method was the Hot Phenol method, based on the method described by Verwoerd et al. (1989) and developed further by Dr Peter Buchner at Rothamsted Research. The second is a kit-based method. These are described below.

2.2.4.1 Hot Phenol RNA extraction method

Briefly, frozen plant material was ground to a powder under liquid nitrogen and transferred to a 2ml safe-lock micro centrifuge tube. Stock solutions were made in advance and treated with DEPC (0.1%) prior to autoclaving, with the exceptions of the extraction buffer (which was not DEPC treated) and any phenol-containing solutions (which were made on the day of use). Tris-HCl and LiCl solutions were made with DEPC-treated water and added to the extraction buffer after it had been autoclaved. Required solutions: extraction buffer (0.1M Tris/HCl, 0.1M LiCl, 1% SDS, 10mM EDTA, pH8), 3M sodium acetate (pH5.2), 4M LiCl, 70% ethanol (made with DEPC-treated water), RNAse-free DNAse mixed according to manufacturer's instructions (Promega, Madison, U.S.A.), ethanol (100%) and DEPC-treated water. The following work was carried out in a fume hood. Phenol/extraction buffer (1:1) was heated to 80°C in a water bath. 1ml of hot (80°C) phenol/extraction buffer added to the frozen tissue powder and the tube was vortexed to homogenisation, with an additional 30 seconds of vortexing. 500µl chloroform/IAA (isoamyl alcohol) was added and the tube was vortexed for

30 seconds. The sample was then centrifuged at 13,500 rpm for 5 minutes at 4°C. The aqueous phase was transferred to a fresh 2-ml tube and an equal volume (usually 1 ml) chloroform/IAA was added, followed by vortexing for 30 seconds and centrifugation for 5 minutes at 4°C. The aqueous phase was then transferred to a fresh 2ml tube and mixed well with an equal volume of 4M LiCl, followed by incubation overnight at 4°C. The next day, the sample was centrifuged at 13,500 rpm for 20 minutes at 4°C and the supernatant discarded

The remaining pellet was then washed with 1ml 70% ethanol, the supernatant discarded and the pellet allowed to dry. Once the pellet was fully dried 150 µl DNase mix (15 µl 10x DNase buffer, 130 µl DEPC-treated water, 5 µl RNase-free DNAse) was added and the sample left on ice for 15-30 min prior to incubation at 37°C for 30 minutes. Subsequently 150 µl of DEPC-treated water was added and the reaction mixed before the additions of 300 µl phenol/chloroform/IAA (25:24:1). The reaction was then vortexed and centrifuged at 13,500 rpm for 5 minutes and the aqueous phase was transferred to a new 2-ml tube. A further extraction was then conducted by adding 300 µl Chloroform/IAA (24:1) and repeating the extraction procedure above, the resulting aqueous phase was transferred to a fresh 1.5-ml tube, 30 µl of 3M sodium acetate (pH 5.2) and 750 µl ethanol added and the reaction left overnight at -20°C. The reaction was then centrifuged at 13,500 rpm for 20 minutes and the pellet washed with 1ml 70% ethanol. Supernatant was discarded and the pellet was dried prior to dissolving in 100 µl DEPC-treated water.

2.2.4.2 RNeasy Kit RNA extraction method

The second method for RNA extraction used was the RNeasy Mini Kit, used according to manufacturer's instructions (QIAGEN GmbH, Hilden, Germany). Additionally, an on-column DNase treatment was conducted with the optional RNase-free DNase Set (QIAGEN GmbH, Hilden, Germany), as per manufacturer's instructions. Sample material was flash frozen in liquid nitrogen and stored at -80°C until grinding in pestles and mortars (that had been baked and autoclaved) under liquid nitrogen prior to RNA extraction.

2.2.4.3 cDNA synthesis for qRT-PCR

After RNAse extraction (with described DNase treatment) a second, cautionary, DNAse treatment was conducted on samples prior to cDNA synthesis. There were two DNase methods used. The first was treatment of the entire RNA aliquot with Turbo DNAse (Thermo Fisher Scientific, Waltham, U.S.A.), according to manufacturer's instructions (with the rigorous option of adding a further 1 µl of DNase after the initial 30 minutes of incubation). The alternative DNase method used was with RQ1 DNase (Promega, Madison, U.S.A.). This included taking a 2µg aliquot of an RNA sample and drying it down in a vacuum concentrator prior to following manufacturer's instructions.

After removal or deactivation of the DNase (depending on the method used), the RNA was quantified using either a Nanodrop ND-1000 or a Nanodrop ND-2000c spectrophotometer (Thermo Fisher Scientific, Waltham, U.S.A.) and the integrity of the RNA checked by running 500 ng on a 1xTBE agarose electrophoresis gel and inspecting the ethidium bromide-stained gel for the presence of the expected ribosomal RNA bands.

When using the Turbo DNase, an aliquot of RNA (e.g.1.5 µg) was made up to 15 µl with nuclease-free water, 10 µl of this was used in the RT (reverse transcriptase) reaction and 2.5 µl was used in the nonRT control reaction. When using the RQ1 DNase, an aliquot of RNA (e.g. 1.5 µg) was down in a vacuum concentrator and then the sample had 15 µl nuclease-free water added.

The reverse transcriptase used for cDNA synthesis was Superscript III Reverse Transcriptase (Invitrogen/ Thermo Fisher Scientific, Waltham, U.S.A.). Each RT reaction consisted of 0.5 µl of Oligo dTV [V, any deoxynucleotide except T, was placed at the end of an oligo dT] (100 µM) (Eurofins Genomics, Ebersberg, Germany), 1 µl dNTPs (10mM), 10 µl DNase-treated RNA (e.g. 1 µg) and 1.5 µl nuclease-free water. Each nonRT reaction consisted of 0.25 µl Oligo dTV (100 µM), 0.5 µl dNTPs (10mM), 2.5 µl DNase-treated RNA (e.g. 250 ng) and 3.25 µl nuclease-free water. A control reaction with water instead of RNA was performed for RT and nonRT conditions, for each batch of cDNA samples produced. The pre-RT mixes were heated at 65°C for 5 minutes in a thermocycler (PTC-100; MJ Research, Inc., Waltham, U.S.A.) and put on ice for 2 minutes. Subsequently, 4 µl First Strand Buffer (5x), 1 µl DTT (0.1M), 1 µl nuclease-free water and 1 µl Superscript III (200 U/µl) were added to RT reactions. 2 µl First Strand Buffer, 0.5 µl DTT (0.1M) and 1 µl nuclease-free water were added to the half-scale non-RT reactions.

Samples were then put in the thermocycler (PTC-100; MJ Research, Inc., Waltham, U.S.A.) and heated to 25°C for 5 minutes, 50°C for 60 minutes, 70°C for 15 minutes and then held at 4°C until transfer to -20°C for storage.

2.2.4.4 qRT-PCR

A 1/20 dilution of cDNA was made for each sample from the reactions described in 2.2.4.3, typically 10 µl cDNA in 190 µl nuclease-free water and aliquoted into to a strip of eight PCR tubes to serve as template in qRT-PCR reactions. A single 20 µl qRT-PCR reaction contained 5 µl primer mix (0.24 µl of 10 µM forward primer stock, 0.24 µl of 10 µM reverse primer stock, 2.4 µl 25 mM $MgCl^2$, 0.02 µL 100X reference dye (Rox) and 2.1 µl nuclease-free water); 5 µl 1:20 cDNA (section 2.2.4.3) and 10 µl SYBR mix (SYBR Green JumpStart Taq ReadyMix without $MgCl_2$ in buffer, Sigma-Aldrich Co., St Louis, U.S.A.). A 100x stock of primer mix was made dispensed in 60 µl aliquots eight-strip PCR tubes and stored at -20°C. Only one batch of a given primer pair was used within any experiment. Aliquots of SYBR (SYBR Green JumpStart Taq ReadyMix without $MgCl_2$ in buffer, Sigma-Aldrich Co., St Louis, U.S.A.) sufficient for the plate(s) were also dispensed into a strip of eight PCR tubes and stored at -20^{o}C. White 96-well, semi-skirted PCR plates (STARLAB International, Hamburg, Germany) and qPCR adhesive seals (4titude, Wotton, U.K.) were used for qRT-PCR. Appropriate multichannel pipettes were used to transfer (from thawed aliquots) 10 µl SYBR into each well of the plate, followed by 5 µl of relevant primer mix to relevant wells, followed by 5 µl relevant cDNA to relevant wells. For each RT sample, the nonRT sample was tested at least once for each primer pair used. Additionally the no-RNA controls of each cDNA synthesis batch were tested. Once a plate was completed and sealed, the plate was spun briefly in a either a Labnet Mini Plate Spinner MPS 1000 (Sigma-Aldrich Co., St Louis, U.S.A.) or a Sigma 4-15C centrifuge (QIAGEN GmbH, Hilden, Germany) and run on a 7500 Real Time PCR System (Applied Biosystems, part of Thermo Fisher Scientific, Waltham, U.S.A.). Primers were optimised to run on a standard schedule of 95°C for 10 minutes (x1 round), 95°C 15 seconds and then 60°C 1 minute (x40-45 cycles) followed by a default dissociation stage.

2.2.4.5 qRT-PCR analysis

For analysis of qRT-PCR data, expression values were determined using threshold cycle (Ct) values obtained for each replicate using the Applied Biosystems 7500 software v2.01 (Life Technologies Corporation, California, U.S.A.) and individual PCR efficiencies calculated using the LinRegPCR programme v12.3 (HFRC, Amsterdam, the Netherlands (Ruijter et al., 2009; Tuomi et al., 2010)). Target gene expression was normalised against geometric means of expression of two or three reference genes.

Relative Quantity (RQ) values were calculated for individual reactions using the formula (RQ= Efficiency^-Ct). For each gene (primer pair) technical replicates were then averaged to provide the RQ values for each sample. A Normalised Relative Quantities (NRQ, also known as NE – Normalised Expression) value was calculated by dividing the RQ of the target genes against the geometric mean of RQs of multiple reference genes, where the geometric mean of three reference genes was calculated as (RQref1x RQref2 x RQref3)^(1/3). Biological replicates were not measured for the data presented in this thesis, thus statistical analysis was inappropriate.

2.2.5 Transformation of wheat by bombardment

All wheat transformation and initial PCR analysis was carried out by the Cereal Transformation Unit at Rothamsted Research: Amanda Riley carried out the bombardment, Melloney St Leger the leaf sampling and Caroline Sparks confirmed transformation of plants by PCR. The work was in accordance with the materials and methods described in detail in Sparks and Jones (2014). In brief, gold particles were coated with plasmid DNA (1 mg/ml) [a mixture of the RNAi construct plus a vector containing the selectable marker gene *bar*] and bombarded into immature wheat embryos with a Bio-Rad PDS-1000/He particle gun. After several rounds of selection in tissue culture, surviving plantlets were potted on and grown in soil. These T_0 plants were grown to provide T_1 seed.

2.3 Hormone extraction and quantification

2.3.1 GA and ABA extraction and quantification

Leaf four (fourth leaf in Figure 1.3B) of wheat plants samples were individually harvested while still in the process of elongation from plants grown in weak and strong soil under conditions described in section 2.1.5, weighed and placed in a 15-ml plastic tube (Greiner) and immersed in liquid

N_2. Fresh weights of individual leaves ranged from 0.05-0.41g. Samples were subsequently stored at -80°C prior to lyophilisation. Leaves were pooled (six leaves per pool, three pools/replicates per weak and strong soil treatment) and sample weights were recorded. The lyophilised and pooled sample weights ranged from 0.130g to 0.299g. Samples were then homogenized in liquid N_2 with a pestle and mortar. GA content was analysed as described by Griffiths et al. (2006) with the modifications that the GC-MS equipment used in the current study was a Thermo Finnigan MAT95 GC-MS and ABA was also quantified (ABA was analysed as the methyl ester prior to the addition of *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA)). Four samples of leaf material (two replicates per treatment of plants grown in weak or strong substrate) were processed together. The two remaining samples (one replicate of each treatment) were processed in a further round of the method.

In brief, samples were extracted overnight at 4°C in 80% methanol-water (circa 100 ml) after addition of ^3H- and $^2H_2$-labelled GA and $^2H_3$-labelled ABA standards. An acidic fraction containing GAs and ABA was obtained by chromatography on QAE Sephadex anion-exchange columns and C_{18} Sep-Pak cartridges. Samples were then methylated in ethereal diazomethane and partitioned between ethyl acetate and water, the organic phase passed through a Bond Elut NH_2 cartridge and reduced to dryness. The samples were separated by reverse-phase HPLC with a C_{18} column. Grouped fractions (based on the retention times of the ^3H-GA standards) were trimethylsilylated (by heating with MSTFA) and analysed by GC-MS with selected ion monitoring. Concentrations of endogenous GAs and ABA were determined by comparison to established calibration curves for unlabelled and deuterated analytes (where peak area ratios were plotted against a range of molar ratios of the two compounds).

2.3.2 Strigolactone extraction

The following method was used to simultaneously extract three strigolactones (−)-ent-2'-epi-5-deoxystrigol (herein referred to as (−)-ent-2'-epi-5DS), orobanchyl acetate and orobanchol. The extraction method was developed by Dr Yoshiya Seto at Tohoku University, Sendai, Japan and adapted for local equipment at Rothamsted Research by Prof. Peter Hedden and the author.

Fresh root samples were weighed and added to enough acetone to cover the tissue (approximately 10x v/w). Samples $\leq$2 g were added to sterile 50-ml PP (polypropylene) tubes (Greiner Bio-One GmbH, Frickenhausen, Germany), while samples >2 g were added to 250-ml glass jars (Wheaton wide-mouth bottles, Sigma-Aldrich Co., St Louis, U.S.A.). Samples in 250-ml glass jars (>2 g) were cut into small pieces with scissors (under the acetone) and then homogenised with a Polytron PT3100 fitted with a medium sized dispersing aggregate (PT-DA 3012/2). After homogenisation the sample volume was measured in a glass measuring cylinder and a volume equivalent to 1 g Fresh Weight (FW) of sample was transferred to a sterile 50-ml PP tube. Internal standards (d_1-(−)-ent-2'-epi-5DS, d_1-orobanchol, d_1-orobanchyl acetate) were added to the acetone extracts in the 50-ml PP tubes. Samples $\leq$2 g were homogenized with a Polytron PT3100 with medium sized aggregator after addition of the internal standards. 100 pg (10 µl of 10 pg/µl solution) of internal standards were added to wild-type (WT) and *d10* mutant samples. 1000 pg (10 µl of 100 pg/µl solution) were added to *d3* mutant samples.

Samples were then filtered with Bond Elute reservoirs. Filtrates were then taken to dryness under N_2 gas, dissolved in sterile deionized water (4 ml) and extracted with ethyl acetate (2x 2 ml). The combined ethyl acetate phases were taken to dryness under N_2. The extracts were then dissolved in 150 µl acetone and 850 µl water was added to bring them to 15% acetone. The samples were loaded on to Oasis HLB 1cc cartridges (Waters, Wexford, Ireland), washed with 2x 1 ml 15% acetone and eluted with 3x1 ml 80% acetone. After evaporating to dryness under N_2, the extracts were dissolved in 150 µl ethyl acetate before adding 850 µl *n*-hexane to make them up to ethyl acetate:*n*-hexane (15:85), which was loaded onto Sep-Pak Silica 1cc cartridges (Waters, Wexford, Ireland). After washing with ethyl acetate:*n*-hexane (15:85; 2x1 ml), (−)-ent-2'-epi-5DS and orobanchyl acetate were eluted with ethyl acetate:*n*-hexane (35:65; 3x1 ml), followed by elution of orobanchol with ethyl acetate:*n*-hexane (50:50; 3x1 ml). The two pools of eluates were taken to dryness under N_2, dissolved in acetone (2x 250 µl), transferred to glass ampules and then evaporated to dryness under N_2 for storage and transfer.

2.3.3 Strigolactone quantification

Samples (extracted as described section 2.3.2) were dissolved in ethyl acetate (2x100 µl), transferred to LC-MS/MS sample tubes (300 µl screw neck PP vial) (Waters, Wexford, Ireland) and then dried down with a vacuum concentrator at 40°C. Extracts were then dissolved in 15 µl acetonitrile followed by addition of 15 µl deionized water to take the solution to 50% acetonitrile. LC-MS/MS analysis of (−)-ent-2'-epi-5DS, orobanchol and orobanchyl acetate was carried out using a quadrupole/time-of-flight tandem mass spectrometer (TripleTOF5600; AB SCIEX, Framingham, USA) and ultra-high performance liquid chromatography (Nexera; Shimadzu, Kyoto, Japan), with an Acquity UPLC BEH-C18, ϕ2.1×50 mm, 1.7 µm column (Waters). The analysis method is the same as described in the supplementary information of Seto et al. (2014), where further details can be found. The positive mode for ionization was used, so that chemicals were detected as m/z (mass/charge) at $[M+1]^+$. Parent ions selected with quadrupole mass spectrometry were 331 m/z for (−)-ent-2'-epi-5DS, 347m/z for orobanchol and 349 m/z for orobanchyl acetate. The m/z values for 6'-d_1 labelled equivalents equaled m/z of endogenous chemical +1. Fragment ions of 234.1 m/z for endogenous (−)-ent-2'-epi-5DS and 235.1m/z for [6'-d_1]-(−)-ent-2'-epi-5DS. Quantification of strigolactones was performed by Dr Yoshiya Seto by calculating strigolactone quantities from calibration curves for known quantities.

2.3.4 Global Hormone Profiling

2.3.4.1 Preparation for Global Hormone Profiling

Approximately 1 g Dry Weight (DW) (exact weights were recorded) samples of freeze dried root and leaf material were weighed into 50-ml PP tubes (Corning; Corning, U.S.A.) with a digital balance (CPA225D; Sartorius, Göttingen, Germany), the lids were closed and the samples were stored at -40°C until analysis. Hormones tested were salicylic acid (SA), jasmonic acid (JA), jasmonate-isoleucine conjugate (JA-Ile), trans-zeatin (tZ), dihydrozeatin (DHZ), isopentenyladenine (iP), indole acetic acid (IAA), abscisic acid (ABA) and fourteen GAs (GA_{12}, GA_{15}, GA_{24}, GA_9, GA_4, GA_{34}, GA_{51}, GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, GA_8, GA_{29}). Separate major mixes of internal standards (IS) were made for root and leaf samples and a further mix of SA, JA and JA-Ile was made for both root and leaf samples. Most internal standards were labelled with a particular number of deuterium atoms, while JA-Ile was labelled with ^{13}C. 510 µl of root major IS mix gave 50 ng d_2-IAA, 5 ng d_6-ABA, 1 ng d_5-tZ, 1 ng d_3-DHZ, 500 pg d_6-iP, 500 pg of each of fourteen d_2-GAs. 50 µl of the SA, JA and JA-Ile IS mix for roots provided 50 ng d_6-SA, 500 ng d_2-JA, 50 ng $^{13}C_6$-JA-Ile. 510 µl of leaf major IS mix gave 50 ng d_2-IAA, 50 ng d_6-ABA, 1 ng d_5-tZ, 1 ng d_3-DHZ, 500 pg d_6-iP, 500 pg of each of fourteen d_2-GAs. 50 µl of the SA, JA and JA-Ile IS mix for leaves provided 500 ng d_6-SA, 50 ng d_2-JA and 5 ng $^{13}C_6$-JA-Ile.

2.3.4.2 Extraction for Global Hormone Profiling

The details of the global hormone extraction method were developed by Takafumi Shimizu and Yuri Kanno at the Riken Institute, Yokohama, Japan.

Overall, four fractions were collected during the global hormone extraction method: an acidic & neutral fraction was sub-divided into a GA fraction and an IAA & ABA fraction, a basic fraction was collected and a SA, JA & JA-Ile fraction was also collected.

35 ml of aqueous acetonitrile (80%) containing 1% acetic acid was added to the sample. 510 µl of relevant (leaf or root) major IS mix was added to the sample. The sample was vortexed, covered in foil and left to stand for four hours at 4°C. Subsequently, the sample was centrifuged at 3,000 rpm and supernatant was transferred to a new 50-ml PP tube. 15 ml of aqueous acetonitrile (80%) containing 1% acetic acid was added to sample remnants. The sample was then vortexed, covered with foil and left at 4°C for one hour before centrifuging at 3,000 rpm and the supernatants combined. The sample was dried under N_2 gas (overnight and some of the following day). 10 ml of aqueous methanol (50%) was added to the sample before vortexing three times. **SA, JA & JA-Ile fraction taken:** 100 µl of the sample was transferred to a 2-ml microcentrifuge tube for assay of SA, JA and JA-Ile and 50 µl of the SA/JA/JA-Ile IS mix was added prior to being dried down in a vacuum concentrator and then stored at 4°C. **Main extraction continued:** To the remaining 9.9 ml of the sample, 10 ml of hexane was added before vortexing, centrifuging at 5,000 rpm for 5 minutes and discarding supernatant. A further 10 ml of hexane was then added to the sample and vortexing, centrifugation and discarding of supernatant were repeated. The sample was dried down under N_2 gas (ca. 4 hours). An HLB (6cc) cartridge (Waters) was washed with 6 ml acetonitrile, 6 ml methanol and then equilibrated with 6 ml aqueous

acetic acid (1%). The sample was taken to ca. 4 ml with distilled water and loaded to the conditioned HLB cartridge. 2 ml of aqueous acetic acid (1%) was added to the remaining sample, vortexed and loaded to the HLB cartridge to increase recovery. Sample was eluted into new 50-ml PP tubes with aqueous acetonitrile (80%) containing 1% acetic acid (4 ml x3), before drying down under N_2 gas (overnight). An MCX (3cc) cartridge (Waters) was washed with acetonitrile (3 ml), then methanol (3 ml) before being regenerated with 0.1M hydrochloric acid (1.5 ml). Litmus paper was used to check that through-flow was pH 1-2. 2 ml aqueous acetic acid acetic acid (1%) was added to the dry sample, before vortexing and loading onto the conditioned MCX cartridge. A further 3 ml aqueous acetic acid (1%) was added to the sample vial, vortexed and added to the MCX cartridge to increase recovery. **Acidic & neutral fraction taken:** Acids and neutral chemicals were eluted from the MCX cartridge with aqueous acetonitrile (80%) containing 1% acetic acid (3 ml x2) which were pooled in a glass tube. The acid and neutral extract was mixed by pipetting and was dried down at 40°C in a vacuum concentrator to <1 ml (to avoid problems with redissolving a dry sample), covered with foil and left at 4°C (overnight).

Basic fraction taken: The MCX cartridge was washed (in a fume hood) with aqueous ammonia (5%) (3 ml), prior to elution of bases with aqueous ammonia (5%) and acetonitrile (60%) solution. The basic fraction was dried down under N_2 gas until at least 1 ml had gone (to ensure ammonia had been removed) before being completely dried down at 40°C in a vacuum concentrator, covered with foil and left at 4°C (overnight).

Acidic & neutral extraction continued: A WAX (3cc) cartridge (Waters) was washed with acetonitrile (3 ml) then methanol (3 ml), followed by equilibration with 0.1M potassium hydroxide (1.5 ml). Litmus paper was used to check that through-flow was pH 11. The WAX cartridge was then washed with 3 ml aqueous acetic acid (1%). For the acidic and neutral extract, 1 ml aqueous acetic acid (1%) was added, mixed by pipetting and then loaded to the conditioned WAX cartridge. 3 ml aqueous acetic acid (1%) was added to the acidic and neutral extract, mixed by vortexing and loaded to the cartridge. The cartridge was washed with 3 ml aqueous acetic acid (1%) and then aqueous acetonitrile (80%) (3 ml x2). The sample was eluted with aqueous acetonitrile (80%) containing 1% acetic acid (3 ml x2). The eluate was mixed by pipetting and 600 µl (10% of volume) was transferred to a new glass tube. **GA fraction and IAA & ABA fractions separated:** The smaller fraction (10%) (for IAA and ABA) and remaining 90% (for GAs) of the eluate were then dried down at 40°C in a vacuum concentrator.

Basic extraction continued: For the basic extract, 300 µl of methanol was added, vortexed and 150 µl was transferred to a well in a polypropylene (PP) plate (Agilent) before being dried down in a vacuum concentrator. 100 µl of methanol was added to the 10% fraction of acidic and neutral extract, vortexed and transferred to a well in a PP plate before being dried down in a vacuum concentrator.

GA extraction continued: The 90% (for GAs) fraction of acidic and neutral extract was further purified on a silica cartridge. The Sep-pak (3cc) cartridge (Waters) was washed with 3 ml chloroform (with any remaining in cartridge after running through pushed out with a syringe), then equilibrated with 3 ml of 50% chloroform 50% ethyl acetate containing 1% acetic acid. 2 ml of 50% chloroform 50% ethyl acetate containing 1% acetic acid was added to the 90% fraction of acidic and neutral extract, vortexed, sonicated and loaded to the Sep-pak cartridge (collecting the eluate). The Sep-pak cartridge was eluted with 2 ml 50% chloroform 50% ethyl acetate containing 1% acetic acid twice (with the first 2 ml also being used to load any remaining sample in the previous tube). The eluate was dried down in a vacuum concentrator, covered with foil and left at 4°C. Methanol (200 µl x2) was added to the sample, vortexed and transferred to a well of a PP plate, before being dried down in a vacuum concentrator.

Three of the four fractions (basic extract containing cytokinins, 10% fraction of acidic and neutral extract containing IAA and ABA and the 90% fraction of acidic and neutral extract containing GAs) were then ready for analysis.

SA, JA and JA-Ile extraction continued: The fraction taken for SA, JA and JA-Ile was purified through a WAX (1cc) cartridge (Waters). The WAX cartridge was washed with acetonitrile (1 ml), then methanol (1 ml) before regeneration with 500 µl 0.1M potassium hydroxide and equilibration with aqueous acetic acid (1%) (1 ml). Aqueous acetic acid (1%) was added to the SA, JA and JA-Ile fraction, vortexed, sonicated, vortexed, mixed by pipetting and then loaded on to the conditioned WAX cartridge. The cartridge was washed with aqueous acetic acid (1%) (1 ml), followed by aqueous methanol (80%) (1 ml x2). The SA, JA and JA-Ile were eluted into a new glass tube with 5% formic acid 80% acetonitrile (1 ml x2). The sample was dried down in a vacuum concentrator, covered in foil and stored at 4°C. The sample was transferred to a well of a PP plate with 100 µl methanol (with vortexing prior to transfer).

All leaf samples were processed as a batch and all root samples were processed as another batch.

2.3.4.3 Quantification for Global Hormone Profiling

The quantification of all hormones was performed by Yuri Kanno with time of flight LC-MS/MS, using a TripleTOF5600 (AB SCIEX, Framingham, USA). Under the guidance of Yuri Kanno and Dr Takafumi Shimizu, the author used MultiQuant 2.0.2 (AB SCIEX, Framingham, USA) to determine the sample and IS peak area for each sample. The quantity of each hormone was calculated as (sample peak area/IS peak area)*(IS amount/DW) for each sample.

2.4 Bioinformatics and data analysis

2.4.1 Bioinformatics

The software packages Primer3Plus and Geneious were used for primer design. Geneious was also used for sequence alignments, assemblies and to produce images of assemblies. The IWGSC (International Wheat Genome Sequencing Consortium) wheat chromosome arm survey sequence databank was interrogated by BLASTing with a sequence of interest and the results were imported into Geneious. The BLASTN function on the NCBI website was used to identify sequence data for sequences of interest. In silico identification of contigs of 454 data (from http://www.cerealsdb.uk.net/) were produced by Dr Andy Phillips as initial sequences for multiple genes of interest and included exon locations. IWGSC sequence data was compiled by Rothamsted Research bioinformatics staff, enabling the author to identify wheat sequence data by BLASTing with Brachypodium nucleotide sequences for genes of interest. Relevant genome sequences were de novo assembled (in Geneious) with annotated Brachypodium genes to identify exon boundaries. See Chapter 4 (Section 4.3.1) for more details.

2.4.2 Data Analysis

Statistical analysis was conducted with GenStat data analysis tool (VSNI International Ltd., Hemel Hempstead, U.K.). Due to a balanced design, most experiments were analysed with analysis of variance (ANOVA). Where the parameters of ANOVA were not met (for example, where residuals were not normally distributed) the data for a given phenotypic characteristic were transformed prior to statistical analysis. Least significant differences (LSD) with a significance threshold of 5% were used to assess significant differences between treatments or pairs of treatments. Where experimental design required, a different analysis method was used.

Chapter 3: The Role of Strigolactones in Mediating Strong Soil Effects on Rice Architecture

3.1 Introduction

Strigolactones (SLs) are plant hormones occurring in dicots and monocots, including petunia, pea, Arabidopsis, rice, chrysanthemum and willow (Snowden et al., 2005; Gomez-Roldan et al., 2008; Umehara et al., 2008; Liang et al., 2010; Ward et al., 2013) and play a variety of roles in plant biology including plant-plant interactions and plant-microbe interactions (Yoneyama et al., 2008; Proust et al., 2011). Of most relevance to this project is the developmental control of root and shoot architecture by SLs, particularly the control of shoot branching – specifically tillering in cereals. SLs inhibit shoot branching by curtailing outgrowth of axillary buds in Arabidopsis, pea and rice (Gomez-Roldan et al., 2008; Umehara et al., 2008).

This chapter focuses on the role SL plays in the response of rice plants to changes in substrate strength. It is known that strong substrate/soil results in fewer tillers in both rice and wheat (Clark et al., 2002; Whalley et al., 2006; Whalley et al., 2008) and that rice plants respond to the abiotic stress of phosphate deficiency with an increase in SL biosynthesis that results in reduced tillering via inhibition of axillary bud outgrowth (Umehara et al., 2010). It is possible that SL biosynthesis and signalling is also involved in the response to the abiotic stress of strong soil. There are no known wheat mutants for the SL biosynthesis and signalling pathways. However, rice mutants described by Umehara et al. (2008) offered an excellent opportunity to test the hypothesis that SLs are involved in mediating the tillering response to strong soil.

Mutations in rice (*Oryza sativa* L. cv. Shiokari) plants in *D10* (the rice gene encoding the SL biosynthesis gene carotenoid cleavage dioxygenase OsCCD8) and *D3* (encoding an F-box protein crucial to SL signalling) were identified by Arite et al. (2007) and Ishikawa et al. (2005), respectively (see Figures 1.4 and 1.5). The d*10* and d*3* mutants display high tillering dwarf phenotypes and the inhibitor of shoot branching was identified as strigolactones or their derivatives (Ishikawa et al., 2005; Arite et al., 2007; Umehara et al., 2008). The cause of CCD8 loss of function in *d10* Shiokari rice plants is a nucleotide base change from T to C that converts the 112th Leucine to a Proline (Arite et al., 2007). The loss of function of D3 in *d3* Shiokari rice plants is due to an insertion of a transposon at the 154th amino acid that modifies the amino acid sequence and caused a stop codon (Ishikawa et al., 2005). Figure 3.1A indicates these mutations and is based on a supplemental figure by Umehara et al. (2008). Figure 3.1B and 3.1C demonstrate the high tillering dwarf phenotypes of *d10* and *d3* plants.

This chapter seeks to test the hypothesis that SLs are involved in mediating the tillering response to strong soil in rice. This hypothesis is tested by examination of the genetic resources outlined above, measurement of SLs and related gene expression analysis under weak and strong soil conditions.

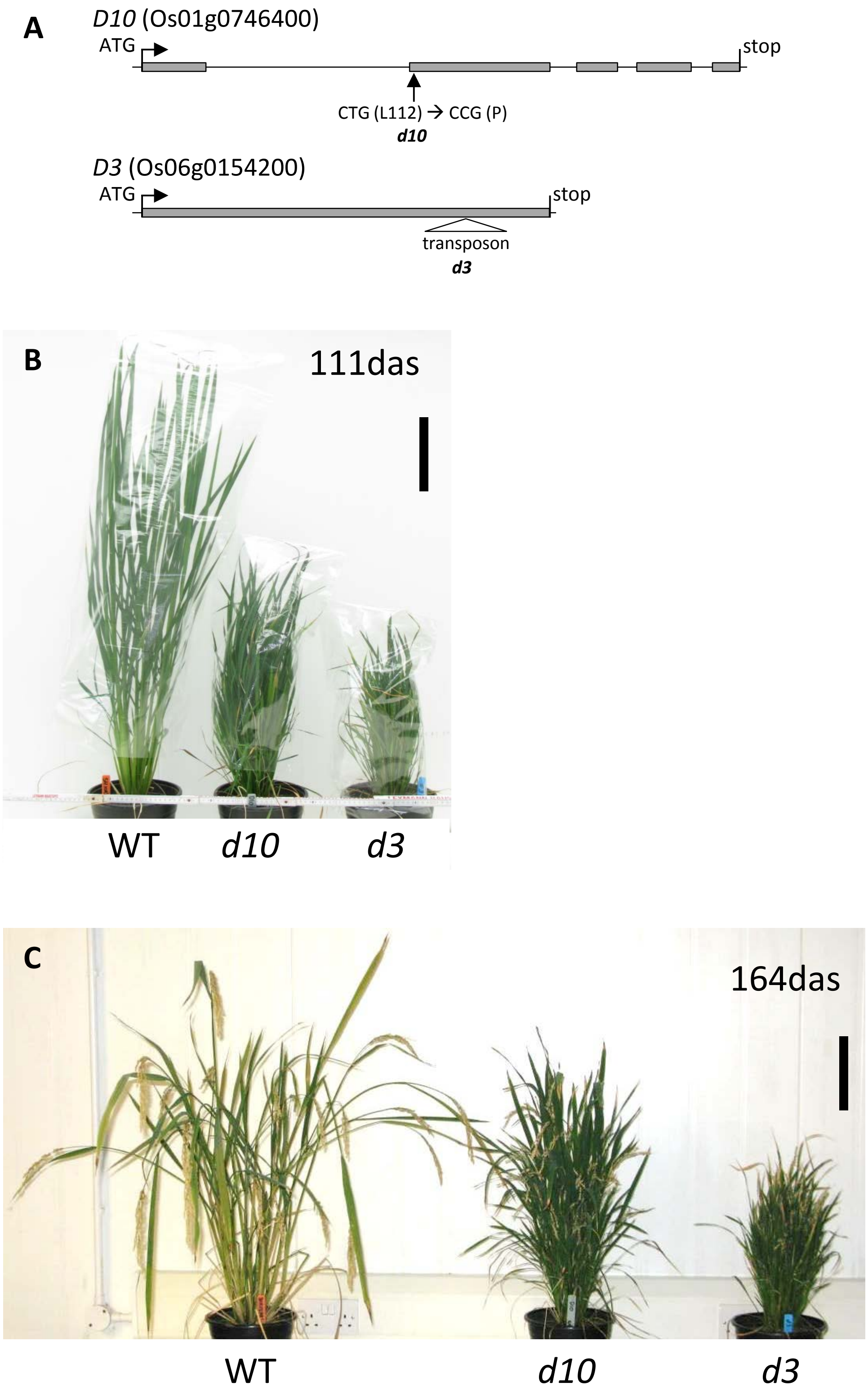


Figure 3.1: Rice strigolactone mutants used in this study.
(A) Graphical representation of mutations in *D10* and *D3,* strigolactone biosynthesis and perception genes, respectively. B) Phenotypes of rice (*Oryza sativa* L. cv. Shiokari) plants with wild-type (WT), *d10* (deficient in the biosynthesis gene D10) and *d3* (deficient in the perception gene D3) genotypes at 111 days after sowing (das); scale bar=15cm. C) Phenotypes of Shiokari rice plants with WT, *d10* and *d3* genotypes at 164 das; scale bar=15cm. Information for A from Ishikawa et al. (2005), Arite et al. (2007) and Umehara et al. (2008).

3.2 Materials and methods

Wild-type (WT) rice plants (*Oryza sativa* L. cv. Shiokari) and two high tillering dwarf mutants (*d3* and *d10*) in the Shiokari background were used in this study. These plants have been described in Ishikawa et al. (2005), Arite et al. (2007) and Umehara et al. (2008). Figure 3.1 demonstrates the mutations involved and the resulting phenotypes.

Soil strength apparatus, sowing of rice plants, growth conditions, nutrient solution, plant measurements, RNA extraction, strigolactone extraction, strigolactone quantification and were performed as described in sections 2.1.5, 2.1.2, 2.1.4, 2.1.6, 2.1.7, 2.2.4.2, 2.3.2 and 2.3.3, respectively.

3.3 Results

3.3.1 Experiment 3.1: The response of strigolactone mutants in rice to increased soil strength

The aim of Experiment 3.1 was to test the hypothesis that rice mutants defective in strigolactone biosynthesis or perception would be less responsive to strong soil, especially in terms of tiller generation, than WT plants. The experimental design of Experiment 3.1 was a Randomised Complete Block Design (RCB) with 3 genotypes, 2 soil strengths (weak and strong) and 4 replicates. All plants were grown in individual sand columns, using the strong soil apparatus described in Section 2.1.5. Leaf lengths, leaf widths and tillering were recorded daily, while shoot biomass, root biomass and root architecture were assessed at harvest for modification of response to strong soil in strigolactone mutants. The experiment continued until 51 days after sowing (das).

Table 3.1 contains statistically significant comparisons for Experiment 3.1, after analysis by 2-way ANOVA and accompanied Fisher's LSD test. In the following graphs, the full set of means $\pm$ SE are shown as well as effects shown to be significant (as listed in Table 3.1). LSDs enable comparison between all bars within a graph.

Table 3.1 Statistically Significant Results of Experiment 3.1

Results of 2-way ANOVA. Where * indicates the interaction between the two factors. d.f.=degrees of freedom; F=F statistic; *p*=*p*-value; LSD=Fisher's Least Significant Difference value at 5% level (allowing comparison of means)

Variable	Effect	d.f.	F	*p*	LSD (5%)
Leaf 3 length	genotype	2,14	3.06	0.079	13.71
	soil strength	1,14	0.26	0.617	11.20
	genotype*soil strength	2,14	0.29	0.750	19.39
Leaf 4 length	**genotype**	**2,14**	**11.14**	**0.001**	**22.44**
	soil strength	**1,14**	**21.47**	**<0.001**	**18.32**
	genotype*soil strength	2,14	1.05	0.377	31.74
Leaf 5 length	**genotype**	**2,14**	**10.96**	**0.001**	**33.63**
	soil strength	**1,14**	**46.00**	**<0.001**	**27.46**
	genotype*soil strength	2,14	0.23	0.800	47.56
Leaf 6 length	**genotype**	**2,14**	**11.33**	**0.001**	**34.24**
	soil strength	**1,14**	**77.59**	**<0.001**	**27.96**
	genotype*soil strength	2,14	0.85	0.447	48.42
Leaf 7 length	genotype	2,14	8.87	0.003	36.96
	soil strength	1,14	42.67	<0.001	30.18
	genotype*soil strength	**2,14**	**4.60**	**0.029**	**52.28**
Leaf 3 width	**genotype**	**2,14**	**4.92**	**0.024**	**0.544**
	soil strength	1,14	1.15	0.301	0.444
	genotype*soil strength	2,14	0.55	0.592	0.769
Leaf 4 width	**genotype**	**2,14**	**5.32**	**0.019**	**0.825**
	soil strength	**1,14**	**5.05**	**0.041**	**0.673**
	genotype*soil strength	2,14	0.07	0.930	1.166
Leaf 5 width	**genotype**	**2,14**	**7.84**	**0.005**	**1.216**
	soil strength	**1,14**	**26**	**<0.001**	**0.993**
	genotype*soil strength	2,14	0.10	0.907	1.720
Leaf 6 width	**genotype**	**2,14**	**9.31**	**0.003**	**0.958**
	soil strength	**1,14**	**46.19**	**<0.001**	**0.782**
	genotype*soil strength	2,14	1.49	0.259	1.354
Leaf 7 width	**genotype**	**2,14**	**6.48**	**0.010**	**1.11**
	soil strength	**1,14**	**36.12**	**<0.001**	**0.906**
	genotype*soil strength	2,14	1.30	0.303	1.569
Leaf 3 area	**genotype**	**2,14**	**6.47**	**0.010**	**61.9**
	soil strength	1,14	0.98	0.340	50.5
	genotype*soil strength	2,14	0.48	0.629	87.5
Leaf 4 area	**genotype**	**2,14**	**10.85**	**0.001**	**125.9**
	soil strength	**1,14**	**14.41**	**0.002**	**102.8**
	genotype*soil strength	2,14	0.66	0.534	178.0
Leaf 5 area	**genotype**	**2,14**	**14.66**	**<0.001**	**184.5**
	soil strength	**1,14**	**51.42**	**<0.001**	**150.6**
	genotype*soil strength	2,14	1.24	0.318	260.9
Leaf 6 area	genotype	2,14	24.25	<0.001	188.2
	soil strength	1,14	129.07	<0.001	153.7
	genotype*soil strength	**2,14**	**5.44**	**0.018**	**266.2**

Variable	Effect	d.f.	F	*p*	LSD (5%)
Leaf 7 area	genotype	2,14	15.27	<0.001	276.3
	soil strength	1,14	64.52	<0.001	225.6
	genotype*soil strength	**2,14**	**4.15**	**0.038**	**390.8**
# primary tillers	genotype	2,14	22.35	<0.001	0.883
	soil strength	1,14	29.72	<0.001	0.721
	genotype*soil strength	**2,14**	**6.14**	**0.012**	**1.249**
# leaves on main tiller	genotype	2,14	0.60	0.561	0.640
	soil strength	**1,14**	**15.37**	**0.002**	**0.523**
	genotype*soil strength	2,14	0.38	0.688	0.905
Site filling	genotype	2,14	24.50	<0.001	0.1031
	soil strength	1,14	16.65	0.001	0.0842
	genotype*soil strength	**2,14**	**6.97**	**0.008**	**0.1458**
# secondary tillers	**genotype**	**2,14**	**11.87**	**<0.001**	**1.286**
	soil strength	**1,14**	**15.42**	**0.002**	**1.05**
	genotype*soil strength	2,14	3.57	0.056	1.818
# total tillers	**genotype**	**2,14**	**21.03**	**<0.001**	**1.86**
	soil strength	**1,14**	**28.13**	**<0.001**	**1.519**
	genotype*soil strength	2,14	1.17	0.339	2.631
Shoot fresh weight	genotype	2,14	18.53	<0.001	1.654
	soil strength	1,14	89.46	<0.001	1.350
	genotype*soil strength	**2,14**	**16.30**	**<0.001**	**2.339**
Shoot dry weight	genotype	2,14	13.64	<0.001	0.5030
	soil strength	1,14	77.54	<0.001	0.4107
	genotype*soil strength	**2,14**	**13.44**	**<0.001**	**0.7113**
Root fresh weight	genotype	2,14	7.65	0.006	2.944
	soil strength	1,14	119.62	<0.001	2.404
	genotype*soil strength	**2,14**	**4.53**	**0.030**	**4.163**
Root dry weight	genotype	2,14	3.01	0.082	1.269
	soil strength	**1,14**	**48.33**	**<0.001**	**1.036**
	genotype*soil strength	2,14	1.31	0.301	1.794
# primary roots	**genotype**	**2,14**	**4.06**	**0.041**	**8.82**
	soil strength	**1,14**	**40.99**	**<0.001**	**7.20**
	genotype*soil strength	2,14	1.90	0.186	12.47
Max root depth	genotype	2,14	0.13	0.883	8.95
	soil strength	**1,14**	**14.74**	**0.002**	**7.31**
	genotype*soil strength	2,14	0.26	0.775	12.66

3.3.1.1 Leaf architecture

Final lengths and widths were determined when all replicates for a given leaf number had finished elongating. Leaf 3 length was not affected by either genotype or soil strength and there was no interaction between the two factors (Figure 3.2A, see Table 3.1 for F statistic, *p*-value and LSD). No interaction was shown between genotype and strong soil for lengths of leaves 4, 5 and 6; meaning that, statistically, these leaves responded similarly to strong soil for all genotypes. However, the final lengths of leaves 4, 5 and 6 were significantly longer in WT plants than in either *d10* or *d3* plants, which did not differ from each other (Figure 3.2B, C and D, see Table 3.1 for F statistic, *p*-value and LSD). The final lengths of leaves 4, 5 and 6 were also reduced in strong soil (Figure 3.2B, C and D , see Table 3.1 for F statistic, *p*-value and LSD). Leaf 7 final length was statistically significantly reduced by strong soil for WT and *d10* plants but was not reduced for *d3* plants (Figure 3.2E, see Table 3.1 for F statistic, *p*-value and LSD).

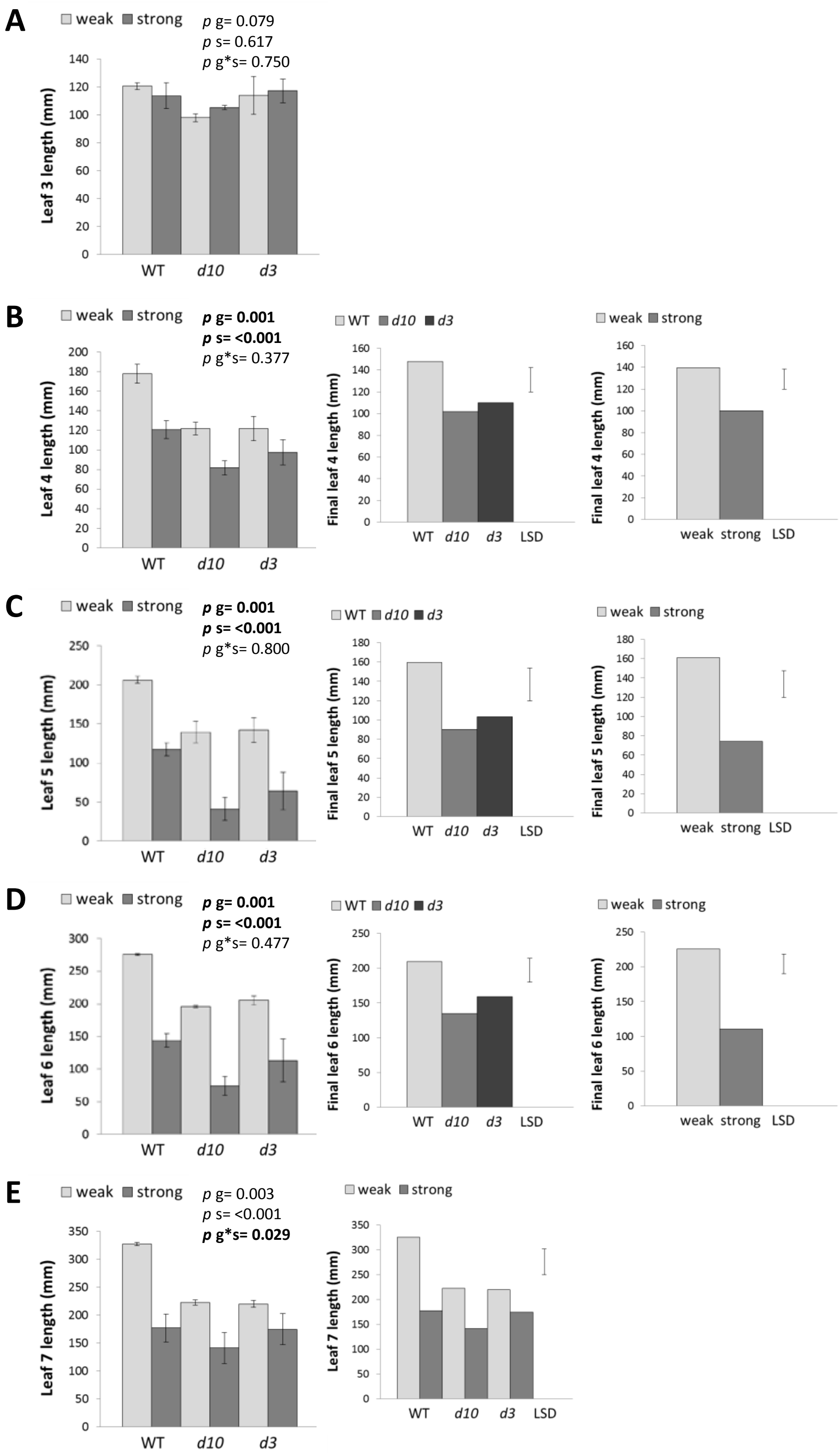


Figure 3.2: Final leaf blade lengths of rice plants grown in strong and weak soil.
A) Leaf 3 length, no significant effects. B) Leaf 4 length, significant effects of genotype and soil strength. C) Leaf 5 length, significant effects of genotype and soil strength. D) Leaf 6 length, significant effects of genotype and soil strength. E) Leaf 7 length, significant interaction between genotype and soil strength. Data given are means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.1). *n*= 3-4.

Leaf 3 width was statistically significantly smaller in *d10* plants than in WT or *d3* plants, which did not differ from each other (Figure 3.3A, see Table 3.1 for F statistic, *p*-value and LSD). No interaction was demonstrated between genotype and soil strength for widths of any of leaves 4, 5, 6 or 7 (Table 3.1). Over both soil strength treatments, WT plants had statistically significantly wider leaves for leaves 4, 5, 6 and 7 than for either *d10* or *d3* plants, which did not differ from each other (Figure 3.3B, C, D, and E, see Table 3.1 for F statistic, *p*-value and LSD). Additionally, final widths of leaves 4, 5, 6 and 7 were statistically significantly reduced in strong soil for all genotypes (Figure 3.3B, C, D, and E, see Table 3.1 for F statistic, *p*-value and LSD).

Leaf 3 area was statistically significantly smaller in *d10* plants than in WT or *d3* plants, which did not differ from each other (Figure 3.4A, see Table 3.1 for F statistic, *p*-value and LSD). Final leaf areas of leaves 4 and 5 were larger for WT plants than for either *d10* or *d3*, which did not differ from each other (Figure 3.4B and C, see Table 3.1 for F statistic, *p*-value and LSD). Leaf 4 and 5 final leaf areas were reduced by strong soil (Figure 3.4B and C , see Table 3.1 for F statistic, *p*-value and LSD). Strong soil reduced leaf 6 area for all genotypes but had a bigger impact on *d10* plants than WT and *d3* plants (Figure 3.4D, see Table 3.1 for F statistic, *p*-value and LSD). Compared to weak soil values leaf 6 area was reduced by 43% in strong soil for WT plants, 54% for *d3* plants and 78% for *d10* plants.

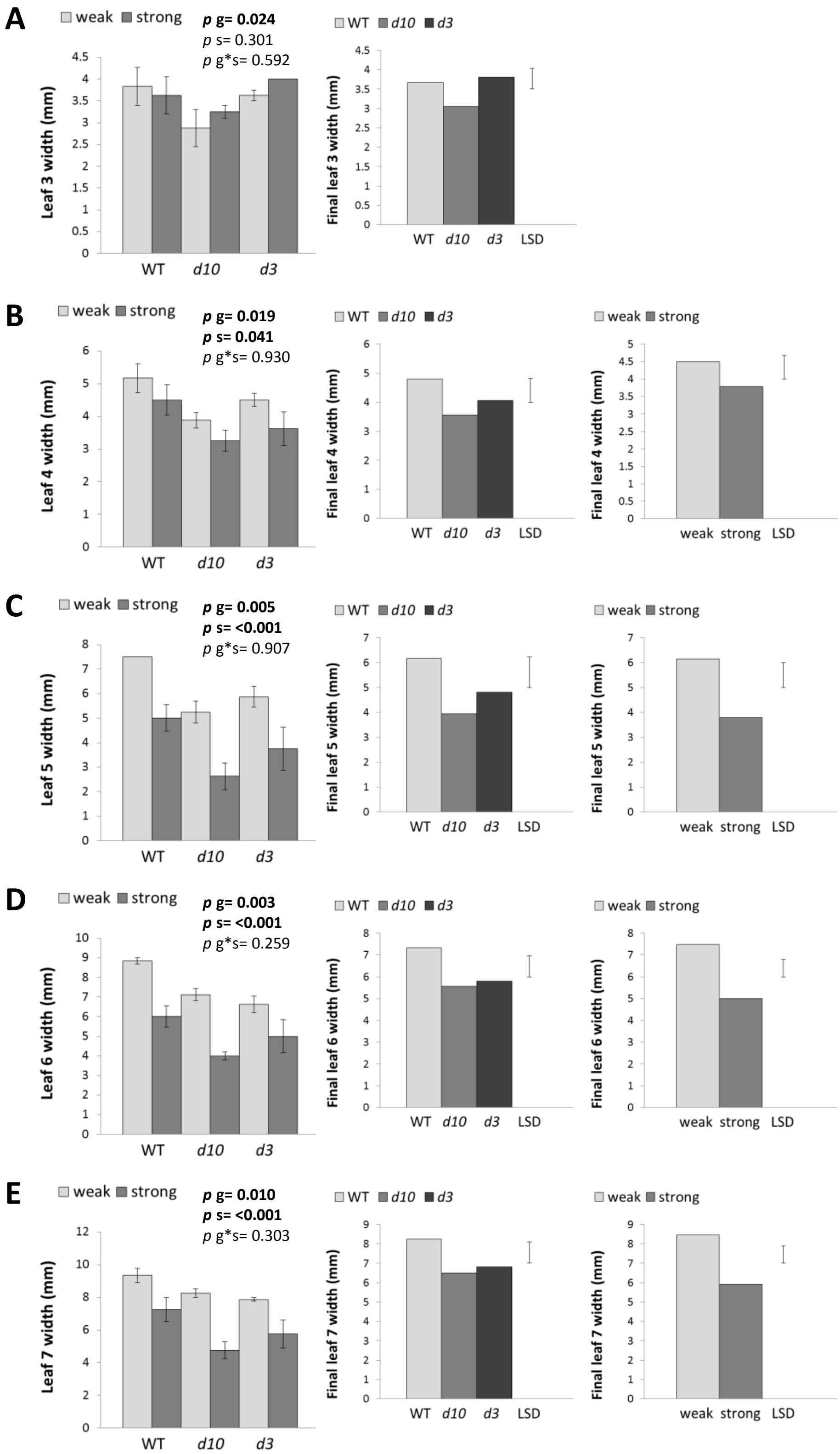


Figure 3.3: Final leaf blade widths of rice plants grown in strong and weak soil.
A) Leaf 3 width, significant effect of genotype. B) Leaf 4 width, significant effects of genotype and soil strength. C) Leaf 5 width, significant effects of genotype and soil strength. D) Leaf 6 width, significant effects of genotype and soil strength. E) Leaf 7 width, significant effects of genotype and soil strength. Data given are means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.1). *n*= 3-4.

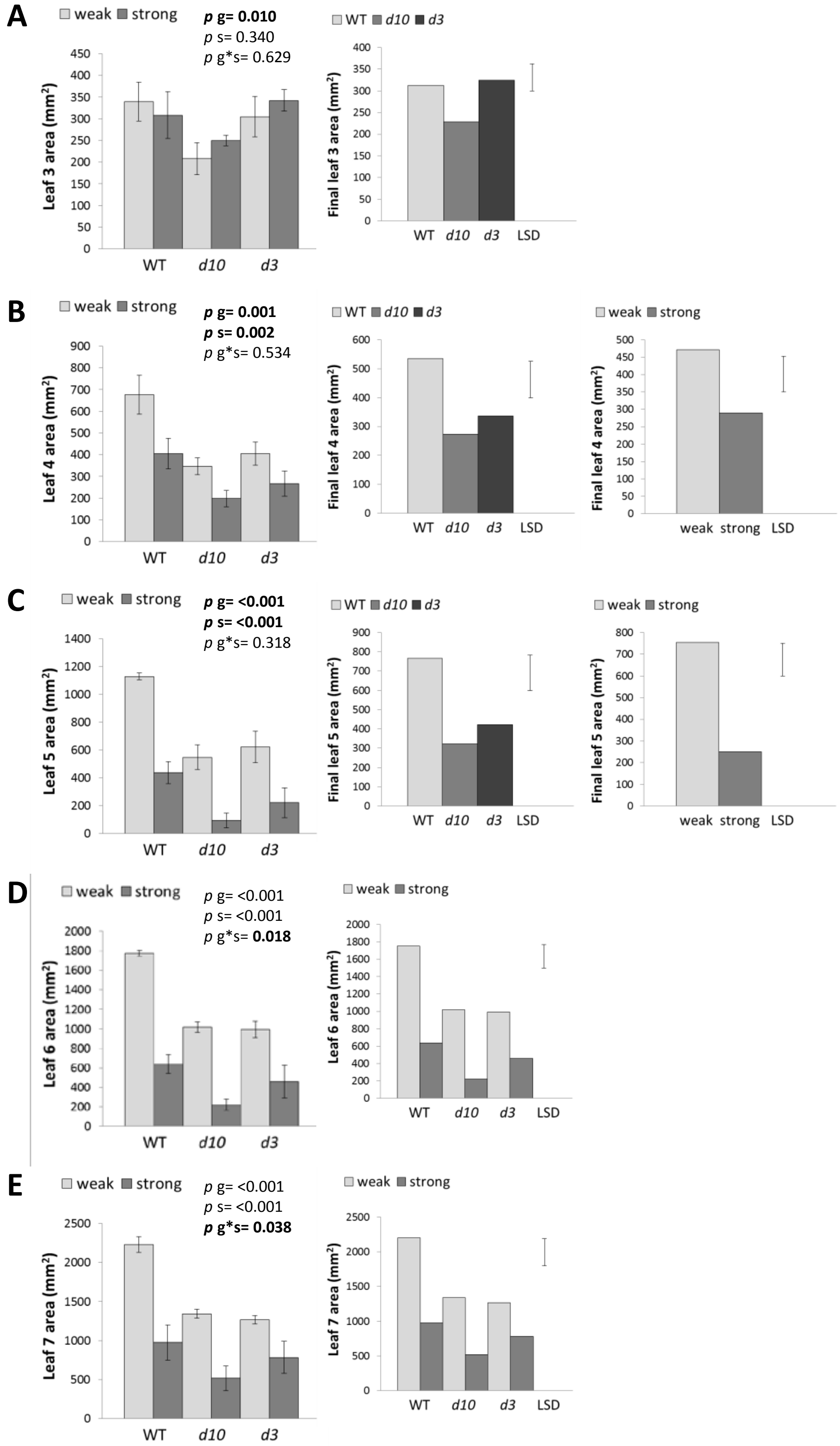


Figure 3.4: Final leaf blade areas of rice plants grown in strong and weak soil.
A) Leaf 3 areas, significant effect of genotype. B) Leaf 4 areas, significant effects of genotype and soil strength. C) Leaf 5 areas, significant effects of genotype and soil strength. D) Leaf 6 areas, significant interaction between genotype and soil strength. E) Leaf 7 areas, significant interaction between genotype and soil strength. Data given are means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.1). *n*= 3-4.

Strong soil reduced leaf 7 area for all genotypes but impacted *d3* plants less than WT and *d10* plants (Figure 3.4E, see Table 3.1 for F statistic, *p*-value and LSD). Leaf 7 area was reduced by 56% for WT plants, 61% for *d10* plants and 38% for *d3* plants.

All of leaves 4, 5, 6 and 7 showed reductions of length, width and area in response to strong soil. Genotype differences tended to be WT plants having bigger leaves than the *d10* and *d3* mutants. However, interactions between genotypes and soil strength were only demonstrated for leaf 7 length, leaf 6 area and leaf 7 area. When such an interaction was seen, it was the *d3* mutants that showed no difference between weak and strong soil treatments. Overall, strigolactone (SL) signalling showed little to no involvement in the response of leaf growth to strong soil although the areas of leaves 6 and 7 were reduced by a smaller extent in *d3* plants (Figure 3.4D and E).

3.3.1.2 Tiller architecture

Primary tillers are those tillers emerging from the axils of leaves on the main tiller/culm (Figure 1.3). In this experiment both genotype and soil strength affected the number of primary tillers and there was a significant interaction ($p=0.012$) between the two main effects (Figure 3.5A, see Table 3.1 for all F statistics, *p*-values and LSDs). WT plants showed an 87.5% reduction in primary tillering while *d10* and *d3* plants showed only a 20% and 19% reduction, respectively, when grown in strong soil compared with counterparts grown in weak soil. WT plants showed a statistically significant reduction in primary tillering in response to strong soil, while *d3* and *d10* plants did not.

As developmental stunting of plants could reduce the potential for primary tiller emergence it is important to check the number of leaves on the main tiller (culm) to assess the potential and actual utilisation of leaf axils (site filling). Soil strength reduced the number of leaves on the main tiller, although genotype had no effect and there was not an interaction between the two factors (Figure 3.5B, see Table 3.1 for F statistic, *p*-value and LSD). However, site filling was affected by genotype and soil strength and there was a significant interaction ($p=0.008$) between these effects (Figure 3.5C, see Table 3.1 for F statistics, *p*-values and LSDs). WT plants showed an 87% reduction in site filling when grown in strong soil compared with weak soil but the SL mutants showed no statistically significant difference between the two treatments. Comparison of site filling allowed the plants in weak and

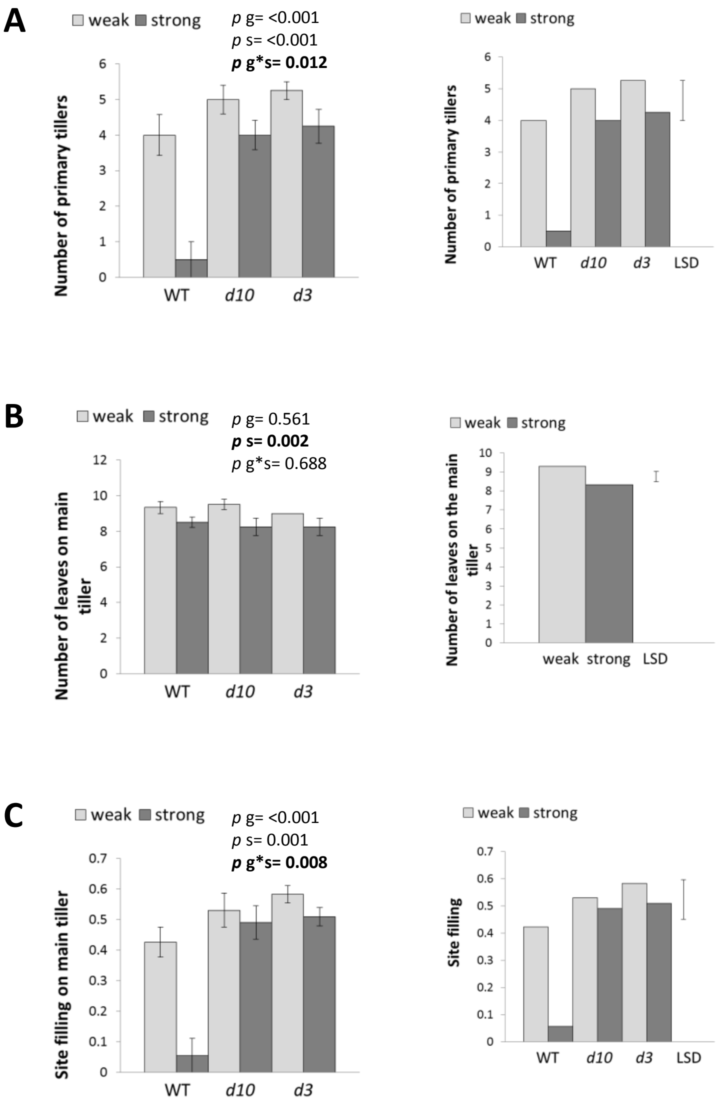


Figure 3.5: Final primary tillering and site filling of rice plants grown in strong and weak soil. A) Final primary tillering. B) Final number of leaves on main tiller. C) Site Filling on main tiller (site filling=number of primary tillers/number of leaves on main tiller). Data given are means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.1). *n*= 3-4.

strong soil to be compared in a developmentally equivalent manner (primary tiller per leaf on the main culm), confirming reduced outgrowth of buds instead of only delayed plant development.

The number of secondary tillers was affected by genotype and soil strength (Figure 3.6A, see Table 3.1 for F statistic, *p*-value and LSD), with an almost significant interaction (p=0.056) between genotype and soil strength. WT plants had statistically significantly fewer secondary tillers than *d3* and *d10* plants, which did not differ between each other while the number of secondary tillers was statistically significantly reduced in plants grown in strong soil (Figure 3.6A). WT plants had very few secondary tillers in either treatment and large reductions in the secondary tillering were seen for both *d10* and *d3* plants.

Total tillering was affected by both genotype and strong soil (Figure 3.6B, see Table 3.1 for F statistic, *p*-value and LSD) but there was no interaction between these effects. WT plants had a significantly lower total number of tillers than *d10* and *d3* plants, which did not differ from each other and strong soil significantly reduced the total number of tillers (Figure 3.6B).

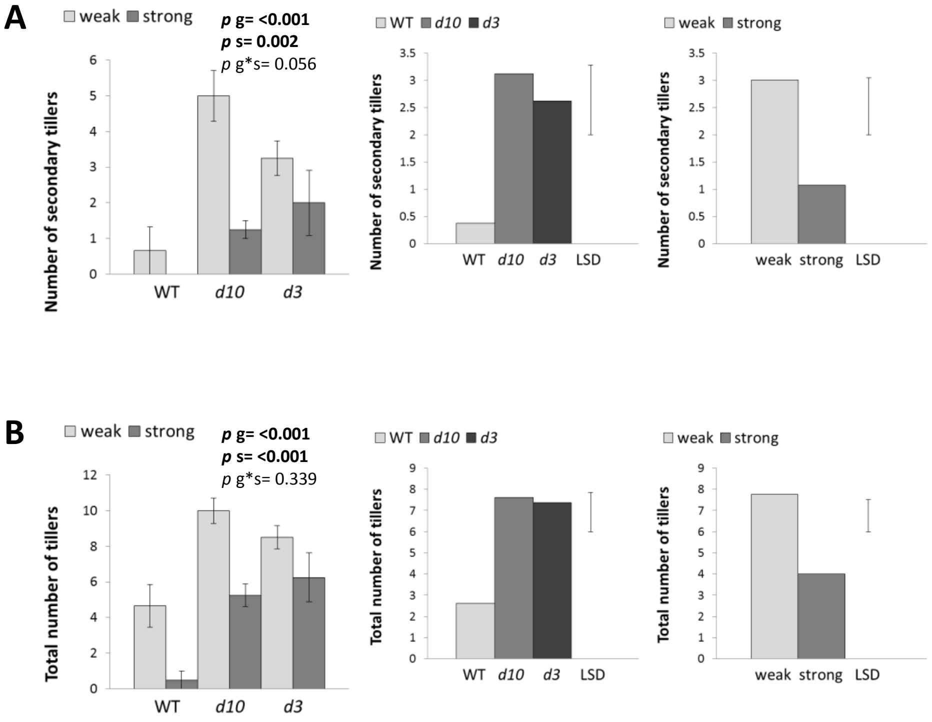


Figure 3.6: Final secondary and total tillering of rice plants grown in strong and weak soil. A) Final number of secondary tillers (tillers arising from axils of leaves on primary tillers), significant effects of genotype and soil strength. B) Final total number of tillers, significant effects of genotype and soil strength. Data given are means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.1). *n*= 3-4.

3.3.1.3 Shoot and root Biomass

Shoot fresh weight was affected by both genotype and soil strength and showed an interaction between the two (Figure 3.7A, see Table 3.1 for F statistic, *p*-value and LSD). There was an 83% reduction in shoot fresh weight when WT plants were grown in strong soil, a 79% reduction for *d10* plants and a 49% reduction for *d3* plants. These differences were statistically significant for WT and *d10* plants but not *d3* plants (Figure 3.7A).

Shoot dry weight was affected by both genotype and soil strength and showed an interaction between the two (Figure 3.7B, see Table 3.1 for F statistic, *p*-value and LSD). WT plants showed an 84% reduction in shoot dry weight in strong soil relative to weak soil, *d10* plants showed a 78% reduction and *d3* plants showed a 50% reduction. These differences were significant for WT and *d10* plants but not *d3* plants (Figure 3.7B).

Root fresh weight was affected by both genotype and soil strength and a statistically significant interaction was shown between the two main effects (Figure 3.7C, see Table 3.1 for F statistic, *p*-value and LSD). An 80% reduction in root fresh weight was seen for WT plants grown in strong soil compared to ones grown in weak soil, an 81% reduction was seen for *d10* and a 70% reduction was seen for *d3* (Figure 3.7C, LSD=4.163). It seems that the slightly smaller reduction in root fresh weight in *d3* plants was the reason for the interaction between genotype and soil strength. However, all genotypes show reduced root fresh weight in strong soil.

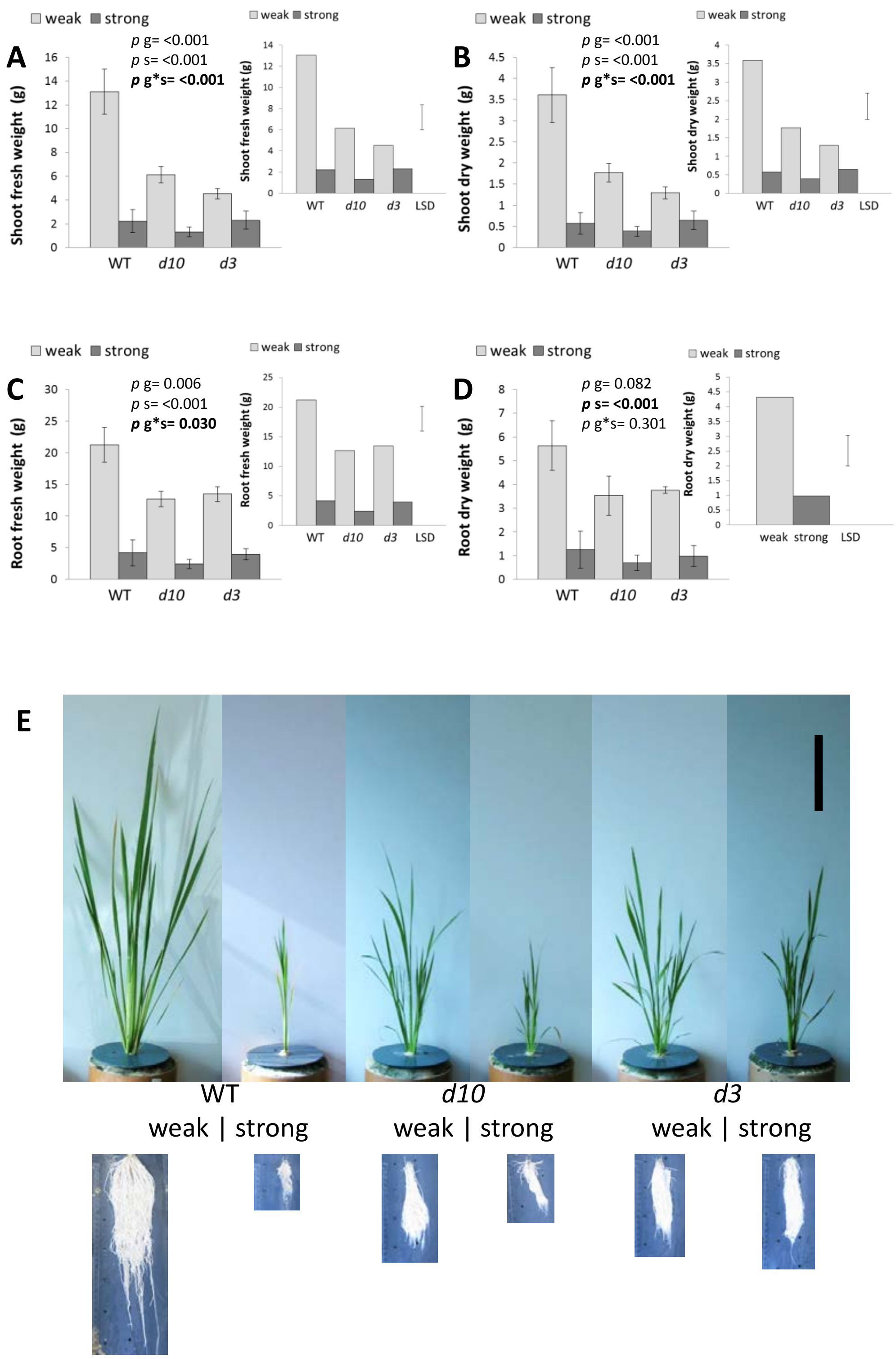


Figure 3.7: Shoot and root fresh and dry weight of rice plants grown in strong and weak soil. A) Shoot fresh weight (g), significant interaction between genotype and soil strength. B) Shoot dry weight (g), significant interaction between genotype and soil strength. C) Root fresh weight (g), significant interaction between genotype and soil strength. D) Root dry weight (g), significant effect of soil strength. E) Plants representing the shoot and root systems of each treatment combination. Data given are means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.1). *n*= 3-4. Scale bar=14cm.

Root dry weight was statistically significantly affected by soil strength (see Table 3.1 for F statistic, *p*-value and LSD) but not genotype, and there was no interaction between the two factors. Strong soil reduced the root dry weight of plants (Figure 3.7D, see Table 3.1 for LSD).

Figure 3.7E compares root and shoot architecture of plants from each genotype and soil strength combination.

3.3.1.4 Root architecture

The number of primary roots was affected by genotype and soil strength (Figure 3.8A, see Table 3.1 for F statistic, *p*-value and LSD) although there was no interaction between the two. WT plants had statistically significantly more primary roots than *d3* plants but the number of primary roots in *d10* plants did not differ from either WT or *d3* plants (Figure 3.8A). There were statistically significantly fewer primary roots in plants grown in strong soil than in plants grown in weak soil, (Figure 3.8A).

Soil strength statistically significantly reduced maximum root depth (Figure 3.8B, see Table 3.1 for F statistic, *p*-value and LSD), although genotype had no effect and there was not an interaction between the two factors.

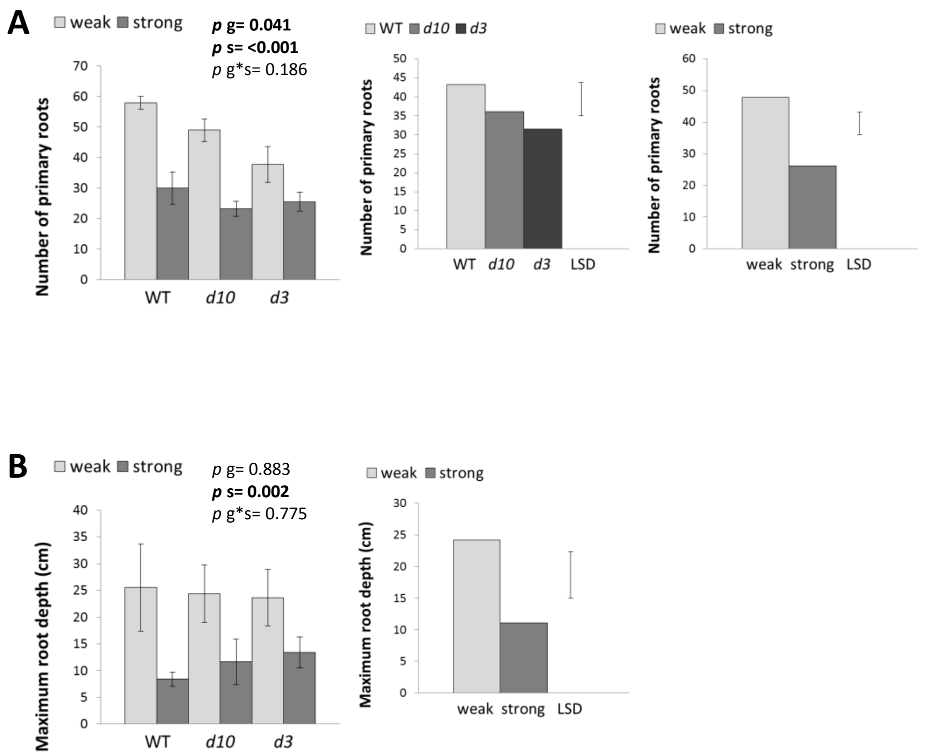


Figure 3.8: Number of primary roots and maximum root depth of rice plants grown in weak and strong soil.
A) Number of primary roots, significant effects of genotype and soil strength. B) Maximum root depth (cm), significant effect of soil strength. Data given are means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.1). *n*= 3-4.

Root to shoot ratio (root dry weight/shoot dry weight) was not affected by genotype or soil strength and no interaction was seen; as such, these data have been omitted here.

3.3.2 Experiment 3.2: Strigolactone mutants in strong soil and quantifying endogenous strigolactone levels

The aims of Experiment 3.2 were to confirm the diminished tillering inhibition of SL mutants in response to strong soil observed in experiment 3.1 and to quantify the levels of strigolactone in the roots of rice plants grown in strong and weak soil.

The experimental design of Experiment 3.2 (as for Experiment 3.1) used a Randomised Complete Block Design (RCB) with 3 genotypes, 2 soil strengths and 4 replicates. However, less exhaustive phenotypic measurements were made in Experiment 3.2 (Table 3.2) as only leaf length was measured daily and ceased to be measured after the next leaf on the main stem had finished expanding. Tillering was recorded weekly and at harvest. For this experiment, plants were grown until 61 days after sowing (das). Additionally, fresh root samples were collected for SL concentration analysis at harvest of the experiment (61 das).

Data were analysed with ANOVA and Fisher's LSD to the 5% level (results shown in Table 3.2)

Table 3.2 Statistically Significant Results of Experiment 3.2

Results of 2-way ANOVA. Where * indicates the interaction between the two factors. d.f.=degrees of freedom; F=F statistic; *p*=*p*-value; LSD=Fisher's Least Significant Difference value at 5% confidence (allowing comparison of means).

Variable	Effect	d.f.	F	*p*	LSD (5%)
Leaf 2 length	**genotype**	**2,15**	**37.29**	**<0.001**	**3.064**
	soil strength	1,15	0.99	0.336	2.502
	genotype*soil strength	2,15	0.70	0.512	4.333
Leaf 3 length	genotype	2,15	0.70	0.511	14.78
	soil strength	1,15	0.94	0.347	12.07
	genotype*soil strength	2,15	0.48	0.630	20.91
Leaf 4 length	**genotype**	**2,14**	**3.97**	**0.043**	**21.85**
	soil strength	1,14	0.01	0.907	17.84
	genotype*soil strength	2,14	0.69	0.517	30.90
Leaf 5 length	**genotype**	**2,15**	**20.47**	**<0.001**	**19.07**
	soil strength	**1,15**	**16.31**	**0.001**	**15.57**
	genotype*soil strength	2,15	0.39	0.686	26.97
Leaf 6 length	genotype	2,15	11.04	0.001	26.26
	soil strength	1,15	23.09	<0.001	21.44
	genotype*soil strength	**2,15**	**5.06**	**0.021**	**37.14**
Leaf 7 length	**genotype**	**2,15**	**28.57**	**<0.001**	**27.01**
	soil strength	**1,15**	**11.44**	**0.004**	**22.06**
	genotype*soil strength	2,15	0.56	0.584	38.20
# primary tillers	**genotype**	**2,15**	**21.15**	**<0.001**	**0.899**
	soil strength	**1,15**	**39.61**	**<0.001**	**0.734**
	genotype*soil strength	2,15	1.46	0.262	1.271
# leaves on main tiller	**genotype**	**2,15**	**7.12**	**0.007**	**0.3552**
	soil strength	**1,15**	**54.00**	**<0.001**	**0.2901**
	genotype*soil strength	2,15	3.37	0.062	0.5024
Site filling	**genotype**	**2,15**	**20.93**	**<0.001**	**0.103**
	soil strength	**1,15**	**17.98**	**<0.001**	**0.0841**
	genotype*soil strength	2,15	2.36	0.128	0.1457
# secondary tillers	**genotype**	**2,15**	**8.92**	**0.003**	**1.406**
	soil strength	**1,15**	**67.2**	**<0.001**	**1.148**
	genotype*soil strength	2,15	1.03	0.381	1.989
# total tillers	**genotype**	**2,15**	**30.96**	**<0.001**	**1.703**
	soil strength	**1,15**	**123.54**	**<0.001**	**1.390**
	genotype*soil strength	2,15	0.93	0.416	2.408
(log) root fresh weight	**genotype**	**2,15**	**9.57**	**0.002**	**0.2901**
	soil strength	**1,15**	**50.33**	**<0.001**	**0.2369**
	genotype*soil strength	2,15	0.60	0.562	0.4103
(log) max root depth	**genotype**	**2,15**	**7.16**	**0.007**	**0.1400**
	soil strength	**1,15**	**35.04**	**<0.001**	**0.1143**
	genotype*soil strength	2,15	1.00	0.393	0.1979
(log χ+1) 5DS level	genotype	2,15	459.78	<0.001	0.1858
	soil strength	1,15	56.20	<0.001	0.1517
	genotype*soil strength	**2,15**	**18.20**	**<0.001**	**0.2627**

3.3.2.1 Leaf architecture

Leaf 2 was affected by genotype in Experiment 3.2, with WT and *d10* not being different from one another but *d3* leaf 2 length being significantly longer than either WT or *d10* leaf 2 (Figure 3.9A, see Table 3.2 for F statistic, *p*-value and LSD).

As in Experiment 3.1, no effect of genotype or soil strength was seen on leaf 3 length and there was no interaction between the two factors (Figure 3.9B, see Table 3.2 for F statistic, *p*-value and LSD).

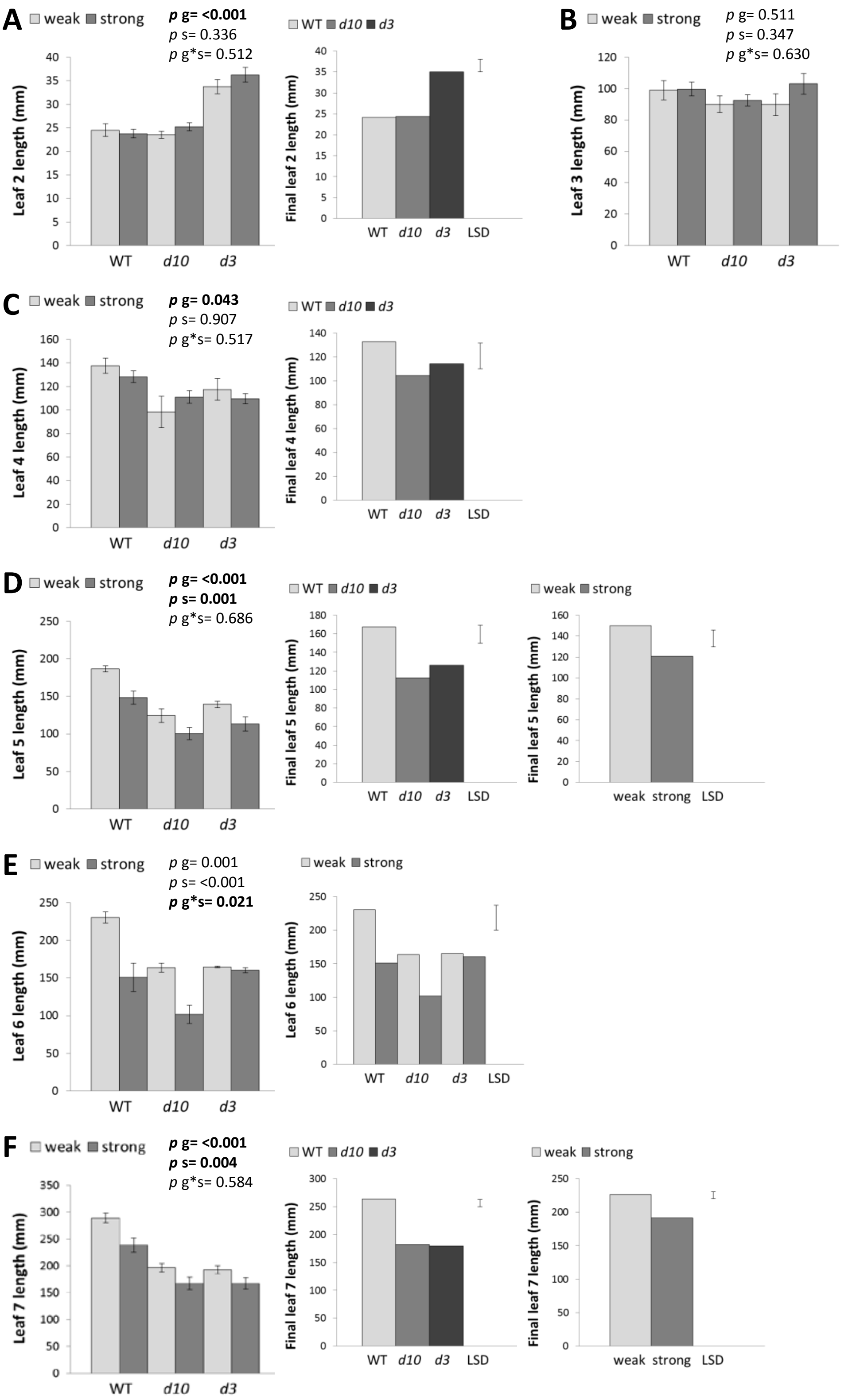


Figure 3.9: Final leaf blade lengths of rice plants grown in strong and weak soil.
A) Leaf 2 length, significant effect of genotype. B) Leaf 3 length, no significant effects. C) Leaf 4 length, significant effect of genotype. D) Leaf 5 length, significant effects of genotype and soil strength. E) Leaf 6 length, significant interaction between genotype and soil strength. F) Leaf 7 length, significant effects of genotype and soil strength. Data given are means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.2). *n*= 3-4.

In Experiment 3.2 leaf 4 WT leaves were statistically significantly longer than those of *d10* plants but no effect of soil strength and no interaction was seen between genotype and soil strength (Figure 3.9C, see Table 3.2 for F statistic, *p*-value and LSD). As in Experiment 3.1, no interaction between genotype and soil strength was seen in leaf 5, although leaf 5 was significantly longer in WT plants than in either *d10* or *d3* plants, which did not differ from each other (Figure 3.9D, see Table 3.2 for F statistic, *p*-value and LSD). Leaf 5 was shorter in rice plants grown in strong soil than plants grown in weak soil (Figure 3.9D, see Table 3.2 for F statistic, *p*-value and LSD).

Unlike in Experiment 3.1, there was a statistically significant interaction between genotype and soil strength for leaf 6 length (see Table 3.2 for F statistic, *p*-value and LSD). Soil strength reduced leaf 6 length for both WT and *d10* plants but did not affect *d3* plants (Figure 3.9E, see Table 3.2 for F statistic, *p*-value and LSD).

Unlike in Experiment 3.1, no interaction between genotype and soil strength was detected for leaf 7 length although both genotype and soil strength individually affected leaf 7 length (see Table 3.2 for F statistic, *p*-value and LSD). Leaf 7 was longer in WT plants than in either of *d10* or *d3* plants, which did not differ from each other (Figure 3.9F, see Table 3.2 for F statistic, *p*-value and LSD). Leaf 7 was shorter in plants grown in strong soil compared to those in weak (Figure 3.9F, see Table 3.2 for F statistic, *p*-value and LSD).

As in Experiment 3.1, generally no interaction between genotype and soil strength was seen for effects on leaf length, although an interaction was seen for leaf 6. It seems that SLs do not influence the response of leaf length to strong soil.

3.3.2.2 Tiller architecture

As seen in Experiment 3.1, both genotype and strong soil affected the number of primary tillers in Experiment 3.2 (see Table 3.2 for F statistic, *p*-value and LSD). However, no statistically significant interaction between genotype and strong soil was seen for primary tillering in Experiment 3.2 (p=0.262). WT plants had fewer primary tillers than *d10* and *d3* plants which did not differ from each other (Figure 3.10A, see Table 3.2 for F statistic, *p*-value and LSD). Strong soil reduced the number of primary tillers for all genotypes (Figure 3.10A, see Table 3.2 for F statistic, *p*-value and LSD) and the difference in response to strong soil between SL mutants and WT plants was not big enough to be statistically significant in Experiment 3.2. Despite the lack of a statistically significant interaction, the difference between genotypes in response to strong soil is noteworthy. Between weak and strong there was a 27% reduction in primary tillering for *d3* and *d10* plants (a difference of 1.75 tillers), while for WT plants the reduction was 63% (a difference of 3 tillers) (Figure 3.10A). While the difference between a 27% and a 63% reduction in strong soil was not enough to provide statistical support, it does imply a genuine difference in response.

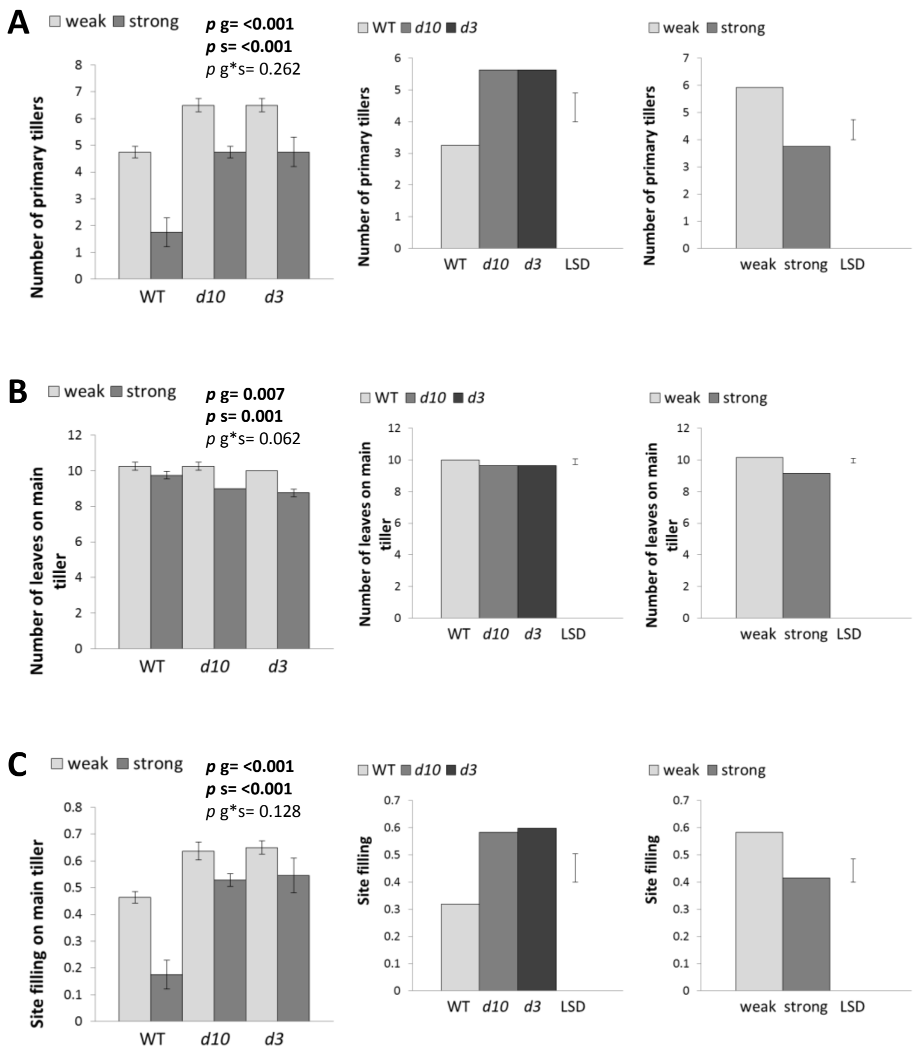


Figure 3.10: Final primary tillering and site filling of rice plants grown in strong and weak soil. A) Final primary tillering, significant effects of genotype and soil strength. B) Final number of leaves on main tiller, significant effects of genotype and soil strength. C) Final site filling (site filling=number of primary tillers/number of leaves on main tiller), significant effects of genotype and soil strength. Data given are means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.2). *n*=4.

The number of leaves on the main tiller was affected by both genotype and soil strength (see Table 3.2 for F statistic, *p*-value and LSD). There were slightly more leaves on WT plants than on *d3* or *d10* plants, which did not differ between each other (Figure 3.10B, see Table 3.2 for F statistic, *p*-value and LSD). Plants grown in strong soil had one less leaf on the main tiller than those in weak soil (Figure 3.10B, see Table 3.2 for F statistic, *p*-value and LSD).

For site filling, unlike Experiment 3.1, there was no significant interaction between genotype and soil strength although both individually affected site filling (see Table 3.2 for F statistic, *p*-value and LSD). WT site filling was less than that of *d10* or *d3*, which did not differ from each other (Figure 3.10C, see Table 3.2 for F statistic, *p*-value and LSD). Soil strength reduced site filling (Figure 3.10C, see Table 3.2 for F statistic, *p*-value and LSD). Despite the lack of a statistically significant interaction, effects of strong soil did seem to differ between genotype. Site filling was reduced in strong soil by 17% for *d10* plants (difference of 0.108), 16% for *d3* plants (difference of 0.105) and by 62% for WT (difference of 0.289), but the difference in response was not enough to give a statistically significant interaction between genotype and soil strength (p=0.128).

As for primary tillering and site filling, no significant interaction was seen in Experiment 3.2 between genotype and soil strength for secondary tillering. However, secondary tillering was statistically significantly lower in WT plants than in *d10* and *d3* plants, which did not differ between each other and soil strength statistically significantly reduced secondary tillering (Figure 3.11A, see Table 3.2 for F statistic, *p*-value and LSD).

As seen in experiment 3.1, total tillering was affected by both genotype and soil strength individually but the two factors did not significantly interact in Experiment 3.2. Total tillering was statistically significantly lower in WT plants than in *d10* or *d3* plants, which did not differ from each other and soil strength statistically significantly reduced total tillering overall (Figure 3.11B, see Table 3.2 for F statistic, *p*-value and LSD).

3.3.2.3 Biomass

Poor homogeneity of variance meant that ANOVA was not valid for raw root fresh weight data in Experiment 3.2, leading to their log transformation prior to statistical analysis. No significant interaction between genotype and soil strength was demonstrated for log transformed root fresh weight (see Table 3.2 for F statistic, *p*-value and LSD). However, WT plants had statistically significantly larger root systems than *d10* or *d3* plants, which did not differ from each other and soil strength reduced the size of the root system (Figure 3.12A, see Table 3.2 for F statistic, *p*-value and LSD).

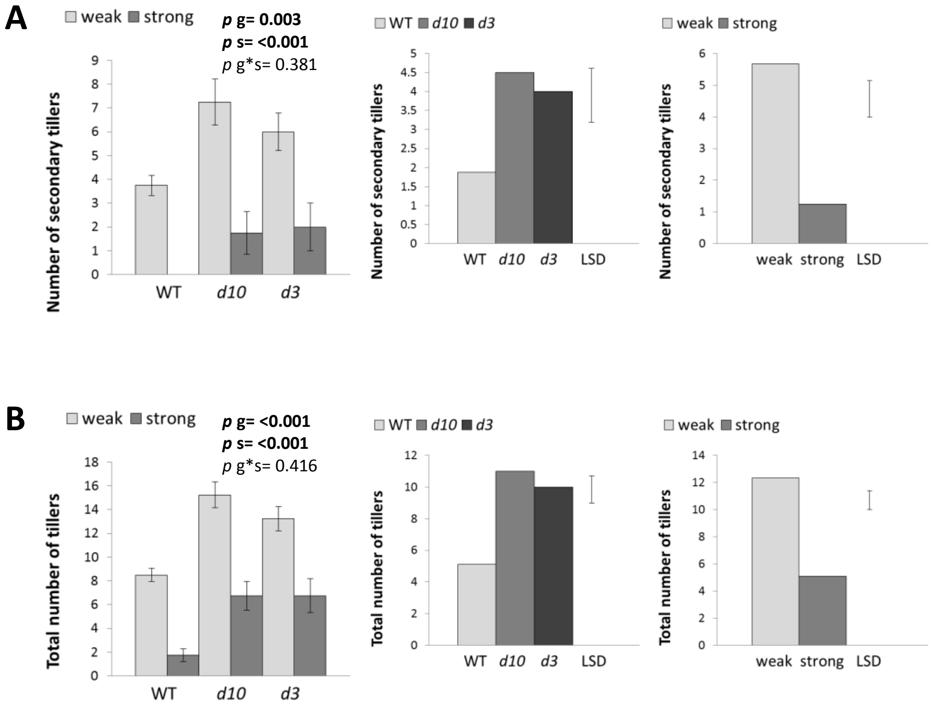


Figure 3.11: Final secondary and total tillering of rice plants grown in strong and weak soil. A) Final number of secondary tillers (tillers arising from axils of leaves on primary tillers), significant effects of genotype and soil strength. B) Final total number of tillers (primary and secondary tillers), significant effects of genotype and soil strength. Data given are means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.2). *n*=4.

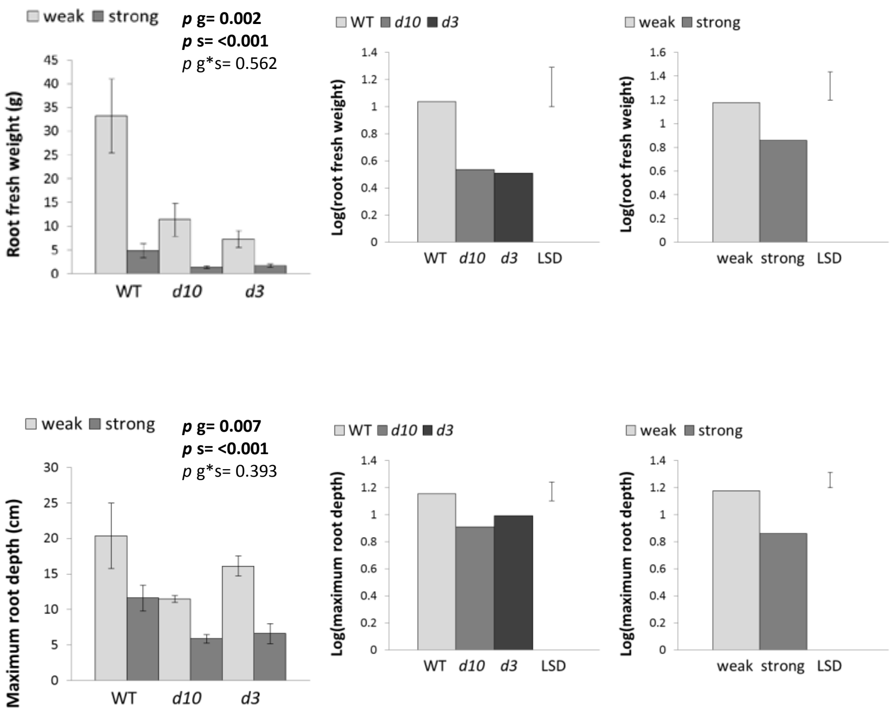


Figure 3.12: Root fresh weight and maximum root depth of rice plants grown in strong and weak soil.

A) Root fresh weight, significant effects of genotype and soil strength (data log transformed for statistical analysis). B) Maximum root depth (cm), significant effects of genotype and soil strength (data log transformed for statistical analysis). Data given are means and log transformed means. Standard Error (SE) bars given on far left panels. Results of a two-way ANOVA are given and significant results are presented with accompanying LSD for each graph (also see Table 3.2). *n*=4.

3.3.2.4 Root architecture

Attempts at ANOVA for maximum root depth data showed that residuals were not normally distributed. To allow for analysis, maximum root depth data were log transformed. No significant interaction between genotype and soil strength was shown for log transformed maximum root depth, although both genotype and soil strength had statistically significant effects individually (see Table 3.2 for F statistic, *p*-value and LSD). Roots of WT plants reached a greater maximum depth than those of *d10* or *d3* plants, which did not differ from each other (Figure 3.12B, see Table 3.2 for F statistic, *p*-value and LSD). Soil strength statistically significantly reduced the log transformed maximum depth of roots (Figure 3.12B, see Table 3.2 for F statistic, *p*-value and LSD).

3.3.2.5 Strigolactone levels

One of the main aims of Experiment 3.2 was to examine SL levels in rice plants (cv. Shiokari WT, *d10* and *d3* mutants in Shiokari background) grown in weak and strong soil. Strigolactones were extracted from fresh root samples placed in acetone as described in Section 2.3.2. Internal standards of (−)-*ent*-2'-*epi*-5-deoxystrigol (herein referred to as (−)-*ent*-2'-*epi*-5DS), orobanchol and orobanchyl acetate were added to extraction samples. Upon quantification, (−)-*ent*-2'-*epi*-5DS) was the only one of the 3 strigolactones tested found to be at quantifiable levels. (−)-*ent*-2'-*epi*-5DS is the only endogenous isomer of the strigolactone 5DS produced by Shiokari rice and is bioactive (Seto et al., 2014). The amount of (−)-*ent*-2'-epi-5DS was calculated by fitting the measured amount to a calibration curve (Materials and Methods Section 2.3.3). Values of (−)-*ent*-2'-*epi*-5DS for *d10* plants, which are deficient in SL biosynthesis, were too low to be above any baseline noise and deemed to be 0. Table 3.3 shows the calculated concentrations of (−)-*ent*-2'-*epi*-5DS.

Table 3.3 Calculated (-)-*ent*-2'-*epi*-5DS concentrations (pg/gFW) in rice plants grown in weak and strong soil.

Genotype	Soil Strength	Mean (−)-*ent*-2'-*epi*-5DS concentration (pg/gFW)	Standard Deviation
WT	weak	95	49
	strong	318	73
d10	weak	0	0
	strong	0	0
d3	weak	75	40
	strong	846	490

To allow statistical analysis by ANOVA, homogeneity of variance was improved by log transformation of the calculated concentrations of (−)-*ent*-2'-*epi*-5DS. To account for values of 0 pg/gFW for *d10* roots the function log(χ+1) was needed. There was a significant interaction between genotype and soil strength on the log(χ+1) of 5DS concentration ($F(2,15)=18.20$, $p=<.001$). In strong soil, log(5DS concentration+1) was statistically significantly increased for both WT and *d3* plants, while *d10* plants did not produce (−)-*ent*-2'-*epi*-5DS in either soil strength (Figure 3.13, see Table 3.3 for F statistic, *p*-value and LSD).

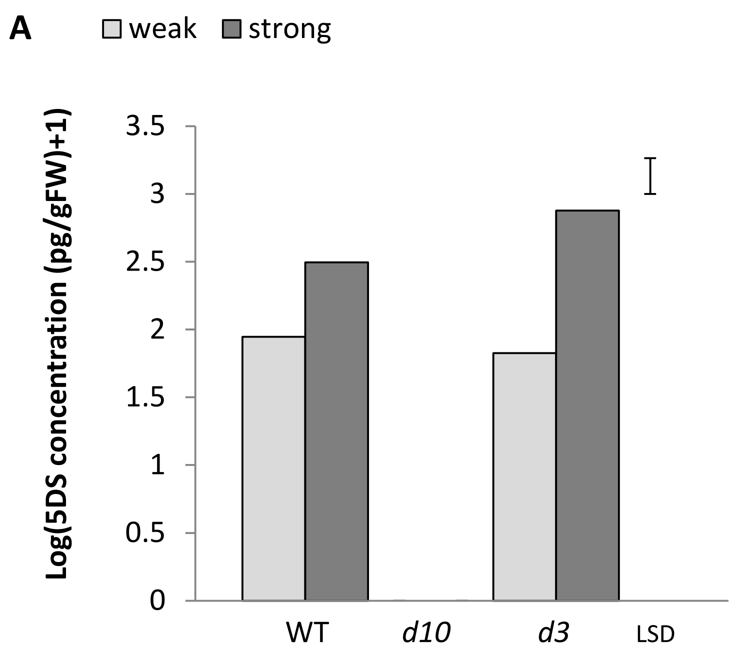


Figure 3.13: Levels of the natural strigolactone 5DS in rice plants grown in strong and weak soil

A) 5DS levels extracted from the roots of WT, *d10* and *d3* rice plants grown in weak and strong conditions.
Data given are log transformed means. LSD shown for each graph. *n*= 4.

3.3.2.6 Global gene expression analysis in rice by RNA-seq

In order to assess changes in gene expression between rice plants grown in weak and strong soil, root RNA was extracted (see Section 2.2.4.2) from 3 WT (wild-type) *Oryza sativa* (cv. Shiokari) plants per soil strength treatment (from Experiment 3.2) and sent to The Genome Analysis Centre (TGAC) for RNA sequencing (RNA-seq). Illumina RNA-seq libraries were produced from RNA samples from three individual plants grown in two soil strengths (weak and strong) using the TruSeq mRNA sample kit (Illumina, San Diego, CA, U.S.A.) using 4 µg total RNA and 100bp reads were generated on an Illumina HiSeq 2500 sequencer (by staff of TGAC). The resulting RNA-seq single-end reads were processed within Galaxy (Goecks et al., 2010) by Dr Andy Phillips. The single end reads were mapped to a reference transcriptome for rice (Oryza_sativa_Japonica.IRGSP-1.0.28.cdna.all.fa) with BWA for Illumina (Li and Durbin, 2010). The mapped reads were sorted in Galaxy by transcript and read location within each transcript. The sorted mapped reads were then processed through eXpress (Roberts and Pachter, 2013) to quantify abundances of target sequences from sampled subsequences. EdgeR (Robinson et al., 2010) was used to identify differentially expressed genes, with a Benjamini and Hochberg correction used to account for the large number of tests. A results table was exported to Excel and gene descriptions were added (from http://rice.genomics.org.cn/). The differentially expressed genes were mapped to a collection of known genes in hormone metabolism and signalling from Hirano et al. (2008) as well as genes from the SL biosynthesis and signalling pathways. The differentially expressed genes were assessed for Gene Ontology (GO) term enrichment using Singular Enrichment Analysis (SEA) and the *Oryza sativa* species selection at http://bioinfo.cau.edu.cn/agriGO/analysis.php. (Du et al., 2010). The GO term analysis provided categories or families of genes that were differentially expressed in rice roots between weak and strong soil. If a category is enriched it means that the percentage of the genes annotated to the term that were differentially expressed between treatments was higher than would be expected due to chance (Loraine et al., 2015).

Figure 3.14A demonstrates the similarity of the samples and Figure 3.14B shows the relationship between overall expression and differential expression. Not all samples of each treatment were very similar to each other, potentially reducing the power of analysis to detect differentially expressed genes (see Figure 3.14A). Figure 3.14B demonstrates that expression was changed for many genes. For a table showing the results of the GO term analysis conducted by Dr Andy Phillips see Appendix 1. Key GO terms for differentially expressed genes between weak and strong soil included carbohydrate metabolic process, response to stress, response to oxidative stress, response to stimulus, response to chemical stimulus, cell wall macromolecule metabolic process, nitrogen compound metabolic process and extracellular region (see Appendix 1). Figure 3.15 shows the number of differentially expressed genes between weak and strong soil that map to GO terms at a specificity level of 3. This analysis was completed by Dr. Rob King at Rothamsted Research, using the data analysed by Dr. Andy Phillips.

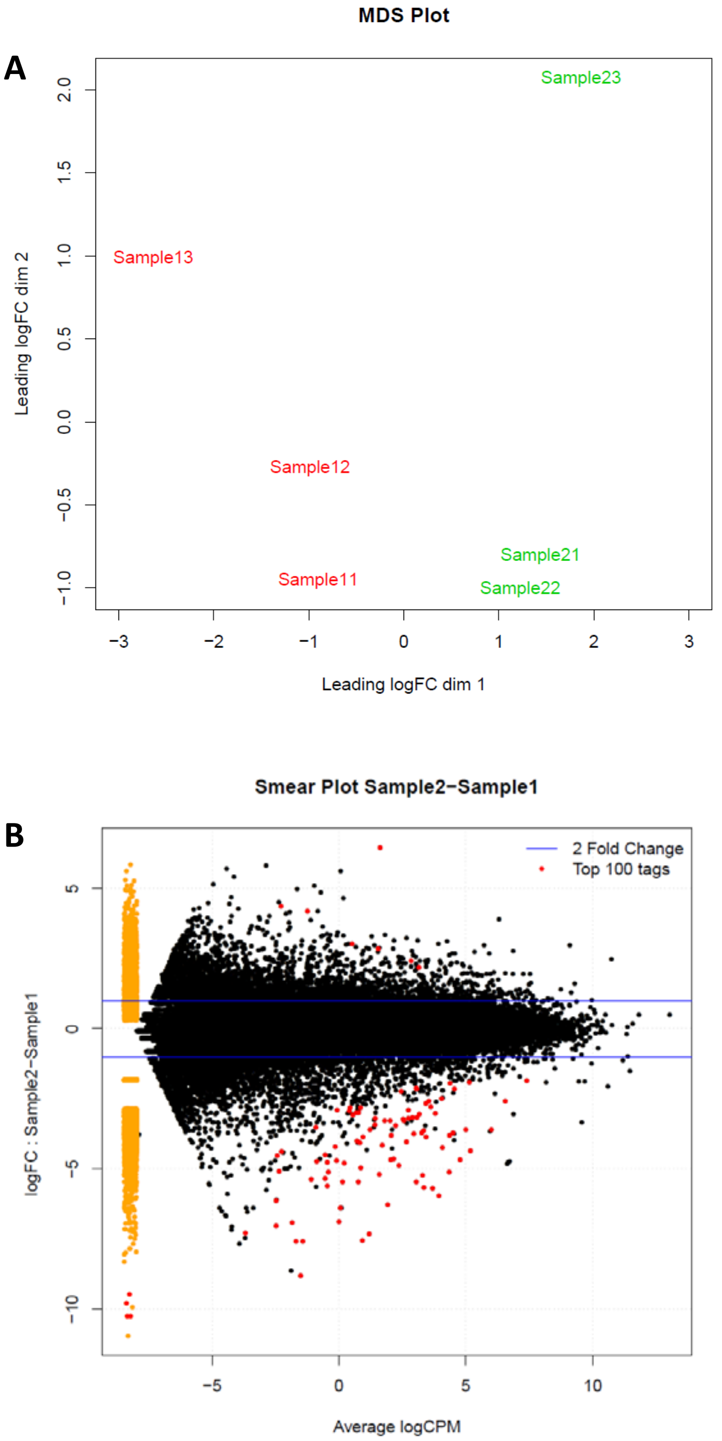


Figure 3.14: MDS plot and Smear Plot of RNAseq transcript data for roots of rice plants grown in weak and strong soil.

A) Multidimensional scaling (MDS) plot showing that not all samples of each treatment are very similar. Samples 11, 12 and 13 were in weak soil and samples 21, 22 and 23 were in strong soil. B) Smear plot showing how overall expression (in Average logCountsPerMillion) relates to log Fold Change of weak to strong soil.

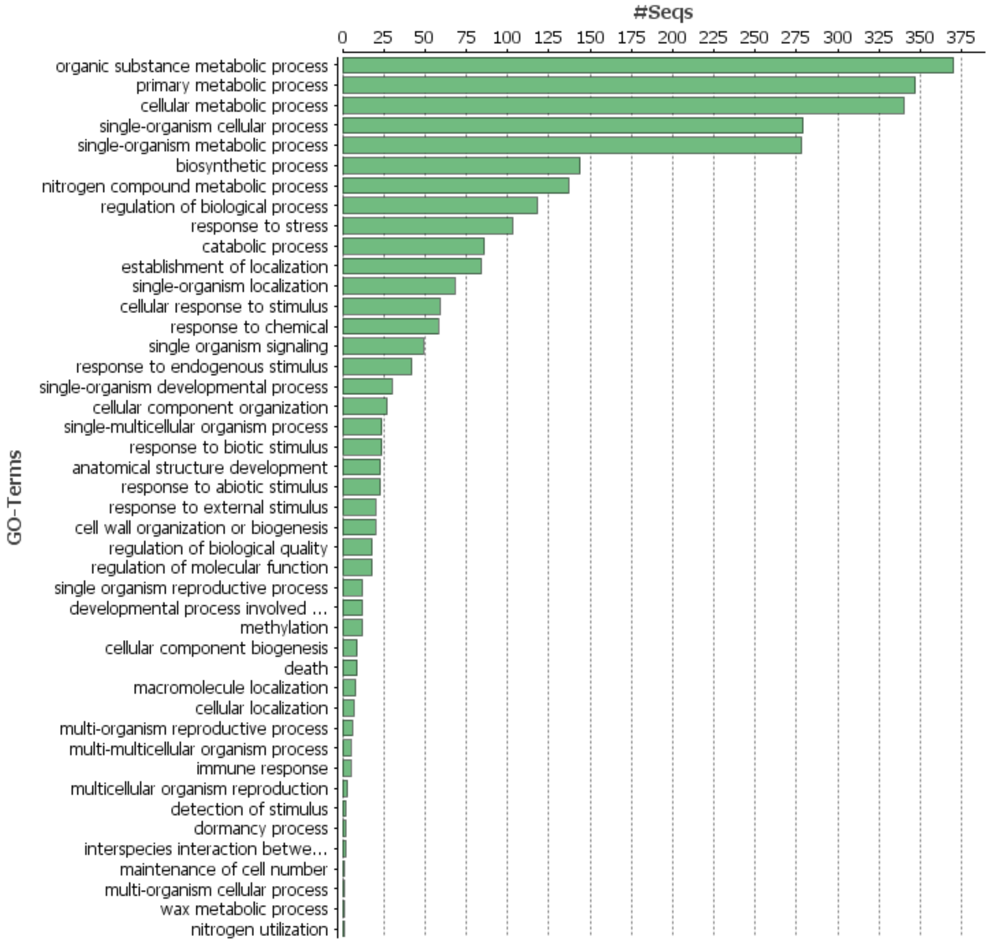


Figure 3.15: Bar chart showing the number of differentially expressed genes between weak and strong soil per GO Term (at a GO term specificity level of 3).

A) Differentially expressed genes between weak and strong soil mapped to a range of GO (gene ontology) terms including metabolic processes, response to stress, response to abiotic stress and cellular response to stimulus. #Seqs: number of sequences (genes).

As well as an assessment of the types of processes that differentially expressed genes are involved in, it is of interest to examine specific genes that are differentially expressed between strong and weak soil. Figures 3.16 and 3.17 show the top 50 up- and down-regulated genes in rice roots in strong soil compared to weak soil conditions, respectively. For a complete table of differentially expressed genes with false discovery rates see Appendix 2. Proteins encoded by up-regulated genes of interest in Figure 3.16 included three putative germin-like proteins which may be involved in plant defence, a drought-induced S-like ribonuclease and a putative pathogenesis-related protein. The top 50 down-regulated genes included cinnamyl alcohol dehydrogenase 7 (CAD7) and cinnamoyl-CoA reductase (Figure 3.17). These two genes are involved in lignin biosynthesis. *OsCAD7* (Os04g0612700/ Loc_Os04g52280) encodes a protein termed FLEXIBLE CULM 1 (note that the abbreviation FC1 used elsewhere in this thesis refers to FINE CULM 1, so FLEXIBLE CULM 1 is referred to here as OsCAD7) ((Li et al., 2008)). Delayed growth and reduction of the mechanical strength of the main culm due to altered cell wall composition were key phenotypes caused by the loss of function of OsCAD7. Loss of function in *Arabidopsis thaliana* of cinnamoyl-CoA reductase 1 resulted in delayed development, a dwarf phenotype and collapsed xylem ((Patten et al., 2005; Ruel et al., 2009)).

Table 3.4 shows the genes involved in hormone metabolism and signalling that were significantly differentially expressed in rice roots between weak and strong soil.

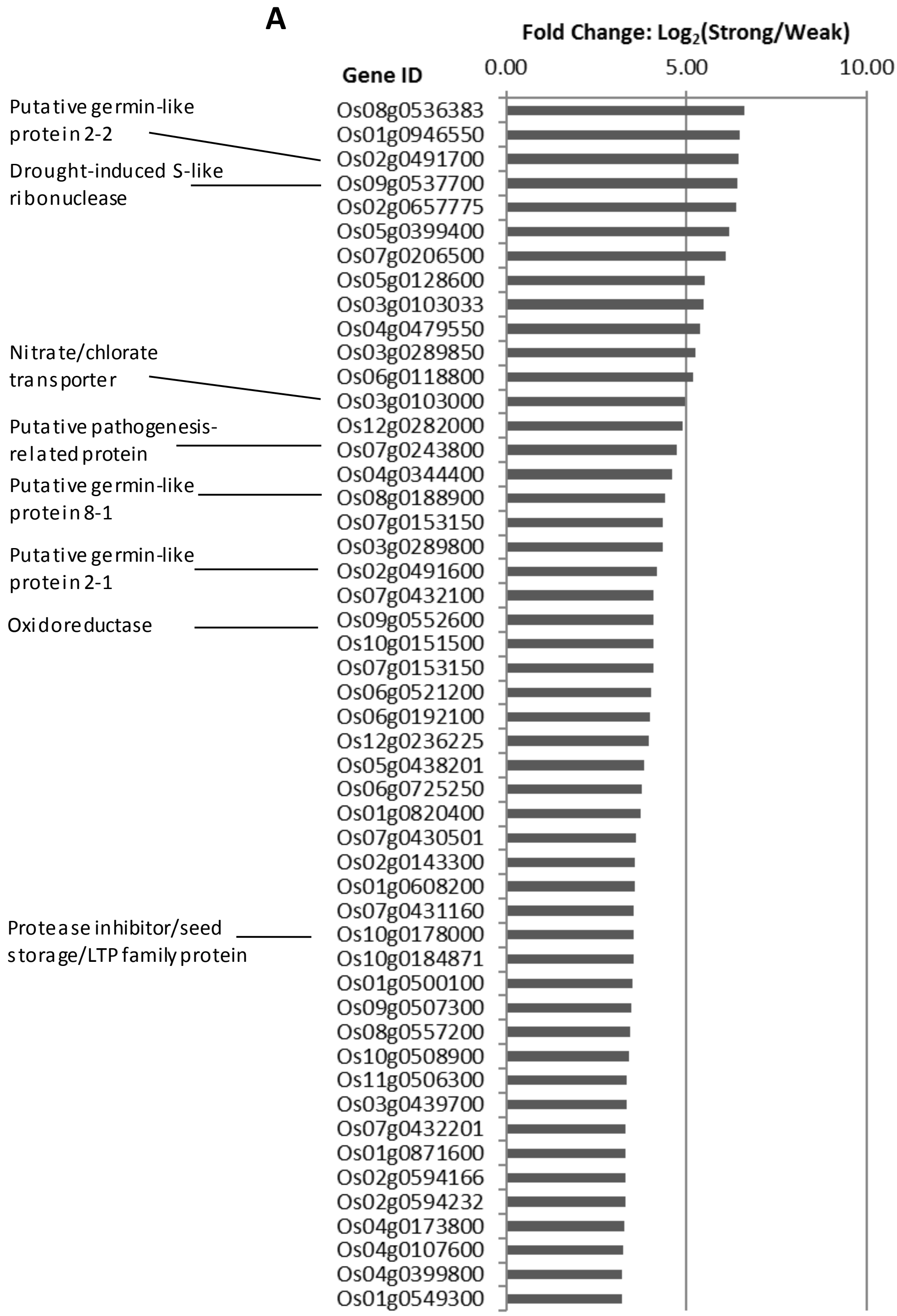


Figure 3.16: Top 50 Up-Regulated gene-coding transcripts.

A) The top 50 most up-regulated gene-coding transcripts showed $\log_2$ fold changes from 6.60 to 3.21 in strong soil compared to weak soil (97.1 to 9.2 times as many in reads in strong soil). All genes presented have a false discovery rate (FDR) of <0.05.

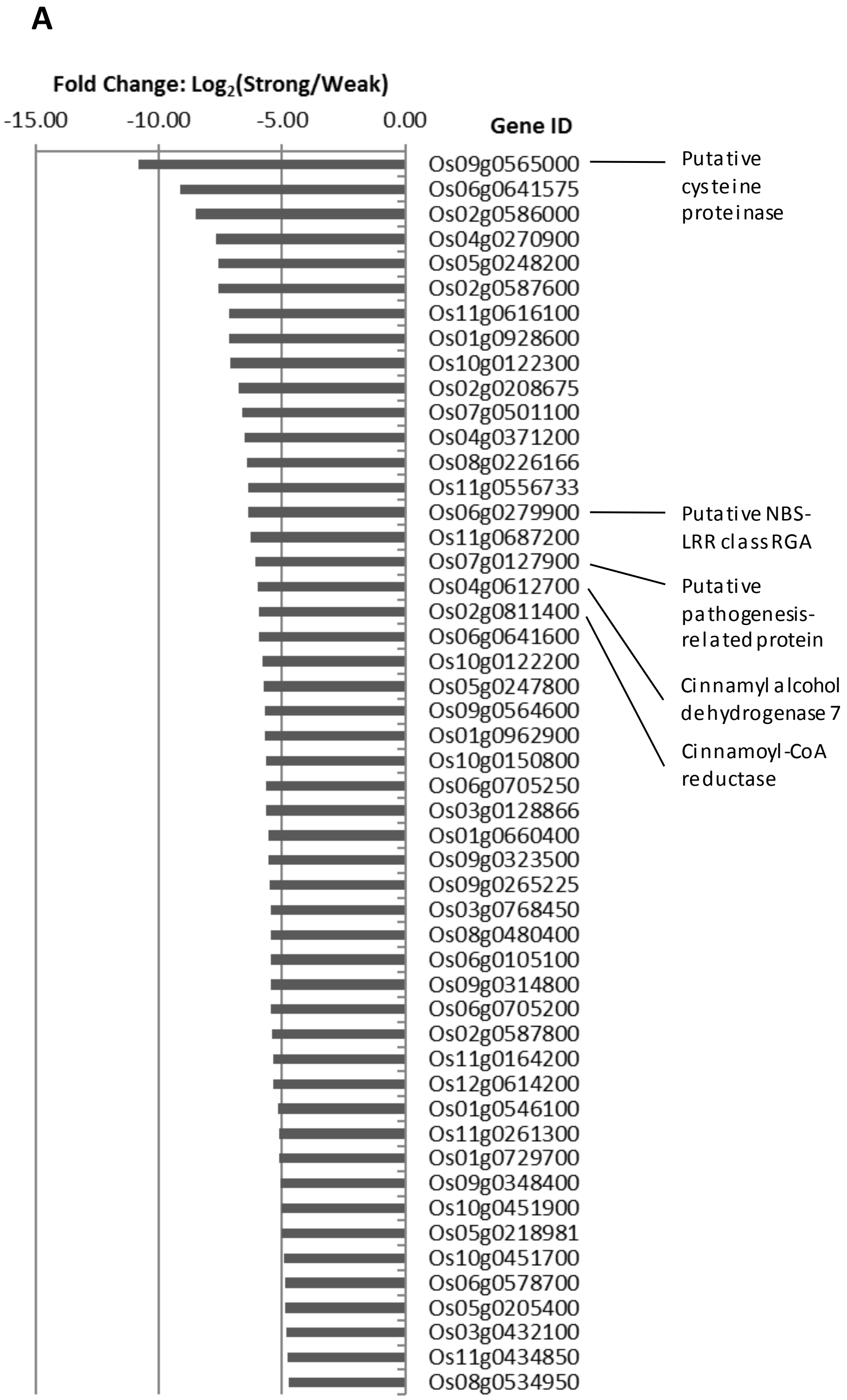


Figure 3.17: Top 50 Down-Regulated gene-coding transcripts.

A) The top 50 most down-regulated gene-coding transcripts showed $\log_2$ fold changes from -10.82 to -4.73 in strong soil compared to weak soil (1804.8 to 26.5 times fewer reads in strong soil). All genes presented have a false discovery rate (FDR) of <0.05.

Table 3.4: Genes involved in hormone metabolism and signalling that were differentially expressed in rice roots between weak and strong soil.

FDR=false discovery rate

Hormone pathway and Gene name	MSU gene name	Weak	Strong	Fold-change	Up/ Down	FDR
Auxin						
OsTIR1;4	LOC_Os11g27440	3.20	1.15	2.78	Down	5.02E-06
OsARF10	LOC_Os04g43910	7.91	14.66	1.84	Up	2.46E-02
GA						
GA2ox7	LOC_Os05g48700	1.69	0.49	3.44	Down	2.02E-04
GA2ox1	LOC_Os05g06670	2.39	0.63	3.75	Down	1.39E-03
Ethylene						
OsACO3	LOC_Os09g27750	2.80	8.03	2.86	Up	8.52E-03
OsACS3	LOC_Os05g10780	0.09	0.48	4.62	Up	1.06E-02
OsACS5	LOC_Os01g09700	0.49	2.65	5.28	Up	2.94E-07
OsACO7	LOC_Os01g39860	3.48	1.75	2.00	Down	3.93E-02
OsEIN3;3	LOC_Os07g48630	6.99	16.09	2.32	Up	3.83E-03
ABA						
OsPLDα6	LOC_Os06g40170	2.55	7.78	3.02	Up	2.46E-03
OsPLDα7	LOC_Os06g40180	2.24	6.88	3.03	Up	3.95E-04
OsABA8ox3	LOC_Os09g28390	0.08	0.60	7.93	Up	8.83E-03
JA						
OsAOS2	LOC_Os03g55800	2.82	7.42	2.60	Up	1.94E-02
OsJAZ7	LOC_Os09g26780	4.36	16.08	3.66	Up	7.75E-04
OsJAZ2	LOC_Os10g25290	8.33	23.19	2.76	Up	3.98E-03
BR						
OsCPD2/ CYP90A4	LOC_Os12g04480	2.86	7.46	2.59	Up	6.72E-03
OsBRL3	LOC_Os08g25380	14.36	7.13	2.02	Down	4.95E-04
Other						
OsD10-like	LOC_Os01g38580	0.55	3.99	7.23	Up	2.91E-13

Although no genes directly involved in the SL biosynthesis pathway were differentially expressed in rice roots between weak and strong soil, *OsD10-like* (the orthologue of the SL biosynthesis gene *OsD10*) was shown to be up regulated in strong soil (see Table 3.4). The hormonal biosynthesis and signalling roles of the remaining genes in Table 3.4 were identified from (Hirano et al., 2008). Additionally, genes involved in auxin signalling (known to be involved in SL responses) were differentially expressed with *OsTIR1;4* downregulated and *OsARF10* upregulated in strong soil. Components of other hormone metabolism and signalling pathways were also identified. *OsGA2ox7* and *OsGA2ox1* (involved in deactivating GA) were downregulated. Genes involved in ethylene biosynthesis were mostly upregulated (*OsACO3*, *OsACS3*, *OsACS5*) although *OsACO7* was downregulated in strong soil. The downstream transcription factor of ethylene signalling *EIN3;3* was upregulated in rice roots grown in strong soil. Genes involved in abscisic acid (ABA) signalling (*OsPLDα6* and *OsPLDα7*) were upregulated in strong soil, as was *OsABA8ox3* (involved in ABA inactivation). The jasmonate (JA) biosynthesis gene *OsAOS2* was upregulated in strong soil, as were the JA signalling genes *OsJAZ7* and *OsJAZ2*. Finally, the brassinosteroid (BR) biosynthesis gene *OsCPD2* was upregulated in strong soil while *OsBRL3* was downregulated, which Nakamura et al. (2006) demonstrated to be involved in perception of BR in rice roots.

3.4 Discussion

As described in Section 1.3.2, CCD8 (carotenoid cleavage dioxygenase 8) is needed for strigolactone biosynthesis at the step of converting 9-*cis*-β-apo-10’-carotenal to the strigolactone precursor carlactone (Figure 3.18A) (Umehara et al., 2008; Seto et al., 2014). In contrast, D3 is an F-box protein required for the perception of strigolactones (Figure 3.18B) (Ishikawa et al., 2005; Umehara et al., 2008). Upon binding of SL by its receptor, D14, they associate in an SCF complex that includes D3, targeting the SL signalling repressor D53 for degradation by ubiquitination (Jiang et al., 2013; Zhou et al., 2013). The *d10* and *d3* mutants used in the experiments described in this chapter were lacking in SL biosynthesis and perception, respectively, causing high tillering dwarf phenotypes (Figure 3.1).

Goss (1977) showed that in barley the length of leaf 1 was reduced by increased mechanical impedance to roots grown in a pressure chamber. Similarly, the leaf area was reduced for tomato plants grown in compacted soil (that would include an increase in soil strength) (Hussain et al., 1999). Masle and Passioura (1987) showed that increasing soil strength resulted in reduced leaf 1 length and leaf 1 area in wheat plants. The data presented here show that leaf length, width and area of rice were all reduced by strong soil (Figures 3.2, 3, 4 and 11). The reduction in leaf length was consistent for leaf 4 through to leaf 7 in Experiment 3.1 and for leaf 5 through to leaf 7 for Experiment 3.2 (Figures 3.3 and 11). It is noteworthy that soil strength does not influence the length of the first 3 leaves in the work presented here.

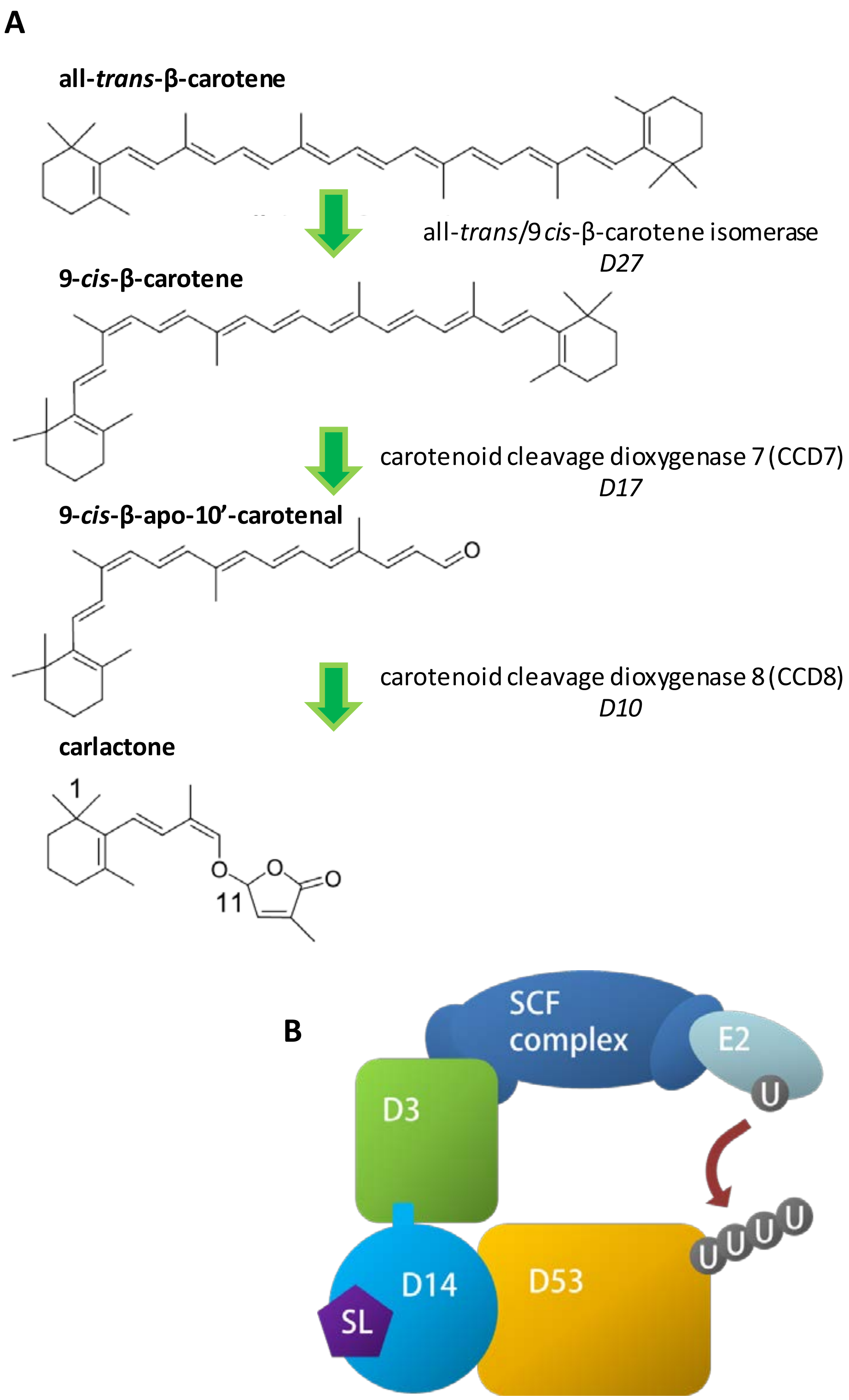


Figure 3.18: Locations of D10 and D3 in strigolactone biosynthesis and signalling mechanisms. A) *D10* encodes a carotenoid cleavage dioxygenase that is required for the production of carlactone (a strigolactone precursor) from 9-*cis*-β-apo-10’-carotenal. Precursors are in bold, enzymes are in standard font, italics are used for rice gene names. B) D3 is required to bind the SL receptor D14 to an SCF complex, enabling ubiquitination (and subsequent degradation) of the SL signalling inhibitor D53. SL=strigolactone, U=ubiquitin. Information for A from Umehara et al. (2008) and Seto et al. (2014). Information for B from Jiang et al. (2013) and Zhou et al. (2013).

This difference to the results of Goss (1977) and Masle and Passioura (1987) could be due to either differences in methodology or differences in the species under examination. Goss (1977) used a pressure chamber with glass ballotini beads as substrate to examine barley, and Masle and Passioura (1987) used packed soil columns to examine wheat, while the experiments in this chapter used sand columns with either 17kg or foam (only a few grams) weights to vary the impedance to the root system to examine rice. The method used in this study avoids damage to the root system, water deficit and anoxia, which can be flaws of the respective methods of Goss (1977) and Masle and Passioura (1987). It is possible that hypoxia or drought is the actual cause of reduced leaf elongation for Masle and Passioura (1987).

In the work presented here, increased soil strength (the strength of the sand that the plants are growing in) reduced leaf length, width and area regardless of genotype (except for leaf 7 length of *d3* plants in Experiment 3.1 and leaf 6 length of *d3* plants in Experiment 3.2). This demonstrates that a lack of strigolactone biosynthesis or signalling does not diminish the ability of rice plants to respond to strong soil by reducing leaf length. Strigolactones thus do not appear to be involved in the leaf length response to strong soil. The role of an alternative hormone in mediating leaf length is examined in Chapter 5 of this thesis.

High mechanical impedance to the root system has previously been show to result in reduced tillering in rice and wheat plants (Clark et al., 2000; Whalley et al., 2006). Clark et al. (2000) grew rice plants in sand columns with weak and strong wax layers, while Whalley et al. (2006) grew wheat plants in the same sand columns with heavy or control weights as used in the experiments described in this chapter. In Experiments 3.1 and 3.2, tillers were separated into primary or secondary tillers. This added a level of detail that previous work did not include, which may have provided new insights as to which aspect of tillering is affected by strong soil.

In Experiment 3.1 and Experiment 3.2 strong soil reduced primary and secondary tillering. However, there was a difference in the response to strong soil between genotypes. In Experiment 3.1, SL *d3* and *d10* mutant rice plants developed more primary tillers than WT plants in weak sand columns and the reduction in tillering due to strong soil was not statistically significant for SL mutants but was statistically significant for WT plants. Indeed, SL mutants had primary tillering in strong soil at a similar level to WT primary tillering in weak soil. In Experiment 3.2, no statistically significant interaction between strong soil and genotype was revealed by 2-way ANOVA of primary tillering. However, the fact that strong soil reduced the number of primary tillers in WT plants by 63% and only by 27% for SL mutants suggests that the response to strong soil (of reduced primary tillering) is lessened in SL mutant plants even in Experiment 3.2. The observation that mutations in SL biosynthesis or signalling reduce the plant's ability to respond to strong soil by decreasing the number of tillers strongly suggests a role for SL in this response and is the first demonstration of such a role.

Secondary tillering was reduced by increased substrate strength in all genotypes and WT plants had fewer secondary tillers than mutants across both strength treatments. However, there was an almost significant interaction for genotype and soil strength for secondary tillering in Experiment 3.1 (p=0.056). It is interesting that it was primary tillering and not secondary tillering that responded differently to strong soil between WT and SL mutant plants, as Zhou et al. (2013) showed no difference in the number of primary tillers between *d53* and WT plants, but showed large changes in the numbers of secondary and tertiary tillers. However, Umehara et al. (2010) saw differences in the number of primary tillers between SL mutants (*d3* and *d10*) and WT plants, as well as nullification of the primary tiller reduction caused by phosphate deficiency in SL mutants.

Assuero et al. (2012) highlighted that a change in tillering between treated and control plants does not necessarily equate to a change in apical dominance. This is because a modified phyllochron (i.e. a difference in the rate of leaf emergence) can also be present when tiller numbers are modified. To see if apical dominance of rice plants was affected by strong soil in Experiment 3.1 and Experiment 3.2, site filling was examined. This was carried out only for primary tillers and the number of leaves on the main tiller. In Experiment 3.1, WT plants had statistically significantly reduced site filling in strong soil whereas the SL mutants did not. In Experiment 3.2, no interaction between genotype and soil strength showed that all genotype responded similarly to the strong soil. However, a reduction of site filling of

62% for WT plants but only 17% for *d10* plants and 16% for *d3* plants suggests a different degree of response to increased soil strength, even if analysis by ANOVA is unable to separate these responses. Although ANOVA allowed all data for a particular variable to be analysed at the same time, there were a surprising number of occasions when no interaction between the genotype and soil strength treatments was seen. Perhaps it would have been more appropriate to analyse results of each genotype for each variable in separate T-tests. Overall, it seems that strong soil increased apical dominance in WT rice plants (with reduced utilisation of potential tiller sites) but was not affected as much in SL mutants.

Whalley et al. (2006) showed that shoot and root dry weight was approximately halved when wheat was grown in strong soil. In Experiment 3.1, the reduction in root and shoot biomass was even greater than that seen by Whalley et al. (2006) and occurred for each genotype. This was with the exception of shoot fresh and dry weight in *d3* plants, where an approximate halving of shoot biomass was not a statistically significant change. Unexpectedly, the slightly smaller effect of strong soil on *d3* biomass resulted in a statistically significant interaction between genotype and soil strength for shoot fresh weight, shoot dry weight and root fresh weight. The only biomass parameter to be tested in Experiment 3.2 was root fresh weight and did not show such an interaction, with all genotypes showing a reduction in root fresh weight due to strong soil. Overall, it seems unlikely that a lack of SL influences the impact of strong soil on biomass. This is because when the genotypes do differ from each other in their biomass response to strong soil, only *d3* is less responsive while *d10* plants respond similarly to WT plants.

The root elongation rate is known be reduced in response to increased soil strength in barley, peanut, cotton and maize (Bengough et al., 2011). It is no surprise to see that strong soil reduced maximum root depth and the number of primary roots in the experiments described in this chapter. However, it is of interest that these responses to strong soil were consistent for WT and SL mutant rice, suggesting no clear role for SLs in root growth responses to strong soil. The SL mutants had fewer primary roots than WT plants but soil strength affected the number of primary roots regardless of the presence of SLs.

Umehara et al. (2010) showed that SL production is increased in rice roots in response to phosphate deficiency, resulting in inhibition of tillering. The roots of rice plants grown in strong soil in Experiment 3.2 contained statistically significantly higher levels of the endogenous rice SL (−)-*ent*-2'-*epi*-5DS, compared to the root systems of plants in control weak soil (Table 3.3, Figure 3.13). This was the case for both WT and *d3* plants. The fact that values of (−)-*ent*-2'-*epi*-5DS for *d10* plants (which are perturbed in SL biosynthesis) were below quantifiable levels validated the results of the WT and *d3* plants. The fact that SL levels were higher in *d3* plants than WT plants, and that the increase in response to strong soil seemed to be greater for *d3* plants than WT plants is likely to be linked to feedback mechanisms. Mashiguchi et al.

(2009) showed through qRT-PCR (quantitative Real Time PCR) that *D10* and *D17* orthologues in Arabidopsis were down regulated in response to application of SL, but that this negative feedback mechanism relied on the functionality of the *D3* orthologue. However, *d3* mutants in rice showed the same increase in *OsD10* transcript as other mutants in the SL biosynthesis and signaling pathways (Arite et al., 2007), implying that Os*D3* is not required for the negative feedback mechanisms involved with high levels of SL. Whether this is a genuine interspecific difference in SL feedback mechanisms or if the greater sensitivity of qRT-PCR compared to the semi-quantitative reverse transcriptase PCR used by Arite et al. (2007) allowed more accurate results to be achieved by Mashiguchi et al. (2009) is unclear.

It is noteworthy that the differences in (−)-*ent*-2'-*epi*-5DS between weak and strong soil for WT and *d3* plants could be even greater than these results have shown. The elevated levels of (−)-*ent*-2'-*epi*-5DS found in strong soil especially in the *d3* mutant, resulted in problems with accurate quantification of SL in some samples. An insufficient amount of internal standard was added to some samples to accurately calculate the, unexpectedly high, concentrations of endogenous (−)-*ent*-2'-*epi*-5DS. A calculated concentration of (−)-*ent*-2'-*epi*-5DS is obtained by comparing the peak area ratio (endogenous peak area/internal standard peak area) to an established calibration curve for concentration ratio. The concentration ratio can then be used to calculate the concentration of endogenous (−)-*ent*-2'-*epi*-5DS. In order to accurately make these calculations, the endogenous peak area must be less than or equal to the internal standard peak area. The reason for this is that naturally present ^{13}C in the unlabeled endogenous (−)-*ent*-2'-*epi*-5DS contributes to the peak area of the d_1-labelled internal standard (−)-*ent*-2'-*epi*-5DS. This causes the endogenous (−)-*ent*-2'-*epi*-5DS level to be increasingly underestimated at peak area ratios >1. One WT weak soil replicate, all four WT strong soil replicates and one *d3* strong soil replicate had peak area ratios over 1. Therefore, the accuracy of the results presented here are suboptimal but can be assumed to underestimate the actual endogenous level of (−)-*ent*-2'-*epi*-5DS in strong WT root samples. Although a repeated experiment is required to be certain of the actual amounts of (−)-*ent*-2'-*epi*-5DS in rice roots grown in strong and weak soil, it can still be validly concluded from these results that roots grown in strong soil contain more (−)-*ent*-2'-*epi*-5DS than roots in weak soil.

Global expression analysis was conducted on rice plants grown in weak and strong soil (Section 3.3.2.6). None of the genes known to be in the SL pathway were identified as differentially expressed between the treatments. However, *OsD10-like* (the paralogue of the SL biosynthesis gene *OsD10*) was shown to be up-regulated in strong soil. *OsD10* was expressed in panicle, leaf, root and bud samples while *OsD10-like* was absent from roots and mostly expressed in the panicle (Arite et al., 2007). Additionally, *OsD10* expression was inducible by auxin, although *OsD10-like OsD3* and *OsFC1* were not (Arite et al., 2007). While *OsD10* suppression by RNAi in rice successfully resulted in increased high tillering dwarf plants, no changes were seen in tiller number or plant height for RNAi lines containing *OsD10-like* (Ngo, 2011). It does not seem that *OsD10-like* is involved in the production or signalling of SLs. It is therefore quite surprising that it was one of the genes to be upregulated in rice roots in response to strong soil, especially considering that (Arite et al., 2007) showed no expression in roots using semi-quantitative reverse transcriptase-PCR. Karrikins are a hormone group structurally related to SLs whose signalling also require D3, a paralogue of D14 (KAI2) and a paralogue of D53 (SMAX1) but are not produced via carlactone (Waters et al., 2014). Perhaps *OsD10-like* is responsible for biosynthesis of karrikins. Importantly, karrikins show a feedback effect on expression of the *D10* homologue in Arabidopsis (*MAX4*) and can be taken up by roots (Nelson et al., 2011). As karrikins stimulate seed germination and cotyledon expansion it is unclear why rice plants subjected to strong soil would show increased expression of a gene that may be involved in karrikin biosynthesis, indicating that other roles for karrikins exist (perhaps controlling root architecture) or that *OsD10-like* is unlikely to be involved in karrikin biosynthesis.

In addition response to stress, response to oxidative stress, response to stimulus and response to chemical stimulus, GO terms enriched for differentially expressed genes from RNAseq analysis of rice roots grown in weak and strong soil included nitrogen compound metabolic process. This adds support to the importance of nutrient availability signalling and may well involve SLs. Indeed, Yoneyama et al. (2012) were able to demonstrate increased production of various SLs for multiple species in response to deficiency in either nitrogen or phosphorus. Considering that SLs have already been implicated in tillering responses to phosphate deficiency and nitrogen deficiency (Yoneyama et al., 2007; Umehara et al., 2010; Yoneyama et al., 2012), they may even be part of a signalling cascade for abiotic stress in general.

In addition to the roles of SLs and the potential role of the related karrikins, the RNAseq analysis for roots of rice plants grown in weak and strong soil implicated a number of hormones in response to strong soil, including auxin, ABA, ethylene, JA, BR and GA. These hormones are likely to be involved in mediating some of the other architectural responses to strong soil that SLs biosynthesis and perception did not influence (such as leaf elongation that could involve GA).

3.5 Conclusion

The results of this chapter suggest that SLs are involved in the reduction of primary tillering caused by strong soil and that apical dominance has increased in WT plants in response to strong soil. There is a greatly diminished reduction of primary tillering in SL rice mutants compromised in SL biosynthesis and perception when grown in strong soil. Furthermore, roots grown in strong soil contain more (−)-*ent*-2’-*epi*-5DS than roots in weak soil. The results presented in this chapter have also demonstrated that SLs do not seem to be involved in the responses to strong soil of reduced leaf length and area, fewer primary roots or shallower maximum root depth.

Chapter 4: The Role of Strigolactones in Mediating Strong Soil Effects on Wheat Architecture

4.1 Introduction

Tillering is known to be reduced as a consequence of strong soil in both rice and wheat (Clark et al., 2000; Whalley et al., 2006; Whalley et al., 2008). In the previous chapter it was shown that rice mutants with impaired strigolactone (SL) biosynthesis or signalling had a reduced tillering response to strong soil. It was also shown that the levels of epi-5DS increased in roots of rice plants subjected to strong soil. Taken together, these results provided convincing evidence for the involvement of SL in the mediation of the strong soil stimulus resulting in reduced tillering in rice, and suggested that SLs might similarly be involved in wheat. However, at the time of conception, there were no known wheat mutants modified in SL biosynthesis or perception; to the best of the author's knowledge, this remains the case. For this reason a transgenic approach was taken to reduce the expression of two wheat genes from the SL biosynthetic pathway in wheat. For the purposes of clarity, terminology used in this chapter regarding homologues is briefly defined here. Homologue is a catch-all term referring to DNA or protein sequences with a high degree of similarity. An orthologue is a homologue in another species. A paralogue is a homologue with a different (divergent) function. Homoeologue refers to a homologous sequence (i.e. for the same gene, either complete or incomplete) located on another genome of a polyploid species (such as on the A, B or D genomes of wheat). The genes in question, *TaD10* (or *TaCCD8*) and *TaD17* (*TaCCD7*), are orthologous to rice genes *OsD10* and *OsD17*, respectively (see Figure 1.5). Biosynthetic genes were selected as targets because it would be possible to rescue any phenotypes with application of GR24 (a synthetic SL), providing further evidence for the role of SLs in the observed response. The transgenic lines generated thus offered the chance to explore the role of SL in response to strong soil in wheat. Furthermore, reduced SL production (and exudation from roots) may give the transgenic lines more resistance to SL-responsive parasitic plants such as *Striga spp*. These plants are major cereal pathogens in sub-Saharan Africa and wheat was shown by Vasey et al. (2005) to be highly susceptible to the *Striga hermonthica*.

The method chosen to reduce expression of the selected genes in wheat was RNA interference (RNAi) which has been shown to facilitate the generation of dominant loss-of-function mutants in polyploid species; bypassing the problems of gene redundancy associated with other methods of gene knockout (Lawrence and Pikaard, 2003). RNAi constructs consist of hairpin DNA sequences that comprise a promoter, inverse repeat sequence of a transcribed fragment of the target gene, a spacer region (or intron) between the inverted repeats and a terminator (Gil-Humanes et al., 2008). Upon transcription, the resulting RNA forms a hairpin shape due to dimerization of the inverted repeats (Gil-Humanes et al., 2008). This double-stranded RNA (dsRNA) is detected by an RNase called Dicer that cleaves the dsRNA into short interfering RNA sequences (siRNAs) that enable an RNA-induced silencing complex (RISC) to target mRNA sequences with a high degree of similarity to the dsRNA for degradation (Lawrence and Pikaard, 2003; Gil-Humanes et al., 2008). Travella et al. (2006) refer to two types of siRNA: the approximately 21-mer siRNAs that result in the degradation of mRNA with high sequence similarity and the 24-mer siRNAs that invoke silencing and methylation of homologous DNA (resulting in systemic silencing). The production of an RNAi construct with target regions long enough to contain multiple 21-mer or 24-mer sequence similarity to all three homoeologues should ensure silencing through at least one of the RNAi mechanisms described by Travella et al. (2006).

4.2 Materials and Methods

4.2.1 SL extraction from wheat roots

Extraction of SLs from wheat roots was conducted as described for rice in Section 2.3.2, with the following exception. Instead of using fresh roots placed directly into acetone after weighing, here the wheat roots were frozen in liquid nitrogen and stored at -80°C. To begin the extraction process, the frozen root material was ground under liquid nitrogen and an aliquot was weighed out and placed into acetone. SLs were extracted and then quantification was conducted by Dr Yoshiya Seto, as outlined in Section 2.3.3.

4.2.2 Global gene expression analysis in wheat by RNA-seq

To investigate changes in wheat gene expression resulting from growth under different soil strengths RNA was extracted from root tissues of wheat plants (*T.aestivum*, cv. Mercia) grown in weak and strong soil (2 biological replicates were used) (see Section 2.2.4.1) and sent to the Transcriptomics Facility at University of Bristol for RNA sequencing (RNA-seq). Illumina RNA-seq libraries were produced from duplicate RNA samples from individual plants grown in the two soil strengths (weak and strong) using the TruSeq mRNA sample kit (Illumina, San Diego CA, USA) using 4µg total RNA and 100bp reads were generated on an Illumina GAII sequencer (by staff of the Transcriptomics Facility at University of Bristol). The resulting RNAseq paired-end read data were processed within Galaxy (Goecks et al., 2010). The reads were mapped to a reference transcriptome for wheat (developed by bioinformaticians at Rothamsted Research) with BWA (Li and Durbin, 2010) for Illumina, followed by processing through a counts tool. The resulting counts were normalised for read library size.

4.3 Results

4.3.1 Identification of wheat orthologues of SL genes

As stated in the Introduction to this Chapter, no wheat mutants defective in SL biosynthesis or signalling exist that would enable investigation of the role of SLs in the response to soil strength; a reverse genetics approach was therefore taken using transgenic lines. The first step in this approach was to identify the wheat sequences of the genes of interest. The steps involved in the identification of wheat sequences are outlined in Figure 4.1.

Strategies to identify wheat sequences

Select rice target genes (from Ensembl Plants)

Enter rice protein sequence into BLASTP (at Phytozome)

Align returned sequences with MUSCLE (in Geneious) and build tree to identify closest Brachypodium sequence

First identification

BLAST Brachypodium DNA against 454 reads of *T. aestivum* from cerealsdb

Assemble reads to Brachypodium sequence to give wheat sequence (annotate intron-exon sequence boundaries)

Extract coding regions of wheat sequence

Translate protein sequence and align to Brachypodium and rice protein sequences before making a tree to confirm homology

Homoeologue-specific

BLAST Brachypodium DNA sequence against IWGSC CSS assemblies of Illumina reads of *T.aestivum*

If necessary to complete sequence, BLAST CSS sequence against 454 reads from cerealsdb and assemble reads to the CSS sequence

Align sequence to Brachypodium sequence to provide intron-exon boundaries

Figure 4.1: Strategies for identification of target gene sequences in wheat.

4.3.2 Identification of Triticum *aestivum D10* and *D17* sequences

The process used to identify the first *Triticum aestivum* sequences for the wheat orthologues of the rice genes *D10* (*CCD8*) and *D17* (*CCD7*) involved homology searches via Brachypodium. First, the rice sequence data were extracted from Ensembl Plants. The Rice gene names were Os01g0746400 (*D10*) and Os04g0550600 (*D17*). For each gene, the rice protein sequence was used in the BLASTP tool of Phytozome (http://www.phytozome.net/) to identify orthologues (and paralogues) in other grass species. D10 homologues were identified in maize, sorghum, Brachypodium and Foxtail millet while D17 orthologues were identified in maize, sorghum, Brachypodium, Foxtail millet and switchgrass. In the bioinformatics package Geneious (Biomatters, Aukland, New Zealand), MUSCLE (Edgar, 2004) alignments of the protein sequences were converted into phylogenetic trees by Geneious Tree builder (Jukes-Cantor, neighbour-joining method with no outgroup). This allowed the most closely related Brachypodium sequences to be identified as the Brachypodium *D10* (Bradi2g49670) or *D17* (Bradi5g17657). However, BLAST searches on wheat cDNA and genomic sequence at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) did not identify any homologous sequences.

Candidate wheat genomic sequences for *D10* and *D17* were developed by Dr. Andy Phillips using 454 shotgun sequences from cv. Chinese Spring (Brenchley et al 2012) identified by BLASTN at http://www.cerealsdb.uk.net/). Matching 454 reads were assembled using the De Novo Assembly function of Geneious at high stringency (5% mismatch, 5% gaps allowed). Candidate genomic assemblies (TaD10-454 and TaD17-454) were aligned with the Brachypodium orthologues of D10 and D17 and the intron-exon boundaries annotated. The coding regions of the wheat genes were extracted and translated into protein sequences. Figure 4.2 shows peptide alignments for *TaD10-454*, *OsD10* and *BdD10*. *TaD10-454* and *BdD10* shared 87% pairwise similarity and *TaD10-454* and *OsD10* shared 86% pairwise identity. Figure 4.3 show peptide alignments for *TaD17-454*, *OsD17* and *BdD17*. *TaD17-454* and *BdD17* shared 80% pairwise similarity and *TaD10-454* and *OsD17* shared 72% pairwise identity.

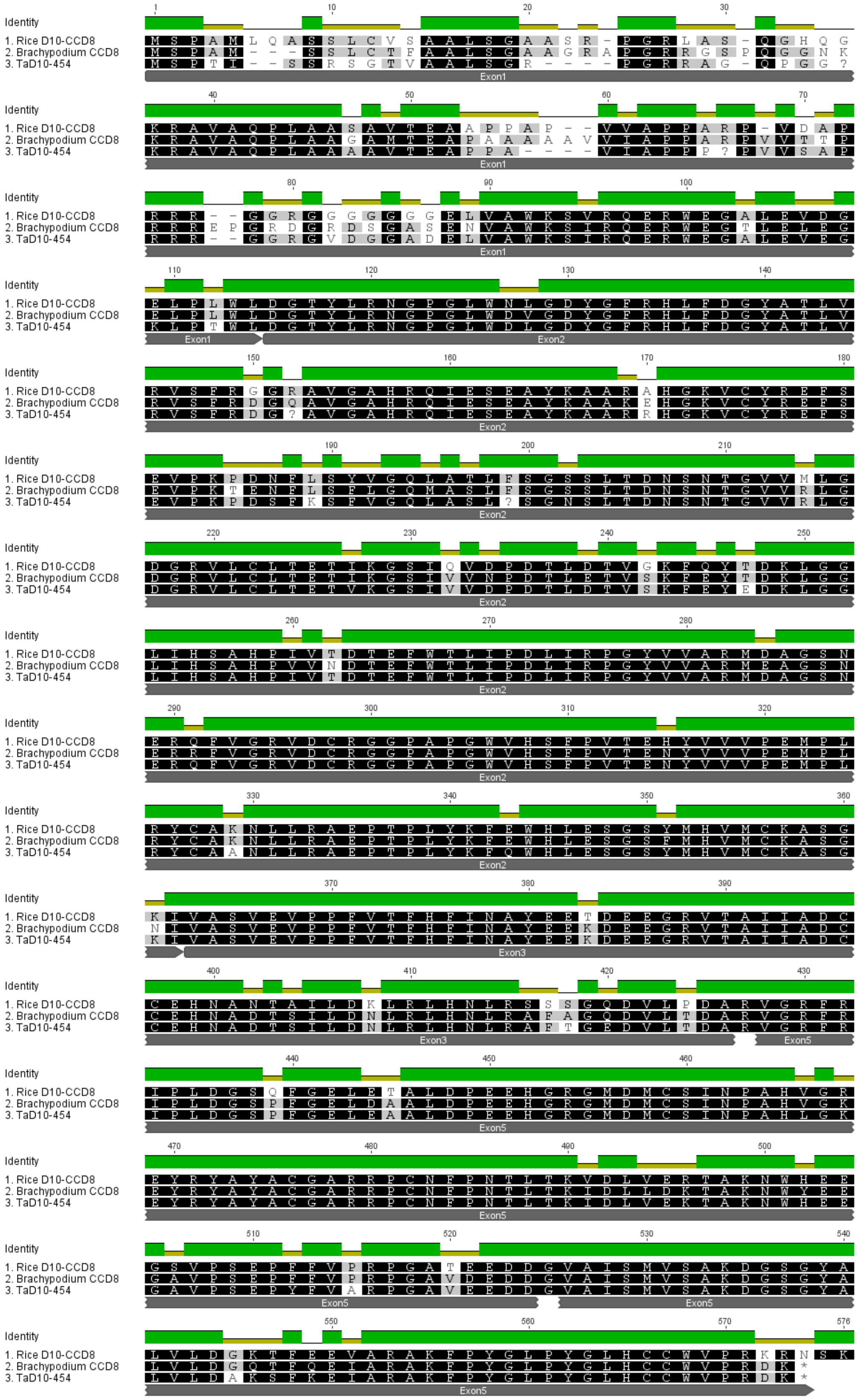


Figure 4.2 Peptide sequence of the coding sequence region (cds) of *TaD10-454* aligned to rice D10 and brachypodium D10. A similarity colour scheme was used, where black equals the same and white equals very different

Figure 4.3 Peptide sequence of the coding sequence region (cds) of *TaD17-454* aligned to rice D17 and brachypodium D17. A similarity colour scheme was used, where black equals the same and white equals very different

Figure 4.4 shows the genomic sequences of *TaD10-454* and *TaD17-454* aligned to their respective rice and Brachypodium orthologue cds regions. Phylogenetic analysis (in Section 4.3.1.3) provided good evidence that the wheat candidates were true orthologues of the known *D10* and *D17* genes from other monocot and dicot species.

Inspection of the consensus genomic sequence of the 454 assemblies for both *D10* and *D17* showed that they contained multiple nucleotide ambiguities, suggesting that each was a composite of more than one homoeologue. The opportunity to identify homoeologue-specific sequences came about with the IWGSC (International Wheat Genome Sequencing Consortium) Chromosome Sequence Survey (CSS) assemblies of Illumina reads of *T.aestivum*.

4.3.1.2 Homoeologue-specific sequences

The rice genes (Os01g0746400 (*D10*) and Os04g0550600 (*D17*)) were obtained from Ensembl Plants, using the rice protein sequence with the BLASTP tool of Phytozome to identify orthologues (and paralogues) in other grass species. D10 homologues were identified in maize, sorghum, Brachypodium and Foxtail millet while D17 orthologues were identified in maize, sorghum, Brachypodium, Foxtail millet and switchgrass.

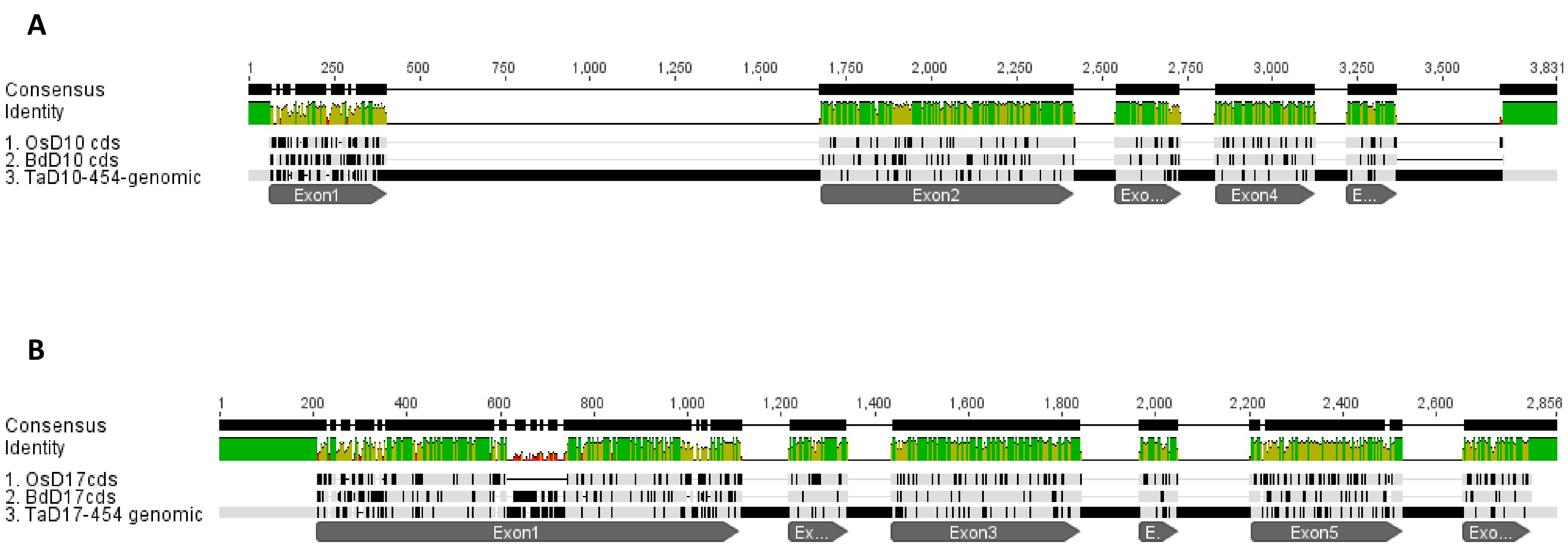


Figure 4.4 Genomic sequences of *TaD10-454* and *TaD17-454*

A) *TaD10-454* genomic sequence aligned to rice *D10* cds and Brachypodium *D10* cds.

B) *TaD17-454* genomic sequence aligned to rice *D17* cds and Brachypodium *D17* cds.

MUSCLE alignments and phylogenetic trees (by Geneious Tree builder using the Jukes-Cantor neighbour-joining method with no outgroup) identified the Brachypodium orthologues to be *D10* (Bradi2g49670) and *D17* (Bradi5g17657).

The nucleotide sequences for the Brachypodium *D10* (Bradi2g49670) and *D17* (Bradi5g17657) orthologues were blasted against the Triticaceae nucleotide database through NCBI Blast and no wheat orthologues were identified. Rothamsted Research bioinformatics staff had collated data from the IWGSC (International Wheat Genome Sequencing Consortium) Chromosome Sequence Survey (CSS) assemblies of Illumina reads of *T.aestivum* gDNA. These data were accessed by TERA-BLASTN within the DeCypher package (TimeLogic, Carlsbad, CA, USA). The Brachypodium cDNA sequences of *D10* and *D17* were blasted against the wheat CSS data using DeCypher and the highest-scoring hits were individually aligned to the respective Brachypodium cDNA sequences in Geneious in order to annotate the intron-exon boundaries. The IWGSC CSS data were chromosome and homoeologue specific. Initially only partial sequence confirmation was obtained for *TaD10*, on chromosome 3B (scaffold 10647166). However, performing BLASTN with scaffold 3B 10647166 at the CerealsDB portal to the IWGSC database (http://www.cerealsdb.uk.net/) and mapping of the returned Illumina 454 reads allowed the exon 1 region of *TaD10* to be identified and a 50% consensus sequence was extracted and termed *TaD10_3B*. Figure 4.5A shows this genomic sequence for *TaD10* aligned to rice and Brachypodium cds sequnces.

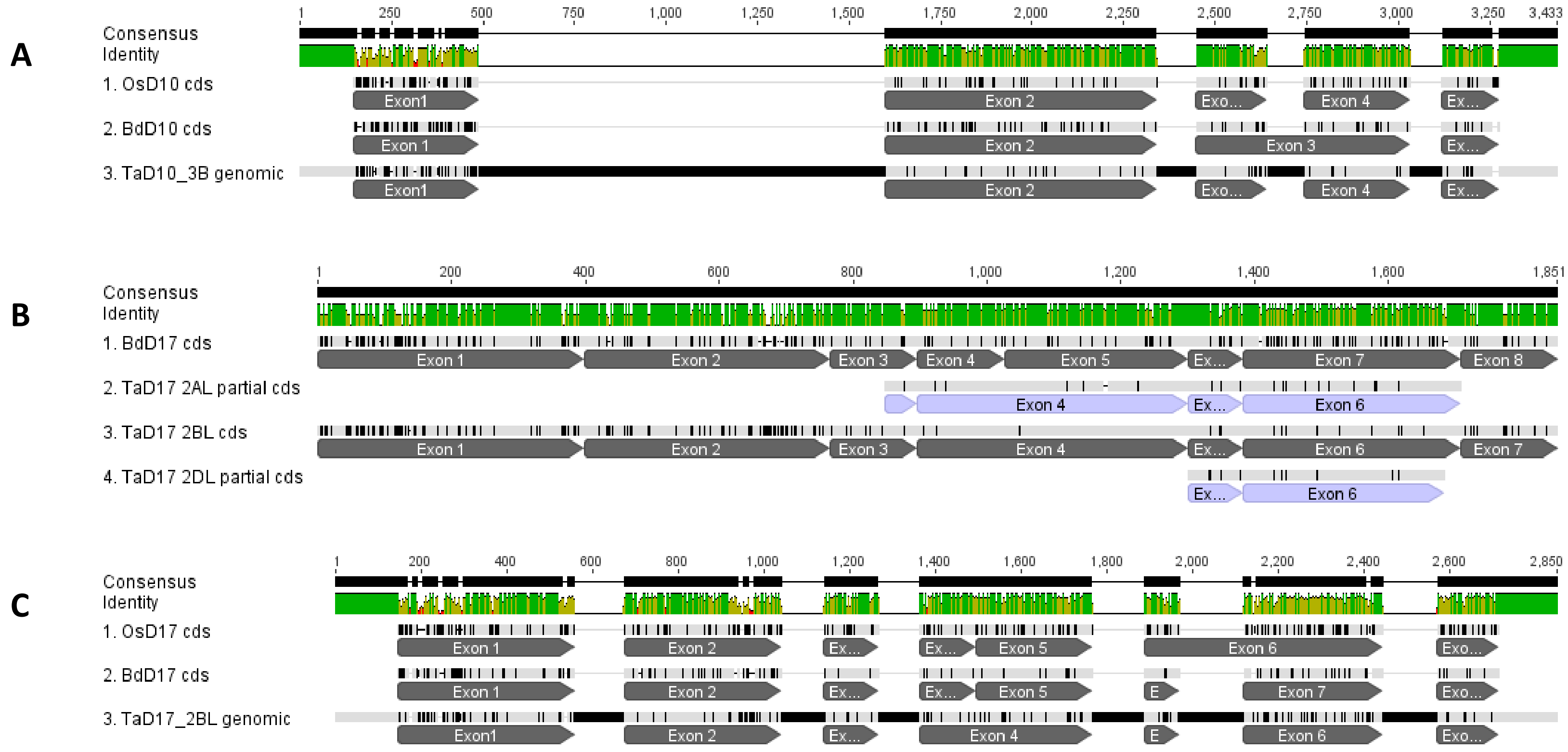


Figure 4.5 Genomic sequences of *TaD10* and *TaD17* according to IWGSC CSS assembly sequences
A) *TaD10_3B* genomic sequence aligned to rice *D10* cds and Brachypodium *D10* cds. B) *TaD17_2AL*, *_2BL* and *_2DL* cds sequence aligned to Brachypodium *D17* cds. C) *TaD17-2BL* genomic sequence aligned to rice *D17* cds and Brachypodium *D17* cds.

The IWGSC CSS data provided a full genomic wheat sequence for *TaD17* on chromosome 2BL (scaffold 7910419) as well as partial sequences of *TaD17* on chromosome 2AL (scaffold 6401380) and chromosome 2DL (scaffold 9700821) (see Figure 4.5B). Figure 4.5C shows the genomic sequence for *TaD17* (on chromosome 2BL) aligned to rice and Brachypodium cds sequnces. For *D17*, it seems that exons 4 and 5 of rice have merged in wheat while exon 7 of rice has split, leaving 7 exons in *OsD17* and *TaD17* but 8 exons in the Brachypdodium orthologue. The chromosomes that provided full sequence data were used to provide cds data (with exons copied over from aligned Brachypodium cds). The cds data was translated to give protein sequences for *TaD10_3B* and *TaD17_2BL*.

4.3.1.3 Sequence confirmation

In order to obtain more reliable versions of the wheat *D10* and *D17* genes, corresponding to the *in silico* sequences developed in section 4.3.1.1 and 4.3.1.2, partial sequences of *TaD10* and *TaD17* were cloned from wheat (cv. Cadenza) genomic DNA and cDNA and sequenced.

Primer pairs were designed against the *in silico* sequences identified above with some primer pairs targeting the entire genes and some designed to amplify targeted sub sections of the genes. For both *TaD10* and *TaD17*, partial sequences covering the 3’ half of the genes were successfully obtained from both gDNA and cDNA. The primers used to amplify these products are shown in Table 4.1 below.

Table 4.1 Cloning primers for confirming TaD10 and TaD17 sequences

Primer name	Sequence (5' to 3'; sense)
D10MF3	ACGGAGACCGTCAAGGGCTC
D103'2	AGAGAAACAATGGCACCGGTTG
D17MF2	AACGCCTTCGAGGAGGACAAC
D173'1	TGCGATTTTTGCCATTCATTC
M1360F	GCCAGGGTTTTCCCAGTCACGAC
M1360R	ACAGCTATGACCATGATTACGCCAAGC

For *TaD10*, the primer pair D10MF3 & D103'2 amplified a product of 1,388bp in length from gDNA and 1,090bp in length from cDNA (see Table 4.1 for primer sequences).

For D17, the primer pair of D17MF2 & D173'1 amplified a product of 1,078bp in length from gDNA template and 664bp in length from cDNA (see Table 4.1 for primer sequences).

Amplification products were generated using the proofreading DNA polymerase described in Section 2.2.1.1. Thermocycling included an annealing temperature of 66°C and an extension time of 1min 30 secs. Products were gel electrophoresed, excised, purified (see Sections 2.2.1.2 and 2.2.1.3) and inserted into pSC-B amp/kan (StrataClone Blunt End Cloning Kit, Stratagene) and inserted into competent cells, as per manufacturer's instructions. Transformed bacteria were cultured on agar plates at 37°C overnight (under selection from carbenicillin (100µg/ml) and X-gal (40µg/ml)) (see Section 2.2.2.2). White colonies (and some light blue colonies) were streaked onto fresh agar plates (8 per plate) and colony PCR was conducted to identify clones containing the desired inserts. The colony PCR used the robust DNA polymerase described in Section 2.2.1.1 and primers M1360F & M1360R (see Table 4.1). An annealing temperature of 60°C and extension times of 4 minutes were used for colony PCR. A selection of clones containing products of the expected size were cultured in liquid medium (containing carbenicillin at 100µg/ml) overnight and DNA was then purified with a QIAprep Spin Miniprep Kit (see sections 2.2.2.1 and 2.2.1.7). A diagnostic digest (with *Hin*dIII and *Bam*HI) (see Section 2.2.1.4) confirmed that selected clones were likely to contain the expected products and this was confirmed further by sequencing (conducted by Eurofins Genomics, Ebersberg, Germany).

Figure 4.6 shows the amplified products and the sequences obtained from individual clones for *TaD10* aligned to genomic and cds *in silico* sequences. Clones were sequenced in forward and reverse directions with M13uni21 and M13rev29 primers produced by the sequencing company (Eurofins Genomics). Clones pSC17c5 and pSC17c3 showed 95% and 94% pairwise identity to the predicted *in silico* genomic sequence, respectively. Clones pSC19c4 and pSC19c14 showed 98% and 99.9% identity to the cds, respectively.

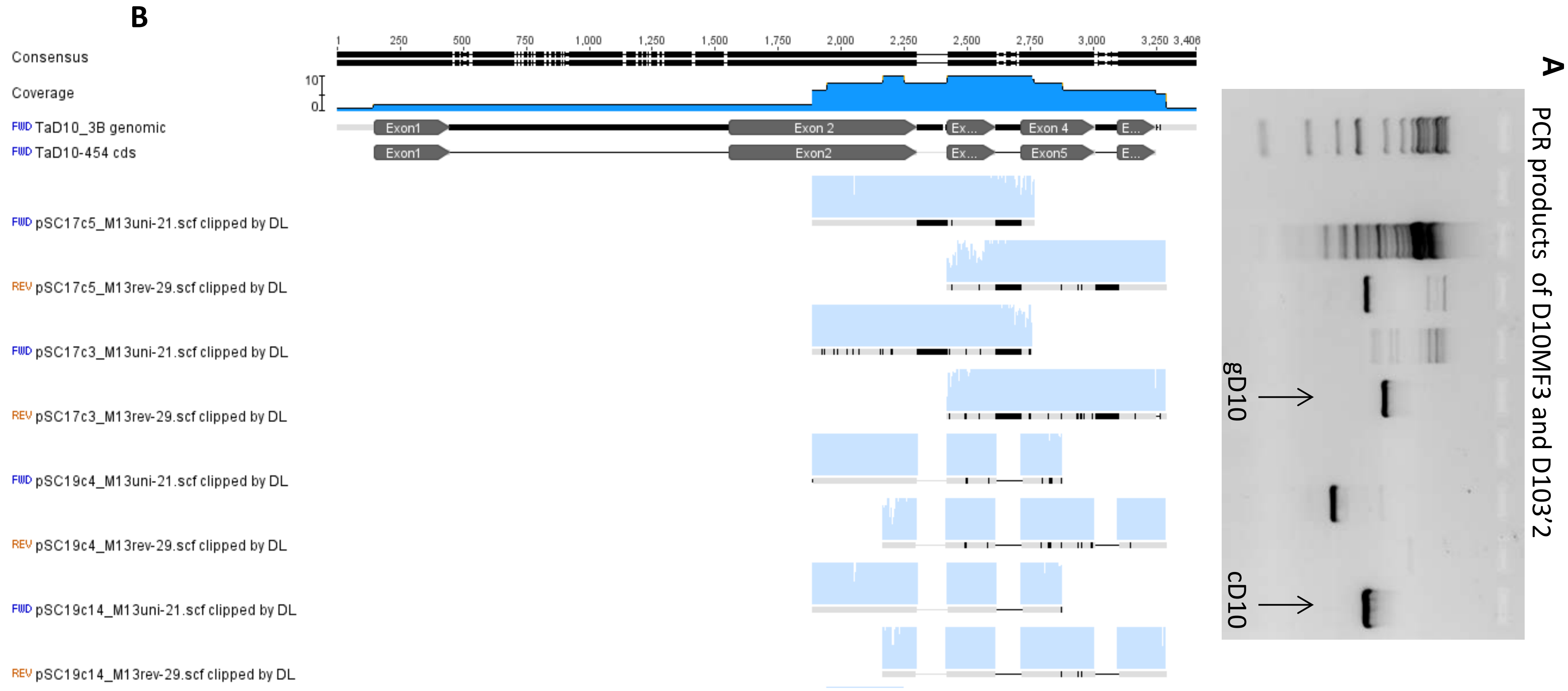


Figure 4.6 Cloned genomic and cds sequences of *TaD10*
A) TaD10 PCR products using primers D10MF3 and D103’2. B) Forward and reverse sequencing results of *TaD10* clones pSC17c5. pSC17c3, pSC19c4 and pSCc14. pSC17 clones contained genomic sequences and pSC19 clones contained cds sequences.

Figure 4.7 shows the amplified products and the sequences obtained from individual clones for *TaD17* aligned to genomic and cds *in silico* sequences. Clone pSC18c2 showed 97% pairwise identity to the cds for 2BL. Clones pSC16c14 and pSC16c13 both showed 99.8% pairwise identity to the genomic sequence for 2AL (but only 94.8% pairwise identity to the genomic sequence for 2BL). Clone pSC18c8 showed 99.5% pairwise identity to the 2BL cds. Importantly, clone sequences of both *TaD10* and *TaD17* confirmed the predicted intron-exon structure, from exon 2 to exon 5 for *TaD10* and exon 4 to exon 7 for *TaD17*. Although unsuccessful attempts were made to amplify the 5' ends of the genes, the partial sequences that were identified and isolated provide confirmation of the sequences generated via *in silico* methods.

By the time of completion of this thesis, IWGSC CSS contigs were added to the Ensembl Plants database. Selecting orthologues of *OsD10* or performing a BLAST returned a *T.aestivum* orthologue of *D10* called Traes_3B_ BDF4FBBCE, located on IWGSC CSS scaffold 10668854. Traes_3B_ BDF4FBBCE shared 95.2% pairwise amino acid identity with the *TaD10_3B* sequence (as translated from the predicted cds) outlined in Section 4.3.1.2 but lacked exon 1 and the beginning of exon 2. Traes_3B_ BDF4FBBCE was also returned by Ensembl Plants as an orthologue of *OsD10-like*. Arite et al. (2007) determined that only *OsD10* was involved in SL biosynthesis and the paralogue *OsD10-like* was not. Alternative names for these two genes include *D10*/*CCD8*/*CCD8b* and *D10-like*/*CCD8-like*/*CCD8a*, respectively.

Figure 4.7 Cloned genomic and cds sequences of *TaD17*

A) TaD17 PCR products using primers D10MF2 and D103’1. B) Forward and reverse sequencing results of *TaD17* clones. pSC16c14, pSC16c13 and pSC18c8. pSC18c2 only required sequencing in one direction. pSC16c14 and pSC16c13 clones contained genomic sequences matching ch2AL. pSC18c2 contained cds sequence matching ch2AL. pSC18c8 contained cds sequence matching ch2BL.

To confirm the identity of the *TaD10* (*CCD8*) and *TaD17* (*CCD7*) sequences identified above, protein alignments and phylogenetic trees were produced using the known orthologues of the target genes from a range of plant species. Protein sequences were collated from the Ensembl Plants and NCBI databases. The alignments were created with MUSCLE (Edgar, 2004) within Geneious, manually edited to remove gapped and non-aligned regions and phylogenetic trees were generated using PHYML (Guindon and Gascuel, 2003) within the TOPALi package (Milne et al., 2009). The model selection tool within TOPALi was used and the resulting optimal model was WAF+I+G (Whelan and Goldman + Invariable sites + Gamma). Trees were displayed in MEGA6 (Tamura et al., 2013). Figure 4.8 shows the resulting phylogenetic tree. As you would expect, all CCD7 sequences diverge from CCD8 sequences and the monocot species sequences diverge from the dicot species sequences. Wheat D10 and D17 sequences were most similar to the other wheat sequences. For both D10 and D17, Brachypodium was the most similar species. TaD103B-1 and TaD103B-2 refer to the TaD10_3B sequence identified by the author and the Traes_3B_ BDF4FBBCE on Ensembl Plants, respectively. The Traes_3B_ BDF4FBBCE sequence on Ensembl Plants actually seems to be the wheat orthologue of OsD10-like.

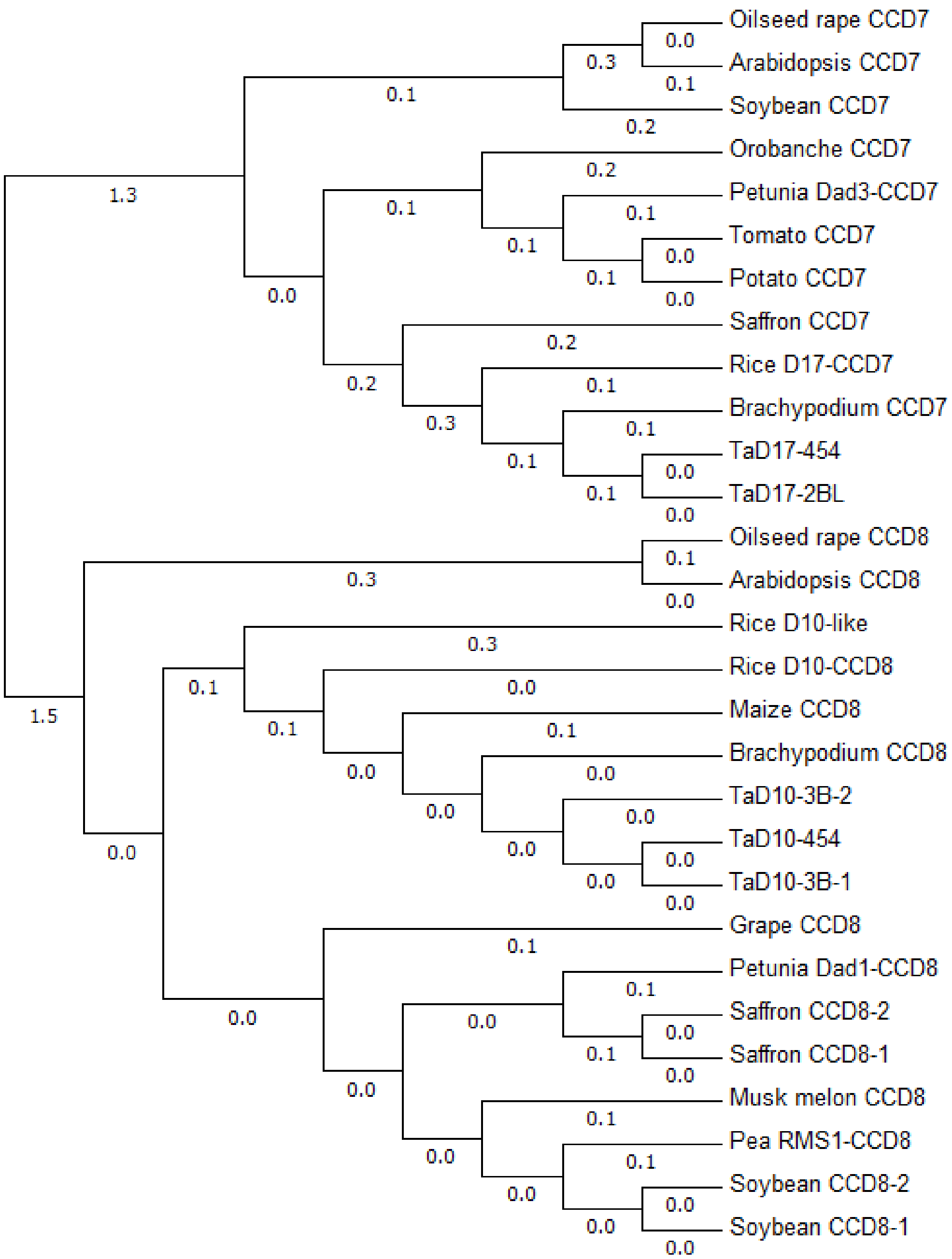


Figure 4.8 Phylogentic tree of *TaD17* and *TaD10*

Relationship between a collection of available *TaD10* and *TaD17* sequences and their orthologues in Brachypodium, rice, maize, grape, petunia, oilseed rape, Arabidopsis, saffron, musk melon, pea, soybean, potato and tomato.

4.3.3 RNAi construct production

Two RNAi constructs were produced targeting *TaD10* and *TaD17*, respectively, for degradation by the *in planta* silencing machinery. The design of the RNAi constructs included sense and antisense fragments of the target coding region DNA either side of an intron (see schematic in Figure 4.9A).

The regions of the *in silico TaD10* and *TaD17* genes selected for use as RNAi targets were chosen before the entirety of the sequence data described in Section 4.3.1 was known. The target regions were selected to be in coding regions that would allow qRT-PCR primers to be designed either side of the RNAi target to allow for subsequent expression analysis in the transgenic lines. The availability of more recent sequence information (paralogue information for TaD10 and homoeologue information for TaD17) allowed the RNAi target region used to be subsequently examined in more detail. For instance, we now know that the amplified target region for *TaD10* showed 98.7% pairwise identity to *TaD10_3B*, 98.4% pairwise identity to *TaD10-454* and 94.8% pairwise identity to the *TaD10_3B* sequence from Ensemble Plants (see Figure 4.10). The amplified *TaD17* target region is likely to be from the B genome (see Figure 4.11).

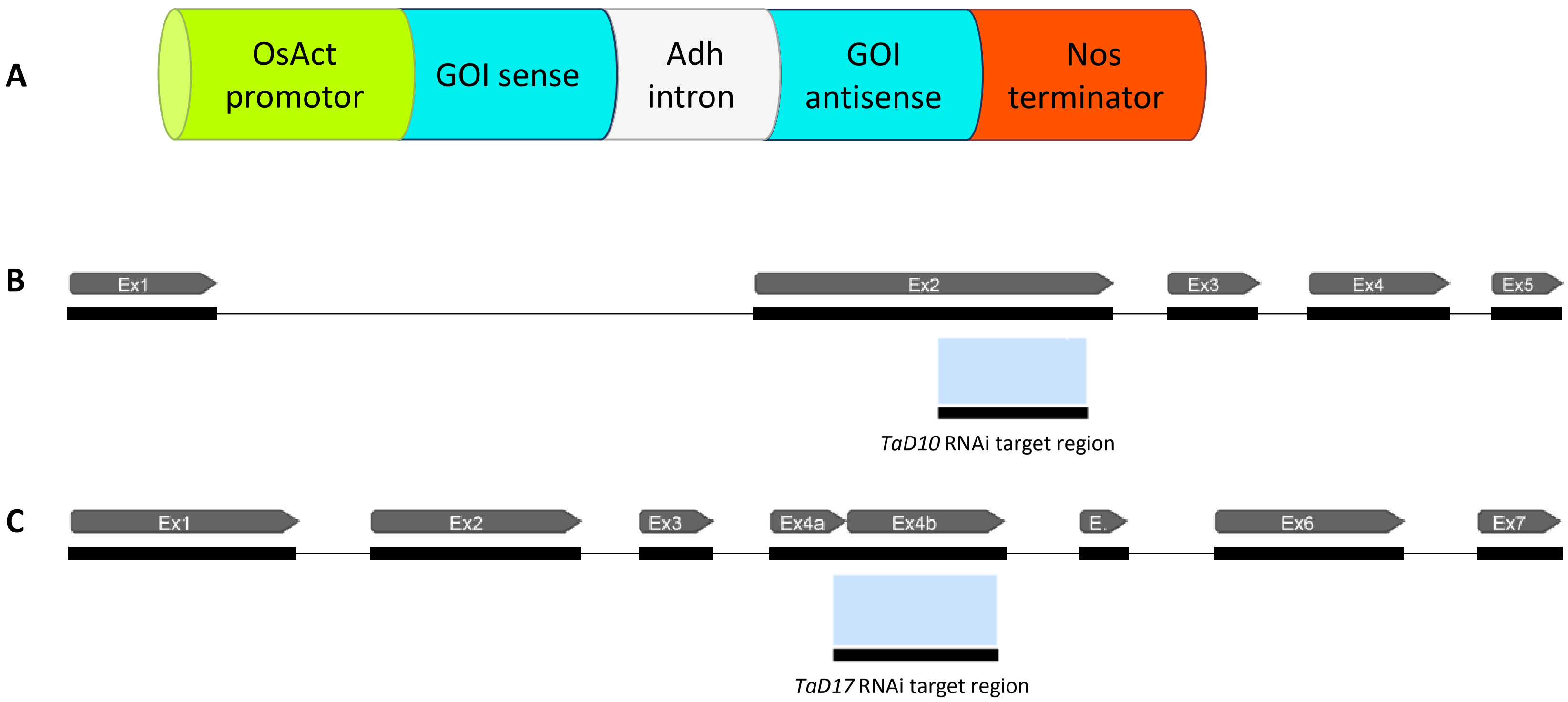


Figure 4.9: Graphical representations of an RNAi construct and the locations of RNAi target regions within *TaD10* and *TaD17*. A) Diagram of the key elements of an RNAi construct. GOI = gene of interest target region. B and C) Locations of *TaD10* and *TaD17* RNAi target regions within their respective genes

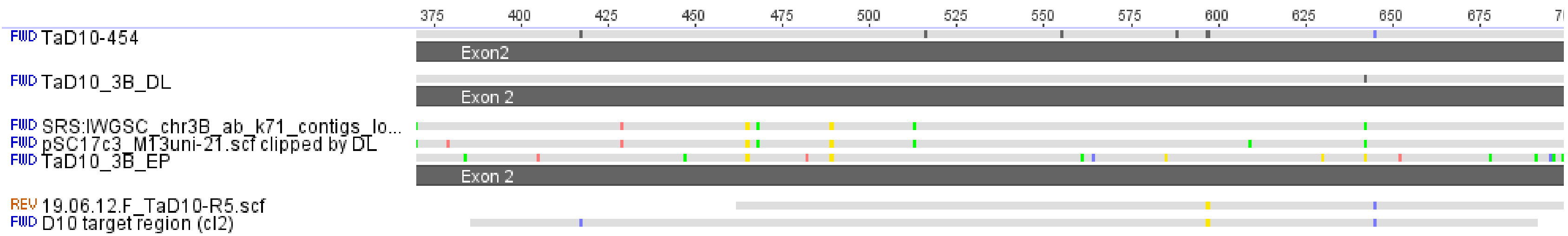


Figure 4.10: Alignment of the TaD10 RNAi target region and available TaD10 sequences
TaD10-454 = an *In silico* sequence from Dr Andy Phillips, TaD10_3B_DL is an *In silico* sequence from the author; TaD10_3B_EP is an *In silico* sequence on the Ensembl plants database.

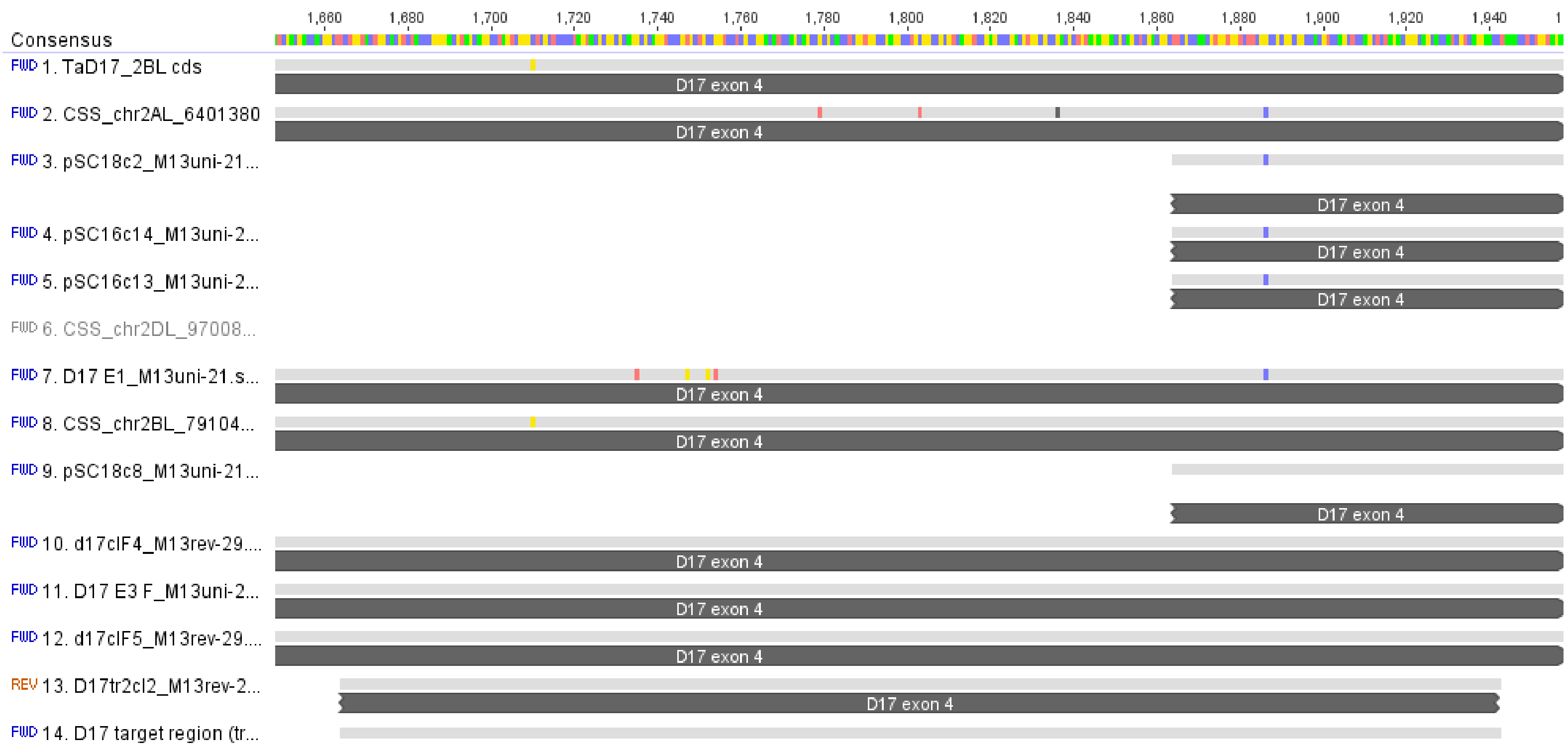


Figure 4.11: Alignment of the TaD17 RNAi target region and available TaD17sequences
TaD17-2BL cds = an *In silico* sequence from the author; CSS_ch2AL, CSS_ch2BL and CSS_ch2DL are chromosome sequence assembly sequences from International Wheat Genome Sequence Consortium (IWGSC) identified by the author to be orthologues of the Brachypodium D10 sequence. Cloned sequence data has been included in the alignment to enable comparison between all three homoeologues.

However, for both genes, target regions were likely to produce enough 21mer regions of dsRNA to knock-down expression in all paralogues and homoeologues. The *TaD10* RNAi target had 29mer, 71mer, 20mer, 32mer and 25mer sections identical to the TaD103B sequence from Ensembl Plants (likely to be *TaD10-like*), with 31mer, 35mer, 20mer, 23mer, 38mer,20mer, 32mer and 39mer sections with no difference to any homoeologue sequence. The *TaD17* RNAi target had 46mer, 24mer, 24mer, 23mer, 32mer, 49mer and 56mer sections with no difference to any homoeologue sequence.

Figures 4.8B and C show the locations of the target regions for RNAi for *TaD10* and *TaD17*. The primers for obtaining the RNAi target regions were designed against the *TaD10-454* and *TaD17-454* sequences and are shown in Table 4.2. The primers added *Bgl*II sites at the 5' ends and *Bam*HI sites at the 3' ends of the amplified sequences to enable the sub-cloning procedures involved in RNAi construct production to occur.

Table 4.2 Cloning primers for obtaining RNAi target regions

Primer name	**Final primer Sequence (5' to 3'; sense) PolyA sequence in italics, addition for cloning strategy underlined, target binding sequence in bold.**	**Expected size of fragment**
D10cloneF1	*AAA*<u>AGATCT</u>**AGTTCGAGTACGAGGACAAGC**	317 bp
D10cloneR1	*AAA*<u>GGATCC</u>**GCCACTGGAACTTGTACAGC**	
D17cloneF1	*AAA*<u>AGATCT</u>**ACAGGATCAGGCTCGACATC**	289 bp
D17cloneR1	*AAA*<u>GGATCC</u>**GGAACCAGTCGTAGGAGCAG**	

The *TaD10* target sequence (of 317bp in size) was amplified from gDNA with the primers D10cloneF1 &D10cloneR1 (see Table 4.2), using a proofreading

DNA polymerase PCR reaction with a 55°C annealing temperature for the first cycle and a 68°C annealing temperature for subsequent cycles as well as a 1 min extension time (see Section 2.2.1.1 for PCR method details). The amplified product was then inserted into a pSC-B amp/kan holding vector with the StrataClone Blunt cloning kit (as per manufacturer's instructions) (Stratagene). Diagnostic digests with *Bgl*II and *Bam*HI were used to identify clones containing the RNAi target, based product size (see Section 2.2.1.4 for restriction digest methods). Selected clones were then sequenced to confirm they contained the desired RNAi target region. Figure 4.10 shows the gene targeting region for two clones aligned to *TaD10-454*. The sequence of D10 clone 2 had 99.0% identity to that of the *in silico* sequence and was selected for use as the source of the RNAi target region for the *TaD10* RNAi construct (Figure 4.10).

The TaD17 target sequence (of 289bp in size) was amplified with the primers D17cloneF1 &D17cloneR1 (see Table 4.2)), using a proofreading DNA polymerase PCR reaction with a 60°C annealing temperature for the first cycle and a 68°C annealing temperature for subsequent cycles as well as a 1 min extension time (see Section 2.2.1.1 for PCR method details). As described above for D10, the product was inserted into a holding vector and clones were selected with a *Bgl*II and *Bam*HI diagnostic digests. The sequence of D17 clone 2 had 99.6% identity to the *in silico* sequence and was selected for use as the source of the RNAi target region for the *TaD17* RNAi construct (Figure 4.11).

To produce a final construct, the target gene sequence of *TaD10* or *TaD17* was inserted in sense orientation between the seed-specific *High Molecular Weight glutenin (HMW)* promoter and the *adh* intron of *pHMWadhNos*, which also contained the *Nos* terminator (obtained from Dr Alison Huttly). The target gene sequence was then inserted in an antisense orientation between the *adh* intron and the *Nos* terminator. The *HMW* promoter was then removed and replaced with the *rice actin* (*OsAct*) promoter from the plasmid *pGEMOsAct* (obtained from Dr Simon Vaughan), which had been shown to be expressed throughout wheat plants (including the roots where SL biosynthesis would be assumed to take place) (S. Vaughan, unpublished data). A full schematic of the assembly stages of the RNAi constructs is given in Figure 4.12.

All cloning stages were verified by diagnostic restriction endonuclease digests and sequencing to ensure correct assembly of the expected constructs; pRNAiTaD10 and pRNAiTaD17. Maps of the completed RNAi constructs targeting *TaD10* and *TaD17* can be seen in Figure 4.13A and B.

The constructs pRNAiTaD10 and pRNAiTaD17 were bulked up and purified (as described Sections 2.2.2.1 and 2.2.1.7) and 50 µg at a concentration of 1mg/ml were supplied to the Cereal Transformation Unit at Rothamsted Research who generated transgenic lines as described in Section 2.2.5.

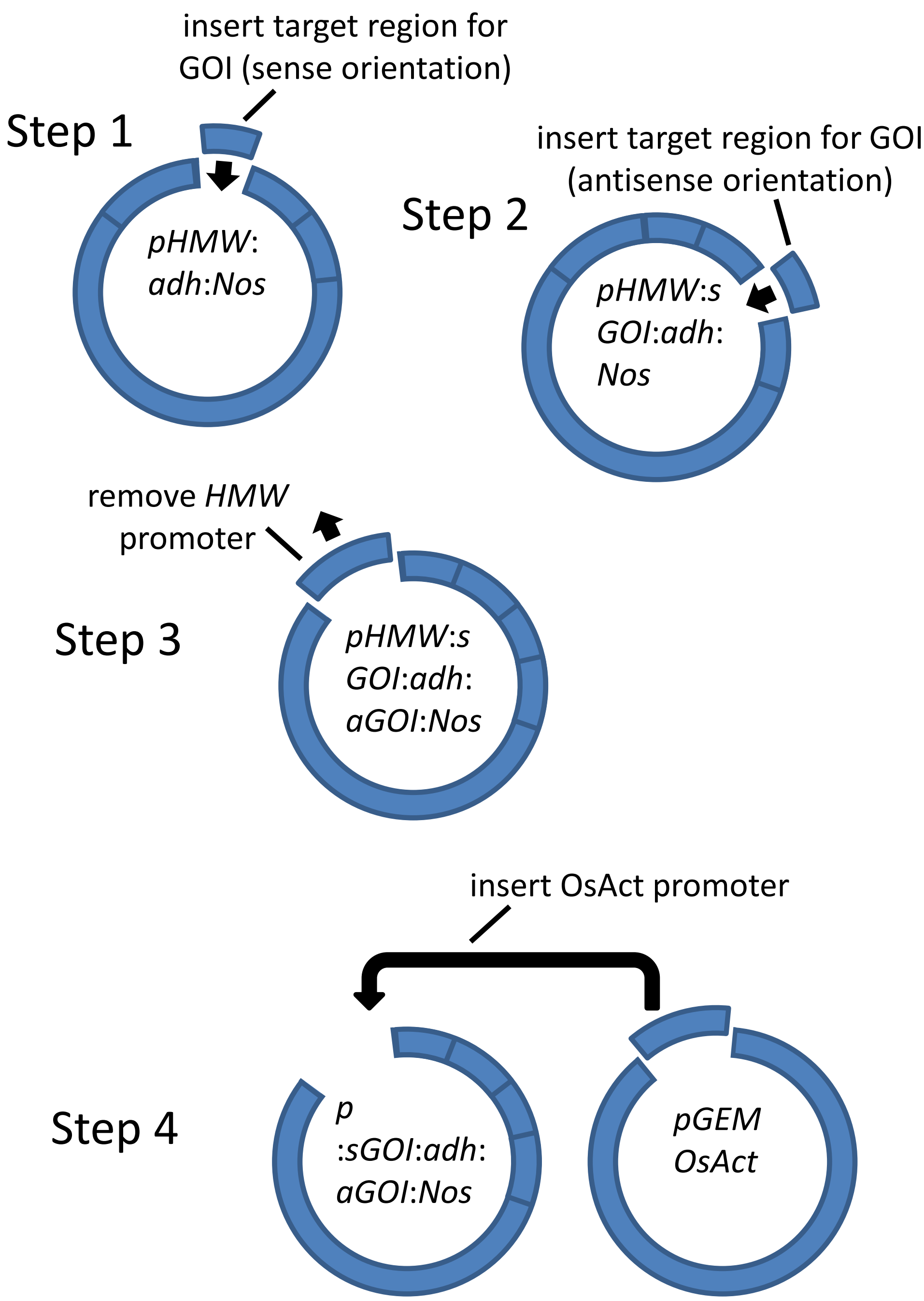


Figure 4.12: Cartoon of the RNAi construction process.
Step1: insertion of the target region of the gene of interest (GOI) in sense orientation between the promoter and the intron. Step 2: insertion of the target region of the gene of interest (GOI) in antisense orientation between the intron and the terminator. Step 3: removal of the old promoter (*HMW*). Step 4: insertion of the desired promoter (*OsAct* obtained from the donor construct *pGEMOsAct*), after which the RNAi construct is complete.

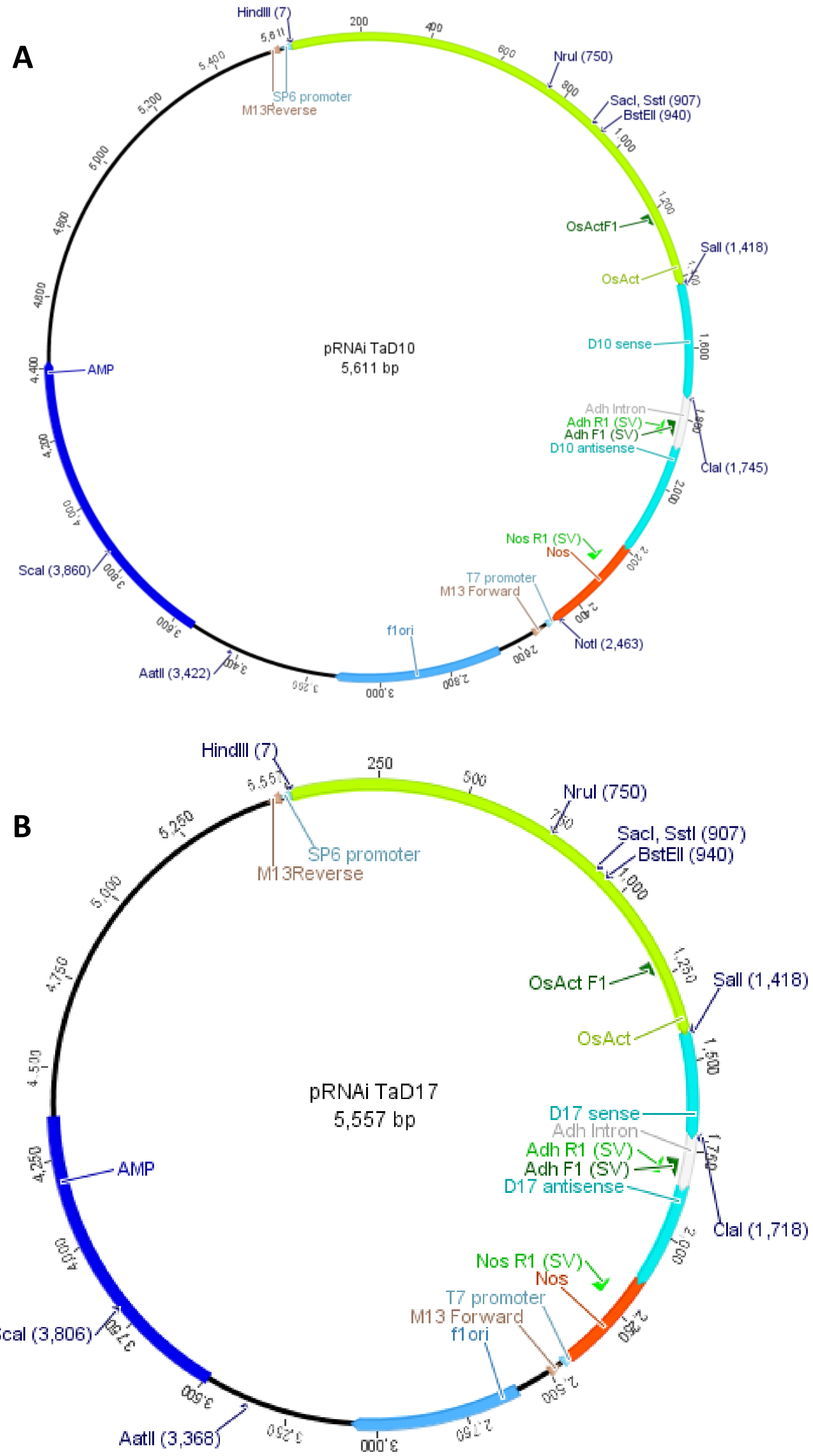


Figure 4.13: Graphical representation of the final RNAi constructs targeting strigolactone biosynthesis
A) pRNAiTaD10. B) pRNAiTaD17. 1= *OsAct*, 2 = target sequence in sense orientation, 3 = *adh* intron, 4 =target sequence in antisense orientation, 5 = *Nos* terminator. Binding sites of single-site restriction enzymes are labelled.

For both pRNAiTaD10 and pRNAiTaD17, putative independent transgenic lines were generated with 17 lines containing the RNAi construct and one control line containing only the selective marker. Leaf tissue samples were collected for PCR analysis at the T0 stage and genotyping was conducted using the diagnostic primers in Table 4.3. Genotyping involved DNA extraction (Section 2.2.3) followed by PCR with robust DNA polymerase (Section 2.2.1.1) with the annealing temperature of 52°C and extension time of 1 min. The ACTIN F and R primers were to confirm the presence of DNA while the GosactF1 and R1 primers confirmed the present of the rice actin promoter and the GnosF1 and GnosR2 primers confirmed the presence of the Nos terminator.

Table 4.3 Genotyping primers for confirming presence of RNAi hairpins

Primer name	Sequence (5' to 3'; sense)
GosactF1	AAAGGTGGCCCAAAGTGAAA
GosactR1	GCGGACAACTCACGGTGATA
GnosF1	GCCGGTCTTGCGATGATTA
GnosR2	CGGCCGCGATCTAGTAACA
ACTIN F	ATACACGCTTCCTCATGCTATCC
ACTIN R	GCTAAGAGAGGCCAAAATAGAGC

At the T0 stage no high tillering or reduced height phenotypes were observed, although some individual plants did show high levels of adventitious (of stem origin) rooting. However, at the T0 stage, there can be no replication of transgenic events and anomalous effects from the tissue culture process on individual plants cannot be ruled out so these observations required verification in subsequent generations under controlled environmental conditions.

4.3.4 Growth of T_1 SL gene-targeted RNAi wheat lines

The experiments with rice SL mutants described in the previous chapter demonstrated that the difference in tiller number between wild-type and mutant lines increased under strong soil conditions, with mutant line being more resistant to the stress. For this reason it was decided to grow multiple independent lines in strong soil with the hypothesis that difference in tillering between positive transgenic lines and null siblings would be exaggerated.

T_1 plants of nine of the 18 independent transgenic lines of each construct were grown in soil under standard glasshouse conditions (see Section 2.1.5 for growth conditions). The chosen lines included the bombardment only control (containing only the selectable marker) and 8 transgenic lines shown by the Cereal Transformation Unit to contain both the *OsAct* promoter and the *Nos* terminator regions of the RNAi construct. Leaf material was collected and genotyping was conducted in the same way as described above for T_0 plants, using primers in Table 4.3. Due to a problem with mechanical threshing many seeds were damaged which contributed to variable germination rates for the lines and meant that not all lines were available for study in subsequent experiments. Variable segregation of PCR-positive (transgenic) to PCR-negative (null sibling) plants was observed between lines. While some plants containing the construct had more tillers than their PCR-negative null siblings, this was uncommon and no transgenic plants had dramatically more tillers than the WT (wild-type) *T. aestivum* (cv. Cadenza) plants or untransformed controls.

Due to time and methodology constraints only T_1 plants of three independent lines of each construct could be tested in a replicated experiment under strong soil conditions. To assist in the selection of which lines should be in this experiment a preliminary analysis of expression levels for multiple lines was conducted, which required the development of qRT-PCR for both TaD10 and TaD17.

The methods required for qRT-PCR (including RNA extraction, cDNA synthesis, qRT-PCR and subsequent analysis) are described in Sections 2.2.4.2 to 2.2.4.5. For this work the relative quantities of the target genes (*TaD10* and *TaD17*) were both normalised against the geometric mean of relative quantities of three reference genes, namely *Ta2526* (a probable 26S proteasome subunit), *TaSDH* (*succinate dehydrogenase 3*) and *TaGAPDH* (*glyceraldehyde 3 phosphate dehydrogenase*) (see Section 2.2.4.5 for the analysis method). See Table 4.4 for target gene and reference gene primers. The selection of reference genes was based on availability of established genes in the lab, which were confirmed to have stable expression across samples. Multiple primer pairs were designed for qRT-PCR for both *TaD10* and *TaD17*. The best performing primer pairs were used to determine expression in the following work (see Table 4.4). Primers were designed to amplify a product downstream of the RNAi target region for each gene. The primers were designed to produce a product between 60 and 170bp in length and to have a Tm compatible with the Tm of primers for reference genes. In the process of testing primers it was determined that *TaD10* and *TaD17* were not expressed in leaf material, requiring the use of root material for RNA extraction and cDNA synthesis.

Table 4.4 qRT-PCR primers used for *TaD10*, *TaD17* and reference genes

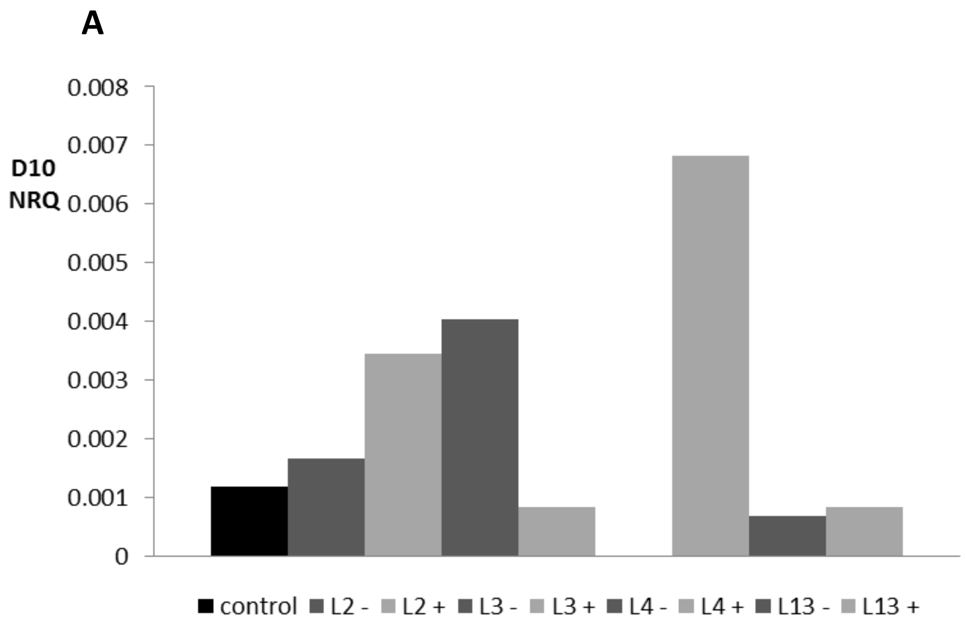
Primer name	Sequence	Product size
qTaD10_Fx5	ATGGTGAGCGCCAAGGAC	115 bp
qTaD10_Rx5	AGCAGCAGTGCAAGCCGTAG	
qRTTaD17F2	CAACTGGAAGAACAAGAAGCTG	68 bp
qRTTaD17R2	GAGGCAGCATTTCCTTGG	
qTa2526-Fext	GATCGACCAAGAATGGGATGACAAGGAAGATG	67 bp
qTa2526-Rext	CCAGTCATCCTCCCATTGCTGGACAG	
qSDH-01F	GAGACGCTCCATTTGCTCTCCGTG	137 bp
qSDH-01R	ATGTCGCAGAGCTCCTAAGGG	
qGAPDH-ex3F	ACCCCTTCATCACCACCGACTACATGACC	160 bp
qGAPDH-ex4R	GGATCTCCTCAGGGTTCCTGCAGCC	

The effectiveness of RNAi suppression of *TaD10* and *TaD17* was tested in root material with qRT-PCR. Root material was harvested from selected plants (based on the genotyping results) from each line 30 days after sowing (das). At this stage, most plants had five leaves on the main stem and had tillered from nodes of leaf 1 and leaf 2. There were too many samples to carry out qRT-PCR analysis on each individual and therefore root samples from plants testing positive or negative for the presence of the construct were pooled before RNA extraction. Additionally, not all lines could be tested due to time constraints. For the lines tested, RNA was extracted according to Section 2.2.4.2 and cDNA was produced with 2µg of RNA used for each reverse transcriptase reaction (Section 2.2.4.3). To examine expression levels of the lines, qRT-PCR for *TaD10* and *TaD17* was conducted on these cDNA samples, using the primers in table 4.4 (with Section 2.2.4.4).

The resulting gene expression estimates for the tested pRNAiTaD10 lines and the pRNAiTaD17 lines are presented in Figure 4.14. As the PCR-positive and -negative plants in each line were pooled, error bars are not presented and a full statistical analysis is not appropriate. These results may therefore be considered indicative rather than conclusive. It should also be noted that there was considerable variation in WT expression of *TaD10* and *TaD17* between PCR-negative lines. The cDNA sample for a negative plant of L4 (line four) did not provide analysable data and is thus missing from the results. pRNAiTaD10 line L3 seemed to show the expected reduction of expression of *TaD10* in the positive plants compared to the null siblings (see Figure 4.14A). However, this was not the case for *TaD10* lines L2 and L13 and (see Figure 4.14A). pRNAiTaD17 L22, L23 and L33 all seemed to show reduced expression of *TaD17*, while TaD17 line L30 showed no difference between the positive and null plants (see Figure 4.14B).

Remaining positive and negative plants of each line were grown to seed under standard glasshouse conditions and showed no clear phenotypes.

The qRT-PCR results for lines targeting *TaD10* were not particularly encouraging, with only line L3 potentially having reduced expression. Unfortunately, due to seed damage described earlier, there were not enough viable seed of this line to take forward to replicated experiments. As such, pRNAiTaD10 lines L4, L15 and L18 were selected despite preliminary expression data for these lines being missing or inconclusive.



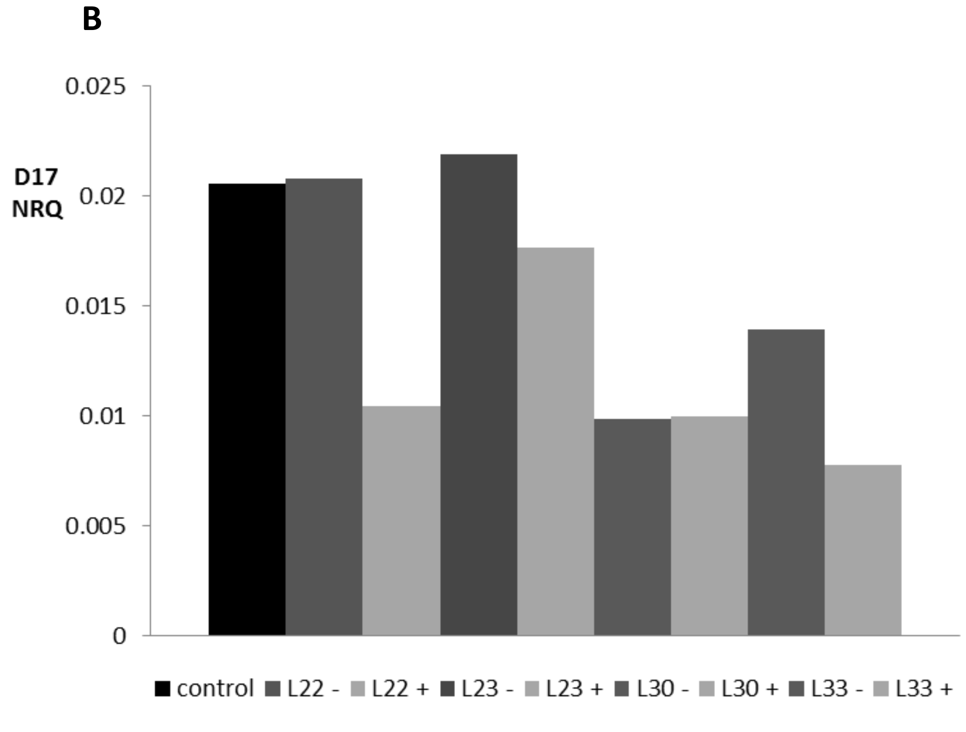


Figure 4.14: Gene expression of D10 and D17 in respective RNAi lines
A) TaD10 qRT-PCR results of a selection of pRNAiTaD10 lines. B) TaD17 qRT-PCR results of a selection of pRNAiTaD17 lines. Normalised relative quantification results of qRT-PCR are presented (where relative quantities of target genes were normalised to the geometric mean of three reference genes, *Ta2526*, *TaSDH* and *TaGAPDH*). Control = cadenza WT (bombardment only control), - = PCR-negative sample (null segregant), + = PCR-positive (trangenic) sample. Lines were represented by single pooled samples and no error is presented.

The qRT-PCR results for lines targeting *TaD17* were more promising, with three lines showing reduced expression. Lines L22 and L23 (targeting *TaD17*) were selected to be part of the replicated experiment testing 3 independent RNAi lines per construct in strong soil for tillering phenotypes.

Ideally a further round of segregation and analysis would have been carried out to identify homozygous lines to take forward, but the timing of the project did not allow for this. As such, the following experiment compares PCR-positive plants, which are likely to be a mix of homozygous or heterozygous material, with PCR-negative (null segregant) plants of each line in the T_1 generation.

48 seedlings of lines L4, L15, L18 (for *TaD10*), L22, L23 and L32 (for *TaD17*) were germinated in square repliplates (25 squares per plate) by imbibing with water. Leaf tissue was sampled 9 days after imbibing and the plates were sealed and stored at 4°C while DNA was extracted and genotyping was conducted as outlined previously using primers in Table 4.3. After genotyping for the presence of the transgenes, 4 positive seedlings and 4 negative seedlings for each of the 3 lines per construct were transplanted to sand columns in a replicated experiment using the soil strength apparatus (Section 2.1.2). As the number of sand columns available was limited, and to allow for adequate replication, only strong soil conditions were used. The plants were grown in strong soil conditions for 61 days and monitored for leaf length and tiller number weekly. At harvest, root and shoot fresh weights were recorded

and root samples were taken (with weights recorded). The maximum root depth, the number of primary roots and the number of tillers were recorded at harvest. Shoot and root dry weights were determined as described in Section 2.1.7.

Two-way *t*-tests were used to test for differences between PCR-positive and PCR-negative plants within lines for each architectural variable presented in the figures to follow. Figure 4.15 shows the final length of leaves for PCR-positive (transgenic) and PCR-negative (null segregant) plants of the T_1 RNAi plants grown in strong soil. Final leaf lengths for line L4 (a pRNAiTaD10 line) were similar between PCR-positive and PCR-negative plants (Figure 4.15A). Leaves of line L15 (a pRNAiTaD10 line) PCR-positive plants tended to be shorter than the PCR-negative plants of L15, with leaf 7 statistically significantly so (Figure 4.15B). Final leaf lengths for line L18 (a pRNAiTaD10 line) were similar between PCR-positive and PCR-negative plants (Figure 4.15C). From leaf 5 the leaves of PCR-positive plants of line L22 (a pRNAiTaD17 line) were longer than the PCR-negative plants, with leaves 7 and 8 showing statistically significant differences between PCR-positive and PCR-negative plants (Figure 4.15D). Early leaves of PCR-positive plants of line L23 (a pRNAiTaD17 line) were marginally longer than the leaves of PCR-negative plants (with a statistically significant difference in leaf 3), while leaves 8, 9 and 10 were shorter in the PCR-positive plants (with leaves 8 and 9 statistically significantly so) (Figure 4.15E).

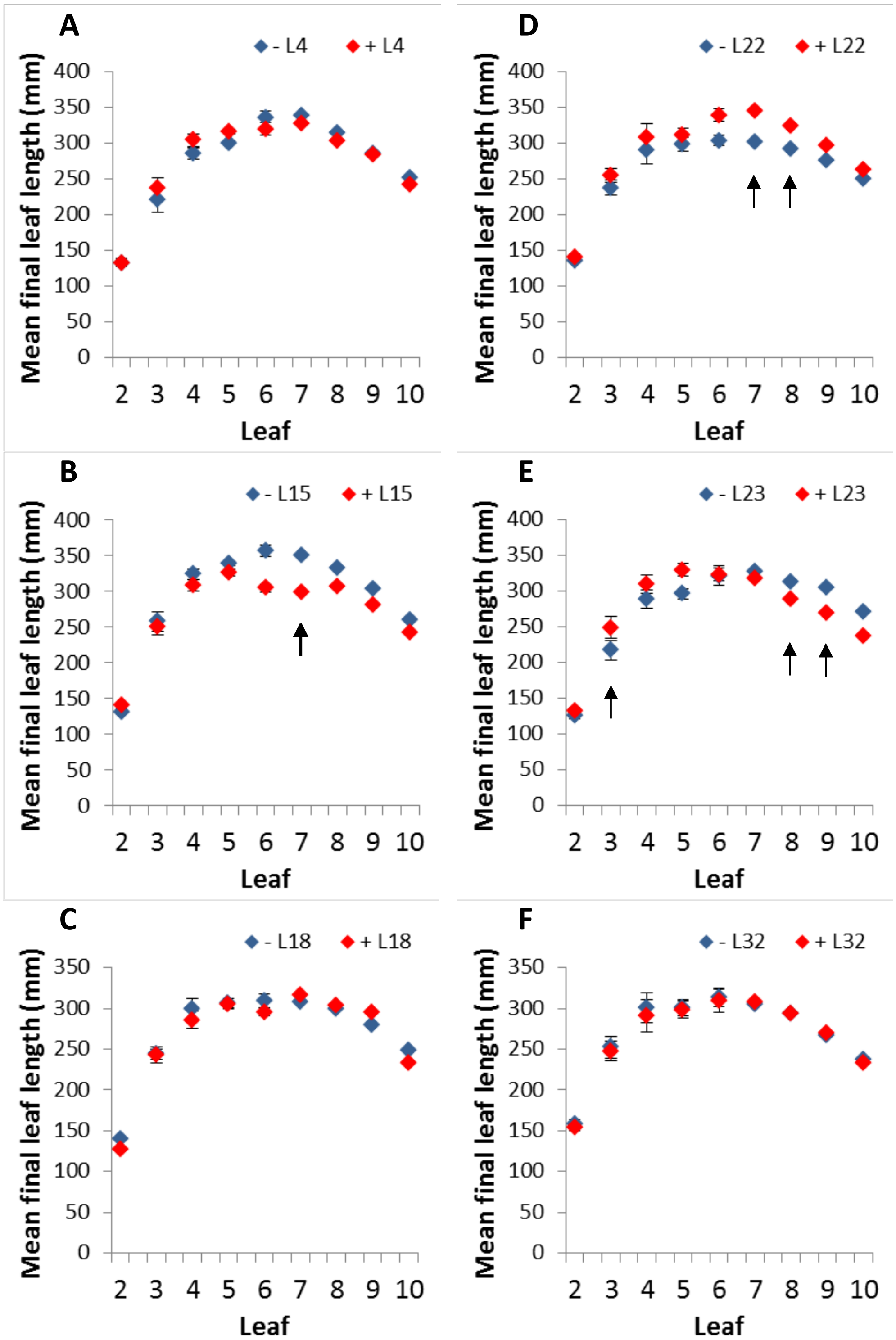


Figure 4.15 Leaf length phenotypes of T1 transgenic plants targeted to be SL deficient grown in strong soil.
A, B and C) Final leaf length of plants containing pRNAiTaD10 (L4, L15, L18). D, E and F) Final leaf length of plants containing pRNAiTaD17 (L22, L23 and L32). Positive transgenic plant = +; null sibling = -. Error bars are given as standard error of the means. *n*=3-4. Black arrows indicate statistically significant (p<0.05) differences between PCR negative (-) and PCR positive (+) plants, tested with Two-way *t*-tests.

All leaves of PCR-positive and PCR-negative plants were the same length for line L32 (a pRNAiTaD17 line) (Figure 4.15F).
Apart from PCR-positive plants for line L15, plants showed maximal leaf length for 7 and a subsequent decline of leaf length in later leaves.

No general differences between PCR-positive plants and their null siblings were seen for maximum root depth, although lines L4 (containing pRNAiTaD10) and L22 (containing pRNAiTaD17) showed shallower maximal root depth in PCR-positive plants than PCR-negative plants in the respective lines (but not statistically significantly so) (Figure 4.16A). The number of primary roots was not statistically significantly different between PCR-positive and PCR-negative plants for any line. Tillering related phenotypes were the key data from this experiment to assess the functionality of the RNAi constructs and the role of SL in wheat responses to strong soil. The total number of tillers were higher in PCR-positive plants compared to PCR-negative plants for both line L18 (containing pRNAiTaD10) and L22 (containing pRNAiTaD17) (Figure 4.16C). However, none of these differences were statistically significant, as tested with Two-way *t*-tests. In the knowledge that the later leaves of all plants showed reductions in length compared to leaves 6 and 7 (implying some form of stress was present), site filling was assessed for a time when leaves were reaching maximal lengths; at 26 days after sowing

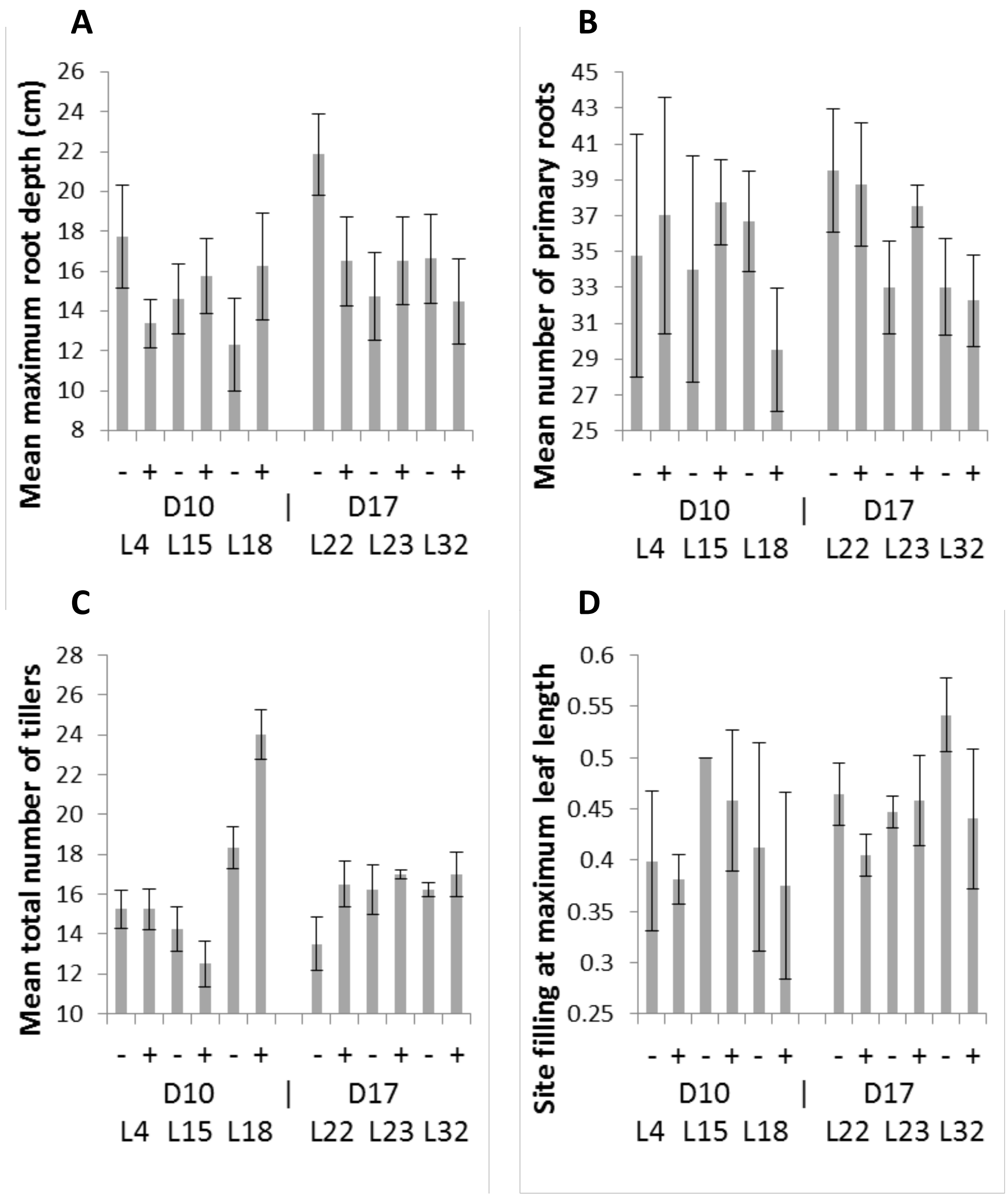


Figure 4.16 Root and shoot architecture phenotypes of T1 transgenic plants targeted to be SL deficient grown in strong soil.
A) Maximum root depth (cm) of plants containing either pRNAiTaD10 or pRNAiTaD17. B) Number of primary roots of plants containing either pRNAiTaD10 or pRNAiTaD17. C) Total number of tillers of plants containing either pRNAiTaD10 or pRNAiTaD17. D) Site filling of plants containing either pRNAiTaD10 or pRNAiTaD17. Positive transgenic plant = +; null sibling = -. Error bars are given as standard error of the means. *n*=3-4. A-C show results at harvest (61 days after sowing), D shows results 26 days after sowing (when leaves 6 & 7 were actively growing).

(when leaf 6 or 7 was extending). Differences in site filling were not able to support the differences seen in total tillering, with line L18 showing no difference in site filling and L22 possibly showing reduced site filling in PCR-positive plants relative to PCR-negative plants, with no statistically significant differences observed for any lines.

A general reduction of shoot biomass (fresh and dry weight) was seen in lines containing pRNAiTaD10 for PCR-positive plants compared to PCR-negative plants (Figures 4.17A and B). Reductions of shoot dry weight were seen in PCR-positive plants of lines L15 and L18. Line L23 (containing pRNAiTaD17) showed reduced shoot biomass in PCR-positive plants relative to PCR-negative plants (Figures 4.17A and B). However, no statistically significant differences in any biomass measurement were seen between PCR-positive and PCR-negative plants for any line. Root biomass showed no general trend for differences between PCR-positive and PCR-negative plants containing either RNAi construct (Figures 4.16C and D), although PCR-positive plants of line L15 showed a reduction in root fresh weight and in PCR-positive plants of line L23 showed an increase in root fresh weight, although not significantly so (Figure 4.17C).

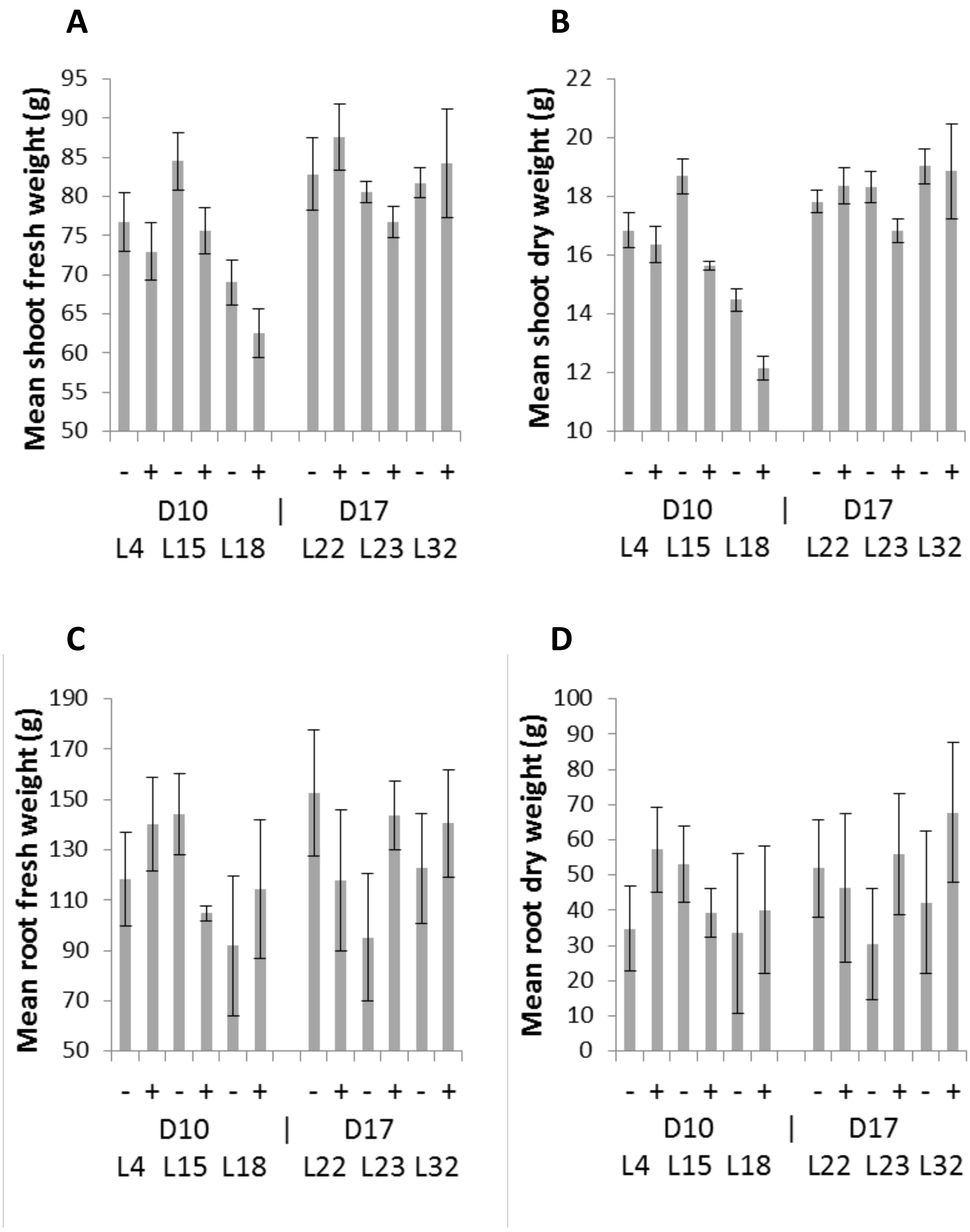


Figure 4.17: Biomass of T1 transgenic plants targeted to be SL deficient grown in strong soil.
A) Mean shoot fresh weight (g) of plants containing either pRNAiTaD10 or pRNAiTaD17. B) Mean shoot dry weight (g) of plants containing either pRNAiTaD10 or pRNAiTaD17. C) Mean root fresh weight (g) of plants containing either pRNAiTaD10 or pRNAiTaD17. D) Mean root dry weight (g) of plants containing either pRNAiTaD10 or pRNAiTaD17. Positive transgenic plant = +; null sibling = -. Error bars are given as standard error of the means. *n*=3-4. A-D show results at harvest (61 days after sowing).

Whilst expression data is not yet available to indicate in which lines gene silencing has been most efficient and the zygosity of individual plants is not yet know, it is essential to recognise at this stage that the performance of individual lines is of major interest. The fact that one line for each construct (L18 for pRNAiTaD10 and L22 for pRNAiTaD17) showed increased total tillering in PCR-positive plants relative to PCR-negative plants of respective lines was encouraging, despite the lack of a statistically significant difference (Figure 4.16C). Although preliminary expression data was not available for pRNAITaD10 L18, target gene expression for pRNAITaD17 L22 was shown to be reduced by almost 50% (Figure 4.14B), suggesting that *TaD17* may well have a role in mediating the response of wheat to strong soil.

4.3.5 Quantification of SL in wheat roots from weak and strong soil

Preliminary assessments of the feasibility of using frozen wheat root material confirmed orobanchol to be at high enough endogenous levels to quantify, while (−)-*ent*-2'-*epi*-5-deoxystrigol and orobanchyl acetate were below detectable limits. With a view to using frozen wheat material so that it could be used for multiple tests, samples of root material from *T. aestivum* (cv. Cadenza) plants grown in weak soil (sand columns with foam weights) were either collected directly into acetone or flash frozen in liquid nitrogen and then processed according to Section 2.3.2 (Section 4.2.5 outlines the modification to that process), using 500pg of internal d_1-orobanchol standard. Very low endogenous levels of orobanchol could be detected but not reliably quantified in both frozen and acetone stored samples, indicating that freezing of root material before extraction did not affect the ability to extract SLs. Due to availability of frozen root samples from an earlier experiment, samples of root material from WT *T. aestivum* (cv. April Bearded) plants grown in either weak soil or strong soil were tested, with a pool of 4 plants making up each sample and using a range of internal standard levels (10pg, 100pg and 500pg of d_1-orobanchol). It was determined that 500pg of the internal standard d_1-orobanchol was suitable to allow for quantification.

Table 4.5 shows that there was no striking difference between the root concentration of orobanchol between weak and strong soil treatments. If anything, the orobanchol level was slightly higher in wheat roots grown in weak soil than in those grown in strong soil, but the lack of replication emphasizes the preliminary nature of this result.

Table 4.5 Orobanchol levels in wheat roots grown in strong and weak soil
Samples were pools of 4 *T.aestivum* (cv. April Bearded) plants

Treatment	Orobanchol concentration (pg/gFW)
Weak	113
Strong	97.5

A replicated experiment was designed to measure not only the levels of SL but also a variety of other hormones in a global hormone analysis of samples of root tips, root system, expanding leaves and fully elongated leaves. Further details of this experiment are presented in Section 5.2.2. For the purposes of the current chapter, SL was extracted from frozen *T. aestivum* (cv. Cadenza) root material in accordance with Sections 4.2.5 and 2.3.2. No SL was detected at quantifiable levels (with orobanchol, (−)-*ent*-2'-*epi*-5-deoxystrigol and orobanchyl acetate all tested using 500pg of each internal standard).

4.3.6 SL gene expression in wheat roots under weak and strong soil

In order to assess gene expression changes between weak and strong soil conditions, root samples were collected from plants grown in one of the experiments described in (Coelho Filho et al., 2013). The experiment included WT (wild-type) plants of *T. aestivum* (cvs. Mercia and April Bearded) which carried the fully functioning *Rht-B1a* allele of *Rht*. Other plants in the experiment carried the *Rht-B1b* and *Rht-B1c*. It was decided to test WT (*Rht-B1a*) plants of the Mercia background for differences in gene expression of roots grown in weak and strong soil. To limit costs only four samples could be tested. Two biological samples (from individual plants) were selected for each of the two soil strength treatments. The alternative strategy would have been to pool multiple plants and lose all biological replication. RNA was extracted as described in Section 2.2.4.1 and sent to the Transcriptomics Facility at University of Bristol for RNA-seq analysis (see Section 4.2.2 for more detail). Reads were mapped to a reference transcriptome and read counts were normalised for the read library size (see Section 4.2.2). Dr Andy Phillips had identified wheat orthologues for a number of genes in the SL pathway, using similar methods to those described in Section 4.3.1. The normalised read counts for each of those genes were identified. It was not possible to carry out statistical analyses on these results due to only having two biological replicates and two soil strength levels (leaving only 1 degree of freedom). Therefore, the Log_{10} of mean counts and standard error of the means are presented in Figure 4.18. There are no striking differences in the expression of any of the genes of the wheat SL pathway between roots grown in weak and strong soil. There is some indication of reduced expression of the biosynthesis genes *TaD27*, *TaD10*, *TaD17* and *TaMAX1*. The SL perception genes, *TaD3* and *TaD14* seem to be identically expressed in weak and strong soil.

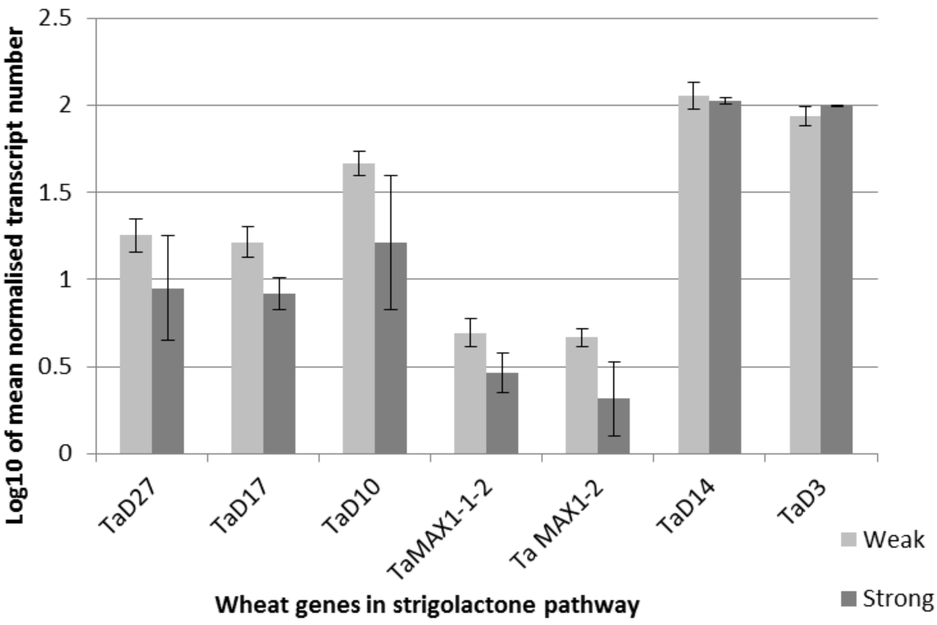


Figure 4.18: Wheat SL gene expression in weak and strong soil, as determined by mapping of RNAseq reads from root material.
n=2. Error bars are given as standard error of the means.

4.4 Discussion

The use of mutants to assess the role of individual genes or signalling pathways in developmental processes in plants is well established. For example, the previous chapter describes work using existing SL biosynthetic and signalling mutants of rice to demonstrate a role for this hormone in the mediation of strong soil effects on architecture. However, as a polyploid species, mutagenesis and forward genetics have made little contribution to our understanding of wheat biology, as mutations in one homologue may be complemented by other homoeologues and therefore have little effect on the phenotype. As a result, there are very few characterised mutants in developmental pathway in wheat. It was therefore clear that to develop such resources in wheat, a reverse genetics strategy such as RNAi would be the best approach.

Many plant species, including cereal crops, have fully sequenced and annotated genomes, including *Arabidopsis thaliana, Brassica rapa*, *Solanum lycopersicum*, *Solanum tuberosum*, and *Oryza sativa*. Additionally, seed collections of T-DNA insertions lines and RNAi lines as well as mutant libraries (including the Nottingham Arabidopsis Stock Centre, RIKEN BRC Bioresource, Agrikola and the International Rice Genebank) are available for many species. A fully sequenced genome and available mutants make genetic studies of such a plant species relatively straightforward. *Triticum aestivum* is a more complicated experimental candidate. The very large hexaploid genome of bread wheat (*T. aestivum*) has until very recently been an obstacle to genome sequencing, and few resources were available at the start of this project.

However, there have been rapid developments in wheat genomics during the course of this project. As the project started, Brenchley et al. (2012) publically released a 5-fold sequence coverage using 454 pyrosequencing technology of cv. Chinese Spring and also a partial assembly of this data. Due to the limited coverage, the *de novo* sequence assembly was relatively unsuccessful and the resulting contigs were mostly very limited in extent, having an N_{50} of only 481 bp (i.e. 50% of the assembled genome was represented by contigs shorter than 481bp). However, by selecting individual 454 reads by BLASTN to the unassembled reads using probes from the related grass Brachypodium, Pearce et al. (2015) showed that it was possible in many cases to develop accurate assemblies of target genes, although in many cases lack of coverage resulted in the assemblies being composites or chimaeras of two or more homoeologues.

To develop wheat sequences for SL genes, Dr Andy Phillips used this custom assembly approach to develop genomic sequences of putative orthologues to a number of rice SL genes, including D10 and D17. Mapping of RNA-seq reads from wheat showed that these sequences were expressed and also confirmed that the assemblies were accurate representations of the genes. In the absence of a source of higher quality assemblies, these draft genomic sequences were used as the basis for the design of RNAi constructs designed to supress the endogenous genes when used to transform wheat.

While the RNAi constructs were being prepared and wheat transformation undertaken, a strategy was put in place to confirm the draft sequences by amplifying the corresponding sequence from the target cultivar, Cadenza. At this point, the International Wheat Genome Sequencing Consortium (IWGSC) carried out a shotgun survey of each chromosome arm of cv. Chinese Spring and pre-released the draft sequence assemblies of each of the seven chromosomes for all three sub-genomes (A, B and D genomes) (Mayer KF, 2014). These assemblies had an average L50 of 2,400 bp (Mayer KF, 2014), but many genes were still missing or present as partial sequences. Thus, when searched with the Brachypodium sequences for *D10* and *D17* full length gene sequences were only identified for one homoeologue of *TaD10* (on chromosome 3B) and a partial sequence for *TaD17* (on chromosome 2BL) and location of Exon 1 for *TaD10* required a BLASTN search of 454 data with the 3B sequence. In addition, the IWGSC assemblies contained partial assemblies of *TaD17* from chromosomes 2AL and 2DL. During the search for *TaD10* an additional partial sequence on chromosome 3B was identified, lacking the exon 1 and part of exon 2 and showing more similarity to dicot *D10* sequences than to the Brachypodium *D10* sequence. This latter result strongly suggested the presence of two paralogues of *TaD10* on the group 3 chromosomes. After IWGSC sequence data were made available on Ensembl Plants, this second paralogue of *TaD10* was published on Ensembl Plants and as can be returned as *T. aestivum* orthologue Os*D10* and *OsD10-like* sequences. Despite the presence of two paralogues for *TaD10*, at least five regions in the RNAi target region of pRNAiTaD10 were long enough (21mer to 24mer) to be expected to cause siRNAs to knock-down the *TaD10*/*TaD10-like* sequence from Ensembl Plants, and six regions were long enough to cause siRNAs to knock-down all three *TaD10* homoeologues. No paralogue was identified for *TaD17* and seven regions of the RNAi target region of pRNAiTaD17 were at least 21 mer to 24mer in length and expected to result in siRNAs capable of knocking-down *TaD17* expression in all three homoeologues.

The use of RNAi to reduce gene expression has been a useful tool in reverse genetics, especially for polyploid species where gene redundancy reduces the effectiveness of other methods. Multiple authors have successfully used RNAi to knock down expression of target genes and showed effects on various target signalling pathways in wheat (Travella et al., 2006; Gil-Humanes et al., 2008; Sestili et al., 2010). This includes lines produced by the Cereal Transformation Unit at Rothamsted Reasearch (Lovegrove et al., 2013).

The time taken to identify SL genes in wheat, generate RNAi constructs and go through the necessary generation times to produce transgenic lines limited the depth of analysis that was possible on the resulting plants. Ideally, multiple lines would have been taken through to a least the T_2 generation, and zygosity, assessed by real-time PCR, determined to identify homozygous lines. These would have been assessed in replicated experiments for suppression of the target genes and the phenotype fully characterised before assessing the response to soil strength. In the event, it was necessary to foreshorten this analysis and the qRT-PCR analysis was carried out on pooled transgene-positive and -negative plants in the segregating T_1 generation. From this analysis there was a tentative assessment that one line for *TaD10* may have had successfully altered gene expression, *TaD10* was reduced in positive L3 plants (containing the pRNAiTaD10 construct) and the positive plant grown to seed had more tillers than the negative plant. For *TaD17*, there were 3 lines that showed reduced expression of *TaD17* in the positive plants (containing the pRNAiTaD17 construct) (L22, L23 and L33).

In an attempt to highlight any phenotypic differences between RNAi and wild-type plants, a subset of lines was tested in strong soil using the strong soil apparatus, which, by analogy with the rice experiment in Chapter 3, would be expected to supress tillering more strongly in wild-type lines (PCR-negative) than in SL-deficient lines (PCR-positive). For *TaD10*, L18 showed increased final tillering in PCR-positive lines. For *TaD17*, L22 showed a similar increase in final tillering in PCR-positive line. Despite only being able to grow three lines of each construct in strong soil, and without having yet been able to determine expression levels of the target genes, one line for each construct has been shown to have increased tillering (as would be expected from successfully knocking-down expression of SL biosynthesis genes). However, this was not at a statistically significant level. The lack of significant differences may well have been due to sample size, as a reliable *t*-test result generally requires 5 or more samples. With more time it would have been possible to thoroughly evaluate all 17 transgenic lines of each construct and take selected lines (such as L22 and L18) through to T_2 homozyogus plants that could be tested in strong soil.

Most of the work to establish the response of wheat to strong soil was carried out in the cultivars Mercia or April Bearded. The switch to cv. Cadenza for the transgenic work was forced by the lack of established protocols for transformation of the two former varieties. In the event, this work found barely detectable (unquantifiable) levels of orobanchol in cv. Cadenza; unlike those of April Bearded which can be seen in Table 4.5. This may indicate that cv. Cadenza does not produce high levels of this SL. However, Vasey et al. (2005) showed that root exudates from cv. Cadenza are capable of promoting germination of Striga seeds, but did not specify the presence of SLs in their samples. The number of tillers produced by wild-type Cadenza plants is higher than many varieties so it is plausible that the strigolactones they produce are biologically active for Striga germination but do not inhibit tiller bud outgrowth, perhaps by lacking the *R'*-orientation of the D ring that Umehara et al. (2015) showed was required for tiller inhibition in rice. Interestingly, cv. Cadenza showed low build-up of inoculum of the wheat fungal disease take all (McMillan et al., 2011). SLs are known to stimulate branching of arbuscular mychorrizae and germination of parasitic seeds (Akiyama et al., 2005; Yoneyama et al., 2008). It is possible that that *Gaeumannomyces graminis* var. *tritici* (the fungus responsible for take-all in wheat) responds to SLs in a similar manner to arbuscular mychorrizae and seeds of parasitic plants, using SLs as indicators of the presence of host plants. If this were to be the case then the reduced build-up of inoculum for cv. Cadenza seen by McMillan et al. (2011) could be another indication of limited SL production in this cultivar. The specific functioning of different SLs in wheat, and especially cv. Cadenza, is certainly worthy of further investigation. Indeed, application of different levels of the synthetic SL GR24 should result in a range of tillering responses and offers a simple and effective method of testing the hypothesis that Cadenza may be non-perceptive to SL for tillering. If cv. Cadenza were in some way limited in the production or perception of SLs in general it would have been surprising to see the differences in tillering between PCR-positive and PCR-negative plants seen in the RNAi lines L18 and L22 that targeted *TaD10* and *TaD17*, respectively.

The number of copies of the transgene (RNAi construct) will vary between lines and different individuals will be heterozygous and homozygous at the T_1 stage. Variation in transgene copy number can account for some of the variation in the effects of RNAi and the level of phenotype severity is often greater in homozygous individuals (Travella et al., 2006). This highlights some of the reasons for variation between RNAi lines and the need to take lines to at least the T_2 stage, and link any phenotypes to zygosity and expression level of the target gene. Considering the expected variability between lines it is quite promising that any lines were shown to have phenotypes in strong soil (of greater tillering in PCR-positive plants than in PCR-negative plants) that suggest reductions of SL biosynthesis. It is likely that pRNAiTaD10 would cause knock-down of both *TaD10* and the paralogue *TaD10-like*. This will potentially complicate the search for lines demonstrating reduced *TaD10* expression. The lack of such a paralogue for *TaD17* may explain why a clearer pattern of reduced expression was seen for PCR-positive plants carrying pRNAiTaD17 (Figure 4.14).

The expression of most of the SL biosynthesis genes was reduced for WT wheat plants (cv. Mercica) grown in strong soil compared to those grown in weak soil (Figure 4.18). Initially it would be expected that high levels of SL biosynthesis genes would to cause inhibition of tillering in response to strong soil as high levels of SL would ensue. The reduction of expression of these biosynthesis genes would imply a reduction of SL levels. However, it has been shown that feedback mechanisms exist in both Arabidopsis and rice that mean that biosynthesis genes can show reduced expression in response to high levels of SL (Arite et al., 2007; Mashiguchi et al., 2009). It is therefore imperative that a replicated experiment measuring the levels of SLs in the roots of a range of wheat cultivars grown in weak and strong soil is conducted before making conclusions about the meaning of the reduction of expression of SL biosynthesis genes in strong soil.

High nucleotide and protein sequence similarity to rice and Brachypodium *D10* and *D17* genes suggests that *TaD10-454*, *TaD10_3B*, *TaD17-454* and *Ta-D17_2BL* sequences do represent wheat orthologues of their respective genes. Furthermore, the successful cloning of the 3’ end of each gene and confirmation of expected intron-exon boundaries strengthened the case. However, to address whether or not the genes truly are correct it would be important to test if TaD17 can perform the conversion of 9-*cis*-β-carotene to 9-*cis*-β-apo-10'-carotenal and if TaD10 can convert that further to carlactone. It would be interesting to conduct feeding experiments similar to those used by (Seto et al., 2014) where labelled precursors were used to demonstrate that *d10* rice mutants could not produce carlactone. Or perhaps *TaD17* and *TaD10* could be even be used to recover the *d17* and d*10* phenotypes of rice mutants deficient in the respective orthologues.

4.5 Conclusion

The results of this investigation into the role of SL in mediating strong soil reduction of tillering in wheat are somewhat inconclusive. Orobanchol seems to be at equal concentrations in roots grown in weak and strong soil (Table 4.5). Two out of three lines containing RNAi constructs targeting expression of *TaD10* or *TaD17* showed no indication of modified levels of tillering in transgenic (PCR-positive) plants compared to null segregant (PCR-negative) plants when grown in strong soil. These results indicate that SLs are not involved in mediating reduction of tillering in response to strong soil in wheat. However, one line containing the RNAi constructs targeting *TaD10* or *TaD17* showed more tillering in PCR-positive plants than PCR-negative plants grown in strong soil, although not significant it remains a promising result considering the experiment only used T_1 plants with few replicates. Whether a reduction in the expression of SL biosynthesis genes in response to strong soil is indicative of a cue for reduced SL levels or a response to existing high levels of SL is unclear without accompanying data for endogenous SL levels. The conclusion here is that more work is required to ascertain whether or not SL mediates reduction of tillering in response to strong soil. Perhaps future analysis of lines produced here (using the T_2 generation) will demonstrate the function of *TaD10* and *TaD17*.

Chapter 5: The Roles of Other Hormones in Mediating Strong Soil Effects on Wheat Architecture

5.1 Introduction

Chapter 3 described strong evidence for the involvement of strigolactones (SLs) in the reduced tillering response to strong soil in rice, but SLs did not seem to be involved in leaf elongation and root growth responses to strong soil (see Chapter 3). There was also preliminary evidence suggesting that SL may also be involved in the tillering response in wheat (see Chapter 4), further investigation and analysis will be required to validate this. To examine the mediators of other architectural responses to strong soil it was important to explore the roles of other hormones. Could typical stress response hormones such as ABA, ethylene and jasmonic acid be involved? Perhaps the auxins, cytokinins and gibberellins (GAs) that regulate so much of development are responsible for some of the changes to architecture brought about by strong soil. As described in Section 1.4, there is some evidence that ethylene may be involved in modifying crop growth in response to strong soil although there is some disagreement on the subject (Moss et al., 1988; Sarquis et al., 1991; Hussain et al., 1999). In published work contributed to as part of this PhD study, Coelho Filho et al. (2013) showed that the GA signalling system was involved in the reduction in leaf elongation in response to strong soil. The paper abstract is provided for reference in Appendix 3. In summary, Coelho Filho et al. (2013) found that the length of leaves of GA-sensitive (*Rht-B1a*) wheat plants grown in strong soil was reduced, but could be restored by application of GA_3 (a bioactive GA produced by culture of the fungus *Gibberella fujikuroi*). In contrast, GA-insensitive (*Rht-B1c*) plants did not show reduced leaf elongation in response to strong soil (see Figure 5.1). Furthermore, GA-sensitive plants grown in strong soil became hyper-responsive to added GA, suggesting that the reduction in leaf length in the absence of GA was due to a reduction in bioactive GA levels in leaves rather than a reduction in the GA sensitivity of leaf growth.

Despite this role for GA in the leaf elongation response to strong soil, the authors found no interaction between the treatments of soil strength, GA_3 application or GA sensitivity of the plants for the number of tillers, indicating that GA had no role in the tillering response to strong soil (Coelho Filho et al., 2013). Indeed, tillering was reduced by application of GA_3 and reduction of leaf elongation in response to strong soil would require reduced levels of GA rather than an increase (Coelho Filho et al., 2013).

The roles of GA in mediating architectural responses to strong soil are examined further in the current Chapter. See Figure 5.2 for a diagram of how selected key cytoplasmic GAs are related and the enzymes involved in the conversion steps required. Additionally, the emerging tools of simultaneous analysis of multiple hormones (global hormone profiling) and whole transcriptome analysis (RNAseq) are used to explore the involvement of other hormones in mediating wheat architectural responses to strong soil.

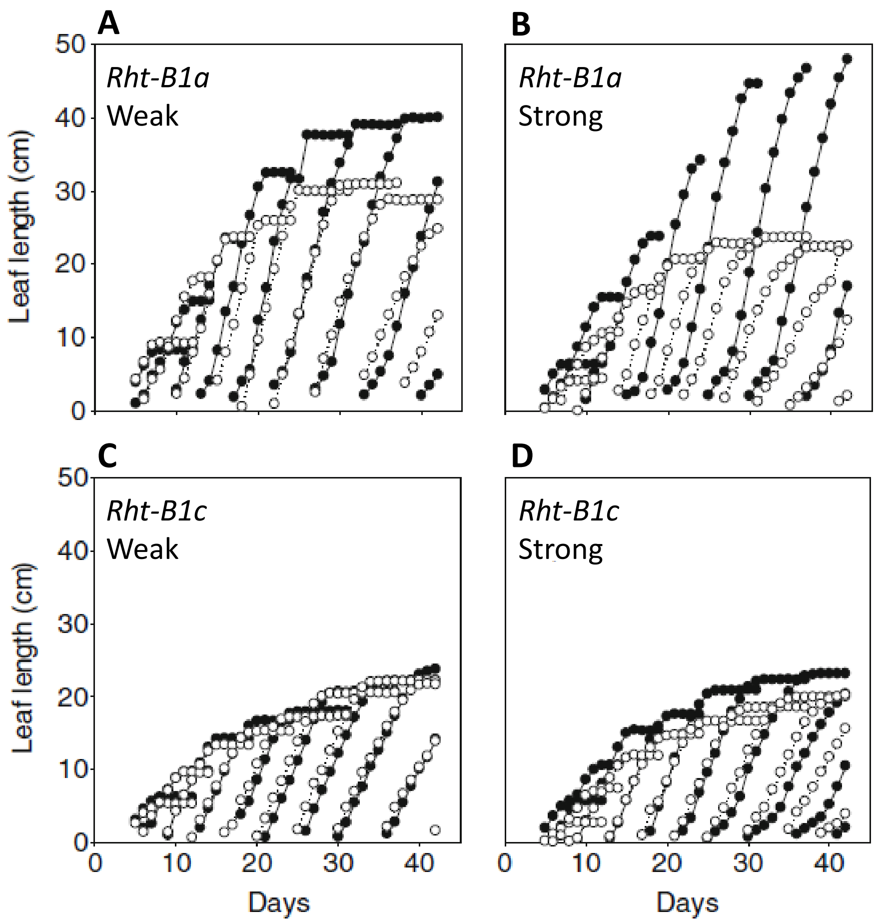


Figure 5.1 Leaf elongation in GA sensitive and GA-insensitive plants in strong and weak soil, with and without application of GA_3.

A) Leaf length in GA-sensitive plants in weak soil with and without GA_3 application. B) Leaf length in GA-sensitive plants in strong soil with and without GA_3 application. C) Leaf length in GA-insensitive plants in weak soil with and without GA_3 application. D) Leaf length in GA-insensitive plants in strong soil with and without GA_3 application

Trititcum aestivum (cv. Mercia) *Rht B1a* plants were GA-sensitive while *Rht B1c* was a GA-insensitive NIL. Each line represents the growth of particular leaf number of the main tiller (Leaf 1 to Leaf 9).

Open circles = no added GA_3. closed circles = 100µM GA_3 added. This image is adapted from work described in Coelho Filho et al. (2013).

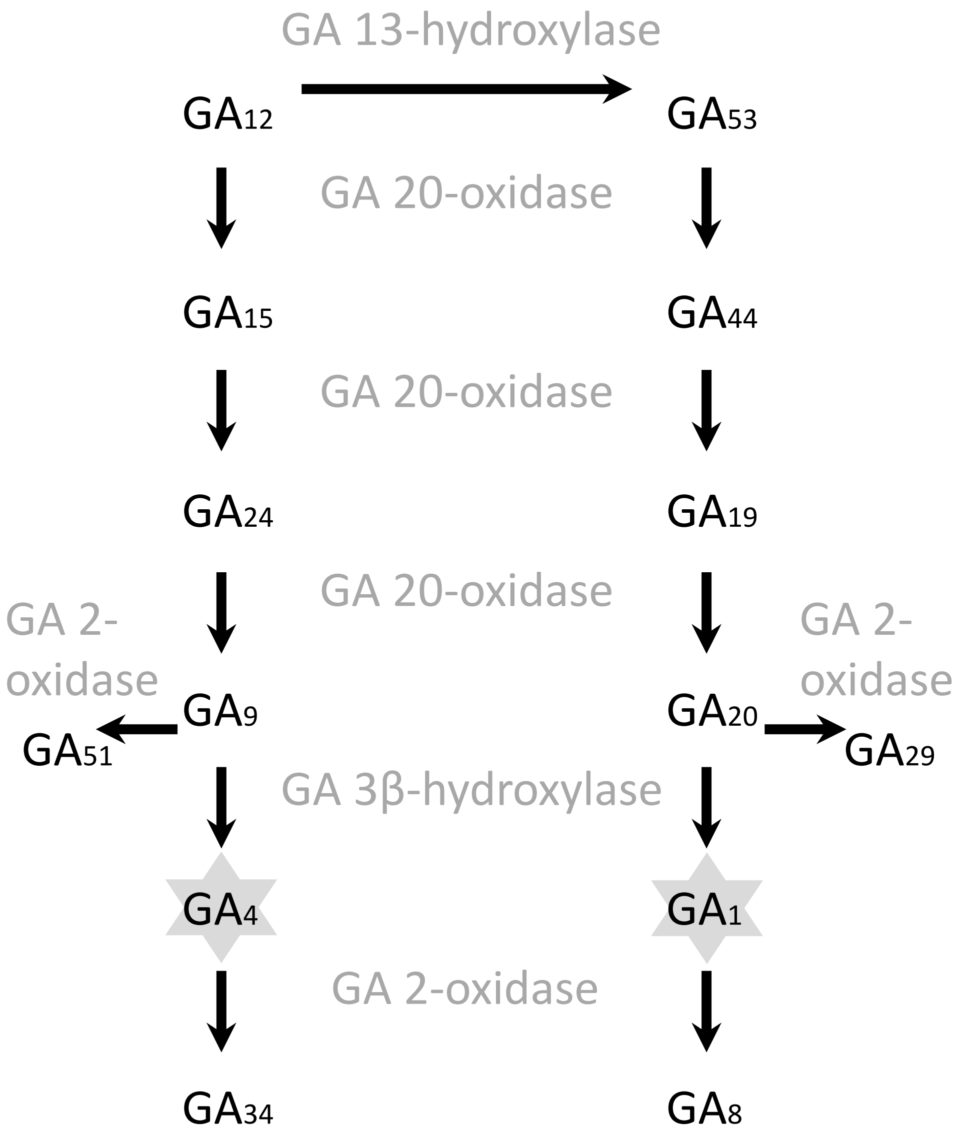


Figure 5.2 Key cytoplasmic steps of gibberellin (GA) metabolism
The 13-hydroxy GAs and 13-H GAs are in black text. Enzymes families responsible for catalysing each step are in grey text. The two bioactive GAs presented here (GA_4 and GA_1) are highlighted with stars.
Information is from Hedden and Phillips (2000).

5.2 Materials and Methods

See Sections 2.1.5, 2.2.1.1, 2.2.5, 2.3.1, 2.3.4.1, 2.3.4.2 and 2.3.4.3 for methods used in this chapter.

5.3 Results

5.3.1 Experiment 5.1: GA levels in actively growing leaves of wheat plants in weak and strong soil

The findings of Coelho Filho et al. (2013) that the GA signalling pathway was involved in the reduction of wheat leaf elongation in strong soil, coupled with the observation that strong soil increased the sensitivity of leaf elongation to GA (Figure 5.1), suggested that bioactive GA levels might be lower in the leaves of plants grown in strong soil than those grown in weak soil. To examine whether that was indeed the case, GA-sensitive wheat (*T.aestivum* cv. April-bearded, *Rht-B1a*) plants were grown in strong and weak soil (see Section 2.1.5) and GA levels were determined in actively growing leaf material (see Section 2.3.1). Three replicates of each treatment were used, with each replicate consisting of pooled samples of leaf 4 of the main tiller from six plants. Leaf sampling was carried out when all sampled leaves were actively growing (ensuring developmental similarity). This included a one day delay for the sampling of the strong soil leaves to account for the lag in leaf emergence in those plants. Leaf 4 was selected for sampling as it was the first leaf to show reduced leaf elongation in response to strong soil by Coelho Filho et al. (2013) (see Figure 5.1). At harvest maximum root depth, the number of primary roots and root fresh weight were all reduced in wheat plants grown in strong soil (see Figure 5.3A to C). Multiple GAs and ABA were extracted and quantified according to Section 2.3.1 (see Figure 5.2 for a diagram of the steps between these GAs). ABA and those GAs (the key 13-hydroxy GAs GA_{44}, GA_{19}, GA_{20} and GA_1) that were at quantifiable levels in all three replicates are presented in Figure 5.3D to H. Results of statistical analyses by ANOVA are presented in Table 5.1. The level of ABA did not statistically significantly differ between actively growing leaves from plants grown in weak soil and those grown in strong soil (Figure5.3D). As shown in Figures 5.3E to H, the mean levels of GA_{44}, GA_{19}, GA_{20} and GA_1 were lower in actively growing leaves of plants grown in strong soil than those of plants grown in weak soil (GA_1 is the prevalent bioactive GA in wheat) (see Figure 5.2 for an overview how a selection of key GAs relate to each other). However, the reduction of GA was only statistically significant for GA_{44} (as highlighted by the asterisk in Figure 5.3A). The number of primary roots, root fresh weight and maximum root depth were all statistically significantly reduced in plants grown in strong soil compared to plants grown in weak soil (Figures 5.2F to H). It was not possible re-verify the response of leaf elongation to soil strength in this experiment, as it has been previously shown that in this cultivar leaves 1-3 do not respond significantly to the stress (Coelho Filho et al., 2013) and leaf 4 was collected for GA analysis before it had reached its full length.

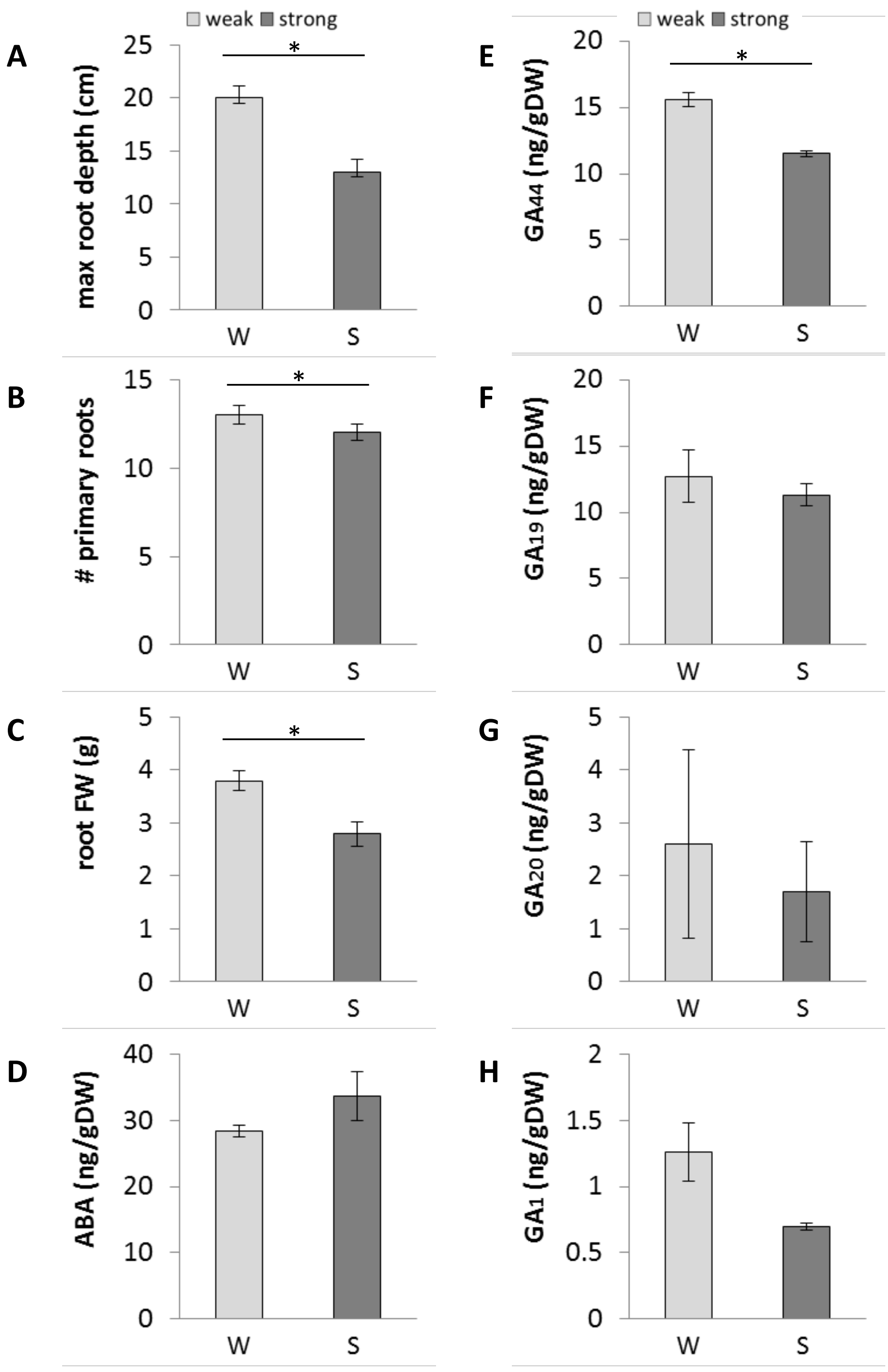


Figure 5.3 GA, ABA and architecture results from an experiment on young wheat plants in weak and strong soil.

A to C) The maximum root depth (cm), the number of primary roots, root fresh weight (FW, g) and in plant grown in weak and strong soil at 18 and 20 DAS, respectively. D to H) ABA, GA_{44}, GA_{19}, GA_{20}, GA_{1} levels in samples of actively growing leaf four from plants in weak (W) and strong (S) soil. Leaf four was sampled at 16 days after sowing (DAS) for weak soil plants and 18 DAS for strong soil plants. Asterisk and connecting bar indicates a statistically significant difference where LSDs (5% level) = 5.7 (maximum root depth), 0.5 (number of primary roots), 0.831 (root fresh weight), 2.026 (GA_{44}).

Thus, as all GAs measured were reduced in expanding leaves of plants grown in strong soil and, despite only the levels of GA_{44} being statistically significant, GA remains a strong candidate for controlling leaf elongation response to strong soil.

Table 5.1 Output from statistical analysis of Experiment 5.1 data for GA, ABA and architecture.
ANOVA and Fisher's LSD were used. d.f. = degrees of freedom; F=F-statistic; *p* = *p*-value; LSD=Fisher's Least Significant Difference value.

Hormones and architecture (variable)	Effect	d.f.	F	*p*	LSD (5% level)
Maximum root depth	Strength	1, 4	12.06	0.026	5.7
Number of primary roots	Strength	1, 4	36.00	0.004	0.5
Root fresh weight	Strength	1, 4	11.12	0.029	0.831
GA1	Strength	1, 4	4.33	0.106	0.742
GA19	Strength	1, 4	0.30	0.612	7.25
GA20	Strength	1, 4	0.16	0.711	6.85
GA44	Strength	1, 4	31.12	0.005	2.026
ABA	Strength	1, 4	1.26	0.324	12.858

5.3.2 Experiment 5.2: The response of wheat cv. Cadenza to weak and strong soil

As described above, Coelho Filho et al. (2013) found, using the weighted sand column method, that strong soil reduced leaf elongation in wheat cvs. Mercia and April Bearded, that GA treatment of these lines could reverse the effect of soil strength on the leaves, and that GA-insensitive lines were less responsive to the increased soil strength. As an aim of this project was to explore the role of GAs in the response to strong soil by manipulation of the GA pathway in transgenic plants, it was necessary to demonstrate that these responses were also observed in cv. Cadenza, a GA-responsive cultivar and the only such cultivar routinely used at Rothamsted for wheat transformation.

A soil strength experiment was undertaken with weak and strong soil conditions and three concentrations of GA_3 application (10 µM, 100 µM and a control of sterile distilled water containing 0.1% ethanol) twice a week. Sand columns were set up as described in Section 2.1.5 including the optional funnel for hormone application to roots, with the following exceptions: to enable different concentrations of GA_3 to be applied, the columns were placed in individual containers (plastic buckets) rather than the usual tanks and to enable nutrient solution to flow up into a column two 6 mm x 37 mm slits were made in each plastic tube and were covered by mesh to prevent escape of sand. Buckets were arranged in a complete randomised block design with three replicates per treatment and strength combination. The author received assistance from Hugo Emerit who aided experiment establishment and measured leaf lengths throughout the experiment. As GA-insensitive near-isogenic lines (NILs) are not available in cv. Cadenza, only the GA-sensitive cultivar itself was used. Leaf blade length was measured for each leaf on a daily basis at least until extension had completed. Table 5.2 contains all final leaf length means and Table 5.3 provides the results of statistical analysis with ANOVA and includes Fisher's LSD statistics to determine if particular means are statistically significantly different from each other.

Table 5.2 Final leaf blade length data for Experiment 5.2

		Leaf blade length (mm)		
Leaf number	**Strength**	**Control**	**10μM**	**100μM**
1	Weak	123.7	125.3	134
1	Strong	128.7	136.3	120.7
2	Weak	168.3	188.7	190.3
2	Strong	167.3	189.3	186.7
3	Weak	207.7	271.3	280.7
3	Strong	190.3	279	300.7
4	Weak	256.3	383.7	429.7
4	Strong	215	382.3	410.3

Table 5.3 Output from statistical analysis of Experiment 5.2 data for final leaf blade length.

ANOVA and Fisher's LSD were used. d.f. = degrees of freedom; F=F-statistic; *p* = *p*-value; LSD=Fisher's Least Significant Difference value.

(variable)	**Effect**	**d.f.**	**F**	***p***	**LSD (5% level)**
Final leaf blade length	Leaf number	3, 40	924.64	<0.001	10.76
	Strength	1, 40	0.72	0.403	10.55
	GA_3 concentration	2, 40	78.03	<0.001	12.93
	Leaf number* strength	3, 40	1.11	0.356	17.37
	Leaf number* GA_3 concentration	6, 40	23.16	<0.001	22.81
	Strength*GA_3 concentration	2, 40	1.01	0.374	18.28
	Leaf number* strength*GA_3 concentration	6, 40	0.61	0.721	34.43
		Except when comparing means with the same level(s) of leaf number or leaf number*strength or leaf number*GA_3 concentration			36.56

At harvest, the number of leaves on the main tiller, the number of primary tillers, the total number of tillers, shoot dry weight (DW), shoot fresh weight (FW), root dry weight (DW), root fresh weight (FW), maximum root depth and the number of primary roots were measured (see Table 5.4 for results of statistical analysis with ANOVA and Fisher's LSD statistics).

Table 5.4 Output from statistical analysis of Experiment 5.2 data for shoot and root architecture.

ANOVA and Fisher's LSD were used. d.f. = degrees of freedom; F=F-statistic; *p* = *p*-value; LSD=Fisher's Least Significant Difference value.

(variable)	Effect	d.f.	F	*p*	LSD (5% level)
Number of leaves at harvest	Strength	1, 10	11.36	0.007	0.3672
	GA_3 concentration	2, 10	1.82	0.212	0.4497
	Strength*GA_3 concentration	2, 10	1.82	0.212	0.6360
Number of primary tillers at harvest	Strength	1, 10	160.56	<0.001	0.3322
	GA_3 concentration	2, 10	71.67	<0.001	0.4068
	Strength*GA_3 concentration	2, 10	13.89	0.001	0.5753
Total number of tillers at harvest	Strength	1, 10	164.80	<0.001	0.791
	GA_3 concentration	2, 10	67.35	<0.001	0.968
	Strength*GA_3 concentration	2, 10	0.69	0.526	1.369
Shoot DW	Strength	1, 10	83.49	<0.001	0.2756
	GA_3 concentration	2, 10	3.57	0.068	0.3375
	Strength*GA_3 concentration	2, 10	0.39	0.690	0.4773
Shoot FW	Strength	1, 10	146.45	<0.001	1.093
	GA_3 concentration	2, 10	3.35	0.077	1.338
	Strength*GA_3 concentration	2, 10	1.44	0.282	1.892
Max root depth	Strength	1, 10	30.95	<0.001	1.736
	GA3 concentration	2, 10	1.87	0.205	2.126

	Strength*GA_3 concentration	2, 10	1.39	0.294	3.006
Number of primary roots	Strength	1, 10	54.42	<0.001	2.786
	GA_3 concentration	2, 10	5.85	0.021	3.412
	Strength*GA_3 concentration	2, 10	1.71	0.229	4.825
Root DW	Strength	1, 10	63.53	<0.001	0.1277
	GA_3 concentration	2, 10	5.15	0.029	0.1564
	Strength*GA_3 concentration	2, 10	0.01	0.985	0.2211
Root FW	Strength	1, 10	139.23	<0.001	1.587
	GA_3 concentration	2, 10	13.23	0.002	1.943
	Strength*GA_3 concentration	2, 10	1.08	0.376	2.748

Figure 5.4A to D show final leaf blade lengths for leaves 1 to 4 in strong and weak soil with application of a no GA_3 control treatment, 10µM GA_3 or 100µM GA_3. For leaves 1-3, there was no significant effect of soil strength. For leaf 4, strong soil significantly reduced the final length, but this stunting effect was fully reversed by treatment with either 10µM or 100µM GA_3, as found by Coelho et al for cv. Mercia (see Fig 5.1). However, unlike that cultivar, there was no evidence of any increased sensitivity to GA under the strong soil treatment.

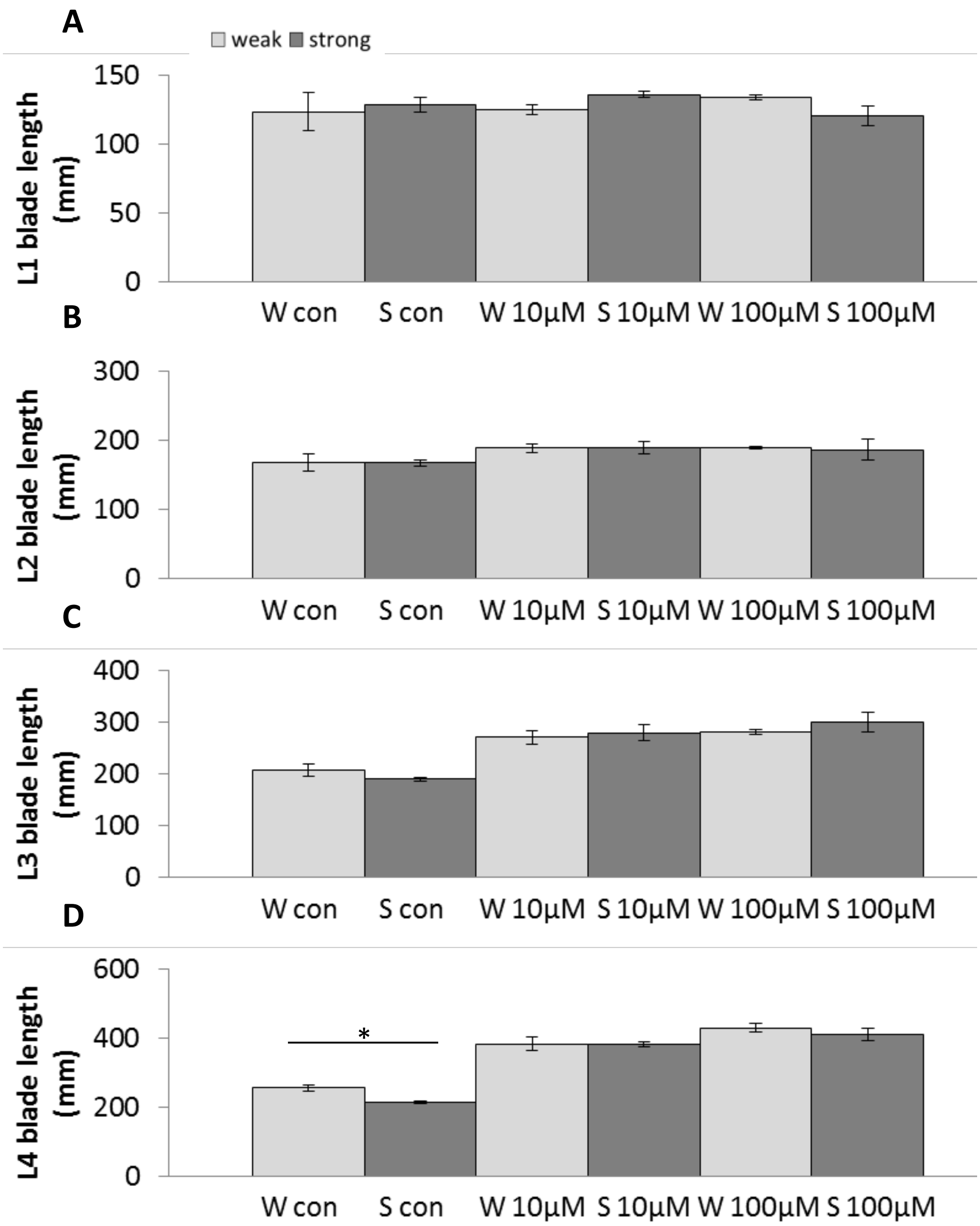


Figure 5.4 Final leaf lengths for wheat plants grown in weak and strong soil with and without GA_3 application.

A to D) Final leaf blade lengths (mm) of leave one, two, three and four of wheat plants grown in weak and strong soil at levels of 0 µM (control), 10 µM and 100 µM of GA_3 applied to the root system. W=weak soil S= strong soil L1 to L4 = leaf one of the main tiller to leaf 4 of the main tiller. *n* = 3. Bars are SE. Asterisk and connecting bar indicate a statistically significant difference where LSD = 36.56.

At the end of the experiment (26 days after sowing), the number of leaves was slightly lower in plants grown in strong soil but this was only statistically significant in plants treated with 100µM GA_3 (Figure 5.5A). The number of primary tillers and the total number of tillers were reduced by strong soil in all treatment levels of GA_3 (Figure 5.5B and C). Shoot dry weight and shoot fresh weight were reduced in strong soil in all treatment levels of GA_3 (Figure 5.5D and E). Maximum root depth was reduced by strong soil in all treatment levels of GA_3 but was not statistically significantly reduced in the 100µM level (Figure 5.5F). The number of primary roots, root dry weight and root fresh weight were reduced by strong soil in all treatment levels of GA_3 (Figure 5.5G to I).

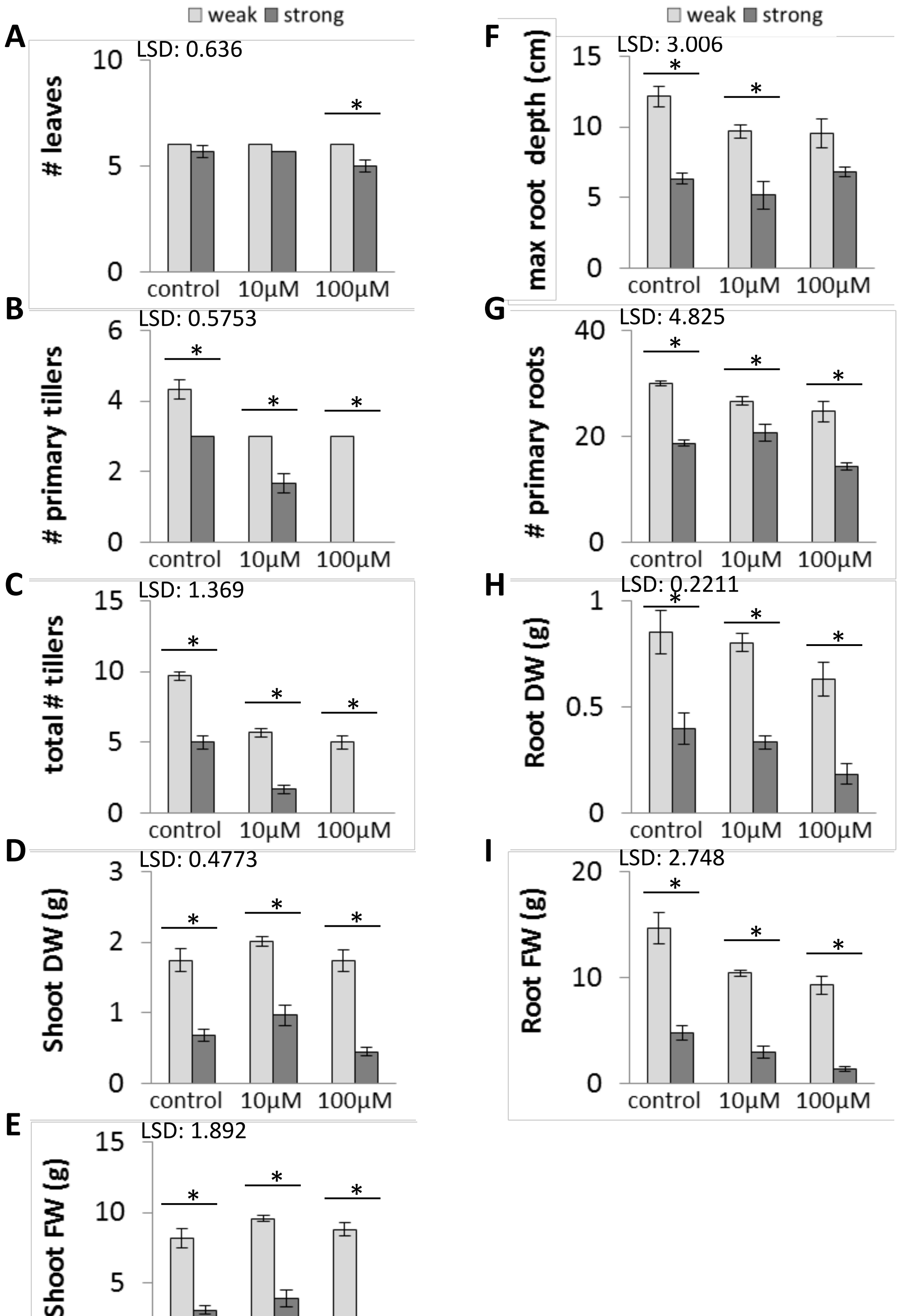


Figure 5.5 Shoot and root architecture of wheat plants in weak and strong soil with and without GA_3 application.

Wheat plants were grown in weak or strong soil, with levels of 0 µM (control), 10 µM and 100 µM of GA_3 applied to the root system twice a week. A) Number of leaves on the main tiller 26 days after sowing (DAS) B) Number of primary tillers 26 DAS C) Total number of tillers 26 DAS D) Shoot dry weight 26 DAS (DW) (g). E) Shoot fresh weight (FW) (g) 26 DAS. F) Maximum root depth (cm) 26 DAS. G) Number of primary roots 26 DAS. H) Root dry weight (DW) (g) 26 DAS. I) Root fresh weight (FW) (g) 26 DAS. *n* = 3. Bars are SE. Asterisk and connecting bar indicate a statistically significant difference with LSD on graph.

5.3.3 Experiment 5.3: Global Hormone Profiling Experiment – GA and leaf elongation results

Global hormone profiling was conducted to confirm the involvement of GA in leaf elongation responses to strong soil and to explore other plant hormones that might be involved in mediating various architectural responses to strong soil. To facilitate this analysis, the author successfully applied for a travel bursary to travel to the Dormancy and Adaptation Research Unit in the RIKEN Centre for Sustainable Resource Science, Yokohama, Japan, where a team led by Dr Mitsunori Seo has developed methodology to allow for extraction and quantification of multiple plant hormones from the same sample. A randomised complete block design was used where wheat plants were grown in strong and weak soil conditions (in line with those described in Section 2.1.5), with six plants per tank (three in weak and three in strong soil) and four tank replicates. Four samples types were collected. The root tip-enriched samples (RT) were composed of approximately 2cm distal edge of the root system. The root system (RS) samples were the rest of the root system. The actively growing leaf samples (AL) were pools of visibly young, still expanding, leaves of the main tiller and any higher order tillers. The fully expanded leaf samples (EL) were pools of leaf (except actively growing leaves) from the main tiller or higher order (secondary or tertiary) tillers. Samples were frozen in liquid nitrogen and freeze dried prior to transport to the host lab. Samples for extraction were pools of plants from a single tank (consisting of three plants). Preparation, extraction and quantification methods for global hormone profiling were as described in Sections 2.3.4.1, 2.3.4.2 and 2.3.4.3.

Sub-samples of the prepared material were also used for SL analysis as reported in Chapter 4, Section 4.3.5. The hormones assessed as part of the global hormone profiling were auxin (indole acetic acid – IAA), cytokinins (isopentenyladenine – iP and *trans*-zeatin – tZ), a range of GAs, jasmonates (jasmonic acid – JA, jasmonate isoleucine –JA-Ile) and salicylic acid (SA) (see Section 2.3.4.1 for more details). The extraction method was developed by Dr Takafumi Shimizu and Yuri Kanno, and conducted by the author. Quantification methodology by tandem LC-MS was performed by Yuri Kanno, while assessment of peak area ratios and calculation of hormone concentrations were conducted by the author, with guidance from Yuri Kanno and Dr Takafumi Shimizu. Only results that passed quality control are presented in this chapter. The author received assistance from Hugo Emerit who measured leaf lengths throughout the experiment.

For the purposes of this section only the GA and leaf growth results are reported, while the remaining results from the global hormone profiling experiment are provided in Section 5.3.6. The concentrations of a range of precursor and bioactive GAs and GA metabolites were determined in samples of root tip (RT), root system (RS), actively growing leaves (AL) and fully expanded leaves (EL) of wheat (*T.aestivum* cv. Cadenza) plants grown in weak and strong soil. There were four biological replicates (each was a pool of material from three plants and each plant provided a sample of each tissue type). Endogenous gibberellins from plants fall into two broad classes based on the hydroxylation state at Carbon-13; wheat contains predominantly 13-hydroxy GAs and lower levels of 13-H types. Although internal standards for

GA_{12}, GA_{15}, GA_{24}, GA_9, GA_4, GA_{34}, GA_{51}, GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, GA_8 and GA_{29} were added to all samples, it was not possible to quantify the 13-H GAs GA_4, GA_{34} or GA_{51} in any sample, no 13-H GAs were quantifiable in root tissues and the hydroxy GA GA_{29} was not quantifiable in any sample (see Figure 5.2 for a diagram of how these GAs relate to each other). The only bioactive GA present at quantifiable levels was GA_1. Statistical analyses of leaf growth measurements, leaf GA levels and root GA levels were conducted with ANOVA and results are presented in Tables 5.5, 5.6 and 5.7.

Table 5.5 Output from statistical analysis of Experiment 5.3 data for final leaf blade length, width and area of multiple leaves.
ANOVA and Fisher's LSD were used. d.f. = degrees of freedom; F=F-statistic; *p* = *p*-value; LSD=Fisher's Least Significant Difference value.

Leaf measurement (variable)	Effect	d.f.	F	*p*	LSD (5% level)
Blade length	Leaf number	6,132	254.57	<0.001	12.65
	Strength	1,19	29.14	<0.001	11.48
	Leaf number* Strength	6,132	16.18	<0.001	19.82
		Except when comparing means with the same level of strength			17.89
Blade width	Leaf number	6,132	271.68	<0.001	0.3291
	Strength	1,19	27.90	<0.001	0.5095
	Leaf number* Strength	6,132	8.32	<0.001	0.6542
		Except when comparing means with the same level of strength			0.4654
Blade area	Leaf number	6,132	333.02	<0.001	106.7
	Strength	1,19	38.24	<0.001	145.7
	Leaf number* Strength	6,132	18.02	<0.001	197.9
		Except when comparing means with the same level of strength			150.9

Table 5.6 Output from statistical analysis of Experiment 5.3 data for GA in leaf samples.

ANOVA and Fisher's LSD were used. d.f. = degrees of freedom; F=F-statistic; *p* = *p*-value; LSD=Fisher's Least Significant Difference value.

Leaf GA (variable)	Effect	d.f.	F	*p*	LSD (5% level)
GA_1	Strength	1,3	6.55	0.083	0.03520
GA_8	Tissue	1,9	72.48	<0.001	0.2088
	Strength	1,9	2.17	0.175	0.2088
	Tissue*Strength	1,9	4.20	0.071	0.2953
GA_9	Tissue	1,9	36.04	<0.001	0.2927
	Strength	1,9	10.58	0.010	0.2927
	Tissue*Strength	1,9	9.88	0.012	0.4139
GA_{12}	Tissue	1,9	188.58	<0.001	0.1020
	Strength	1,9	30.57	<0.001	0.1020
	Tissue*Strength	1,9	15.99	0.003	0.1442
GA_{15}	Tissue	1,9	130.32	<0.001	0.0953
	Strength	1,9	27.83	<0.001	0.0953
	Tissue*Strength	1,9	18.86	0.002	0.1347
GA_{19}	Tissue	1,9	11.98	0.007	0.3361
	Strength	1,9	1.07	0.328	0.3361
	Tissue*Strength	1,9	1.12	0.317	0.4753
GA_{20}	Tissue	1,9	99.70	<0.001	0.1268
	Strength	1,9	3.02	0.116	0.1268
	Tissue*Strength	1,9	2.72	0.133	0.1793
GA_{24}	Tissue	1,9	72.37	<0.001	0.0882
	Strength	1,9	31.39	<0.001	0.0882
	Tissue*Strength	1,9	15.95	0.003	0.1247
GA_{44}	Tissue	1,9	316.57	<0.001	0.814
	Strength	1,9	6.92	0.027	0.814
	Tissue*Strength	1,9	8.07	0.019	1.151
GA_{53}	Tissue	1,9	150.86	<0.001	0.2473
	Strength	1,9	0.24	0.635	0.2473
	Tissue*Strength	1,9	0.01	0.939	0.3497

Table 5.7 Output from statistical analysis of Experiment 5.3 data for GA in root samples.

ANOVA and Fisher's LSD were used. d.f. = degrees of freedom; F=F-statistic; *p* = *p*-value; LSD=Fisher's Least Significant Difference value.

Root GA (variable)	Effect	d.f.	F	*p*	LSD (5% level)
GA_1	Tissue	1,9	1.65	0.232	0.801
	Strength	1,9	0.50	0.497	0.801
	Tissue*Strength	1,9	0.62	0.451	1.132
GA_8	Tissue	1,9	6.20	0.034	0.02619
	Strength	1,9	0.65	0.440	0.02619
	Tissue*Strength	1,9	4.70	0.058	0.03703

Root GA (variable)	Effect	d.f.	F	*p*	LSD (5% level)
GA_{19}	Tissue	1,9	24.32	<0.001	0.4105
	Strength	1,9	10.70	0.010	0.4105
	Tissue*Strength	1,9	8.28	0.018	0.5806
GA_{20}	Tissue	1,9	10.29	0.011	0.04060
	Strength	1,9	18.09	0.002	0.04060
	Tissue*Strength	1,9	1.88	0.203	0.05742
GA_{53}	Tissue	1,9	23.08	<0.001	0.0486
	Strength	1,9	9.04	0.015	0.0486
	Tissue*Strength	1,9	5.35	0.046	0.0688

Table 5.5 shows that soil strength and leaf number both statistically significantly affected leaf length, width and area. Furthermore, an interaction between soil strength and leaf number existed, meaning that leaf length, width and area did not respond to soil strength in the same way for the entirety of the experiment. Figure 5.6A shows that final leaf length did not differ between weak and strong soil for leaves 1 and 2, was reduced in strong soil for leaves 3, 4 and 5 and then reverted back to not differing between soil strengths for leaves 6 and 7. Leaf width and area were also not different between strong and weak soil for leaves 1 and 2 but then remained reduced in strong soil for leaves 3 to 7 (see Figures 5.6B and C).

Figure 5.7 shows the results of GA quantification in leaf material. The Fisher's LSD values presented in Table 5.6 were used to determine whether or not levels of each GA were different between weak and strong soil conditions in leaves. Figures 5.7A to D show that the 13-H precursor GAs (GA_{12}, GA_{15}, GA_{24} and GA_9) were at statistically significantly higher levels in actively growing leaves grown in strong soil compared to actively growing leaves grown in weak soil.

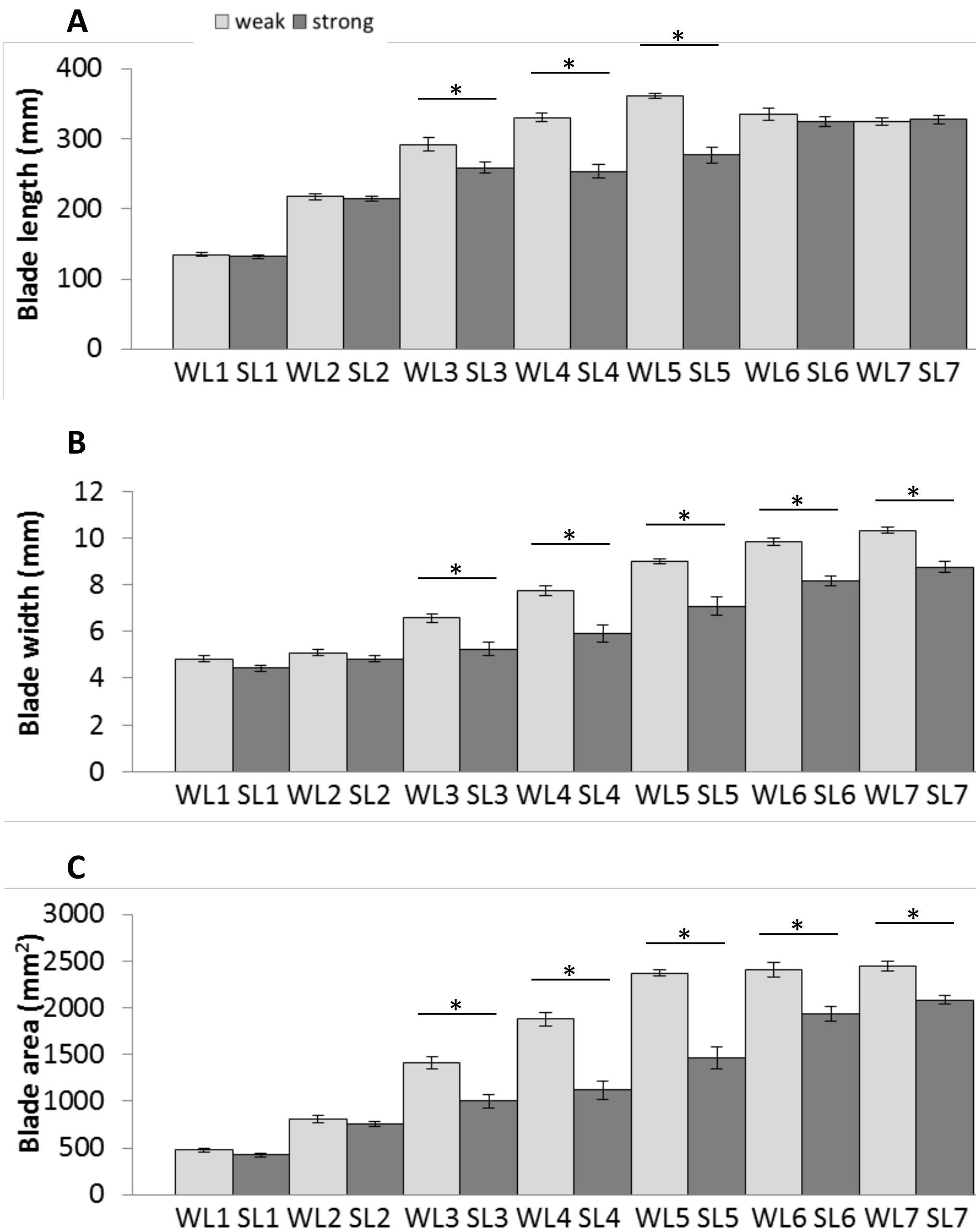
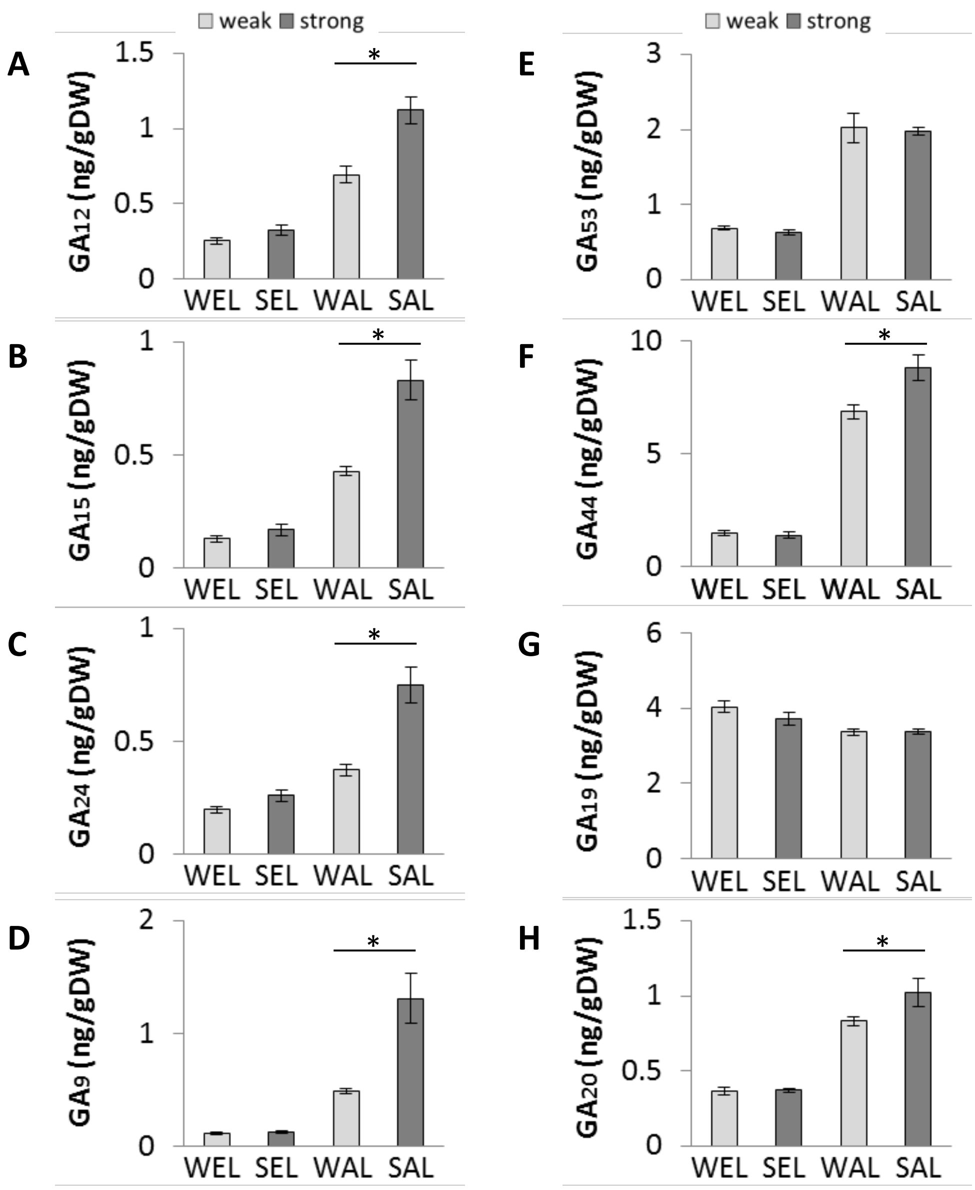


Figure 5.6 Final leaf lengths, widths and areas for wheat plants grown in weak and strong soil from a global hormone profiling experiment.
A) Final leaf blade length (mm) of leaves one to seven of wheat plants grown in weak and strong soil. B) Final leaf blade width (mm) of leaves one to seven of wheat plants grown in weak and strong soil. C) Final leaf blade area (mm^2) of leaves one to seven of wheat plants grown in weak and strong soil. W=weak soil S= strong soil L1 to L7 = leaf one of the main tiller to leaf seven of the main tiller. *n* = 4. Bars are SE. Asterisk and connecting bar indicate a statistically significant difference where LSDs = 19.82 (blade length), 0.6542 (blade width), 197.9 (blade area).



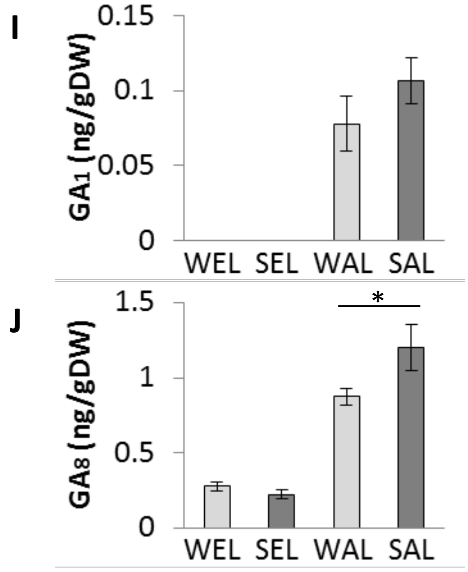


Figure 5.7 GA measurements in leaf samples of wheat plants grown in weak and strong soil from a global hormone profiling experiment. A to D) Levels of the 13-H GAs GA_{12}, GA_{15}, GA_{24} and GA_9. E to J) Levels of the 13-OH GAs GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1 and GA_8. Samples were collected 43 days after sowing (DAS). WEL = Weak soil Expanded Leaf. SEL = Strong soil Expanded Leaf. WAL = Weak soil Actively growing leaf. SAL = Strong soil Actively growing leaf. *n* = 4. Bars are SE. Asterisk and connecting bars indicate a statistically significant difference where LSDs = 0.1442 (GA_{12}), 0.1347 (GA_{15}), 0.1247 (GA_{24}), 0.4139 (GA_9), 1.151 (GA_{44}), 0.1793 (GA_{20}), 0.2953 (GA_8).

Figures 5.7E to J show that the levels of the 13-hydroxy-GAs GA_{53}, GA_{19} and GA_1 showed no significant difference between actively growing leaves grown in weak and strong soil – although all except GA_{19} were elevated in the latter – while others (GA_{44}, GA_{20} and GA_8) were significantly increased in growing leaves in strong soil.

The Fisher's LSD values presented in Table 5.7 were used to determine whether or not levels of each GA were different between weak and strong soil conditions in roots. Results of quantification of 13-hydroxy-GAs in roots are presented in Figure 5.8. GA_{53}, GA_{19} and GA_{20} were all reduced in the root tips of plants grown in strong soil, while GA_1 and GA_8 did not differ significantly between weak and strong soil treatments in root tip or root system samples (see Figures 5.8A to E). It was also notable that levels of bioactive GA (GA_1) were very low in root tips.

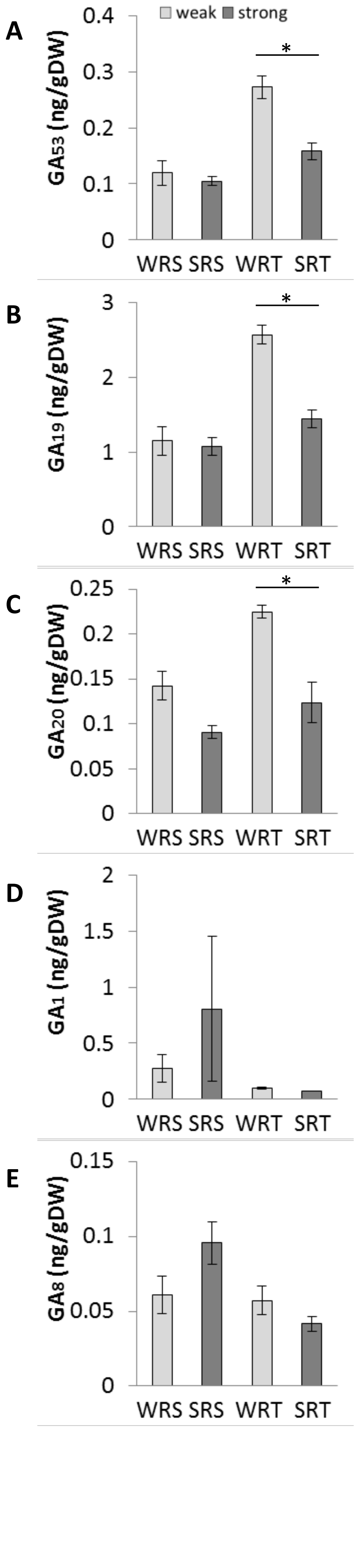


Figure 5.8 GA levels in root samples of wheat plants grown in weak and strong soil from a global hormone profiling experiment.
A to E) Levels of the 13-OH GAs GA_{53}, GA_{19}, GA_{20}, GA_1 and GA_8. Samples were collected 43 days after sowing (DAS). WRS = Weak soil Root System. SRS = Strong soil Root system. WRT = Weak soil Root Tip enriched. SRT = Strong soil Root Tip enriched. *n* = 4. Bars are SE. Asterisk and connecting bar indicate a statistically significant difference where LSDs = 0.0688 (GA_{53}), 0.5806 (GA_{19}), 0.05742(GA_{20}).

5.3.4 Production of an RNAi construct targeting *Ta2ox3*

Coelho Filho et al. (2013) showed that GA was involved in the reduction in leaf elongation in response to strong soil, and this was supported by the results presented above (Figures 5.3 and 5.7). These results also indicated that this stunting effect could be reversed by treatment with exogenous GA, suggesting that manipulation of the endogenous levels of GA might allow leaf elongation to continue normally even under high strength soil. Modification of the GA biosynthetic pathway may therefore be an efficient way to maintain GA levels even in strong soil. As discussed above in section 5.3.1, the levels of all GA intermediates appeared to be reduced in leaves under strong soil stress, implying reduced flux into the later part of the pathway. The effect of the stress may therefore be to reduce the levels of enzymes earlier in the pathway, or to increase the activity of enzymes that remove GA intermediates. This suggests the involvement of *GA2ox* genes, which encode enzymes involved in both GA inactivation (Thomas et al., 1999) and also removal of earlier C_{20} intermediates from the pathway (Schomburg et al., 2003). Furthermore, *GA2ox* genes have been shown to play a role in the response to various abiotic stresses including freezing and high salinity (Achard et al., 2008; Magome et al., 2008).

Wheat contains at least 10 paralogues of *GA2ox* (Pearce et al., 2015) of which *TaGA2ox3*, *TaGA2ox4* and *TaGA2ox9* are the most highly expressed in vegetative tissues. *TaGA2ox3* encodes a GA 2-oxidase with activity against both C_{19} (i.e. bioactive) and C_{20} (i.e. precursor) GAs and is normally expressed in high levels in roots and at lower levels in leaves; drought treatment increases expression in wheat leaves while reducing root expression (Li and Phillips, unpublished). This suggests that *TaGA2ox3* expression and modulation of GA levels is involved in modifying shoot and root growth under drought. Furthermore, *TaGA2ox3* is closely associated with a height QTL in wheat (Prosser and Phillips, unpublished). Although there is no direct evidence that *TaGA2ox3* is affected by soil strength, the endogenous expression pattern of this gene and the roles of *GA2ox* proteins in response to other abiotic stresses makes this gene a strong candidate for involvement in the reduction of leaf elongation due to strong substrate conditions. It was under this premise that an RNAi construct targeting the expression of *TaGA2ox3* was designed (herein referred to as *Ta2ox3*).

As for the RNAi constructs described in Section 4.3.2, a final RNAi construct for *Ta2ox3* included the following components: a rice actin promoter (*OsAct*), the *Adh* intron and *Nos* terminator and a sequence of the target gene (inserted in a sense orientation between the promoter and the intron and in an antisense orientation between the intron and the terminator) (see Figure 4.9A). Although the production of the RNAi construct targeting *Ta2ox3* was very similar to the process for producing the RNAi constructs targeting SL biosynthesis genes, there were two key differences. Firstly, *Ta2ox3* was a previously characterised gene with existing knowledge of paralogues. Secondly, unlike the SL RNAi target regions, the selected RNAi target region of *Ta2ox3* contained a *Sal*I binding site, precluding the use of that restriction endonuclease for removing the *HMW glutenin* promoter and replacing it with the *OsAct* promoter. This necessitated the use of an alternative restriction endonuclease (*Bcl*I) and the design of a suitable primer pair to amplify the *OsAct* promoter with a *Hind*III site at the 5' end and a *Bcl*I site at the 3' end (see Table 5.8).

Table 5.8 Cloning primers for obtaining *Ta2ox3* RNAi target and *OsAct* (with *Bcl*I at 3' end).

Primer name	Final primer Sequence (5' to 3'; sense) PolyA sequence in italics, addition for cloning strategy underlined, target binding sequence in bold.	Expected size of fragment
Ta2ox3cloneF1	*AAA*AGATCT**AGGCCGTCAGGTTCTTCTCG**	303 bp
Ta2ox3cloneR1	*AAA*GGATCC**CGTCACCATCGCGCTCAG**	
OsActcloneF1	*AA*AAGCTT**GTCATTCATATGCTTGAGAAGAGA**	1,405 bp
OsActcloneR1	*AA*TGATCA**CATTGTTGGGTCTACCTACAAAAA**	

As a large number of GA2ox enzymes were known in wheat, it was important to avoid targeting expression of paralogous *GA2ox* genes. This involved individually aligning the *Ta2ox3* sequence (a consensus sequence of the *Ta2ox3* cds sequences of genomes A, B and D) to the sequences of *GA2ox1*, *2*, *4*, *6*, *8*, *9* and *10* genes using the MUSCLE algorithm within the Geneious package. The alignments were examined to avoid regions of 100% identity between *Ta2ox3* and the other paralogues over 21 or more base pairs, as these regions represent areas that could lead to RNAi suppression of the non-target gene. The primers for amplification of the *TaGA2ox3* target region (see Table 5.8) were, hence, designed to avoid the danger of non-target-gene suppression. With this in mind, the primers targeted a coding region across exon boundaries, necessitating the use of cDNA as template for target amplification (the proofreading DNA polymerase described in section 2.2.1.1

was used). Sequencing confirmed that the correct gene fragment had been amplified. Both clones sequenced showed 99.1% identity to the reference sequence. All three disagreements to reference in the cloned sequences were identical to the A genome of wheat. The G at nucleotide position 138 and the C at nucleotide position 154 both result in genome A-specific amino acid changes in any translated protein.

The *Ta2ox3* target sequence was excised from pSC-B amp/kan using a *Bgl*II & *Bam*HI double digest and inserted at the *Bgl*II site between the *HMW glutenin* promoter and the *Adh* intron in plasmid pHMWadhNos. Ligated plasmids were screened using a *Bgl*II & *Bam*HI double digest to identify positive clones and confirm that the gene fragment had been inserted in a sense orientation between the promoter and the intron. These constructs were then linearized with *Bam*HI and a further *Ta2ox3* gene fragment was inserted between the *Adh* intron and the *Nos* terminator. A diagnostic double digest with *Bam*HI and *Eco*RI was then carried out to ensure that the second fragment was inserted in the antisense orientation.

Because *Bcl*I digestion is blocked by *dam* methylase regulated N6 methylation of adenine in DNA (www.neb.com, accessed 28/8/13), after the *Hin*dIII and *Bcl*I version of the *OsAct* promoter fragment had been subcloned into pSC-B amp/kan, the purified plasmid was transformed into competent *dam*$^{-}$ *E. coli* cells (of the strain ER2925 (NEB), obtained from Dr Pippa Madgwick). Similarly, the RNAi construct containing the two RNAi target region inserts and the undesired *HMW* promoter was transformed into competent *dam*$^-$ *E. coli* cells (of the strain ER2925). It was then possible to excise the two promoters by sequential *Hin*dIII and *Bcl*I digestion (Step 3 in Figure 4.12). The *OsAct* promoter (with *Bcl*I at the 3' end) was then ligated into the *Ta2ox3* RNAi construct (Step 4 in Figure 4.6C). Positive clones were identified and sequencing was used to confirm the final *pRNAiTa2ox3* construct had been produced correctly. It was also confirmed by PCR that the entire *OsAct* sequence was present in pRNAiTa2ox3.

The completed construct pRNAiTa2ox3 was supplied to the Cereal Transformation Unit at Rothamsted Research (5µg at mg/ml). The Cereal Transformation Unit transformed wheat cv. Cadenza embryos by bombardment with pRNAiTa2ox3, co-bombarded with pAHC20 containing the *bar* gene selectable marker. The *bar* selectable marker provided resistance to glufosinate ammonium-based herbicides such as phosphinothricin (PPT) and enabled successfully transformed embryos to be selected. The Cereal Transformation Unit grew transformed plants to seed, providing T1 seeds (see Section 2.2.5 for further details on the transformation and selection process).

5.3.5 T_1 2ox3 RNAi plants

Due to time and space constraints, only a limited number of independent *Ta2ox3* RNAi lines could be assessed for reduced expression using qRT-PCR.

T_1 plants (i.e. segregating progeny of the primary transformed lines) were first tested for the presence of the RNAi construct as described for previous constructs in Section 4.3.3. PCR testing for the presence of the transgene in segregating T_1 *Ta2ox3* RNAi lines allowed the plants to be categorized as PCR-negative (null segregants lacking the transgene) or PCR-positive (homozygous or heterozygous for the transgene). The expression of the endogenous *Ta2ox3* genes was assessed in root material of a T_1 plants containing the pRNAiTa2ox3 construct and their null siblings, for a selection of lines. Expression was examined with qRT-PCR for *Ta2ox3*, amplifying a 144bp product using the primers q2ox3-04F (ATGACACAGAGGATTGCACCATTGCC) and q2ox3-04R (GCTACCTGTGGAACTGAGCCAG) which were already in use within the lab and designed by Dr Simon Vaughan. This primer pair was designed to be a perfect match for the 3' end of the transcripts of the B and D homoeologues of *TaGA2ox3* (Pearce et al 2015) that were available at the time but more recent genomic sequence data from wheat (Chapman et al., 2015) shows a single base mismatch of primer q2ox3-04R against the A homoeologue; nevertheless, these primers should allow expression across all three homoeologues to be assessed. The genes used as reference genes for qRT-PCR analysis of *Ta2ox3* were *Ta2526* (a probable 26S proteasome subunit) and *TaGAPDH* (*glyceraldehyde 3 phosphate dehydrogenase*); see Table 4.4 for primer sequences. Figure 5.9 shows *Ta2ox3* expression of PCR-positive and PCR-negative (null) plants of 4 independent RNAi lines for *Ta2ox3*. None of the lines showed a large reduction of expression in PCR-positive plants, as shown by the NRQs (Normalized Relative Quantities),

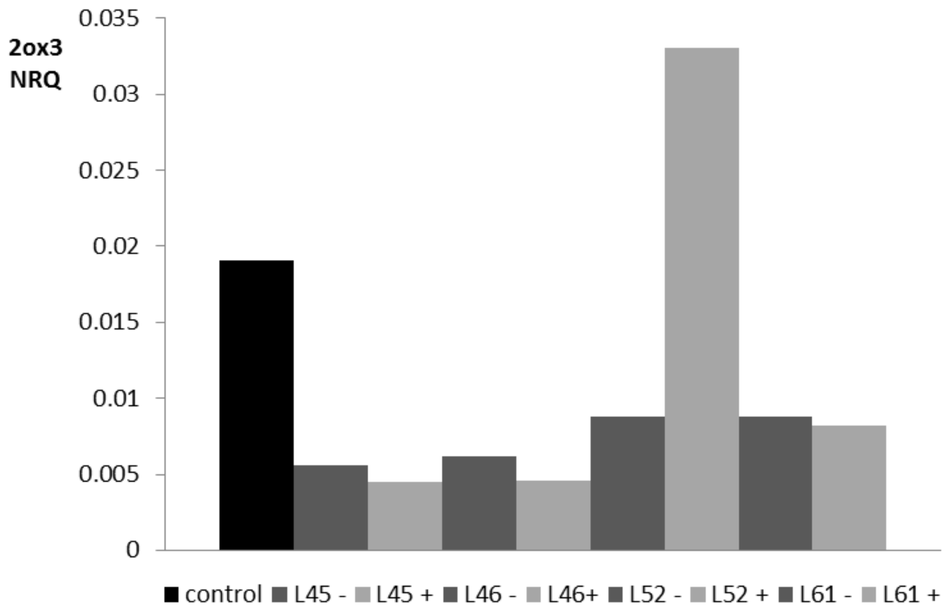


Figure 5.9 *Ta2ox3* expression in roots of T_1 RNAi lines targeting *Ta2ox3*.

Normalised relative quantification results of qRT-PCR are presented (where relative quantities of target genes were normalised to the geometric mean of two reference genes, *Ta2526* and *TaGAPDH*). L45, L46, L52 and L61 were individual T_1 lines. Control = cadenza WT (bombardment only control), - = PCR-negative sample (null segregant), + = PCR-positive (trangenic) sample. Lines were represented by single pooled samples and no error is presented.

compared to their PCR-negative siblings. Furthermore, no clear or consistent plant height phenotypes were observed at the T_1 stage and these plants were taken no further.

5.3.6 Experiment 5.3: Global Hormone Profiling Experiment – non-GA hormones and architecture results

The work described in the preceding chapters and earlier in the current chapter investigated the roles of SL and GA in the response of plants to soil strength, and demonstrated that SL was clearly involved in the reduced tillering of rice under strong soil, and there was good evidence that GA was involved in the leaf elongation response of wheat to the same stress. The following section explores the potential involvement of other plant hormones in the response of wheat to weak or strong soil by measuring their levels in leaf and root samples. These results are from the Global Hormone Profiling experiment, with analysis carried out by the author at the RIKEN in Yokohama, Japan, described in Section 5.2.2. As described previously, the experiment included 4 replicates for each sample type (each consisting of pools of tissue from three plants from the same tank). Samples of actively growing leaves (AL), fully expanded leaves (EL), root-tip enriched (RT) samples and root system (RS) samples were tested. The samples were processed according to Sections 2.3.4.1, 2.3.4.2 and 2.3.4.3, with multiple hormones being measured from the same samples. The GAs that were measured and leaf growth results have been described in Section 5.3.2.

SL analysis was carried out separately (from sub-samples of the material) but did not reveal quantifiable levels of SL, as described in Section 4.3.5. Here in Section 5.3.6, the levels of auxin (indole acetic acid – IAA), cytokinins (isopentenyladenine – iP and *trans*-zeatin – tZ), jasmonates (jasmonic acid – JA, jasmonate isoleucine –JA-Ile) and salicylic acid (SA) are analysed and described. The results of ANOVA and Fisher's LSD are given in Table 5.9 (for tillering data), Table 5.10 (for remaining architecture data), Table 5.11 (for hormone data in leaf samples) and Table 5.12 (for hormone data in root samples).

Table 5.9 Output from statistical analysis of Experiment 5.3 data for tillering.

ANOVA and Fisher's LSD were used. d.f. = degrees of freedom; F=F-statistic; *p* = *p*-value; LSD=Fisher's Least Significant Difference value.

Tiller measurement (variable)	Effect	d.f.	F	*p*	LSD (5% level)
Total tillering	Days after sowing (DAS)	4, 88	287.52	<0.001	0.966
	Strength	1, 19	52.32	<0.001	1.466
	Strength*DAS	4, 88	30.42	<0.001	1.869
		Except when comparing means with the same level of strength			1.365
Primary tillering	DAS	4, 88	478.09	<0.001	0.1878
	Strength	1, 19	20.72	<0.001	0.2989
	Strength*DAS	4, 88	10.18	<0.001	0.3741
		Except when comparing means with the same level of strength			0.2656

Table 5.10 Output from statistical analysis of remaining Experiment 5.3 data for shoot and root architecture.

ANOVA and Fisher's LSD were used. d.f. = degrees of freedom; F=F-statistic; *p* = *p*-value; LSD=Fisher's Least Significant Difference value.

Other Architecture (variable)	Effect	d.f.	F	*p*	LSD (5% level)
Maximum root depth	Strength	1, 19	69.72	<0.001	1.629
Shoot fresh weight	Strength	1, 19	76.40	<0.001	4.71
Root fresh weight	Strength	1, 19	21.48	<0.001	31.21
Leaf number at harvest	Strength	1, 19	52.78	<0.001	0.2401

Table 5.11 Output from statistical analysis of Experiment 5.3 data for non-GA hormones in leaf samples.

ANOVA and Fisher's LSD were used. d.f. = degrees of freedom; F=F-statistic; *p* = *p*-value; LSD=Fisher's Least Significant Difference value.

Leaf (variable)	Effect	d.f.	F	*p*	LSD (5% level)
IAA	Tissue	1,9	82.97	<0.001	3.139
	Strength	1,9	0.83	0.386	3.139
	Tissue*Strength	1,9	1.05	0.333	4.439
iP	Tissue	1,9	337.41	<0.001	0.0841
	Strength	1,9	0.50	0.496	0.0841
	Tissue*Strength	1,9	0.00	0.950	0.1190
tZ	Tissue	1,9	48.63	<0.001	0.3754
	Strength	1,9	0.16	0.699	0.3754
	Tissue*Strength	1,9	2.30	0.164	0.5309
ABA	Tissue	1,9	11.44	0.008	10.01
	Strength	1,9	3.27	0.104	10.01
	Tissue*Strength	1,9	0.01	0.930	14.15
SA	Tissue	1,9	1.07	0.327	228.2
	Strength	1,9	0.38	0.551	228.2
	Tissue*Strength	1,9	1.66	0.229	322.8
JA	Tissue	1,9	3.62	0.090	44.91
	Strength	1,9	1.62	0.235	44.91
	Tissue*Strength	1,9	0.01	0.915	63.52
JA-Ile	Tissue	1,9	10.92	0.009	2.279
	Strength	1,9	0.66	0.438	2.279
	Tissue*Strength	1,9	0.74	0.412	3.222

Table 5.12 Output from statistical analysis of Experiment 5.3 data for non-GA hormones in root samples.

ANOVA and Fisher's LSD were used. d.f. = degrees of freedom; F=F-statistic; *p* = *p*-value; LSD=Fisher's Least Significant Difference value.

Root (variable)	Effect	d.f.	F	*p*	LSD (5% level)
IAA	Tissue	1,9	62.83	<0.001	7.19
	Strength	1,9	28.34	<0.001	7.19
	Tissue*Strength	1,9	25.08	<0.001	10.16
iP	Tissue	1,9	0.00	0.975	0.0722
	Strength	1,9	0.02	0.880	0.0722
	Tissue*Strength	1,9	23.66	<0.001	0.1022
tZ	Tissue	1,9	0.04	0.846	0.0929
	Strength	1,9	9.62	0.013	0.0929
	Tissue*Strength	1,9	5.64	0.042	0.1314
ABA	Tissue	1,9	21.55	0.001	0.499
	Strength	1,9	0.13	0.730	0.499
	Tissue*Strength	1,9	5.45	0.044	0.706
SA	Tissue	1,9	21.01	0.001	15.11
	Strength	1,9	0.00	0.972	15.11
	Tissue*Strength	1,9	0.21	0.661	21.37
JA	Tissue	1,9	2.24	0.169	620.5
	Strength	1,9	6.02	0.037	620.5
	Tissue*Strength	1,9	0.05	0.834	877.5
JA-Ile	Tissue	1,9	18.81	0.002	2.217
	Strength	1,9	4.77	0.057	2.217
	Tissue*Strength	1,9	1.19	0.303	3.135

Significant effects of days after sowing (das) and soil strength, as well as an interaction between das and soil strength, were shown on both total tillering and primary tillering (Table 5.9). Total tillering was reduced in strong soil conditions from 21 das until the final measurements at 42 das (Figure 5.10A), as previously reported by (Coelho Filho et al., 2013). Primary tillering was reduced in strong soil at 14, 21 and 28 das but was equal in weak and strong soil at 35 and 42 das (see Figure 5.10B).

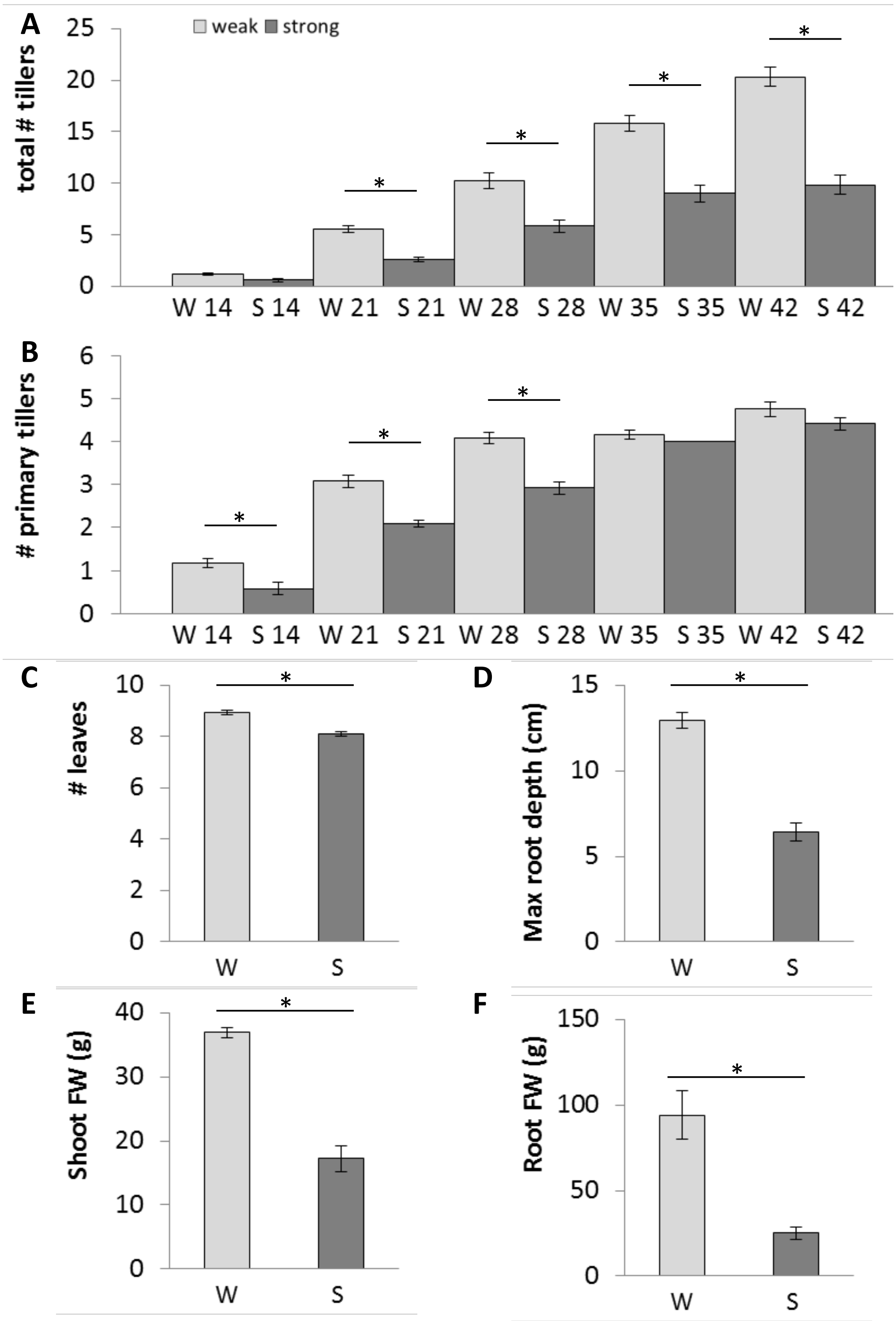


Figure 5.10 Shoot and root architecture of wheat plants in weak and strong soil from a global hormone profiling experiment.

A) Total number of tillers at 14, 21, 28, 35 and 42 days after sowing (DAS). B) Number of primary tillers 14, 21, 28, 35 and 42 DAS. C) Number of leaves on the main tiller 43 DAS. D) Maximum root depth 43 DAS. E) Shoot fresh weight (FW) (g) 43 DAS. F) Root fresh weight (FW) (g) 43 DAS. *n* = 4. Bars are SE. Asterisk and connecting bar represents a statistically significant difference where LSDs = 1.869 (total tillers), 0.3741 (primary tillers), 0.2401 (number of leaves), 1.629 (maximum root depth), 4.71 (shoot fresh weight), 31.21 (root fresh weight).

At the end of the experiment, the number of leaves on the main tiller, the maximum root depth, shoot fresh weight and root fresh weight were all statistically significantly reduced in strong soil (see Table 5.10 and Figures 5.10C to F).

Although tissue type was shown to significantly affect leaf levels of IAA, iP, tZ, JA-Ile, no significant effects of soil strength were seen (Table 5.11). As seen in Figure 5.11, the levels of IAA, iP, tZ, ABA, SA, JA and JA-Ile did not statistically significantly differ between weak and strong soil samples within leaf tissue type (AL or EL). However, differences were seen in root samples between weak and strong soil (see Figure 5.12). IAA, iP, tZ and JA-Ile were statistically significantly reduced in the root tips of plants grown in strong soil (see Figures 5.12A, B, C and G). ABA, SA and JA showed no statistically significant difference between weak and strong soil samples (see Figures 5.12D, E and F). There was also a statistically significant increase in JA-Ile in the root system of plants grown in strong soil (see Figure 5.12G). These results explain the significant interactions between the effects of tissue and soil strength on roots levels of IAA, iP, tZ shown in Table 5.12. The ANOVA results for ABA showed a significant interaction between tissue and soil strength but not a significant effect of soil strength on root ABA levels (Table 5.12); explained by the fact that ABA levels went up in root system samples and down in root tip samples in response to strong soil although neither change was significant (Figure 5.12D).

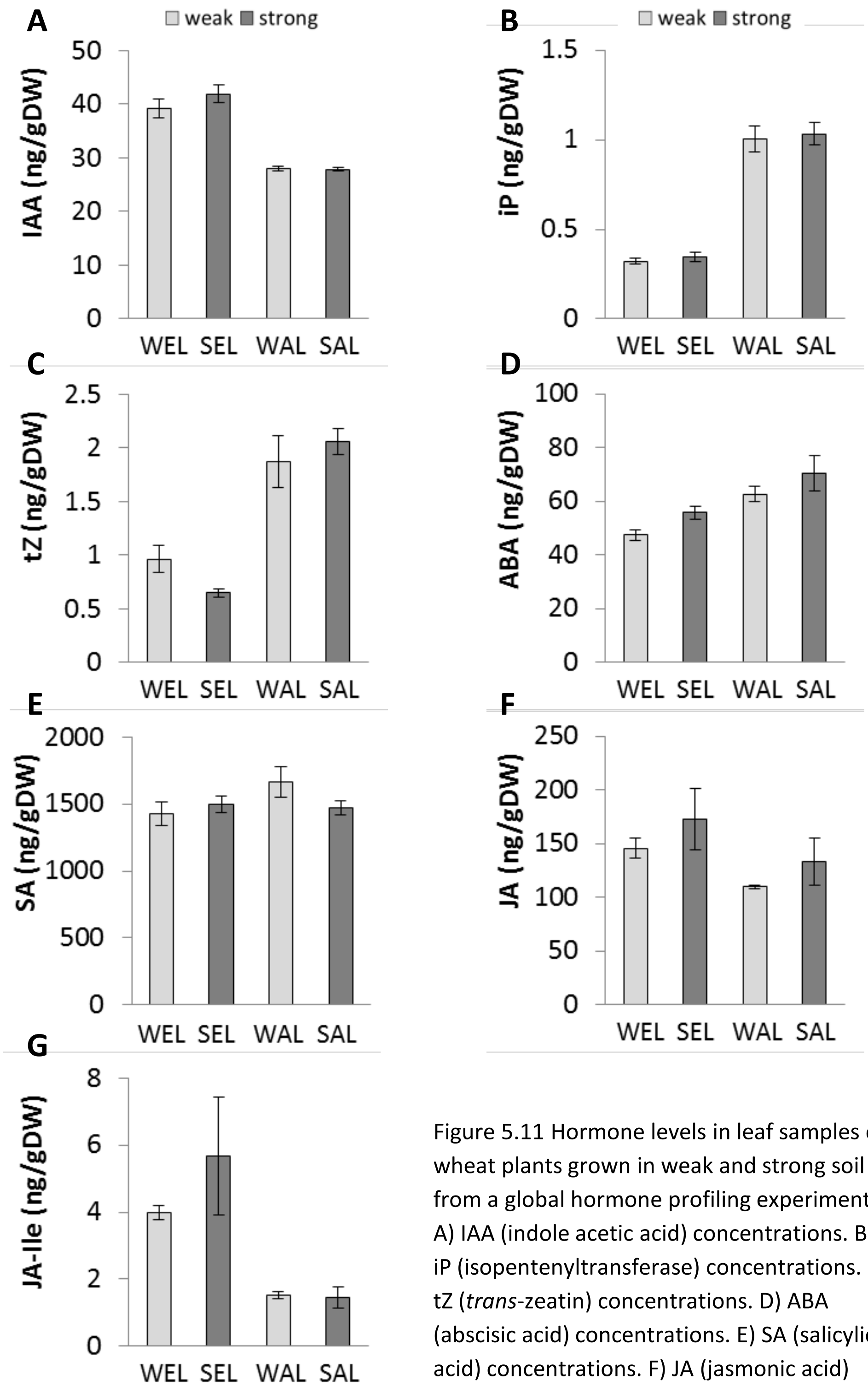
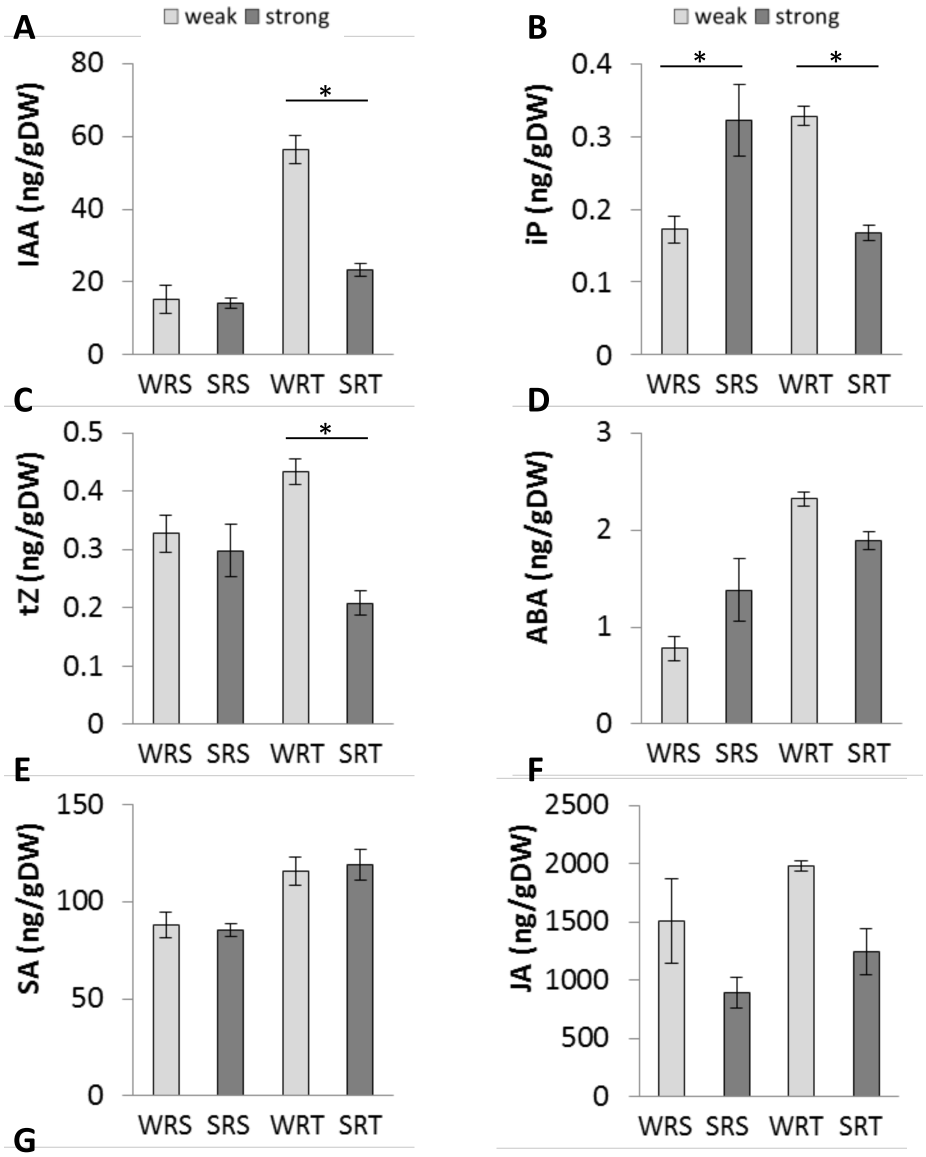


Figure 5.11 Hormone levels in leaf samples of wheat plants grown in weak and strong soil from a global hormone profiling experiment. A) IAA (indole acetic acid) concentrations. B) iP (isopentenyltransferase) concentrations. C) tZ (*trans*-zeatin) concentrations. D) ABA (abscisic acid) concentrations. E) SA (salicylic acid) concentrations. F) JA (jasmonic acid) concentrations. G) JA-Ile (jasmonate isoleucine) concentrations. Samples were collected 43 days after sowing (DAS). WEL = Weak soil Expanding Leaf. SEL = Strong soil Expanding Leaf. WAL = Weak soil Active leaf. SAL = Strong soil Active leaf. *n* = 4. Bars are SE. No statistically significant difference were seen between weak and strong soil.



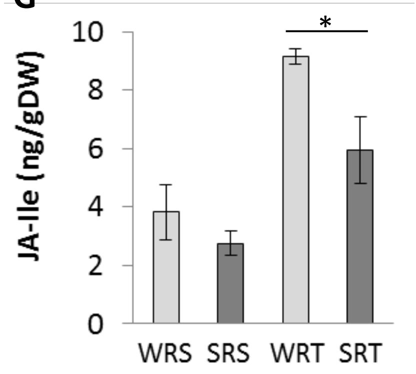


Figure 5.12 Hormone levels in root samples of wheat plants grown in weak and strong soil from a global hormone profiling experiment.
A) IAA (indole acetic acid) concentrations. B) iP (isopentenyladenine) concentrations. C) tZ (*trans*-zeatin) concentrations. D) ABA (abscisic acid) concentrations. E) SA (salicylic acid) concentrations. F) JA (jasmonic acid) concentrations. G) JA-Ile (jasmonate isoleucine) concentrations. Samples were collected 43 days after sowing (DAS). WRS = Weak soil Root System. SRS = Strong soil Root system. WRT = Weak soil Root Tip enriched. SRT = Strong soil Root Tip enriched. *n* = 4. Bars are SE. Asterisk and connecting bar represents a statistically significant difference where LSDs = 10.16 (IAA), 0.1022 (iP), 0.1314 (tZ), 3.135 (JA-Ile).

5.4 Discussion

The work in this chapter partially supports the observations of Coelho Filho et al. (2013) that GA is involved in mediating leaf elongation in response to strong soil. This paper (contributed to by the author during the PhD) showed that the reduction in leaf length in wheat plants (cvs. Mercia and April Bearded) grown on strong soil was dependent on a functional GA signalling system (Figure 5.1). It was also shown that application of GA_3 was able to recover leaf elongation to non-stressed levels in GA-sensitive plants grown in strong soil. In the work presented here, the reduction in leaf elongation response of wheat on strong soil and its restoration by exogenous GA_3 was reproduced in a third wheat variety, Cadenza, supporting the original conclusions and suggesting that this response is widespread in UK varieties of bread wheat. Furthermore, root measurements showed that strong soil had a range of other effects on plant growth as the number of primary roots, root biomass (fresh and dry weight) and the maximum root depth were all reduced (see Figure 5.5).

To provide further support for the role of GA in the leaf elongation response, the levels of the hormone were measured in actively growing leaves of wheat plants (cv. April-bearded, *Rht-B1a*) grown in weak and strong soil. Reductions of GA_{44}, GA_{19}, GA_{20} and GA_1 were seen (see Figures 5.3E to H). These are sequential intermediates in the biosynthetic pathway leading to GA_1. Although the reduction in GA levels was only statistically significant for GA_{44}, the result added another layer of support to the idea that strong soil modifies leaf elongation via GA levels. Leaf four was the leaf selected for the GA measurements as it was shown to be the first leaf to be affected by strong soil in the work by Coelho Filho et al. (2013) (see Figure 5.1A). In retrospect, it may have been better to confirm reduced leaf elongation in response to strong soil in Experiment 5.1 (in leaves 4 and 5) and to have collected actively growing leaf 6 samples. ABA levels did not differ greatly between weak and strong soil, although there was a non-statistically significant increase in leaves of plants grown in strong soil (see Figure 5.3D).

The analysis of GAs was extended further as part of a global hormone profiling experiment in cv. Cadenza. In contrast to the study above, the levels of GAs in actively growing leaves were either similar between weak and strong soil or higher in strong soil (Figure 5.7). At first sight, this casts doubt upon the hypothesis that GA levels mediate the leaf elongation response to strong soil. However, it is clear from Figure 5.6A that, in this experiment, only leaves 3, 4 and 5 were significantly reduced in length by the strong soil: the later leaves, 6 and 7, showed no difference in length between weak and strong soil. As the global hormone analysis was carried out on actively growing leaves after leaf 7, this analysis was not carried out on leaves showing a length difference between strong and weak soil, a clear difference with the previous experiment where GA analysis was carried out on leaf 4. This raises the question of why leaves 6 and 7 (and presumably the later leaves that were collected) of plants in strong soil were apparently not affected by the stress as had been observed in cvs. Mercia and April Breaded.

One possibility is that all the plants, in both weak and strong soil, were subject to another stress, such as nutrient availability, and were therefore all restricted in growth. Certainly the final lengths of leaves 6 and 7 in this experiment were shorter than the preceding leaves, whereas in Coelho Filho et al. (2013) the later leaves were as least as long as leaves 4 and 5 (see Figure 5.1). This seems unlikely, as a fresh batch of nutrient solution had been supplied to the tank 44 das, although diffusion of the nutrients in the sand column could limit access to the new supply. Plants may also have been suffering from apoxia, although there was no evidence of this when harvesting the plants. It is possible that a viral or fungal disease may have taken hold and been manipulating hormone levels *in planta*. Although many plants had patches of necrosis on their leaves by the time of sampling it was not clear if this was due to programmed death of older leaves, an infection or another stress. It may even be possible that wheat plants of the Cadenza cultivar are able to overcome the stress of strong soil and elongate later leaves as normal.

It is also noteworthy that precursor GAs were at lower concentrations in root tips of plants growing in strong soil. Shani et al. (2013) demonstrated GA accumulation in the endodermal cells of the root elongation zone of Arabidopsis plants. It is possible that the reduction of GA in the root tip samples of wheat seen here is responsible for reduced root growth (as seen in a reduced maximum root depth in wheat plants growing in strong soil). However, as the bioactive GA GA_1 was at equal levels in the root tips of plants in weak and strong soil this link is by no means conclusive and needs further investigation.

In light of the apparent differences in the responses between the Cadenza cultivar used in the global hormone analysis and the cultivars used by Coelho Filho et al. (2013), it was important to demonstrate a connection between GA and the leaf elongation response in cv. Cadenza. In Section 5.3.2 Cadenza plants subjected to weak and strong soil showed a significant stunting response by leaf 4 (see Figure 5.4D). Leaf four was longer in plants treated with 10 µM or 100 µM than plants not treated with GA_3 and there was no difference in final leaf length between weak and strong soil conditions for those plants that received GA_3 application, demonstrating that GA_3 could reverse the effects of strong soil on leaf elongation. It was also noted by the author that the phylochron was extended for plants treated with GA_3, as continued elongation of a leaf prevented the elongation of a subsequent leaf. In addition, the plants became spindly and some could not remain upright. For these reasons, the application of GA_3 as a remedy for reduced leaf elongation in strong soil would not seem to be appropriate. Furthermore, it is clear from the results in Figure 5.5 that GA_3 application did not prevent the other effects of strong soil on shoot and root architecture; these plants still had reduced tillering, fewer roots and lower shoot and root biomass in strong soil.

As the exogenous application of GA_3 was unlikely to be viable as a method for alleviating reduced leaf elongation in response to strong soil, an alternative approach was considered to maintain the endogenous levels of GA_1 on strong soil and thereby avoid leaf stunting and consequent effects on biomass and yield. The broad pattern of reduction of GA levels observed under strong soil in Section 5.3.1 suggested the involvement of a known endogenous mechanism for reduction in GA levels involving 2-hydroxylation of early (C_{20}) intermediates in the biosynthetic pathway, catalysed by GA 2-oxidases (Schomburg et al., 2003) of Class III (of the classes proposed by Lee and Zeevaart (2005)). In wheat, the best candidate for such a role is *TaGA2ox3*, which although by sequence homology is a member of GA2ox Class I (active against C_{19}-GAs), also catalyses the hydroxylation (and removal from the pathway) of earlier C_{20} GAs such as GA_{12} and GA_{53} (Pearce et al., 2015). Furthermore, *TaGA2ox3* was observed to be induced in wheat leaves by drought in sandy soil, a stress that not only reduces water availability but also increases soil resistance to root penetration (Li and Phillips, unpublished). There is, unfortunately, no direct evidence that *TaGA2ox3* is involved in the response to soil strength, but suppression of this homoeologous gene family, which is one of the more highly-expressed members of the wheat GA2ox superfamily in vegetative tissues (Pearce et al), would be expected to reduce GA turnover and thereby increase leaf elongation.

The production of an RNAi construct designed to suppress *Ta2ox3* transcript accumulation is described in Section 5.3.4 and this was introduced into wheat by bombardment. The T_1 plants of a selection of independent transformed lines were examined for reduced expression of *Ta2ox3* in PCR-positive plants compared to their PCR-negative siblings. However, no reduction in expression of *Ta2ox3* was observed and no clear phenotype of PCR-positive plants growing in soil was noted. RNAi suppression of target genes has previously been shown to be successful in wheat (eg (Travella et al., 2006; Lovegrove et al., 2013)); however, variable levels of success have been reported (McGinnis, 2010), the reasons for which are largely unknown. Due to time and space constraints the *Ta2ox3* RNAi lines could not be tested for response to soil strength in the soil strength apparatus, as described in Section 2.1.5). However, the lack of reduced expression and any height leaf elongation phenotype suggests that this was unlikely to have generated positive results. Furthermore, had time allowed, the selection of T_2 plants homozygous for the transgene would have allowed a more robust examination of the effects on *Ta2ox3* expression, leaf elongation and the response to the strong and weak soil conditions.

Aside from the GA-mediated effects of strong soil on leaf elongation in wheat, the mediators of effects of strong soil on shoot and root architecture are unknown. Chapter 4 explored the role of SLs (strigolactones) in the reduction of tillering in wheat but was unable to categorically confirm that such a role existed, unlike the case of rice where this thesis has provided strong evidence for the role of this hormone. A very interesting opportunity to perform global hormone analysis arose. While the GA results that came from this experiment (Experiment 5.3) have already been discussed, the remaining results are also of interest. Compellingly, the first key result was that none of the non-GA hormones measured in actively growing or fully expanded leaves differed between weak and strong soil conditions. This adds further weight to the belief that GA is the key hormonal mediator of leaf length in response to strong soil. Having said that, it is arguable that the lack of a difference in hormone levels in active leaves is linked to the lack of an effect of strong soil on the elongation of later leaves in this experiment as was argued for the GA results above.

Roots also show a very significant response to soil strength and global hormone analysis was also performed on root tissues: auxin (IAA), cytokinin (iP and tZ) and JA-Ile were all statistically significantly reduced in the root tips of wheat plants grown in strong soil compared to those grown in weak soil (see Figure 5.10). Additionally, iP was higher in the remaining root system of wheat plants grown in strong soil compared to those grown in weak soil. It is surprising that auxin and cytokinin were both reduced in root tips in strong soil. These two hormones tend to act antagonistically. Root formation on plant cuttings and adventitious root formation would be expected from low levels of cytokinin and high levels of auxin (George and Sherington, 1984). Therefore, a reduction of auxin concentration may be expected to accompany reduced root growth. However, it is not clear why the level of cytokinin would be reduced in root tips at the same time. Of course a balance must be struck between these two important regulators of development and perhaps the cytokinin to auxin ratio must be maintained to enable growth to continue. One interesting point is that, with IAA remaining stable between weak and strong root system samples and iP being increased in root systems of plants grown in strong soil the cytokinin to auxin ratio was higher in the root systems of plants grown in strong soil compared to plants in weak soil. Smigocki and Owens (1989) noted increase shoot proliferation, loss of apical dominance and inhibition of rooting in transformed *Nicotiana* tissue growing in conditions of elevated cytokinin to auxin ratios. Could it be that the reduction in the size of the root system of wheat plants growing in strong soil is mediated by an increase in the cytokinin to auxin ratio in the root system? This idea explored in greater detail in Chapter 6, along with a general discussion of the results of this thesis.

5.5 Conclusion

Chapter 5 demonstrates that GA is involved in the response of leaf elongation to strong soil. Reductions of GA in response to strong soil have been seen (Figure 5.3) and application of GA_3 can preclude a reduction of leaf elongation in wheat plants growing in strong soil (Figure 5.4). Furthermore, when leaf length stops responding to strong soil there are no longer reductions in GA (Figures 5.6 and 5.7). Reduced levels of GA in root tips of plants in strong soil may play a role in reducing root growth but the association needs further investigation. In addition to the role of GA in mediating architectural responses to strong soil, the potential roles of auxin and cytokinin have been uncovered. It is possible that an increased cytokinin (iP) to auxin (IAA) ratio could be responsible for reduced root growth of wheat plants in strong soil. However, this is a somewhat speculative assessment and further work would need to be conducted to demonstrate the importance of cytokinin to auxin ratios in response to strong soil.

Chapter 6: General Discussion

6.1 Plant growth in resistant soil.

Crops experiencing strong soil in a field situation are met by hard pans and localised patches of clods and rocks. The root systems will seek out a path of least resistance and avoid growing through the hardest of substrates. As ever, crops must adapt to the circumstances in which they find themselves. It is this plasticity that enables plants to survive times of hardship and flourish in times of abundance. However, this ability to adapt can occasionally be too finely tuned. For instance, when a crop senses moderately strong soil without any desiccation it may demonstrate reduced growth (perhaps responding to strong soil as a primer for greater stress). Such a response was seen with wheat growth being sensitive to increased soil strength in the absence of water stress (Whalley et al., 2006). As the crop still has access to sufficient water supplies (especially when irrigated), it may be useful to reduce the responsiveness of the plants to strong soil to allow growth and yield to be maintained. Indeed, for Whalley et al. (2006) the rooting depth of autumn-sown wheat was determined before the soil began to dry in spring. It is known that deep roots provide water and N in many agricultural environments (Lynch and Wojciechowski, 2015). However, while a bigger root system and deeper roots can improve yield via improved water and nitrogen uptake, the risk of depleting water resources is increased in environments dependent on stored soil water (Palta et al., 2011). Nevertheless, in agricultural systems with high inputs of fertilizer and water it is important that plants make maximal use of available resources without overreacting to mild stresses of strong soil. A greater understanding of the molecular mechanisms involved in architectural responses to strong soil could lead to fine-tuned modifications to signalling pathways, enabling crop varieties that ignore mild stresses to be grown in high-input systems. Furthermore, greater understanding of architectural responses to strong soil could enable the development of drought-tolerant crop varieties adapted to less favourable conditions.

Pre-existing knowledge

Before the start of this work, it was known that seminal roots of barley were shorter in response to mechanical impedance and showed an increased lateral root density (Goss, 1977). It was suggested that ethylene may control these effects on root architecture (Kays et al., 1974; Moss et al., 1988; Sarquis et al., 1991; Hussain et al., 1999). While some uncertainty remained as to whether or not wounding played a role in the production of ethylene in these experiments, it seemed that ethylene did play some role at least in the early sensing of mechanical impedance (Moss et al., 1988; Sarquis et al., 1991). As plant roots are in contact with the stress of high mechanical impedance from strong soil, the roots seem to respond directly to the stress. However, it was also known that leaf elongation and tillering are reduced in wheat in response to high mechanical impedance (Masle and Passioura, 1987; Whalley et al., 2008). This could either be due to nutrient deficiency caused by a small root system depleting local reserves of N and P or due to a root-to-shoot signal which would most likely be a plant hormone. Indeed, even if nutrient deficiency were a factor that would not rule out a role for plant hormones as strigolactone (SL) production is increased in crops (including sorghum and wheat) in response to nitrogen and phosphorus deficiency (Yoneyama et al., 2007; Yoneyama et al., 2012). The role of SLs in inhibiting tillering of rice and shoot branching in dicot species became of great interest and led to the investigations in Chapter 3 of this thesis and subsequently Chapter 4. A similar level of interest was taken in the role of gibberellins (GAs) in promoting leaf elongation and their potential requirement for mediating this effect in response to strong soil.

6.2 The work described in this thesis

The work of this thesis was able to link SLs with a role in mediating the reduction of tillering in rice in response to strong soil (see Chapter 3). The ability of rice mutants lacking the biosynthesis (*d10*) or perception (*d3*) of SL to tiller more in strong soil than the WT plants suggested that SLs were at least partially responsible for mediating the reduction of tillering in response to strong soil. When this finding was combined with the fact that the concentrations of the strigolactone (−)-*ent*-2'-*epi*-5DS was increased in the roots of rice plants grown in strong soil it became quite convincing that SLs are the signal for reduced tillering in rice plants in response to strong soil. When RNAi lines targeting wheat orthologues of the SL biosynthesis genes *TaD10* and *TaD17* were grown in strong soil the effects on tillering were not replicated in all three lines of each construct tested (see Chapter 4).

However, lines L18 (containing pRNAiTaD10) and L22 (containing pRNAiTaD17) did show increases in tillering for plants containing an RNAi construct compared to PCR-negative plants (although not statistically significantly) (Figure 4.16C). It is possible that a thorough assessment of all transgenic lines would have strongly supported the role of SL in reducing tillering of wheat in strong soil. Further evidence that SLs are not required for wheat to respond to strong soil is that SLs were not shown to be increased in roots of wheat (cv. April Bearded) plants grown in strong soil compared to plants grown in weak soil. Whether or not this is related to the fact that orobanchol is the most prevalent SL in wheat, compared to the (−)-*ent*-2'-*epi*-5DS prevalent in rice is unknown. These two SLs do differ structurally and a growing body of evidence is emerging that particular roles of strigolactones require specific structural characteristics. For instance, while the coupling of C- and D-rings was required for both the stimulation of branching of arbuscular mycrorrhizae and the inhibition of tillering in rice, an enol ether bond, alkoxy ether or an imino ether were suitable for the response of arbuscular mycorrhizae but an enol bond was required for full inhibition of shoot branching in rice (Akiyama et al., 2010; Umehara et al., 2015). Importantly, an *R* configuration of C-2' of the D-ring was found to be essential for SL biological activity in rice and both orobanchol and 5DS have stereoisomers of *R* configuration (Umehara et al., 2015). Orobanchol was highly active in stimulating seed germination of the parasitic plant *Orobanche minor* while 5DS showed some of the least activity of those SLs tested (Kim et al., 2010). It seems possible that the increase of orobanchol levels in response to N and P deficiency seen by Yoneyama et al. (2012) is more to do with attempting to increase colonization by arbuscular mychorrizae instead of directly influencing the tillering. Indeed, Yoneyama et al. (2012) did not find a negative correlation between orobanchol levels and the number of tillers in wheat (as both were reduced in N starvation relative to P starvation). Factors other than simply SLs, including N deficiency, strongly regulate shoot branching (Yoneyama et al., 2012). Perhaps further structural components of SL are required for control of tillering in wheat, or perhaps the cultivars used in this thesis (cv. Cadenza) and by Yoneyama et al. (2012) (c.v Chinese Spring) are somehow defective in SL signaling.

In addition to reduced tillering, the effect of strong soil on leaf elongation was studied in this thesis. In Chapter 5, it was confirmed that the level of bioactive GA is reduced in actively growing leaves (leaf four) of wheat plants in strong soil. It was also found that this reduction in GA was not seen when wheat plants did not respond to strong soil with a reduction in leaf elongation. What is unclear is what caused the *T.aestivum* (cv. Cadenza) plants grown for global hormone profiling (in Experiment 5.3) to show no difference in leaf length between weak and strong soil treatments for leaves six and seven. It is possible that this was due to a difference in response between cultivars, as we previously showed that wheat plants of April Bearded and Mercia cultivars continued to respond to strong soil for leaves six and seven (Coelho Filho et al., 2013). Although work in Chapter 5 showed that Cadenza responded to GA_3 application with increased leaf elongation the experiment was ended prior to development of leaf six and seven. It plausible that Cadenza plants may develop some level of resistance to strong soil by the time leaves six and seven develop although it seems unlikely and the mechanism by which this would occur is not known. It is also important to recognize that leaf area of leaves six and seven remained smaller in strong soil than in weak soil conditions and a lack of a difference in leaf length between weak and strong soil was due to both an increase in leaf length in strong soil (relative to length of leaf five) and a reduction in leaf length in plants grown in weak soil. It is for this reason that the most likely cause for this peculiarity is an unknown confounding variable. The possibility of such an unaccounted stress may undermine the results of the global hormone analysis and has been discussed in Chapter 5. It was interesting to note that differentially expressed genes in RNAseq analysis of global gene expression of rice roots grown in weak and strong soil returned GO (Gene Ontology) enrichment for terms including "response to stress", "response to oxidative stress", "cellular nitrogen compound metabolic process" and "apoplast" (Appendix 1). This underlines that plants growing in strong soil are certainly under stressful conditions but also suggests that nitrogen signaling may be playing a role in response to strong soil.

Assuming that there were no confounding variables in the global hormone profiling experiment, the results for ABA, Ja-Ile, auxin and cytokinin provided interesting insights into potential mechanisms of architectural responses to strong soil.

The work described in this thesis showed no statistically significant change in ABA concentration in leaves or roots between wheat plants grown in weak and strong soil. ABA has been shown to control stomatal conductance but not leaf growth in response to abiotic stresses of water and nutrient deficiencies (Hussain et al., 1999; Dodd et al., 2003; Dodd, 2005). ABA increases in roots were shown in response to multiple stimuli linked to arid regions (including soil strength) but ABA accumulation was not always seen in the leaves (Davies et al., 2005). Furthermore, an initial increase in xylem nitrate concentration has been shown in response to soil drying which could increase sensitivity to ABA (Perez-Alfocea et al., 2011). It is possible that strong soil also causes plants to initially increase xylem concentrations of nitrates leading to increased ABA-responsiveness without accumulation of ABA in the leaves. This would be a potential route of future enquiry, but the hormone quantification results of this thesis do not suggest a role for ABA in mediating architectural responses to strong soil. Having said this, there is evidence that MAX2 (the D3 orthologue in Arabidopsis) functions upstream of the ABA pathway component ABI (Bu et al., 2014). Stomatal closure in *max2* Arabidopsis plants was less sensitive to ABA than in WT plants (Bu et al., 2014). ABA had been shown to inhibit bud outgrowth in Arabidopsis, possibly by mediating auxin and cell cycle regulation but downstream of MAX2 and BRC1 (Yao and Finlayson, 2015). It is possible that the occasions in this thesis when *d3* rice plants responded to strong soil differently to *d10* and WT plants (such in Figure 3.2E and Figure 3.9E) were linked to modified sensitivity to ABA. While tillering effects would not be expected to differ between *d10* and *d3* plants (if ABA is acting downstream of BRC1), differing response to ABA for stomatal conductance could alter transpiration rates and potentially the rate at which signaling components are transported. Perhaps the *d3* rice mutants, in addition to having reduced closure of stomata, produced fewer desiccation resistance compounds and spent the saved resources on continued leaf growth in strong soil. The link between ABA and D3 in rice would need to be verified but if similar results are found to those of Bu et al. (2014) another layer of complexity would be unveiled in the signaling processes of SLs in rice.

Jasmonate has been shown to be required for touch-induced morphological changes and to reduce rosette diameter and delay flowering in Arabidopsis (Chebab et al., 2012). One Jasmonate transcription factor (MYC2) plays roles in controlling the synthesis of auxin, ethylene and jasmonates, responses to wounding and drought stress and a role in JA-induced root growth inhibition (Wasternack and Hause, 2013). JA-Ile (the biologically active compound in jasmonate signaling) has been shown to be synthesized rapidly in response to wounding (Kazan and Manners, 2013). Therefore, it is surprising to see that JA-Ile levels were in fact reduced in root tips of plants growing in strong soil. However, it is possible that the reduction of JA-Ile is an attempt by the plant to increase root growth to counteract the strong soil stress, only for the plant to be unable to accomplish the feat.

It was found in Chapter 5 that both cytokinin and auxin levels were reduced in root tips of wheat plants grown in strong soil. Additionally, the cytokinin to auxin ratio was higher in root systems of wheat plants grown in strong soil than plants grown in weak soil. In other work, inhibition of rooting was seen in *Nicotiana* under conditions of increased cytokinin to auxin ratios (Smigocki and Owens, 1989). A general pattern of a transient (<3 hr) peak in cytokinin production/accumulation in response to abiotic stress has been identified and cytokinin levels then either subside to below-baseline levels in response to sustained moderate stress or continue to rise if the stress is severe or increased (Zwack and Rashotte, 2015). It is known that cytokinin and auxin signalling converge at the point of *SHY2* which negatively regulates expression of *PINs* (responsible for auxin transport) in response to a cytokinin-response transcription factor (ARR1), resulting in redistribution of auxin and cell differentiation in neighbouring tissues while reducing the size of the root meristem (Moubayidin et al., 2015). When considered in the context of this knowledge, it seems possible that the reductions of both cytokinin and auxin in root tips of wheat plants grown in strong soil are results of reduced sizes of root meristems and that the increased cytokinin to auxin ratio in root systems grown in strong soil is indicative of an ongoing abiotic stress. As the axillary shoot buds of rice and wheat are located below ground it is plausible that these neighbouring tissues could be also be affected by the reduction of auxin transport, possibly making initiation of a polar auxin flux more difficult for dormant buds. Although these results are only preliminary they suggest clear options for hormones to explore in future work. It would be interesting to demonstrate how reduction of auxin transport affects the response of crops to strong soil. Use of the fluorescent auxins described by Hayashi et al. (2014) and treatment with strong soil could provide very interesting results.

The role of ethylene was not investigated in this thesis due to extensive previous work on the role of ethylene in reduced plant growth in response to high impedance conditions (Kays et al., 1974; Moss et al., 1988; Sarquis et al., 1991; Hussain et al., 1999), as well as the requirement for specialist equipment to be able to quantify and apply ethylene in a controlled manner. The general findings of those authors that ethylene did play a role in root growth responses to high impedance conditions. Therefore, it is likely that ethylene would be involved in responses to strong soil and that hormone cross-talk would be occurring. Ethylene has been shown to inhibit root growth of Arabidopsis, at least in part, in a DELLA-dependent manner (Achard et al., 2003). In addition to ethylene, DELLA proteins are known to be part of signaling pathways for GA, ABA, auxin and JA (Yang et al., 2012). Integrators of hormonal signaling like DELLAs and MYC2 are likely to play important roles in plant architectural responses to strong soil.

6.3 Future work

Future work should investigate the roles of auxin, cytokinins and Ja-Ile in architectural responses to strong soil in wheat as well as further explore the role of SL in reduced tillering in rice. An experiment assessing the effects of addition of GR24 (a synthetic SL analogue) on rice would provide another layer of support for the hypothesis that tillering reduction due to strong soil involves SLs. There would also seem to be some work to be done on assessing the availability of nutrients in plants growing in strong soil. Although Masle and Passioura (1987) found that shoot levels of N and P remained similar to levels in control-treated plants during the early stages of reduction in shoot growth in response to strong soil, Yoneyama et al. (2012) showed that N deficient conditions results in reduced SL exudation and reduced tillering which, along with results of this thesis, suggests that other signaling mechanisms (not just strigolactones) are mediating the tillering responses of wheat to nutrient deprivation and strong soil. A key premise of the sand column system used in this thesis for examining soil strength is that nutrients remain available in weak and strong soil. However, it is likely that a smaller root system, as a result of strong soil, will be able to access and accumulate fewer nutrients than a larger root system. Similarly, the volume of sand explored by the roots in strong soil is much lower, and while we assume that diffusion of nutrients within the medium will reduce the effect of root volume, this has not been investigated. This will slow the speed with which a plant will be able to grow and thus the yield potential. The subtleties of responses to strong soil will take a considerable amount of work to elucidate.

A number of potential improvements to the methodology could be made if future work were to be conducted. The sand column system used for comparing soil strengths was limited in numbers of columns (and hence plants) that could be used. It would be exciting to build a large facility where hundreds of sand columns could be built and moved robotically with continuous data logging of nutrient availability and where variable known mechanical impedances could be applied to each column. If this growth facility were attached to a state-of-the art automated X-ray CT facility (similar to one at the Sutton Bonington campus of University of Nottingham) a very high-throughput and rapid discovery method could be developed. However, such a facility would require blocking of nutrient containers, vast amounts of computing power (as well as advances in the automation of image processing) and a very substantial level of funding. A more feasible improvement to the soil strength experiments described in this thesis would be to use more than two different weights, allowing for a gradient of soil strength effects to be examined. Additionally, a system where automated measurements of shoot architecture could be made after addition of a soil strength stimulus would be very interesting. This could even demonstrate a spike of cytokinin production before sustained reduction in response to strong soil, in line with the general pattern of cytokinin production in response to abiotic stresses highlighted by (Zwack and Rashotte, 2015).

Sampling of active leaves for comparison of GA levels differed between Experiment 5.1 and 5.3 that both studied the levels of the hormone under weak and strong soil. In Experiment 5.1 active leaves from the main tiller (specifically leaf four) were sampled at equal developmental stages, with a delay in collecting strong soil leaves and no leaves were at their predicted final length. For Experiment 5.3 samples from weak and strong soil were taken at the same time, with any actively growing leaf potentially being collected. Both of these methods have pros and cons. Selecting developmentally equal leaves ensured samples were directly comparable but may not have fully represented the effects of the soil strength treatments as timing accounted for the delay in growth seen in strong soil. Collecting any actively growing leaves gave a more global estimation for GA levels in actively growing leaves but did not account for the proportions of samples that came from the main tiller or higher order tillers. Sampling for weak and strong soil treatments at the same time did seem to be a less biased method but may not be the most appropriate biologically.

When investigating the role of SL in wheat the decision was made to use RNAi technology to produce transgenic lines containing constructs that targeted the genes of interest for silencing. RNAi silencing has been used successfully by other authors. Gil-Humanes et al. (2008) successfully reduced the levels of specific gluten proteins in wheat (*T.aestivum*) grain. Sestili et al. (2010) successfully reduced amylose levels in *T. aestivum* by targeting starch branching enzymes for silencing RNAi. Johnson et al. (2008) used RNAi in wheat (*T. aestivum*) grains to target a signalling component involved in ABA-suppression after GA-induced gene expression. In this last case, while *TaABF1* mRNA levels were reduced by the RNAi construct it was not possible to prevent normal functioning of other signalling components (Johnson et al., 2008). The lack of effect was attributed to an unknown redundant protein that could perform the functions of *TaABF1*, while also noting that modification of mRNA levels of *TaABF1* (*ABA response element binding factor*) did not seem to be important in GA and ABA signalling (Johnson et al., 2008). It is possible that the very low concentrations at which plant hormones function make them particularly difficult targets for RNAi. RNAi rarely totally abolishes expression and a small amount of the hormone may allow normal function to be maintained. However, Travella et al. (2006) were able to target a component of ethylene signalling with RNAi in *T.aestivum* and successfully caused total ethylene insensitivity with 70% reduction of mRNA levels. Plant hormone signalling can also be modified by RNAi. The attribution of a redundant protein by Johnson et al. (2008) holds particular relevance to the work in this thesis. It has been described in Chapter 4 that two paralogues of *D10* seem to exist in wheat. *D10* and *D10-like* exist in rice and RNAi has been used to reduce expression of these two genes in rice (Ngo, 2011). While *D10* suppression by RNAi in rice successfully resulted in increased tiller number and decreased plant height, no changes were seen in tiller number or plant height for *D10-like* (Ngo, 2011). The *TaD10* sequence used for RNAi in Chapter 4 has sufficient homology to likely be able to knock-down both *TaD10* (as per *TaD10-454* and *TaD10_3B_DL*) and *TaD10-like* (the *TaD10* orthologue on Ensembl Plants). If the pTaRNAiD10 construct did inadvertently target multiple *D10* and *D10-like* paralogues a phenotypic response would still be expected in plants with reduced levels of *TaD10* and *TaD10-like* mRNA. It was encouraging that one of the three T_1 lines for each wheat SL biosynthesis gene targeted for RNAi (L18 containing pRNAiTaD10 and L22 containing pRNAiTaD17) demonstrated increased tillering for PCR-positive plants compared to PCR-negative plants grown in strong soil. Follow-on work from this thesis would need to include a thorough assessment of the RNAi lines developed to knock-down expression of wheat SL biosynthesis genes, in terms of expression, zygosity and phenotype in the T_2 generation. It would be interesting to use qRT-PCR to examine the expression of each paralogue and homoeologue in T_2 homozygous plants for all lines containing the SL RNAi constructs produced in this thesis, providing conclusive evidence of the effectiveness of the constructs and enabling the responses of the plants to strong soil to be examined thoroughly. These assessments would cost both money and time but could provide extremely useful insights into the roles of SLs in wheat development and responses to strong soil.

An alternative method for assessing the role of SL (and GA) genes in wheat would have been to use TILLING (Targeting Induced Local Lesions IN Genomes). This is a reverse genetics strategy where chemically induced point mutations are screened for in genes of interest (Till et al., 2004). TILLING projects for Arabidopsis and maize have been established (Greene et al., 2003; Till et al., 2004). The John Innes Centre has combined TILLING efforts in a number of plant species with RevGenUK. Included in the list of mutagenized species are *Brassica rapa*, *Triticum aestivum* and *Oryza sativa*. The recently added hexaploid *T. aestivum* population for this collection was supplied by Dr Andy Phillips, Dr Archana Patil, Dr Steve Thomas and colleagues at Rothamsted Research. A searchable database of hexaploid wheat TILLING mutants, identified by exon capture and next-generation sequencing, will be available online imminently. With this advancement, TILLING is likely to be the method of choice for reverse genetic experiments in wheat. Indeed, had this database been available at the beginning of this PhD, TILLING lines in the SL pathway would certainly have been characterised and tested for a role in mediating tillering in strong soil. It is important to recognise that, even with the development of a database of TILLING mutations in wheat, a considerable amount of time will be needed to produce a complete null phenotype. The hexaploid nature of *T. aestivum* allows accumulation of mutations, increasing the chance of finding a mutation of interest (Borrill et al., 2015). To account for any redundancy between homoeologues, truncation mutations in all three homoeologues would need to be identified and a 12-16 month crossing programme established to eventually produce F_2 progeny of a line containing a mutation in all three genomes (Borrill et al., 2015). However, it is not certain that even these genetic resources would have resulted in anticipated outcomes. As outlined earlier, Chapter 4 of this thesis and Yoneyama et al. (2012) suggest that factors other than SL are also controlling tillering in strong soil and nutrient deficient conditions.

Finally, future work can build on the results of the RNA-seq experiment that assessed differential gene expression between rice roots grown in weak and strong. Some level of variability was seen between samples within strength treatments (Figure 3.14A), However, considering that the samples were from individual plants, the amount of variability was very reasonable. For all samples between 88.4% and 90.5% of reads mapped to the reference so a variation in mapping efficiency would not seem to be the cause of the variability shown. The total number of reads mapped did vary between samples and may have contributed to some of the variability in the weak samples, as there was a positive correlation between number of mapped reads and the logFC on dimension 2 for weak soil samples, although this was not the case for the strong soil samples.

6.4 Conclusions

The work in this thesis established the involvement of strigolactones (SLs) in the reduction of tillering by rice plants in response to strong soil, demonstrated by reduced responsiveness to strong soil in SL rice mutants and increased endogenous levels of SL in rice plants grown in strong soil. RNAi lines were produced to target SL biosynthesis genes in wheat. Time constraints limited the assessment of these lines and precluded establishment of a role for SLs in response to strong soil in wheat. However, further characterisation of the transgenic plants demonstrating knocked-down SL biosynthesis could elucidate numerous roles for SLs in wheat. The work in this thesis also supported the findings of other research that gibberellin (GA) is involved in mediating leaf elongation responses to strong soil, with reduction of bioactive GA in actively growing leaves required for the response to occur. Finally, the involvement of JA-Ile, auxin and cytokinin in mediating plant architectural responses to strong soil have been identified, providing rich sources for further investigations into this important abiotic stress.

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Appendix 1

Table of results from GO term analysis of all significantly differentially expressed genes from RNAseq of wild-type Shiokari rice plants grown in weak and strong soil.

GO terms are divided by ontology into biological processes (P), molecular functions (F) and cellular components. FDR= false discovery rate.

GO term	Ontology	Description	*p*-value	FDR	Number in input list	Number in BG/Ref
GO:0005975	P	carbohydrate metabolic process	5.00E-13	3.90E-10	61	864
GO:0006950	P	response to stress	4.30E-09	1.70E-06	53	883
GO:0009308	P	amine metabolic process	9.20E-09	2.00E-06	33	414
GO:0006979	P	response to oxidative stress	1.00E-08	2.00E-06	22	194
GO:0050896	P	response to stimulus	3.10E-08	4.80E-06	56	1022
GO:0044281	P	small molecule metabolic process	7.60E-08	9.80E-06	57	1079
GO:0006519	P	cellular amino acid and derivative metabolic process	2.20E-07	2.50E-05	30	408
GO:0042221	P	response to chemical stimulus	1.40E-06	0.00014	24	308
GO:0044106	P	cellular amine metabolic process	2.60E-06	0.00022	26	366
GO:0034641	P	cellular nitrogen compound metabolic process	3.90E-06	0.0003	30	473
GO:0044036	P	cell wall macromolecule metabolic process	7.00E-06	0.00049	9	48
GO:0042180	P	cellular ketone metabolic process	9.30E-06	0.0006	30	496
GO:0019318	P	hexose metabolic	1.20E-05	0.00069	14	134

		process				
GO:0006520	P	cellular amino acid metabolic process	1.40E-05	0.00078	23	332
GO:0043436	P	oxoacid metabolic process	1.90E-05	0.00081	29	490
GO:0006082	P	organic acid metabolic process	2.00E-05	0.00081	29	491
GO:0019752	P	carboxylic acid metabolic process	1.90E-05	0.00081	29	490
GO:0005976	P	polysaccharide metabolic process	1.80E-05	0.00081	14	140
GO:0006066	P	alcohol metabolic process	1.60E-05	0.00081	17	199
GO:0016998	P	cell wall macromolecule catabolic process	3.20E-05	0.0012	8	45
GO:0009309	P	amine biosynthetic process	3.40E-05	0.0013	14	149
GO:0044262	P	cellular carbohydrate metabolic process	3.70E-05	0.0013	24	379
GO:0005996	P	monosaccharide metabolic process	3.90E-05	0.0013	14	151
GO:0006006	P	glucose metabolic process	3.80E-05	0.0013	12	112
GO:0044283	P	small molecule biosynthetic process	7.30E-05	0.0023	20	297
GO:0044271	P	cellular nitrogen compound biosynthetic process	9.70E-05	0.0029	17	232
GO:0000097	P	sulfur amino acid biosynthetic process	0.00011	0.003	6	27
GO:0009064	P	glutamine family amino acid metabolic process	0.00011	0.003	7	40

GO:0055114	P	oxidation reduction	0.00013	0.0033	18	261
GO:0008652	P	cellular amino acid biosynthetic process	0.00013	0.0034	12	129
GO:0000096	P	sulfur amino acid metabolic process	0.00015	0.0037	6	29
GO:0006022	P	aminoglycan metabolic process	0.00015	0.0037	6	29
GO:0044092	P	negative regulation of molecular function	0.0003	0.0068	7	48
GO:0043086	P	negative regulation of catalytic activity	0.0003	0.0068	7	48
GO:0006952	P	defense response	0.00033	0.0072	24	442
GO:0044272	P	sulfur compound biosynthetic process	0.00033	0.0072	7	49
GO:0016052	P	carbohydrate catabolic process	0.00071	0.014	11	135
GO:0006026	P	aminoglycan catabolic process	0.00073	0.014	5	26
GO:0006030	P	chitin metabolic process	0.00073	0.014	5	26
GO:0006032	P	chitin catabolic process	0.00073	0.014	5	26
GO:0044282	P	small molecule catabolic process	0.0011	0.021	11	143
GO:0015833	P	peptide transport	0.0012	0.022	9	101
GO:0006857	P	oligopeptide transport	0.0012	0.022	9	101
GO:0006790	P	sulfur metabolic process	0.0013	0.023	7	63
GO:0010876	P	lipid localization	0.0014	0.024	9	104
GO:0006869	P	lipid transport	0.0014	0.024	9	104
GO:0016053	P	organic acid biosynthetic process	0.002	0.032	13	203
GO:0046394	P	carboxylic acid biosynthetic	0.002	0.032	13	203

		process				
GO:0000272	P	polysaccharide catabolic process	0.0027	0.042	5	36
GO:0016051	P	carbohydrate biosynthetic process	0.0029	0.045	9	116
GO:0006073	P	cellular glucan metabolic process	0.0031	0.046	8	95
GO:0044042	P	glucan metabolic process	0.0031	0.046	8	95
GO:0016491	F	oxidoreductase activity	7.70E-20	4.20E-17	120	1995
GO:0005506	F	iron ion binding	4.20E-17	1.10E-14	63	733
GO:0009055	F	electron carrier activity	2.30E-16	4.20E-14	70	919
GO:0020037	F	heme binding	5.80E-15	8.00E-13	54	622
GO:0046906	F	tetrapyrrole binding	8.90E-15	9.80E-13	54	629
GO:0004553	F	hydrolase activity, hydrolyzing O-glycosyl compounds	5.30E-11	4.90E-09	39	454
GO:0045735	F	nutrient reservoir activity	5.40E-10	4.20E-08	16	77
GO:0016798	F	hydrolase activity, acting on glycosyl bonds	8.40E-10	5.50E-08	39	503
GO:0016209	F	antioxidant activity	8.90E-10	5.50E-08	25	219
GO:0016684	F	oxidoreductase activity, acting on peroxide as acceptor	4.00E-09	2.00E-07	23	201
GO:0004601	F	peroxidase activity	4.00E-09	2.00E-07	23	201
GO:0004497	F	monooxygenase activity	4.80E-08	2.20E-06	32	423
GO:0003824	F	catalytic activity	1.40E-07	5.80E-06	388	13462
GO:0030145	F	manganese ion binding	9.40E-07	3.70E-05	11	61
GO:0016829	F	lyase activity	1.40E-06	4.90E-05	23	286

GO:0048037	F	cofactor binding	1.40E-06	4.90E-05	34	547
GO:0016831	F	carboxy-lyase activity	6.70E-05	0.0022	9	66
GO:0019842	F	vitamin binding	0.00014	0.0044	16	217
GO:0016769	F	transferase activity, transferring nitrogenous groups	0.00016	0.0046	8	58
GO:0016830	F	carbon-carbon lyase activity	0.00021	0.0057	10	96
GO:0050662	F	coenzyme binding	0.00025	0.0066	22	380
GO:0004867	F	serine-type endopeptidase inhibitor activity	0.00046	0.012	8	69
GO:0004866	F	endopeptidase inhibitor activity	0.00048	0.012	9	88
GO:0030414	F	peptidase inhibitor activity	0.00056	0.013	9	90
GO:0004568	F	chitinase activity	0.00073	0.016	5	26
GO:0042802	F	identical protein binding	0.00076	0.016	7	57
GO:0070279	F	vitamin B6 binding	0.00094	0.018	11	140
GO:0005507	F	copper ion binding	0.00089	0.018	11	139
GO:0030170	F	pyridoxal phosphate binding	0.00094	0.018	11	140
GO:0000287	F	magnesium ion binding	0.001	0.019	11	142
GO:0016705	F	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.0013	0.023	7	63
GO:0004713	F	protein tyrosine kinase activity	0.0017	0.028	53	1426
GO:0004674	F	protein serine/threonine kinase activity	0.0016	0.028	53	1424
GO:0016758	F	transferase activity, transferring hexosyl groups	0.0021	0.033	22	450

GO:0030554	F	adenyl nucleotide binding	0.0027	0.042	102	3210
GO:0001883	F	purine nucleoside binding	0.0027	0.042	102	3210
GO:0001882	F	nucleoside binding	0.003	0.044	102	3220
GO:0005576	C	extracellular region	3.10E-11	2.40E-09	30	269
GO:0048046	C	apoplast	4.30E-08	1.60E-06	15	94

Appendix 2:
Differential gene expression (FDR<0.05) in rice roots.

		log2(strong/	log2(strong	Up or	Fold change	Weak FPKM		Strong FPKM	
Transcript	Gene RAP ID	weak) FC	/weak) FDR	Down	(FC)	mean	SE	mean	SE
OS08T0536383-00	Os08g0536383	6.60	2.86E-05	Up	97.1	0.0	0.0	1.0	0.3
OS01T0946550-00	Os01g0946550	6.48	2.17E-05	Up	89.3	0.0	0.0	0.9	0.1
OS02T0491700-00	Os02g0491700	6.44	6.79E-05	Up	86.9	0.0	0.0	0.2	0.0
OS09T0537700-02	Os09g0537700	6.42	9.97E-15	Up	85.9	0.4	0.2	31.7	8.9
OS02T0657775-00	Os02g0657775	6.39	1.57E-02	Up	83.7	0.0	0.0	0.8	0.3
OS05T0399400-00	Os05g0399400	6.19	9.72E-07	Up	72.9	0.1	0.0	6.1	4.0
OS07T0206500-00	Os07g0206500	6.07	1.07E-03	Up	67.4	0.0	0.0	0.2	0.1
EPIOSAT00000004985		5.98	1.07E-03	Up	63.3	0.0	0.0	0.7	0.1
OS05T0128600-00	Os05g0128600	5.50	1.58E-02	Up	45.3	0.0	0.0	0.4	0.1
OS03T0103033-00	Os03g0103033	5.48	1.34E-07	Up	44.7	0.0	0.0	0.2	0.1
OS04T0479550-00	Os04g0479550	5.36	2.53E-02	Up	41.2	0.0	0.0	0.4	0.0
OS03T0289850-01	Os03g0289850	5.26	4.21E-03	Up	38.3	0.0	0.0	0.3	0.2
OS06T0118800-00	Os06g0118800	5.17	2.76E-05	Up	35.9	0.0	0.0	0.5	0.2
OS03T0103000-01	Os03g0103000	4.95	2.29E-05	Up	30.8	0.0	0.0	0.4	0.2
OS12T0282000-01	Os12g0282000	4.91	4.47E-03	Up	30.0	0.2	0.1	6.9	3.2
OS07T0243800-00	Os07g0243800	4.74	1.25E-04	Up	26.8	0.2	0.0	5.6	2.8
OS04T0344400-00	Os04g0344400	4.61	7.86E-04	Up	24.5	0.0	0.0	0.2	0.1
EPIOSAT00000030778		4.57	4.94E-02	Up	23.8	0.1	0.0	1.4	0.6
OS08T0188900-00	Os08g0188900	4.40	3.43E-06	Up	21.1	0.1	0.0	1.6	0.6
OS07T0153150-01	Os07g0153150	4.35	1.20E-04	Up	20.4	0.0	0.0	0.8	0.3
OS03T0289800-01	Os03g0289800	4.34	6.22E-03	Up	20.3	0.1	0.0	1.2	0.8
OS02T0491600-00	Os02g0491600	4.17	6.19E-13	Up	18.0	0.1	0.0	1.7	0.1
OS07T0432100-00	Os07g0432100	4.09	1.33E-03	Up	17.1	17.6	5.0	302.3	123.5
OS09T0552600-01	Os09g0552600	4.08	7.61E-04	Up	17.0	0.0	0.0	0.2	0.1
OS10T0151500-00	Os10g0151500	4.08	3.36E-02	Up	16.9	0.0	0.0	0.1	0.0
OS07T0153150-02	Os07g0153150	4.08	2.29E-03	Up	16.9	0.0	0.0	0.9	0.4
OS06T0521200-00	Os06g0521200	4.00	3.84E-02	Up	16.0	0.0	0.0	0.2	0.1
OS06T0192100-00	Os06g0192100	3.99	6.04E-03	Up	15.8	0.0	0.0	0.1	0.0
OS12T0236225-00	Os12g0236225	3.96	7.96E-04	Up	15.6	2.3	0.6	35.9	14.2
OS05T0438201-00	Os05g0438201	3.82	4.26E-02	Up	14.2	0.0	0.0	0.2	0.1
OS06T0725250-00	Os06g0725250	3.76	2.09E-02	Up	13.5	0.0	0.0	0.3	0.1
OS01T0820400-00	Os01g0820400	3.73	5.17E-05	Up	13.2	0.2	0.0	2.6	1.3
OS07T0430501-00	Os07g0430501	3.59	1.11E-02	Up	12.0	61.9	20.6	746.1	305.1
OS02T0143300-00	Os02g0143300	3.56	4.22E-03	Up	11.8	0.0	0.0	0.4	0.1
OS01T0608200-00	Os01g0608200	3.56	2.38E-02	Up	11.8	0.0	0.0	0.2	0.1
OS07T0431160-00	Os07g0431160	3.54	2.95E-03	Up	11.6	5.3	1.1	61.4	25.1
OS10T0178000-00	Os10g0178000	3.52	1.97E-02	Up	11.5	0.0	0.0	0.4	0.1
OS10T0184871-00	Os10g0184871	3.52	3.79E-02	Up	11.5	0.0	0.0	0.3	0.1
OS01T0500100-00	Os01g0500100	3.50	2.21E-02	Up	11.3	0.0	0.0	0.3	0.1
OS09T0507300-01	Os09g0507300	3.47	5.23E-03	Up	11.0	0.0	0.0	0.1	0.0
OS08T0557200-01	Os08g0557200	3.44	9.75E-03	Up	10.9	0.0	0.0	0.5	0.2
OS10T0508900-01	Os10g0508900	3.41	3.77E-02	Up	10.6	0.0	0.0	0.1	0.0
OS11T0506300-00	Os11g0506300	3.35	1.57E-03	Up	10.2	0.0	0.0	0.2	0.0
OS03T0439700-01	Os03g0439700	3.34	4.80E-03	Up	10.1	0.2	0.0	2.3	0.9
OS07T0432201-01	Os07g0432201	3.32	1.58E-02	Up	10.0	221.9	69.0	2219.2	904.7
OS01T0871600-01	Os01g0871600	3.32	1.50E-02	Up	10.0	0.7	0.2	7.0	2.9
OS02T0594166-01	Os02g0594166	3.31	1.16E-03	Up	9.9	1.7	0.5	16.6	6.1
OS02T0594232-00	Os02g0594232	3.31	5.83E-04	Up	9.9	1.7	0.4	16.7	6.1
OS04T0173800-01	Os04g0173800	3.26	1.37E-05	Up	9.6	0.9	0.3	8.2	2.1
OS04T0107600-01	Os04g0107600	3.23	4.42E-03	Up	9.4	0.2	0.0	1.5	0.8
OS04T0399800-00	Os04g0399800	3.22	3.16E-02	Up	9.3	0.0	0.0	0.2	0.1
OS01T0549300-01	Os01g0549300	3.21	3.16E-04	Up	9.2	0.0	0.0	0.2	0.0
OS04T0532400-01	Os04g0532400	3.18	2.77E-02	Up	9.1	0.0	0.0	0.2	0.1
OS04T0308400-00	Os04g0308400	3.18	2.50E-03	Up	9.1	0.8	0.3	7.3	3.1
OS04T0107600-02	Os04g0107600	3.18	2.54E-02	Up	9.0	0.1	0.1	1.3	0.6
OS11T0259700-01	Os11g0259700	3.13	2.35E-02	Up	8.7	0.2	0.1	2.0	0.9
OS10T0116701-00	Os10g0116701	3.12	3.25E-03	Up	8.7	0.1	0.0	0.9	0.3
OS07T0290500-00	Os07g0290500	3.11	4.73E-07	Up	8.6	0.6	0.2	5.5	0.1
OS04T0310200-00	Os04g0310200	3.09	9.38E-03	Up	8.5	0.8	0.3	6.6	2.9
OS01T0647200-01	Os01g0647200	3.09	2.03E-04	Up	8.5	0.1	0.0	0.6	0.2
OS04T0310100-01	Os04g0310100	3.06	1.99E-02	Up	8.3	0.9	0.4	7.8	3.4
OS01T0495701-00	Os01g0495701	3.05	2.44E-03	Up	8.3	3.8	1.0	31.7	11.7
OS06T0521500-01	Os06g0521500	3.05	9.79E-07	Up	8.3	0.2	0.0	1.8	0.5

OS07T0432333-01	Os07g0432333	3.05	9.78E-03	Up	8.3	43.5	11.1	358.1	143.2
OS10T0195250-01	Os10g0195250	3.01	4.53E-03	Up	8.1	22.4	7.7	182.3	65.7
OS06T0294950-01	Os06g0294950	3.01	1.11E-02	Up	8.0	0.8	0.3	6.9	2.5
OS01T0608000-01	Os01g0608000	3.00	4.19E-03	Up	8.0	3.6	1.1	28.9	10.4
OS01T0608101-00	Os01g0608101	2.99	3.43E-03	Up	7.9	3.5	1.1	28.0	10.1
OS08T0151900-00	Os08g0151900	2.96	1.77E-06	Up	7.8	0.1	0.0	1.1	0.1
OS12T0548501-01	Os12g0548501	2.95	7.88E-04	Up	7.7	3.3	1.3	26.3	7.5
OS06T0521500-02	Os06g0521500	2.95	7.92E-06	Up	7.7	0.2	0.0	1.5	0.5
OS09T0457100-01	Os09g0457100	2.94	3.40E-02	Up	7.7	0.1	0.0	0.6	0.3
OS11T0262600-00	Os11g0262600	2.94	8.36E-04	Up	7.7	6.2	1.0	47.8	17.3
OS06T0177600-00	Os06g0177600	2.94	3.84E-02	Up	7.7	0.1	0.0	0.6	0.3
OS11T0553500-00	Os11g0553500	2.91	3.02E-03	Up	7.5	0.1	0.0	0.8	0.3
OS01T0952900-01	Os01g0952900	2.89	6.98E-03	Up	7.4	0.7	0.2	5.6	2.0
OS01T0227350-00	Os01g0227350	2.89	1.34E-02	Up	7.4	0.0	0.0	0.1	0.0
OS01T0566500-01	Os01g0566500	2.89	1.22E-15	Up	7.4	1.0	0.1	7.4	0.7
OS03T0340300-01	Os03g0340300	2.89	2.86E-05	Up	7.4	0.1	0.0	0.8	0.2
OS06T0336150-01	Os06g0336150	2.88	2.53E-03	Up	7.4	0.4	0.2	2.9	0.8
OS07T0258501-00	Os07g0258501	2.88	1.50E-02	Up	7.4	1.3	0.5	9.9	3.4
OS08T0414500-00	Os08g0414500	2.87	2.21E-02	Up	7.3	0.1	0.1	1.3	0.4
OS02T0731900-01	Os02g0731900	2.87	5.01E-05	Up	7.3	0.1	0.0	1.0	0.3
OS08T0168700-00	Os08g0168700	2.84	1.08E-03	Up	7.2	0.2	0.1	1.7	0.4
OS08T0178900-00	Os08g0178900	2.84	5.54E-06	Up	7.1	0.0	0.0	0.3	0.0
OS08T0254300-00	Os08g0254300	2.83	1.08E-02	Up	7.1	0.5	0.2	3.7	1.2
OS11T0549615-01	Os11g0549615	2.83	1.19E-05	Up	7.1	0.2	0.0	1.2	0.3
OS03T0409400-00	Os03g0409400	2.82	2.84E-02	Up	7.1	0.4	0.1	2.8	1.4
OS01T0138800-01	Os01g0138800	2.82	2.83E-05	Up	7.0	2.2	0.5	15.7	6.3
OS06T0215500-01	Os06g0215500	2.81	2.58E-03	Up	7.0	0.0	0.0	0.2	0.0
OS01T0813800-02	Os01g0813800	2.80	2.32E-04	Up	7.0	0.1	0.0	0.6	0.1
OS02T0270800-00	Os02g0270800	2.79	6.86E-03	Up	6.9	0.1	0.0	0.5	0.2
OS05T0448300-01	Os05g0448300	2.74	8.41E-03	Up	6.7	0.0	0.0	0.1	0.0
OS10T0379854-00	Os10g0379854	2.74	1.72E-04	Up	6.7	0.1	0.0	0.5	0.1
OS02T0649900-00	Os02g0649900	2.74	6.89E-03	Up	6.7	17.4	4.2	116.5	42.5
OS01T0206300-00	Os01g0206300	2.70	2.89E-02	Up	6.5	0.0	0.0	0.1	0.0
OS02T0320800-01	Os02g0320800	2.70	7.69E-03	Up	6.5	0.0	0.0	0.2	0.0
OS07T0258400-01	Os07g0258400	2.68	2.42E-03	Up	6.4	6.2	1.3	40.0	14.1
OS01T0444100-00	Os01g0444100	2.68	4.26E-02	Up	6.4	17.6	5.6	114.0	43.0
OS04T0688500-01	Os04g0688500	2.67	9.48E-03	Up	6.3	0.1	0.0	0.6	0.2
OS03T0658800-02	Os03g0658800	2.66	7.69E-04	Up	6.3	0.1	0.0	0.5	0.0
OS06T0208951-00	Os06g0208951	2.66	3.81E-05	Up	6.3	0.1	0.0	0.4	0.0
OS01T0813800-01	Os01g0813800	2.66	1.36E-08	Up	6.3	0.1	0.0	0.5	0.0
OS07T0258400-02	Os07g0258400	2.65	1.68E-03	Up	6.3	5.8	1.2	36.8	12.6
OS05T0510600-01	Os05g0510600	2.65	5.30E-04	Up	6.3	0.0	0.0	0.3	0.1
OS09T0518000-00	Os09g0518000	2.62	4.41E-02	Up	6.2	0.1	0.0	0.4	0.2
OS03T0431600-00	Os03g0431600	2.62	5.32E-03	Up	6.2	2.5	0.6	15.3	5.4
OS12T0154700-01	Os12g0154700	2.61	6.71E-07	Up	6.1	10.2	1.9	63.0	16.3
OS02T0270200-01	Os02g0270200	2.61	8.29E-05	Up	6.1	0.5	0.1	3.2	1.2
OS01T0192900-00	Os01g0192900	2.60	1.90E-08	Up	6.1	0.4	0.1	2.7	0.2
OS08T0138100-00	Os08g0138100	2.60	1.45E-05	Up	6.1	1.5	0.3	9.1	2.6
OS11T0594000-01	Os11g0594000	2.60	2.43E-04	Up	6.0	0.2	0.1	1.4	0.3
OS11T0282700-01	Os11g0282700	2.58	1.52E-04	Up	6.0	0.1	0.0	0.4	0.1
OS10T0534700-01	Os10g0534700	2.55	3.59E-02	Up	5.9	0.0	0.0	0.1	0.0
OS03T0296600-01	Os03g0296600	2.54	3.81E-03	Up	5.8	0.1	0.0	0.5	0.0
OS01T0860601-01	Os01g0860601	2.54	1.23E-04	Up	5.8	2.1	0.7	12.5	2.1
OS05T0179950-00	Os05g0179950	2.54	1.47E-04	Up	5.8	0.1	0.0	0.3	0.0
OS03T0609500-01	Os03g0609500	2.52	6.40E-03	Up	5.8	1.0	0.4	5.8	1.2
OS01T0910450-00	Os01g0910450	2.51	3.22E-02	Up	5.7	0.1	0.0	0.3	0.1
OS05T0219900-01	Os05g0219900	2.51	6.67E-04	Up	5.7	1.3	0.3	7.4	2.8
OS02T0178100-01	Os02g0178100	2.48	9.17E-06	Up	5.6	0.3	0.1	1.7	0.1
OS03T0577300-00	Os03g0577300	2.48	3.47E-02	Up	5.6	0.1	0.0	0.5	0.1
OS09T0116000-00	Os09g0116000	2.48	2.72E-02	Up	5.6	0.1	0.0	0.4	0.0
OS01T0773800-00	Os01g0773800	2.47	1.84E-05	Up	5.6	0.2	0.0	1.2	0.2
OS06T0677000-00	Os06g0677000	2.47	9.33E-03	Up	5.5	0.2	0.0	0.9	0.3
OS10T0116650-00	Os10g0116650	2.47	2.94E-02	Up	5.5	0.2	0.1	1.0	0.3
OS06T0518100-01	Os06g0518100	2.46	1.54E-02	Up	5.5	0.1	0.0	0.5	0.2
OS11T0553666-00	Os11g0553666	2.46	7.07E-03	Up	5.5	0.1	0.0	0.7	0.3
OS08T0106000-00	Os08g0106000	2.45	2.02E-05	Up	5.5	0.5	0.1	2.6	0.5
OS08T0431700-01	Os08g0431700	2.45	1.26E-04	Up	5.5	5.5	0.8	30.5	13.1
OS12T0154800-00	Os12g0154800	2.44	7.42E-11	Up	5.4	4.9	0.5	26.7	3.7
OS11T0291900-01	Os11g0291900	2.44	9.75E-03	Up	5.4	0.1	0.0	0.6	0.1

OS03T0197300-01	Os03g0197300	2.44	1.97E-02	Up	5.4	0.0	0.0	0.2	0.0
OS07T0129700-01	Os07g0129700	2.43	5.72E-06	Up	5.4	0.3	0.0	1.4	0.2
OS10T0527601-00	Os10g0527601	2.42	5.04E-04	Up	5.4	10.2	2.6	55.3	14.9
OS01T0515300-01	Os01g0515300	2.42	2.77E-05	Up	5.3	0.1	0.0	0.5	0.1
OS12T0503000-01	Os12g0503000	2.42	2.77E-08	Up	5.3	4.5	1.1	24.4	1.5
OS04T0469300-01	Os04g0469300	2.41	1.15E-02	Up	5.3	0.1	0.0	0.4	0.0
OS12T0503000-05	Os12g0503000	2.41	3.55E-07	Up	5.3	3.2	0.8	17.3	1.0
OS10T0116600-00	Os10g0116600	2.39	5.68E-03	Up	5.3	0.4	0.0	2.0	0.6
OS01T0606200-00	Os01g0606200	2.39	3.77E-02	Up	5.2	0.1	0.1	0.7	0.1
OS07T0588400-00	Os07g0588400	2.39	7.89E-08	Up	5.2	0.6	0.1	3.1	0.3
OS10T0380100-01	Os10g0380100	2.38	1.61E-03	Up	5.2	0.2	0.1	1.0	0.1
OS03T0658800-01	Os03g0658800	2.38	5.39E-10	Up	5.2	0.6	0.0	3.0	0.4
OS12T0503000-04	Os12g0503000	2.37	1.09E-06	Up	5.2	2.4	0.6	12.8	0.8
OS03T0658800-03	Os03g0658800	2.37	9.04E-09	Up	5.2	0.6	0.1	3.2	0.4
OS06T0305400-01	Os06g0305400	2.36	6.83E-03	Up	5.1	0.1	0.0	0.3	0.1
OS01T0124401-01	Os01g0124401	2.36	3.74E-07	Up	5.1	12.4	1.6	64.0	15.7
OS03T0559900-01	Os03g0559900	2.35	1.53E-04	Up	5.1	1.1	0.0	5.9	2.3
OS03T0237201-00	Os03g0237201	2.34	2.99E-02	Up	5.1	2.7	0.8	13.8	4.8
OS03T0117100-01	Os03g0117100	2.33	1.31E-06	Up	5.0	0.9	0.2	4.4	0.3
OS12T0503000-03	Os12g0503000	2.33	9.38E-08	Up	5.0	5.0	1.2	25.5	2.1
OS05T0206800-00	Os05g0206800	2.33	4.34E-02	Up	5.0	0.0	0.0	0.2	0.1
OS01T0860450-01	Os01g0860450	2.33	6.16E-10	Up	5.0	1.2	0.2	6.2	0.6
OS12T0154900-00	Os12g0154900	2.33	1.34E-06	Up	5.0	3.8	0.5	19.3	4.2
OS04T0482100-00	Os04g0482100	2.32	3.06E-06	Up	5.0	1.0	0.2	5.1	0.3
OS01T0891700-01	Os01g0891700	2.32	1.57E-03	Up	5.0	0.4	0.1	1.8	0.5
OS12T0155000-00	Os12g0155000	2.31	5.71E-06	Up	5.0	3.0	0.3	14.7	4.0
OS06T0331900-01	Os06g0331900	2.31	1.53E-02	Up	5.0	0.4	0.1	2.2	0.9
OS05T0478800-00	Os05g0478800	2.28	1.08E-02	Up	4.9	0.1	0.0	0.6	0.1
OS08T0189100-01	Os08g0189100	2.27	3.63E-04	Up	4.8	5.4	1.5	26.9	8.6
OS07T0417600-01	Os07g0417600	2.27	1.71E-03	Up	4.8	0.1	0.0	0.5	0.1
OS12T0548700-01	Os12g0548700	2.26	2.95E-03	Up	4.8	21.7	9.2	106.4	20.2
OS02T0179200-01	Os02g0179200	2.26	1.92E-02	Up	4.8	2.1	0.5	10.2	4.8
OS03T0117100-02	Os03g0117100	2.26	1.07E-04	Up	4.8	1.0	0.3	4.7	0.4
OS12T0548401-01	Os12g0548401	2.26	1.00E-03	Up	4.8	22.6	7.8	110.4	26.8
OS06T0668400-01	Os06g0668400	2.26	5.38E-06	Up	4.8	0.3	0.0	1.7	0.3
OS03T0559900-02	Os03g0559900	2.24	3.97E-03	Up	4.7	0.7	0.0	3.3	1.4
OS06T0278150-00	Os06g0278150	2.23	1.28E-04	Up	4.7	0.2	0.0	1.1	0.2
OS10T0554200-02	Os10g0554200	2.22	1.77E-02	Up	4.6	2.1	0.8	10.0	3.1
OS03T0709300-01	Os03g0709300	2.21	1.37E-09	Up	4.6	2.9	0.3	13.5	1.0
OS06T0278000-00	Os06g0278000	2.20	3.60E-06	Up	4.6	0.3	0.0	1.6	0.2
OS04T0618800-00	Os04g0618800	2.20	1.24E-04	Up	4.6	0.4	0.0	1.9	0.6
OS07T0127500-01	Os07g0127500	2.20	2.11E-03	Up	4.6	0.7	0.2	3.4	0.9
OS04T0320700-02	Os04g0320700	2.19	1.37E-04	Up	4.6	0.3	0.1	1.3	0.2
OS08T0231400-01	Os08g0231400	2.19	3.74E-06	Up	4.5	14.5	1.9	66.0	16.8
OS06T0209100-00	Os06g0209100	2.18	3.86E-04	Up	4.5	0.1	0.0	0.5	0.1
OS01T0860500-02	Os01g0860500	2.17	8.94E-09	Up	4.5	2.1	0.3	9.5	0.2
OS02T0270350-00	Os02g0270350	2.17	2.52E-02	Up	4.5	0.3	0.1	1.4	0.5
OS08T0138200-00	Os08g0138200	2.16	8.15E-03	Up	4.5	0.2	0.0	0.8	0.2
OS08T0323400-01	Os08g0323400	2.16	2.13E-02	Up	4.5	0.2	0.0	0.8	0.2
OS03T0237100-01	Os03g0237100	2.14	4.54E-02	Up	4.4	13.0	3.8	58.2	20.2
OS08T0376550-00	Os08g0376550	2.14	2.67E-02	Up	4.4	0.6	0.2	2.7	0.7
OS07T0677400-00	Os07g0677400	2.14	5.05E-04	Up	4.4	0.2	0.0	0.8	0.2
OS11T0709000-00	Os11g0709000	2.13	4.38E-02	Up	4.4	0.1	0.0	0.6	0.2
OS10T0554200-01	Os10g0554200	2.13	1.48E-03	Up	4.4	2.7	0.8	12.1	3.0
OS04T0618700-01	Os04g0618700	2.12	1.11E-04	Up	4.3	0.5	0.0	2.0	0.6
OS04T0423400-01	Os04g0423400	2.12	5.22E-04	Up	4.3	8.6	2.6	37.9	4.6
OS08T0114400-01	Os08g0114400	2.12	1.57E-04	Up	4.3	3.4	1.1	14.9	2.1
OS01T0124100-01	Os01g0124100	2.10	4.71E-05	Up	4.3	0.5	0.1	2.3	0.4
OS05T0135450-00	Os05g0135450	2.09	5.43E-03	Up	4.2	7.4	2.2	31.9	7.5
OS02T0535901-00	Os02g0535901	2.08	1.29E-02	Up	4.2	0.4	0.2	1.7	0.1
OS01T0248300-00	Os01g0248300	2.08	5.99E-07	Up	4.2	8.4	1.6	36.2	4.1
OS02T0107800-00	Os02g0107800	2.07	1.68E-02	Up	4.2	0.3	0.1	1.1	0.2
OS05T0196600-00	Os05g0196600	2.07	1.05E-02	Up	4.2	0.1	0.0	0.5	0.1
OS07T0115500-01	Os07g0115500	2.06	1.54E-09	Up	4.2	6.7	0.9	28.4	0.5
OS08T0114300-01	Os08g0114300	2.06	3.70E-04	Up	4.2	3.2	1.1	13.5	1.8
OS03T0660050-00	Os03g0660050	2.04	4.61E-02	Up	4.1	0.1	0.0	0.5	0.1
OS08T0376500-00	Os08g0376500	2.04	2.47E-04	Up	4.1	1.4	0.2	5.7	1.2
OS05T0417700-01	Os05g0417700	2.03	1.29E-02	Up	4.1	2.9	0.6	12.0	4.7
OS01T0963000-04	Os01g0963000	2.03	3.95E-07	Up	4.1	1.4	0.2	5.9	0.2

OS12T0640950-00	Os12g0640950	2.02	1.44E-05	Up	4.1	3.7	0.2	15.0	3.0
OS11T0664800-00	Os11g0664800	2.02	3.61E-02	Up	4.1	4.9	0.8	20.0	10.1
OS10T0413700-01	Os10g0413700	2.02	2.33E-02	Up	4.1	0.0	0.0	0.2	0.0
OS03T0680700-00	Os03g0680700	2.02	1.49E-02	Up	4.1	0.1	0.0	0.4	0.0
OS05T0179100-01	Os05g0179100	2.01	4.34E-03	Up	4.0	2.2	0.6	9.1	1.6
OS01T0248350-00	Os01g0248350	2.01	1.28E-04	Up	4.0	2.3	0.5	9.6	1.1
OS11T0113700-02	Os11g0113700	2.01	1.95E-05	Up	4.0	10.6	2.6	43.2	3.5
OS09T0400100-01	Os09g0400100	2.01	6.82E-04	Up	4.0	0.2	0.0	0.7	0.0
OS01T0127600-01	Os01g0127600	2.00	2.75E-02	Up	4.0	1.3	0.5	5.2	1.4
OS02T0174100-03	Os02g0174100	2.00	3.71E-02	Up	4.0	0.1	0.1	0.6	0.1
OS04T0649500-01	Os04g0649500	1.99	9.51E-06	Up	4.0	13.5	3.0	54.3	5.4
OS06T0567900-01	Os06g0567900	1.99	2.89E-02	Up	4.0	0.9	0.2	3.5	1.4
OS11T0113700-01	Os11g0113700	1.98	1.23E-05	Up	3.9	11.3	2.6	45.0	4.1
OS04T0423400-02	Os04g0423400	1.98	2.11E-03	Up	3.9	13.0	4.1	51.7	6.9
OS12T0573600-01	Os12g0573600	1.98	3.50E-02	Up	3.9	1.3	0.2	5.3	2.2
OS01T0892800-01	Os01g0892800	1.97	5.44E-04	Up	3.9	1.0	0.1	4.2	1.2
OS04T0419750-00	Os04g0419750	1.97	1.15E-02	Up	3.9	0.6	0.0	2.2	0.9
OS04T0347200-01	Os04g0347200	1.95	1.58E-04	Up	3.9	0.9	0.1	3.4	0.5
OS04T0419700-00	Os04g0419700	1.95	1.35E-02	Up	3.9	0.7	0.1	2.6	1.0
OS02T0220000-00	Os02g0220000	1.95	2.69E-04	Up	3.9	0.4	0.1	1.5	0.1
OS05T0399300-01	Os05g0399300	1.95	8.40E-04	Up	3.9	35.7	10.5	139.5	20.2
OS12T0268100-01	Os12g0268100	1.95	9.75E-03	Up	3.9	0.3	0.1	1.1	0.2
OS12T0236100-00	Os12g0236100	1.94	4.78E-04	Up	3.8	11.5	0.8	44.4	12.2
OS09T0400000-00	Os09g0400000	1.94	1.04E-03	Up	3.8	0.2	0.1	1.0	0.1
OS04T0320700-01	Os04g0320700	1.94	3.22E-03	Up	3.8	0.3	0.1	1.2	0.2
OS06T0212600-00	Os06g0212600	1.94	2.72E-02	Up	3.8	0.1	0.0	0.4	0.1
OS03T0693900-00	Os03g0693900	1.94	4.65E-03	Up	3.8	5.2	1.1	20.1	7.1
OS01T0963000-01	Os01g0963000	1.94	7.43E-05	Up	3.8	1.4	0.3	5.5	0.2
OS04T0597600-01	Os04g0597600	1.93	3.73E-03	Up	3.8	0.1	0.0	0.4	0.1
OS04T0584100-01	Os04g0584100	1.93	4.20E-03	Up	3.8	0.4	0.1	1.6	0.3
OS03T0807600-00	Os03g0807600	1.92	4.84E-02	Up	3.8	0.1	0.0	0.4	0.1
OS07T0677350-01	Os07g0677350	1.92	3.60E-02	Up	3.8	0.2	0.1	0.7	0.1
OS06T0642600-00	Os06g0642600	1.92	2.58E-02	Up	3.8	0.1	0.0	0.3	0.0
OS12T0113500-02	Os12g0113500	1.90	2.76E-05	Up	3.7	11.7	2.5	44.1	6.9
OS01T0124200-02	Os01g0124200	1.90	2.15E-06	Up	3.7	26.3	4.3	99.0	17.7
OS03T0694000-01	Os03g0694000	1.90	1.80E-02	Up	3.7	2.8	0.7	10.6	3.7
OS04T0689300-01	Os04g0689300	1.89	3.04E-02	Up	3.7	0.1	0.0	0.2	0.0
OS01T0892800-02	Os01g0892800	1.89	6.00E-04	Up	3.7	1.9	0.2	7.0	2.1
OS07T0227300-00	Os07g0227300	1.88	6.02E-03	Up	3.7	0.1	0.0	0.3	0.0
OS08T0137800-01	Os08g0137800	1.88	3.38E-05	Up	3.7	2.6	0.4	9.8	1.4
OS11T0557500-00	Os11g0557500	1.87	6.46E-04	Up	3.7	0.2	0.0	0.8	0.1
OS03T0643000-00	Os03g0643000	1.87	1.77E-02	Up	3.7	0.2	0.0	0.6	0.1
OS02T0719600-01	Os02g0719600	1.87	1.95E-02	Up	3.7	1.8	0.2	6.6	2.6
OS12T0113500-01	Os12g0113500	1.87	2.67E-05	Up	3.7	12.6	2.7	46.6	6.8
OS06T0109000-00	Os06g0109000	1.87	3.79E-02	Up	3.7	1.4	0.4	5.0	0.7
OS06T0199402-00	Os06g0199402	1.86	4.76E-03	Up	3.6	0.3	0.0	1.3	0.1
OS03T0734000-01	Os03g0734000	1.86	4.80E-03	Up	3.6	12.2	2.7	44.5	11.4
OS07T0664000-00	Os07g0664000	1.85	2.45E-02	Up	3.6	0.5	0.1	1.7	0.3
OS09T0439200-01	Os09g0439200	1.85	2.24E-03	Up	3.6	5.2	0.7	19.2	6.7
OS10T0416800-03	Os10g0416800	1.84	5.96E-04	Up	3.6	8.6	2.8	31.4	1.2
OS07T0537701-01	Os07g0537701	1.84	1.11E-02	Up	3.6	0.1	0.0	0.4	0.0
OS11T0181900-01	Os11g0181900	1.84	1.16E-04	Up	3.6	0.5	0.0	1.9	0.3
OS08T0296600-01	Os08g0296600	1.83	4.22E-04	Up	3.6	1.7	0.1	6.3	1.9
OS08T0540400-01	Os08g0540400	1.83	3.58E-02	Up	3.5	5.5	0.9	19.9	9.0
OS03T0718800-01	Os03g0718800	1.81	3.38E-05	Up	3.5	2.8	0.2	9.7	1.4
OS06T0356800-01	Os06g0356800	1.80	6.13E-04	Up	3.5	1.0	0.2	3.7	0.6
OS06T0645650-00	Os06g0645650	1.80	1.73E-02	Up	3.5	0.1	0.0	0.3	0.0
OS01T0141450-00	Os01g0141450	1.79	3.29E-02	Up	3.5	0.3	0.0	1.0	0.3
OS04T0104900-01	Os04g0104900	1.79	7.19E-04	Up	3.5	0.5	0.1	1.6	0.2
OS04T0415100-01	Os04g0415100	1.79	4.77E-03	Up	3.5	0.3	0.1	1.2	0.2
OS09T0543400-00	Os09g0543400	1.79	3.53E-03	Up	3.4	1.6	0.4	5.6	0.6
OS03T0216500-00	Os03g0216500	1.78	2.42E-03	Up	3.4	0.4	0.1	1.5	0.3
OS07T0538000-01	Os07g0538000	1.77	3.89E-02	Up	3.4	0.9	0.1	3.0	0.9
OS09T0518266-00	Os09g0518266	1.77	1.57E-03	Up	3.4	8.3	2.1	28.7	4.5
OS07T0241866-00	Os07g0241866	1.77	1.84E-02	Up	3.4	0.3	0.1	1.0	0.2
OS07T0586200-02	Os07g0586200	1.76	9.91E-03	Up	3.4	0.9	0.2	3.2	0.6
OS06T0711000-01	Os06g0711000	1.76	4.80E-03	Up	3.4	2.2	0.5	7.8	1.8
OS03T0734100-02	Os03g0734100	1.76	8.63E-03	Up	3.4	65.8	16.0	224.9	55.2
OS08T0519100-02	Os08g0519100	1.76	1.46E-02	Up	3.4	0.4	0.1	1.5	0.2

OS10T0550900-01	Os10g0550900	1.75	4.11E-02	Up	3.4	1.3	0.3	4.5	1.7
OS09T0399800-01	Os09g0399800	1.75	2.11E-03	Up	3.4	11.1	3.3	37.9	5.2
OS06T0294775-00	Os06g0294775	1.74	7.96E-04	Up	3.3	0.4	0.0	1.5	0.2
OS01T0849000-02	Os01g0849000	1.74	4.11E-02	Up	3.3	1.0	0.1	3.3	1.2
OS04T0415800-00	Os04g0415800	1.74	3.24E-03	Up	3.3	2.4	0.3	8.1	2.0
OS07T0586200-01	Os07g0586200	1.74	1.90E-02	Up	3.3	0.7	0.1	2.2	0.5
OS02T0589450-00	Os02g0589450	1.74	4.87E-02	Up	3.3	1.0	0.3	3.5	0.6
OS05T0402900-01	Os05g0402900	1.73	3.06E-04	Up	3.3	4.3	0.5	14.6	3.7
OS11T0701100-02	Os11g0701100	1.73	3.18E-02	Up	3.3	1.5	0.1	4.9	1.4
OS04T0568950-00	Os04g0568950	1.73	1.97E-02	Up	3.3	0.9	0.2	3.3	0.6
OS12T0227500-00	Os12g0227500	1.73	4.24E-02	Up	3.3	4.5	0.6	15.3	6.4
OS06T0693600-00	Os06g0693600	1.72	4.99E-08	Up	3.3	5.5	0.4	18.3	1.2
OS09T0547500-01	Os09g0547500	1.72	4.03E-03	Up	3.3	0.6	0.0	1.9	0.4
OS03T0738600-01	Os03g0738600	1.72	4.93E-05	Up	3.3	17.1	3.0	57.2	9.3
OS09T0400200-01	Os09g0400200	1.72	1.11E-02	Up	3.3	0.1	0.0	0.4	0.0
OS03T0287750-00	Os03g0287750	1.71	1.53E-02	Up	3.3	1.0	0.3	3.5	0.3
OS10T0339650-01	Os10g0339650	1.71	2.80E-02	Up	3.3	0.3	0.1	0.9	0.1
OS04T0584201-01	Os04g0584201	1.70	1.04E-02	Up	3.3	0.8	0.1	2.7	0.6
OS03T0838800-00	Os03g0838800	1.70	6.66E-03	Up	3.3	0.7	0.1	2.4	0.5
OS03T0199300-01	Os03g0199300	1.70	1.67E-02	Up	3.3	0.1	0.0	0.5	0.1
OS09T0441000-00	Os09g0441000	1.69	3.31E-02	Up	3.2	0.4	0.1	1.2	0.2
OS12T0553800-00	Os12g0553800	1.69	4.30E-02	Up	3.2	0.2	0.1	0.7	0.0
OS12T0555300-00	Os12g0555300	1.69	3.77E-02	Up	3.2	1.7	0.5	5.7	1.1
OS05T0135400-01	Os05g0135400	1.68	8.34E-04	Up	3.2	13.4	2.1	43.5	9.4
OS11T0701100-01	Os11g0701100	1.68	1.06E-02	Up	3.2	1.3	0.1	4.1	1.0
OS01T0314800-01	Os01g0314800	1.68	4.07E-03	Up	3.2	23.2	1.7	75.7	26.4
OS03T0291500-01	Os03g0291500	1.68	3.80E-02	Up	3.2	40.0	12.2	129.3	25.2
OS02T0765925-00	Os02g0765925	1.67	4.05E-02	Up	3.2	1.0	0.3	3.3	0.5
OS04T0639000-01	Os04g0639000	1.67	4.84E-02	Up	3.2	4.1	0.4	13.4	5.1
OS02T0151350-00	Os02g0151350	1.67	4.83E-03	Up	3.2	0.7	0.2	2.2	0.1
OS10T0545500-00	Os10g0545500	1.67	2.09E-03	Up	3.2	1.3	0.3	4.3	0.5
OS02T0181200-00	Os02g0181200	1.67	1.44E-02	Up	3.2	3.7	1.0	11.9	1.7
OS09T0484900-01	Os09g0484900	1.67	6.29E-03	Up	3.2	0.8	0.2	2.7	0.2
OS02T0829000-01	Os02g0829000	1.67	3.16E-02	Up	3.2	1.7	0.1	5.4	2.1
OS08T0359200-01	Os08g0359200	1.67	3.10E-02	Up	3.2	2.9	0.1	9.3	3.5
OS01T0207600-00	Os01g0207600	1.66	2.22E-02	Up	3.2	0.1	0.0	0.3	0.1
OS03T0672900-00	Os03g0672900	1.66	3.84E-02	Up	3.2	0.3	0.1	0.8	0.1
OS03T0418000-00	Os03g0418000	1.66	1.97E-04	Up	3.2	0.7	0.1	2.1	0.1
OS08T0489100-00	Os08g0489100	1.66	2.40E-02	Up	3.2	0.2	0.1	0.7	0.1
OS09T0400300-01	Os09g0400300	1.65	2.51E-02	Up	3.1	0.2	0.1	0.6	0.0
OS04T0532100-01	Os04g0532100	1.65	3.41E-06	Up	3.1	12.3	1.8	39.2	4.0
OS02T0185200-01	Os02g0185200	1.65	3.51E-04	Up	3.1	2.6	0.3	8.3	1.8
OS11T0209900-00	Os11g0209900	1.65	3.71E-02	Up	3.1	0.1	0.0	0.4	0.1
OS03T0734100-01	Os03g0734100	1.64	1.60E-02	Up	3.1	83.8	19.1	263.0	65.6
OS07T0636800-01	Os07g0636800	1.64	6.06E-04	Up	3.1	8.0	1.6	25.3	4.1
OS03T0854250-00	Os03g0854250	1.64	1.54E-03	Up	3.1	0.5	0.0	1.6	0.0
OS02T0834300-01	Os02g0834300	1.64	1.07E-02	Up	3.1	0.7	0.2	2.3	0.3
OS07T0461950-00	Os07g0461950	1.63	1.25E-03	Up	3.1	2.0	0.2	6.5	0.8
OS09T0485050-00	Os09g0485050	1.63	4.24E-03	Up	3.1	0.2	0.0	0.7	0.1
OS06T0697000-02	Os06g0697000	1.62	4.03E-02	Up	3.1	23.2	6.6	72.2	25.0
OS04T0306400-01	Os04g0306400	1.61	2.53E-03	Up	3.1	11.2	2.9	34.9	4.7
OS10T0191300-01	Os10g0191300	1.61	2.92E-02	Up	3.0	596.8	181.9	1836.4	262.6
OS09T0518200-01	Os09g0518200	1.60	7.08E-04	Up	3.0	17.3	3.8	53.4	7.7
OS09T0486900-00	Os09g0486900	1.60	1.05E-02	Up	3.0	0.3	0.1	1.0	0.1
OS03T0819600-01	Os03g0819600	1.60	2.97E-02	Up	3.0	7.6	2.7	23.5	2.8
OS10T0415100-01	Os10g0415100	1.60	2.90E-02	Up	3.0	1.1	0.3	3.5	0.5
OS03T0819600-02	Os03g0819600	1.59	2.62E-02	Up	3.0	7.0	2.4	21.6	2.7
OS03T0712800-02	Os03g0712800	1.59	1.49E-03	Up	3.0	6.1	0.2	18.6	5.3
OS02T0136000-00	Os02g0136000	1.59	1.06E-03	Up	3.0	2.5	0.5	7.7	0.8
OS06T0294600-02	Os06g0294600	1.59	1.11E-02	Up	3.0	0.7	0.1	2.2	0.4
OS10T0478300-01	Os10g0478300	1.59	1.98E-02	Up	3.0	3.4	0.9	10.3	0.6
OS06T0604300-01	Os06g0604300	1.59	1.36E-03	Up	3.0	2.3	0.1	7.0	1.6
OS12T0614600-01	Os12g0614600	1.58	3.55E-02	Up	3.0	0.1	0.0	0.4	0.1
OS01T0915666-00	Os01g0915666	1.58	7.75E-04	Up	3.0	2.5	0.4	7.6	0.6
OS06T0604200-02	Os06g0604200	1.58	7.06E-03	Up	3.0	2.6	0.1	8.0	2.6
OS12T0170800-01	Os12g0170800	1.58	2.26E-04	Up	3.0	7.4	1.3	22.3	2.4
OS07T0412100-01	Os07g0412100	1.58	2.13E-02	Up	3.0	20.2	4.7	60.8	18.3
OS12T0611300-00	Os12g0611300	1.57	2.08E-02	Up	3.0	0.2	0.1	0.7	0.1
OS07T0676600-01	Os07g0676600	1.57	3.76E-02	Up	3.0	28.9	1.7	87.7	36.7

OS12T0625200-02	Os12g0625200	1.57	4.07E-06	Up	3.0	1.9	0.1	5.7	0.5
OS02T0271000-01	Os02g0271000	1.56	1.57E-03	Up	3.0	0.2	0.0	0.7	0.1
OS03T0376000-01	Os03g0376000	1.56	1.28E-03	Up	2.9	0.6	0.1	1.9	0.2
OS12T0625200-01	Os12g0625200	1.56	1.73E-05	Up	2.9	1.7	0.1	5.0	0.3
OS04T0441700-01	Os04g0441700	1.56	1.51E-02	Up	2.9	0.4	0.1	1.1	0.1
OS10T0527800-01	Os10g0527800	1.55	1.73E-03	Up	2.9	123.1	13.1	363.6	81.7
OS12T0135800-01	Os12g0135800	1.55	4.84E-02	Up	2.9	0.5	0.1	1.5	0.2
OS12T0630500-01	Os12g0630500	1.55	2.10E-02	Up	2.9	9.2	2.7	27.6	4.8
OS06T0264432-00	Os06g0264432	1.55	1.36E-02	Up	2.9	1.6	0.4	4.9	0.5
OS10T0361900-00	Os10g0361900	1.55	1.55E-04	Up	2.9	2.5	0.2	7.3	0.5
OS02T0770800-01	Os02g0770800	1.54	1.30E-04	Up	2.9	3.9	0.6	11.5	1.7
OS10T0478350-00	Os10g0478350	1.54	3.22E-02	Up	2.9	0.7	0.2	2.1	0.1
OS06T0199400-00	Os06g0199400	1.54	2.49E-02	Up	2.9	1.5	0.5	4.6	0.1
OS03T0817200-01	Os03g0817200	1.54	3.96E-02	Up	2.9	2.1	0.3	6.3	2.1
OS07T0170000-00	Os07g0170000	1.54	5.52E-04	Up	2.9	1.1	0.1	3.2	0.2
OS01T0910500-01	Os01g0910500	1.54	4.77E-03	Up	2.9	1.1	0.2	3.1	0.1
OS04T0631200-01	Os04g0631200	1.53	3.27E-02	Up	2.9	0.6	0.0	1.9	0.5
OS06T0522300-01	Os06g0522300	1.53	1.76E-03	Up	2.9	3.5	0.5	10.2	1.5
OS12T0438600-01	Os12g0438600	1.53	5.14E-03	Up	2.9	9.7	1.5	28.0	6.5
OS02T0246900-01	Os02g0246900	1.52	3.94E-02	Up	2.9	0.7	0.1	1.9	0.5
OS06T0241200-01	Os06g0241200	1.52	4.25E-02	Up	2.9	0.1	0.0	0.4	0.1
OS01T0205600-01	Os01g0205600	1.51	2.89E-02	Up	2.9	0.5	0.1	1.5	0.2
OS11T0151200-01	Os11g0151200	1.51	2.87E-03	Up	2.8	1.9	0.4	5.4	0.1
OS09T0451133-00	Os09g0451133	1.51	3.01E-02	Up	2.8	1.2	0.1	3.4	0.9
OS08T0482600-01	Os08g0482600	1.51	1.81E-02	Up	2.8	4.8	1.0	13.9	2.7
OS02T0131850-00	Os02g0131850	1.50	1.58E-04	Up	2.8	5.0	0.7	14.2	0.3
OS11T0438700-01	Os11g0438700	1.50	3.45E-03	Up	2.8	1.3	0.2	3.9	0.6
OS01T0141300-00	Os01g0141300	1.49	2.21E-02	Up	2.8	1.9	0.2	5.5	1.3
OS10T0146901-00	Os10g0146901	1.49	4.67E-02	Up	2.8	0.7	0.2	1.9	0.3
OS01T0557100-01	Os01g0557100	1.48	2.98E-05	Up	2.8	9.9	1.3	27.8	2.1
OS12T0260500-01	Os12g0260500	1.48	3.70E-03	Up	2.8	3.6	0.7	10.2	0.9
OS11T0438700-02	Os11g0438700	1.48	6.47E-03	Up	2.8	1.1	0.1	3.1	0.6
OS03T0700750-00	Os03g0700750	1.47	7.14E-03	Up	2.8	6.4	0.2	18.0	5.0
OS09T0451000-01	Os09g0451000	1.47	4.93E-02	Up	2.8	2.3	0.4	6.3	1.8
OS11T0275200-01	Os11g0275200	1.47	1.34E-03	Up	2.8	8.9	1.8	25.0	1.5
OS02T0605900-01	Os02g0605900	1.46	1.16E-03	Up	2.7	7.1	1.1	19.7	2.5
OS01T0868000-00	Os01g0868000	1.45	4.98E-02	Up	2.7	0.6	0.1	1.7	0.4
OS01T0723500-00	Os01g0723500	1.45	2.26E-02	Up	2.7	1.2	0.3	3.4	0.4
OS01T0946700-01	Os01g0946700	1.45	2.99E-02	Up	2.7	7.5	1.7	20.5	4.5
OS10T0392400-01	Os10g0392400	1.45	1.17E-02	Up	2.7	9.0	1.1	25.0	6.9
OS09T0481400-01	Os09g0481400	1.44	3.66E-02	Up	2.7	2.9	0.8	7.9	0.4
OS07T0502550-00	Os07g0502550	1.44	1.41E-02	Up	2.7	0.6	0.1	1.7	0.2
OS10T0550500-01	Os10g0550500	1.44	2.19E-02	Up	2.7	0.7	0.1	1.8	0.3
OS09T0540400-02	Os09g0540400	1.44	4.46E-03	Up	2.7	0.6	0.1	1.6	0.1
OS01T0776500-01	Os01g0776500	1.44	2.23E-03	Up	2.7	0.7	0.0	1.8	0.3
OS06T0289900-01	Os06g0289900	1.44	4.10E-02	Up	2.7	0.7	0.1	1.9	0.4
OS04T0639200-01	Os04g0639200	1.43	3.66E-02	Up	2.7	0.8	0.1	2.0	0.4
OS01T0357150-00	Os01g0357150	1.43	5.44E-03	Up	2.7	22.2	4.8	60.5	6.9
OS09T0454600-00	Os09g0454600	1.43	7.63E-04	Up	2.7	5.0	0.6	13.8	2.0
OS11T0696900-01	Os11g0696900	1.42	2.26E-02	Up	2.7	2.0	0.1	5.3	1.5
OS01T0173600-00	Os01g0173600	1.42	4.11E-02	Up	2.7	0.7	0.2	1.9	0.2
OS02T0205700-00	Os02g0205700	1.41	2.55E-02	Up	2.7	4.8	0.7	13.0	3.6
OS03T0794200-00	Os03g0794200	1.41	1.64E-02	Up	2.7	0.6	0.1	1.5	0.1
OS09T0298200-01	Os09g0298200	1.41	2.50E-03	Up	2.7	12.8	1.0	34.5	8.3
OS06T0666600-01	Os06g0666600	1.41	1.16E-03	Up	2.7	2.5	0.4	6.8	0.2
OS01T0357100-02	Os01g0357100	1.40	4.13E-03	Up	2.6	25.9	5.2	69.2	8.3
OS01T0973500-01	Os01g0973500	1.40	4.54E-03	Up	2.6	5.2	0.3	13.8	3.5
OS04T0679050-00	Os04g0679050	1.40	3.11E-02	Up	2.6	0.8	0.1	2.1	0.3
OS09T0130300-01	Os09g0130300	1.40	1.31E-02	Up	2.6	11.7	0.8	31.0	7.2
OS11T0707050-00	Os11g0707050	1.40	3.18E-02	Up	2.6	2.1	0.2	5.7	0.9
OS01T0357100-01	Os01g0357100	1.40	3.85E-03	Up	2.6	21.2	4.2	56.2	6.4
OS05T0427400-00	Os05g0427400	1.39	6.86E-04	Up	2.6	17.1	1.8	45.6	8.9
OS11T0484000-01	Os11g0484000	1.39	1.49E-02	Up	2.6	166.9	25.7	440.9	96.7
OS05T0580000-01	Os05g0580000	1.39	1.13E-02	Up	2.6	16.1	3.3	42.7	8.4
OS09T0298266-00	Os09g0298266	1.39	1.87E-02	Up	2.6	4.4	0.3	11.7	3.1
OS10T0189100-01	Os10g0189100	1.38	1.00E-02	Up	2.6	6.7	1.1	17.7	4.0
OS03T0187800-01	Os03g0187800	1.38	2.59E-02	Up	2.6	3.9	0.5	10.4	2.8
OS09T0506700-00	Os09g0506700	1.37	7.07E-03	Up	2.6	0.5	0.1	1.2	0.1
OS01T0687400-01	Os01g0687400	1.37	3.32E-03	Up	2.6	1.9	0.3	5.1	0.3

OS10T0508700-01	Os10g0508700	1.37	1.05E-02	Up	2.6	1.6	0.2	4.3	0.7
OS05T0431750-00	Os05g0431750	1.37	4.42E-02	Up	2.6	2.0	0.5	5.2	0.1
OS01T0854500-01	Os01g0854500	1.37	4.21E-02	Up	2.6	0.3	0.0	0.9	0.1
OS01T0736000-01	Os01g0736000	1.37	1.09E-02	Up	2.6	0.7	0.1	2.0	0.2
OS03T0431200-00	Os03g0431200	1.37	1.43E-02	Up	2.6	7.7	0.4	20.0	5.9
OS07T0539100-02	Os07g0539100	1.36	2.14E-06	Up	2.6	33.8	1.6	87.6	6.3
OS07T0539100-01	Os07g0539100	1.36	1.36E-06	Up	2.6	31.4	1.0	81.3	5.7
OS01T0168100-02	Os01g0168100	1.36	1.54E-02	Up	2.6	38.4	4.6	100.0	28.1
OS07T0529600-01	Os07g0529600	1.36	1.45E-05	Up	2.6	88.6	6.1	229.1	22.2
OS01T0977100-00	Os01g0977100	1.35	4.98E-02	Up	2.6	0.4	0.0	1.1	0.2
OS12T0582000-00	Os12g0582000	1.35	2.35E-02	Up	2.6	0.4	0.0	1.0	0.1
OS01T0910400-00	Os01g0910400	1.35	1.09E-02	Up	2.5	0.6	0.0	1.4	0.2
OS09T0298200-02	Os09g0298200	1.35	4.29E-03	Up	2.5	13.4	1.2	34.6	8.0
OS09T0403300-00	Os09g0403300	1.35	1.68E-03	Up	2.5	2.0	0.3	5.3	0.3
OS06T0666800-01	Os06g0666800	1.35	2.75E-02	Up	2.5	1.7	0.2	4.4	0.7
OS09T0490400-00	Os09g0490400	1.34	4.99E-02	Up	2.5	0.3	0.1	0.8	0.1
OS04T0465100-01	Os04g0465100	1.34	2.89E-02	Up	2.5	0.7	0.1	1.7	0.2
OS12T0139300-01	Os12g0139300	1.34	1.70E-02	Up	2.5	3.1	0.1	8.1	2.1
OS01T0327100-02	Os01g0327100	1.34	3.25E-03	Up	2.5	3.4	0.6	8.9	0.1
OS04T0422000-03	Os04g0422000	1.34	4.02E-02	Up	2.5	0.3	0.0	0.9	0.1
OS03T0700700-04	Os03g0700700	1.34	1.00E-02	Up	2.5	10.1	0.5	26.0	7.0
OS07T0469100-02	Os07g0469100	1.33	4.99E-02	Up	2.5	0.3	0.0	0.9	0.0
OS07T0448150-00	Os07g0448150	1.33	1.65E-02	Up	2.5	3.6	0.7	9.1	0.8
OS01T0327250-00	Os01g0327250	1.33	3.27E-02	Up	2.5	1.7	0.4	4.3	0.1
OS01T0207400-01	Os01g0207400	1.33	1.83E-02	Up	2.5	2.3	0.3	5.9	0.9
OS04T0535200-02	Os04g0535200	1.33	8.58E-03	Up	2.5	1.1	0.2	2.7	0.3
OS02T0131800-01	Os02g0131800	1.33	7.12E-05	Up	2.5	31.3	3.7	79.2	5.7
OS01T0155300-00	Os01g0155300	1.32	2.73E-02	Up	2.5	0.5	0.1	1.4	0.1
OS11T0645400-01	Os11g0645400	1.32	3.55E-02	Up	2.5	3.3	0.8	8.4	0.8
OS11T0438700-03	Os11g0438700	1.32	6.02E-03	Up	2.5	0.6	0.1	1.4	0.2
OS08T0366000-03	Os08g0366000	1.32	2.11E-03	Up	2.5	3.7	0.7	9.4	0.4
OS01T0871500-01	Os01g0871500	1.32	4.22E-02	Up	2.5	35.5	8.3	89.5	15.4
OS03T0369600-00	Os03g0369600	1.32	4.16E-02	Up	2.5	4.3	0.9	10.8	1.7
OS05T0192200-00	Os05g0192200	1.32	4.27E-02	Up	2.5	4.3	0.6	10.8	1.7
OS01T0313050-00	Os01g0313050	1.32	4.50E-02	Up	2.5	0.3	0.1	0.8	0.1
OS02T0115801-00	Os02g0115801	1.31	1.84E-02	Up	2.5	112.6	24.0	284.8	54.7
OS08T0366050-00	Os08g0366050	1.31	1.99E-03	Up	2.5	4.0	0.7	10.1	0.5
OS06T0712250-00	Os06g0712250	1.31	4.40E-02	Up	2.5	1.0	0.2	2.5	0.3
OS06T0336200-01	Os06g0336200	1.31	1.37E-02	Up	2.5	181.1	41.1	452.4	44.4
OS06T0606700-00	Os06g0606700	1.30	2.76E-05	Up	2.5	6.4	0.1	16.1	0.7
OS07T0529600-02	Os07g0529600	1.30	4.43E-05	Up	2.5	80.2	4.8	199.9	20.9
OS07T0677100-01	Os07g0677100	1.30	6.08E-03	Up	2.5	9.7	1.4	24.3	3.9
OS07T0582450-00	Os07g0582450	1.30	6.24E-03	Up	2.5	15.9	3.5	39.7	1.0
OS10T0101200-01	Os10g0101200	1.30	3.07E-03	Up	2.5	6.7	0.9	16.7	2.0
OS08T0485000-01	Os08g0485000	1.29	3.69E-02	Up	2.5	2.6	0.2	6.5	1.5
OS02T0254600-01	Os02g0254600	1.29	2.65E-04	Up	2.5	10.5	0.7	26.1	2.5
OS07T0169600-01	Os07g0169600	1.29	9.32E-04	Up	2.5	13.4	2.2	33.3	0.9
OS09T0551000-00	Os09g0551000	1.29	3.25E-03	Up	2.4	1.1	0.1	2.6	0.3
OS08T0366000-02	Os08g0366000	1.28	2.25E-03	Up	2.4	5.5	1.0	13.6	0.7
OS08T0366000-04	Os08g0366000	1.28	5.06E-03	Up	2.4	5.4	1.0	13.3	0.7
OS05T0135000-01	Os05g0135000	1.28	1.29E-02	Up	2.4	16.6	3.3	40.6	5.6
OS10T0527900-01	Os10g0527900	1.28	3.02E-02	Up	2.4	0.9	0.1	2.2	0.3
OS01T0327100-01	Os01g0327100	1.27	3.85E-03	Up	2.4	2.4	0.3	5.9	0.2
OS11T0147700-01	Os11g0147700	1.27	3.19E-02	Up	2.4	4.8	1.0	11.6	0.4
OS03T0103300-00	Os03g0103300	1.27	1.32E-03	Up	2.4	33.0	1.1	80.5	14.1
OS02T0580500-01	Os02g0580500	1.27	1.58E-02	Up	2.4	1.4	0.2	3.5	0.3
OS03T0749300-04	Os03g0749300	1.26	4.48E-03	Up	2.4	81.1	12.1	197.9	33.9
OS08T0366000-01	Os08g0366000	1.26	1.34E-03	Up	2.4	5.9	0.9	14.4	0.9
OS02T0326100-01	Os02g0326100	1.26	1.33E-02	Up	2.4	0.8	0.1	1.9	0.0
OS07T0580700-01	Os07g0580700	1.26	2.99E-02	Up	2.4	0.2	0.0	0.4	0.0
OS06T0264500-01	Os06g0264500	1.25	1.37E-03	Up	2.4	15.8	2.7	38.2	2.0
OS09T0517900-01	Os09g0517900	1.25	1.63E-02	Up	2.4	0.6	0.0	1.4	0.1
OS02T0700500-01	Os02g0700500	1.25	9.65E-03	Up	2.4	3.4	0.5	8.2	1.0
OS12T0268000-01	Os12g0268000	1.25	2.55E-02	Up	2.4	1.8	0.1	4.2	0.7
OS06T0486800-01	Os06g0486800	1.25	7.46E-03	Up	2.4	66.1	6.3	159.0	29.7
OS12T0638300-01	Os12g0638300	1.25	1.97E-02	Up	2.4	1.3	0.1	3.0	0.5
OS01T0128100-01	Os01g0128100	1.25	5.67E-03	Up	2.4	0.7	0.1	1.6	0.1
OS06T0336200-02	Os06g0336200	1.25	1.50E-02	Up	2.4	202.4	43.0	485.7	46.5
OS01T0168100-01	Os01g0168100	1.24	2.62E-02	Up	2.4	49.6	6.6	119.6	30.2

OS11T0644800-00	Os11g0644800	1.24	1.01E-02	Up	2.4	1.0	0.0	2.5	0.3
OS09T0379900-02	Os09g0379900	1.24	4.01E-04	Up	2.4	51.4	6.2	122.8	12.1
OS02T0637000-01	Os02g0637000	1.23	3.79E-02	Up	2.4	30.9	7.3	73.6	8.9
OS06T0666650-00	Os06g0666650	1.23	4.20E-02	Up	2.3	1.1	0.2	2.7	0.1
OS05T0593000-01	Os05g0593000	1.23	3.20E-03	Up	2.3	5.8	0.7	13.9	1.3
OS10T0411650-00	Os10g0411650	1.22	3.07E-02	Up	2.3	3.7	0.3	8.6	1.6
OS01T0369800-00	Os01g0369800	1.22	1.70E-03	Up	2.3	74.2	11.3	174.8	10.2
OS04T0418100-00	Os04g0418100	1.22	3.09E-02	Up	2.3	2.3	0.3	5.3	0.4
OS10T0147200-01	Os10g0147200	1.22	3.74E-02	Up	2.3	3.6	0.6	8.5	0.8
OS03T0802400-01	Os03g0802400	1.21	2.34E-02	Up	2.3	3.9	0.6	9.2	1.2
OS02T0670900-05	Os02g0670900	1.20	3.91E-03	Up	2.3	13.2	1.9	30.9	4.0
OS09T0394300-01	Os09g0394300	1.20	3.82E-02	Up	2.3	4.4	0.5	10.3	2.4
OS11T0474800-01	Os11g0474800	1.20	1.57E-04	Up	2.3	14.1	1.2	32.8	0.8
OS03T0281466-01	Os03g0281466	1.20	4.76E-03	Up	2.3	5.0	0.8	11.7	0.9
OS03T0700700-03	Os03g0700700	1.19	4.90E-02	Up	2.3	11.3	0.6	26.3	7.6
OS04T0447566-00	Os04g0447566	1.19	4.27E-02	Up	2.3	3.0	0.3	7.0	1.2
OS07T0518100-01	Os07g0518100	1.19	4.10E-02	Up	2.3	0.9	0.1	2.1	0.2
OS04T0612500-01	Os04g0612500	1.19	6.02E-03	Up	2.3	4.4	0.4	10.2	0.5
OS02T0670900-02	Os02g0670900	1.19	3.97E-03	Up	2.3	12.8	1.8	29.7	3.7
OS02T0716500-01	Os02g0716500	1.18	7.02E-04	Up	2.3	44.2	6.0	101.6	2.6
OS06T0214300-01	Os06g0214300	1.18	4.49E-02	Up	2.3	2.3	0.4	5.4	0.6
OS07T0461900-01	Os07g0461900	1.18	2.24E-02	Up	2.3	25.6	2.7	58.6	13.7
OS07T0147500-01	Os07g0147500	1.18	4.99E-02	Up	2.3	1.4	0.1	3.3	0.4
OS01T0945100-00	Os01g0945100	1.18	7.16E-03	Up	2.3	3.5	0.4	8.0	0.6
OS02T0756850-01	Os02g0756850	1.18	1.05E-02	Up	2.3	16.8	2.9	38.5	2.4
OS11T0178800-01	Os11g0178800	1.17	1.29E-02	Up	2.3	12.0	2.2	27.5	2.1
OS07T0685700-01	Os07g0685700	1.17	1.35E-02	Up	2.2	13.3	1.9	30.5	5.2
OS04T0406600-01	Os04g0406600	1.17	2.42E-02	Up	2.2	7.4	1.3	17.0	2.3
OS04T0474800-03	Os04g0474800	1.16	2.04E-03	Up	2.2	47.1	7.0	106.7	4.1
OS12T0607000-02	Os12g0607000	1.16	2.03E-04	Up	2.2	19.8	0.6	44.7	3.9
OS08T0518600-01	Os08g0518600	1.16	4.67E-02	Up	2.2	1.3	0.0	2.9	0.5
OS01T0954500-01	Os01g0954500	1.16	2.33E-03	Up	2.2	5.2	0.4	11.7	1.1
OS11T0673200-01	Os11g0673200	1.15	1.08E-02	Up	2.2	1.6	0.2	3.6	0.2
OS03T0281500-01	Os03g0281500	1.15	7.80E-03	Up	2.2	5.5	0.8	12.4	1.1
OS05T0403300-01	Os05g0403300	1.14	4.82E-02	Up	2.2	3.4	0.1	7.7	1.8
OS01T0913100-01	Os01g0913100	1.14	4.47E-02	Up	2.2	5.4	1.1	12.1	0.9
OS09T0345300-01	Os09g0345300	1.14	2.26E-02	Up	2.2	0.6	0.1	1.4	0.1
OS01T0546000-01	Os01g0546000	1.14	8.69E-03	Up	2.2	1.3	0.1	2.8	0.1
OS03T0802500-01	Os03g0802500	1.13	1.38E-02	Up	2.2	10.3	1.8	22.9	1.7
OS03T0734300-01	Os03g0734300	1.13	1.97E-02	Up	2.2	125.2	14.8	276.7	46.8
OS03T0788650-00	Os03g0788650	1.13	3.07E-02	Up	2.2	10.2	2.1	22.6	2.1
OS03T0832200-01	Os03g0832200	1.13	1.22E-02	Up	2.2	7.0	1.1	15.4	1.2
OS04T0640900-01	Os04g0640900	1.12	2.12E-04	Up	2.2	51.8	3.7	114.3	5.5
OS07T0535800-01	Os07g0535800	1.12	3.37E-03	Up	2.2	9.0	1.0	19.8	1.8
OS07T0187000-01	Os07g0187000	1.12	1.77E-02	Up	2.2	1.6	0.2	3.5	0.1
OS05T0431700-01	Os05g0431700	1.12	3.58E-02	Up	2.2	3.6	0.7	8.0	0.4
OS08T0480050-00	Os08g0480050	1.12	3.50E-02	Up	2.2	4.8	0.2	10.6	2.3
OS04T0475600-01	Os04g0475600	1.12	5.68E-03	Up	2.2	5.7	0.6	12.5	1.1
OS04T0474800-01	Os04g0474800	1.12	1.05E-02	Up	2.2	35.6	6.2	78.2	5.9
OS04T0474800-02	Os04g0474800	1.12	3.92E-03	Up	2.2	43.1	6.7	94.7	4.2
OS07T0537900-02	Os07g0537900	1.11	9.04E-03	Up	2.2	1.5	0.1	3.3	0.1
OS03T0604566-01	Os03g0604566	1.11	4.98E-02	Up	2.2	1.4	0.0	3.2	0.4
OS01T0945150-00	Os01g0945150	1.11	1.53E-02	Up	2.2	2.2	0.2	4.8	0.2
OS12T0106200-01	Os12g0106200	1.11	1.74E-02	Up	2.2	2.3	0.1	5.0	0.3
OS03T0189600-02	Os03g0189600	1.11	1.14E-02	Up	2.2	7.0	0.6	15.2	1.9
OS11T0106900-01	Os11g0106900	1.11	1.00E-02	Up	2.2	2.6	0.1	5.6	0.2
OS12T0607000-01	Os12g0607000	1.11	8.83E-04	Up	2.2	14.8	0.4	32.3	3.3
OS07T0240300-01	Os07g0240300	1.10	7.96E-04	Up	2.1	11.4	0.9	24.7	0.3
OS05T0111200-01	Os05g0111200	1.10	5.90E-04	Up	2.1	65.3	3.2	141.0	11.5
OS03T0146200-01	Os03g0146200	1.09	3.20E-02	Up	2.1	62.8	13.9	135.9	8.7
OS06T0618700-01	Os06g0618700	1.09	2.19E-02	Up	2.1	12.8	2.0	27.5	2.2
OS01T0959750-00	Os01g0959750	1.09	6.65E-03	Up	2.1	21.4	2.0	45.9	3.1
OS02T0115700-02	Os02g0115700	1.09	4.21E-02	Up	2.1	197.0	31.4	425.2	82.1
OS07T0537900-01	Os07g0537900	1.08	4.77E-03	Up	2.1	2.5	0.1	5.3	0.3
OS06T0218600-01	Os06g0218600	1.08	2.16E-02	Up	2.1	19.7	2.4	42.4	6.2
OS05T0214300-00	Os05g0214300	1.08	3.58E-02	Up	2.1	15.9	0.8	33.9	7.0
OS08T0105700-01	Os08g0105700	1.07	4.98E-02	Up	2.1	1.3	0.1	2.7	0.3
OS12T0555000-01	Os12g0555000	1.07	1.05E-02	Up	2.1	331.5	46.2	705.2	73.3
OS03T0186900-01	Os03g0186900	1.06	1.85E-02	Up	2.1	7.8	0.7	16.5	2.2

OS03T0795350-00	Os03g0795350	1.05	1.82E-02	Up	2.1	13.5	2.0	28.4	1.8
OS10T0554300-00	Os10g0554300	1.05	4.20E-02	Up	2.1	1.5	0.2	3.2	0.2
OS01T0327400-01	Os01g0327400	1.05	2.90E-03	Up	2.1	35.6	4.1	74.6	2.0
OS04T0121800-01	Os04g0121800	1.04	7.96E-03	Up	2.1	20.1	0.5	41.7	3.8
OS03T0700700-01	Os03g0700700	1.04	3.72E-02	Up	2.1	17.3	0.7	36.2	8.3
OS01T0369700-03	Os01g0369700	1.04	4.86E-03	Up	2.1	80.4	9.8	167.5	9.0
OS12T0571700-01	Os12g0571700	1.04	3.56E-02	Up	2.1	9.2	0.5	19.2	2.7
OS03T0189600-01	Os03g0189600	1.04	3.06E-02	Up	2.1	6.0	0.6	12.5	1.6
OS10T0411500-01	Os10g0411500	1.03	3.37E-02	Up	2.0	7.2	0.8	14.9	2.7
OS10T0530800-01	Os10g0530800	1.03	3.21E-03	Up	2.0	31.1	0.9	64.2	7.7
OS07T0523600-01	Os07g0523600	1.03	2.72E-02	Up	2.0	10.8	0.6	22.4	3.8
OS08T0495866-00	Os08g0495866	1.03	4.52E-02	Up	2.0	1.7	0.1	3.5	0.4
OS10T0530900-03	Os10g0530900	1.02	1.34E-02	Up	2.0	31.5	1.0	64.7	10.0
OS01T0369700-01	Os01g0369700	1.02	5.47E-03	Up	2.0	76.8	8.9	157.6	7.9
OS02T0716500-04	Os02g0716500	1.02	2.04E-03	Up	2.0	48.8	4.1	100.3	4.1
OS05T0165200-00	Os05g0165200	1.02	2.47E-02	Up	2.0	4.5	0.1	9.3	0.8
OS09T0433600-01	Os09g0433600	1.02	3.22E-02	Up	2.0	16.7	2.4	34.4	1.3
OS09T0392666-01	Os09g0392666	1.02	3.48E-02	Up	2.0	11.4	1.7	23.3	1.2
OS06T0547400-01	Os06g0547400	1.01	3.93E-03	Up	2.0	40.5	3.6	82.5	5.7
OS06T0505700-01	Os06g0505700	1.01	3.07E-02	Up	2.0	4.2	0.3	8.6	0.3
OS11T0474900-00	Os11g0474900	1.01	1.84E-02	Up	2.0	20.8	2.2	42.3	2.8
OS02T0756800-01	Os02g0756800	1.01	4.68E-02	Up	2.0	27.0	4.5	54.9	3.9
OS07T0691200-01	Os07g0691200	1.01	6.11E-03	Up	2.0	8.3	0.5	17.0	1.8
OS10T0530900-01	Os10g0530900	1.00	8.99E-03	Up	2.0	72.3	2.7	146.5	21.9
OS03T0259100-01	Os03g0259100	1.00	1.81E-02	Up	2.0	247.7	15.1	503.2	85.0
OS05T0134700-02	Os05g0134700	1.00	3.82E-02	Up	2.0	15.6	1.9	31.6	4.2
OS04T0690100-01	Os04g0690100	1.00	1.17E-02	Up	2.0	3.1	0.1	6.4	0.2
OS11T0133800-00	Os11g0133800	1.00	5.00E-03	Up	2.0	11.2	0.4	22.6	1.8
OS03T0218400-01	Os03g0218400	1.00	4.54E-03	Up	2.0	16.4	1.5	33.2	1.5
OS07T0523400-01	Os07g0523400	1.00	2.59E-02	Up	2.0	3.9	0.1	8.0	1.2
OS02T0115700-01	Os02g0115700	1.00	4.57E-02	Up	2.0	278.7	44.2	565.9	82.0
OS09T0248900-01	Os09g0248900	1.00	2.90E-03	Up	2.0	6.8	0.0	13.7	0.9
OS04T0558400-01	Os04g0558400	1.00	2.45E-02	Up	2.0	16.4	0.3	33.2	6.0
OS07T0582400-01	Os07g0582400	1.00	1.40E-02	Up	2.0	43.9	6.0	88.6	5.8
OS02T0139650-00	Os02g0139650	0.99	1.38E-02	Up	2.0	6.3	0.6	12.8	0.6
OS04T0558400-02	Os04g0558400	0.99	4.52E-02	Up	2.0	17.5	0.2	35.1	7.1
OS11T0133850-00	Os11g0133850	0.98	3.01E-02	Up	2.0	9.8	0.5	19.5	1.6
OS10T0542900-01	Os10g0542900	0.98	1.33E-02	Up	2.0	7.8	0.4	15.6	1.0
OS06T0215750-00	Os06g0215750	0.98	5.51E-03	Up	2.0	14.9	1.0	29.7	0.9
OS01T0723600-01	Os01g0723600	0.98	3.04E-03	Up	2.0	13.8	0.4	27.5	1.3
OS07T0691200-02	Os07g0691200	0.98	7.54E-03	Up	2.0	8.7	0.2	17.4	2.0
OS09T0272600-01	Os09g0272600	0.97	3.83E-03	Up	2.0	193.4	11.0	382.6	32.6
OS08T0331000-01	Os08g0331000	0.97	4.41E-03	Up	2.0	100.8	2.9	198.9	15.4
OS02T0581200-01	Os02g0581200	0.96	1.33E-02	Up	2.0	16.0	1.0	31.6	1.6
OS01T0609900-01	Os01g0609900	0.96	7.80E-03	Up	2.0	22.8	0.2	44.9	5.9
OS07T0691200-03	Os07g0691200	0.96	2.77E-02	Up	1.9	4.2	0.1	8.3	0.7
OS09T0110300-01	Os09g0110300	0.96	4.50E-02	Up	1.9	34.7	5.5	68.3	5.6
OS07T0502200-01	Os07g0502200	0.96	2.85E-02	Up	1.9	5.3	0.3	10.5	1.0
OS03T0733900-01	Os03g0733900	0.96	2.68E-02	Up	1.9	12.2	0.6	23.9	2.1
OS03T0725800-01	Os03g0725800	0.95	8.85E-03	Up	1.9	28.0	2.3	54.9	4.4
OS02T0532500-01	Os02g0532500	0.95	3.08E-02	Up	1.9	8.3	0.7	16.3	1.1
OS02T0324400-01	Os02g0324400	0.95	2.97E-02	Up	1.9	12.9	1.4	25.2	1.5
OS03T0300400-01	Os03g0300400	0.95	1.21E-02	Up	1.9	88.7	4.0	173.0	21.3
OS03T0133400-01	Os03g0133400	0.95	4.19E-03	Up	1.9	46.5	3.1	90.5	3.1
OS07T0691200-04	Os07g0691200	0.94	1.84E-02	Up	1.9	10.5	0.4	20.4	2.3
OS04T0663700-01	Os04g0663700	0.94	8.10E-03	Up	1.9	8.2	0.5	15.9	0.4
OS05T0362100-01	Os05g0362100	0.94	3.56E-02	Up	1.9	8.3	0.7	16.1	1.9
OS08T0482700-01	Os08g0482700	0.93	1.75E-02	Up	1.9	56.2	7.1	108.8	1.8
OS04T0543900-02	Os04g0543900	0.93	3.51E-02	Up	1.9	19.4	1.3	37.5	5.6
OS01T0609900-02	Os01g0609900	0.93	1.43E-02	Up	1.9	25.1	0.2	48.4	6.6
OS08T0525050-00	Os08g0525050	0.93	4.39E-02	Up	1.9	19.0	0.9	36.7	4.8
OS04T0552300-01	Os04g0552300	0.92	3.41E-02	Up	1.9	7.9	0.3	15.1	1.5
OS08T0395700-01	Os08g0395700	0.91	1.63E-02	Up	1.9	35.0	3.9	66.3	3.7
OS06T0215600-02	Os06g0215600	0.91	7.18E-03	Up	1.9	24.7	1.8	46.8	0.5
OS03T0301700-01	Os03g0301700	0.91	3.77E-02	Up	1.9	6.4	0.2	12.1	1.4
OS02T0562600-01	Os02g0562600	0.90	7.80E-03	Up	1.9	25.1	0.6	47.4	3.6
OS11T0639300-01	Os11g0639300	0.90	1.25E-02	Up	1.9	8.7	0.4	16.5	0.3
OS04T0165700-01	Os04g0165700	0.89	3.62E-02	Up	1.9	95.0	2.0	178.2	27.2
OS01T0126600-01	Os01g0126600	0.89	3.55E-02	Up	1.9	5.8	0.2	10.8	0.9

OS02T0819450-00	Os02g0819450	0.89	3.61E-02	Up	1.9	13.6	0.3	25.5	3.5
OS07T0677200-02	Os07g0677200	0.88	2.82E-02	Up	1.8	47.4	5.3	88.2	3.5
OS02T0151300-01	Os02g0151300	0.87	2.97E-02	Up	1.8	6.8	0.3	12.5	0.7
OS02T0324700-01	Os02g0324700	0.84	2.82E-02	Up	1.8	58.0	3.9	105.2	7.5
OS04T0491500-02	Os04g0491500	0.83	3.06E-02	Up	1.8	10.4	0.6	18.7	1.0
OS02T0139600-01	Os02g0139600	0.83	2.21E-02	Up	1.8	17.6	0.8	31.6	1.3
OS04T0434800-02	Os04g0434800	0.83	1.68E-02	Up	1.8	55.4	2.4	99.4	4.7
OS04T0493100-01	Os04g0493100	0.82	2.25E-02	Up	1.8	52.9	2.5	94.4	4.6
OS02T0716500-03	Os02g0716500	0.81	4.50E-02	Up	1.8	62.0	2.7	109.5	7.6
OS07T0235800-01	Os07g0235800	0.80	2.80E-02	Up	1.7	16.3	0.6	28.6	0.6
OS04T0434800-01	Os04g0434800	0.80	2.84E-02	Up	1.7	43.9	2.4	77.3	3.5
OS01T0323775-00	Os01g0323775	0.78	4.64E-02	Up	1.7	114.9	7.2	199.0	12.2
OS06T0528600-01	Os06g0528600	0.77	3.29E-02	Up	1.7	48.9	2.3	83.9	1.6
OS07T0622250-00	Os07g0622250	0.77	3.15E-02	Up	1.7	64.5	1.6	110.8	4.0
OS07T0622200-01	Os07g0622200	0.76	3.56E-02	Up	1.7	143.7	3.6	245.3	11.4
OS08T0400300-01	Os08g0400300	-0.76	3.95E-02	Down	1.7	48.2	1.7	28.6	0.9
OS06T0247500-02	Os06g0247500	-0.78	3.77E-02	Down	1.7	273.5	17.5	160.9	3.9
OS11T0525200-02	Os11g0525200	-0.78	3.82E-02	Down	1.7	50.6	1.0	29.8	1.8
OS05T0426400-01	Os05g0426400	-0.82	3.38E-02	Down	1.8	14.9	0.4	8.5	0.4
OS04T0610800-01	Os04g0610800	-0.82	1.99E-02	Down	1.8	100.8	5.2	57.4	1.7
OS10T0503800-01	Os10g0503800	-0.83	2.89E-02	Down	1.8	41.8	2.1	23.7	0.7
OS05T0426350-00	Os05g0426350	-0.84	3.58E-02	Down	1.8	13.4	0.5	7.6	0.3
OS02T0104100-00	Os02g0104100	-0.84	3.95E-02	Down	1.8	74.6	4.9	42.0	3.3
OS10T0390500-02	Os10g0390500	-0.84	1.09E-02	Down	1.8	243.6	7.9	137.5	4.4
OS02T0736200-01	Os02g0736200	-0.84	4.68E-02	Down	1.8	5.6	0.1	3.2	0.1
OS06T0129900-01	Os06g0129900	-0.84	1.51E-02	Down	1.8	121.1	2.5	68.2	4.5
OS06T0129900-02	Os06g0129900	-0.84	1.14E-02	Down	1.8	127.4	1.6	71.6	3.8
OS05T0592300-02	Os05g0592300	-0.85	2.81E-02	Down	1.8	30.2	1.2	16.8	1.1
OS11T0498600-01	Os11g0498600	-0.86	2.28E-02	Down	1.8	66.0	2.5	36.8	3.0
OS03T0293500-01	Os03g0293500	-0.86	2.09E-02	Down	1.8	60.4	2.5	33.6	2.9
OS02T0773200-01	Os02g0773200	-0.87	1.85E-02	Down	1.8	80.3	1.0	44.5	4.2
OS09T0480600-01	Os09g0480600	-0.87	2.72E-02	Down	1.8	126.1	10.1	69.5	5.3
OS07T0175500-00	Os07g0175500	-0.87	3.15E-02	Down	1.8	68.3	4.0	37.7	3.6
OS05T0380300-01	Os05g0380300	-0.88	4.26E-02	Down	1.8	6.8	0.2	3.8	0.4
OS06T0591600-01	Os06g0591600	-0.88	1.44E-02	Down	1.8	110.3	4.9	60.6	3.6
OS05T0302300-01	Os05g0302300	-0.88	2.65E-02	Down	1.8	17.6	0.2	9.6	1.0
OS07T0421300-01	Os07g0421300	-0.88	2.97E-02	Down	1.8	69.4	6.0	38.0	3.4
OS03T0128100-02	Os03g0128100	-0.89	9.40E-03	Down	1.9	44.0	2.2	23.9	1.2
OS12T0145100-01	Os12g0145100	-0.89	1.27E-02	Down	1.9	447.6	43.7	243.0	10.9
OS11T0160100-01	Os11g0160100	-0.90	1.44E-02	Down	1.9	14.0	0.9	7.6	0.3
OS03T0231700-01	Os03g0231700	-0.90	1.69E-02	Down	1.9	12.0	0.8	6.5	0.3
OS08T0557800-01	Os08g0557800	-0.90	1.08E-02	Down	1.9	196.6	3.2	105.8	9.6
OS03T0255500-01	Os03g0255500	-0.91	3.99E-02	Down	1.9	75.5	3.2	40.6	5.5
OS05T0280500-01	Os05g0280500	-0.91	6.13E-03	Down	1.9	48.8	2.7	26.2	0.5
OS03T0250000-01	Os03g0250000	-0.91	1.49E-02	Down	1.9	8.4	0.2	4.5	0.2
OS03T0128100-01	Os03g0128100	-0.91	5.57E-03	Down	1.9	52.4	2.5	28.0	1.4
OS09T0564800-01	Os09g0564800	-0.92	4.87E-02	Down	1.9	6.6	0.7	3.5	0.2
OS03T0293450-00	Os03g0293450	-0.92	3.30E-02	Down	1.9	83.0	8.8	44.5	5.6
OS11T0569500-01	Os11g0569500	-0.92	3.49E-02	Down	1.9	9.3	0.1	5.0	0.6
OS03T0231750-00	Os03g0231750	-0.92	4.00E-02	Down	1.9	8.0	0.4	4.3	0.3
OS02T0104150-00	Os02g0104150	-0.92	3.49E-02	Down	1.9	88.5	7.8	47.0	4.2
OS07T0671800-01	Os07g0671800	-0.92	3.29E-03	Down	1.9	171.2	6.6	91.4	1.6
OS08T0159500-01	Os08g0159500	-0.92	8.69E-03	Down	1.9	51.9	4.3	27.6	0.9
OS11T0590700-01	Os11g0590700	-0.92	5.17E-03	Down	1.9	50.8	2.0	27.0	1.6
OS06T0247500-01	Os06g0247500	-0.92	4.17E-03	Down	1.9	384.4	22.6	204.2	8.0
OS11T0551800-02	Os11g0551800	-0.92	3.37E-02	Down	1.9	37.7	1.8	20.0	2.4
OS03T0293500-02	Os03g0293500	-0.92	3.67E-02	Down	1.9	148.2	16.6	79.0	10.8
OS06T0605950-00	Os06g0605950	-0.93	3.12E-02	Down	1.9	37.4	2.0	19.8	1.5
OS12T0145150-00	Os12g0145150	-0.93	1.02E-02	Down	1.9	81.6	7.6	43.3	0.4
OS12T0193800-01	Os12g0193800	-0.94	2.47E-02	Down	1.9	4.0	0.1	2.1	0.1
OS08T0345700-01	Os08g0345700	-0.94	3.51E-03	Down	1.9	245.9	12.2	129.4	6.1
OS08T0345700-02	Os08g0345700	-0.95	3.21E-03	Down	1.9	285.0	16.1	148.9	6.8
OS03T0231700-02	Os03g0231700	-0.95	8.73E-03	Down	1.9	15.3	0.5	8.0	0.6
OS06T0247651-00	Os06g0247651	-0.96	2.60E-03	Down	1.9	111.0	5.6	57.7	2.7
OS03T0255500-02	Os03g0255500	-0.96	9.41E-03	Down	1.9	95.5	4.6	49.6	4.3
OS02T0131050-01	Os02g0131050	-0.96	1.15E-02	Down	1.9	41.7	4.7	21.7	1.4
OS03T0250000-02	Os03g0250000	-0.96	1.50E-02	Down	1.9	17.5	0.4	9.1	0.7
OS01T0685100-02	Os01g0685100	-0.96	4.73E-02	Down	1.9	3.1	0.3	1.6	0.1
OS10T0390500-01	Os10g0390500	-0.97	5.16E-03	Down	2.0	218.9	7.5	112.9	11.0

OS01T0815400-01	Os01g0815400	-0.97	1.60E-02	Down	2.0	71.5	4.8	36.8	4.0
OS01T0905800-03	Os01g0905800	-0.97	1.92E-03	Down	2.0	1377.9	61.2	707.8	34.2
OS01T0905800-01	Os01g0905800	-0.97	1.86E-03	Down	2.0	1325.8	58.1	680.9	32.5
OS01T0795300-02	Os01g0795300	-0.98	4.06E-02	Down	2.0	6.5	0.9	3.3	0.2
OS06T0605900-01	Os06g0605900	-0.98	7.13E-03	Down	2.0	57.7	2.7	29.4	3.1
OS03T0387900-01	Os03g0387900	-0.99	3.72E-02	Down	2.0	15.3	2.5	7.7	0.6
OS03T0128200-01	Os03g0128200	-0.99	1.33E-03	Down	2.0	38.9	1.8	19.7	0.5
OS07T0268800-01	Os07g0268800	-1.00	6.88E-03	Down	2.0	15.4	0.7	7.8	0.6
OS04T0403900-00	Os04g0403900	-1.00	2.36E-02	Down	2.0	101.0	5.8	50.9	7.5
OS03T0387900-02	Os03g0387900	-1.00	3.16E-02	Down	2.0	15.3	2.4	7.7	0.5
OS03T0387900-03	Os03g0387900	-1.00	4.20E-02	Down	2.0	15.6	2.6	7.8	0.7
OS07T0268400-00	Os07g0268400	-1.00	7.45E-03	Down	2.0	13.0	0.4	6.5	0.5
OS01T0113500-01	Os01g0113500	-1.00	3.33E-02	Down	2.0	2.5	0.1	1.3	0.1
OS07T0659400-00	Os07g0659400	-1.01	5.03E-03	Down	2.0	29.3	1.3	14.7	0.7
OS06T0605900-02	Os06g0605900	-1.01	4.80E-03	Down	2.0	58.7	3.6	29.4	2.6
OS09T0122300-01	Os09g0122300	-1.01	2.27E-02	Down	2.0	1.5	0.1	0.8	0.1
EPIOSAT00000046001		-1.02	2.97E-02	Down	2.0	35.0	1.5	17.5	1.4
OS06T0298200-02	Os06g0298200	-1.02	3.82E-02	Down	2.0	14.9	1.1	7.4	1.2
OS03T0367900-01	Os03g0367900	-1.03	3.85E-03	Down	2.0	53.1	1.3	26.3	2.5
OS07T0605200-01	Os07g0605200	-1.03	3.49E-02	Down	2.0	29.0	3.1	14.3	2.0
OS07T0294100-00	Os07g0294100	-1.03	1.94E-02	Down	2.0	10.6	1.5	5.2	0.2
OS06T0298200-01	Os06g0298200	-1.03	4.89E-02	Down	2.0	16.0	1.3	7.9	1.3
OS05T0495200-02	Os05g0495200	-1.04	4.52E-02	Down	2.1	42.0	1.1	20.6	3.9
OS02T0574500-03	Os02g0574500	-1.04	2.38E-02	Down	2.1	4.8	0.2	2.3	0.2
OS04T0438600-01	Os04g0438600	-1.04	3.64E-03	Down	2.1	36.8	1.9	18.0	1.6
OS08T0342300-00	Os08g0342300	-1.04	1.41E-03	Down	2.1	14.9	0.8	7.3	0.4
OS01T0330650-01	Os01g0330650	-1.04	2.90E-02	Down	2.1	2.2	0.2	1.1	0.0
OS05T0578500-01	Os05g0578500	-1.04	2.57E-02	Down	2.1	6.9	0.8	3.4	0.1
OS05T0273500-01	Os05g0273500	-1.05	5.95E-03	Down	2.1	6.7	0.4	3.2	0.1
OS05T0401500-01	Os05g0401500	-1.05	1.16E-03	Down	2.1	148.2	2.7	72.2	5.6
OS01T0795300-01	Os01g0795300	-1.06	2.91E-02	Down	2.1	9.9	1.3	4.8	0.1
OS08T0170700-00	Os08g0170700	-1.06	8.64E-03	Down	2.1	17.8	1.2	8.6	1.1
OS02T0813166-00	Os02g0813166	-1.06	5.29E-03	Down	2.1	28.3	2.2	13.7	0.9
OS03T0367900-02	Os03g0367900	-1.06	2.17E-03	Down	2.1	59.3	1.9	28.7	2.7
OS01T0192300-01	Os01g0192300	-1.06	1.13E-02	Down	2.1	15.9	1.3	7.7	0.8
OS10T0406300-01	Os10g0406300	-1.06	1.53E-02	Down	2.1	21.6	1.3	10.4	1.5
OS01T0905800-02	Os01g0905800	-1.07	1.07E-03	Down	2.1	920.9	45.4	441.7	38.9
OS06T0166000-02	Os06g0166000	-1.07	3.82E-02	Down	2.1	25.6	0.7	12.2	2.4
OS01T0728300-01	Os01g0728300	-1.07	4.33E-02	Down	2.1	5.2	0.3	2.5	0.4
OS04T0542000-01	Os04g0542000	-1.07	3.63E-03	Down	2.1	23.1	2.6	11.0	0.7
OS01T0283600-00	Os01g0283600	-1.07	1.46E-02	Down	2.1	9.5	0.7	4.6	0.4
OS05T0405000-01	Os05g0405000	-1.08	3.22E-02	Down	2.1	142.3	30.4	67.7	4.7
OS11T0147800-01	Os11g0147800	-1.08	3.41E-03	Down	2.1	1080.8	149.6	514.7	31.5
OS04T0637000-01	Os04g0637000	-1.09	4.21E-02	Down	2.1	38.3	0.9	18.1	3.9
OS01T0957200-01	Os01g0957200	-1.09	3.44E-02	Down	2.1	15.1	3.0	7.2	0.5
OS05T0318600-01	Os05g0318600	-1.09	4.40E-02	Down	2.1	6.2	0.1	2.9	0.6
OS05T0405000-02	Os05g0405000	-1.09	2.83E-02	Down	2.1	161.9	34.4	76.4	5.5
OS01T0149500-01	Os01g0149500	-1.10	1.43E-02	Down	2.1	3.9	0.1	1.8	0.2
OS05T0119100-01	Os05g0119100	-1.10	2.16E-02	Down	2.1	82.9	7.3	39.1	6.5
OS02T0200300-01	Os02g0200300	-1.10	4.98E-02	Down	2.1	1.5	0.0	0.7	0.1
OS07T0618400-01	Os07g0618400	-1.10	1.25E-04	Down	2.1	27.0	0.8	12.7	0.1
OS05T0428100-00	Os05g0428100	-1.10	4.00E-03	Down	2.1	6.2	0.2	2.9	0.3
OS03T0172100-01	Os03g0172100	-1.10	8.35E-03	Down	2.1	104.3	7.3	49.3	8.1
OS01T0809000-01	Os01g0809000	-1.11	4.25E-02	Down	2.2	0.9	0.0	0.4	0.1
OS01T0865100-01	Os01g0865100	-1.11	1.58E-02	Down	2.2	153.7	13.5	71.8	12.3
OS11T0147901-00	Os11g0147901	-1.11	3.39E-03	Down	2.2	216.4	29.8	101.1	7.6
OS11T0156401-01	Os11g0156401	-1.11	3.54E-02	Down	2.2	3.7	0.3	1.7	0.2
OS04T0439100-03	Os04g0439100	-1.11	6.28E-03	Down	2.2	18.9	0.5	8.8	1.3
OS11T0147800-02	Os11g0147800	-1.12	4.79E-03	Down	2.2	174.1	22.1	81.0	8.6
OS04T0525200-01	Os04g0525200	-1.12	3.38E-02	Down	2.2	0.6	0.0	0.3	0.0
OS04T0439100-01	Os04g0439100	-1.12	1.33E-02	Down	2.2	13.7	0.4	6.3	1.0
OS04T0559400-02	Os04g0559400	-1.12	1.38E-02	Down	2.2	13.4	1.5	6.2	0.3
OS07T0110000-01	Os07g0110000	-1.12	1.07E-02	Down	2.2	15.6	2.5	7.2	0.6
OS11T0668650-01	Os11g0668650	-1.12	3.25E-03	Down	2.2	9.8	0.5	4.5	0.4
OS02T0601300-01	Os02g0601300	-1.12	2.51E-04	Down	2.2	3055.8	179.7	1414.2	96.7
OS04T0439100-02	Os04g0439100	-1.13	5.16E-03	Down	2.2	18.6	0.6	8.6	1.3
OS08T0465800-02	Os08g0465800	-1.13	7.44E-04	Down	2.2	45.3	3.2	20.9	1.9
OS08T0465800-01	Os08g0465800	-1.13	4.97E-04	Down	2.2	43.2	2.8	19.9	1.7
OS02T0152400-01	Os02g0152400	-1.14	2.83E-02	Down	2.2	61.2	10.0	28.0	5.3

OS07T0618450-00	Os07g0618450	-1.15	6.64E-04	Down	2.2	10.7	0.5	4.9	0.1
OS04T0186800-00	Os04g0186800	-1.15	4.10E-02	Down	2.2	4.8	0.7	2.2	0.2
OS11T0112000-01	Os11g0112000	-1.15	7.65E-03	Down	2.2	95.6	7.8	43.4	7.1
OS04T0538000-01	Os04g0538000	-1.15	4.42E-03	Down	2.2	3.5	0.1	1.6	0.1
OS04T0560100-02	Os04g0560100	-1.15	4.30E-02	Down	2.2	3.5	0.3	1.6	0.2
OS07T0129900-00	Os07g0129900	-1.15	3.22E-02	Down	2.2	1.0	0.1	0.5	0.0
OS06T0683700-01	Os06g0683700	-1.16	2.19E-02	Down	2.2	67.9	11.9	30.7	3.0
OS02T0601350-00	Os02g0601350	-1.17	2.69E-04	Down	2.2	634.0	47.7	284.3	23.7
OS05T0421600-01	Os05g0421600	-1.17	4.81E-02	Down	2.2	2.7	0.2	1.2	0.2
OS04T0607200-02	Os04g0607200	-1.17	4.70E-02	Down	2.3	0.4	0.0	0.2	0.0
OS06T0125500-01	Os06g0125500	-1.18	1.01E-02	Down	2.3	2.5	0.3	1.1	0.0
OS10T0419400-01	Os10g0419400	-1.18	4.88E-02	Down	2.3	49.8	3.0	22.1	5.0
OS04T0559400-01	Os04g0559400	-1.18	1.68E-03	Down	2.3	9.9	0.8	4.4	0.3
OS01T0948600-01	Os01g0948600	-1.18	3.41E-03	Down	2.3	257.3	34.7	114.1	15.1
OS02T0731500-02	Os02g0731500	-1.19	1.90E-02	Down	2.3	2.2	0.2	0.9	0.0
OS11T0139700-01	Os11g0139700	-1.19	8.98E-05	Down	2.3	407.6	19.3	180.1	17.0
OS11T0695800-01	Os11g0695800	-1.19	4.34E-02	Down	2.3	0.6	0.1	0.3	0.0
OS07T0618400-02	Os07g0618400	-1.19	1.95E-05	Down	2.3	30.2	1.2	13.4	0.2
OS11T0569600-01	Os11g0569600	-1.19	4.50E-02	Down	2.3	3.8	0.2	1.7	0.3
OS07T0180300-01	Os07g0180300	-1.20	1.00E-03	Down	2.3	8.9	0.5	3.9	0.4
OS02T0618100-01	Os02g0618100	-1.21	1.70E-02	Down	2.3	90.0	13.9	39.2	6.7
OS05T0524400-02	Os05g0524400	-1.21	3.35E-05	Down	2.3	70.8	3.5	30.9	1.8
OS03T0803500-01	Os03g0803500	-1.21	4.73E-04	Down	2.3	62.7	7.6	27.4	1.5
OS02T0519700-01	Os02g0519700	-1.21	3.78E-02	Down	2.3	518.0	61.2	226.0	50.4
OS07T0605400-01	Os07g0605400	-1.21	2.33E-02	Down	2.3	8.8	0.9	3.8	0.5
OS10T0447900-01	Os10g0447900	-1.21	3.64E-03	Down	2.3	4.7	0.4	2.0	0.2
OS06T0109600-01	Os06g0109600	-1.22	4.67E-02	Down	2.3	73.5	1.3	31.6	8.5
OS02T0173600-01	Os02g0173600	-1.22	1.14E-02	Down	2.3	3.5	0.2	1.5	0.2
OS01T0795300-03	Os01g0795300	-1.23	8.84E-03	Down	2.3	16.8	2.5	7.2	0.8
OS06T0264700-01	Os06g0264700	-1.23	3.52E-02	Down	2.3	3.5	0.4	1.5	0.2
OS05T0524400-01	Os05g0524400	-1.23	2.98E-05	Down	2.3	65.9	3.6	28.3	1.8
EPIOSAT00000011696		-1.23	4.64E-02	Down	2.3	18.6	3.6	7.9	0.7
OS09T0376350-00	Os09g0376350	-1.23	7.56E-03	Down	2.4	24.6	2.0	10.5	1.0
OS05T0533500-01	Os05g0533500	-1.24	1.07E-03	Down	2.4	20.9	1.0	8.9	1.1
OS01T0142600-01	Os01g0142600	-1.24	2.86E-02	Down	2.4	1.3	0.1	0.6	0.1
OS02T0332450-00	Os02g0332450	-1.24	4.74E-02	Down	2.4	2.6	0.6	1.1	0.1
OS06T0714800-01	Os06g0714800	-1.24	3.97E-04	Down	2.4	35.9	4.0	15.3	0.7
OS04T0554600-00	Os04g0554600	-1.24	4.40E-02	Down	2.4	201.7	39.5	86.8	18.3
OS01T0795400-01	Os01g0795400	-1.24	4.77E-03	Down	2.4	56.5	9.3	24.0	3.5
OS06T0128700-01	Os06g0128700	-1.25	9.50E-04	Down	2.4	16.3	2.2	6.9	0.2
OS11T0293800-00	Os11g0293800	-1.25	1.45E-02	Down	2.4	21.8	4.3	9.3	0.6
OS01T0880800-01	Os01g0880800	-1.25	6.89E-03	Down	2.4	8.7	1.3	3.7	0.2
OS07T0530600-01	Os07g0530600	-1.25	5.25E-05	Down	2.4	60.8	6.3	25.8	0.6
OS06T0701700-02	Os06g0701700	-1.26	2.53E-03	Down	2.4	53.8	3.8	22.6	3.7
OS11T0528500-01	Os11g0528500	-1.26	3.97E-02	Down	2.4	2.1	0.1	0.9	0.1
OS01T0666000-01	Os01g0666000	-1.27	1.27E-03	Down	2.4	46.7	5.2	19.6	3.0
OS07T0541850-00	Os07g0541850	-1.27	1.82E-03	Down	2.4	3.0	0.2	1.3	0.1
OS09T0354100-00	Os09g0354100	-1.27	3.27E-02	Down	2.4	2.9	0.3	1.2	0.2
OS04T0513550-00	Os04g0513550	-1.28	3.27E-02	Down	2.4	1.1	0.2	0.5	0.0
OS08T0124750-00	Os08g0124750	-1.28	8.30E-03	Down	2.4	5.7	0.7	2.3	0.3
OS07T0629500-01	Os07g0629500	-1.28	4.74E-02	Down	2.4	1.3	0.2	0.5	0.1
OS06T0125500-02	Os06g0125500	-1.29	1.08E-02	Down	2.4	2.8	0.5	1.2	0.1
OS01T0899275-00	Os01g0899275	-1.29	4.34E-02	Down	2.4	5.5	0.6	2.3	0.3
OS01T0690600-01	Os01g0690600	-1.29	3.22E-02	Down	2.4	0.9	0.1	0.4	0.0
OS04T0249500-00	Os04g0249500	-1.29	1.14E-03	Down	2.5	2.6	0.2	1.1	0.1
OS07T0673200-01	Os07g0673200	-1.29	3.52E-03	Down	2.5	19.5	2.6	8.0	1.1
OS11T0462900-00	Os11g0462900	-1.30	2.78E-04	Down	2.5	3.1	0.3	1.3	0.0
OS09T0425500-01	Os09g0425500	-1.30	2.72E-02	Down	2.5	17.2	3.9	7.1	0.6
OS04T0593500-00	Os04g0593500	-1.30	4.11E-02	Down	2.5	1.6	0.1	0.7	0.0
OS06T0701700-01	Os06g0701700	-1.31	2.04E-03	Down	2.5	81.3	6.9	32.9	5.9
OS09T0564450-01	Os09g0564450	-1.32	2.96E-02	Down	2.5	2.4	0.1	1.0	0.2
OS08T0124700-01	Os08g0124700	-1.32	2.50E-03	Down	2.5	6.4	0.9	2.6	0.3
OS01T0658900-01	Os01g0658900	-1.32	3.05E-04	Down	2.5	29.3	3.6	11.8	1.1
OS03T0131100-01	Os03g0131100	-1.33	1.07E-05	Down	2.5	39.1	3.2	15.7	1.1
OS07T0620700-01	Os07g0620700	-1.33	1.61E-02	Down	2.5	1.3	0.2	0.5	0.1
OS01T0940000-01	Os01g0940000	-1.34	8.41E-05	Down	2.5	9.5	0.8	3.8	0.3
OS06T0230100-00	Os06g0230100	-1.34	1.77E-02	Down	2.5	2.3	0.4	0.9	0.1
OS11T0462850-00	Os11g0462850	-1.34	4.54E-04	Down	2.5	2.4	0.1	1.0	0.1
OS08T0457400-01	Os08g0457400	-1.35	2.89E-02	Down	2.5	16.6	2.4	6.6	1.6

OS09T0442100-01	Os09g0442100	-1.35	5.63E-03	Down	2.6	15.9	2.5	6.3	1.1
OS01T0656600-01	Os01g0656600	-1.36	1.61E-05	Down	2.6	54.8	5.0	21.7	1.8
OS11T0138300-01	Os11g0138300	-1.36	4.05E-02	Down	2.6	1.3	0.1	0.5	0.1
OS12T0541350-00	Os12g0541350	-1.36	1.71E-04	Down	2.6	6.0	0.4	2.4	0.2
OS07T0482900-00	Os07g0482900	-1.36	2.06E-02	Down	2.6	1.0	0.1	0.4	0.0
OS04T0513400-01	Os04g0513400	-1.37	3.43E-03	Down	2.6	5.3	0.9	2.1	0.1
OS04T0679500-00	Os04g0679500	-1.37	2.10E-02	Down	2.6	14.8	2.0	5.8	1.2
OS07T0673900-01	Os07g0673900	-1.37	1.50E-02	Down	2.6	155.1	36.6	60.6	12.9
EPIOSAT00000050720		-1.38	1.31E-02	Down	2.6	21.3	4.1	8.2	0.6
OS03T0638400-01	Os03g0638400	-1.38	1.23E-02	Down	2.6	0.6	0.1	0.2	0.0
OS11T0471100-01	Os11g0471100	-1.38	1.07E-03	Down	2.6	4.7	0.1	1.8	0.1
OS11T0668300-00	Os11g0668300	-1.39	9.12E-03	Down	2.6	9.7	1.0	3.7	0.7
OS04T0481000-00	Os04g0481000	-1.39	2.15E-02	Down	2.6	18.8	0.8	7.2	1.8
OS04T0477000-01	Os04g0477000	-1.40	1.15E-02	Down	2.6	0.7	0.0	0.3	0.0
OS01T0128000-01	Os01g0128000	-1.40	3.11E-04	Down	2.6	5.8	0.6	2.2	0.1
OS10T0112100-01	Os10g0112100	-1.40	4.12E-02	Down	2.6	61.4	2.8	23.4	6.5
OS02T0191000-00	Os02g0191000	-1.40	2.32E-04	Down	2.6	9.1	0.4	3.5	0.5
OS11T0579900-01	Os11g0579900	-1.41	2.44E-03	Down	2.7	0.7	0.0	0.3	0.0
OS07T0638400-01	Os07g0638400	-1.41	9.38E-03	Down	2.7	554.1	53.1	209.1	49.6
OS09T0345600-00	Os09g0345600	-1.42	2.81E-02	Down	2.7	1.5	0.1	0.5	0.1
OS05T0554000-02	Os05g0554000	-1.42	4.77E-04	Down	2.7	37.9	8.4	14.3	0.1
OS10T0163040-02	Os10g0163040	-1.43	1.96E-03	Down	2.7	1.0	0.1	0.4	0.0
OS05T0560100-01	Os05g0560100	-1.43	2.62E-02	Down	2.7	1.8	0.3	0.7	0.1
OS08T0495500-01	Os08g0495500	-1.44	1.03E-07	Down	2.7	76.7	3.8	28.6	1.0
OS12T0622900-01	Os12g0622900	-1.44	1.48E-02	Down	2.7	2.9	0.5	1.1	0.1
OS10T0333700-00	Os10g0333700	-1.44	4.12E-05	Down	2.7	135.8	12.1	50.7	7.0
OS05T0136200-02	Os05g0136200	-1.44	1.44E-05	Down	2.7	8.6	0.5	3.2	0.1
OS09T0311600-01	Os09g0311600	-1.44	3.73E-05	Down	2.7	39.5	0.6	14.6	2.3
OS01T0734000-01	Os01g0734000	-1.45	7.79E-07	Down	2.7	30.1	1.6	11.1	0.4
OS05T0525900-01	Os05g0525900	-1.45	3.82E-03	Down	2.7	10.1	1.3	3.7	0.7
OS04T0120300-00	Os04g0120300	-1.45	9.26E-04	Down	2.7	2.4	0.1	0.9	0.1
OS05T0136200-01	Os05g0136200	-1.45	2.91E-05	Down	2.7	8.6	0.4	3.2	0.3
OS03T0627900-01	Os03g0627900	-1.46	1.83E-02	Down	2.7	0.5	0.1	0.2	0.0
OS03T0826800-01	Os03g0826800	-1.46	1.50E-02	Down	2.8	204.5	47.2	74.5	18.5
OS01T0218500-03	Os01g0218500	-1.47	3.94E-02	Down	2.8	0.8	0.1	0.3	0.0
OS07T0267200-01	Os07g0267200	-1.47	1.44E-02	Down	2.8	1.8	0.4	0.6	0.0
OS01T0127200-01	Os01g0127200	-1.47	8.07E-05	Down	2.8	65.4	6.3	23.9	4.0
OS03T0724500-01	Os03g0724500	-1.47	8.17E-06	Down	2.8	31.2	3.1	11.3	0.4
OS06T0304700-01	Os06g0304700	-1.48	1.72E-05	Down	2.8	8.0	0.4	2.9	0.3
OS09T0474400-00	Os09g0474400	-1.49	3.29E-02	Down	2.8	1.7	0.1	0.6	0.1
OS10T0163040-01	Os10g0163040	-1.49	4.09E-04	Down	2.8	0.9	0.1	0.3	0.0
OS04T0356600-01	Os04g0356600	-1.49	1.05E-02	Down	2.8	0.7	0.1	0.3	0.0
OS08T0387050-01	Os08g0387050	-1.49	3.25E-03	Down	2.8	1.5	0.3	0.5	0.0
OS05T0554000-01	Os05g0554000	-1.50	1.11E-04	Down	2.8	58.5	12.5	20.9	0.5
OS03T0388600-01	Os03g0388600	-1.52	1.44E-02	Down	2.9	8.7	1.7	3.0	0.6
OS11T0172300-01	Os11g0172300	-1.53	5.38E-06	Down	2.9	13.9	1.2	4.9	0.5
OS07T0482887-00	Os07g0482887	-1.53	1.92E-02	Down	2.9	0.6	0.0	0.2	0.0
OS07T0541800-01	Os07g0541800	-1.54	5.53E-06	Down	2.9	17.6	1.9	6.1	0.6
OS01T0706600-00	Os01g0706600	-1.54	4.15E-05	Down	2.9	18.0	1.5	6.2	0.6
OS06T0495800-01	Os06g0495800	-1.55	5.09E-06	Down	2.9	18.6	0.8	6.4	0.8
OS06T0548200-01	Os06g0548200	-1.55	1.11E-03	Down	2.9	5.7	0.2	2.0	0.4
OS01T0888900-01	Os01g0888900	-1.55	8.76E-03	Down	2.9	17.8	0.9	6.1	1.5
OS11T0210300-02	Os11g0210300	-1.55	9.77E-03	Down	2.9	172.1	41.7	59.3	15.8
OS04T0359700-00	Os04g0359700	-1.56	1.74E-02	Down	2.9	4.9	0.4	1.7	0.4
OS10T0109900-00	Os10g0109900	-1.56	1.49E-05	Down	2.9	5.7	0.3	2.0	0.2
OS03T0425300-02	Os03g0425300	-1.56	1.17E-02	Down	3.0	2.5	0.5	0.8	0.1
OS01T0135600-01	Os01g0135600	-1.57	1.70E-02	Down	3.0	0.4	0.0	0.1	0.0
OS12T0189300-01	Os12g0189300	-1.57	3.80E-08	Down	3.0	105.7	9.1	35.9	1.8
OS05T0555600-02	Os05g0555600	-1.57	2.14E-06	Down	3.0	25.9	3.5	8.8	0.6
OS12T0541300-01	Os12g0541300	-1.58	3.60E-07	Down	3.0	20.5	1.6	6.9	0.6
OS12T0123700-00	Os12g0123700	-1.58	3.18E-02	Down	3.0	10.3	0.4	3.4	1.2
OS12T0189300-02	Os12g0189300	-1.58	1.08E-08	Down	3.0	145.8	5.0	49.0	2.2
OS11T0513900-01	Os11g0513900	-1.59	1.45E-02	Down	3.0	1.6	0.1	0.5	0.1
OS11T0210300-01	Os11g0210300	-1.59	7.26E-03	Down	3.0	161.8	40.7	54.5	14.0
OS06T0523300-01	Os06g0523300	-1.59	2.30E-04	Down	3.0	6.8	0.4	2.3	0.2
OS03T0182800-01	Os03g0182800	-1.59	1.55E-04	Down	3.0	115.1	0.9	38.5	8.0
OS01T0155800-01	Os01g0155800	-1.59	2.47E-02	Down	3.0	46.6	9.9	15.5	4.1
OS07T0459500-00	Os07g0459500	-1.59	6.93E-03	Down	3.0	1.0	0.1	0.3	0.1
OS02T0511100-00	Os02g0511100	-1.59	2.15E-03	Down	3.0	14.2	1.2	4.8	1.3

OS03T0425300-01	Os03g0425300	-1.60	8.85E-03	Down	3.0	2.4	0.5	0.8	0.2
OS01T0947500-00	Os01g0947500	-1.60	3.61E-02	Down	3.0	1.3	0.2	0.4	0.1
OS05T0486300-01	Os05g0486300	-1.61	3.17E-02	Down	3.0	0.4	0.0	0.1	0.0
OS10T0109801-01	Os10g0109801	-1.61	6.99E-03	Down	3.1	0.5	0.1	0.2	0.0
OS01T0220700-00	Os01g0220700	-1.62	4.18E-05	Down	3.1	4.8	0.3	1.6	0.1
OS06T0569500-01	Os06g0569500	-1.63	3.10E-03	Down	3.1	0.6	0.0	0.2	0.0
OS01T0642200-01	Os01g0642200	-1.63	4.03E-02	Down	3.1	260.3	67.9	84.9	23.3
OS02T0575700-01	Os02g0575700	-1.64	3.88E-04	Down	3.1	15.8	0.1	5.1	1.2
OS01T0313300-01	Os01g0313300	-1.65	5.00E-07	Down	3.1	53.2	7.0	17.2	0.4
OS12T0221400-01	Os12g0221400	-1.65	4.39E-02	Down	3.1	1.4	0.2	0.4	0.1
OS01T0635200-01	Os01g0635200	-1.65	7.43E-03	Down	3.1	2.5	0.3	0.8	0.1
OS11T0462600-01	Os11g0462600	-1.65	4.79E-04	Down	3.1	2.3	0.1	0.7	0.1
OS01T0895200-01	Os01g0895200	-1.65	3.45E-05	Down	3.1	16.1	1.5	5.2	0.8
OS04T0489600-02	Os04g0489600	-1.66	1.99E-04	Down	3.2	33.8	2.9	10.8	2.3
OS06T0665500-01	Os06g0665500	-1.66	2.65E-07	Down	3.2	71.2	11.2	22.7	1.1
OS07T0673400-02	Os07g0673400	-1.66	3.22E-02	Down	3.2	0.5	0.1	0.2	0.0
OS01T0642801-00	Os01g0642801	-1.67	6.07E-04	Down	3.2	8.0	0.9	2.5	0.3
OS05T0312600-01	Os05g0312600	-1.67	1.67E-04	Down	3.2	16.0	1.0	5.1	1.0
OS01T0719350-00	Os01g0719350	-1.67	1.50E-02	Down	3.2	6.4	1.8	2.1	0.4
OS04T0150300-01	Os04g0150300	-1.67	3.39E-03	Down	3.2	0.9	0.2	0.3	0.0
OS06T0162550-00	Os06g0162550	-1.68	2.08E-02	Down	3.2	13.6	2.1	4.3	1.2
OS04T0360400-00	Os04g0360400	-1.68	1.98E-02	Down	3.2	1.4	0.2	0.4	0.1
OS11T0465200-02	Os11g0465200	-1.69	1.40E-02	Down	3.2	0.4	0.0	0.1	0.0
OS10T0551800-00	Os10g0551800	-1.69	2.52E-03	Down	3.2	52.8	10.5	16.5	3.6
OS12T0442800-01	Os12g0442800	-1.70	9.96E-06	Down	3.3	119.2	22.4	36.8	4.4
OS11T0648400-01	Os11g0648400	-1.70	6.98E-03	Down	3.3	4.3	0.5	1.3	0.3
OS06T0188400-01	Os06g0188400	-1.71	2.65E-02	Down	3.3	1.2	0.2	0.4	0.1
OS05T0434800-00	Os05g0434800	-1.71	2.51E-04	Down	3.3	5.5	0.6	1.7	0.2
OS01T0283300-01	Os01g0283300	-1.72	9.24E-03	Down	3.3	127.9	22.1	38.9	13.0
OS03T0100030-01	Os03g0100030	-1.72	9.55E-05	Down	3.3	18.8	1.6	5.7	1.1
OS02T0609200-00	Os02g0609200	-1.72	1.05E-02	Down	3.3	343.5	14.6	104.5	31.3
OS04T0635500-01	Os04g0635500	-1.72	4.99E-02	Down	3.3	23.5	4.0	7.2	2.2
OS01T0104200-00	Os01g0104200	-1.72	9.35E-05	Down	3.3	11.9	0.2	3.6	0.7
OS04T0635100-00	Os04g0635100	-1.72	1.51E-02	Down	3.3	35.0	7.5	10.7	2.6
OS01T0283300-02	Os01g0283300	-1.73	7.45E-03	Down	3.3	145.0	27.1	43.8	14.1
OS04T0180500-00	Os04g0180500	-1.74	3.74E-02	Down	3.3	0.3	0.0	0.1	0.0
OS06T0206700-01	Os06g0206700	-1.74	6.50E-07	Down	3.3	4.1	0.2	1.2	0.1
OS12T0134900-01	Os12g0134900	-1.75	2.23E-03	Down	3.4	0.7	0.1	0.2	0.0
OS11T0145200-01	Os11g0145200	-1.75	6.29E-09	Down	3.4	50.8	6.0	15.3	1.0
OS04T0635450-00	Os04g0635450	-1.75	2.90E-02	Down	3.4	2.5	0.3	0.7	0.2
OS05T0555600-01	Os05g0555600	-1.75	3.05E-08	Down	3.4	27.2	3.6	8.2	0.6
OS06T0152400-01	Os06g0152400	-1.75	2.78E-04	Down	3.4	13.6	3.7	4.1	0.3
OS02T0523100-00	Os02g0523100	-1.76	2.06E-02	Down	3.4	0.9	0.2	0.3	0.0
OS04T0340300-01	Os04g0340300	-1.76	1.57E-03	Down	3.4	20.9	4.1	6.3	1.4
OS01T0802700-01	Os01g0802700	-1.76	2.36E-08	Down	3.4	26.2	3.0	7.8	0.4
OS11T0173432-00	Os11g0173432	-1.76	1.48E-05	Down	3.4	1.7	0.2	0.5	0.0
OS01T0895300-01	Os01g0895300	-1.77	2.20E-09	Down	3.4	42.8	3.9	12.7	0.6
OS03T0561400-01	Os03g0561400	-1.77	8.20E-04	Down	3.4	2.0	0.4	0.6	0.0
OS11T0679700-01	Os11g0679700	-1.77	1.28E-03	Down	3.4	5.1	0.3	1.5	0.4
OS04T0341500-01	Os04g0341500	-1.78	1.02E-02	Down	3.4	0.2	0.0	0.1	0.0
OS01T0719300-01	Os01g0719300	-1.78	6.44E-03	Down	3.4	42.3	13.2	12.4	3.4
OS11T0489250-00	Os11g0489250	-1.79	1.39E-09	Down	3.5	20.0	0.3	5.8	0.6
OS04T0489600-01	Os04g0489600	-1.80	4.02E-05	Down	3.5	3.5	0.2	1.0	0.2
OS01T0837200-01	Os01g0837200	-1.80	1.21E-05	Down	3.5	27.1	4.1	7.9	1.0
OS08T0543050-00	Os08g0543050	-1.80	1.70E-02	Down	3.5	2.9	0.4	0.8	0.2
OS05T0560900-01	Os05g0560900	-1.80	1.22E-03	Down	3.5	0.8	0.1	0.2	0.0
OS02T0163500-01	Os02g0163500	-1.81	1.17E-07	Down	3.5	11.2	0.4	3.2	0.2
OS04T0581700-01	Os04g0581700	-1.81	4.28E-03	Down	3.5	6.4	0.8	1.8	0.4
OS04T0125700-01	Os04g0125700	-1.83	1.57E-07	Down	3.6	24.7	3.8	7.0	0.6
OS04T0679400-02	Os04g0679400	-1.83	7.01E-06	Down	3.6	24.1	3.6	6.8	1.0
OS05T0560900-02	Os05g0560900	-1.83	5.29E-04	Down	3.6	0.9	0.1	0.3	0.0
OS11T0172350-00	Os11g0172350	-1.83	1.45E-06	Down	3.6	6.0	0.5	1.7	0.2
OS05T0548300-01	Os05g0548300	-1.83	1.17E-03	Down	3.6	5.0	0.1	1.4	0.4
OS02T0253150-01	Os02g0253150	-1.84	2.85E-04	Down	3.6	1.2	0.1	0.3	0.1
OS01T0956500-01	Os01g0956500	-1.86	2.62E-02	Down	3.6	0.9	0.2	0.2	0.1
OS04T0679400-04	Os04g0679400	-1.86	2.23E-06	Down	3.6	33.6	4.4	9.3	1.4
OS06T0591200-01	Os06g0591200	-1.87	1.03E-07	Down	3.6	64.9	10.8	18.0	0.9
OS01T0736100-01	Os01g0736100	-1.87	3.39E-03	Down	3.7	1.0	0.3	0.3	0.0
OS11T0210500-01	Os11g0210500	-1.88	1.50E-03	Down	3.7	1464.2	166.7	399.9	119.7

OS10T0116800-01	Os10g0116800	-1.88	2.09E-12	Down	3.7	318.1	15.8	87.0	7.0
OS04T0135400-00	Os04g0135400	-1.88	5.61E-03	Down	3.7	4.0	0.5	1.1	0.4
OS08T0465700-01	Os08g0465700	-1.88	2.58E-04	Down	3.7	5.2	0.3	1.4	0.3
OS03T0826900-01	Os03g0826900	-1.89	5.83E-03	Down	3.7	1.0	0.1	0.3	0.1
OS06T0230201-00	Os06g0230201	-1.89	1.45E-02	Down	3.7	0.9	0.1	0.2	0.0
OS01T0718500-01	Os01g0718500	-1.89	1.21E-08	Down	3.7	15.8	0.7	4.3	0.2
OS06T0128600-01	Os06g0128600	-1.89	1.25E-04	Down	3.7	2.2	0.3	0.6	0.1
OS06T0178450-00	Os06g0178450	-1.90	1.07E-03	Down	3.7	3.5	0.2	0.9	0.2
EPIOSAT00000011908		-1.90	1.62E-03	Down	3.7	3.4	0.4	0.9	0.0
OS04T0679400-03	Os04g0679400	-1.90	7.65E-06	Down	3.7	33.8	4.7	9.1	1.5
OS11T0462950-00	Os11g0462950	-1.90	1.34E-06	Down	3.7	3.0	0.3	0.8	0.1
EPIOSAT00000045689		-1.90	3.15E-03	Down	3.7	3.9	0.4	1.0	0.3
OS09T0334600-00	Os09g0334600	-1.91	1.83E-06	Down	3.8	6.5	0.8	1.7	0.1
OS07T0438600-01	Os07g0438600	-1.92	1.09E-02	Down	3.8	2.8	0.8	0.7	0.1
OS11T0278300-00	Os11g0278300	-1.93	3.21E-03	Down	3.8	0.5	0.1	0.1	0.0
OS02T0570800-00	Os02g0570800	-1.94	2.82E-04	Down	3.8	1.4	0.2	0.4	0.1
OS11T0210500-02	Os11g0210500	-1.94	2.84E-04	Down	3.8	1382.4	181.0	360.8	101.4
OS02T0570700-01	Os02g0570700	-1.95	8.80E-05	Down	3.9	2.5	0.4	0.7	0.1
OS04T0635650-00	Os04g0635650	-1.95	1.19E-03	Down	3.9	6.9	0.7	1.8	0.4
OS04T0679400-01	Os04g0679400	-1.95	2.82E-06	Down	3.9	33.1	4.6	8.6	1.7
OS01T0947000-01	Os01g0947000	-1.95	1.02E-13	Down	3.9	22.2	1.0	5.8	0.1
OS03T0277700-01	Os03g0277700	-1.95	4.19E-03	Down	3.9	11.9	2.9	3.1	0.7
OS04T0387300-01	Os04g0387300	-1.96	5.16E-07	Down	3.9	9.8	1.6	2.5	0.1
OS01T0760000-01	Os01g0760000	-1.97	7.88E-05	Down	3.9	38.4	9.2	10.0	1.2
OS02T0634100-00	Os02g0634100	-1.97	4.21E-02	Down	3.9	0.9	0.2	0.2	0.1
OS02T0214766-00	Os02g0214766	-1.98	4.38E-03	Down	3.9	2.0	0.2	0.5	0.1
OS02T0649800-01	Os02g0649800	-1.98	1.55E-04	Down	3.9	44.3	3.9	11.3	3.0
OS04T0268700-01	Os04g0268700	-1.99	4.07E-06	Down	4.0	7.2	1.2	1.8	0.1
OS07T0291400-01	Os07g0291400	-2.00	8.08E-03	Down	4.0	1.3	0.5	0.3	0.0
OS02T0215300-00	Os02g0215300	-2.00	3.88E-04	Down	4.0	5.2	0.9	1.3	0.3
OS08T0155900-00	Os08g0155900	-2.01	2.17E-02	Down	4.0	2.7	0.5	0.7	0.2
OS12T0509200-01	Os12g0509200	-2.02	2.22E-02	Down	4.1	0.2	0.0	0.0	0.0
OS02T0694100-01	Os02g0694100	-2.03	9.42E-06	Down	4.1	6.1	1.3	1.5	0.1
OS11T0540900-00	Os11g0540900	-2.04	4.12E-02	Down	4.1	0.3	0.1	0.1	0.0
OS11T0160000-00	Os11g0160000	-2.04	2.02E-02	Down	4.1	3.5	1.0	0.9	0.2
OS04T0180400-01	Os04g0180400	-2.04	4.13E-03	Down	4.1	0.4	0.1	0.1	0.0
OS04T0219600-01	Os04g0219600	-2.05	3.10E-03	Down	4.1	0.9	0.1	0.2	0.1
OS12T0540000-01	Os12g0540000	-2.05	7.01E-03	Down	4.2	0.4	0.1	0.1	0.0
OS06T0578100-00	Os06g0578100	-2.05	2.62E-02	Down	4.2	0.3	0.0	0.1	0.0
OS07T0124100-01	Os07g0124100	-2.06	1.63E-02	Down	4.2	33.8	5.5	8.2	2.7
OS06T0683800-01	Os06g0683800	-2.06	3.43E-06	Down	4.2	20.9	3.1	5.1	1.0
OS02T0215200-03	Os02g0215200	-2.06	2.29E-04	Down	4.2	14.0	3.2	3.4	0.8
OS05T0533450-00	Os05g0533450	-2.06	2.05E-03	Down	4.2	1.3	0.2	0.3	0.1
OS08T0372900-02	Os08g0372900	-2.06	1.94E-05	Down	4.2	4.2	0.7	1.0	0.2
OS08T0462800-01	Os08g0462800	-2.07	6.70E-04	Down	4.2	0.7	0.0	0.2	0.0
OS01T0736100-02	Os01g0736100	-2.07	3.14E-04	Down	4.2	1.0	0.2	0.2	0.0
OS07T0673400-01	Os07g0673400	-2.07	4.29E-04	Down	4.2	0.9	0.1	0.2	0.0
OS02T0215200-01	Os02g0215200	-2.08	3.75E-05	Down	4.2	9.2	1.8	2.2	0.4
OS11T0208651-00	Os11g0208651	-2.08	1.73E-02	Down	4.2	0.3	0.1	0.1	0.0
OS02T0163300-01	Os02g0163300	-2.08	6.95E-08	Down	4.2	7.8	0.8	1.9	0.2
OS01T0704000-01	Os01g0704000	-2.08	4.45E-02	Down	4.2	0.5	0.1	0.1	0.0
OS08T0372900-01	Os08g0372900	-2.09	5.25E-04	Down	4.3	4.0	1.1	1.0	0.1
OS05T0244700-02	Os05g0244700	-2.10	6.48E-08	Down	4.3	8.6	0.9	2.0	0.3
EPIOSAT00000017695		-2.10	1.33E-02	Down	4.3	2.6	0.2	0.6	0.1
OS01T0269550-01	Os01g0269550	-2.10	4.25E-05	Down	4.3	3.9	1.1	0.9	0.1
OS12T0456783-00	Os12g0456783	-2.10	3.50E-02	Down	4.3	0.1	0.0	0.0	0.0
OS11T0462500-00	Os11g0462500	-2.11	2.36E-10	Down	4.3	3.0	0.2	0.7	0.0
OS03T0130100-01	Os03g0130100	-2.11	2.77E-05	Down	4.3	1.2	0.2	0.3	0.0
OS11T0559366-00	Os11g0559366	-2.12	3.85E-03	Down	4.3	1.9	0.3	0.4	0.1
OS08T0128000-02	Os08g0128000	-2.12	5.41E-07	Down	4.3	6.0	1.0	1.4	0.2
OS02T0215200-02	Os02g0215200	-2.12	6.72E-05	Down	4.4	13.7	2.5	3.2	0.7
OS04T0458600-01	Os04g0458600	-2.12	4.58E-02	Down	4.4	0.4	0.1	0.1	0.0
OS06T0210000-00	Os06g0210000	-2.13	6.19E-03	Down	4.4	0.8	0.2	0.2	0.0
OS07T0587300-01	Os07g0587300	-2.13	2.87E-04	Down	4.4	2.0	0.4	0.5	0.0
OS02T0160900-01	Os02g0160900	-2.15	8.15E-05	Down	4.4	1.1	0.2	0.2	0.0
OS08T0404700-00	Os08g0404700	-2.15	2.99E-02	Down	4.4	1.3	0.2	0.3	0.1
OS03T0421000-01	Os03g0421000	-2.16	1.05E-02	Down	4.5	0.8	0.3	0.2	0.0
OS04T0680400-01	Os04g0680400	-2.16	2.64E-10	Down	4.5	30.4	5.3	6.9	0.6
OS07T0542067-00	Os07g0542067	-2.16	1.77E-07	Down	4.5	1.2	0.1	0.3	0.0

OS04T0249600-01	Os04g0249600	-2.16	2.32E-08	Down	4.5	6.3	1.1	1.4	0.1
OS11T0568800-00	Os11g0568800	-2.16	8.58E-03	Down	4.5	0.5	0.2	0.1	0.0
OS02T0570400-01	Os02g0570400	-2.16	2.13E-04	Down	4.5	3.2	0.7	0.7	0.2
OS06T0695001-00	Os06g0695001	-2.17	3.68E-02	Down	4.5	4.6	0.8	1.0	0.4
OS05T0244700-01	Os05g0244700	-2.18	1.47E-08	Down	4.5	8.3	1.1	1.8	0.2
OS02T0620500-00	Os02g0620500	-2.19	4.11E-02	Down	4.5	13.5	1.9	3.0	1.1
OS08T0128000-01	Os08g0128000	-2.21	3.44E-09	Down	4.6	4.5	0.6	1.0	0.0
OS11T0524601-00	Os11g0524601	-2.22	1.87E-06	Down	4.6	8.1	1.3	1.7	0.3
OS08T0150700-01	Os08g0150700	-2.22	2.60E-03	Down	4.7	2.7	0.6	0.6	0.1
OS06T0695300-01	Os06g0695300	-2.22	1.52E-04	Down	4.7	31.2	2.9	6.7	2.0
OS10T0351200-00	Os10g0351200	-2.22	1.97E-02	Down	4.7	0.5	0.1	0.1	0.0
OS10T0161400-01	Os10g0161400	-2.23	2.40E-09	Down	4.7	12.6	1.6	2.7	0.4
OS06T0695350-00	Os06g0695350	-2.23	2.29E-03	Down	4.7	7.3	0.9	1.6	0.5
OS05T0228000-02	Os05g0228000	-2.24	7.43E-04	Down	4.7	0.7	0.1	0.1	0.0
OS03T0619875-01	Os03g0619875	-2.25	1.48E-03	Down	4.8	26.5	5.8	5.6	1.7
OS02T0214900-01	Os02g0214900	-2.26	2.32E-04	Down	4.8	3.8	0.4	0.8	0.2
OS12T0489800-01	Os12g0489800	-2.26	6.51E-13	Down	4.8	4.1	0.2	0.9	0.1
OS03T0215600-00	Os03g0215600	-2.26	1.22E-02	Down	4.8	3.0	1.0	0.6	0.1
OS06T0176500-00	Os06g0176500	-2.27	2.11E-03	Down	4.8	1.8	0.5	0.4	0.1
OS06T0177700-00	Os06g0177700	-2.27	2.66E-04	Down	4.8	1.5	0.3	0.3	0.1
OS06T0272800-00	Os06g0272800	-2.28	1.50E-03	Down	4.8	0.5	0.0	0.1	0.0
OS01T0916100-01	Os01g0916100	-2.28	8.86E-03	Down	4.9	3.4	0.3	0.7	0.2
EPIOSAT00000021254		-2.29	3.66E-02	Down	4.9	8.8	4.6	1.8	0.3
OS06T0707300-02	Os06g0707300	-2.29	1.21E-05	Down	4.9	3.2	0.8	0.7	0.0
OS12T0594950-01	Os12g0594950	-2.30	2.56E-06	Down	4.9	8.4	1.6	1.7	0.2
OS04T0152000-00	Os04g0152000	-2.30	1.34E-06	Down	4.9	5.3	0.4	1.1	0.2
OS09T0564700-00	Os09g0564700	-2.30	9.33E-03	Down	4.9	11.6	1.1	2.4	0.8
OS02T0620533-00	Os02g0620533	-2.30	3.72E-02	Down	4.9	14.3	2.1	2.9	1.2
OS04T0249600-02	Os04g0249600	-2.31	5.24E-10	Down	4.9	30.1	5.4	6.1	0.2
OS04T0635700-01	Os04g0635700	-2.31	1.80E-06	Down	5.0	1.3	0.1	0.3	0.0
OS01T0720700-00	Os01g0720700	-2.31	5.87E-11	Down	5.0	94.8	18.1	19.3	0.7
OS05T0361700-02	Os05g0361700	-2.31	2.32E-05	Down	5.0	16.7	3.2	3.4	1.0
OS02T0811800-01	Os02g0811800	-2.31	1.53E-02	Down	5.0	41.3	16.3	8.4	3.5
OS03T0805600-00	Os03g0805600	-2.32	4.24E-02	Down	5.0	0.2	0.0	0.0	0.0
OS07T0638300-01	Os07g0638300	-2.32	5.70E-05	Down	5.0	1.3	0.3	0.3	0.0
OS06T0199000-01	Os06g0199000	-2.33	1.49E-06	Down	5.0	169.0	24.3	33.9	8.3
OS05T0361700-01	Os05g0361700	-2.34	7.39E-05	Down	5.1	14.4	3.2	2.9	0.9
EPIOSAT00000017650		-2.34	5.25E-04	Down	5.1	17.4	1.1	3.3	1.3
OS03T0254250-00	Os03g0254250	-2.35	1.39E-02	Down	5.1	2.7	0.5	0.5	0.2
OS06T0222100-00	Os06g0222100	-2.36	2.65E-03	Down	5.1	8.1	0.7	1.6	0.6
EPIOSAT00000021231		-2.37	3.79E-02	Down	5.2	15.4	8.2	3.0	0.6
OS11T0559200-00	Os11g0559200	-2.38	3.88E-06	Down	5.2	1.5	0.3	0.3	0.1
OS02T0571100-01	Os02g0571100	-2.38	3.22E-02	Down	5.2	1.6	0.5	0.3	0.2
OS01T0266400-01	Os01g0266400	-2.38	8.06E-08	Down	5.2	15.2	2.8	2.9	0.3
OS09T0483100-01	Os09g0483100	-2.38	4.43E-07	Down	5.2	10.0	1.3	2.0	0.4
OS10T0504100-00	Os10g0504100	-2.39	1.08E-03	Down	5.2	2.2	0.2	0.4	0.2
OS01T0269500-01	Os01g0269500	-2.39	1.46E-08	Down	5.2	9.0	2.4	1.7	0.0
OS05T0228000-01	Os05g0228000	-2.39	1.63E-03	Down	5.2	0.7	0.1	0.1	0.0
OS04T0607500-02	Os04g0607500	-2.39	1.29E-07	Down	5.3	2.9	0.6	0.6	0.1
OS07T0194550-00	Os07g0194550	-2.40	1.31E-06	Down	5.3	4.3	0.4	0.8	0.2
OS07T0566500-01	Os07g0566500	-2.41	1.96E-02	Down	5.3	0.1	0.0	0.0	0.0
OS11T0514500-01	Os11g0514500	-2.42	1.82E-03	Down	5.4	11.0	4.4	2.1	0.4
OS04T0634900-01	Os04g0634900	-2.42	3.71E-02	Down	5.4	4.8	0.7	0.9	0.4
EPIOSAT00000006653		-2.43	4.70E-05	Down	5.4	4.7	0.1	0.8	0.1
OS04T0607450-01	Os04g0607450	-2.43	5.03E-06	Down	5.4	1.1	0.2	0.2	0.0
OS01T0847100-01	Os01g0847100	-2.44	4.38E-06	Down	5.4	32.9	1.1	6.1	1.7
OS12T0456700-01	Os12g0456700	-2.44	4.50E-02	Down	5.4	0.2	0.0	0.0	0.0
OS11T0127800-01	Os11g0127800	-2.45	1.29E-05	Down	5.5	0.8	0.1	0.1	0.0
OS01T0803900-01	Os01g0803900	-2.46	1.20E-03	Down	5.5	25.3	0.9	4.6	1.6
OS04T0516600-01	Os04g0516600	-2.46	8.34E-03	Down	5.5	2.0	0.9	0.4	0.0
OS06T0141700-01	Os06g0141700	-2.46	1.70E-09	Down	5.5	6.2	0.7	1.1	0.1
OS02T0616100-04	Os02g0616100	-2.47	4.14E-02	Down	5.5	0.3	0.1	0.1	0.0
OS07T0541900-01	Os07g0541900	-2.48	2.24E-10	Down	5.6	4.4	0.4	0.8	0.1
OS04T0481800-01	Os04g0481800	-2.49	4.93E-05	Down	5.6	0.8	0.1	0.1	0.0
OS09T0526700-01	Os09g0526700	-2.49	5.02E-16	Down	5.6	16.2	1.3	2.9	0.2
OS09T0526700-02	Os09g0526700	-2.50	4.60E-14	Down	5.7	16.6	2.0	2.9	0.2
OS08T0544250-00	Os08g0544250	-2.51	6.06E-04	Down	5.7	1.7	0.2	0.3	0.1
OS04T0607500-01	Os04g0607500	-2.51	1.07E-08	Down	5.7	3.6	0.7	0.6	0.1
OS05T0424700-01	Os05g0424700	-2.51	3.58E-02	Down	5.7	5.0	1.0	0.9	0.3

OS10T0341750-00	Os10g0341750	-2.53	1.73E-02	Down	5.8	3.0	0.6	0.5	0.2
OS06T0662550-01	Os06g0662550	-2.53	3.57E-07	Down	5.8	3.0	0.8	0.5	0.0
OS10T0136500-01	Os10g0136500	-2.56	1.51E-02	Down	5.9	0.1	0.0	0.0	0.0
OS04T0635000-01	Os04g0635000	-2.56	1.72E-03	Down	5.9	3.3	0.6	0.6	0.2
OS02T0163533-01	Os02g0163533	-2.57	4.97E-04	Down	5.9	1.8	0.4	0.3	0.1
OS06T0491566-01	Os06g0491566	-2.59	4.66E-03	Down	6.0	0.9	0.4	0.1	0.0
OS08T0544400-01	Os08g0544400	-2.61	4.58E-05	Down	6.1	5.7	0.6	0.9	0.3
OS07T0194500-01	Os07g0194500	-2.61	1.07E-08	Down	6.1	15.8	1.0	2.6	0.6
OS03T0803300-01	Os03g0803300	-2.62	1.83E-10	Down	6.2	7.2	0.3	1.2	0.2
OS11T0608300-01	Os11g0608300	-2.63	6.78E-04	Down	6.2	0.4	0.1	0.1	0.0
OS09T0250800-00	Os09g0250800	-2.63	3.94E-02	Down	6.2	0.1	0.0	0.0	0.0
OS03T0674900-00	Os03g0674900	-2.64	2.11E-03	Down	6.2	0.6	0.1	0.1	0.0
OS06T0695200-00	Os06g0695200	-2.64	5.99E-09	Down	6.2	14.6	1.9	2.4	0.5
OS01T0937500-01	Os01g0937500	-2.66	2.70E-06	Down	6.3	1.1	0.2	0.2	0.0
OS03T0432277-00	Os03g0432277	-2.66	4.27E-05	Down	6.3	4.9	1.3	0.8	0.2
OS01T0318202-01	Os01g0318202	-2.66	3.99E-06	Down	6.3	3.8	0.7	0.6	0.1
OS01T0522400-00	Os01g0522400	-2.67	2.27E-06	Down	6.3	2.3	0.4	0.4	0.1
OS03T0305400-01	Os03g0305400	-2.67	2.20E-15	Down	6.4	18.0	1.5	2.9	0.3
OS11T0701500-01	Os11g0701500	-2.67	1.67E-05	Down	6.4	1.7	0.4	0.3	0.1
OS06T0125200-01	Os06g0125200	-2.68	4.20E-15	Down	6.4	31.4	2.5	4.9	0.7
OS12T0576750-00	Os12g0576750	-2.68	1.22E-09	Down	6.4	8.6	1.6	1.3	0.2
OS05T0312500-01	Os05g0312500	-2.68	2.50E-08	Down	6.4	3.3	0.4	0.5	0.1
OS01T0318400-00	Os01g0318400	-2.68	5.36E-05	Down	6.4	6.6	1.5	1.0	0.3
OS07T0127700-01	Os07g0127700	-2.72	5.02E-08	Down	6.6	30.9	7.3	4.7	0.7
OS01T0185500-00	Os01g0185500	-2.72	9.68E-19	Down	6.6	139.0	21.3	21.2	1.4
OS10T0159033-01	Os10g0159033	-2.75	4.07E-06	Down	6.7	4.7	1.4	0.7	0.1
OS10T0452300-01	Os10g0452300	-2.76	3.20E-04	Down	6.8	14.0	5.9	2.1	0.5
OS09T0287000-01	Os09g0287000	-2.76	6.90E-04	Down	6.8	8.9	3.5	1.3	0.3
OS04T0127100-01	Os04g0127100	-2.80	1.02E-02	Down	6.9	4.2	0.5	0.6	0.3
OS04T0274250-00	Os04g0274250	-2.80	6.69E-05	Down	7.0	1.5	0.3	0.2	0.1
OS12T0423142-00	Os12g0423142	-2.81	4.98E-02	Down	7.0	12.0	7.5	1.7	0.4
OS12T0576732-01	Os12g0576732	-2.81	4.21E-17	Down	7.0	16.4	2.6	2.4	0.2
OS03T0643250-00	Os03g0643250	-2.83	2.67E-10	Down	7.1	22.6	2.2	3.2	0.7
OS01T0129600-00	Os01g0129600	-2.92	6.69E-14	Down	7.6	4.5	0.4	0.6	0.0
OS06T0142300-00	Os06g0142300	-2.94	1.78E-14	Down	7.7	40.3	4.7	5.3	0.9
OS10T0327000-00	Os10g0327000	-2.95	2.09E-03	Down	7.7	0.4	0.1	0.0	0.0
OS08T0514300-00	Os08g0514300	-2.95	3.17E-02	Down	7.7	0.1	0.0	0.0	0.0
OS11T0484700-00	Os11g0484700	-2.96	1.84E-07	Down	7.8	18.1	5.4	2.3	0.1
OS02T0812600-00	Os02g0812600	-2.96	1.17E-07	Down	7.8	1.1	0.1	0.1	0.0
OS03T0595600-00	Os03g0595600	-2.97	8.18E-06	Down	7.8	0.9	0.1	0.1	0.0
OS08T0424100-00	Os08g0424100	-3.00	1.14E-03	Down	8.0	2.3	0.7	0.3	0.1
OS01T0934900-00	Os01g0934900	-3.00	9.77E-15	Down	8.0	4.9	0.5	0.6	0.0
EPIOSAT00000041375		-3.02	5.82E-03	Down	8.1	1.2	0.2	0.2	0.1
OS10T0407200-01	Os10g0407200	-3.02	3.15E-15	Down	8.1	9.5	0.4	1.2	0.2
OS07T0604700-01	Os07g0604700	-3.02	2.37E-11	Down	8.1	80.9	12.8	10.0	2.3
OS07T0197300-00	Os07g0197300	-3.04	1.34E-07	Down	8.2	0.4	0.1	0.1	0.0
OS05T0526200-01	Os05g0526200	-3.05	1.02E-13	Down	8.3	4.1	0.4	0.5	0.1
OS06T0120200-01	Os06g0120200	-3.06	4.80E-02	Down	8.4	0.1	0.0	0.0	0.0
OS09T0243200-01	Os09g0243200	-3.07	5.52E-04	Down	8.4	0.4	0.1	0.0	0.0
OS02T0742700-00	Os02g0742700	-3.08	2.17E-06	Down	8.5	1.3	0.3	0.2	0.0
OS10T0204400-01	Os10g0204400	-3.09	1.55E-07	Down	8.5	179.5	9.2	21.2	6.8
OS05T0376800-01	Os05g0376800	-3.11	1.77E-02	Down	8.7	0.6	0.3	0.1	0.0
OS11T0224040-00	Os11g0224040	-3.12	2.25E-14	Down	8.7	10.1	1.6	1.2	0.2
OS06T0142400-01	Os06g0142400	-3.14	4.02E-24	Down	8.8	89.2	5.4	10.3	1.4
OS08T0178650-00	Os08g0178650	-3.17	3.74E-02	Down	9.0	0.4	0.0	0.1	0.0
OS11T0265850-00	Os11g0265850	-3.18	1.21E-05	Down	9.1	1.3	0.3	0.1	0.0
OS07T0531400-01	Os07g0531400	-3.18	1.22E-09	Down	9.1	11.8	0.5	1.3	0.4
OS03T0182900-01	Os03g0182900	-3.19	4.98E-19	Down	9.1	46.1	4.9	5.1	0.8
OS02T0263432-00	Os02g0263432	-3.19	4.52E-06	Down	9.1	0.4	0.1	0.0	0.0
OS11T0679000-00	Os11g0679000	-3.20	1.46E-02	Down	9.2	0.2	0.0	0.0	0.0
OS04T0168400-01	Os04g0168400	-3.21	1.93E-09	Down	9.2	3.7	0.5	0.4	0.1
OS01T0522100-01	Os01g0522100	-3.21	6.21E-06	Down	9.3	0.6	0.1	0.1	0.0
OS03T0655100-00	Os03g0655100	-3.22	1.80E-13	Down	9.3	7.9	1.2	0.9	0.1
OS07T0674800-01	Os07g0674800	-3.23	3.86E-09	Down	9.4	8.9	2.2	1.0	0.2
OS09T0483500-01	Os09g0483500	-3.24	1.41E-10	Down	9.4	35.9	11.3	3.9	0.6
OS05T0404700-01	Os05g0404700	-3.26	1.80E-10	Down	9.6	6.2	1.6	0.7	0.0
OS10T0515900-01	Os10g0515900	-3.29	6.79E-03	Down	9.8	0.8	0.3	0.1	0.0
OS06T0142350-01	Os06g0142350	-3.30	2.37E-17	Down	9.8	45.2	2.7	4.7	1.1
OS02T0582900-01	Os02g0582900	-3.30	2.03E-07	Down	9.8	4.1	0.9	0.4	0.1

OS11T0700900-02	Os11g0700900	-3.31	1.18E-15	Down	9.9	17.1	3.0	1.7	0.3
OS03T0766600-01	Os03g0766600	-3.31	7.72E-11	Down	9.9	22.6	4.8	2.3	0.5
OS01T0351500-00	Os01g0351500	-3.32	3.04E-03	Down	10.0	0.6	0.2	0.1	0.0
OS02T0802500-01	Os02g0802500	-3.34	5.24E-10	Down	10.1	472.7	47.4	47.0	13.8
OS02T0802500-02	Os02g0802500	-3.35	1.06E-10	Down	10.2	469.0	46.4	46.1	13.2
OS07T0531400-02	Os07g0531400	-3.36	1.42E-12	Down	10.2	13.0	0.6	1.3	0.3
OS12T0516000-00	Os12g0516000	-3.36	6.78E-06	Down	10.3	55.1	15.0	5.4	1.8
OS07T0559000-01	Os07g0559000	-3.38	2.67E-11	Down	10.4	22.0	3.1	2.1	0.6
OS02T0802575-00	Os02g0802575	-3.40	1.65E-09	Down	10.6	280.0	29.6	26.6	8.1
OS02T0808800-01	Os02g0808800	-3.42	3.22E-06	Down	10.7	20.2	10.2	1.9	0.2
OS12T0440300-01	Os12g0440300	-3.44	2.28E-04	Down	10.8	0.5	0.2	0.0	0.0
OS11T0700900-01	Os11g0700900	-3.45	1.18E-15	Down	10.9	13.9	3.0	1.3	0.2
OS10T0516000-00	Os10g0516000	-3.45	8.30E-03	Down	11.0	0.4	0.2	0.0	0.0
OS01T0910900-01	Os01g0910900	-3.47	3.50E-08	Down	11.1	70.2	14.9	6.4	2.0
OS02T0587300-00	Os02g0587300	-3.47	6.69E-15	Down	11.1	49.1	9.4	4.5	0.8
OS06T0141600-01	Os06g0141600	-3.47	5.03E-06	Down	11.1	13.2	3.7	1.2	0.5
OS01T0715201-01	Os01g0715201	-3.54	5.25E-04	Down	11.7	0.5	0.1	0.0	0.0
OS09T0255200-00	Os09g0255200	-3.56	2.67E-05	Down	11.8	5.7	2.5	0.5	0.0
OS07T0117200-00	Os07g0117200	-3.56	9.60E-05	Down	11.8	0.3	0.1	0.0	0.0
OS12T0555100-01	Os12g0555100	-3.58	2.22E-09	Down	12.0	65.7	7.3	5.5	2.0
OS01T0127100-01	Os01g0127100	-3.59	1.66E-06	Down	12.1	49.7	9.9	4.2	2.2
OS12T0278101-01	Os12g0278101	-3.59	7.95E-19	Down	12.1	91.5	17.6	7.6	1.4
OS06T0195150-00	Os06g0195150	-3.60	1.87E-26	Down	12.2	71.6	7.4	5.9	0.3
OS12T0277900-01	Os12g0277900	-3.62	4.98E-19	Down	12.3	88.7	17.2	7.2	1.3
OS06T0142200-02	Os06g0142200	-3.63	4.63E-22	Down	12.4	99.9	19.4	8.2	1.0
OS06T0142200-01	Os06g0142200	-3.63	2.46E-26	Down	12.4	124.5	19.6	10.2	1.3
OS08T0307601-01	Os08g0307601	-3.63	9.38E-08	Down	12.4	2.9	1.0	0.2	0.0
OS10T0327100-00	Os10g0327100	-3.64	1.53E-02	Down	12.4	0.3	0.1	0.0	0.0
OS12T0515800-01	Os12g0515800	-3.64	3.68E-17	Down	12.5	88.9	9.3	7.1	1.8
OS04T0554800-01	Os04g0554800	-3.68	1.38E-03	Down	12.8	0.7	0.3	0.1	0.0
OS12T0197150-00	Os12g0197150	-3.69	4.17E-29	Down	12.9	74.5	9.3	5.8	0.5
OS07T0531200-01	Os07g0531200	-3.70	2.56E-09	Down	13.0	20.2	8.3	1.6	0.2
OS01T0127000-01	Os01g0127000	-3.70	1.34E-06	Down	13.0	107.2	28.2	8.4	4.2
OS12T0249100-00	Os12g0249100	-3.71	1.35E-10	Down	13.1	4.0	0.4	0.3	0.1
OS01T0498802-01	Os01g0498802	-3.72	7.89E-08	Down	13.2	3.3	1.1	0.3	0.1
OS04T0345800-00	Os04g0345800	-3.74	1.24E-21	Down	13.3	11.7	1.5	0.9	0.1
OS06T0686900-01	Os06g0686900	-3.74	1.09E-02	Down	13.4	0.2	0.0	0.0	0.0
OS07T0604600-01	Os07g0604600	-3.74	2.05E-04	Down	13.4	170.2	17.1	12.8	5.4
OS02T0305950-00	Os02g0305950	-3.75	1.15E-06	Down	13.5	8.9	2.3	0.7	0.3
OS11T0701000-01	Os11g0701000	-3.76	2.92E-10	Down	13.5	3.8	1.3	0.3	0.0
OS12T0197100-01	Os12g0197100	-3.78	5.62E-39	Down	13.8	88.1	9.8	6.5	0.5
OS11T0251200-00	Os11g0251200	-3.79	9.55E-16	Down	13.8	3.5	0.8	0.3	0.0
OS11T0127800-02	Os11g0127800	-3.82	8.87E-12	Down	14.1	0.9	0.1	0.1	0.0
OS03T0341000-01	Os03g0341000	-3.83	4.79E-11	Down	14.2	15.8	4.2	1.1	0.3
OS05T0387300-01	Os05g0387300	-3.84	5.12E-07	Down	14.3	1.4	0.4	0.1	0.0
OS05T0489500-01	Os05g0489500	-3.84	1.05E-02	Down	14.4	0.2	0.1	0.0	0.0
OS10T0452100-01	Os10g0452100	-3.86	8.80E-15	Down	14.5	111.1	40.8	7.7	0.3
OS07T0251900-01	Os07g0251900	-3.96	3.21E-14	Down	15.6	0.4	0.0	0.0	0.0
OS01T0568400-00	Os01g0568400	-4.03	2.36E-08	Down	16.3	3.7	0.5	0.2	0.1
OS01T0899387-00	Os01g0899387	-4.03	8.45E-04	Down	16.3	1.7	0.7	0.1	0.1
OS06T0272700-00	Os06g0272700	-4.04	2.03E-03	Down	16.5	0.2	0.1	0.0	0.0
OS11T0701800-01	Os11g0701800	-4.08	4.24E-08	Down	16.9	6.8	1.7	0.4	0.1
OS03T0282166-00	Os03g0282166	-4.10	6.27E-11	Down	17.2	2.4	0.4	0.1	0.0
OS04T0561800-00	Os04g0561800	-4.14	1.28E-25	Down	17.6	12.0	0.9	0.7	0.1
OS11T0687100-01	Os11g0687100	-4.14	3.70E-08	Down	17.7	1.2	0.4	0.1	0.0
OS03T0269300-01	Os03g0269300	-4.17	1.65E-06	Down	18.0	2.8	1.3	0.2	0.0
OS03T0700100-01	Os03g0700100	-4.20	1.88E-03	Down	18.4	1.3	0.7	0.1	0.0
OS04T0353600-01	Os04g0353600	-4.20	2.89E-02	Down	18.4	0.1	0.0	0.0	0.0
OS06T0592500-01	Os06g0592500	-4.22	3.20E-10	Down	18.6	2.4	0.5	0.1	0.0
OS04T0538300-00	Os04g0538300	-4.22	5.55E-09	Down	18.7	8.8	3.2	0.5	0.1
OS01T0155200-00	Os01g0155200	-4.25	4.83E-04	Down	19.0	0.2	0.1	0.0	0.0
OS06T0104900-01	Os06g0104900	-4.26	7.14E-19	Down	19.1	57.0	6.6	3.0	0.8
OS05T0506500-00	Os05g0506500	-4.27	5.72E-06	Down	19.2	0.5	0.1	0.0	0.0
OS08T0169300-00	Os08g0169300	-4.33	4.78E-04	Down	20.1	0.2	0.0	0.0	0.0
OS05T0469701-00	Os05g0469701	-4.33	5.62E-19	Down	20.1	25.9	5.8	1.3	0.3
OS01T0928700-00	Os01g0928700	-4.34	1.14E-02	Down	20.3	0.2	0.1	0.0	0.0
OS07T0270301-00	Os07g0270301	-4.36	3.18E-02	Down	20.5	0.1	0.0	0.0	0.0
OS11T0212100-00	Os11g0212100	-4.48	5.18E-13	Down	22.3	3.2	0.2	0.1	0.1
OS05T0469600-01	Os05g0469600	-4.50	1.02E-18	Down	22.7	94.2	23.7	4.2	1.0

Transcript	Gene RAP ID	log2(strong/ weak) FC	log2(strong /weak) FDR	Up or Down	Fold change (FC)	Weak FPKM mean	SE	Strong FPKM mean	SE
OS01T0550900-00	Os01g0550900	-4.51	1.14E-04	Down	22.7	0.2	0.0	0.0	0.0
OS03T0282100-00	Os03g0282100	-4.56	9.51E-10	Down	23.5	3.6	0.5	0.2	0.1
OS05T0469812-00	Os05g0469812	-4.56	3.63E-15	Down	23.6	22.5	6.8	1.0	0.2
OS03T0269300-02	Os03g0269300	-4.57	4.09E-06	Down	23.8	0.6	0.3	0.0	0.0
OS07T0270800-00	Os07g0270800	-4.57	2.95E-03	Down	23.8	0.1	0.0	0.0	0.0
OS03T0426300-00	Os03g0426300	-4.58	7.56E-09	Down	23.9	1.6	0.4	0.1	0.0
OS01T0729600-01	Os01g0729600	-4.60	3.59E-09	Down	24.3	2.0	0.7	0.1	0.0
OS08T0534300-00	Os08g0534300	-4.61	5.47E-08	Down	24.5	8.0	2.3	0.3	0.1
OS03T0765500-00	Os03g0765500	-4.65	4.66E-07	Down	25.1	0.6	0.1	0.0	0.0
OS11T0212000-00	Os11g0212000	-4.65	2.84E-10	Down	25.2	1.3	0.3	0.0	0.0
OS08T0518900-01	Os08g0518900	-4.69	1.58E-11	Down	25.8	17.8	8.5	0.7	0.1
EPIOSAT00000004002		-4.71	1.00E-02	Down	26.1	1.3	0.7	0.0	0.0
OS05T0469800-00	Os05g0469800	-4.73	4.98E-19	Down	26.5	79.3	25.8	3.0	0.6
OS08T0534950-00	Os08g0534950	-4.73	5.79E-12	Down	26.5	8.3	2.7	0.3	0.1
OS11T0434850-00	Os11g0434850	-4.78	9.33E-03	Down	27.5	0.2	0.1	0.0	0.0
OS03T0432100-01	Os03g0432100	-4.80	3.14E-09	Down	27.9	638.8	115.7	23.0	8.4
OS05T0205400-00	Os05g0205400	-4.87	2.71E-06	Down	29.3	1.7	0.6	0.0	0.0
OS06T0578700-01	Os06g0578700	-4.88	2.60E-05	Down	29.4	0.8	0.4	0.0	0.0
OS10T0451700-01	Os10g0451700	-4.89	2.95E-10	Down	29.7	35.2	14.3	1.2	0.4
OS05T0218981-00	Os05g0218981	-5.01	4.06E-16	Down	32.2	3.9	0.5	0.1	0.1
OS10T0451900-01	Os10g0451900	-5.01	7.01E-06	Down	32.3	3.9	2.0	0.1	0.0
OS09T0348400-00	Os09g0348400	-5.09	1.20E-05	Down	33.9	0.3	0.1	0.0	0.0
OS01T0729700-01	Os01g0729700	-5.13	1.11E-10	Down	35.1	0.8	0.2	0.0	0.0
OS11T0261300-00	Os11g0261300	-5.13	2.70E-04	Down	35.1	0.1	0.0	0.0	0.0
OS01T0546100-01	Os01g0546100	-5.16	1.51E-10	Down	35.9	4.2	0.6	0.1	0.1
OS12T0614200-00	Os12g0614200	-5.34	3.62E-02	Down	40.5	0.1	0.0	0.0	0.0
OS11T0164200-00	Os11g0164200	-5.35	2.14E-11	Down	40.8	1.3	0.4	0.0	0.0
OS02T0587800-01	Os02g0587800	-5.41	2.03E-04	Down	42.4	1.5	0.8	0.0	0.0
OS06T0705200-00	Os06g0705200	-5.43	2.47E-07	Down	43.2	0.2	0.0	0.0	0.0
OS09T0314800-00	Os09g0314800	-5.44	3.78E-15	Down	43.5	1.2	0.3	0.0	0.0
OS06T0105100-00	Os06g0105100	-5.44	1.71E-05	Down	43.5	3.3	2.2	0.1	0.0
OS08T0480400-01	Os08g0480400	-5.45	2.17E-14	Down	43.8	3.3	0.8	0.1	0.0
OS03T0768450-00	Os03g0768450	-5.47	3.83E-02	Down	44.2	0.3	0.1	0.0	0.0
OS09T0265225-00	Os09g0265225	-5.49	9.97E-15	Down	45.0	4.5	1.2	0.1	0.0
OS09T0323500-01	Os09g0323500	-5.53	4.82E-22	Down	46.1	13.4	4.1	0.3	0.1
OS01T0660400-01	Os01g0660400	-5.56	3.18E-06	Down	47.1	1.0	0.3	0.0	0.0
OS03T0128866-00	Os03g0128866	-5.62	1.80E-02	Down	49.3	0.1	0.0	0.0	0.0
OS06T0705250-00	Os06g0705250	-5.65	7.75E-03	Down	50.2	0.1	0.0	0.0	0.0
OS10T0150800-01	Os10g0150800	-5.67	9.73E-06	Down	50.8	1.3	0.6	0.0	0.0
OS01T0962900-00	Os01g0962900	-5.69	6.71E-03	Down	51.7	0.4	0.1	0.0	0.0
OS09T0564600-01	Os09g0564600	-5.70	7.01E-42	Down	52.1	77.6	3.2	1.5	0.4
OS05T0247800-01	Os05g0247800	-5.72	5.81E-18	Down	52.8	74.0	12.8	1.4	0.5
OS10T0122200-00	Os10g0122200	-5.80	3.03E-08	Down	55.7	0.4	0.1	0.0	0.0
OS06T0641600-00	Os06g0641600	-5.91	1.01E-15	Down	60.3	1.5	0.3	0.0	0.0
OS02T0811400-00	Os02g0811400	-5.95	3.18E-06	Down	61.9	6.6	4.1	0.1	0.0
OS04T0612700-01	Os04g0612700	-5.97	1.28E-12	Down	62.7	32.1	13.7	0.5	0.2
OS07T0127900-00	Os07g0127900	-6.09	1.28E-08	Down	68.2	1.9	0.8	0.0	0.0
EPIOSAT00000027275		-6.15	5.17E-04	Down	70.9	0.8	0.2	0.0	0.0
OS11T0687200-02	Os11g0687200	-6.29	9.72E-03	Down	78.0	0.1	0.0	0.0	0.0
EPIOSAT00000045543		-6.31	1.85E-02	Down	79.4	0.7	0.3	0.0	0.0
OS06T0279900-00	Os06g0279900	-6.37	3.78E-15	Down	82.9	12.1	5.4	0.1	0.1
OS11T0556733-00	Os11g0556733	-6.39	3.16E-02	Down	83.6	0.1	0.1	0.0	0.0
OS08T0226166-00	Os08g0226166	-6.44	2.60E-03	Down	86.9	0.3	0.2	0.0	0.0
OS04T0371200-00	Os04g0371200	-6.51	1.61E-20	Down	91.3	3.4	0.9	0.0	0.0
EPIOSAT00000045416		-6.52	7.53E-03	Down	92.1	0.8	0.4	0.0	0.0
OS07T0501100-01	Os07g0501100	-6.60	4.01E-04	Down	97.3	0.5	0.3	0.0	0.0
OS02T0208675-00	Os02g0208675	-6.78	1.84E-05	Down	109.9	0.2	0.1	0.0	0.0
OS10T0122300-00	Os10g0122300	-7.12	1.97E-04	Down	139.0	0.2	0.1	0.0	0.0
OS01T0928600-01	Os01g0928600	-7.14	4.01E-04	Down	140.9	0.6	0.4	0.0	0.0
OS11T0616100-00	Os11g0616100	-7.14	2.67E-05	Down	140.9	0.3	0.1	0.0	0.0
OS02T0587600-00	Os02g0587600	-7.57	2.26E-20	Down	190.0	12.8	4.1	0.1	0.0
OS05T0248200-01	Os05g0248200	-7.57	2.29E-26	Down	190.2	26.1	10.2	0.1	0.0
OS04T0270900-00	Os04g0270900	-7.67	7.22E-08	Down	204.3	0.3	0.1	0.0	0.0
OS02T0586000-01	Os02g0586000	-8.50	1.23E-11	Down	361.3	12.7	7.1	0.0	0.0
OS06T0641575-00	Os06g0641575	-9.12	9.62E-18	Down	557.3	0.8	0.2	0.0	0.0
OS09T0565000-00	Os09g0565000	-10.82	5.43E-12	Down	1804.8	4.4	2.5	0.0	0.0

Appendix 3

Coelho Filho, M., Colebrook, E., **Lloyd, D. P. A.**, Webster, C., Mooney, S., Phillips, A., Hedden, P. & Whalley, W. (2013). The involvement of gibberellin signalling in the effect of soil resistance to root penetration on leaf elongation and tiller number in wheat. *Plant and Soil*, **371**, 81-94.

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REGULAR ARTICLE

The involvement of gibberellin signalling in the effect of soil resistance to root penetration on leaf elongation and tiller number in wheat

Mauricio A. Coelho Filho · Ellen H. Colebrook · David P. A. Lloyd · Colin P. Webster · Sacha J. Mooney · Andrew L. Phillips · Peter Hedden · William R. Whalley

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Abstract
Background and aims The concept of root-sourced chemical signals that affect shoot growth in response to drought is widely reported; in particular the role of ABA in regulating stomatal conductance has received much attention. ABA, alone, does not fully explain all the effects of abiotic stresses in the root zone on shoot architecture. An increase in mechanical impedance, which can occur on even relatively modest soil drying, results in reduced root and shoot growth, processes that are potentially regulated by gibberellins (GAs).
Methods In this study we explored the role of mechanical impedance and exogenous gibberellin (GA_3) on root and shoot architecture in wheat seedlings containing the *Rht-B1a* (tall), *Rht-B1b* (semi-dwarf) or *Rht-B1c* (dwarf) alleles in the April-Bearded or Mercia backgrounds. Our experiments were based on the use of the sand culture system which allows the mechanical impedance of the root growth environment to remain constant and independent of water and nutrient availability. We investigated the effects of the application of exogenous GA_3 to the root system.
Results We found that impeding soil reduced leaf elongation in the tall and semi-dwarf lines, confirming the stunting effect of mechanical impedance which is widely reported. However, leaf elongation in the dwarf lines was not affected by root impedance. Application of GA_3 to the roots restored leaf elongation in the tall and semi-dwarf lines growing in impeding soil, with some growth response even in the dwarf line, the longest leaves being obtained when GA was applied to impeded roots of a tall line. Both exogenous GA and root impedance reduced the number of tillers, but there was no interaction with the *Rht* genotype. The genetic background did not affect the results.
Conclusion We suggest that the GA signalling pathway has an unidentified role in the leaf elongation response to mechanical impedance to root growth.

Keywords Root · Leaf elongation · Tiller · Impedance · GA · Dwarfing alleles

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M. A. Coelho Filho
Embrapa Cassava and Fruits,
Rua Embrapa s/n, C.P. 07,
CEP 44380 000 Cruz das Almas, Bahia, Brazil

M. A. Coelho Filho · E. H. Colebrook · D. P. A. Lloyd · C. P. Webster · A. L. Phillips · P. Hedden · W. R. Whalley (✉)
Rothamsted Research,
Harpenden, Hertfordshire AL5 2JQ, UK
e-mail: richard.whalley@rothamsted.ac.uk

D. P. A. Lloyd · S. J. Mooney
University of Nottingham,
Sutton Bonington LE12 5RD, UK

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