The role of *SDG8i* from the resurrection grass *Sporobolus stapfianus* in ectopic expression system of *Arabidopsis*

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This thesis is presented in total fulfillment of the requirements of the degree of Doctor of Philosophy

May 2014

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Abstract

Isolation of gene transcripts from desiccated leaf tissue of the resurrection grass *Sporobolus stapfianus* Gandoger, resulted in the identification of the gene, *SDG8i*, encoding a Group 1 glycosyltransferase (UGT). Glycolsyltransferases transfer a sugar to a number of acceptor molecules, including hormones and secondary metabolites, changing the solubility, stability and biological activity of these compounds. Functional analysis of the *SDG8i* was undertaken in *Arabidopsis thaliana* because no protocol for transformation of resurrection grasses exists. The phenotype of transgenic *A. thaliana* plants constitutively over-expressing the *SDG8i* UDP-glucosyltransferase under the control of the CaMV 35S promoter indicates that the glucosyltransferase may modulate the activity of both growth- and stress-related hormones.

Plants overexpressing the UGT show elevated auxin levels and results suggest that the enzyme acts downstream of ABA to reduce drought-induced senescence. *In vitro* analysis of the activity of the UGT recombinant protein product indicates that *SDG8i* encodes a glucosyltransferase (SL-UGT) that potentially can glycosylate the synthetic analogue GR24, suggesting a link with strigolactone-related processes. As well as mediating the interactions of host plants with symbiotic fungi or parasitic plants, the well-conserved strigolactone hormone signalling system contributes to environmental regulation of plant growth. The phenotypes of *SDG8i* transgenic plants exhibit hyperbranching and a reduced ability to stimulate strigolactone-dependent germination of *Orobanchaceae* (*Phelipanche ramosa*) seed. The results indicate that *SDG8i* expression negatively affects the bioactivity of a strigolactone-like compound/s produced by *Arabidopsis* that mediates environmentally-induced repression of cell division and expansion, both during normal development and in response to stress. This leads to enhanced growth, reduced senescence, and a substantial improvement in protoplasmic drought tolerance in *SDG8i* transgenic plants. Therefore, overexpression of *SDG8i* confers substantial increases in growth rate and seed yield under non-stress conditions as well as large improvements in survival under cold-, salt- and drought-stress, thus providing a novel means of improving productivity in crop plants. Two putative transcription factors, *SDG7y* and *SDG10y* that may regulate *SDG8i* expression have previously been identified by the yeast-one

hybrid system. The transgenic plants over-expressing *SDG7y* and *SDG10y* displayed similar phenotypes to that of *SDG8i* transgenics with substantial increases in shoot growth and shoot branching, suggesting they are driving expression of an *Arabidopsis SDG8i* orthologue.

Declaration

I hereby declare that this thesis has been written by me, that it contains no material which has been accepted for the award of any previous degree and that, to the best of my knowledge and belief, this thesis contains no material previously published, except where due reference is made in the text of the thesis.

Sharmin Islam
22 May 2014

Acknowledgements

I would like to extend my sincere thanks to my supervisor, Dr. Cecilia Blomstedt for her patient guidance, stimulating academic deliberations and kind support throughout my doctoral research and thesis writing.

I would like to express my profound gratitude to my co-supervisor, Dr. Alan Neale for his splendid supervision and support during my research life at Monash University. His passion for mentoring young researchers helped me immensely in completing my research journey.

I am very grateful to my co-supervisor, Dr. Ros Gleadow for her guidance, advice, support and assistance. My special thanks to Ros for her continuous encouragement particularly during the thesis completion phase.

I am thankful to my former supervisor, Prof. John Hamill for his excellent supervision, support and assistance. A big thank you goes to Dr. Don Gaff for his time and valuable suggestions on planning and design of the drought tolerance experiment.

I wish to thank all my lab colleagues, especially Cara Griffiths for her research support and also other members of the department for their friendship, support and laughter.

I am indebted to my parents for their love and understanding. A special thank you to my dad, who has been a source of encouragement for my PhD.

My beloved husband Akhter deserves a special mention for his love, care, understanding and much needed support throughout my PhD program, and most importantly his sacrifice during the second half of my candidature. A very special thank you to Akhter.

Finally, our always joyful son Rayyan has helped me reach the pinnacle of my drive, energy and inspiration to complete my thesis so that I could give him more time that he needs in the prime time of his early childhood. And this thesis is dedicated to Rayyan.

Chapter 1

Literature Review

1.1 Introduction

Drought is a serious environmental problem that limits crop yield. Understanding the underlying mechanisms of drought tolerance and identifying genes for improving this important trait, is therefore essential as it may ultimately reveal strategies for the production of drought resistant crops. Desiccation-tolerant plants that can adapt to extremely dehydrated environments have been studied at molecular levels. Among the few species studied, the monocotyledonous *Sporobolus stapfianus* Gandoger plant offers several advantages for examining gene expression in relation to desiccation tolerance. *S. stapfianus* has the ability to remain viable in the desiccated state for several years and is able to fully recover upon rehydration (Gaff and Ellis, 1974). The rehydrated *Sporobolus* plant grows very quickly restoring normal metabolism within 24 hours and is therefore useful as a versatile research tool for investigating genes for increased growth rate (Blomstedt *et al.*, 2010) as well as stress tolerance (Neale et al., 2000; Oliver et al., 2011b).

The characterization of drought-responsive genes that are expressed in desiccation-tolerant *Sporobolus* tissue has the potential to reveal some of the mechanisms which this plant utilizes to cope with environmental stress; such as the protection of cellular components during dehydration and rehydration, ability to rapidly adjust growth in response to dehydration, to inhibit dehydration-induced senescence programs, and to reinstitute photosynthesis upon rehydration (Gaff *et al.*, 2009; Blomstedt *et al.*, 2010). The *Sporobolus SDG8i* gene encodes a UGT whose transcript levels increase significantly under severe water deficit (Le *et al.*, 2007). In plants, UGTs are involved in the biosynthesis of plant natural products and in the regulation of plant hormones and therefore play important roles in regulating cell homeostasis, plant growth and development and environmental stress responses (Lim and Bowles, 2004). Hence, the study of UGTs and their roles in plants may contribute to further improvement in crop.

1.2 Drought-tolerance versus desiccation-tolerance

Drought decreases soil water potential which restricts a plants ability to take up water by causing an internal water deficit (Munns, 2002). In most plants, drought induces various physiological and biochemical responses including closing of stomata which restricts photosynthesis as well as slowing plant growth and development. This in turn may evoke a number of responses at the whole plant and cellular levels which allow plants to avoid water loss, take up water at decreased soil water potential or tolerate low water content (Verslues *et al.*, 2006). Drought escape, avoidance and tolerance are important adaptive mechanisms of plants exposed to drought (Levitt, 1972; Turner, 1986). Drought escape is a phenomenon found in some desert plants which exhibit very short life cycle, and denotes the ability of a plant to complete its life cycle before the outset of drought and to go in a state of dormancy before the outset of the dry season (Levitt, 1980). When both soil water content and water potential are low, plants must maintain a low water potential in the tissue to avoid water loss either by increasing water uptake from soil or reducing transpiration (Blum, 2005). This balance can be achieved by stomatal closure in the short term and also by increasing root/shoot ratio in the longer term and are known as dehydration avoidance (Liu *et al.*, 2011). The best examples of dehydration tolerance are desiccation-tolerant plants that can regain normal function from the fully air-dried state after rehydration (Oliver *et al.*, 2000; Vicre *et al.*, 2004).

During desiccation, cells retain only a small quantity of water due to loss of a major portion of the protoplasmic water (Bartels and Salamini, 2001). It is therefore important to maintain the integrity of the cell membrane and to prevent denaturation of some important proteins to tolerate such severe water deficit condition. Desiccation tolerance is commonly found in the reproductive tissue (pollen and seed) in a large number of plants (Oliver, 1996) whilst the vegetative tissue of most plants are unable to survive desiccation to the air-dry state. However, some plant species from every major class, except gymnosperms, can tolerate desiccation in vegetative tissue. This response is cell autonomous, with each individual cell capable of becoming desiccation tolerant (Bewley and Krochko, 1982). Such plants are termed poikilohydric or resurrection plants (Gaff, 1971). About 100 angiosperms have been classified as resurrection plants and include monocotyledonous plants, such as *S. stapfianus* and

Xerophyta viscose, and dicotyledonous plants, such as *Myrothamnus flabellifolia* and *Craterostigma plantagineum* (Gaff *et al.*, 1997).

Both drought-tolerance and desiccation-tolerance mechanisms in plants have been studied at the molecular level. When water stress experienced by non-desiccation tolerant plants is too severe or too prolonged, due to tissue dehydration plants die. Desiccation-tolerant plants under this condition go into a state of anhydrobiosis, their normal cellular functions being completely stopped (Rascio and Rocca, 2005). Desiccation-tolerant plants can stay in this state for years and once tissues are rehydrated they can resume their normal metabolic activities (Gaff, 1977). Desiccation tolerance mechanisms allow cells to deal with dehydration-induced changes in macromolecules and membranes, the accumulation of toxic substances and free radicals, and turgor loss (Rascio and Rocca, 2005). In angiosperms, the molecular systems required for cell recovery following rehydration and repair phases must be induced during dehydration (Dace *et al.*, 1998; Oliver *et al.*, 1998; Neale *et al.*, 2000; Cooper and Farrant, 2002; Blomstedt *et al.*, 2010). Protective mechanisms used by the desiccation-tolerant plants to prevent dehydration stress have been widely reviewed which includes accumulation of proteins, sugars, compatible solutes and free radical scavenging systems, shown to be involved in the maintenance of subcellular integrity (Farrant, 2000; Oliver *et al.*, 2000; Scott, 2000; Vicre *et al.*, 2004; Farrant *et al.*, 2007; Gaff *et al.*, 2009).

Lower order resurrection plants, such as lichens, algae and bryophytes use different mechanisms to tolerate desiccation. Lower order resurrection plants, in particular the bryophytes (mosses and liverworts), appear to employ repair mechanisms following rehydration which reverse the damage suffered during the desiccation and rehydration stages (Oliver and Bewley, 1997; Oliver *et al.*, 1998). This allows frequent and rapid desiccation to be tolerated. Studies suggest that these mechanisms include constitutive (rather than inductive) cellular protection components and a rehydration-induced recovery process to repair cellular damage associated with rapid desiccation and subsequent rehydration (Oliver *et al.*, 1998). A good example here is the desiccation-tolerant moss *Tortula ruralis*, which has developed a rapid desiccation tolerance mechanism (Proctor, 2001). In the natural habitats of these plants, the air-dried state may be reached in less than one hour (Bohnert, 2000).

1.3 Regulation of drought responses in plants

1.3.1 Genes involved in the response to drought

Desiccation tolerance is controlled by many genes or proteins. Some resurrection species have been extensively studied for understanding the molecular basis of desiccation tolerance. Most resurrection plants with large genomes are unsuitable for genetic approaches. However, recent advances in sequencing technologies now allow large-scale analyses of gene expression even without a reference genome (Dinakar and Bartels, 2013). Transcriptomics or mRNA expression profiling captures spatial and temporal gene expression and quantifies RNAs under different conditions (Dinakar and Bartels, 2013). Deep RNA sequencing (RNA-seq) have been used in several resurrection plants such as transcriptome and metabolomic analysis in *C. plantagineum* (Rodriguez *et al*., 2010), proteome analysis in *Haberlea rhodopensis* (Gechev *et al.*, 2012), *Selaginella tamariscina* (Wang *et al.*, 2010), *Xerophyta viscosa* (Ingle *et al.*, 2007), *Boea hygrometrica* (Jiang *et al.*, 2007), *S. stapfianus* (Oliver *et al.*, 2011a; Oliver *et al.*, 2011b) and metabolomic analysis in *Selaginella lepidophylla* (Yobi *et al*., 2013). Microarray studies in several plant species have identified hundreds of genes which are induced under drought (Bockel *et al.*, 1998; Seki *et al.*, 2001; Seki *et al.*, 2002; Shinozaki and Yamaguchi-Shinozaki, 2007). However, expressed sequence tags (ESTs) were used most extensively for transcriptome analysis which is restricted to the number of sequenced cDNAs (Dinakar and Bartels, 2013). Gene expression studies and EST sequencing have been performed in some resurrection species. Global analyses of transcriptome have thus far been reported for *C. plantagineum* and *H. rhodopensis* (Rodriguez *et al.*, 2010; Gechev *et al.*, 2013). The products of genes which are induced or upregulated by dehydration may be grouped into different categories which include: proteins with protective properties, membrane proteins involved in transport process, enzymes related to carbohydrate metabolism, and compatible osmolyte production or to cell antioxidant systems, regulatory molecules such as transcription factors, kinases or other putative signaling molecules and also proteins with unknown function. The up-regulation and down-regulation of the various genes involved in the molecular responses to dehydration play an important part in the ability of resurrection plants to survive desiccation.

1.3.2 Drought influences on plant metabolism and secondary metabolite production

Drought-induced gene expression may lead to substantial changes in carbohydrate metabolism and photosynthesis-related processes. Drought causes stomata to close and decrease the intracellular CO_2 concentration resulting in the inhibition of carbon assimilation and loss of photosynthetic capacity (Schwab *et al.*, 1989). In many plants these changes are mediated by increased accumulation of ABA during water deficit (Wilkinson and Davies, 2002; Lee *et al.*, 2006). In some, but not all, resurrection plants, the changes in the expression of most genes related to desiccation-tolerance are ABA-dependent (Bray, 2002; Wilkinson and Davies, 2002). The steady-state levels of transcripts related to photosynthesis are often down-regulated in response to drought, for example, in *C. plantagenium* the transcript for the small subunit of the Rubisco enzyme decreases (Bernacchia *et al.*, 1996). However, resurrection plants have the ability to resume full photosynthetic activity upon rehydration. For example, this has been shown in *C. plantagineum,* where respiration is quickly restored when water content in the rehydrating plant reaches about 20% of its initial value (Bartels and Salamini, 2001).

Many plants accumulate non-toxic 'compatible' solutes such as proline and glycine-betaine during drought, which results in increased cellular osmolarity leading to an influx, or reduced efflux, of water from cells (Hare *et al.*, 1998; Hasewaga *et al.*, 2000; Ashraf and Harris, 2004; Rascio and Rocca, 2005; Ashraf and Foolad, 2007). These compatible solutes protect cells during drought stress through stabilization of cytoplasmic constituents, ion sequestration and increased water retention. Water deficit in plants also results in accumulation of sugars, most common of which are sucrose and trehalose, a non-reducing disaccharide of glucose (Scott, 2000; Illing *et al.*, 2005; Toldi *et al.*, 2009). During dehydration, resurrection plant *C. plantagenium* shows a significant change in carbohydrate metabolism (Bartels and Salamini, 2001). Photosynthetically active leaves contain large amount of the unusual C8 sugar octulose, which comprises 90% of soluble sugars in this species, corresponding to up to 400 mg g^{-1} of lyophilized leaf material. The level of octulose declines and sucrose accumulates upon dehydration whereas the reverse is observed during rehydration (Bianchi *et al.*, 1991). Most plants including, resurrection plants, accumulate sucrose as the major sugar during dehydration, but trehalose accumulation has also been detected in a number of resurrection

plants (Wingler, 2002). *A. thaliana* has at least one gene which encodes trehalose-6-phosphate phosphatase is required for trehalose synthesis (Vogel *et al.*, 1998). These disaccharides may act as a polar replacement for water within the cell, where the hydrogen bonding from the sugars helps maintain cell function and protein structure (Allison *et al.*, 1999; Goyal *et al.*, 2005). At increased levels of dehydration, the efflux of water continues and causes the sugars within the cell to vitrify into a glassy state. The glassy state causes reduced molecular mobility within the cell, protecting protein structure and membrane integrity (Crowe *et al.*, 1997; Buitink and Leprince, 2004). A number of researches have delineated the importance of sugars in drought stress tolerance. Overexpression of genes encoding trehalose biosynthetic enzymes from *E. coli* accumulate higher levels (3-10 fold) of trehalose and showed increased tolerance to salt, drought and low temperature stresses in transgenic rice (Garg *et al.*, 2002).

In addition to the accumulation of osmolytes, the ability of a plant to survive drought stress depends upon the induction of a number of proteins which protect the cells from damage during water stress (Bray, 1997; Bray, 2007; Farooq *et al.*, 2009). Resurrection plants use a similar set of proteins and metabolites to combat the cellular stresses associated with both the early stages of mild water stress and the later stages of severe water deficit. Late embryogenesis abundant (LEA) proteins are commonly synthesized in both desiccation-tolerant and desiccation-sensitive plants under drought (Close, 1997; Garay-Arroyo *et al.*, 2000; Battaglia *et al.*, 2008; Hundertmark and Hincha, 2008). Several cDNAs representing dehydration-induced transcripts in *S. stapfianus* has been shown to code for proteins related to LEAs (Blomstedt *et al.*, 1998a). LEA proteins are classified into six different groups based on their expression pattern and sequence (Cuming, 1999; Tunnacliffe and Wise, 2007; Hundertmark and Hincha, 2008). LEAs are usually produced during late embryo development, and can comprise up to 4% of cellular protein (Roberts *et al.*, 1993). However, their accumulation was also seen in vegetative organs that experienced water-deficit in response to ABA (Wolkers *et al.*, 2001; Wakui and Takahata, 2002; Liang *et al.*, 2004; Grelet *et al.*, 2005). LEA proteins are believed to play various roles during desiccation, which include protection of cells by sequestering ions, binding water, acting as free radical scavengers and as molecular chaperones (Umezawa *et al.*, 2006). LEA proteins are thought to act in combination with different compatible solutes to help maintain protein and membrane structure and

renaturation of unfolded proteins (Dure *et al.*, 1989; Close, 1996; Hara *et al.*, 2004). Transgenic rice, tobacco and *Arabidopsis,* over-expressing LEA genes, have been shown to improved drought tolerance (Figueras *et al.*, 2004; Wang *et al.*, 2006; Xiao *et al.*, 2007). The expression of *HVA1*, a group 3 *LEA* gene from barley, which protects cell membrane from injury during drought, showed drought tolerance in transgenic rice and wheat plants, respectively (Xu *et al.*, 1996; Sivamani *et al.*, 2000; Chandra Babu *et al.*, 2004). Dehydrins, the group 2 LEA proteins, have been identified in cyanobacteria and plants, including gymnosperms and angiosperms (Close, 1996) and, like other LEA proteins, are hydrophilic in nature and remain soluble at high temperatures and can reduce cellular damage caused by dehydration (Reyes *et al.*, 2008). During drought, production of dehydrins by accumulated ABA improves drought tolerance in plants (Xiao and Nassuth, 2006). In transgenic *A. thaliana*, constitutive expression of the cold-responsive *Arabidopsis COR15A* dehydrin gene results in an increase in the freezing tolerance of transgenic plants (Artus *et al.*, 1996). In wheat, the level of dehydrin expression in response to water stress is directly correlated with the level of drought tolerance in several cultivars (Lopez *et al.*, 2003). Recent studies have indicated that many LEAs may be categorized as intrinsically disordered proteins (IDP), that is, they are a member a group of proteins where the structure is formed only as the plant dehydrates and cellular components require protection against desiccation induced damage (Uversky *et al.*, 2009; Tompa, 2012). A class 2 LEA, CDeT11-24, from *C. plantaginium* has been shown to be an IDP which is able to protect two enzymes, citrate synthase and lactate dehydrogenase, during desiccation (Petersen *et al.*, 2012).

1.3.3 Stress signal transduction pathways

The signal transduction pathways in response to drought and cold stress in *A. thaliana* are shown in Fig. 1.1. Several pathways may operate independently of the perception of an abiotic stress signal. For example, the *Arabidopsis EARLY RESPONSIVE TO DEHYDRATION* STRESS 1 (*ERD1*) gene, encodes a putative protein protease targeted to the chloroplast, is developmentally up-regulated by senescence, but is also induced by salinity and drought (Nakashima *et al.*, 1997). Analysis of *ERD1* promoter shows the involvement of two different *cis*-acting elements in induction of senescence and drought stress (Simpson *et al.*, 2003).

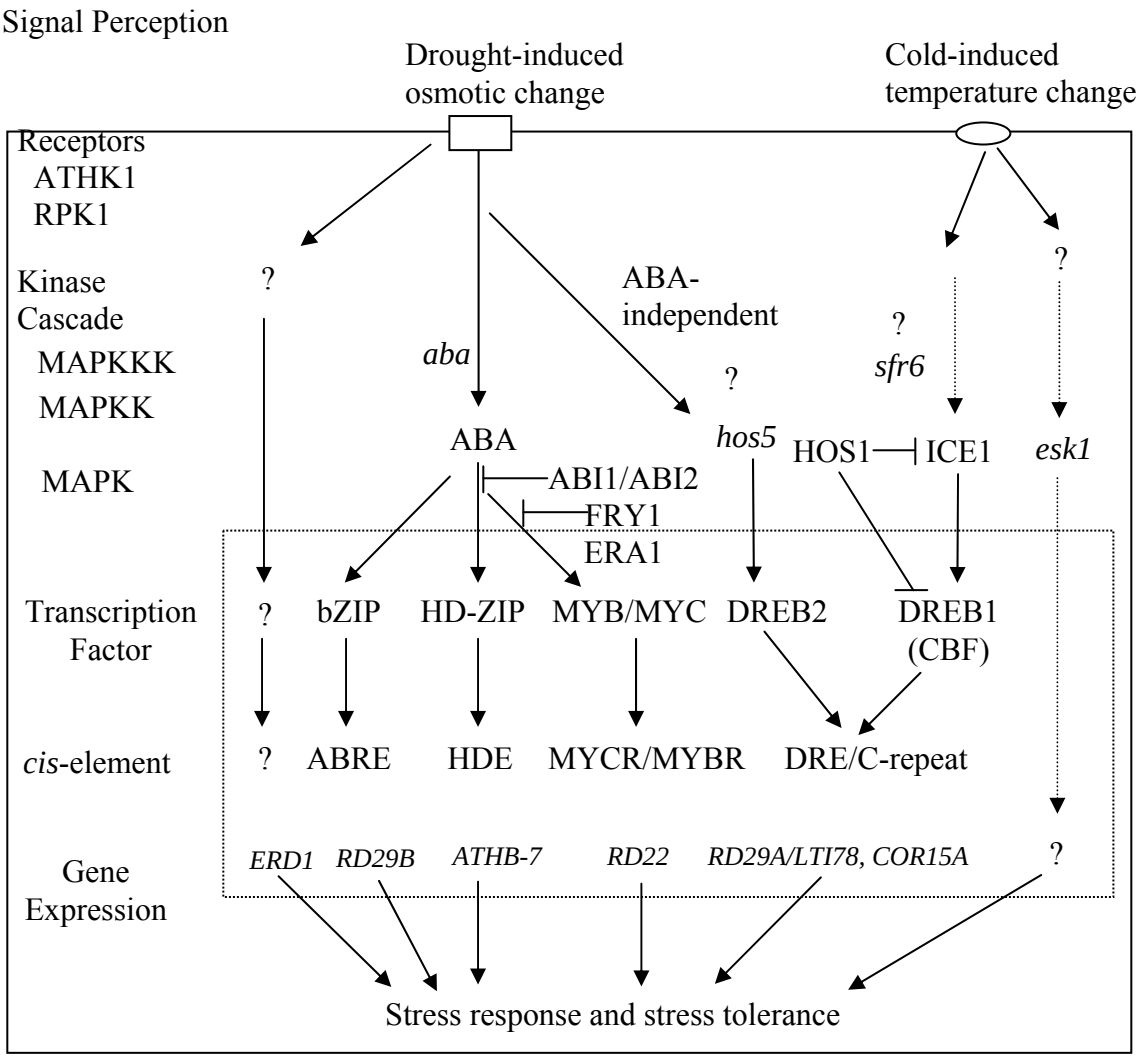


Fig. 1.1 Drought and Cold Stress Signal Transduction Pathways in *A. thaliana*
The signal generated by an osmotic or temperature change is perceived by receptors (square and oval boxes) located on the cell membranes such as ATHK1 and RPK1. The signal is transduced by kinases such as MAPK cascades. MAPKs enter the nucleus (dotted box) to activate transcription factors that, in turn, bind to distinct *cis*-elements in the promoters of stress-responsive genes and activate their transcription. Some pathways are ABA dependent, whilst others are ABA-independent. Positive and negative factors regulate the stress signal transduction pathways. ERA1 enhances the ABA-mediated response, while ABI1, ABI2 and FRY1 are negative regulators of the ABA-dependent pathway. HOS1 is a negative regulator of the ICE1 and DREB1/CBF transcription factors that mediate the cold stress response. Figure adapted from (Shinozaki and Yamaguchi-Shinozaki, 2000).

eskimo1 (*esk1*) mutant in *Arabidopsis* is involved in responses to cold (Xin and Browse, 1998; Xin *et al.*, 2007) via a dehydration-responsive element (DRE)-independent process (Knight *et al.*, 1999). In *A. thaliana*, there appears to be four separate signal transduction pathways: two ABA-dependent and two ABA-independent pathways (Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2000, 2007) (Fig. 1.1). These signaling pathways

do not work in isolation from each other, rather, significant cross-talk occurs between the pathways (Ishitani *et al.*, 1997; Yamaguchi-Shinozaki and Shinozaki, 2005).

The stress signals are perceived at the cell surface by receptors ATHK1 and RPK1. As well as being involved in abiotic stress mitogen-activated protein kinases (MAPKs) are involved in the signal transduction pathways in response to wounding, mechanical stimuli such as touch and fungal pathogen attack and plant hormones, such as ABA, auxin and ethylene in model and crop plants (Hirt, 1997). A MAPK cascade simply consists of three kinases: a MAP3K, a MAP2K and a MAPK (Wrzaczek and Hirt, 2001; Colcombet and Hirt, 2009). MAP3K (MAPK kinase kinases) are activated following the perception of an extracellular signal by various mechanisms including phosphorylation. Analysis of the *Arabidopsis* and rice genomes show that plants in general have at least 20 MAPK pathways involved in stress signal transduction (Wrzaczek and Hirt, 2001). Genes encoding MAPKKKs, *OsEDR1* (rice) and *NPK1* (tobacco) have been shown to play a role in defense signaling pathways (Kim *et al.*, 2003) and in drought tolerance in transgenic maize plants (Shou *et al.*, 2004). Recent studies show that MAPK-based signalling increases accumulation of proline and enhances oxidative stress tolerance (Kong *et al.*, 2011; Zhang *et al.*, 2011).

1.3.4 ABA responsive gene expression

Studies of ABA-related mutants in the model plant *A. thaliana* have helped to explain the role of ABA in mediating the drought response in plants. Such mutants, isolated as ABA-deficient (*aba*) and ABA-insensitive (*abi*) mutants, are affected in the ABA response of vegetative tissue to water stress and show an increased tendency to wilt, suggesting a defect in stomatal regulation (Leung and Giraudat, 1998; Assmann et al., 2000; Shinozaki et al., 2003; Verslues and Bray, 2006). The ABA-deficient mutants have been very useful for understanding the mechanisms of ABA in regulating stomatal closure and other physiological processes related to the desiccation response (Hull *et al.*, 1993). The *abi* mutants contain normal endogenous level of ABA and their phenotypes cannot be reverted to wild-type by exogenous ABA. The *viviparous 1* (*vp1*) mutant in maize has an ABA insensitive phenotype (Suzuki *et al.*, 2001). *Arabidopsis abi1*, *abi2*, and *abi3* and *VP1* ABA-response mutants have defects in various

components of signal transduction pathways and these defects generally cause pleiotropic effects in stress responses (Leung and Giraudat, 1998).

Many genes which are responsive to ABA in mature plant embryos are also responsive to water deficit. Promoter analysis of ABA-responsive genes demonstrates the role of ABA in mediating drought-responsive gene expression. The presence of *cis*-acting ABA-responsive elements (ABRE) has been shown by functional analysis of the promoters of ABA-responsive genes (Izawa *et al.*, 1993; Yamaguchi-Shinozaki and Shinozaki, 1994; Yamaguchi-Shinozaki and Shinozaki, 2005). The best characterized examples of ABREs are the *Em1a* element from wheat *Em* gene (Marcotte *et al.*, 1989) and the Motif 1 element from rice *rab16A* gene (Mundy *et al.*, 1990). Transcription factors belonging to the bZIP family, EmBP-1 and TAF-1 have subsequently been shown to bind the *Em1a* element and Motif 1, respectively (Guiltinan *et al.*, 1990; Oeda *et al.*, 1991). Some motifs related to the G-box play a role in mediating plant responses to a variety of environmental stimuli (Leung and Giraudat, 1998). The presence of an ABRE within a promoter may not always be sufficient to confer ABA-responsiveness. Analysis of ABA-induced genes in barley has revealed that ABREs and coupling elements are required for ABA induction (Shen and Ho, 1995). Two coupling elements, CE1 (TGCCACCGG) and CE3 (ACGCGTGTCCTC), have been identified in the barley *HVA22* and *HVA1* genes, respectively, (Shen and Ho, 1995; Shen *et al.*, 1996). A bZIP protein, TRAB1, interacts with both the ABRE and CE3 elements and binds to the seed-specific VP1 transcription factor to mediate ABA-induced gene expression (Hobo *et al.*, 1999). Not all ABA-response genes have ABRE elements. In the promoter region of the carrot late embryogenesis abundant *Dc3* gene, the sequence ACACNNG confers ABA-responsiveness and is recognised by the bZIP transcription factors, DPBF-1 and DPBF-2 (Kim *et al.*, 1997). Other types of *cis*-acting ABA-responsive elements distinct from G-boxes have been characterized, including the hex3 sequence and the Sph element from maize. Tetramers of the synthetic hex3 element, GGACGCGTGGC, show transcriptional activity upon a truncated (-90) *CaMV 35S* promoter in transgenic tobacco which is induced by ABA (Lam and Chua, 1991). *C1*, a regulatory gene of the anthocyanin biosynthetic pathway, appears to be controlled by ABA and VP1 through a sequence containing a Sph element, CATGCATG, within its promoter (Hattori *et al.*, 1992).

1.3.5 The Drought Responsive Element (DRE)

Studies have shown that the expression of drought-responsive genes in several plants is mediated by ABA-independent as well as ABA-dependent signal transduction pathways (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2005). A common effect of drought and also other abiotic stresses, such as salinity and freezing, is the reduced water availability within cells. The *Arabidopsis RD29A* gene is differentially induced by dehydration, salt, cold stress and ABA treatment. Expression of *RD29A* is induced by dehydration and high salinity stresses rapidly within 20 minutes (Yamaguchi-Shinozaki and Shinozaki, 1993), but elevated ABA levels are not detected until after 2 hours of exposure (Kiyosue *et al.*, 1994). This suggests that *RD29A* does not require ABA synthesis for rapid induction, which has been confirmed by the induction of *RD29A* during cold- and drought-stress in *abi1* and *aba1* mutants (Shinozaki and Yamaguchi-Shinozaki, 2000). Analysis of the *RD29A* promoter has identified the presence of three copies of a DRE TACCGACAT involved in an ABA-independent initial rapid response to drought and cold or salt stress (Yamaguchi-Shinozaki and Shinozaki, 1994). The promoter also contains one ABRE which is essential for the *RD29A* gene expression in response to ABA that occurs more slowly than the response to drought and high salinity stress (Yamaguchi-Shinozaki and Shinozaki, 1993). DRE acts as a coupling element for the ABRE and is required for the slow induction of *RD29A* by ABA (Narusaka *et al.*, 2003). The ABA response of *RD29A* is mediated by two transcription factors; a DREB (DRE-binding protein) and an AREB (ABRE-binding protein) belonging to the AP2 and bZIP family of transcription factors, respectively (Narusaka *et al.*, 2003; Yamaguchi-Shinozaki and Shinozaki, 2005). Transgenic wheat overexpressing *Arabidopsis* transcription factor DREB1A under the control of a *RD29A* promoter showed improved tolerance to drought (Pellegrineschi *et al.*, 2004).

Many genes which are responsive to high salt or drought stress are also responsive to cold stress (Shinozaki and Yamaguchi-Shinozaki, 2000). The primary cause of freezing injury in plants is membrane destabilization as a result of freeze-induced dehydration (Steponkus, 1984). Microarray analyses in *A. thaliana* have revealed significant cross-talk between the drought, cold, high salinity and ABA stress signal transduction pathways (Seki *et al.*, 2001; Seki *et al.*, 2002; Shinozaki and Yamaguchi-Shinozaki, 2007).

1.3.6 AP2 transcription factors

Many of the *Arabidopsis* cold- and drought-responsive genes which have DRE/CRT (dehydration-responsive element/C-repeat) elements within their promoters are regulated by DREB/CBF (DRE-binding protein/C-repeat binding factor) AP2 type transcription factors (Stockinger *et al.*, 1997; Liu *et al.*, 1998; Yamaguchi-Shinozaki and Shinozaki, 2005, 2006; Mizoi *et al.*, 2011). CBF1/DREB1B, CBF2/DREB1C, and CBF3/DREB1A show significant roles in the transcriptional response to osmotic stress (Shinozaki and Yamaguchi-Shinozaki, 2007; Thomashow, 2010). Transgenic *A. thaliana* plants constitutively over-expressing these transcription factors exhibited tolerance to salinity, drought and freezing stress (Kasuga *et al.*, 1999; Gilmour *et al.*, 2000; Gilmour *et al.*, 2004). ICE1 (INDUCER OF CBF EXPRESSION 1) and CAMTA also regulate the expression of *DREB1A/CBF3* or *DREB1C/CBF2* at the transcriptional level (Chinnusamy *et al.*, 2003; Doherty *et al.*, 2009). CAMTA, calmodulin binding transcription activator, provides a link with the activated Ca^{2+} signaling during abiotic stress. A zinc finger protein, ZAT12, also regulates the expression of *DREB/CBF* (Vogel *et al.*, 2005). The *Arabidopsis CBF4* gene, belonging to the DREB1 subclass, is induced by drought stress, but not by low temperature (Haake *et al.*, 2002). Homologues of the *Arabidopsis DREB/CBF* genes were isolated from some flowering plants, including monocotyledonous plants such as rice (Dubouzet *et al.*, 2003), maize (Qin *et al.*, 2003), wheat and rye, and dicotyledonous plants such as *Brassica napus* and tomato (Jaglo *et al.*, 2001). In these plants, as in *A. thaliana*, the *DREB2* homologues are involved in the drought/salinity response pathway, while the *DREB1/CBF* homologues in the cold response pathway. CBF transcription factors have been shown to act in the cold response pathway in plants such as *Arabidopsis* that exhibit cold acclimation (Shinozaki and Yamaguchi-Shinozaki, 2000; Chinnusamy *et al.*, 2003; Thomashow, 2010) and also in plants, such as tomato, that do not cold acclimate (Jaglo *et al.*, 2001). In addition, multiple low-temperature regulatory pathways exist in *A. thaliana* and also in other angiosperms (Fowler and Thomashow, 2002). Non-acclimated *eskimo1 (esk1)* mutant of *Arabidopsis* (Xin and Browse, 1998; Xin *et al.*, 2007) display a similar level of freezing tolerance as control plants which have been cold acclimated. In absence of cold acclimation, the *esk1* plants accumulate high levels of soluble sugars and proline but do not express any of the cold-regulated genes such as *COR15A*, *RAB18* and *COR78 (RD29A)*.

1.3.7 Other drought stress-related transcription factors

In addition to the bZIP and AP2 families, drought stress-related transcription factors belonging to the MYB, MYC and homeobox families have been isolated from *A. thaliana*. The promoter of the drought and ABA-responsive *RD22* gene possesses two recognition sites for the MYC and MYB transcription factors but contains no ABRE (Iwasaki *et al.*, 1995; Abe *et al.*, 2003). Both RD22BP1 (MYC) and ATMYB2 (MYB) have been shown to function as transcriptional activators controlling the expression of *RD22* (Abe *et al.*, 1997; Abe *et al.*, 2003). The *ATMYB2* gene is induced by dehydration and salt stress, as well as by exogenously applied ABA (Urao *et al.*, 1993; Abe *et al.*, 2003). The *Arabidopsis* homeodomain-leucine zipper (HD-Zip) ATHB-7 protein is also induced by dehydration, osmotic stress and ABA (Soderman *et al.*, 1996). Induction of ATHB-7 was shown to be mediated via ABA, because its expression was impaired in the *abi1* mutant and was not detected in the *aba-3* mutant subjected to drought stress (Soderman *et al.*, 1996). Constitutive expression of *ATHB-7* in transgenic *Arabidopsis* caused a reduction in the rate of leaf and inflorescence stem growth (Hjellstrom *et al.*, 2003). *RD26* gene which encodes a NAC transcription factor is induced by drought, high salinity, ABA, and JA (Fujita *et al.*, 2004). NAC proteins recognize a MYC-like target sequence and activate ERD1 (Tran *et al.*, 2004; Tran *et al.*, 2006). Some members of the zinc-finger type TF such as WRKY have been shown to be involved in defense-related plant responses (Ülker and Somssich, 2004).

1.3.8 Activation/repression of drought-responsive pathways

Several positive and negative regulators of the stress response pathways have also been identified. Analysis of *sfr6*, a freezing-sensitive mutant, suggests that the SFR6 protein functions as a positive regulator of the DREB1/CBF factors (Knight *et al.*, 1999). The *ice1* mutant in *Arabidopsis*, is defective in cold-regulated expression of *CBF3/DREB1A* and its target *COR* genes. ICE1 encodes a MYC-type bHLH transcription factor (Chinnusamy *et al.*, 2003). ICE1 is inactive under non-stress conditions, but is activated by cold temperature and activates the expression of *CBF3/DREB1A*, but does not interact with the promoters of other *CBF/DREB1* genes. ICE1 also regulates stomata formation (Kanaoka *et al.*, 2008). The *fiery1* (*fry1*) mutation induced tolerance to drought, freezing and salt stress. FRY1 encodes an

inositol polyphosphate 1-phosphatase and is proposed to be a negative regulator of ABA and stress signaling (Xiong *et al.*, 2001). ICE1 was demonstrated to be under the control of HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1), a RING finger protein (Dong *et al.*, 2006). In the *hos1* mutant, expression of DREB1/CBF genes is super-induced by cold stress, suggesting that HOS1 functions as a negative regulator of the DREB1/CBF transcription factors (Lee *et al.*, 2001). The HOS5 gene product has also been proposed as a negative regulator of osmotic stress-responsive gene expression in both the ABA-dependent and ABA-independent stress signaling pathways. The *hos5* mutation results in increased expression of the stress-responsive gene *RD29A* (Xiong *et al.*, 1999).

1.4 Molecular studies of desiccation tolerance in resurrection plants

Molecular studies of resurrection plants have been performed with a number of species, such as the dicot *C. plantagineum* (Phillips and Bartels, 2000) and *Craterostigma wilmsii* (Farrant *et al.*, 2003), the monocot *S. stapfianus* (Neale *et al.*, 2000), the moss *T. ruralis* (Oliver *et al.*, 2005), the fern *Polypodium virginianum* (Reynolds and Bewley, 1993) and also desiccation-tolerant *Xerophyta viscosa* (Sherwin and Farrant, 1998) and *X. humilis* (Collett *et al.*, 2003).

1.4.1 Desiccation tolerance in *S. stapfianus*

The monocotyledonous resurrection grass *S. stapfianus* (Fig 1.2) is a desiccation tolerant plant which is found in the arid habitats of the Transvaal region in South Africa (Gaff and Ellis, 1974). These plants can withstand a loss of 98% of their water and remain viable in this state for one to two years and can resume their normal metabolism within 24 hours of rehydration (Gaff, 1971; Gaff, 1977; Gaff *et al.*, 1997).

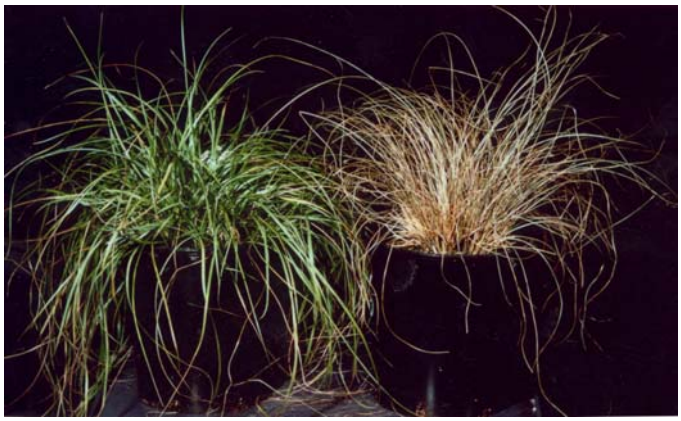


Fig. 1.2 The resurrection grass *S. stapfianus* Gandoger in the hydrated (left) and desiccated (right) state.

A number of physiological changes occur in *S. stapfianus* during dehydration (Table 1.1), which allow the protoplasm of individual cells to tolerate subsequent drying and ensure plant survival during desiccation. *S. stapfianus* requires a slowing drying process in order to establish desiccation tolerance mechanisms. When leaf relative water content (RWC) goes below 60%, desiccation tolerance is induced in *S. stapfianus* (Gaff and Loveys, 1993). Drying from full turgor to 60% RWC usually takes between 2-5 days in natural environments (Gaff, 1980). When leaves of *S. stapfianus* which are attached to intact plants are detached from dehydrated plant (below 60% RWC) and then further dehydrated to air-dryness, they remain alive when rehydrated. On the other hand, fully-hydrated leaves that are dehydrated at the same rate after being detached from the fully-hydrated plant die during drying (Gaff, 1980; Gaff and Loveys, 1993). This suggests that a hormonal signal from drought-stressed roots is required to travel to the leaves to induce the desiccation tolerance pathway (Ghasempour *et al.*, 2001).

In some plants during soil drying, increased transport of ABA from the root system has an important function in root to shoot signaling (Davies and Zhang, 1991). In the leaves of *Boyra constricta* and the callus of *C. plantagineum*, desiccation tolerance may be induced by application of ABA, which is not seen in *S. stapfianus* leaves (Gaff and Churchill, 1976; Bartels *et al.*, 1990; Gaff and Loveys, 1993). Although ABA is not thought to play a main function initiating desiccation tolerance in *S. stapfianus*, the level of ABA increases 6-fold

during the later stages of dehydration and reaches a maximum during severe desiccation (20-10% RWC) (Gaff and Loveys, 1993; Ghasempour *et al.*, 1998a). In studies using *S. stapfianus* protoplasts, hormones such as brassinosteriods, methyl jasmonate (MeJa) and ethylene promoted protoplasmic drought tolerance (PDT) to a higher degree than ABA (Ghasempour *et al.*, 1998a). MeJa was found to be the most effective but none of these hormones promoted PDT to a sufficient level that would be expected to allow the plant to survive desiccation (Ghasempour *et al.*, 1998a). Recently, the involvement of jasmonate in regulation of the desiccation tolerance mechanism has also been shown in *T. ruralis* (Oliver *et al.*, 2009).

Table 1.1 Major physiological events in intact desiccating leaves of *S. stapfianus* (modified from Gaff, 1989).

Changes in protein expression and the levels of several sugars and hormones such as ABA during desiccation in *S. stapfianus* are summarised. Note that physiological processes are still occurring in *S. stapfianus* during severe drought stress (below ~30 % RWC) where death would occur in most non-resurrection plants (Gaff, 1989).

RWC (%)	Level of Drought stress	Physiological event
100 – 90	Hydrated	- Normal photosynthesis and growth
89 – 80	Mild	- Reduction of photosynthesis - Appearance of some novel proteins (as visualized on 2D gels) including dehydrins
79 – 60	Moderate	- Reduction in starch content - Desiccation tolerant state attained at 60% RWC
59 – 40	Strong	- Maximum accumulation levels of glucose and fructose - ABA level increases 7-fold
39 – 20	Severe	- Synthesis of numerous novel proteins (37% RWC)
19 – 11	Extreme	- Maximum ABA level attained - Fragmentation of vacuole into sub-vacuolar vesicles
≤ 10 – Air dry	Anabiosis	- High sucrose content (~ 100 µmole/g dried weight) - Elevated content of raffinose and stachyose

In *S. stapfianus*, the upper leaf surface is protected by a covering of epicuticular wax that reflects some light (DallaVechia *et al.*, 1998). Drought stress causes the leaf margins of *S.*

stapfianus to curl inwards until the leaf lamina is tightly rolled by the contraction of epidermal cells which are located in the upper leaf surface. The level of dehydration can be determined by this leaf rolling (Gaff and Loveys, 1993). The air-dry live leaves may be easily distinguished by the colour difference between live and dead air-dry senesced leaves. The inrolling leaf surfaces build up a purple color due to accumulation of anthocyanins, intensifying to purple-black under full sunshine, indicating that the airdry leaves of *S. stapfianus* are alive. Cultivated plants dried under low irradiance usually do not become purple, but turn a dark olive green colour and are able to survive and recover from airdryness. *S. stapfianus*, like other resurrection plants, lowers its PSII activity during dehydration, but exhibits only a partial loss of its chlorophyll content (~40% in dry leaves returning to 90% on rehydration), thereby diminishing the potential for photooxidative damage to leaves (Quartacci *et al.*, 1997; DallaVechia *et al.*, 1998). After a dehydration/ rehydration cycle, all photosynthetic activities are restored in *S. stapfianus* (Gaff *et al.*, 2009).

In *S. stapfianus*, the induction of glutathione reductase, ascorbate peroxidase and dehydroascorbate reductase as free radical scavenging enzymes is important to protect membrane lipids and proteins and to remove reactive oxygen species (ROS) (Sgherri *et al.*, 1994). During the recovery phase the intensified oxidative stress causes more damage during rehydration than during dehydration (Sgherri *et al.*, 1994). However, in detached leaves of *S. stapfianus*, antioxidant capacity decreases due to reduced activity of ascorbate peroxidase during dehydration (Sgherri *et al.*, 1994). This is why the detached leaves of *S. stapfianus* possibly do not show any recovery from desiccation.

Although large amounts of glucose, fructose and galactose are found in fully-hydrated *S. stapfianus* leaves, during dehydration sucrose is the major sugar (Ghasempour *et al*., 1998b) (Table 1.1). Recent metabolomic study by (Oliver *et al.*, 2011b) showed that in *S. stapfianus* leaves, sucrose accumulated steadily during dehydration to a maximum elevation of 10.5-fold above control concentrations in the desiccated state. However, sugars, such as trehalose decreases whilst raffinose increases slightly in desiccated *S. stapfianus* leaves (Albini *et al*., 1994). Glucose is the dominant sugar in rehydrated *S. stapfianus* plants after desiccation with trehalose also being unusually high 72 hours after rehydration (Murelli *et al.*, 1996).

Alterations in specific mRNAs and proteins are indicative of the changes in gene expression during the induction of desiccation tolerance in *S. stapfianus* (Kuang *et al.*, 1995; Gaff *et al.*, 1997). These changes are vital for the establishment desiccation tolerance in vegetative tissues of *S. stapfianus* (Neale *et al.*, 2000). Studies examining the levels of protein synthesis during dehydration of *S. stapfianus* leaves indicate that at a RWC of 80-83%, the first proteins thought to be involved in desiccation tolerance, including dehydrins, are produced (Kuang *et al.*, 1995). Several studies have also identified several genes which are strongly induced by water deficit in vegetative tissues of *S. stapfianus* (Blomstedt *et al.*, 1998a; Blomstedt *et al.*, 1998b; Neale *et al.*, 2000; Le *et al.*, 2007). These genes encode proteins with putative protective functions including group 3 LEAs, dehydrins, early light inducible proteins (ELIPs), glyoxalase I and several cell wall and membrane associated proteins (Blomstedt *et al.*, 2010). High levels of transcripts encoding a γ-tonoplastic intrinsic protein (TIP), a member of water channel protein family have also been observed during severe dehydration (Neale *et al.*, 2000).

1.4.2 Differences between water-deficit responses in resurrection plants and non-resurrection plants

Leaf RWC of about 90% is generally found in plants in moist environments (Arvidson, 1951). When RWC goes down to 40%, plants cannot recover due to massive cellular damage (Schnepf, 1961). On the contrary, in resurrection plants, desiccation tolerance does not depend greatly on water conservation. The desiccation-tolerance, a protoplasmic phenomenon, may be reversible at any stage of the dehydration process. Unlike the situation in bryophytes, where desiccation tolerance mechanism is constitutive (Bewley *et al.*, 1978), in angiosperms it is induced during dehydration (Gaff and Churchill, 1976). In both resurrection and non-resurrection plants, the early responses to dehydration show many similarities (Neale *et al.*, 2000). This indicates that in vascular resurrection plants drought avoidance mechanisms are required in the field for slowing down drying rates sufficiently to allow the induction of the genetic mechanism of desiccation-tolerance.

Normally in vascular plants sucrose is found as a main protective sugar. This appears to be the case in *S. stapfianus* and many other desiccation-tolerant grasses as well as all mosses

(Smirnoff, 1992; Ghasempour *et al.*, 1998b) except *Selaginella lepidophylla* in which the trehalose acts as a major protective sugar (Itturriaga *et al.*, 2006; Yobi *et al.*, 2013). However, some resurrection ferns and angiosperms accumulate very small amounts of trehalose (Ghasempour *et al.*, 1998b; Farrant *et al.*, 2009). A *Saccharomyces cerevisiae* mutant defective in trehalose biosynthesis may also maintain desiccation-tolerance (Ratnakumar and Tunnacliffe, 2006). Like other sugars, trehalose may act cooperatively as a chemical chaperone to enhance the molecular shield function of LEA proteins in reducing desiccation-induced aggregation of proteins (Chakrabortee *et al.*, 2012).

A recent study showed that several sugars (e.g. glucose, sucrose), sugar alcohols (e.g. inositol-1-phosphate, myo-inositol, mannitol), and betaine, which act as osmoprotectants or hydroxyl radical scavengers, were more abundant in the desiccation-tolerant *S. lepidophylla* at 100% and 50% RWC than the desiccation-sensitive *S. moellendorffi* (Yobi *et al*., 2012). In the foliage and seed of vascular and cryptogamic desiccation-tolerant plants, LEAs are also found as protective proteins. The transcripts of genes involved in some of these protection processes appear at relatively mild levels of water-deficit in both resurrection and non-resurrection plants (Neale *et al.*, 2000).

Resurrection plants have some unique features which allow them to survive desiccation. Resurrection plants contain both drought- and desiccation-tolerance mechanisms to deal with daily mild and severe intermittent water-deficit. Desiccation survival requires a particular metabolic activities to be turned off which is injurious to the cell along with the establishment of mechanisms to protect cellular components recovering from the air-dry state during rehydration (Di Blasi *et al.*, 1998; Neale *et al.*, 2000). Protein turnover and DNA-repair mechanisms are important. In contrast to the situation in the moss *T. ruralis*, during water-deficit, the environmentally-induced desiccation tolerant plants require transcriptional and translational activity to be maintained (Bewley, 1979). Protein synthesis ceases at mild levels of water-deficit in desiccation-sensitive angiosperm whereas in inducible desiccation-tolerant species it continues to occur until leaves are nearly air-dry (Bartels *et al.*, 1990; Gaff *et al.*, 1997). Likewise, in *S. stapfianus* specific transcript accumulation takes place nearly throughout the whole dry-down process (Neale *et al.*, 2000). Desiccation tolerance may

involve the extension of transcriptional activation of early-response genes into later stages of dehydration (Blomstedt *et al.*, 2010).

In resurrection plants, comprehensive analyses of the transcriptome (Oliver *et al.*, 2004; Rodriguez *et al.*, 2010), proteome (Oliver *et al.*, 2010) and metabolome (Oliver *et al.*, 2011b) in dehydrating and rehydrating tissue have been carried out. Gene expression analysis showed that many novel transcripts are produced during the rehydration phase in *S. stapfianus* (O'Mahony and Oliver, 1999) suggesting that several transcripts that largely accumulated in air-dry tissue during dehydration may play a role during rehydration when most of the cellular damage occurs. Establishment of mechanisms required for survival of rapid rehydration, and restoring rapid re-growth when water is available, before the desiccated state is reached is necessary for plants growing in limited rainfall environments. In *S. stapfianus*, growth analysis following a dehydration-rehydration treatment showed more rapid regrowth with approximately twice the biomass (dry weight) produced when compared to continually well-watered plants (Blomstedt *et al.*, 2010).

1.4.3 Isolation of desiccation-tolerance specific genes associated with growth

The analysis of the transcripts that accumulate to high levels in dried tissues of a *S. stapfianas*, designated *S. stapfianus* Drought-related Genes (SDG), have shown that some of them are related to proteins that have been shown to regulate growth related processes (Blomstedt *et al.*, 2010). One such example related to cell wall modification and signaling, is the endo-ß-1,4-glucanase or glycosyl hydrolase (GH) 9 cellulose encoded by *SDG8s*. As plant cellulases are known to be involved in growth related processes, transgenic approaches using plant GH9 cellulases show considerable improvement in cell growth and organ size (Park *et al.*, 2003; Shani *et al.*, 2004). Another example is a chitinase-like protein encoded by *SDG72s* which in other species has been associated with plant growth and development (Kasprzewska, 2003). High chitinase transcripts in young leaves, stems, flowers and pollen suggest their function in loosening and disruption of the cell wall in rapidly growing tissues (Neale *et al.*, 1990; Campillo and Lewis, 1992; Patil and Widholm, 1997). Another example is the *SDG11s* gene,

the fragment of which translates into a section of a putative glycine rich protein (GRP) of 51 amino acids, involved in cellular responses. In *Arabidopsis*, the function of a wall-associated receptor kinase (WAK1) is regulated by AtGRP3. There are 5 *WAK* genes in *Arabidopsis*. *WAK2* and *WAK4* are shown to be involved in cell expansion and affect growth and development in *Arabidopsis* (Lally *et al.*, 2001; Wagner and Kohorn, 2001). In *S. stapfianus*, the changes to the cell wall and the expression of *SDG11s* during water deficit may activate WAK-regulated mechanism for protection of necessary cellular functions and recovery and growth when water is available.

Another transcript that accumulates in dehydrated *S. stapfianus* tissue is *SDG71s*, which encodes a partial protein with homology to IAA-conjugating enzyme GH3 in *Arabidopsis* (Staswick *et al.*, 2005; Park *et al.*, 2007). Studies have shown that the amount of free auxin (IAA) in different tissues plays a role in cell elongation and division, gravitropism and apical dominance, hence *SDG71s* may be involved in regulating the amount of free IAA and therefore may affect plant growth (Hagen and Guilfoyle, 2002). Another transcript from *S. stapfianus*, *SDG16s* that also accumulates in dehydrated *S. stapfianus* tissue encodes a cysteine protease inhibitor (phytocystatin). The cystatin C-terminus encoded by the *SDG16s* fragment has very high homology to a developmentally regulated cystatin from alfalfa, MsCYS1. This suggests that it is involved in the early stages of leaf and stem growth (Rivard *et al.*, 2007). The *SDG20s* gene product, origin recognition complex subunit 6 (Orc6), is also involved in growth and development (Blomstedt *et al.*, 2010). *SDG15s* which encodes the translation elongation factor eEF1A also plays a role in growth-related cellular processes in both plants and animals. eEF1A may undergo post-translational modifications and is involved in cell proliferation by interacting with different proteins, including tubulin, calmodulin and actin.

1.4.4 Desiccation-specific gene expression in *S. stapfianus*

Further studies in *S. stapfianus* were undertaken by differentially screening a cDNA library constructed from dehydration-stressed leaf tissue, in an attempt to identify genes expressed during severe-dehydration which may have an adaptive role in desiccation tolerance (Le *et al.*,

2007). Four of the genes identified are strongly expressed in the intact leaf tissue of *S. stapfianus* during severe water stress, while three of these genes did not show any expression in hydrated or detached leaf tissue (Fig 1.3). The nature of the putative proteins encoded by these genes suggests their important role in plant protection during desiccation. These include a novel LEA-like protein (SDG2i), a pore-like protein (SDG3i) and also a UDPglucose glucosyltransferase (SDG8i) (Le *et al.*, 2007).

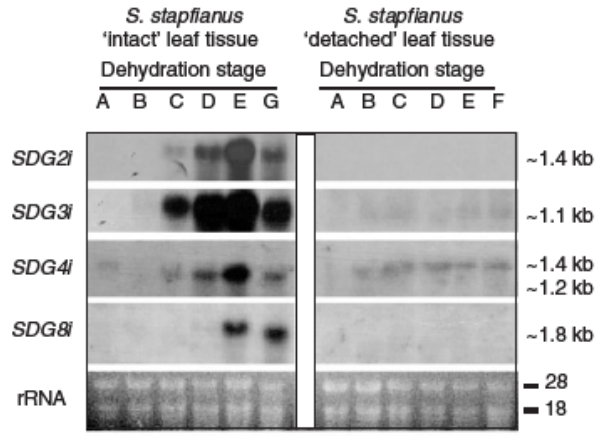


Fig 1.3 Northern Analysis of *SDG* genes at different RWCs. A: ≥90% RWC, B: 89–80% RWC, C: 79–60% RWC, D: 59–40% RWC, E: 39–20% RWC, F 19–10% RWC and G ≤10% RWC (Le *et al.*, 2007).

The present study focuses on *SDG8i*. Sequence analysis shows the presence of a Plant Secondary Product Glycosyltransferase (PSPG) motif in the SDG8i *C*-terminal domain, which indicates that it is a Family 1 UGT and also that it is likely to utilizes a uridine diphosphate (UDP) activated sugar as donor during glycosylation (Le et al 2007). Therefore, *SDG8i* encodes UDP-dependent glycosyltransferase or UGT (Mackenzie *et al.*, 1997; Lim and Bowles, 2004). From the full coding region of *SDG8i* it is shown that there are no introns and encodes a protein of 473 amino acids, with a predicted molecular mass of 52 kDa and a theoretical isoelectric point of 5.66 (Le *et al.*, 2007). By comparing amino acid sequence of SDG8i with UGTs from maize, *Arabidopsis*, tomato and tobacco, high (more than 60% identity) homology with the PSPG motif was found (Le *et al.*, 2007). Less homology (<30%) was found in the *N*-terminal domain of SDG8i with other plant UGTs. The *N*-terminal region

is involved in substrate binding but specificity can not be determined based on the protein sequence.

The effect of exogenous ABA on the expression of *SDG8i* cDNAs was determined by treating fully-hydrated *S. stapfianus* leaf tissues. Initial northern analysis showed that exogenous ABA treatment has no effect on the expression of *SDG8i* after 24 hours (Le *et al.*, 2007), however, more recent experiments indicate a transient induction peaking at 3 hours (Bomstedt personal comunication). The effect of salt and cold stress was also investigated and preliminary results showed that *SDG8i* is induced by cold (4°C) but not salt (300 mM NaCl) (Le, 2004).

To isolate transcription factors involved in the regulation of *SDG8i* in *S. stapfianus*, the promoter region of the *SDG8i* gene was used as bait in the yeast-one hybrid system resulting in identification of *SDG16y*, which was shown to be an AP2/ERF transcription factor (Le, 2004). Screening of a *S. stapfianus* cDNA library with *SDG16y* isolated two full-length cDNA clones *SDG10y*, as well as a related cDNA, *SDG7y* (Le, 2004). Both *SDG10y* and *SDG7y* encode structurally similar AP2/ERF transcription factors, with an acidic region at the C-terminus, an AP2 DNA-binding domain located centrally and a putative nuclear localisation signal located immediately upstream of the AP2 domain. The AP2 domains of SDG10y and SDG7y contain two conserved amino acid residues which are found in ERFs but not in DREB proteins (Liu *et al.*, 1998). The functional significance of this finding is unknown as both *SDG10y* and *SDG7y* transcripts are down-regulated by ethylene treatment as well as dehydration (Fig. 1.4 and 1.5) (Le, 2004). An investigation of the gene regulation activities of the SDG10y and SDG7y transcription factors may reveal new insights into the processes leading to desiccation tolerance in vegetative tissues of *S. stapfianus*.

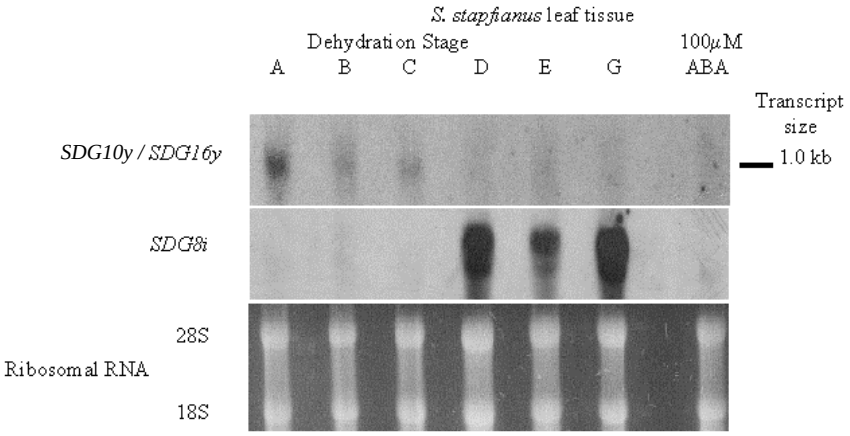


Fig. 1.4 Northern analysis showing expression of *SDG10y* (previously designated *SDG16y*) and *SDG8i* in dehydrated *S. Stapfianus* leaf tissue. A: 90% RWC, B: 89-80% RWC, C: 79-60% RWC, D: 59-40% RWC, E: 39-20% RWC, G ≤11% RWC (Le, 2004).

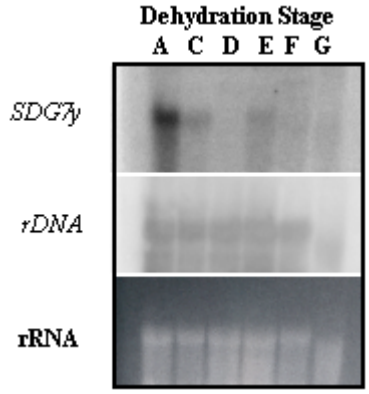


Fig. 1.5 Northern analysis showing expression of *SDG7y*. A: 90% RWC, B: 89-80% RWC, C: 79-60% RWC, D: 59-40% RWC, E: 39-20% RWC, G ≤11% RWC (Le, 2004).

1.5 Plant Glycosyltransferases

Glycosyltransferases (GTs), which catalyze the transfer of a sugar residue from an activated donor to an acceptor molecule, are found in all living organisms. This glycosylation changes the stability, solubility and biological activity of such molecules (Lim and Bowles, 2004). The activity of GTs may be crucial for the biosynthesis of some secondary metabolites, for the

regulation of small molecular compounds active in defense and signaling, as well as for the detoxification and compartmentation of xenobiotics (Jones and Vogt, 2001). This study focuses on the family 1 GTs or UGTs. The model plant *A. thaliana* contain 122 different UGTs which are classified into 14 different phylogenetic groups (Li *et al.*, 2001; Ross *et al.*, 2001). Plant GTs in general show limited sequence similarity (Vogt and Jones, 2000). With their amino-terminal regions being more variable than the carboxy-terminal regions this suggests that the amino-terminal domain may be involved in the recognition and binding of the diverse aglycon acceptor substrates. The more conserved carboxy-terminal region is thought to be involved in binding the nucleotide sugar substrate (Lim *et al.*, 2003a) and contain a highly conserved UDP-Glucose binding sequence found in the C-terminal region of UGTs involved in secondary plant metabolism called the Plant Secondary Product Glycosyltransferase (PSPG)-Box (Hughes and Hughes, 1994). Most of the GTs in the *Arabidopsis* GT family 1 are UGTs, carrying the C-terminal consensus sequence.

1.5.2 Physiological roles of GTs in plants

Studies of GTs and their glycosylation activities in *Arabidopsis* and other plant species have shown that UGTs play important roles in plant growth and development, which are discussed below.

1.5.2.1 The role of UGTs in biosynthesis and storage of secondary metabolites

Plants have developed a number of metabolic pathways to synthesize several thousand different secondary metabolites for environmental adaptation. Glycosides of secondary metabolites identified include phenolics, terpenoids, alkaloids (e.g. betalains), thiohydroximates (glucosinolate precursors), cyanohydrins (cyanogenic glycoside precursors) and steroids (Vogt and Jones, 2000). GTs could play roles in the biosynthesis, alteration, transportation and storage of secondary metabolites (Wang and Hou, 2009). Glycosylation reactions generally lead to stabilization and inactivation and sometimes addition of the sugar conjugates which are highly energetic compounds and biosynthetic intermediates (Bowles *et al.*, 2006).

1.5.2.2 The role of UGTs in defense response and detoxification

Plants have defense responses against different biotic stress such as herbivore attack, bacterial or fungal infections, as well as mechanisms to cope with abiotic stress factors such as drought, heat, cold or oxidative stress. Several UGTs are highly inducible by these environmental stress factors and several recent studies indicate they have important stress related functions (Mazel and Levine, 2002; Langlois-Meurinne *et al.*, 2005; Meissner *et al.*, 2008).

Transgenic tobacco plants overexpressing the tobacco UGT *TOGT1* shows increased resistance to Potato Virus Y (Matros and Mock, 2004) while reduced expression of *TOGTs* impairs plants resistance to Tobacco Mosaic Virus (TMV) by decreasing the levels of scopoletin glucoside (Chong *et al.*, 2002). Overexpression of *UGT74F2* led to increased susceptibility to the hemibiotrophic pathogen *Pseudomonas syringae,* caused by reduced salicylic acid (SA) and its glucoside levels (Song *et al.*, 2008). Two glucosylated forms of SA have been identified in plant species: the glucose ester and the 2-O-glucoside (Vlot *et al.*, 2009). Both the conjugated and the free form are increased upon pathogen infection. Screening of several recombinant UGTs from *Arabidopsis in vitro* revealed two proteins which were active against SA and benzoic acid (Lim *et al.*, 2002). UGT74F1 formed only SA 2-O-β-Dglucose (SAG), while UGT74F2 forms both SAG and the SA glucose ester (SGE). *Arabidopsis* mutant analysis shows that changes in the activity of either *UGT74F1* or *UGT74F2* may have an effect on the metabolism of exogenous SA *in vivo* (Dean and Delaney, 2008). UGT74D1 from *Arabidopsis* recognized jasmonic acid (JA) *in vitro*, but also showed significant activity towards other substrates (Song, 2005). A JA glucoside was also found to accumulate in wounded leaf extracts of *Arabidopsis* (Glauser *et al.*, 2010).

Several UGTs have also been shown to play roles in regulating plant adaptation to different abiotic stresses. For example, ectopic overexpression of *UGT74E2* led to improved survival during drought and salt stress in *Arabidopsis* through its activity toward IBA (Tognetti *et al.*, 2010). Loss-of-function mutations in *UGT73B1*, *UGT73B2*, or *UGT73B3* (tandemly clustered flavonoid *UGTs*) enhanced oxidative stress resistance of plants (Lim *et al.*, 2006). Moreover, loss of *UGT71C1* function in *Arabidopsis* led to increased tolerance to methyl viologen indicating a role in oxidative stress response (Lim *et al.*, 2008). A recent study shows the role

of *UGT85A5* in increasing salt tolerance when overexpressed in tobacco plants (Sun *et al.*, 2013).

1.5.2.3 The role of UGTs in hormone homeostasis

Plant hormones or their precursors play an important role in the regulation of related defense pathways and regulation of plant hormones is crucial to enable adaptation of plants to environmental change. Glycosylation is an important mechanism that controls the levels as well as compartmentation of different hormones within the cells and tissues of a plant.

Most of the hormones except ethylene occur as glycosides *in planta* (Bowles *et al.*, 2006). *IAGLU*, the first gene isolated from maize, encodes a GT that recognizes indole acetic acid (IAA) (Szerszen *et al.*, 1994). It was shown later that UGT84B1 synthesized the 1-O-indole acetyl glucose ester (IAAGlc) by *in vitro* analysis of recombinant *Arabidopsis* GTs. Transgenic plants overexpressing UGT84B1 exhibit a phenotype suggesting a deficiency in auxin (Jackson *et al.*, 2001).

Glycosylation of cytokinins (CKs) involves O-glucosylation, O-xylosylation, and N-glucosylation (Mok and Mok, 2001). Genes encoding GTs recognising CKs have been identified in several plant species (Martin *et al.*, 1999; Martin *et al.*, 2001; Veach *et al.*, 2003). Transgenic plants over-expressing *Arabidopsis* UGT76C1 which has N-glucosyltransferase activity towards CKs showed increased accumulation of trans-zeatin-7-N-glucoside by application of trans-zeatin (Hou *et al.*, 2004).

Glucose ester is the most abundant conjugate among the glycosides of abscisic acid (ABA) in plants (Schwartz and Zeevaart 2004). Among the eight *Arabidopsis* UGTs which are able to glycosylate ABA, UGT71B6 showed enantioselective glycosylation only towards the naturally occurring *cis*-S-(+)-ABA *in vitro* (Lim *et al.*, 2005). UGT71B6 was shown to be able to glycosylate a wide range of ABA analogues *in vitro* (Priest *et al.*, 2005). ABA metabolism was shown to be affected by over-expression of UGT71B6 in *Arabidopsis* during drought

(Priest *et al.*, 2006). Deglycosylation of this hormone has also been shown to have an effect on the pool of free ABA (Lee *et al.*, 2006).

A wide range of glycosides of many different brassinosteroids (BRs) have been identified in plants (Fujioka and Yokota, 2003). *Arabidopsis* UGT73C5, able to catalyze the 23-O-glucosylation of brassinolide (BL) and its biosynthetic precursor, castasterone (CS), when overexpressed, exhibits phenotypes with BR-deficiency (Poppenberger *et al.*, 2005). Over-expression of this GT also reduced the levels of active BRs in transgenic plants, suggesting a reduced bioactivity of BRs by glycosylation. *UGT73C5* has also been shown to glycosylate a fungal toxin (Poppenberger *et al.*, 2003), suggests a dual role of this gene in glycosylating both endogenous and exogenous acceptors.

Salicylic acid (SA) as well as jasmonic acid (JA) are two other important plant hormones, the activity of which seem to be regulated by conjugation (Vlot *et al.*, 2009). As both play important role in plant defense reactions, they were described in the earlier section.

1.6 Hormonal regulation in plant growth and development

Since glycosylation may affect the bioactivity of most plant hormones and many UGTs are environmentally regulated, there may be a role for plant UGTs in regulation of environmentally-induced morphogenesis.

Plant development is characterized by a high degree of plasticity, i.e., the ability to adjust the developmental program to respond to changes in the environment. The overall plant body is defined by the shoot apical meristem at the apex and the root apical meristem at the basal end. During postembryonic development, the activity of these meristems leads to the formation of shoot-and root-specific organs. Moreover, secondary meristems (axillary branches) are formed in both root and shoot with the same developmental potential as the primary meristems (Domagalska and Leyser, 2011). The branching pattern depends on the time and position of branch development and is genetically determined (Halle, 1999). The genetic and environmental interactions involved in determining plant architecture are mediated by plant hormones.

Axillary branching commonly occurs in angiosperms (Sussex and Kerk, 2001) and involves two developmental stages: the formation of axillary meristems in the leaf axils and subsequent axillary bud growth. The growth of axillary meristems is inhibited by the primary inflorescence in many plant species including *Arabidopsis*. This phenomenon, commonly known as apical dominance, is one of the most studied topics of controlling bud outgrowth and involves crosstalk among various hormones and signals that transport within and between the root and shoot (Beveridge, 2006; McSteen, 2009).

Plant hormones (sometimes termed plant growth regulators), have a history of interest to investigators of plant development and several hormones have been implicated in the control of shoot branching and dormancy in axillary meristems. The term hormone in plants is usually applied to the small molecules derived from secondary metabolism which may carry long-range signals and are active at low levels (Santner and Estelle, 2009; Jaillais and Chory, 2010). For many years a set of approximately five substances or substance groups were recognized as hormones – auxins, CKs, gibberellins, ethylene and ABA. More recently the BRs, SA, JA and strigolactone (SL)-related compounds are also included in this group to some degree (Jaillais and Chory, 2010). In many aspects of plant development, these plant hormones interact with each other and this so-called crosstalk has been the subject of several studies (Rock and Sun, 2005; Moubayidin *et al.*, 2009).

1.6.1 Auxin

The hormone auxin is one of the best characterized and the most important signals regulating many environmental and internal developmental processes in plants. Auxin plays an important function in the control of shoot branching (Fig. 1.6) in many seed plants (McSteen and Leyser, 2005; Cline *et al.*, 2006). Auxin is unique among the plant hormones in that it is transported through plant tissues in a polar fashion. This polar auxin transport (PAT) mechanism is a specific, self-regulating and self-organising transport system of plasma-membrane influx and efflux carriers (Benjamins and Scheres, 2008). Auxin is mostly produced in the young expanding leaves of growing shoot apices and is actively transported basipetally in the PAT stream, involving PIN1, a basally localized PIN-FORMED (PIN)-type auxin efflux carrier

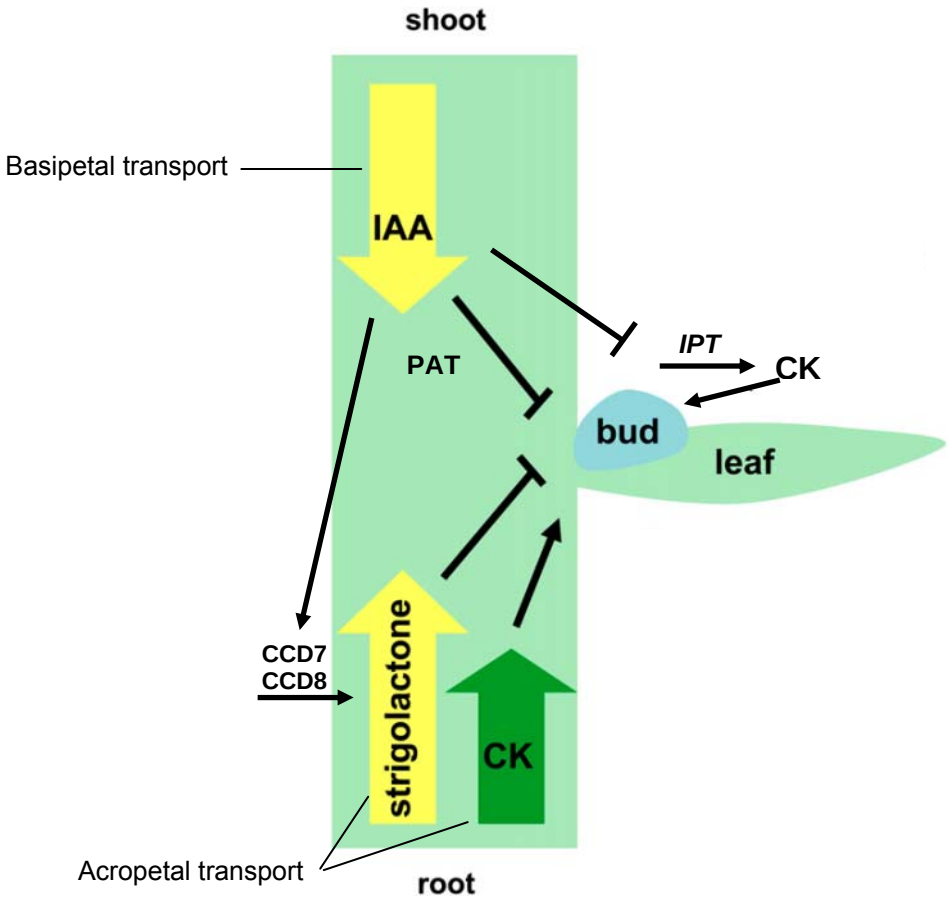


Fig. 1.6. Roles of Auxin, CK and SL on bud outgrowth. These hormones are transported throughout the plant, forming a systemic network. Auxin is actively transported basipetally in the PAT stream, involving basally localized auxin efflux carriers PIN1. SLs and CKs are mainly produced in the root, but also locally in the shoot, and are transported acropetally in the xylem.Auxin acts to independently up-regulate SLs and down-regulate CK level. Auxin and SLs inhibit the progression to growth while CKs promote this progression. Modified from (Brewer *et al.*, 2009; Hayward *et al.*, 2009)

(Gälweiler *et al.*, 1998; Zažímalová *et al.*, 2010). In the control of shoot branching, the polar transport of auxin, travelling from its point of synthesis down the stem to its point of action, is important for the maintenance of dormancy in axillary meristems. Removal of the apex removes the auxin source which then triggers the outgrowth of axillary buds further down the stem. Replacement of this source by application of exogenous auxin may prevent this outgrowth (Thimann and Skoog, 1933). Disruption of polar auxin transport with inhibitors also allows bud outgrowth further down (Panigrahi and Audus, 1966; Chatfield *et al.*, 2000). However, auxin acts indirectly without entering the buds (Booker *et al.*, 2003) and therefore,

the presence of one or more second messengers has been suggested to account for the indirect action of auxin (Booker *et al.*, 2003).

1.6.2 Cytokinin

The actions of CKs are likely to form at least part of the auxin second messenger role (Muller and Leyser, 2011). CKs are synthesized both locally in the bud and also transported acropetally in the transpiration stream from the roots (Sachs and Thimann, 1967; Bangerth, 1994). When applied directly to the bud, CKs promote meristem activity and bud outgrowth (Muller and Leyser, 2011). CKs can activate buds even in the presence of apical auxin when basally applied, and therefore they act antagonistically to auxin in apical dominance (Chatfield *et al.*, 2000). CK biosynthesis is down-regulated by auxin (Fig 1.6), suggesting that auxin inhibits buds partly by reducing their CK supply and this has contributed to a model in which release of CK production from repression by the loss of apical auxin on decapitation promotes bud outgrowth (Tanaka *et al.*, 2006). CKs are implicated in the promotion of meristem identity and outgrowth, partly through their interactions with auxin and through their direct effects on cell cycling (Durbak *et al.*, 2012).

1.6.3 Strigolactone

Studies of branching mutants in a range of species have revealed the existence of another factor, acting together with auxin and CK (Domagalska and Leyser, 2011). These mutants were termed *more axillary growth* (*max*) mutants in *Arabidopsis*, *ramosus* (*rms*) in pea, *decreased apical dominance* (*dad*) in petunia, and *dwarf* (*d*)/*high tillering dwarf* (*htd*) mutants in rice. The *MAX* pathway produces a long-range signal in the root and shoot that travels up towards the shoot apex in the xylem and may act at or near to the bud to repress bud outgrowth (Booker *et al.*, 2005; Stirnberg *et al.*, 2007; Kohlen *et al.*, 2011). This long-distance signal is carotenoid derived and identified as a SL-related group of compounds (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008).

The action of SLs in branching control is to repress outgrowth and their action is proposed to form part of the auxin 'second messenger' function (Fig. 1.6). SL biosynthesis genes are upregulated by auxin (Bainbridge *et al.*, 2005; Johnson *et al.*, 2006; Arite *et al.*, 2007; Foo *et al.*, 2007; Hayward *et al.*, 2009). The precise mechanisms of SL action have not been defined conclusively. In one proposed mechanism for bud repression, SLs act directly, antagonistically to CKs, with the dormancy regulator *BRANCHED1 (BRC1)* in *Arabidopsis* being a putative target in a direct signaling cascade (Dun *et al.*, 2006; Brewer *et al.*, 2009; Dun *et al.*, 2009; Braun *et al.*, 2012). However, in experiments using excised nodes without a natural or supplied auxin source, synthetic SL analogues are incapable of repressing outgrowth (Crawford *et al.*, 2010) which suggests that interaction with other hormones is essential to SL action.

1.6.3.1 Biochemical structure of strigolactone

SLs are comprised of terpenoid lactones (Fig 1.7) with a backbone of four rings. The three ABC rings form a single lactone and are joined to the fourth 'D' ring, a γ-butyrolactone moiety, by an enol ether bond liable to nucleophilic attack. This results in most SL compounds being labile in water and ethanol (Akiyama *et al.*, 2010).

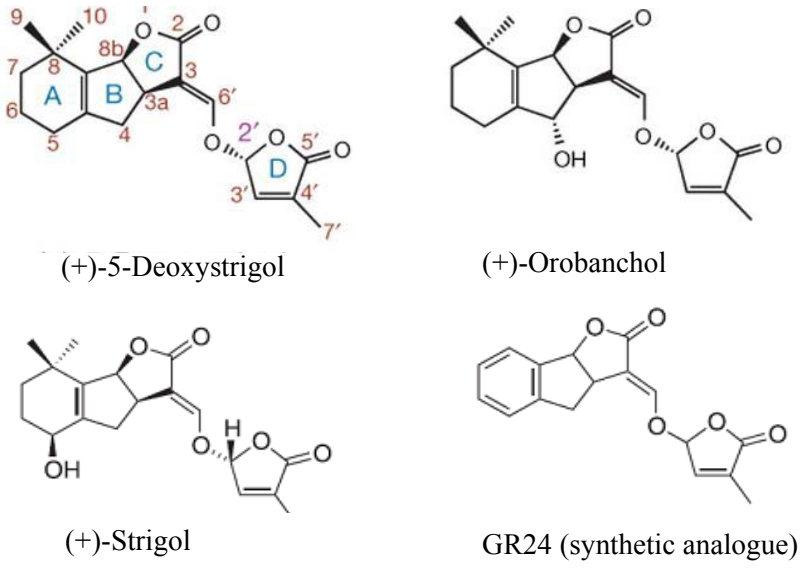


Fig 1.7 Chemical structures of the naturally occurring and the synthetic strigolactones (Umehara *et al.*, 2008).

The C-D ring section is required for the hyphal branching activity of SLs in fungi and for promoting germination activity in parasitic plants (Zwanenburg *et al.*, 2009; Akiyama *et al.*, 2010). There are also isomeric and synthetic forms of SLs with different bioactivities. The SLs strigol, sorgomol, orobanchol and 5-deoxystrigol, have been isolated from plants (Fig 1.7). 5-deoxystrigol is believed to be the synthetisis precursor compound with activity in shoot branching (Rani *et al.*, 2008) and the predominant SL in rice, while orobanchol is probably the main SL in *Arabidopsis* (Goldwasser *et al.*, 2008; Kohlen *et al.*, 2011). GR24 is a synthetic analogue which is increasingly being used to investigate plant architecture and the role of SLs in shoot branching.

1.6.3.2 Biosynthesis of strigolactone

The genes involved in the biosynthesis and signaling of SL have been identified from a diverse range of species and are summerised in Table 1.2. The SL biosynthetic pathway (Fig 1.8) starts with a carotenoid precursor, likely to be β-carotene. Then CAROTENOID CLEAVAGE DIOXYGENASE (CCD) 7 and CCD8 act serially in the plastid and produce carlactone, which is a mobile intermediate in the SL pathway (Alder *et al.*, 2012). DWARF27 (D27), a plastid-localised iron-containing protein, converts *trans*-β-carotene to 9-*cis*-β-carotene in the SL pathway (Lin *et al.*, 2009; Alder *et al.*, 2012). *RMS5/MAX3/DAD3/D17/HTD1* and *RMS1/MAX4/DAD1/D10*, encode CCD7 and CCD8, respectively, are involved in the SL pathway (Sorefan *et al.*, 2003; Snowden *et al.*, 2005; Zou *et al.*, 2006; Arite *et al.*, 2007; Drummond *et al.*, 2009). After CCD7 and CCD8, a cytochrome P450, MAX1, acts in the synthesis of SLs (Booker *et al.*, 2005). *D14/D88/HTD2/DAD2*, encoding an α/β-fold hydrolase, and *RMS4/MAX2/D3*, encoding an F-box protein, are proposed to act downstream of SL synthesis and are required for the SL branching inhibition response (Stirnberg *et al.*, 2002; Ishikawa *et al.*, 2005; Johnson *et al.*, 2006; Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Arite *et al.*, 2009; Gao *et al.*, 2009; Liu *et al.*, 2009; Hamiaux *et al.*, 2012). The transcription factor *BRC1*, a TCP (TB1, CYCLOIDEA, PCF domain) is likely to function downstream of SL to regulate bud outgrowth (Aguilar-Martinez *et al.*, 2007; Finlayson, 2007; Braun *et al.*, 2012).

The SL biosynthesis and response pathway involves feedback regulatory mechanisms (Dun *et al.*, 2009). If mutation of either SL biosynthesis or response genes reduces the SL response the expression of both SL biosynthesis genes, *CCD7* and *CCD8* is enhanced (Foo *et al.*, 2005; Snowden *et al.*, 2005; Johnson *et al.*, 2006; Arite *et al.*, 2007; Drummond *et al.*, 2009; Dun *et al.*, 2009) and the transport of xylem-sap CK is decreased (Beveridge *et al.*, 1997; Foo *et al.*, 2007). This feedback regulation is suggested to involve a novel long-distance signal in pea that moves from shoot-to-root direction and requires *RMS2* (Beveridge *et al.*, 2000; Dun *et al.*, 2009).

Auxin can positively regulate the expression of SL biosynthesis genes *CCD7* and/or *CCD8* in pea, *Arabidopsis*, rice and chrysanthemum (Sorefan *et al.*, 2003; Foo *et al.*, 2005; Zou *et al.*, 2006; Liang *et al.*, 2010). Expression of *CCD7* and/or *CCD8* is enhanced by exogenous treatment with auxin. Decapitation may cause depletion of the expression of *CCD7* and/or *CCD8* but replacement with exogenous auxin may restore the levels of their expression observed in intact wild-type plants (Johnson *et al.*, 2006; Arite *et al.*, 2007; Hayward *et al.*, 2009).

ABA also regulates SL biosynthesis. Both ABA and SL are derived from the carotenoid pathway (Fig 1.8). Epoxycarotenoids' oxidative cleavage produces ABA via functioning of 9-cis-epoxycarotenoid dioxygenase (NCEDs) (Parry and Horgan, 1992; Thompson *et al.*, 2000; Taylor *et al.*, 2005). ABA biosynthesis is inhibited by two carotenoid biosynthesis inhibitors, fluridone and 2-(4-chlorophenylthio)-triethylamine hydrochloride (CPTA) that inhibit phytoene desaturase and lycopene cyclase, respectively (Cazzonelli, 2011). *vp14* in maize and *notabilis* in tomato are the two ABA-deficient mutants, having a mutation in an NCED. It was demonstrated that in both the *vp14* and *notabilis* mutants the ABA content was lower and the production of SLs was decreased (Matusova *et al.*, 2005; López-Ráez *et al.*, 2008). ABA-deficient mutants in tomato such as *sitiens* and *flacca* also showed decreased ABA content and SL production (Taylor *et al.*, 1988; Schwartz *et al.*, 2003). These results suggest that in SL biosynthesis, NCEDs either play a direct role or that ABA play a regulatory role (López-Ráez *et al.*, 2010).

Table 1.2 Genes involved in the biosynthesis and signaling of SL identified in *A. thaliana* (*max*), *Oryza sativa* (*d/htd)*, *Pisum sativum* (*rms*) and *Petunia hybrida* (*dad*)

Order **synth**	**Gene**	**Type**	**Spatial Expression** **organ**	**Tissue /cell**	**highest**	**Mutant phenotype**	**References**
1	**MAX3** DAD3 RMS5 HTD1 D17	CCD7	root (low) shoot	plastid		hyper branched, dwarfed, delayed leaf senescence	Booker *et al*., 2004 Gomez-Roldan *et al*., 2008 Johnson *et al.*, 2006 Zou *et al.*, 2006
2	**MAX4** DAD1 RMS1 D10	CCD8	root shoot (low) not flowers	plastid	root tip	hyper branched, dwarfed; reduced internodes, reduced root mass, delayed flowering, small flowers, reduced leaf senescence, advent roots, reduced axillary meristem, dormancy	Sorefan *et al*., 2003 Snowden 2005 Stirnberg *et al.*, 2002 Arite *et al.*, 2007
3	**D27**	Fe-protein	axillary buds	plastid	axillary buds	hyper branched, dwarfed, reduced culm length, increased auxin content and polar transport in the shoot	Lin *et al.*, 2009
4	**MAX1**	CYP 711A1 P450	whole plant	vascular bundles	lower stem (10x)	hyper branched, dwarfed	Stirnberg *et al.*, 2002
Signal							
	MAX2 RMS4	F-box signal	whole plant; Arab & rice uniform	seedling vascular bundles, inflorescence, stem	pea stipules; (3.5x) Sorghum roots; petunia axillary buds; (2A)	hyper branched, delayed leaf senescence, photomorphogenesis (longer hypocotyl and petiole in light and dark)	Shen 2007 Stirnberg 2007
	DAD2 **RMS3**	signal not identified				dwarfed, hyper branched, delayed leaf senescence	Hamiaux *et al.*, 2012
	D14 D3	signal α/β-fold hydrolase				increased tiller outgrowth, delayed leaf senescence, mesocotyl elongation, defective in sensing karrikins	Arite *et al.*, 2009 Ishikawa *et al*., 2005;

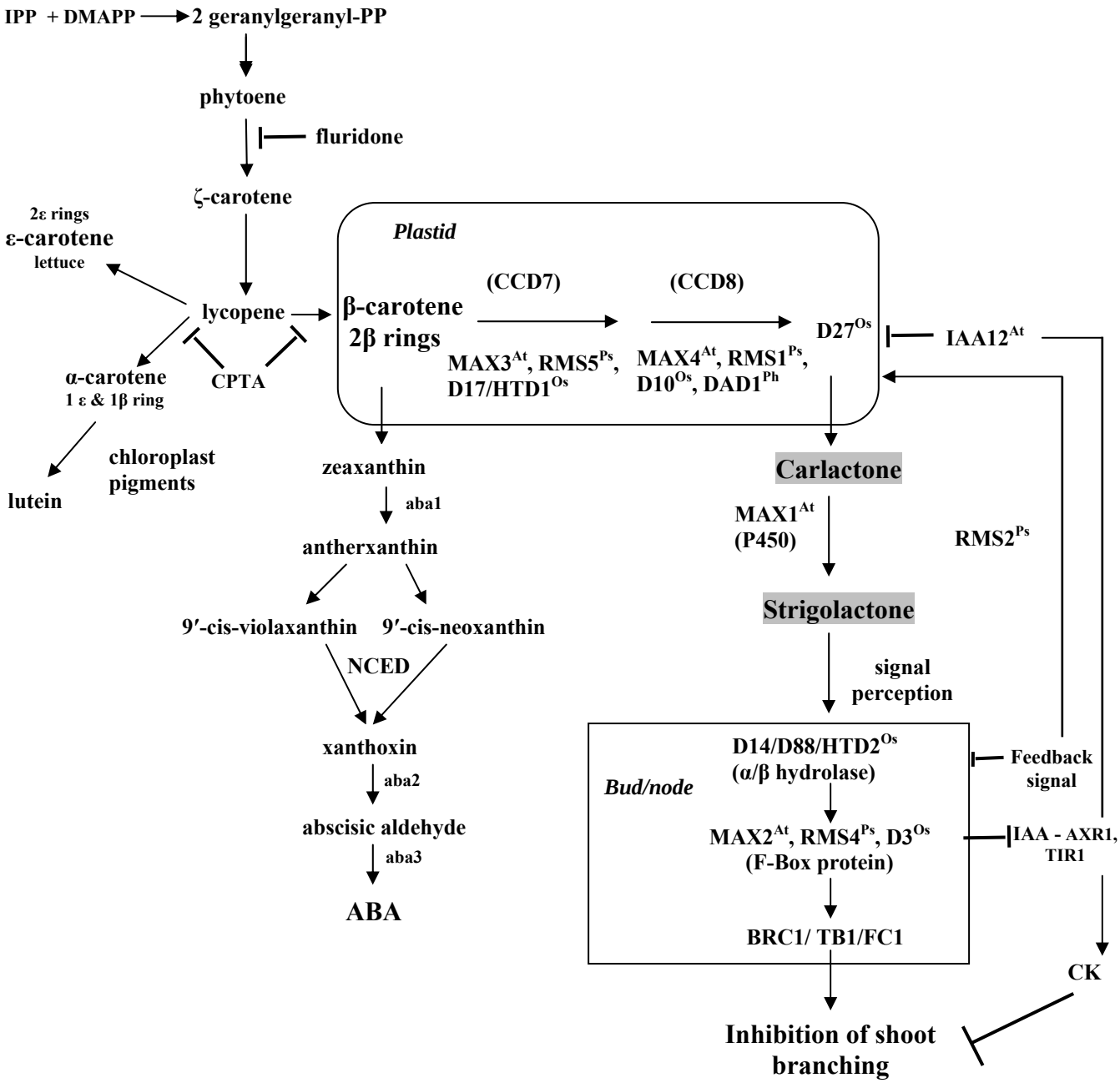


Fig 1.8 Relationship between the ABA and SL pathway resulting in shoot branching inhibition. Genes and their encoded proteins are shown (see text for details); Apart from *RMS2* in pea, all genes shown have been identified at the molecular level; Gene superscripts identify species; At, *A. thaliana*; Ps, *Pisum sativum* (garden pea); Os, *Oryza sativa* (rice); and Ph, *Petunia hybrida*. Modified from (Matusova *et al.*, 2005; Beveridge and Kyozuka, 2010).

1.6.3.3 Different roles of strigolactone

SLs found in root exudates of major plant species were first identified as germination stimulants of the parasitic weed seed including *Striga* and *Orobanche* (Cook *et al.*, 1966; Yokota *et al.*, 1998), which parasitise cereals and leguminous crops, respectively (Parker and Riches, 1993). Due to lack of photosynthetic capability and limited seed reserves, the parasitic plants establish a connection with the host plants within a few days of germination to ensure their survival (Matusova *et al.*, 2005). Parasites later use this connection to acquire nutrients and water (Bouwmeester *et al.*, 2003). Strigol, as a germination stimulant of *Striga hermonthica*, was first identified from cotton (Cook *et al.*, 1972) and later from maize, sorghum and millet (Bouwmeester *et al.*, 2003).

SLs were also characterized as root-derived signals stimulating hyphal branching of arbuscular mycorrhizal (AM) fungi. An endosymbiotic association forms between these fungi in the phylum Glomeromycota and the roots of 80% of plant species (Harrison, 2005). SLs secreted by the host roots stimulate hyphal growth and branching through activation of fungal mitochondrial metabolism (Akiyama *et al.*, 2005; Besserer *et al.*, 2006). The association helps in the acquisition of inorganic soil nutrients, such as phosphate (Pi) and nitrate, by the host plant (Akiyama *et al.*, 2005). Under Pi and nitrate deficient condition, the levels of SLs are significantly increased in roots and root exudates of host plants (Umehara *et al.*, 2008; Yoneyama *et al.*, 2008; Umehara *et al.*, 2010).

SLs or their biosynthetic precursors were shown to negatively regulate shoot branching by inhibiting axillary bud growth and together with auxin and CK, regulate plant architecture (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008), which was discussed in previous section (section 1.6.3). In garden pea, SLs and CKs appear to act together on the bud-specific target gene *BRC1*, which represses bud outgrowth (Aguilar-Martinez *et al.*, 2007; Finlayson, 2007; Braun *et al.*, 2012; Dun *et al.*, 2012). SL or CK also acts on the related genes in maize and rice such as *TEOSINTE BRANCHED1* (*TB1*) and *FINE CULM1*, respectively, in a species specific manner (Doebley *et al.*, 1997; Minakuchi *et al.*, 2010; Muller and Leyser, 2011).

SLs have also been shown to play a role in shaping root system architecture. SLs enhances primary root growth through the control of auxin flux in the roots of *Arabidopsis* (Ruyter-Spira *et al.*, 2011). SLs have been shown to inhibit lateral root formation and promote root hair elongation in response to phosphate and/or carbohydrate availability (Kapulnik *et al.*, 2011; Ruyter-Spira *et al.*, 2011). Auxin and its distribution play an important role in regulating lateral root initiation and formation (De Smet, 2012). Application of GR24 decreases the intensity of PIN1-GFP in lateral root primordia, which suggests that SLs may affect lateral root formation via changes in PIN-related auxin-efflux in the roots (Koltai *et al.*, 2010; Ruyter-Spira *et al.*, 2011).

Recently SLs were found to positively regulate secondary growth in combination with auxin (Agusti *et al.*, 2011). Secondary growth depends on the activity of vascular cambium which has the capacity to divide. Agusti *et al.* (2011) showed that both SL-deficient *max1* and SL-signalling *max2* mutants had decreased cambium activity compared to wild-type *Arabidopsis* plants. Exogenous GR24 application stimulated cell divisions in the interfascicular regions of SL-deficient mutants, which was less effective in SL-signalling mutant. Agusti also showed that SL acts downstream of auxin to induce secondary growth.

SLs were also found to be involved in leaf senescence, an age-dependent deterioration process which finally causes cell death (Noodén, 1988). One of the major changes which occur during senescence is the breakdown of chlorophyll and chloroplasts resulting in the yellowing of the leaf (Gut *et al.*, 1987; Lim *et al.*, 2007). Leaf senescence may be influenced by different external factors including shading, temperature, drought, nutrient deficiency, and pathogen infection. Among the different hormones involved in the regulation of leaf senescence such as ABA, JA, ethylene, and SA, the role of SL was identified through the analysis of mutants and expression of senescence-associated genes (SAGs). The SL signaling mutant, *max2*/ *oresara9 (ore9*) was found to exhibit reduced leaf senescence with reduced expression of some genes suggesting the function of SL as a positive regulator of senescence (Woo *et al.*, 2001; Woo *et al.*, 2004). SL mutant *d3* in rice and *dad1* in petunia also showed reduced leaf senescence (Snowden *et al.*, 2005; Yan *et al.*, 2007).

SLs are found to be involved in the control of photomorphogenesis. *MAX2* is involved in the light signalling and known as *PLEIOTROPIC PHOTO SIGNALING (PPS)* (Stirnberg *et al.*, 2002; Shen *et al.*, 2007; Shen *et al.*, 2012). *max2* mutant is hyposensitive to red/far-red and blue light resulting in longer hypocotyls, which is not seen in other *max* mutants (*max1, max3* and *max4)* (Stirnberg *et al.*, 2002; Shen *et al.*, 2007; Nelson *et al.*, 2011; Shen *et al.*, 2012). Moreover, *max2* shows reduced seed germination and expression of early light-regulated genes under red/ far-red light conditions. This suggests that *MAX2* positively regulates photomorphogenesis but in a SL-independent manner. Tsuchiya *et al.* (2010) has shown that SL inhibits hypocotyl elongation in the dark. Exogenous application of GR24 (10μM) causes stabilization of ELONGATED HYPOCOTYL5 (HY5), a bZIP transcription factor that promotes photomorphogenesis, by nuclear exclusion of CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) (Tsuchiya *et al.*, 2010; Toh *et al.*, 2012). COP1 is an E3 ligase which is known to degrade HY5 protein in the dark. The longer mesocotyl in the dark was also seen in SL mutants in rice and was rescued by exogenous GR24 application in SL deficient mutants, but not in SL-insensitive mutants (Hu *et al.*, 2010).

SLs have also recently been implicated in a wide range of other processes, which include promoting germination in non-parasitic plants (Tsuchiya *et al.*, 2010; Nelson *et al.*, 2011; Toh *et al.*, 2012) and promoting nodulation (the formation of symbioses with nitrogen fixing bacteria) in pea (Foo and Davies 2011). Ancient roles for SLs in plants predate evolution of vascular species and are evident in algae (Delaux *et al.*, 2012) and the moss, *Physcomitrella patens*, where SLs regulate branching of the protonema and have been implicated as quorum sensing-like signals (Proust *et al.*, 2011).

1.7 Aims of the study

An understanding of the function of the *Sporobolus* drought genes, isolated from desiccation-tolerant leaf tissues of dried plants, and how their expression is regulated may throw light on the mysteries of desiccation tolerance. Many major food crops such as rice, wheat and sorghum are monocots and it is expected that advanced knowledge about desiccation tolerance mechanisms in the monocot *S. stapfianus* will allow components of the desiccation tolerance program to be used to alter the stress responses in these crops. Characterization of one of the *Sporobolus* drought genes, UGT *SDG8i*, has the potential to reveal new molecular mechanisms for coping with severe stress in resurrection plants.

Although some dehydration-stress-related transcription factors of resurrection plants have been delineated (Le, 2004; Bartels and Sunkar, 2005), there exists very limited literature on regulatory genes controlling desiccation-tolerant gene expression models. This is mainly due to challenges of mutational approaches and an absence of proven protocols for transformation. *S. stapfianus*, presently recalcitrant to several recent genetic methods is a case in point. On the other hand, due to the small genome (125 Mbp) and the size and the short generation time, the herbaceous dicot *A. thaliana* serves as an ideal model to study developmental processes in plants (Meinke *et al.*, 1998). Its genome has been fully sequenced (AGI, 2000) and a number of reporter lines and molecular tools are available for this species. Since no protocol for transformation of resurrection grasses exists, functional analysis of the *SDG8i* was initially undertaken in *Arabidopsis.* It is possible that over-expression of *SDG8i* in *A. thaliana* may produce a clear or biochemically detectable phenotype, thereby providing insight into the function of *SDG8i*. The main objective of this study was therefore to conduct an analysis of the role of the UGT SDG8i by ectopic overexpression of *SDG8i* in *Arabidopsis* followed by characterization of these transgenic plants to determine the effect of *SDG8i* on plant growth and development, and hormonal and stress responses.

Chapter 2

General materials and methods

2.1 Seed stocks

Arabidopsis, *Sporobolus stapfianus* and Sorghum seed stocks were obtained from laboratory stocks. *Orobanche* seeds were obtained from the South Australian Department of Water, Land and Biodiversity Conservation.

2.2 Growing *Arabidopsis* in soil

Dry *Arabidopsis* seeds were sown by sprinkling them onto a 2:1 mixture of Seed Raising Mix (Debco) and perlite moistened with *Arabidopsis* nutrient solution, covered in plastic wrap and placed at 4°C for 3 days to break dormancy, which is known as stratification. Germinating seedlings were then grown in cycles of 8h day/16h (SD) night or 16h day/8h (LD) night photoperiod at 22^{o}C and approximately 200 μmole m^{-2} sec^{-1} light intensity. After seedlings had reached the cotyledon stage, the plastic wrap was removed, and the plants were watered every two or three days with *Arabidopsis* nutrient solution [100 ml of 1M KNO_3, 50 ml of 20mM FeEDTA, 40 ml of 1M $MgSO_4.7H_2O$, 40 ml of 1M $Ca(NO_3).4H_2O$, 50 ml of 1M Potassium Phosphate Buffer pH 5.5 (1M KH_2PO_4 and 1M K_2HPO_4), and micronutrient solution (70mM H_3BO_3, 14mM $MnCl_2.4H_2O$, 0.5mM $CuSO4.5H_2O$, 1mM $ZnSO_4.7H_2O$, 0.2mM $NaMoO_4.2H_2O$, 10mM NaCl, 0.01mM $CoCl_2.6H_2O$) per 20 L solution] until plants mature and set seeds.

2.3 Growing *Arabidopsis* in media

Arabidopsis seeds were surface-sterilized in 70% ethanol followed by 5 times wash with sterile water and germinated on Murashige and Skoog (MS) medium (pH5.8) containing 1/2 MS with 1% sucrose and 0.8% agar. After stratification at 4°C for 3 days to break dormancy,

the germinating seedlings were then grown in cycles of 8h day/16h night or 16h day/8h night photoperiod at 22°C.

2.4 Growing *N. benthamiana* in soil

Seeds were sprinkled onto a punnet of seed-raising mix (with perlite and osmocote), placed in a tray of water and covered with plastic-wrap. The plants were grown in greenhouse under a light cycle of 14h light/10h dark at approximately 24°C for 7 days. After seedlings had reached the cotyledon stage, the plastic wrap was removed. After that plants were transplanted in individual pots (½ seed raising mix, ½ potting mix with perlite and osmocote) and grown until 3 weeks of age under the same conditions. A week prior to infiltration, individual leaves were covered with foil (as many as required).

2.5 Agrobacterium-mediated transformation of *Arabidopsis thaliana*

A. thaliana ecotype Columbia plants were transformed using the floral dip method (Martinez-Trujillo *et al.*, 2004). Plants were grown in a soil mixture (2 parts soil and 1 part Perlite) at 20^{o}C under constant light at a density of 10-15 plants per punnet. Approximately 2 weeks after germination, the primary inflorescence bolt was cut using a sharp scalpel blade. Three days after cutting, the plants were dipped into a solution of *A tumefaciens* (OD600=1) containing the appropriate binary vector for 5 minutes. The infiltration medium consisted of 10 mM $MgCl_2$, 5% (w/v) sucrose, B5 vitamins (10-6x concentration) and 0.03% (v/v) Silwet L-77 surfactant. The punnet was laid on its side in a tray and covered in clear plastic Cling Wrap^{TM} for 1 day. The plastic cover was removed and the plants were allowed to stand upright. Seeds were collected approximately 3-4 weeks after transformation.

2.6 RNA extraction

Total RNA was isolated using a LiCl/phenol extraction method (Verwoerd *et al.*, 1989). Leaf tissue (0.5-1.0g) was crushed in liquid nitrogen with a mortar and pestle until a fine powder

was obtained. The powder was added to a pre-warmed (80^{o}C) polypropylene tube (Falcon BLUE MAX™) containing equal volume of TLES buffer and phenol (*V*T = 1.5 mL). Samples were mixed by vortexing for 30 seconds. The mixture was added to 750µl of chloroform/IAA (24:1) and centrifuged at 9279 g for 5 minutes in a microcentrifuge. The supernatant was transferred to a microcentrifuge tube and an equal volume of 4M LiCl was added. Samples were kept overnight at 0-4^{o}C and then centrifuged at 9279 g for 30 minutes at 4^{o}C. The RNA pellet was dissolved in 250µl of sterile distilled, de-ionised water. RNA was precipitated with 25µl of 3M sodium acetate (pH 6.0) and 500µl of ethanol and stored at -20^{o}C overnight or -70^{o}C for 1 hour. The RNA precipitate was centrifuged at 9279 g for 20 minutes, washed with 70% ethanol and dissolved in 50µl of sterile distilled water.

2.7 RNA gel electrophoresis

RNA samples were heated at 65^{o}C for 15 minutes in a loading buffer containing 50% glycerol, 1mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF, formaldehyde (8% v/v), formamide (50% v/v) and MOPS (1x). RNA was separated on an agarose gel (1.5% w/v) containing formaldehyde (8% v/v) and MOPS (1×). The relative sizes of the ribosomal RNA bands and gene transcripts were estimated using RNA markers.

2.8 Radioactive labeling of DNA probe fragments

DNA fragments were labelled with radioactive α-^{32}P-dATP nucleotides using the Gigaprime DNA Labelling Kit according to the manufacturer's instructions (Geneworks).

2.9 Transfer of nucleic acids from agarose gels to nylon membranes

Transfer was carried out using Hybond-N+ nylon membranes according to manufacturer's instructions (Amersham Pharmacia Biotech). After electrophoresis of DNA fragments, the gel was rinsed in a 0.125M HCl solution until the bromophenol blue dye changed colour. The gel was then washed in denaturing solution until the dye returned to its original colour. The gel

was submerged in neutraliser solution for 30 minutes with gentle agitation. For Northern analysis, RNA gels were washed in 2×SSC buffer to remove formaldehyde. The treated gel was placed on a wick platform consisting of three sheets of 3MM paper saturated in transfer buffer (10× SSC). The nylon membrane was placed on top of the gel. Three sheets of 3MM paper and a stack of absorbent paper, all cut to size, were successively placed on top of the membrane. A glass plate and a weight were placed on top of the paper stack. The transfer was allowed to proceed overnight in transfer buffer (10× SSC). After transfer, nucleic acids were fixed by exposing the nylon membrane to UV light for 3 minutes.

2.10 Northern blot hybridisation

Following transfer of RNA, the nylon membrane was pre-hybridised in 5 ml of ExpressHyb Hybridisation Solution (Clontech) at 68^{o}C for 30 minutes. Hybridisation was performed in 5 ml of fresh ExpressHyb solution containing the denatured radioactively labelled DNA probe with continuous shaking at 68^{o}C for 1 hour. The membrane was rinsed in wash solution 1 (2× SSC, 0.05% SDS) at room temperature for 30 minutes with continuous shaking and several changes of fresh solution and then in wash solution 2 (0.1× SSC, 0.1% SDS) with continuous agitation at 50^{o}C for 40 minutes with one change of fresh solution. The membrane was then sealed in plastic wrap to prevent drying.

2.11 X-ray film autoradiography

Radioactive membranes were exposed to X-ray film at -70^{o}C with one intensifying screen. The time of exposure varied from 30 minutes to 1 week, depending on the intensity of the radioactive signal.

2.12 Phosphoimaging

Radioactive signals on blots were detected by exposing membranes to a phosphoimaging screen for 1 to 7 days. The signal intensity was scanned using the Storm 860 scanner (Molecular Dynamics) and quantified using the ImageQuant V5.0 software (Molecular

Dynamics). The expression levels of *SDG8i* in the transgenic lines are calculated relative to the ribosomal RNA bands and the highest expression is set at 100%, with the expression in the additional lines calculated relative to this 100%.

2.13 Light microscopy

The microscope used for this work was the Leica Microsystem (Wetzlar, Germany) with a digital camera and the Axioskop (Carl Zeiss, Germany) with normal and Normasky optics and a Zeiss Axiocam digital camera.

Chapter 3

Effect of *SDG8i* on plant architecture and the growth rate of *Arabidopsis*

3.1 Introduction

Recent advances in biochemistry, analytical chemistry and molecular biology, and the use of the plant *Arabidopsis thaliana* as a model system, have significantly increased our knowledge of developmental processes in plants. There is growing insight into how plant hormones affect growth and development and the specific biochemical mechanisms that they regulate. In the present study, *Arabidopsis* plants constitutively over-expressing UGT *SDG8i*, under the control of the CaMV 35S promoter, have been produced to investigate the activity of the gene product *in planta*. This study characterizes the *SDG8i* transgenic plants and compares them with wild-type Col-0 under long and short day photoperiods. The yeast-one hybrid system has previously been used to identify two putative transcription factors, *SDG7y* and *SDG10y* that may regulate *SDG8i* expression (Le, 2004). Transgenic plants, expressing these two transcription factors, SDG10y and SDG7y, driven by the CaMV35S promoter were also generated and characterized.

The development from a seed to a fully-grown plant is a plastic process with the basic architecture of the plant being laid out in the embryo. During plant growth and development the primary and secondary meristems continuously give rise to new leaves and branches, flowers, roots and stem tissue. During germination and subsequent growth, a plant must be able to adapt to a range of environmental conditions in order to develop and reproduce successfully. Environmental signals induce responses in diverse groups of cells and tissues via specific intrinsic signal transduction pathways which influence cell division, cell expansion and cell differentiation processes, and therefore regulates growth and development. Plant hormones or plant growth regulating substances, particularly auxins, cytkinins (CKs) and the recently identified novel carotenoid-derived hormone, strigolactone (SL) have essential roles

in this process (Dun *et al.*, 2009; Leyser, 2009). Auxin was the first hormone identified and shown to have a main role in the regulation of bud outgrowth (Thimann and Skoog, 1933). Experiments and the subsequent development of models indicate that the auxin signal which controls bud outgrowth is produced in the shoot apex and is transported basipetally down the shoot. However, this apically synthesized auxin cannot move into lateral buds and therefore it is considered that there are one or more secondary messenger(s) involved. It has been proposed that SLs and CKs act as these long-distance second messengers, interacting in an antagonistic way to communicate with auxin (Brewer *et al.*, 2009; Dun *et al.*, 2012). CK moves acropetally and promotes bud outgrowth directly whilst SL, which also moves acropetally, inhibits shoot branching (Booker *et al.*, 2005; Muller and Leyser, 2011).

In the regulation of root development, including primary root growth, lateral root initiation and growth as well as root hair initiation, hormones play important roles (Leyser, 2006). Auxins are known for their strong inhibitory effects on root elongation but are the dominant regulators of lateral root initiation (LRI) and development which determines the ability of the plant to adapt to changing environments. Application of auxin at high concentrations has been shown to increase LRI by activating the root pericycle cells adjacent to the xylem, which results in a decrease in the spacing of the lateral root primordia (Himanen *et al.*, 2002; Benková *et al.*, 2003; Dubrovsky *et al.*, 2008). Other hormones, such as ethylene (Ivanchenko *et al.*, 2008) and CK (Laplaze *et al.*, 2007) have been shown to modify the auxin activation of the pericycle cells. SLs are also involved in root growth and development by positively regulating primary root growth in *Arabidopsis* through changes in auxin flux (Koltai, 2011; Ruyter-Spira *et al.*, 2011) but negatively regulating lateral root formation under sufficient phosphate (Pi) conditions (Kapulnik *et al.*, 2011; Ruyter-Spira *et al.*, 2011). This was demonstrated by the analysis of *Arabidopsis* SL-biosynthesis mutants, *max3* and *max4*, or SL-signaling mutants, *max2* which were shown to have a large number of LRs (Kapulnik *et al.*, 2011; Ruyter-Spira *et al.*, 2011). SLs was also shown to promote root hair elongation, which is important for plants to take up water, nutrients and minerals from soil (Kapulnik *et al.*, 2011). CKs have also been shown to have negative effect on LR formation, possibly by interfering with the transport of auxin to the lateral root primordia (Bishopp *et al.*, 2011).

Auxin plays a central role in the hypocotyl elongation. This has been demonstrated by studies of *AUXIN RESPONSE FACTOR 2* (*arf2)* mutants, which have a long hypocotyl phenotype under a wide range of red/far-red light (Rc and FRc) conditions, under continuous blue light, and also in the dark, which may be due to increased auxin content or an alteration in auxin sensing (Jackson *et al.*, 2002). The role of SL in light signaling has come from analysis of hypocotyl development in the *Arabidopsis* F-box *MAX2* mutant, which has a defective response to light and *max2* displays elongated hypocotyls and smaller cotyledons when grown in the light (Stirnberg *et al.*, 2002). The SL pathway promotes photomorphogenesis by inducing nuclear exclusion of CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1). COP1 is an E3 ligase involved in protein degredation. Exclusion of COP1 from the nucleus stabilizes ELONGATED HYPOCOTYL5 (HY5) which is involved in promoting photomorphogenesis (Tsuchiya *et al.*, 2010). However, Hu *et al.* (2010) found that mesocotyl elongation in rice is negatively regulated by SLs in the dark.

In addition to the analyses of the *SDG8i* transgenic plants, the present study also looks at the role of *SDG7y* and *SDG10y*, two AP2/ERF transcription factors (TFs) isolated from the resurrection plant *S. stapfianus.* These two genes, which are highly similar (74% similarity), were isolated using a yeast-one-hybrid screen with 400bp of the *SDG8i* promoter as the target sequence (Le, 2004). The AP2/ERF family are a large plant specific group of TFs with 144 members reported in *Arabidopsis* and have been shown to have roles in plant development and responses to environmental stresses (Sakuma *et al.*, 2002; Nakano *et al.*, 2006; Sharoni *et al.*, 2011; Licausi *et al.*, 2013). The name is based on APETALA2, a homeotic gene involved in flowering in Arabidopsis, and ethylene response factor (ERF) genes and the identification of conserved AP2/ERF binding domains. The AP2/ERF TFs are generally divided into 4 classes, AP2 (14 genes) ERF/DREB (122), RAV (6) and 'others' (4) (Sakuma *et al.*, 2002). Members of the AP2 group possess two AP2/ERF DNA binding domains, the RAV sub-family has a single AP2 domain and a B3 DNA binding domain, whilst the ERF/DREB group has a single AP2 domain and a WLG motif whilst those in the 'other' group also have a single AP2/ERF domain but do not have the WLG sequence. The ERF/DREB group may also be divided into two classes, the ethylene response element binding protein (EREBP) and the dehydration-responsive element binding (DREB) protein classes (Nakano *et al.*, 2006). *SDG7y* and

SDG10y have a single AP2 domain and thus fall into the ERF/DREB transcription factor category and sequence homology suggests they are in the ERF subgroup (Le, 2004). The ERF TFs were initially thought to be involved in ethylene mediated responses but recently have also been shown to be involved in the regulation of genes induced upon drought stress (Mizoi *et al.*, 2013) whilst DREB TFs have been shown to activate several dehydration/cold-regulated (RD/COR) genes (Stockinger *et al.*, 1997; Liu *et al.*, 1998; Lucas *et al.*, 2011). The generation of transgenic wheat and rice plants over-expressing DREB1-type genes has been shown to improve tolerance to abiotic stress (Pellegrineschi *et al.*, 2004; Oh *et al.*, 2005; Ito *et al.*, 2006). Several studies have also showed that ectopic expression of a number of ERF genes from different plants such as *Arabidopsis* and rice confer tolerance to salt and drought stresses (Yi *et al.*, 2004; Seo *et al.*, 2010; Fukao *et al.*, 2011; Mito *et al.*, 2011). Song *et al.* (2005) showed that AtERF7 in *Arabidopsis* plays an inhibitory role in response to ABA during periods of water loss. Overexpression of the ERF/EREBP transcription factor *CAP2* increased the growth levels and tolerance of transgenic tobacco during dehydration and salt stress (Shukla *et al.*, 2006). To date there are no published reports of the isolation of AP2/ERF TFs from desiccation tolerant species, but a *myb* transcription factor has been isolated from *C. plantagineum* (Iturriaga *et al.*, 1996). This TF, CpMYB10, was expressed in *Arabidopsis* and plants were more tolerant to osmotic and salt stress (Villalobos *et al.*, 2004). CpMYB10 was found to upregulate some genes known to be involved in abiotic stress response in *Arabidopsis* such as *RD29A* and downregulate others such as *COR15A*. The preliminary analysis of the phenotypes of *SDG7y* and *SDG10y* is presented in this chapter.

3.2 Materials and methods

3.2.1 Generation of *SDG8i* transgenic plants

Seven independent homozygous transgenic *Arabidopsis* plants constitutively over-expressing *SDG8i* (accession number AM268210) was produced in association with Dr. Cecilia Blomstedt. Gene specific primers were used to amplify the coding region of *SDG8i* using the *Pfu* proofreading polymerase (Promega). To allow the use of the Gateway cloning system (Invitrogen Corporation, Carlsbad, CA, United States of America), the sequences for the recombination sites, attB1 and attB2, of the donor vector pDONR221 were included as part of the primers.

5′attB1 Primer:
GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACGAAGACCGTGGTTCTG
3′attB2 Primer:
GGGGACCACTTTGTACAAGAAAGGTGGGTCTCACGGACGACCGACAGCCTCCA

Following successful generation of the pDONR221:SDG8i construct, the *SDG8i* coding sequence was transferred to the plant expression vector pMDC32 containing the 2 × 35S promoter (Curtis and Grossniklaus, 2003). This construct was then introduced into the wild-type accession Columbia-0 (Col-0) *Arabidopsis* using *Agrobacterium tumerfaciens* (Agli strain) by the floral dip method (Martinez-Trujillo *et al.*, 2004). Transgenic plants were selected for resistance to hygromycin and taken through to the second generation (T2) to generate plants homozygous for *SDG8i*.

3.2.2 Generation of *SDG7y* and *SDG10y* transgenic plants

The complete nucleotide sequences of SDG7y and SDG10y determined by Le (2004) are given in Appendices 2 and 1, respectively. The transgenic *Arabidopsis* plants constitutively over-expressing *SDG7y* and *SDG10y* were produced by Ella Jungereth and Dr. Cecilia Blomstedt using the Gateway cloning system as detailed above for *SDG8i* (Invitrogen Corporation, Carlsbad, CA, United States of America). Except that *SDG10y* was initially ligated into the *Eco*RI and *Xho*I site of the pENTR1A vector. After successful generation of

the pDONR221:SDG7y and the pENTR1A:SDG10y constructs, the *SDG7y* and *SDG10y* coding sequences were transferred to the plant expression vector pMDC32 containing the 2 × 35S promoter (Curtis and Grossniklaus, 2003).

Primers for SDG7y:

5′attB1:

GGGGACCACTTTGTACAAGAAAGCTGGGTACAACTGAATTGGGGCAATC

3′attB1:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGGCTCAACTCAGTCA

The constructs were then introduced into the wild-type accession Columbia-0 (Col-0) *Arabidopsis* using *Agrobacterium* (Agli strain) by the floral dip method (Martinez-Trujillo *et al.*, 2004). Transgenic plants were selected for resistance to hygromycin and taken through to the second generation (T2) to generate plants homozygous for *SDG7y* and *SDG10y*.

3.2.3 RNA extraction and northern analysis of *SDG8i* transgenic plants

Total RNA was isolated from 1.0g of leaf tissue using a LiCl/phenol extraction method (Verwoerd *et al.*, 1989). Following electrophoresis, the RNA was transferred onto a nylon membrane (Hybond N, GE Healthcare) and probed (Sambrook *et al.*, 1989) with a radiolabelled *SDG8i* DNA fragment generated by PCR using pMDC32:SDG8i as a template. The forward primer 5'ACACCGACGTCACTCCCAAGC3' and the reverse primer 5'GCCGACGGCGATGTCATGGAGC3' were used for amplification of the *SDG8i* gene specific probe. The membrane was exposed to a storage phosphor screen (Molecular Dynamics) overnight and visualized using Typhoon Trio Scanner (GE Healthcare). An estimate of the *SDG8i* transcript levels relative to 28s rRNA in the ethidium bromide stained gel was obtained using ImageQuant TL 7.0 software.

3.2.4 Observation of palisade cells in fully expanded leaves

To observe palisade cells, the second fully expanded rosette leaves of wild-type Col-0 and *SDG8i* transgenic plants were immersed in 0.1% Triton X-100 and centrifugation at 10,000 g

for 1 min at room temperature to remove air bubbles from intercellular spaces and sediment the chloroplasts. Leaves were then viewed under a light microscope (Axioskop, Carl Zeiss, Germany) with normal and Normasky optics. Photographs were taken using a Zeiss Axiocam digital camera using Axio Vision software. The area of the leaf blade was measured with Adobe Photoshop CS5.1. Twenty palisade cells in the sub-epidermal layer aligned along the proximo–distal (P–D) and medio–lateral (M–L) axes were used to calculate the average cell area using Image J software. The total cell number per leaf was calculated by dividing the leaf area by the palisade cell area for each leaf.

3.2.5 Examination of root architecture

Plants were grown at 21^{o}C under long day (LD; 8h day/16h night) following stratification for 13 days in vertically orientated petri dishes on MS media (pH5.8) with 1% sucrose, and 0.8% agar. To observe the root architecture tissue was prepared according to (Dubrovsky *et al.*, 2009) which involved fixing the roots in 4% formaldehyde in 0.025 M phosphate buffer (pH 7.2) for at least 4 h at room temperature, or O/N at 4^{o}C. The fixative was replaced with 30% (aq. v/v) glycerol containing 2% (v/v) DMSO and left for 30 min at room temperature. Roots were mounted in a clearing solution composed of 4.2 M NaI, 8 mM $Na_2S_2O_3$ prepared in 65% (v/v) glycerol supplemented with 2% (v/v) DMSO. Roots were observed 1 h after the sample preparation. Root primordia and root cell-length analyses were performed under the microscope (Axioskop, Carl Zeiss, Germany) with normal and Normasky optics. Photographs were taken using a Zeiss Axiocam digital camera using Axio Vision software.

3.2.6 Statistical analysis

All data were examined by analyses of variance using GraphPad Prism software version 5.0. Tukey's Multiple Comparison Test was used for comparison between wild-type and *SDG8i* transgenic means at 5% level of significance.

3.3 Results

3.3.1 Over-expression of *SDG8i* in *Arabidopsis thaliana*

Seven independent *Arabidopsis* lines (F1aD, D1E, F6bA, D5aA, D2E, D4I, D7c) over-expressing SDG8i protein under the control of the 35S promoter were generated and taken to homozygosity. Northern blot analysis was carried out using ten micrograms of total RNA isolated from leaf tissue of wild-type and transgenic seedlings and hybridized with a *SDG8i* gene-specific probe. This analysis confirmed the presence of *SDG8i* transcripts and their steady-state levels in leaf tissues of each of the transgenic lines (Fig 3.1). The expression levels are calculated relative to the ribosomal RNA bands and the highest expression is set at 100% and the others are relative to this. The levels are generally strong and there is no major phenotypic difference between the various transgenic lines.

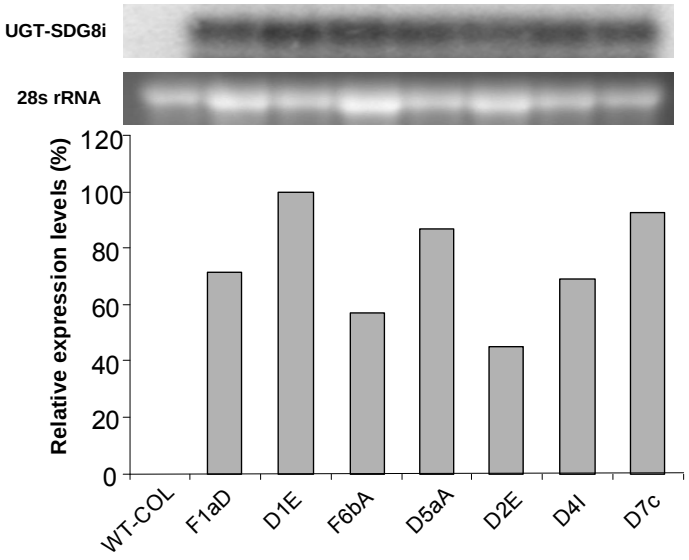


Fig 3.1 Northern analysis showing the presence of SDG8i transcripts in *SDG8i* transgenic lines. Ten micrograms of total RNA was isolated from leaf tissue of wild-type and transgenic seedlings and hybridized with a *SDG8i* gene-specific probe. Ethidium bromide staining was used to estimate levels of RNA loading (This experiment was performed with the assistance of Ms Cara Griffiths).

3.3.2 Phenotypic development of *SDG8i* transgenic plants in long and short day

The *SDG8i* transgenic plants displayed phenotypes which differed from those of the wild-type Col-0 plants. When the plants were grown in long day (LD:16h day/ 8h night) (Fig 3.2) photoperiod, four of the *SDG8i* transgenic plants produced significantly longer leaves (about 1.5 – 1.6 times longer) compared to wild-type Col-0 plants (P<0.05) (Fig 3.3A). No

significant difference between the transgenic lines and wild-type plants was observed in the number of rosette leaves produced before bolting (Fig 3.3B). The first inflorescence was produced 16 days after germination when all the plants were at about 8 to 10 leaf stage indicating that the *SDG8i* gene does not affect flowering time in long day. *SDG8i* transgenic plants also produced a significantly higher number of rosette branches (about 1.3 to 1.7 times) than did wild-type Col-0 plants ($P<0.01$; Fig. 3.3C), which gave them a bushy appearance (Fig. 3.2). Amongst the transgenic plants, D5a, D4I and D7c produced about 1.3 times higher number of inflorescence compared to others ($P<0.01$). The height of the bolting shoots of all the transgenic lines was significantly increased compared to wild-type Col-0 after 35 days of growth in LD ($P<0.01$). The height increase over wild-type Col-0 ranged from 15 to 33% (Fig. 3.3D). The growth rate of the primary bolt was similar in transgenics and wild-type Col-0 controls in LD. The larger leaf size resulted in the average fresh weight of shoot biomass of all LD grown transgenic plants being almost 1.7-1.8 times higher than that of wild-type Col-0 (Fig. 3.3E) ($P<0.001$). Some of the transgenic plants produced a small but significantly higher number of seeds than did WT Col-0 plants ($p<0.05$) (Fig. 3.3F).

WT Col-0 **D5aA**

Fig 3.2 *SDG8i* transgenic and wild-type Col-0 plants growing at 21^{o}C under long day (LD) condition of 16h day/ 8h night after stratification. Bar = 2 cm.

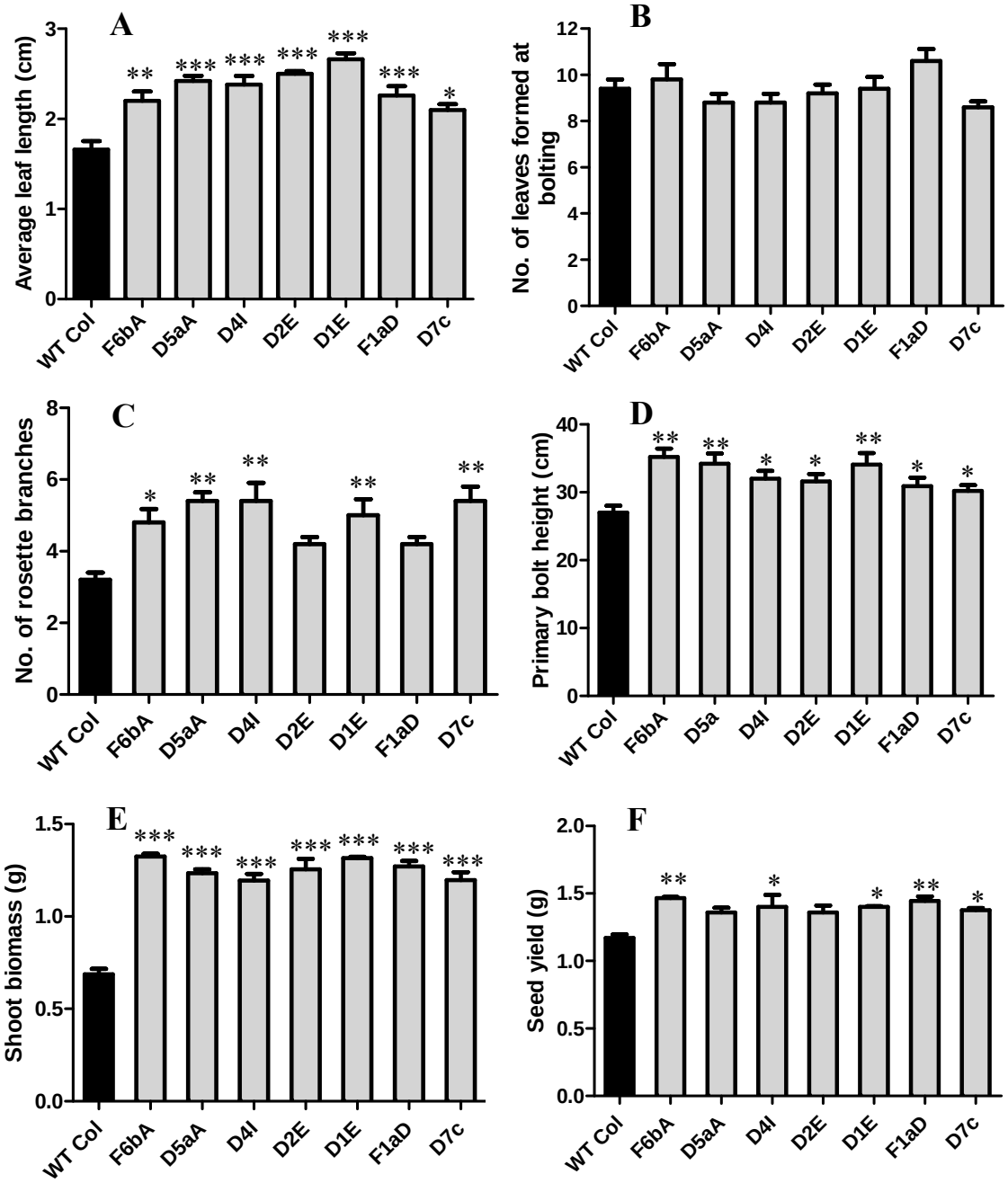


Fig 3.3 A) Average leaf length (cm) calculated 16 days after germination, B) leaf count is the no. of primary rosette leaves formed at the floral initiation stage, C) number of rosette branches and D) primary bolt height measured 35 days after germination, E) shoot biomass (FW) measured 16 days after germination and F) seed yield of *SDG8i* transgenic and wild-type Col-0 plants growing at 21^{o}C under the condition of 16h day/ 8h night. Dry seeds were collected following senescence. Values are the means ± SE of 5 replicates. Significant differences at 5, 1 and 0.1% levels from WT determined by Tukey's Multiple Comparison Test are denoted by *, ** and ***, repectively.

Flower morphology and seed development appeared normal in all the transgenic and wild-type Col-0 plants when grown under LD and there was no significant difference between them in the size of siliques and number of seeds produced per silique (~27 seeds in young silique and ~33 seeds in mature silique) or in the weight of the individual seed produced (Fig 3.4).

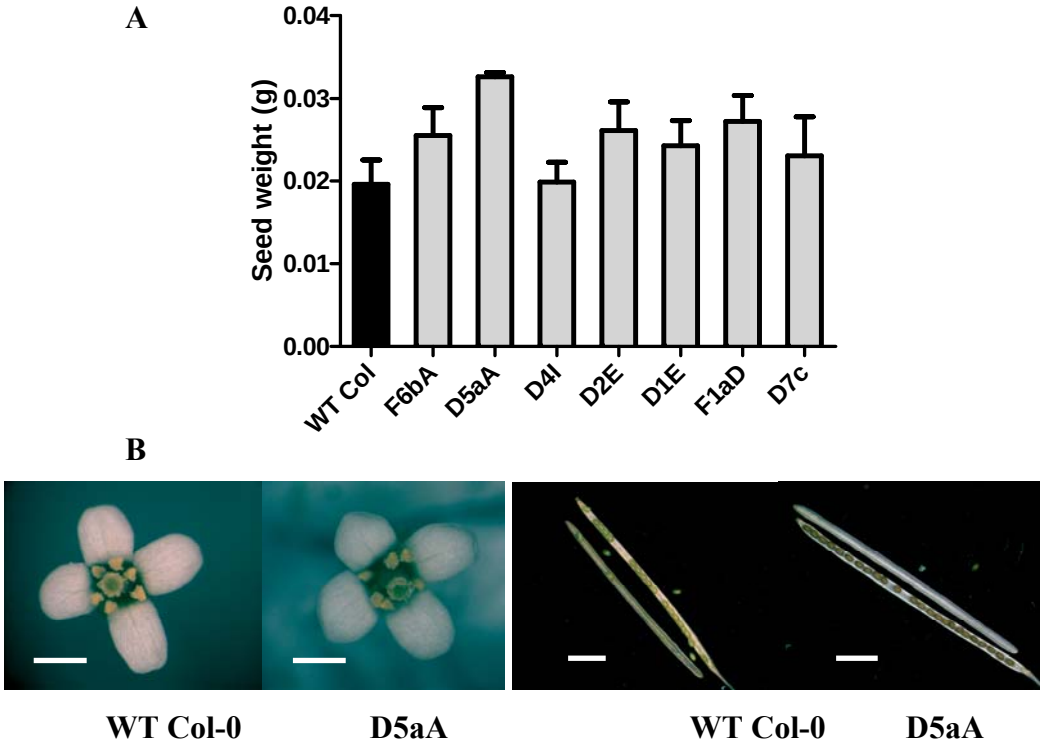


Fig 3.4 A) The seed weight is the weight of 500 seeds. Values are the means ± SE of 5 replicates. B) Flower morphology and seed development of *SDG8i* transgenic and wild-type Col-0 plants growing at 21^{o}C under LD the condition. Bar = 2 mm.

Short day length (SD: 8h day/ 16h night) favored development of rosette leaves and delayed flowering of all the plants (Fig 3.5). Both wild-type Col-0 and *SDG8i* transgenic plants produced the first inflorescence 52 days after the germination. By this time wild-type Col-0 plants had produced around 30-34 primary rosette leaves whereas the transgenic plants had produced approximately 40-42 leaves. A more pronounced difference between wild-type Col-0 and transgenic plants was observed one month after flowering when total number of leaves, including primary rosette leaves and leaves formed in the rosette from axillary meristems by

transgenic plants was about 1.3 to 1.6 times higher than that of wild-type Col-0 plants (P<0.01) (Fig 3.6A) and among the transgenic plants, D5aA produced the highest number of leaves (P<0.05). The average size of the leaves of transgenic plants was significantly larger than that of wild-type Col-0 (P<0.05) (Fig. 3.6B). All the *SDG8i* transgenic plants also produced a significantly higher number of inflorescences which was about 1.4 to 1.6 times higher than that of wild-type Col-0 (P<0.01) (Fig. 3.6C). All *SDG8i* transgenic plants were significantly taller than wild-type Col-0 after 15 days of bolting (P<0.01) and the height increase of the transgenic lines over wild-type Col-0 was 9 to 16% after 21 days of bolting (Fig. 3.6D). The rate of growth of the primary inflorescence of the transgenic plants was around 20% greater (~7.12 cm week^{-1}) than that of wild-type Col-0 plants (5.4 cm week^{-1}).

Fig 3.5 *SDG8i* transgenic plants showing increased shoot growth compared to wild-type Col-0 plants. Plants were grown at 21^{o}C under the short day (SD) condition of 8h day/ 16h night after stratification. Bar = 2 cm.

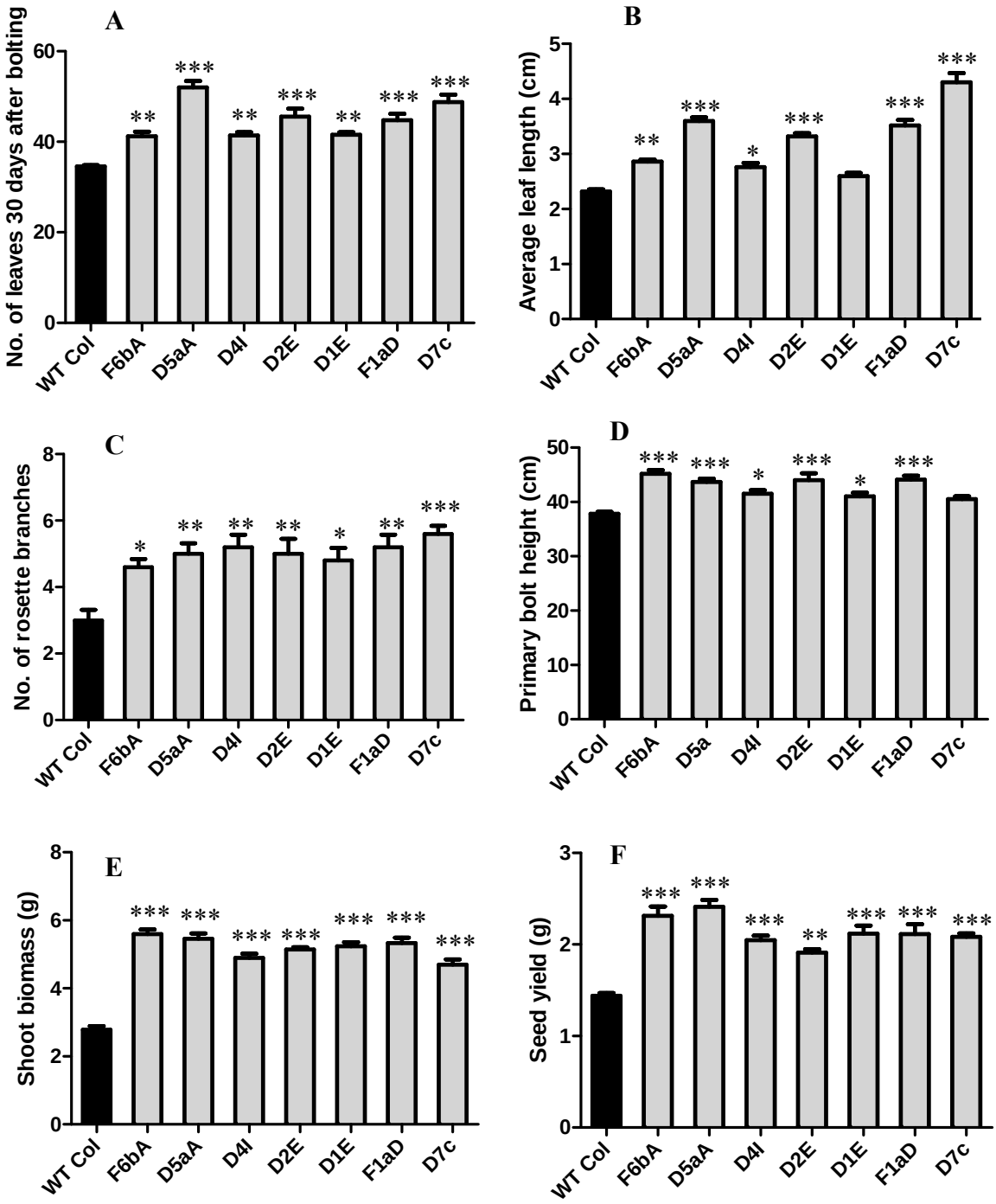


Fig 3.6 The A) number of leaves produced by primary and axillary meristems in the rosette, B) average leaf length, C) number of rosette branches and D) primary bolt height, measured 30 days after bolting, E) shoot biomass (FW) measured 50 days after germination and F) seed yield of *SDG8i* transgenic and wild-type Col-0 plants growing at 21^{o}C under the condition of 8h day/ 16h night after stratification. Dry seeds were collected following senescence. Values are the means ± SE of 5 replicates. Significant differences at 5, 1 and 0.1% levels from WT determined by Tukey's Multiple Comparison Test are denoted by *, ** and ***, repectively.

In both LD and SD, the primary bolt of transgenic plants maintained dominance over secondary bolts throughout the growth of the plant. In SD photoperiods the average fresh weight of shoot biomass of all transgenic plants, measured 50 days after germination, was almost twice that of wild-type Col-0 (Fig. 3.6E). The increased seed yield of *SDG8i* transgenic plants was more pronounced in SD than in LD with the transgenic plants producing about 1.4-1.6 times the more seeds by mass than wild-type Col-0 plants ($p<0.001$) (Fig 3.6F). Among the transgenic plants, F6bA and D5aA produced the highest number of seeds. The average fresh weight of the root biomass of all pre-flowering *SDG8i* transgenic plants was almost twice that of wild-type Col-0 when grown in both LD and SD (Fig. 3.7 A&B).

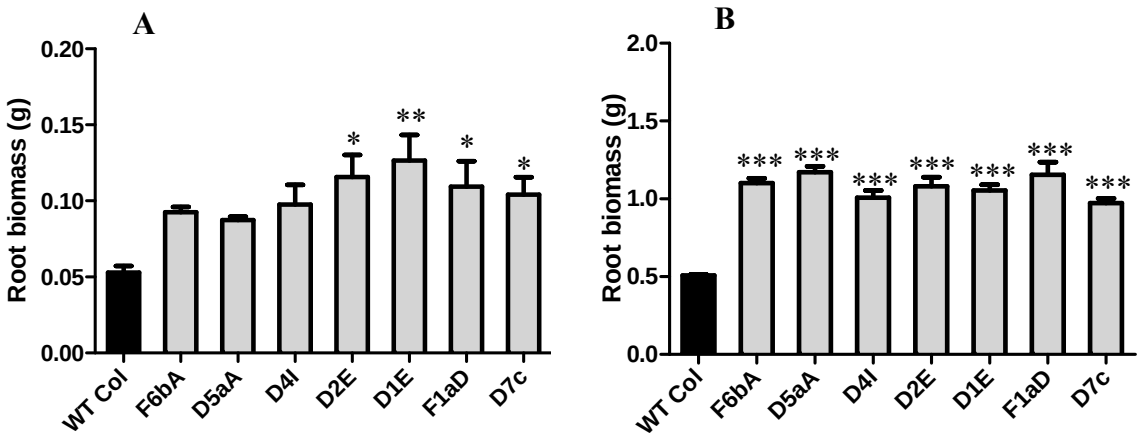


Fig 3.7 Root biomass (FW) of A) long day and B) short day grown pre-flowering *SDG8i* transgenic and wild-type Col-0 plants measured 16 and 50 days after germination, respectively at 21^{o}C. Values are the means ± SE of 4 replicates. Significant differences at 5, 1 and 0.1% levels from WT determined by Tukey's Multiple Comparison Test are denoted by *, ** and ***, repectively.

In SD, flowers of all *SDG8i* transgenic and wild-type Col-0 plants were normal and fertile, and similar in size (Fig 3.8). The size of siliques and number of seeds produced per silique was similar in all transgenic and wild-type Col-0 plants (Fig 3.8).

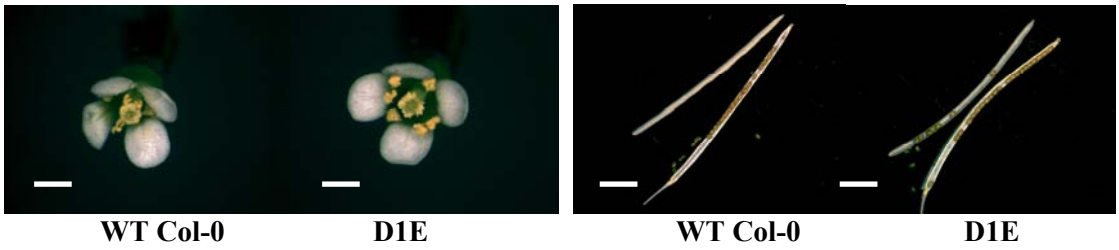


Fig 3.8 Flower morphology and seed development of *SDG8i* transgenic and wild-type Col-0 plants. Plants were grown at 21°C under the SD condition of 8h day/ 16h night after stratification. Bar = 2 mm.

3.3.3 Length of hypocotyl and primary root in the dark

SDG8i transgenic and wild-type Col-0 seedlings were germinated and grown in the dark and the length of the hypocotyl and primary root was measured. While no significant differences were observed in cotyledon development between the dark-grown wild-type Col-0 and transgenic seedlings, all the transgenic plants produced significantly longer hypocotyls and roots that were approximately 1.5 to 1.7 times than those of wild-type Col-0 ($P<0.05$) (Fig. 3.9 A & B).

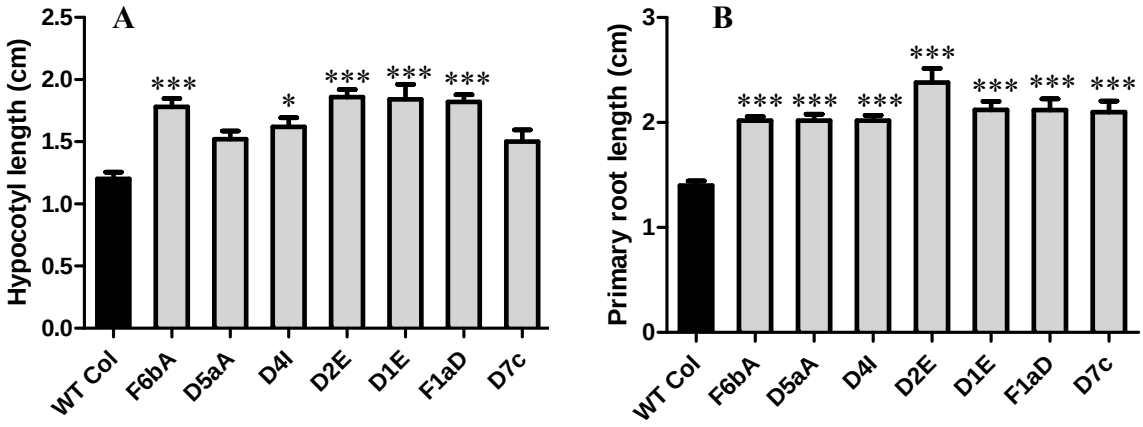


Fig 3.9 Lengths of A) hypocotyl and B) primary root measured from each *SDG8i* transgenic and wild-type Col-0 plants. Plants were grown in the dark at 21°C for 7 days after stratification. Values are the means ± SE of 5 replicates. Significant differences at 5, 1 and 0.1% levels from WT determined by Tukey's Multiple Comparison Test are denoted by *, ** and ***, repectively.

3.3.4 Number and size of leaf cell

To assess whether the changes in overall leaf morphology were supported by changes at the cellular level, the second fully expanded leaves were analyzed by microscopy. The rosette leaves of *SDG8i* transgenic plants are larger than those of control wild-type Col-0 plants in both LD and SD. When the leaf cells of SD soil-grown plants were examined under the light microscope following preparation to visualize the cell, it was found that the leaves of transgenic plants contained more cells than comparable leaves from control plants and the cells were larger (Fig 3.10 & table 3.1) ($p<0.01$). The increase in the number and size of leaf palisade cells of *SDG8i* transgenic plants indicates that the *SDG8i* has a role in promoting leaf cell expansion and cell division.

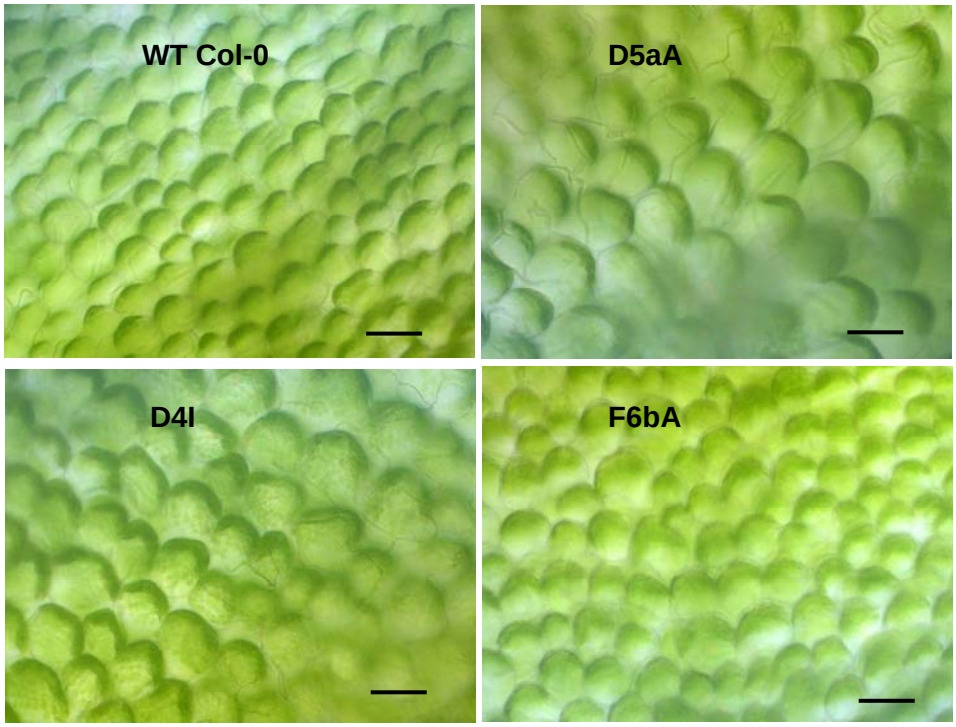


Fig 3.10 Palisade cells in the second fully expanded rosette leaves of wild-type Col-0 and *SDG8i* transgenic plants. Bar = 50 μm.

Table 3.1. Average leaf area, average cell area and average cell number per leaf of wild-type Col-0 and *SDG8i* transgenic *Arabidopsis* plants
Second fully expanded leaves from 25-day-old soil-grown plants in SD were used. The leaf area and cell area were calculated using imaging software. For each leaf, 20 palisade cells were used to determine the average cell area. The average number of palisade cells per leaf was determined by dividing the leaf area by the palisade cell area for each leaf. Values are the means ± SE of 3 replicates.

Name of the plants	Average leaf area (mm^2)	Average cell area (μm^2)	Average cell number/leaf
WT Col-0	47.55 ± 1.98	3160 ± 30	15047 ± 586
D5aA	63.92 ± 0.84	3540 ± 159	18129 ± 845
D4I	62.0 ± 1.4	3575 ± 113	17392 ± 831
F6bA	59.97 ± 3.13	3592 ± 173	16858 ± 885

3.3.5 Root cell length and lateral root initiation

A comparison of the root cell length and lateral root primordia in *SDG8i* transgenic and wild-type Col-0 plants grown *in vitro* in SD was made to determine if *SDG8i* expression also affected root architecture. Roots were mounted on microscope slides and the number of primordia in cleared roots within the lateral-root-formation zone between the most-distal initiated primordium and the most-distal emerged lateral roots (Fig. 3.11A & B) was determined using the protocol of Dubrovsky *et al.* (2009). Lateral root primordium density (*d*) was calculated for each individual primary root as number of primordia per mm. I_{LRI}, (the number of lateral root primordia initiated within a portion of the root which corresponds to the length (*l*, mm) of 100 fully elongated cortical cells in a single file in the same parent root) was determined as 100*dl*, where *l* is the average cortical cell length in mm for each individual primary root.

The primary root of all *SDG8i* transgenic plants was longer than that of wild-type Col-0 plants ($p<0.001$) (Fig. 3.11C). Fully elongated cortical cells in all the transgenic plants were longer than wild-type Col-0 ($p<0.05$) (Fig 3.11D). There was also significant difference in the lateral root primordium density between *SDG8i* transgenic plants and wild-type Col-0 plants ($p<0.05$) (Fig. 3.11E). The higher estimation of I_{LRI} in transgenic plants indicates increased root

branching (Fig. 3.11F). These findings suggest that *SDG8i* activity has a positive role in both primary root growth and lateral root initiation.

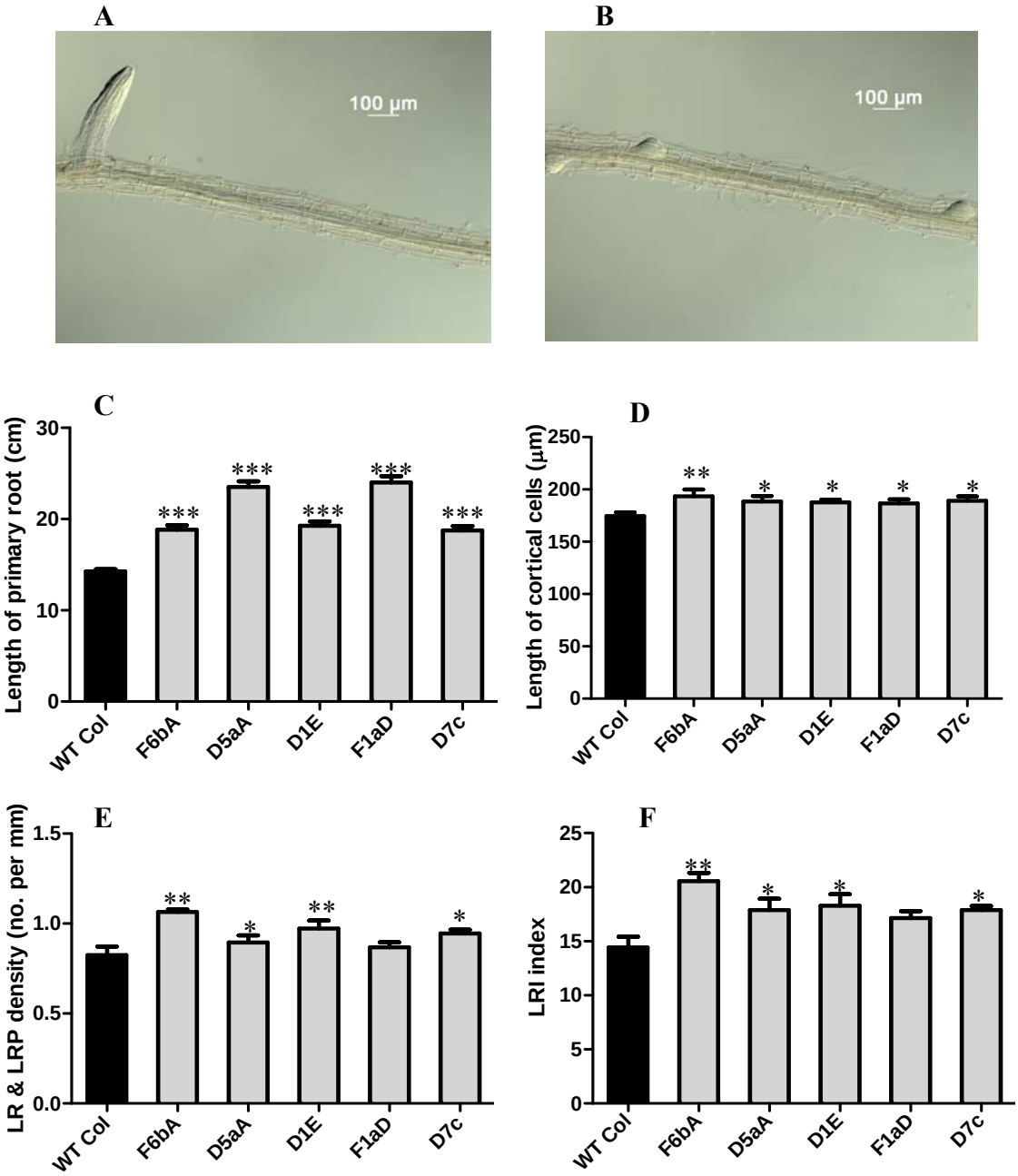


Fig 3.11 Roots cleared using the NaI-based method, showing various stages of lateral root development in 13-d-old *Arabidopsis* plants: A) Young lateral root in WT Col-0 and B) emerging primordium in the *SDG8i* transgenic. Scale bars: 100 µm. C) Length of primary root, D) and of fully elongated cortical cells, E) lateral root primordium density and F) lateral root initiation index in WT Col-0 and *SDG8i* transgenic *Arabidopsis* plants. Plants were grown in vertical orientation at 21^{o}C under SD following stratification for 13 days in MS media. Values are the means ± SE of 4 replicates. Significant differences at 5, 1 and 0.1% levels from WT determined by Tukey's Multiple Comparison Test are denoted by *, ** and ***, repectively.

3.3.6 Characterization of *SDG7y* and *SDG10y* transgenic plants in long and short day

The *S. stapfianus* SDG7y and SDG10y transcription factors (TFs) have been implicated in regulation of desiccation-tolerant specific gene *SDG8i* expression, originally through interactions with the SDG8i promoter in the yeast one-hybrid system (Le, 2004). As regulators of desiccation responsive gene expression in *S. stapfianus*, there is the possibility that these AP2/ERF TFs may influence drought tolerance in plants. An understanding of gene regulation is important for the understanding of desiccation tolerance as the interaction of different dehydration-responsive regulatory pathways determine the expression of a different set of genes (Bartels and Salamini, 2001). Transgenic studies have contributed to the understanding of drought-responsive gene regulation in *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki, 2000). Independent homozygous transgenic *Arabidopsis* lines constitutively overproducing *SDG10y* and *SDG7y* have been produced. These homozygous transgenic plants were characterized by examining the developmental phenotype in the present study in order to see the possible role these TFs play in regulation of gene expression in plants.

WT Col-0 10y8a 7y3

Fig 3.12 *SDG7y* and *SDG10y* transgenic plants showing differences in shoot growth compared to wild-type Col-0 plants. Plants were grown at 21^{o}C under the long day (LD) condition of 16h day/ 8h night after stratification. Bar = 2 cm.

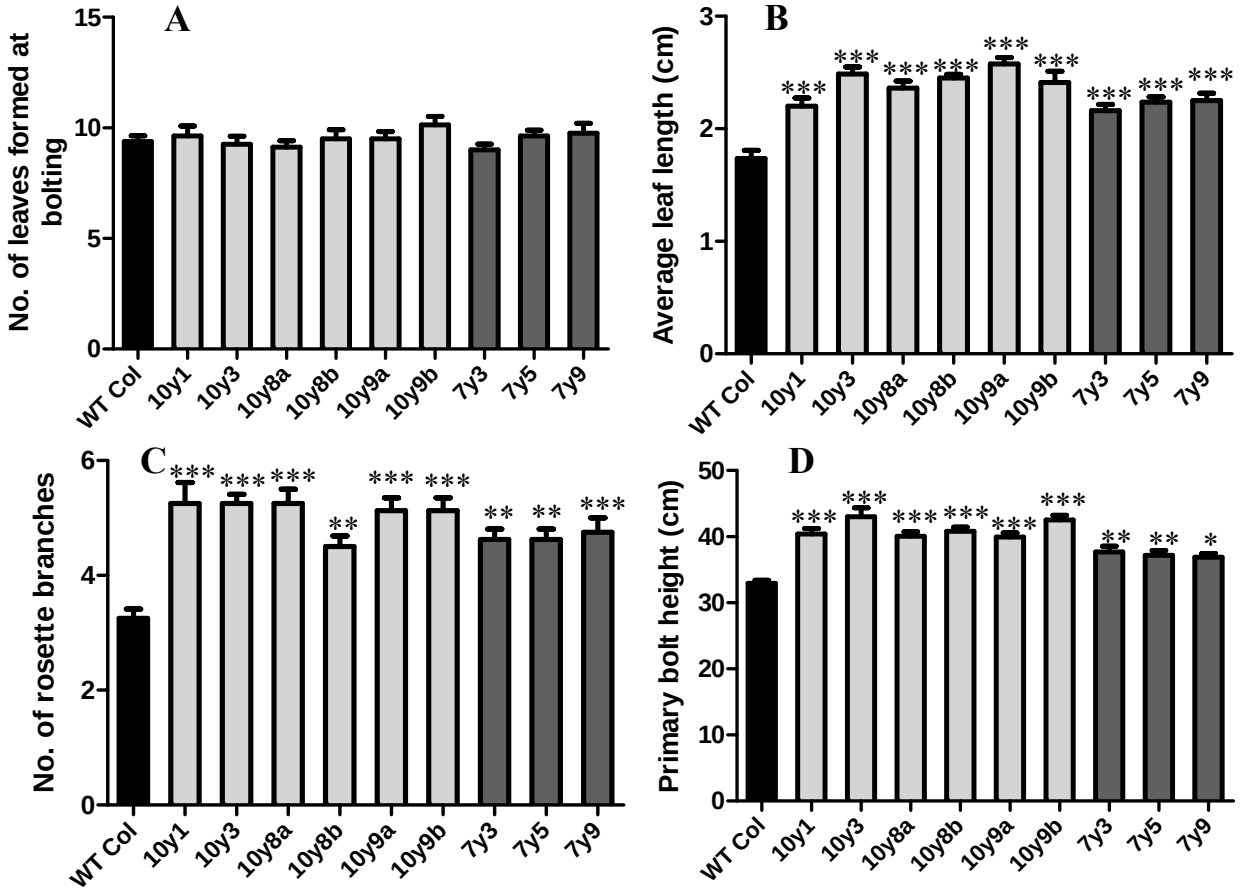


Fig 3.13 A) Number of primary rosette leaves formed at the floral initiation stage, B) average leaf length (cm) calculated 16 days after germination, C) number of rosette branches measured 27 days after germination D) primary bolt height measured 38 days after germination, of *SDG8i* transgenic and WT Col-0 plants growing at 21^{o}C under the long day (LD) condition of 16h day/ 8h night. Values are the means ± SE of 8 replicates. Significant differences at 5, 1 and 0.1% levels from WT determined by Tukey's Multiple Comparison Test are denoted by *, ** and ***, repectively.

All the plants growing in LD photoperiod (16h day/ 8h night) (Fig. 3.12) produced their first inflorescence 20 days after germination at about 12 to 14 leaf stage. No significant difference was observed in the number of rosette leaves produced between the transgenic and wild-type Col-0 plants (Fig. 3.13A). The average length of the leaves of transgenic plants was significantly longer than that of WT (P<0.05) (Fig. 3.13B). Both *SDG10y* and *SDG7y* transgenic plants produced a significantly higher number of rosette branches (about 1.4 to 1.6 times) than that of wild-type Col-0 plants (P<0.01; Fig. 3.13C), which gave them a bushy

appearance. No significant difference was observed between the *SDG10y* and *SDG7y* transgenic plants. However, the height of the bolting shoots of all *SDG10y* and *SDG7y* transgenic plants was significantly higher than that of wild-type Col-0 38 days after germination ($P<0.05$) (Fig. 3.13D). Significant difference was also seen between *SDG10y* and *SDG7y* plants for primary bolt height ($P<0.05$).

In LD, flower morphology and seed development appeared normal in all the transgenic and wild-type Col-0 plants (Fig. 3.14). There was no significant difference between transgenics and wild-type Col-0 plants in the size of siliques and number of seeds produced per silique.

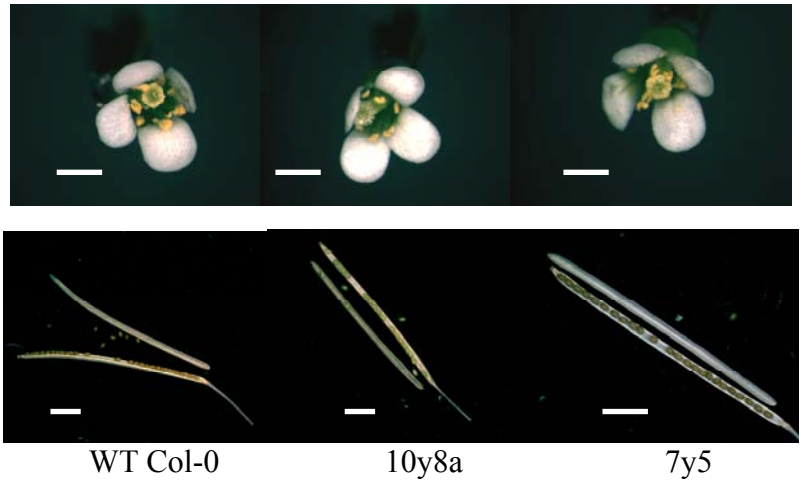


Fig 3.14 Flower morphology and seed development of *SDG7y* and *SDG10y* transgenic and WT Col-0 plants. Plants were grown at 21°C under the long day (LD) condition of 16h day/ 8h night after stratification. Bar = 2 mm.

Short days (8h day/16h night) favored development of rosette leaves and delayed flowering of all the plants (Fig 3.15). The *SDG10y* transgenic plants produced the first inflorescence after 56 to 58 days of their germination while *SDG7y* transgenic and wild-type Col-0 plants produced their first inflorescence 66 to 70 days after their germination. The number of rosette leaves produced by primary and axillary meristems in the rosette before bolting in the transgenic plants was 2 to 3 times higher compared to that of wild-type Col-0 plants ($p<0.001$) (Fig 3.16A). Among the transgenic plants, *SDG10y* plants produced higher number of rosette

leaves formed by primary and axillary meristems in the rosette before bolting compared to that of *SDG7y* transgenic plants ($p<0.05$). Leaf size of all the transgenic plants was larger, which was almost twice the size of wild-type Col-0 ($p<0.001$) (Fig. 3.16B). All transgenic plants produced significantly higher number of rosette branches which was about 1.5 to 2.0 times higher than that of wild-type Col-0 ($P<0.001$) (Fig. 3.16C). Among the transgenic plants 10y3, 10y8a and 10y8b produced significantly higher number of inflorescence compared to the others ($P<0.01$). All transgenic plants were taller and the average height increase of transgenic plants after 40 days of bolting was 1.3 to 1.5 times higher than that of wild-type Col-0 plants (Fig. 3.16D). All *SDG10y* transgenic plants were significantly taller than *SDG7y* transgenic plants ($P<0.05$). The rate of growth of primary inflorescence of the transgenic plants was ~ 25% greater (6.7 to 7.5 cm week^{-1}) that wild-type Col-0 plants (5.7 cm week^{-1}).

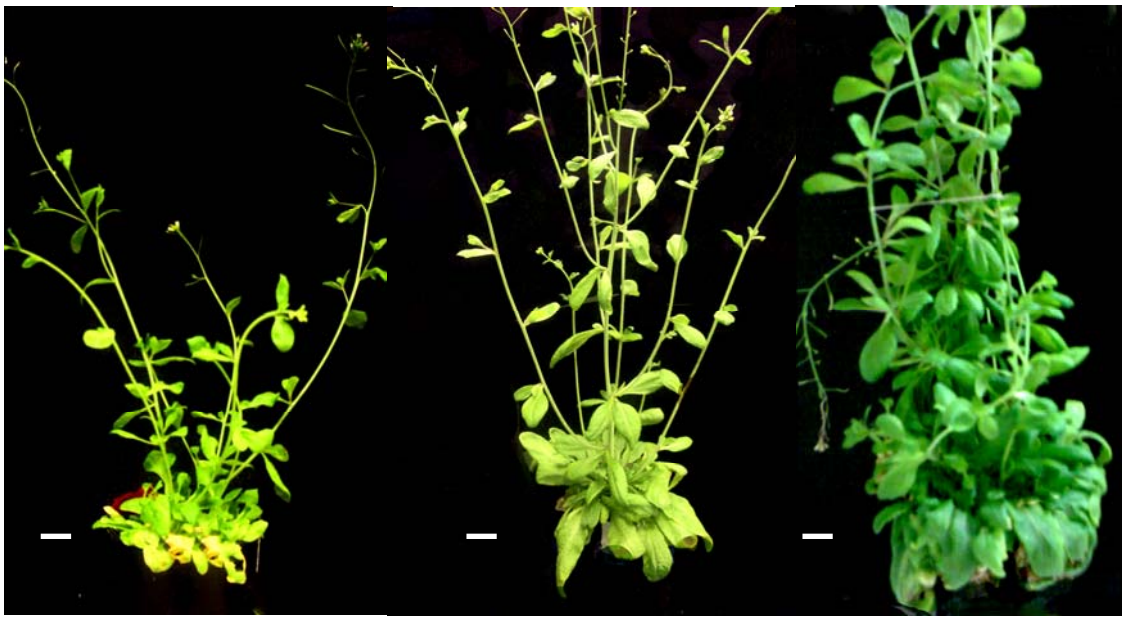


Fig 3.15 *SDG7y* and *SDG10y* transgenic plants showing enhanced shoot growth compared to WT Col-0 plants. Plants were grown at 21^{o}C under the short day (SD) condition of 8h day/ 16h night after stratification. Bar = 2 cm.

In SD, flower morphology and seed development appeared normal in all transgenic and wild-type Col-0 plants (Fig 3.17). No obvious differences were noted in the size of siliques or in the

number of seeds produced per silique between transgenic *SDG10y* and *SDG7y* plants and wild-type Col-0 plants.

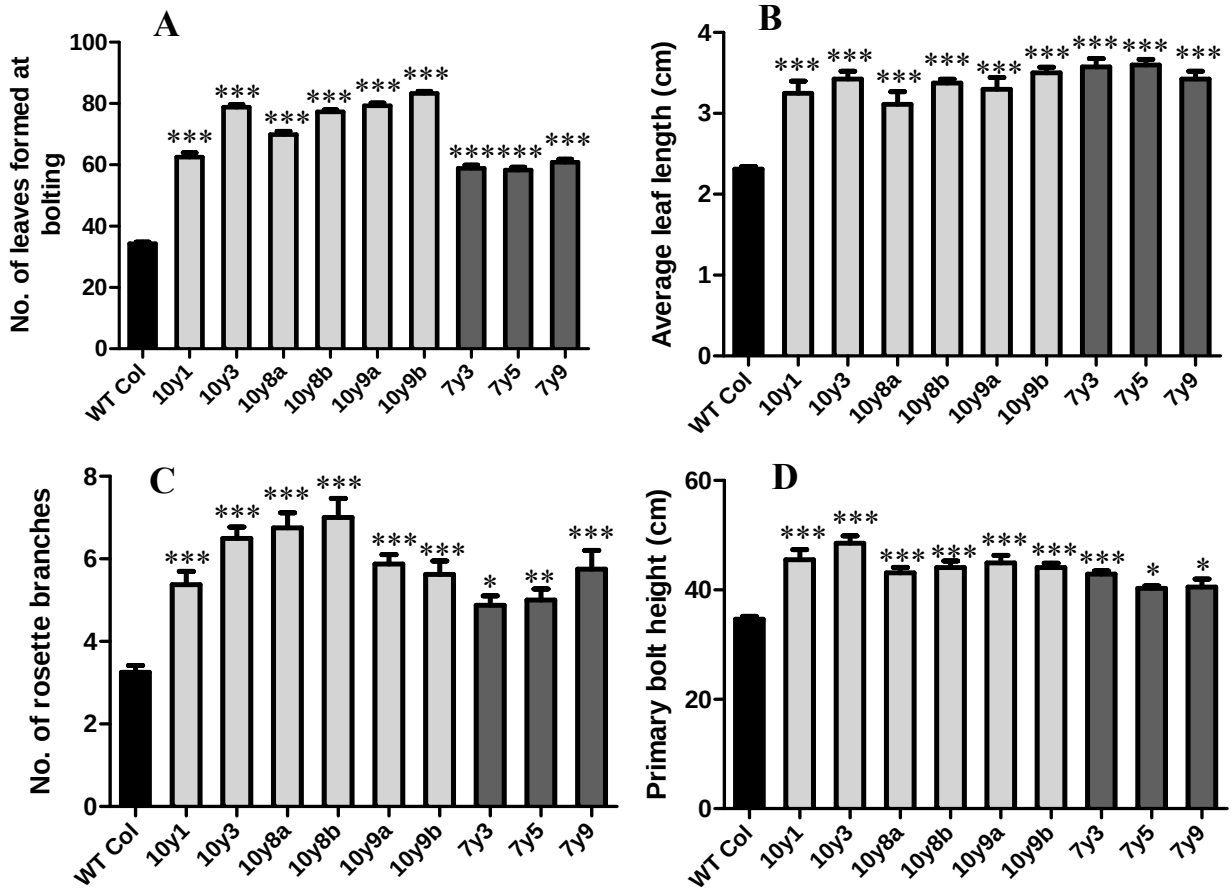


Fig 3.16 A) Number of leaves produced by primary and axillary meristems in the rosette before bolting, B) average leaf length, C) number of rosette branches measured 48 days after bolting and D) primary bolt height measured 40 days after bolting of *SDG7y* and *SDG10y* transgenic and wild-type Col-0 plants growing at 21^{o}C under the condition of 8h day/ 16h night after stratification. Values are the means ± SE of 8 replicates. Significant differences at 5, 1 and 0.1% levels from WT determined by Tukey's Multiple Comparison Test are denoted by *, ** and ***, repectively.

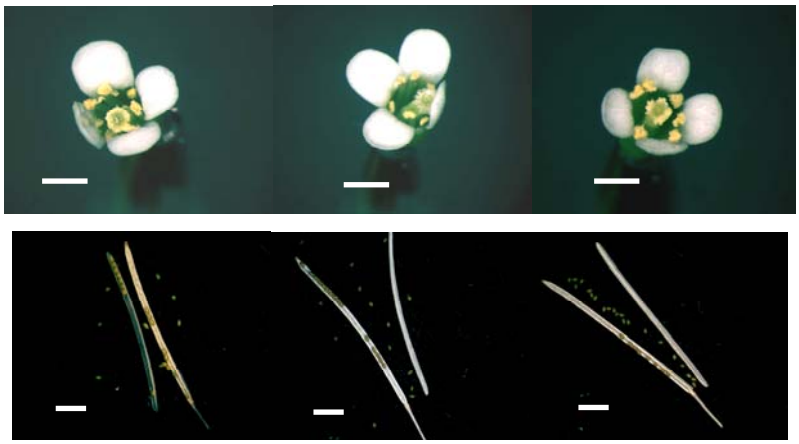


Fig 3.17 Flower morphology and seed development of *SDG7y* and *SDG10y* transgenic and WT Col-0 plants. Plants were grown at 21^{o}C under the SD condition of 8h day/ 16h night after stratification. Bar = 2 mm

3.4 Discussion

Isolation of gene transcripts from desiccated leaf tissues of the resurrection grass *S. stapfianus* resulted in the identification of the gene, *SDG8i*, encoding a Group 1 glycosyl transferase (UGT). UGTs transfer activated sugars to hormones, secondary metabolites and xenobotics, resulting in glycosylation (Wang and Hou, 2009) and may play important roles in maintaining cell homeostasis as well as in the regulation of plant growth, development and in responses to stress (Jones and Vogt, 2001). In the present study, the transgenic plants over-expressing *SDG8i* displayed phenotypes which differed from those of the wild-type plants which were even more pronounced under short-day growth conditions. After primary inflorescence emergence, the *SDG8i* transgenic plants developed a clear shoot branching phenotype. In *SDG8i* transgenic plants, the shoot branching was of a higher order than that of wild-type plants. As auxin is known to inhibit axillary branches, the increased branching of *SDG8i* transgenic plants may suggest a reduced concentration of auxin. *Arabidopsis* auxin resistant mutant, *axr1* shows increased axillary branching (Leyser *et al.*, 1993). Similarly, a phenotype in the *bud1* mutant, which has a decrease in auxin transport also shows increased shoot branching (Dai *et al.*, 2006). SL is also proposed to be involved in inhibiting bud outgrowth. Mutants in SL biosynthesis and signaling increase branching in diverse plant species. CKs, on the other hand, promotes axillary bud outgrowth. Exogenous CK application or overexpression of CK biosynthesis genes or mutants with a greater concentration of CK show more shoot branching (Medford *et al.*, 1989; Miguel *et al.*, 1998; Dun *et al.*, 2006). As the activity of auxin, CK and SL appears linked in the regulation of bud outgrowth, the results presented here suggest that *SDG8i* activity may affect the activity of these hormones. Inactivation of either auxin or SL via glycosylation or an upregulation of CK could lead to the production of a phenotype with increased level of shoot branching. An examination of the endogenous concentration of these hormones in *SDG8i* transgenic plants may elucidate this.

Transcription factors play an important role in the regulation of gene expression. In the present study, the transgenic plants over-expressing *SDG7y* and *SDG10y* displayed similar phenotypes to that of *SDG8i* transgenics. The phenotypic development of *SDG10y* and *SDG7y* transgenic plants also showed substantial increases in shoot growth and shoot branching. This suggests that there is an *Arabidopsis SDG8i* orthologue and that *SDG10y* and *SDG7y* may affect the

expression of this endogenous *SDG8i* in at least a partially similar way. Further analysis of the phenotypes of the *SDG10y* and *SDG7y* transgenic plants is necessary under different abiotic stress conditions and also hormonal treatments to see if they also show similarities in stress and hormonal response observed in *SDG8i* transgenic plants.

The analysis of root development of *SDG8i* transgenic plants suggests that *SDG8i* activity affects not only shoot branching but also root branching. Auxin is known to inhibit primary root elongation but plays an important role in regulation of lateral root initiation in *Arabidopsis*. Analyses of auxin transport mutants, such as the *aux1*, *axr4*, and *pin* multiple mutants, have shown the role of auxin transport in promoting lateral root formation (Marchant *et al.*, 2002; Benková *et al.*, 2003). On the other hand, CKs are known to inhibit lateral root formation, and transgenic *Arabidopsis* plants with decreased CK concentrations display both increased primary root growth and root branching (Werner *et al.*, 2003). SLs enhance primary root growth and inhibit lateral root formation under normal Pi sufficient conditions (Kapulnik *et al.*, 2011; Ruyter-Spira *et al.*, 2011). SL signaling mutant *max2-1* showed increased root branching suggesting the negative role of SL in lateral root growth. Pi starvation enhances lateral root formation and elongation (Péret *et al.*, 2011). Under low Pi condition, ethylene inhibits primary root growth but promote lateral root elongation and root hair formation (Chiou and Lin, 2011). Under high Pi conditions, SLs have been shown to induce ethylene biosynthesis (Kapulnik *et al.*, 2011), which in turn may affect auxin synthesis and transport. Auxin and ethylene may potentially inhibit primary root elongation either independently or together. Stimulation of auxin biosynthesis by SL-mediated ethylene production in the root meristem may lead to auxin transport to the root elongation zone where it inhibits root growth by slowing down cell elongation (Stepanova and Alonso, 2009). Therefore, the longer primary root and increased lateral root branching phenotype of *SDG8i* transgenic plants cannot be easily explained by alteration in the effective concentrations of these hormones currently understood to control root growth and branching. An examination of root growth of *SDG8i* transgenic plants on low Pi media may indicate if SLs are required to induce LR formation under Pi starvation.

Plant growth is regulated by coordinated cell division and expansion (Collett *et al.*, 2000). Our results showed that the effects of *SDG8i* activity on growth are mediated through increases in cell division rate and cell expansion. The majority of the classic plant hormones are referred to as plant growth regulators due to their ability to modulate either cell elongation or proliferation, or both, often in an interdependent or synergistic manner. CKs promote cell division, and application of CK to buds can induce outgrowths (Sachs and Thimann, 1967). A study of ethylene mutants, *etr1* and *ein3*, show that the larger leaf size correlates with increases in both cell number and size, suggesting a negative role of ethylene on leaf blade expansion (León and Sheen, 2003). Analysis of *arf2* mutant exhibits large-leaf phenotype suggesting an increased number and size of cell by the activity of *ARF* gene family member (Okushima *et al.*, 2005). The increase in cell size and division rate in both shoots and roots of *SDG8i* transgenic plants of the present study may also suggest an interaction of plant hormones. The leaves of the *SDG8i* transgenic plants contain more and larger-sized cells, as do plants overexpressing *Gibberellin 20-Oxidase* (Huang *et al.*, 1998). This gene, which regulates the amount of active GA, is reported as being repressed by SL (Mashiguchi, 2009).

SDG8i transgenic plants produce longer hypocotyls both in the light and dark than do wild-type plants. Mutants, such as *arf2*, with altered auxin sensitivity or content exhibit long hypocotyl phenotypes (Jackson *et al.*, 2002). Ethylene is known to inhibit hypocotyl elongation (McKeon and Yang, 1995). Because auxin promotes ethylene synthesis, it is possible that auxin inhibits hypocotyl elongation through alteration in ethylene activity (Smalle *et al.*, 1997). Hypocotyl elongation is independent of auxin in the absence of light as *Arabidopsis* auxin underproducers and overproducers all have similar hypocotyl lengths in the dark. Therefore hypocotyl elongation of *SDG8i* transgenic plants in the dark looks unrelated to altered auxin homeostasis. Brassinosteroid is known to have a major role in hypocotyl elongation in the dark (Jensen *et al.*, 1998). Interestingly, transgenic *Arabidopsis* overexpressing a hydroxysteroid dehydrogenase, involved in brassinosteroid signaling (Li *et al.*, 2007), has a similar growth-enhanced phenotype to the *SDG8i* transgenic plants. Hu *et al.* (2010) found that rice mesocotyl elongation was enhanced in SL-deficient or -insensitive mutants in the dark and application of GR24 could rescue only the SL-deficient mutants. Mesocotyl elongation in the dark was also seen in rice JA-deficient mutant *hebiba* (Riemann

et al., 2003), suggesting the negative role of this hormone in mesocotyl elongation. SL-signaling mutant *max2*, identified as a photomorphogenesis mutant (Shen *et al.*, 2007; Shen *et al.*, 2012), displayed a long hypocotyl phenotype under both continuous white light and dark (Stirnberg *et al.*, 2002; Stirnberg *et al.*, 2007).

As well as affecting branching of both primary roots and shoots, the *SDG8i* activity causes increased growth throughout the entire plant. Larger leaf mass and flowering bolts result in a 65% increase in seed yield of *SDG8i* transgenic plants. As multiple shoot branching phenotypes reproduce healthy and high-yielding plants by increasing vegetative biomass and seed production, it may be a desirable trait in some crop plants, such as rice in which multiple tillers give higher yield. The phenotype of *SDG8i* transgenic plants suggest that high-yield production could be achieved by genetically modifying crop plants to express *SDG8i*.

Chapter 4

Effect of *SDG8i* on hormonal responses of *Arabidopsis*

4.1 Introduction

The phenotypic analysis of transgenic *Arabidopsis* plants constitutively over-expressing *SDG8i* indicates that growth effects may be mediated by an alteration in hormone homeostasis. Therefore, an analysis of hormone responses of *SDG8i* transgenics compared to control plants was conducted. The production of SDG8i recombinant protein also allowed an analysis of the enzyme activity of SDG8i *in vitro* against several candidate plant growth-related hormonal acceptor substrates to be conducted.

Hormonal interactions play a major role in plant growth and development. The biosynthesis, degradation and chemical modification of plant hormones may help plants adapt to different environmental conditions. The study of enzymatic activities and biological roles of plant glucosyltransferases (GTs) which are involved in hormone homeostasis may open up new perspectives into the control of growth and development in plants. Glycosylation may alter the properties of hormones, for instance, the activity may be reduced or lost by glycosylation (Bowles and Lim, 2010). Most of the classical hormones occur as glycosides *in planta* (Bowles *et al.*, 2006) and UGTs capable of glycosylating auxins, CK, ABA, SA, JA and brassinosteroids (BR) or their synthetic precursors have been identified (Jackson *et al.*, 2001; Lim *et al.*, 2002; Hou *et al.*, 2004; Lim *et al.*, 2005; Poppenberger *et al.*, 2005; Song, 2005; Tognetti *et al.*, 2010). An analysis of the acceptor substrate specificity of the multigene family of Group 1 UGTs of *Arabidopsis* identified a gene, *UGT84B1*, whose recombinant product glucosylates indole-3-acetic acid (IAA) *in vitro*. The overexpression of *UGT84B1* in *Arabidopsis* resulted in a phenotype in the aerial parts of the plant similar to that of an auxin-deficient mutant, as well as loss of geotropism in the root system (Jackson *et al.*, 2001; Jackson *et al.*, 2002). Another *Arabidopsis* UGT, *UGT71B6* has been shown to have activity

against ABA and overexpression of *UGT71B6* affected ABA metabolism in *Arabidopsis* with high accumulation of ABA glucose ester in the rosettes leaves (Priest *et al.*, 2006).

Auxin, cytokinin and strigolactone are the three major hormones which are involved in the regulation of axillary branching. Bud outgrowth or shoot branching, an important aspect in crop production, can be activated through environmental stimuli, mostly when the primary shoot apex is damaged (Thimann and Skoog, 1933) which affects the auxin signal coming from the apex of the plant (Tanaka *et al.*, 2006). As mentioned earlier, several mechanisms have been proposed for the roles of auxin, CK and SL in shoot branching. Auxin is thought to regulate shoot branching by influencing the level of other signals acting as second messengers such as CK and SL. Auxin depletion by decapitation results in an increase in the endogenous CK concentrations in axillary buds. The increase in CK may be partially prevented by addition of exogenous auxin to the decapitated apex (Chatfield *et al.*, 2000). CK synthesis in both the node and the root is affected by apically derived auxin (Chatfield *et al.*, 2000). In pea, it was shown that auxin reduces CK synthesis by repressing the expression of *adenosine-phosphate-isopentenyl transferase (IPT)*, a CK biosynthetic gene, in the stem (Tanaka *et al.*, 2006). In chickpeas, after decapitation, CK coming from the root has been shown to accumulate in the bud (Mader *et al.*, 2003). In pea and bean, the increased concentrations of CK after decapitation may be restored to its original level by application of auxin to the decapitated bud (Bangerth, 1994; Li *et al.*, 1995; Bangerth *et al.*, 2000). After decapitation, SL content or SL biosynthesis gene expression is decreased to induce bud outgrowth, which may be repressed by direct application of GR24 at the node below the site of decapitation (Brewer *et al.*, 2009). Hence it is now thought that alterations in auxin, CK and SL levels coordinately regulates bud outgrowth during plant development (Dun *et al.*, 2012). In the present study two methods were used to analyze bud outgrowth of *SDG8i* transgenic plants, namely the growth response of buds on excised nodes to apically and basally supplied hormones and the effect on rosette branching in mature plants from application of the synthetic SL analogue, GR24.

SLs were first identified as germination stimulants for the seed of parasitic plants including *Striga* spp. and *Orobanche* spp. (Cook *et al.*, 1972). Susceptible hosts include staple crops such as maize (*Zea mays*), millet (*Panicum miliaceum* L.), sorghum (*Sorghum bicolor*),

sunflower (*Helianthus annuus*), rapeseed (*Brassica napus*), tomato (*Solanum lycopersicum*) and some legumes (Press *et al.*, 2001; Matusova *et al.*, 2005). From the host plant, *Orobanche* species acquire nutrients and water by the formation of a haustorium that connects to the roots. After that, parasites establish a connection and interaction with the xylem for their growth and production of seeds (Matusova *et al.*, 2005). The secretion of secondary metabolites from the host roots stimulates parasite seed germination. SL-deficient mutants in rice and pea showed reduced stimulation of parasitic weed seed germination (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Work by Umehara *et al.* (2008) has shown that roots of a SL biosynthetic mutant, *d10*, exhibit higher resistance to *S. hermonthica* plants, which suggests that the inhibition of SL biosynthesis may be an efficient way for controlling root parasitic weeds. Because *SDG8i* transgenic *Arabidopsis* lines exhibited increased branching, they were also analysed for the ability to stimulate *Orobanche* germination in axenic culture to determine if *SDG8i* activity has an effect on the root secretion of stimulus signals which are required by parasitic plants to germinate.

Leaf development ends with senescence which is regulated positively and negatively by several plant hormones. It is suggested that these hormones coordinate the senescence process with various physiological responses to environmental stress (Nam, 1997). Senescence is a process of deterioration of cellular components that finally cause cell death (Noodén, 1988). During senescence, leaf cells undergo remarkable changes in cellular metabolism including loss of photosynthetic activity and hydrolysis of macromolecules with the sequential degeneration of cellular structures. The metabolic changes during senescence may also reduce the yield of crop plants (Lim *et al.*, 2007). Leaf senescence occurs not only through normal aging but is also influenced by external factors such as darkness, temperature, drought, ozone, nutrient deficiency and pathogen attack. The hormonal regulation of leaf senescence may be determined by analysis of mutants and expression of senescence-associated genes (SAGs) (Lim *et al.*, 2003b; Li *et al.*, 2012).

Among the plant hormones, the role of ABA in senescence has been characterized most thoroughly at the molecular and physiological levels (Zeevaart and Creelman, 1988; Leung *et al.*, 1997; Fang *et al.*, 2008). In response to environmental stress ABA content increases in

senescing leaves (Gepstein and Thimann, 1980; Yang *et al.*, 2003; Xue-Xuan *et al.*, 2010) and induces expression of several SAGs (Weaver *et al.*, 1998; Xue-Xuan *et al.*, 2010). In senescing leaves, both ABA level and the expression of ABA biosynthesis genes, 9-cisepoxycarotenoid dioxygenase (*NECD*), and two aldehyde oxidase genes *AAO1* and *AAO3* increase (Buchanan-Wollaston *et al.*, 2005; van der Graaff *et al.*, 2006). ABA is also shown to induce accumulation of H_2O_2 in senescing rice leaves, which accelerates leaf senescence (Hung and Kao, 2004; Grennan, 2008) Apart from ABA, other hormones such as ethylene act as endogenous modulators of senescence (Abeles *et al.*, 1988) and ethylene-insensitive mutants such as *etr1*, *er*, *ein2*, and *ein3*, show reduced leaf senescence symptoms (Chao *et al.*, 1997; Oh *et al.*, 1997). As well as being associated with biotic responses, jasmonates also positively regulates senescence (Creelman *et al.*, 1992; He *et al.*, 2002). Application of MeJA to detached *Arabidopsis* leaves reduce photosynthetic parameters and increase expression of SAGs (Lim *et al.*, 2007). During senescence, the endogenous concentration of SA increases in the leaves of *Arabidopsis* which induces several SAGs (Morris *et al.*, 2000). CKs, which are known to control shoot growth and branching in plants, also has a very strong antisenescence effect, with the overproduction of CKs or CK application retarding leaf senescence (Richmond and Lang, 1957; Gan and Amasino, 1995). More recently SLs have shown to act as positive regulators of leaf senescence. The large number of delayed leaf senescence mutants that have been identified in *Arabidopsis* (Lim *et al.*, 2007) includes *max2*, a SL signaling mutant, originally reported as *oresara9* (*ore9*) (Woo *et al.*, 2001; Woo *et al.*, 2004). Delayed leaf senescence has also been reported in some SL-deficient mutants such as *dad1* in petunia and *d3* mutant in rice (Snowden *et al.*, 2005; Yan *et al.*, 2007). Senescence is also delayed in kiwi fruit plants when the SL biosynthetic gene CCD8 is knocked down by RNAi (Ledger *et al.*, 2010). To determine if senescence was affected by *SDG8i* activity, the effect of exogenous ABA on leaf senescence in *SDG8i* transgenics was examined.

4.2 Materials and methods

4.2.1 Excised node experiment (*In vitro* assay system)

Seeds were surface-sterilized in 70% ethanol followed by 5 times wash with sterile distilled water and sown in sterile 1-L jars containing 50 ml of ATS (*Arabidopsis thaliana* salt) medium containing 0.8% agar, 1% sucrose, and mineral nutrients according to (Wilson *et al.*, 1990). After stratification at 4°C for 3 days to obtain uniform germination, the seedlings were incubated at 22°C in a 16h day/8h night photoperiod and approximately 200 μmole m^{-2} sec^{-1} light intensity, until the primary inflorescence elongated. The transgenic lines, F6bA, D5aA and F1aD, were used in the assay, with 10 replicates of each line. Controls were wild type (WT) Columbia and transgenic plants (T) on plates with no hormone added.

This assay to determine the effect of auxin and CK on *SDG8i* the transgenic plants is based on the 'split plate' method described by Chatfield *et al.* (2000). Split agar plates were prepared by dispensing 25-ml aliquots of ATS medium into 9-cm Petri dishes. They were then allowed to solidify for 45 to 60 min in a laminar flow hood with the lid open. A strip of agar was cut out about one-eleventh of the total by weight along the diameter of the plate and removed leaving a gap 6 to 8 mm wide. Dishes were dried further for 30 min. A micropipette was used to apply solutions of naphthalene acetic acid (NAA) and benzyl adenine (BA) onto the separated media blocks, in several combinations; (i) NAA apically only, (ii) BA basally only and (iii) both NAA to the apical and BA to the basal. The plates were then left at $4^{o}C$ for at least 72 hours to allow the hormone to diffuse evenly throughout the media. Individual plant section, incorporating 6-10 mm of stem of either side of a node and an axillary bud of mm <1.5 were excised and positioned over the gap of a split plate. Auxin was applied to the apical section, CK to the basal and also the two together. The plates containing the excised nodes were incubated in near-vertical orientation. The lengths of the axillary shoots (lateral buds) were measured daily for 10-14 days with a ruler. All the data represent the growth responses of lateral buds from node 1.

4.2.2 Crossing *Arabidopsis* plants

Arabidopsis plants were grown until petals were visible. Pollinated and immature flowers within the inflorescence were removed before crossing. Under a binocular microscope, all parts of the most apical recipient flower, except the ovary, were removed with fine forceps. Anthers of mature donor flowers were removed, the pollen collected and the pollen applied to the stigmatic surface of the exposed ovary on the recipient flower. The pollinated ovary was covered with wrap and plastic cling left to develop to maturity.

4.2.3 Histochemical analysis of GUS activity

Seedlings were submerged in GUS staining buffer containing 2 mM 5-bromo-4-chloro-3-indolyl β-d-glucuronidase (Gold BioTechnology, St. Louis, Missouri, USA), 100 mM sodium phosphate (pH 7.5), 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, 10 mM EDTA, and 0.1% (v/v) Triton X-100 (Jefferson, 1987), and incubated at 37^{o}C. After 4 h 4h the seedlings were cleared with 70% (v/v) ethanol. Images were taken with a dissecting microscope with a digital camera (Leica Microsystem, Wetzlar, Germany).

4.2.4 Effect of MeJa on root growth

Seeds were surface-sterilized in 70% ethanol and rinsed 5 times with sterile distilled water. Seeds were sown on standard MS media containing 2% sucrose, 0.8% agar in Petri dishes containing 0, 5 and 10 μM MeJA. After stratification at 4°C for 2/3 days to obtain uniform germination, the plates were transferred to normal *Arabidopsis* growth condition and placed vertically to allow gravitropic root growth along the surface of the agar. Root lengths were measured after 10 days.

4.2.5 *Orobanche* germination assay

Orobanche (*Phelipanche ramosa*) seeds were surface sterilized with 2% sodium hypochlorite containing 0.02% (v/v) Tween 20 for 5 min then rinsed (5×) with sterile ddH_2O and dried for 30 min in a laminar air flow cabinet. Seeds (50 to 100) were spread on a sterile 9-mm glass

fiber disk and placed in petri dishes (9 cm diameter) lined with filter paper wetted with 3 mL of ddH_2O. One ml of GA_3 (30 mg L^{-1}) was applied to the seeds and the petri dishes were sealed with parafilm, and incubated at 18-20^oC in the dark for preconditioning. After 7 days surface-sterilized seeds of *Arabidopsis* wild-type, *SDG8i* transgenics, sorghum and *S. stapfianus* were placed on 5-cm glass microfiber disks and incubated on ¼ MS media with 0.5% sugar and 0.8% agar. For controls, 0.6 ml aqueous solution of GR24 (0.0001 mg L^{-1}) or sterile water was applied to petri dishes with preconditioned *Orobanche* seeds. The plates were then resealed with parafilm, wrapped in foil and incubated at 18-20^oC for 7 days for germination. *Orobanche* seed germination was determined under a stereoscopic microscope by counting the number of seeds having an emerged radicle.

4.2.6 Recombinant UGT production

The constructs and the transient transformation of *N. benthamiana* leaves for production of recombinant UGTSDG8i was performed by Ms Cara Griffiths. The UGT encoded by the *SDG8i* gene was produced by transient transformation of *N. benthamiana* leaf tissue using a viral based MagnICON vector system (Icon Genetics GmbH, Germany). The *SDG8i* cDNA sequence (accession number AM268210) was amplified using RT-PCR from the total RNA isolated from *S. stapfianus* leaf tissue at RWC 60% using the following primers:
Forward: 5′GAGAGAATTCATGACGAAGACCGTGGTTCTGTAC3′
Reverse: 5′GAGAGGATCCTCACGGACGACCGAC3′.

The PCR product was digested with *Eco*R1 and *Bam*H1 (sites underlined) and ligated into the *Eco*R1 and *Bam*H1 sites of pICH11599 to generate pICH11599-*SDG8i*. The plasmid was cultured in *E. coli* and confirmed by sequencing. The three pro-vectors: (i) pICH12190 (the 5′ fragment of the viral vector including a chloroplast signal sequence), (ii) pICH11599-*SDG8i* (the 3′ fragment of the viral vector including *SDG8i*) and (iii) pICH14011 (the integrase) (Marillonnet *et al.*, 2005) were separately electroporated into *Agrobacterium tumefaciens* GV3101. Equal amounts of the three *A. tumefaciens* strains carrying each module were resuspended at an OD_{600} of 0.5 in infiltration medium (10mM 2-*N*-morpholino-ethanesulfonic acid (MES) pH 5.5, 10mM $MgSO_4$ and 200µM acetosyringone) and infiltrated using a syringe

into the abaxial side of *N. benthamiana* leaves that had been covered in aluminum foil for 3 days. The foil covered leaves were left to co-incubate for 5 days. The leaves were then harvested and ground in liquid nitrogen before being transferred into 500µL cold protein extraction buffer (5mM sodium phosphate buffer pH 7.5, 10mM EDTA, 0.1% Triton X-100). The homogenate was then centrifuged for 10 minutes at 13500g and 4ºC to remove cell debris. The total protein content was determined by Bradford assay and extracts were analysed by SDS-PAGE (Sambrook *et al.*, 1989).

4.2.7 UGT enzyme assay

The UGT enzyme assay was performed by Ms Cara Griffiths. Recombinant UGT activity was measured using a coupled enzyme assay with pyruvate kinase (PK) and lactate dehydrogenase (LDH) conducted at pH 7.4 with a final substrate concentration of 1 mM using 25 µg of recombinant UGT protein extract from *Nicotiana* and the reaction rate monitored by the change in NADH absorbance at 340 nm (Jackson *et al.*, 2001). Activity in millikatals kg^{-1} was calculated using the extinction coefficient 6.22 x 10^{-3} M^{-1} cm^{-1} for NADH. Background activity of extracts, monitored by measuring the rate without substrate addition, was subtracted from the reaction rate. The reaction mechanisms are shown as the following:

$$\text{Substrate + UDP-Glc} \xrightarrow{\text{UGT}} \text{Sub-Glc + UDP}$$

$$\text{PEP + UDP} \xrightarrow{\text{PK}} \text{UTP + pyruvate}$$

$$\text{Pyruvate + NADH} \xrightarrow{\text{LDH}} \text{lactate + } NAD^{+}$$

4.2.8 Effect of GR24 on shoot branching

The Effect of GR24 on shoot branching was performed according to the protocol of Gomez-Roldan *et al.* 2008. *Arabidopsis* plants were grown in soil and then treated with GR24 every third day for 20 days from 23 days of age (pre-bolting stage). Treatments were applied to the rosette branches and axillary meristems with 50 µl per plant of 5 µM GR24. Control plants were treated with 50 µl of water. The number of rosette branches (>5 mm) was counted on 48 day old plant.

4.2.9 Effect of GR24 on root growth

Seeds were surface-sterilized in 70% ethanol followed by 5 washes with sterile distilled water. Seeds were sown on standard MS media containing 1% sucrose, 0.8% agar in Petri dishes. After stratification at 4°C for 3 days to obtain uniform germination, the germinated seedlings were then transferred to MS-agar media supplemented with 1% sucrose and either no GR24 or two different concentrations of GR24 (0, 5 and 15 μM). The plates were placed vertically and root lengths were measured after 7 days.

4.2.10 Senescence test

Leaves were detached from soil-grown *Arabidopsis* plants 12 days after leaf emergence by cutting the petioles of the third or fourth foliar leaves with a sharp scalpel to minimize wounding effects. For dark-induced senescence the excised leaves were floated on deionized water in the absence of light. For ABA treatment, detached leaves were floated on 3 mM MES buffer (pH 5.8) solution with the adaxial side up for 5 days at 22°C under continuous light in either the presence or absence of 50 μM ABA. Measurements were taken each day.

4.2.11 Measurement of chlorophyll content and photochemical efficiency

For chlorophyll extraction, approximately 20 mg fresh leaf tissue was ground to a fine powder under liquid nitrogen. The powder was mixed with 1 ml of ice-cold 80% acetone and centrifuged at 4°C for 3 minutes (18188 g). The supernatant was transferred to a new tube, and stored in the dark. The residual aqueous layer was re-extracted with 0.5 ml ice-cold 80% acetone and the supernatants were pooled. The chlorophyll absorbance at 647nm and 664nm was measured using 80% acetone as a blank. Chlorophyll concentration per fresh weight of leaf was calculated as described by (Lichtenthaler, 1987). The ratio of Fv/Fm which is the potential quantum yield of photochemical reactions of PSII was measured after dark adaptation of the leaves for 15 min using a PAM-210 (Teaching-PAM) (Heinz Walz GmbH, Germany).

4.2.12 Statistical analysis

All data were examined by analyses of variance using GraphPad Prism software version 5.0. Tukey's Multiple Comparison Test was further used for comparison between wild-type and *SDG8i* transgenic plants means at 5% level of significance.

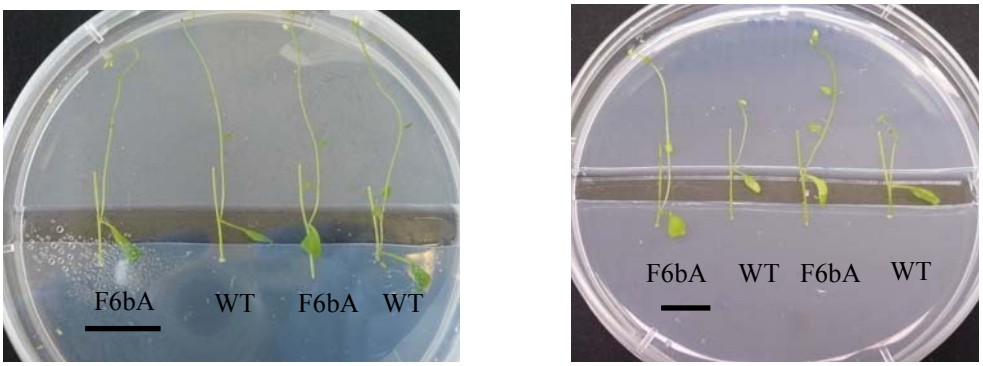
4.3 Results

4.3.1 Effect of exogenous auxin/cytokinin on decapitated plants

The experiment was done to see the effects of both apical and basal applications of auxin and cytokinin, respectively, on the growth of buds of excised nodal sections of *SDG8i* transgenic and wild-type Col-0 *Arabidopsis* plants. NAA (1 μM) in the apical media block (Fig 4.1) was found to completely inhibit lateral bud outgrowth of wild-type Col-0 for 4-5 days and after that the growth proceeded at a greatly reduced rate 0.19 cm day^{-1} of about 42% of the control rate ($p<0.05$). However, lateral buds of all transgenic lines were less inhibited by apical NAA after 2 days compared to wild-type Col-0. The rate of bud extension for transgenic lines was from 0.34 to 0.38 cm day^{-1} which was about 70-78% of the transgenic plants without NAA.

To test the effect of CK, the synthetic CK benzyl adenine (BA) was added to basal media blocks. Basal 1 μM BA caused an increase in the rate of lateral growth of all transgenic lines and wild-type Col-0 compared to untreated control plants (Fig 4.2). The mean rate of lateral outgrowth of treated wild type plants was the same as the untreated plants for first 3 days, after which BA caused gradual increase (0.5 cm day^{-1}) in bud growth. However, application of BA increased the rate of bud outgrowth of all transgenic plants by 0.56 cm day^{-1} in any period compared to wild-type Col-0 and untreated control plants.

A



Control **+ 1µM NAA applied apically**

B

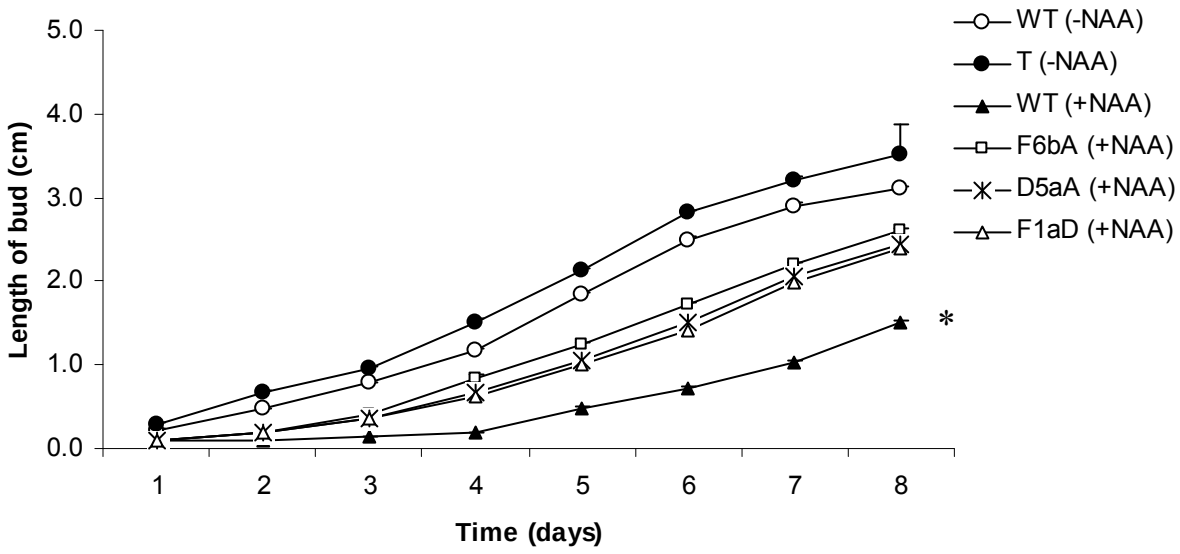


Fig 4.1 (A) The effect of auxin on lateral bud outgrowth from the nodal sections (<1.5 mm) of *SDG8i* transgenics and wild-type Col-0, grown vertically on ATS media containing 1µM NAA on the top block for 25 days. **(B)** Length of lateral buds of *SDG8i* transgenics (T) and wild-type (WT) Col-0, with and without NAA, was measured everyday for 10 days. Values are the means ± SE of 10 replicates**.** Size bar = 1 cm. Asterisk indicates statistically significant decrease in bud outgrowth from control at 5% level.

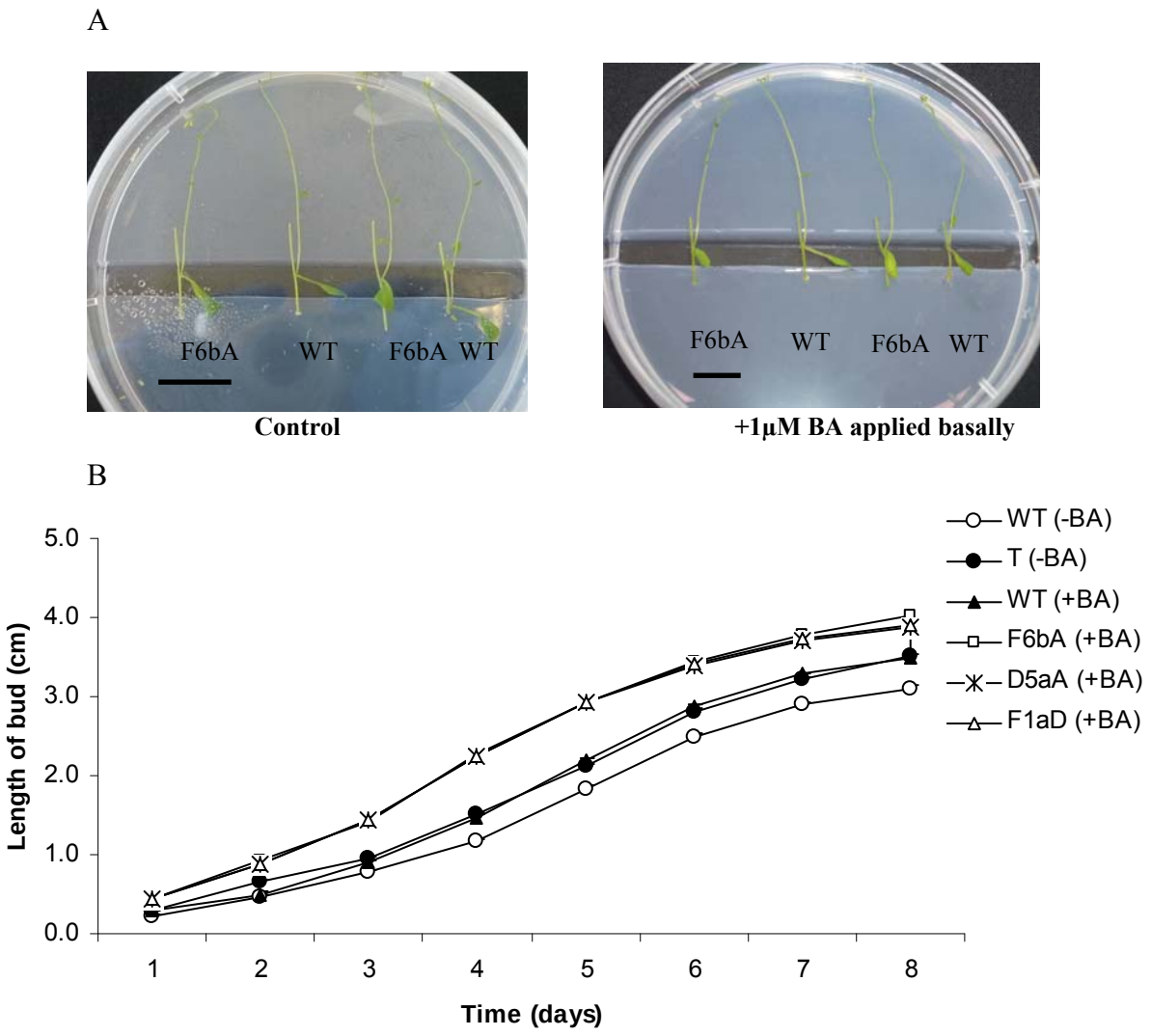


Fig 4.2 (A) The effect of cytokinin on lateral bud outgrowth from the nodal sections (<1.5 mm) of *SDG8i* transgeics and Wild-type Col-0, grown vertically on ATS media containing 1µM BA on the bottom block for 25 days. **(B)** Length of lateral buds was measured everyday for 10 days. Values are the means ± SE of 10 replicates**.** Size bar = 1 cm.

To investigate the interaction of auxin and CK, combinations of apical NAA (1µM) and basal BA (1µM) were applied (Fig 4.3). Basal BA was found to release the inhibition of bud outgrowth by apical NAA. However, the rate of bud growth of all transgenic plants was 20% higher (0.4 cm day^{-1}) compared to wild-type Col-0 plants ($p<0.05$).

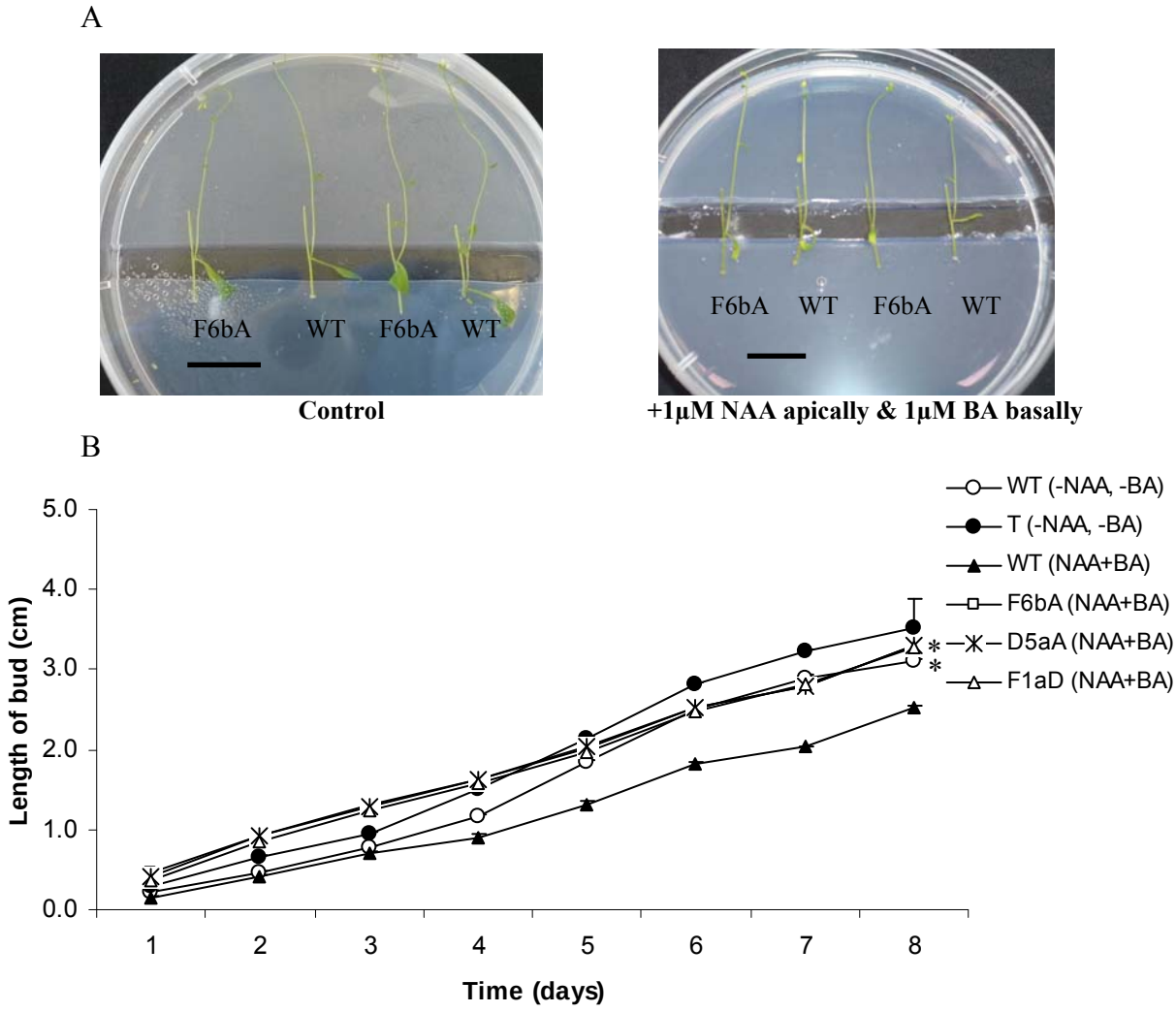


Fig 4.3 (A) The effect of both auxin & cytokinin on lateral bud outgrowth of the nodal sections (<1.5 mm) of *SDG8i* transgenic and wild-type Col-0 plants, grown vertically on ATS media containing 1µM NAA apically & BA basally for 25 days. **(B)** Length of lateral buds was measured everyday for 10 days. Values are the means ± SE of 10 replicates**.** Size bar = 1 cm. Asterisk indicates statistically significant increase in bud outgrowth from WT at 5% level.

4.3.2 Comparison of GUS activity in *Arabidopsis* plants

Temporal and spatial changes in auxin concentrations are believed to regulate many aspects of plant growth and development including apical dominance, growth of vascular tissue, tropism and organ formation (Woodward and Bartel, 2005). An important tool to visualize regions of elevated auxin activity is the synthetic auxin response reporter construct known as DR5::GUS.

To examine the effect of constitutive glycosylation activity of *SDG8i* on auxin levels and distribution, the *SDG8i* transgenic plants were crossed with plants containing the *DR5-GUS* reporter construct and the progeny analyzed for histochemical GUS activity.

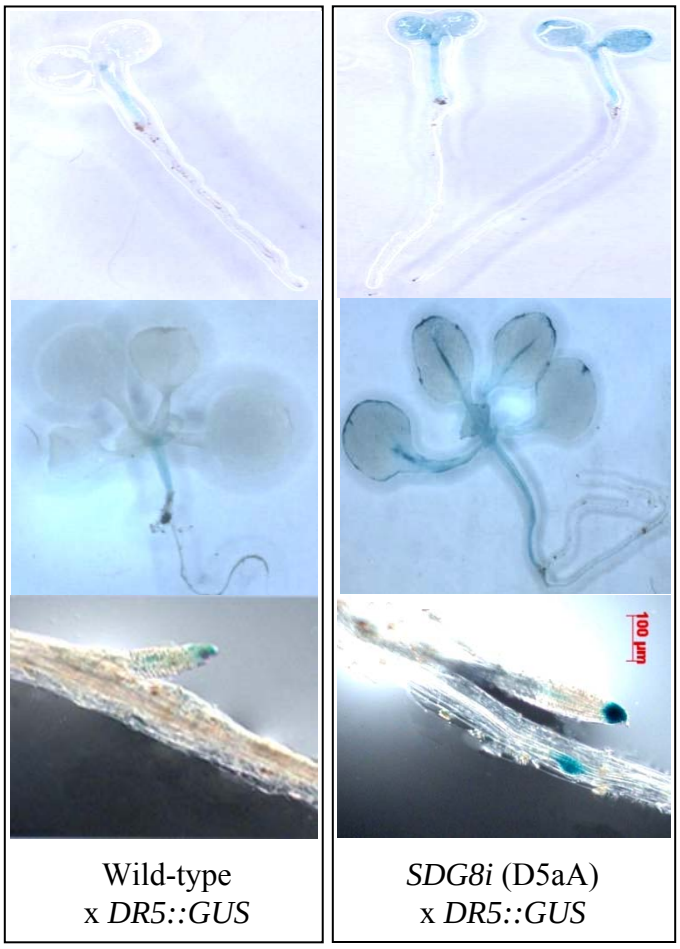


Fig 4.4 GUS activity at various stages of development in 4 day and 12 day old wild-type Columbia and *SDG8i* transgenic plants crossed with *DR5::GUS Arabidopsis* seedlings.

In 4 day old control plants, GUS staining in the hypocotyl was observed, along with very low level staining in the leaf veins and the tips of the cotyledons and root tips (Fig 4.4, left panel). At the same developmental stage the *SDG8i* transgenic plants showed essentially the same spatial pattern of GUS activity (Fig 4.4, right panel) but with a substantially higher level of

staining being observed in the leaf veins. The difference in staining between wild-type Col-0 controls and *SDG8i* transgenics was more pronounced at the four leaf stage (12 day old) with transgenic plants showing much more GUS activity in the root tips and the vascular tissue of shoots and high level GUS activity at the leaf margins (Fig 4.4, left panel). These results suggest that endogenous auxin levels may be elevated in *SDG8i* transgenic plants.

4.3.3 Effect of MeJa on root growth

The phenotypic changes of *SDG8i* transgenic plants compared with wild-type controls include enhanced root growth. Because JA is known to inhibit root growth in *Arabidopsis* (Staswick *et al.*, 1992), the effect of exogenous JA on root elongation was tested in transgenic and control plants. To test for an altered sensitivity to JA, *SDG8i* transgenic plants were assessed for MeJA inhibition of primary root elongation.

SDG8i transgenic and wild-type Col-0 plants were grown in media containing different concentration of MeJa. In the presence of 5 μM and 10 μM MeJA, primary root growth of wild-type Col-0 seedlings was inhibited by about 50 and 71%, respectively (Fig 4.5) ($p<0.001$). The *SDG8i* transgenic plants showed resistance to root growth inhibition compared to that of wild-type Col-0 with about 27 to 40 % inhibition by 5 and 10 μM MeJA, respectively (Fig 4.5) ($p<0.001$).

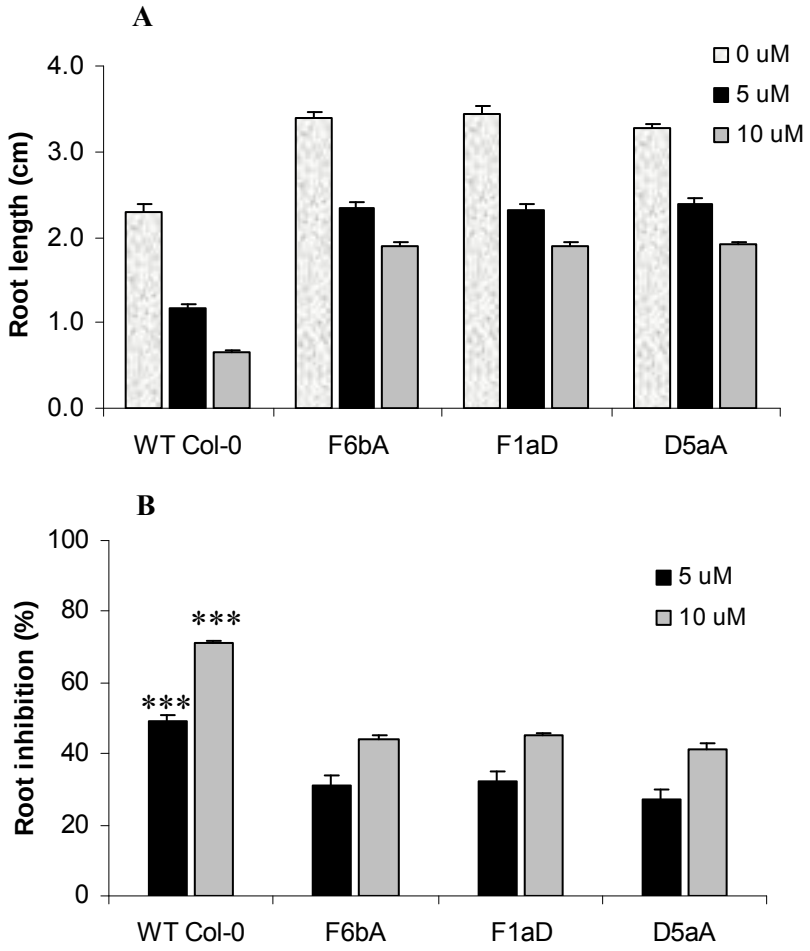


Fig 4.5 Effect of MeJa on root growth of wild-type Col-0 and *SDG8i* transgenic *Arabidopsis*. Plants were grown in normal MS media with 2% sucrose, 0.8% agar in Petri dishes containing 0, 5 and 10 μM MeJA vertically. Root lengths after 10 days **(A)** and the MeJA-induced root inhibition (%) = [(length – MeJA) – (length + MeJA)]/(length – MeJA) **(B)** were calculated. Data are means ± 6 replicates. The asterisk represents significant difference between the wild type and *SDG8i* transgenics determined by Tukey's Multiple Comparison Test at 0.1% level.

4.3.4 The stimulation of *Orobanche* germination

In order to see if *SDG8i* activity affected the ability of *SDG8i* transgenic *Arabidopsis* plants to stimulate *Orobanche* germination, four of the *SDG8i* transgenic *Arabidopsis* plants were compared with wild-type Col-0 *Arabidopsis* plants in axenic culture (Fig 4.6A). GR24, *Sorghum bicolor* and *S. stapfianus* were included as controls. Germination of 95% was

achieved by treatment with GR24, while only 11% germination was observed in the water control (Fig 4.6B).

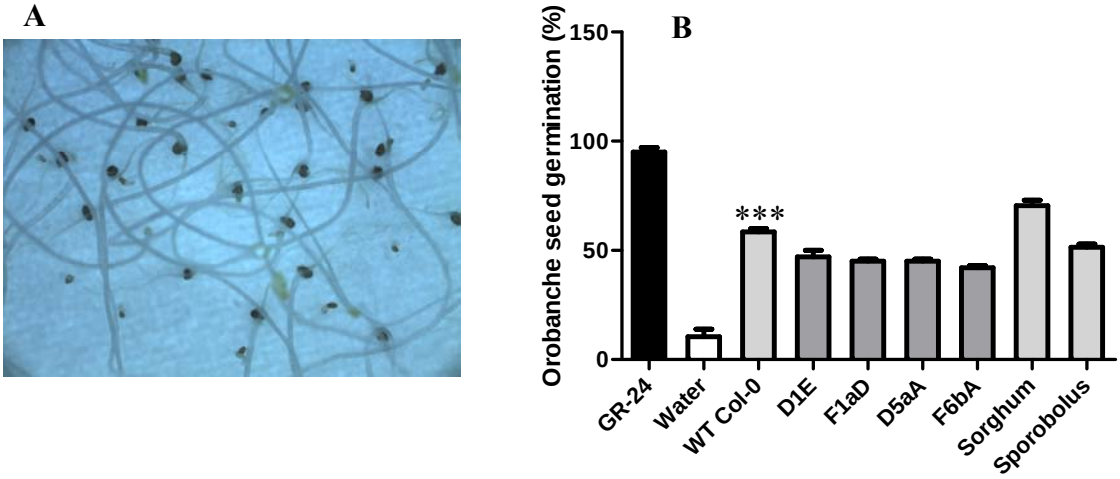


Fig 4.6 Stimulation of germination of *Orobanche* seeds.
(A) *Orobanche* seeds germinating in the presence of wild-type Col-0 *Arabidopsis* seedlings.
(B) Level of stimulation of germination of *Orobanche* seeds in response to root induction by wild-type Col-0 and *SDG8i* transgenic *Arabidopsis*, sorghum and *S. stapfianus* seedlings in *vitro*. The percent germination was calculated by counting the number of seeds having an emerged radicle. Values are the means ± SE of 5 replicates. The asterisk represents significant difference between the wild type and *SDG8i* transgenics determined by Tukey's Multiple Comparison Test at 0.1% level.

The germination percentage of *Orobanche* by sorghum and *S. stapfianus* was 71% and 52% respectively. The 60% germination of *Orobanche* seeds by wild-type Col-0 *Arabidopsis* plants was significantly higher than that of the all the transgenic lines tested ($p<0.001$) with *SDG8i* transgenic lines stimulating 42-47% germination. Amongst the transgenic lines, F6bA showed the highest reduction in *Orobanche* germination (30%) compared with the wild-type Col-0 *Arabidopsis* control. These results suggest that *SDG8i* activity may be reducing the level of germination stimulants secreted from the roots of *Arabidopsis*.

4.3.5 *SDG8i* encodes a SL-UGT that glycosylates GR24 *in vitro*

To investigate the catalytic function of *SDG8i*, the glucosyltransferase activity of *N. benthamiana* leaf protein extracts infiltrated with an actin (*ATACT2*)-promoter driven *SDG8i*

construct, using a viral-based system (Marillonnet *et al.*, 2005), was tested against a number of substrates and compared with UGT activity in extracts infiltrated with a vector-only control. The assay utilized a linked enzymic reaction. The substrates were chosen for their known ability to affect plant growth and development.

The SDG8i extract showed substantial glycosylation activity of strigolactone analogue GR24 ($p<0.05$) (Fig 4.7A) with a Km of 0.349 mM and a Vmax of 5.67 (Fig 4.7B). The activity observed with the other substrates showed very little increase over background endogenous NADH oxidase activity. Similarly, the vector-only control extract showed no substantial activity above background with GR24 or with any of the other substrates. The results indicate that *SDG8i* encodes a glucosyltransferase (SL-UGT) with *in vitro* activity against a strigolactone-like compound.

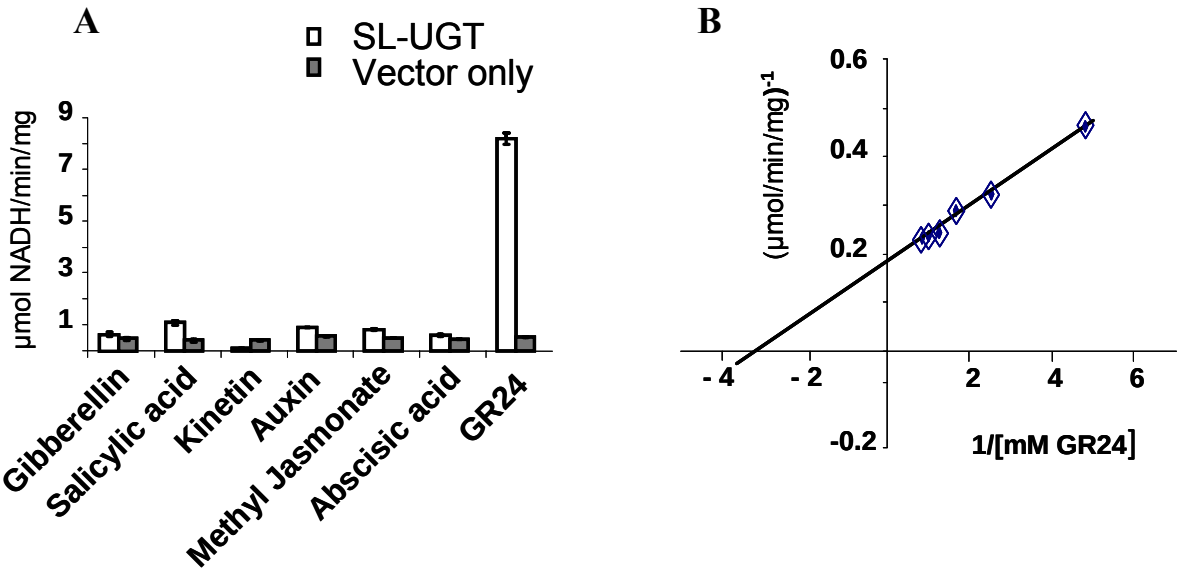


Fig 4.7 Analysis of the enzyme activity of *SDG8i in vitro*.

A) The glycosyltransferase activity of SDG8i recombinant protein extract using the plant hormones Gibberillin (GA_3), Salicyclic acid, Kinetin, Auxin (IAA), Methyl Jasmonate, ABA([±]-cis, trans-abscisic acid and the synthetic strigolactone analogue GR24 as substrate. The coupled enzyme assay (Jackson *et al.*, 2001) was conducted at pH 7.4 using 25µg of protein extract with a final substrate concentration of 1mM. GR24 was obtained from Chiralix B.V. Nijmegen, The Netherlands. All other hormones were obtained from Sigma St. Louis, MO.
B) A Lineweaver-Burke plot of SL-UGT activity with varying concentrations of GR24 indicating a Km of 0.349mM and a Vmax of 5.67. (Data and figure provided by Ms Cara Griffiths)

4.3.6 Effect of GR24 on shoot branching

Gomez-Roldan *et al.* (2008) have shown that the SL-signaling MAX2 protein is required to mediate bud outgrowth inhibition by exogenous GR24. In the present study, the response of wild-type Col-0, *SDG8i* transgenic and the branching mutants *max2 Arabidopsis* plants were tested by the application of GR24 to rosette branches and axillary meristems before and during bolting as described by Gomez-Roldan *et al.* (2008) in order to see whether they are able to respond to SL-mediated shoot branching inhibition (Fig 4.8). In wild-type Col-0 plants, shoot branching decreased by 55% in response to GR24 compared to untreated plants ($p<0.01$). However, in all *SDG8i* transgenic plants, shoot branching was decreased by only 10-15% of the untreated plants and branching increased relative to the wild-type Col-0 by application of GR24 ($p<0.001$). In the absence of GR24 treatment, the *max2* plants exhibited a higher level of branching than *SDG8i* transgenics. The low concentration of GR24 inhibition in *max2* plants was similar to that seen in the *SDG8i* transgenics.

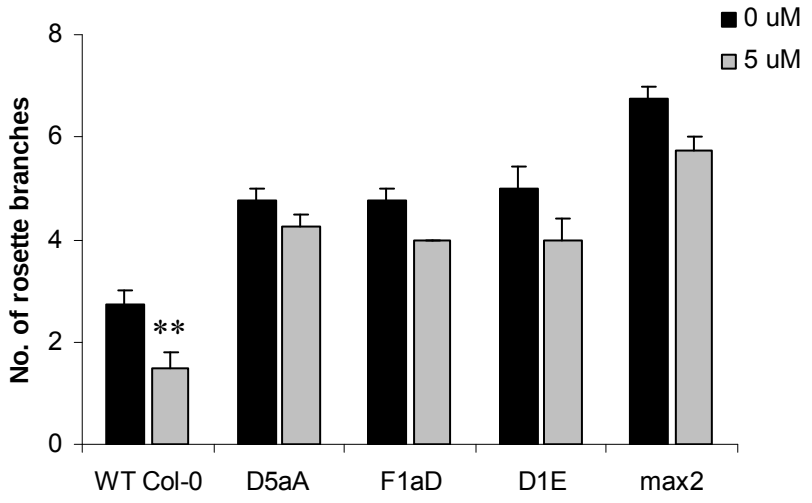


Fig 4.8 Effect of GR24 (0 or 5 µM) on bud outgrowth of wild-type Col-0, *SDG8i* transgenics and *max2 Arabidopsis* plants. Plants were treated with GR24 on the rosette axillary buds and leaf axils every third day for 20 days from 23 days of age and the number of branches was counted on 48 days old plants. Data are means ± 4 replicates. **significant difference at 1% levels from bud outgrowth without GR24 determined by Tukey's Multiple Comparison Test.

4.3.7 Effect of GR24 on root growth

Primary root lengths of 8 day old wild-type Col-0 and *SDG8i* transgenic plants were measured in presence of different concentration of GR24 (Fig 4.9). Application of 5 µM GR24 resulted in an increase in primary root lengths in all the plants. Compared to that of untreated plants, the effect of GR24 was more pronounced with almost twice the increase in root length occurring in *SDG8i* transgenic plants. On the other hand, at the higher concentration of GR24, the length of the primary root of wild-type Col-0 and *SDG8i* transgenic *Arabidopsis* plants were shorter than observed at the low concentration of GR24, but transgenic plants still had longer roots which were almost 1.5 times higher than those of untreated plants ($p<0.01$).

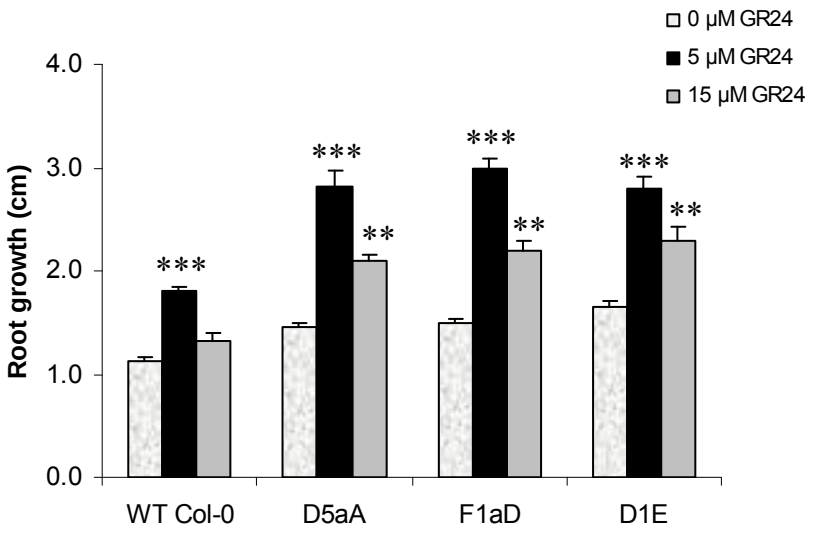


Fig 4.9 Effect of GR24 in a concentration-dependent way on primary root length of 8-d-old wild-type Col-0 and *SDG8i* transgenic *Arabidopsis* plants grown on vertical MS plates containing 1% sucrose and different concentrations of GR24. Data are means ± 4 replicates. ** and ***significant difference at 1 and 0.1% levels from untreated plants determined by Tukey's Multiple Comparison Test.

4.3.8 Effect of *SDG8i* on senescence

To test the effect of *SDG8i* activity on senescence, detached leaves from wild-type and transgenic plants were floated on sterile distilled water in the dark and chlorophyll degradation

monitored over several days. In wild-type Col-0 plants, both chlorophyll content and photochemical efficiency (Fv/Fm) began to decrease after 2 days in darkness (Figure 4.10A, B). After 5 days in darkness the chlorophyll content and the Fv/Fm values of wild-type Col-0 plants decreased to around 20% ($p<0.001$) and the leaves turned completely yellow. In the *SDG8i* transgenic lines the decreases were much less substantial. Leaves of all the transgenic lines tested remained green after 5 days, retaining about 55-60% of their chlorophyll, with Fv/Fm values of about 52-53% of the original reading ($p<0.05$) (Figure 4.10A, B). These results indicate that *SDG8i* transgenic plants exhibit reduced dark-induced senescence.

To test the relationship between *SDG8i* activity and the senescence-promoting phytohormone ABA, we repeated the assay in the presence of the exogenous hormone. ABA-induced senescence was also reduced by overexpression of *SDG8i* activity in transgenic plants (Figure 4.10C, D). When incubated in continuous light without ABA, the chlorophyll content and the photochemical efficiency of excised wild-type Col-0 leaves reduced less rapidly than in darkness.

Over the 5 days the chlorophyll content of control wild-type leaves was gradually reduced to 54%, whereas *SDG8i* transgenic leaves retained on average 71% of their chlorophyll content. However, the Fv/Fm values for both transgenic and control plants remained around 70% of the original readings and the change in photochemical efficiency was not significantly different in wild-type Col-0 and transgenic leaves under these conditions. Upon treatment with ABA, the chlorophyll content of wild-type Col-0 leaves decreased rapidly from day 2 and down to 20% by day 5 ($p<0.001$) (Figure 4.10E). On the other hand, the *SDG8i* transgenic leaves retained about 75% of their chlorophyll up to day 4 and then reduced to around 58% by day 5 ($p<0.05$) (Figure 4.10E). The Fv/Fm value of *SDG8i* transgenics was also substantially higher than control plants on day 5 ($p<0.001$) (Figure 4.10F). These results show that *SDG8i* expression reduces ABA-induced senescence suggesting that the enzyme functions downstream of ABA.

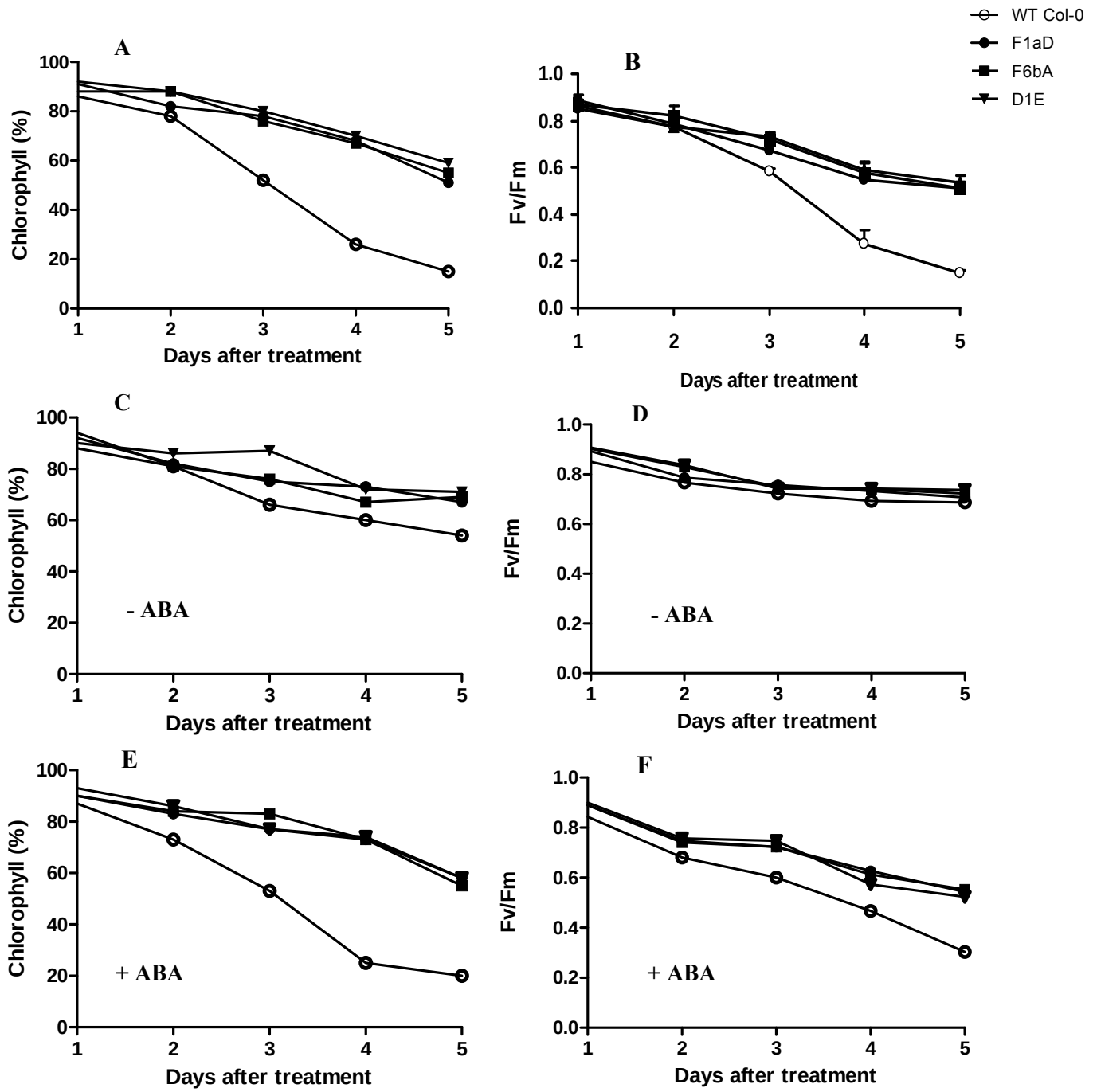


Fig 4.10 Dark- and ABA-induced senescence in wild-type Col-0 and *SDG8i* tansgenic leaves.
(A) Chlorophyll content and (B) photochemical efficiency (Fv/Fm) in dark-treated detached leaves of wild-type Col-0 and *SDG8i* plants. (C) Chlorophyll content and (D) photochemical efficiency (Fv/Fm) in detached leaves of wild-type Col-0 and *SDG8i* plants under continuous light in the absence of ABA or (E) Chlorophyll content and (F) photochemical efficiency (Fv/Fm) in detached leaves of wild-type Col-0 and *SDG8i* plants under continuous light in the presence of 50 μM ABA. Data are means ± 3 replicates.

4.4 Discussion

A network of hormonal interactions regulates shoot branching throughout the plant. Hormonal regulation of apical dominance has been studied for many decades using pea and *Arabidopsis* as models, and the role of auxin, CK and SL in regulating the dormancy and outgrowth of axillary buds has been established (Beveridge *et al.*, 2009; Domagalska and Leyser, 2011). The decapitation experiment of the present study shows that apical auxin inhibits bud outgrowth of both wild-type and *SDG8i* transgenic plants compared to the untreated plants. However, the level of inhibition is reduced in *SDG8i* transgenic plants as they show greater bud outgrowth compared to wild-type plants. Therefore, the reduced effect of auxin on transgenic plants suggests that *SDG8i* affects the ability of auxin to inhibit branching. This could occur by an alteration in auxin bioactivity directly or through an effect on auxin signaling or on the activity of possible second messenger. Loss of apical dominance is a phenotype related to altered auxin homeostasis. For example, the auxin signaling mutants, *auxin-resistance1* (*axr1*), in *Arabidopsis* show increased branching and show resistance to apical auxin (Lincoln *et al.*, 1990; Stirnberg *et al.*, 1999).

The basal application of CK shows a similar growth-promotion effect on the lateral buds of both *SDG8i* transgenic and wild-type plants, suggesting that *SDG8i* activity does not negatively affect CK activity. CK is known to act directly to promote axillary bud outgrowth. Auxin-depletion mutants may exhibit increased branching in response to the removal of CK repression. Decapitation results in an increase in CK concentrations in axillary buds which is partially removed by auxin application (Tanaka *et al.*, 2006). Application of basal CK may overcome the effect of apical auxin in branching inhibition (Chatfield *et al.*, 2000). This was seen in the experiment with the combined application of auxin and CK which shows greater bud outgrowth of *SDG8i* transgenic plants compared to wild-type plants. This indicates again a reduction in auxin response of the transgenic plants. This experiment shows that *SDG8i* activity acts to reduce the repression of bud outgrowth by auxin. This could occur by a direct effect on the bioactivity of auxin itself, or on the bioactivity of SL because the buds of SL pathway mutants are not inhibited by apical auxin (Beveridge *et al.*, 2000; Sorefan *et al.*, 2003; Bennett *et al.*, 2006).

The synthetic auxin response reporter construct *DR5-GUS* has been used in many studies to examine the patterns of auxin distribution (Bai and DeMason, 2008). Auxin may be actively mobilized throughout the plant by polar auxin transport which is known to be down-regulated by the effect of SL on the activity of auxin efflux carrier PIN1 (Crawford *et al.*, 2010). Exogenous GR24 application has also been shown to decrease *DR5-GUS* intensities in the leaves which suggest that SL may act to decrease auxin production (Ruyter-Spira *et al.*, 2011). Increased GUS expression in the vascular tissue of the lower stem was seen in SL-deficient *max* mutants carrying *DR5-GUS* (Bennett *et al.*, 2006), suggesting an elevated auxin concentration correlating with the increased polar transport of auxin (Prusinkiewicz *et al.*, 2009). In the present study, the *SDG8i* transgenic plants showed higher *DR5-GUS* expression in root tips and root primordial compared to control plants. In *SDG8i* transgenic plants, the GUS intensity was clearly elevated in the vascular tissue of both roots and shoots, and was also high in the leaf veins and leaf margins where auxin biosynthesis occurs (Teale *et al.*, 2006), suggesting an elevation of localized endogenous auxin concentrations throughout the plant rather than an effect restricted solely to increased polar transport. The increased intensity of *DR5-GUS* in the leaves of *SDG8i* transgenic plants may suggest a reduced SL bioactivity resulting in elevated auxin biosynthesis and also increased shoot growth of the *SDG8i* transgenic plants.

Phenotypic analysis from the previous chapter showed that the *SDG8i* transgenic plants displayed increased branching phenotype. Using an *SlCCD7* antisense construct it was found that a 90% reduction in SL concentrations was required to produce a strong branching phenotype in tomato and their *in vitro* analysis was validated with bioassays for *O. ramose* germination (Vogel *et al.*, 2010). In the *Orobanche* germination assay of the present study, *SDG8i* transgenic plants exhibited reduced stimulation of seed germination which indicates a reduced concentration of SL and this is not as pronounced as that which occurs in the SL biosynthesis mutants MAX3 and MAX4.

The plant cell contains a large number of acceptor molecules for glycosylation and screening of recombinant UGTs *in vitro* has identified the substrate specificity of many of the enzymes in *Arabidopsis* (Bowles and Lim, 2010). In the present study, the results of *in vitro* analysis of

the enzyme activity of *SDG8i* indicate that SDG8i encodes a glucosyltransferase (SL-UGT) that potentially glycosylates an endogenous SL-like compound as substrate. Genetic analyses with SL mutants may provide additional information, however identification and structural analysis of the endogenous target metabolite/s is required for complete understanding of the mode of SL-UGT action. An effort to identify an endogenous enzyme with similar SL-UGT activity in *Arabidopsis* and other plants may be worthwhile, although substrate specificity of UGTs is not necessarily reflected in gene phylogeny.

To test the SL-mediated shoot branching inhibition, GR24 was applied on the axillary buds of *SDG8i* transgenic, wild-type and SL-signaling mutant, *max2* plants. *SDG8i* transgenic plants showed reduced sensitivity to GR24, which indicates that *SDG8i* activity is reducing the bioactivity of GR24.

SLs enhance primary root growth depending on different growth conditions (Ruyter-Spira *et al.*, 2011). Under normal growth condition, application of GR24 increases primary root growth in a *MAX2*-dependent manner, although relatively higher doses show inhibitory effect which is in a *MAX2*-independent manner. Under low carbohydrate condition, primary root growth is normally decreased and GR24 application may rescue only SL-deficient mutants (Ruyter-Spira *et al.*, 2011). In the present study, application of GR24 affected primary root length of *SDG8i* transgenic plants in a concentration-dependent way. Length of primary root increased more at lower concentration of GR24. The results of the root elongation experiment also suggest that *SDG8i* activity is reducing the bioactivity of GR24. This also suggests optimal concentration of GR24 for increased root growth may be lower than 5 μM. Future experiments to test this are required.

Several mutants affected in JA signaling such as *jar1* (Staswick *et al.*, 1992), *jin1* (Berger *et al.*, 1996), and *coi1* (Feys *et al.*, 1994) showed reduced sensitivity to JA, tested by MeJA inhibition of primary root elongation. In the present study, *SDG8i* transgenic plants also showed a reduced sensitivity to JA, suggesting that *SDG8i* acts downstream or antagonizes the effect of MeJa. The results show that JA inhibition pathway is still working even though level of inhibition was interfering with the pathway. If *SDG8i* activity downstream of JA, then a

reduced inhibition at 5 μM may be expected compared with 10 μM JA. Therefore it could be an antagonistic effect. As JA is involved in senescence, the effect JA on *SDG8i* transgenic plants may be tested.

Leaf senescence may be initiated by different plant hormones although it is a developmentally programmed event. The *SDG8i* transgenic plants of the present study showed reduced senescence symptoms induced by both dark and the hormone ABA, which was shown by analysing changes in chlorophyll content and photochemical efficiency. Therefore, the results presented here suggest *SDG8i* activity acts downstream of ABA and potentially several of the other stress-related hormones to reduce senescence. SL synthesis and signaling mutants have been reported as having reduced senescence (Woo *et al.*, 2001; Snowden *et al.*, 2005). The *SDG8i* transgenic plants have a phenotype consistent with altered ORE9/MAX2 activity which acts as a signaling protein in the strigolactone pathway and promotes senescence (Woo et al., 2001; Stirnberg et al., 2007). MAX2 is also involved in oxidative stress (Woo et al., 2004) and drought responses (Tang et al., 2005). The phenotype of *SDG8i* transgenic plants with reduced senescence and increased branching suggests a reduction in the bioactivity of the signal/s that activate MAX2. *Arabidopsis* produces indeterminate florescence and senesces following flowering, may be because the vegetative tissues act as a source of carbohydrate and nutrients for seed production (Nooden *et al.*, 1997; Masclaux-Daubresse and Chardon, 2011). The reduced senescence, in combination with the larger rosette leaves, may be related to the higher yield of the *SDG8i* transgenic plants.

Chapter 5

Effect of *SDG8i* on different abiotic stress responses of *Arabidopsis*

5.1 Introduction

Abiotic stresses, particularly high and low temperatures, freezing, drought and salinity, are the main causes of crop losses worldwide (Hirayama and Shinozaki, 2010). These stresses impose detrimental effects on plant performance mainly by disrupting plant water status or by osmotic stress (Verslues *et al.*, 2006). Understanding abiotic stress responses in plants is therefore an important and challenging issue in plant research. Le (2004) showed that *SDG8i* expression in *S. stapfianus* is induced by dehydration and cold. Salt appeared to have no effect on *SDG8i* expression. However, these experiments were preliminary, with only one temperature treatment (4°C) and two salt concentrations (150 and 300 mM NaCl) for either 3 or 24 hours. As low temperatures, drought and salt stress all decrease water availability to plant cells these abiotic stresses are of interest and this chapter focuses on drought, salinity and freezing stress and their effect on *SDG8i* transgenic plants.

Soil salinity negatively affects plant growth and development and decreases crop yield by over 20% (Porcel *et al.*, 2012). Salt stress causes physiological drought in plants by reducing osmotic potential. A high uptake of Na^+ and Cl^- ions may lead to toxicity in cells (Munns and Tester, 2008), displace plasma membrane Ca^{2+}, and lower membrane transport activity causing a leakage of K^+ from the cell (Cramer *et al.*, 1985; Cramer and Läuchli, 1986). Na^+ also disrupts ion homeostasis by competing with K^+ for uptake by transport proteins. This negatively affects the plant nutrient balance and vital processes such as cell elongation, leaf and stomatal movements and germination (Kochian and Lucas, 1988). Ion toxicity may also inactivate enzymes, which subsequently may inhibit protein synthesis (Kneera and Zenk, 1992), and photosynthesis and have other secondary effects including accumulation of reactive oxygen species (ROS) in the plant cell, leading to oxidative stress. Morphological responses to

combat osmotic stress conditions include development of extensive root systems to reach deeper into the soil to accumulate nutrients and water, and to reduce transpiration either by decreasing the leaf size or via leaf abscission (Hasewaga *et al.*, 2000).

In response to osmotic stress elicited by water deficit or high salt, the expression of a set of genes is altered (Zhu *et al.*, 1998), some of which are also induced by low temperature stress (Thomashow, 1998). There is an overlap between the responsiveness of many of the stress induced genes, suggesting parallel mechanisms of induction. The analysis of promoters of stress responsive genes revealed a *cis*-acting dehydration-responsive element (DRE) (Yamaguchi-Shinozaki and Shinozaki, 1994) and a similar low-temperature C-repeat (CRT) element (Baker *et al.*, 1994), providing an explanation for the similar stress responses. MAPK cascades have been shown to play role in salt stress by regulating osmolyte biosynthesis (Zhu, 2002; Rodriguez *et al.*, 2010). Overexpression of a tobacco ethylene receptor NTHK1 in *Arabidopsis* showed reduced sensitivity to salt stress (Cao *et al.*, 2007). SALT OVERLY SENSITIVE (SOS) pathway genes -*SOS1*, *SOS2* and *SOS3* have roles in ion homeostasis which improves resistance to salinity in *Arabidopsis* (Mahajan *et al.*, 2008; Luan, 2009).

During freezing stress, ice crystals form in the extracellular space which dehydrates the cell (Verslues *et al.*, 2006). Therefore, tolerance of freezing stress is related to dehydration tolerance. *Arabidopsis* freezing tolerant mutant *eskimo1* (*esk1*; (Xin and Browse, 1998; Xin *et al.*, 2007)) accumulates high total solutes and particularly the compatible solutes, proline, as a process of cold acclimation, which makes *esk1* more resistant to drought-induced water loss. The ICE1–CBF transcriptional cascade plays a role in cold acclimation in diverse plant species. ICE1 protein level and activity are regulated post-translationally by a ring finger protein, HOS1 (HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1)-mediated ubiquitination and proteolysis (Dong *et al.*, 2006) and by SIZ1-mediated sumoylation (Miura *et al.*, 2007). In the *hos1* mutant, expression of DREB1/CBF genes is super-induced by cold stress.

As drought decreases the availability of soil water, the decrease in soil water potential (ψ_w) makes it more difficult for the plant to take up water (Kramer and Boyer, 1995). Plants use

different mechanisms to tolerate water-deficit such as increased root growth, decreased leaf area and transpiration, stomatal closure, leaf curling, wax content, high water-use efficiency and accumulation of compatible solutes. In various species, a large number of different genes respond to drought-stress and the study of stress-responsive transcription factors is an important aspect in the study of drought stress tolerance. In *Arabidopsis*, some members of the AP2/ERF super family have been shown to play important roles in the drought response (Shinozaki and Yamaguchi-Shinozaki, 2000). An *Arabidopsis* NFYB and a rice NAC-type family transcription factors were also shown to confer drought tolerance (Hu *et al.*, 2006; Nelson *et al.*, 2007).

Glycosyltransferases play major roles in plant growth and development and are involved in plant secondary metabolism. Few studies have confirmed that UGTs play important roles in plant defense reactions to biotic stress, such as herbivore attack, and bacterial or fungal infections, as well as in plant responses to abiotic stress, including salinity, drought and cold (Mazel and Levine, 2002; Langlois-Meurinne *et al.*, 2005; Meissner *et al.*, 2008) (discussed in Chapter 1). A study by Tognetti *et al.* (2010) shows a role of UGT74E2, encoding an IBA UGT, in salinity and drought stress in *Arabidopsis*. In addition, loss-of-function mutations in *UGT73B1*, *UGT73B2*, or *UGT73B3* and also *UGT71C1* in *Arabidopsis* enhanced plant resistance to oxidative stress (Lim *et al.*, 2006; Lim *et al.*, 2008).

As signaling molecules, phytohormones form a complex network and play an important role in adaptation to environmental changes. The manipulation of phytohormones and other plant growth promoting substances has gained much attention, with the potential to facilitate plant growth under complex environmental conditions, such as salt and drought stress, or as a means to study the basic molecular mechanisms underlying the improvement of plant resistance to these stresses (Van Staden *et al.*, 1995; Jain *et al.*, 2006; Soós *et al.*, 2009). Among the phytohormones, ABA plays an important role in regulating plants response to abiotic stress. ABA accumulates in both salinity and drought stress, and cause a significant change in the gene expression profile and cellular processes (Kempa *et al.*, 2008). The genes for ABA biosynthetic and catabolic pathway, such as NCEDs and P450 CYP707, respectively, are shown to be activated by various stress treatments (Umezawa *et al.*, 2006). At low water

potentials, ABA promotes root growth and inhibits shoot growth (Creelman *et al.*, 1990) and also plays an integral role in stomatal closure and transpiration efficiencies under these conditions (Zimmermann and Sentenac, 1999).

Other plant hormones in particular CK, SA, ethylene and JA play substantial roles in abiotic stress, either directly or indirectly (Fujita *et al.*, 2006; Grant and Jones, 2009; Pieterse *et al.*, 2009). Increased concentrations of CK promote survival under water-stress conditions and inhibit leaf senescence (Peleg *et al.*, 2011). The interaction between ABA and ethylene has been shown in several studies (Gazzarrini and McCourt, 2003; Fujita *et al.*, 2006). Exogenous BR application induced the expression of stress-related genes (Zhang *et al.*, 2009). ABA was shown to inhibit BR-induced responses during abiotic stress (Divi *et al.*, 2010) suggesting complex hormonal responses to abiotic stresses in plants. Recently, the auxin responsive gene, *TLD1/OsGH3.13*, was shown to enhance the expression of *LEA* genes, increasing the drought tolerance of rice seedlings (Zhang *et al.*, 2009). There is little evidence as yet of a role for SL in salt, drought or cold stress. Though there is some evidence which suggest that SLs may have a function in nutrient stress. For example, production of *orobanchol*, a natural SL first isolated from red clover root extracts, was significantly stimulated upon limited-phosphate (Pi) conditions, suggesting that Pi availability regulates SL production to some extent (Yoneyama *et al.*, 2007). Similarly, SL biosynthesis was also promoted in tomato plants exposed to Pi starvation conditions (López-Ráez *et al.*, 2008). A recent study has shown that the SL production is increased by salinity which positively regulates AM symbiosis (Aroca *et al.*, 2012).

5.2 Materials and methods

5.2.1 Salt stress assay

Salt stress assay was performed according to Verslues *et al*., 2006. Seeds were surface-sterilized in 70% ethanol and rinsed (5×) with sterile ddH_2O. Seeds were then sown on MS media containing 0.5% sucrose and 0.8% agar in approximately 200 µmole $m^{-2}sec^{-1}$ light intensity and stratified at 4°C for 2-4 days to obtain uniform germination. Seedlings were allowed to grow for four to six days with the plates in a vertical orientation before transferred to salt containing MS media (0, 50, 100, 125, 150 and 175 mM NaCl) using forceps. Salt-containing media was prepared by adding an appropriate amount of salt to regular germination media followed by autoclaving. The position of the root tips was marked immediately after transfer and then the agar plates were placed in vertical orientation and inverted to allow roots to grow downward in the shape of a hook. Root elongation was quantified by measuring the position of the root tips over 7 days. The transgenic lines, F6bA, D5aA, D1E, F1aD and D7c were used in the experiment and there were 10 replicates of WT and each transgenic line for each treatment.

5.2.2 Freezing stress assay

The freezing tolerance test was performed as described by (Xin and Browse, 1998) with some modifications. Plants (25 seedlings /plate) were grown on MS media-agar (½ MS, 0.5% sucrose, pH 5.7, 0.8% agar) for 3 weeks at 22^oC and approximately 200 µmole $m^{-2}sec^{-1}$ light intensity after stratification. Petri dishes of plants were then transferred to a chamber (Sanyo Incubator) set to -1±0.1°C. Petri dishes of plants were cold-acclimated at -1^oC with ice chips to achieve uniform freezing at least for 16 hours before further lowering the temperature. The chamber was then programmed to cool at 2^oC per hour until it reached -4^oC. Petri dishes containing seedlings were then exposed to -8^oC for 1 hour and to -12^oC for a further hour. A subset of the plants was removed periodically at -4^oC, -8^oC and -12^oC and transferred to 4^oC overnight for recovery. Plants were returned to 22^oC and survival was scored 7 days later. Plants, which were bleached of chlorophyll and appeared white, were scored as dead, while green plants were scored as having survived the freezing test. The transgenic lines, F6bA, D5aA, D1E and F1aD, were used in the assay, with 3 replicates of each line.

5.2.3 Water withholding test

Wild-type Col-0 and *SDG8i* transgenic lines were grown in separate 5cm diameter pots placed on the same tray under optimal growth conditions in a controlled growth chamber under a 16h light regime at 22^{o}C and approximately 200 μmole $m^{-2}sec^{-1}$ light intensity for 2 weeks. Plants were kept fully watered by sub-irrigation until the 6-7 leaf pre-flowering stage. Water was then withheld for a period of 14 days. The plants were observed over this period and after 14 days, the drought treated plants were re-watered and the recovery was checked 24 hours later.

The experiment was repeated over a longer time frame to see how long transgenic plants could survive without water. Five plants per line grown in separate 5cm diameter pots were used and experiment was done in duplicate. The survival of plants was calculated following exposure to dehydration over a period of 3 weeks. A subset of plants were removed at various time intervals over the 3 weeks drought period and rewatered. The plants were then checked for survival over the following days. The same amount and type of soil was used throughout the experiment and saturated to uniform conditions.

5.2.4 Water vapor equilibration test

Soil grown pre-flowering plants were washed gently and soaked in water for 2 hours until turgid and then blotted dry. For protoplasmic drought tolerance (PDT) determination in shoot tissue, shoots were detached and the initial turgid weight (TWt_o) recorded. Transgenic and wild-type Col-0 shoots (four per line) were used and the experiment was done in duplicate. Shoots were placed on mesh inside closed chambers above different concentrations of $CaCl_2$ solutions, avoiding contact between the leaf and solution, and equilibrated to differing RH. Chambers containing $CaCl_2$ solutions at 98% (-2.8 MPa), 96% (-5.5 MPa), 94% (-8.4 MPa), 92% (-11.3 MPa), 90% (-14.4 MPa), 88% (-17.4 MPa) and 86% (-20.5 MPa) RH at 25^{o}C were used. The relative humidity of air in equilibrium with the $CaCl_2$ solutions are calculated from freezing point depression data using the formula in (Owen, 1952). A preliminary test of the time course of the decrease in the fresh weight of initially fully turgid shoots showed that equilibrium was first reached at one day exposure to 90% RH. The chambers containing shoots were kept in a closed insulator box in a constant temperature room at 20°C for at least 3

days until equilibration was reached ($\psi_{leaf} = \psi_{solution}$). After equilibration the shoots were soaked in water for 24 hours to rehydrate and the final turgid weight (TWt_F) was recorded. Survival of plants was determined by: Method A, where survival was judged subjectively by the recovery of the healthy coloration and crisp texture seen in the pre-equilibration plants (dead and live leaves are readily distinguished in the thin fibre-poor tissues of *Arabidopsis*) and the number of leaves alive per plant recorded; Method B, an objective test, depends on the loss of semipermeability in dead cells which prevents osmotic absorption of water, thus the apparent "turgid" weight per dry weight of dead tissue is less than the turgid weight per dry weight of live tissue before vapor-equilibration. A difficulty of vapor-equilibration methods is that tissues equilibrated in 100% RH in darkness usually suffer injury, often fatal, which is not incurred by samples at lower humidities, a phenomenon extensively shown by Pruzsinsky (1960). The ratio TWt_F/TWt_o was plotted against % RH and the PDT was determined as the % RH at which 50% of the tissue is alive.

5.2.5 Neutral red uptake

Neutral red (NR) uptake was used to check live versus dead leaf tissue and to compare cell damage in the leaves of transgenic and wild-type plants. $CaCl_2$ solution (29 ml) was added to 1 ml of neutral red stock solution. The pH was adjusted by adding NaOH to the NR-$CaCl_2$ solution until the color changed from purple red to orange red. Fresh hand sections of leaves of about 2-3 cells thick were stained immediately with the neutral red solution and left for 60 minutes in a covered Petri dish with moist Kleenex paper under 100% RH to prevent the NR solution from drying. The tissue was then viewed under a microscope. Live cells exhibit an intense red color in the vacuole while dead cells have a diffuse light orange color.

5.2.6 Statistical analysis

All data were examined by analyses of variance using GraphPad Prism software version 5.0. Tukey's Multiple Comparison Test was further used for comparison between wild-type and *SDG8i* transgenic means at 5% level of significance.

5.3 Results

5.3.1 Salt stress

The response of plants to salt stress was assessed by the root growth of *SDG8i* transgenic plants compared to roots of wild-type control plants on different concentrations of NaCl. A comparison of the response of *SDG8i* transgenics and wild-type Col-0 plants to growth on high salt media was made (Fig. 5.1A).

No significant difference was observed between the *SDG8i* transgenic and wild-type Col-0 seedlings at salt concentrations below 100 mM after 7 days (Fig. 5.1B). At 125 mM NaCl, all transgenic lines except D7c showed higher salt tolerance forming significantly longer roots compared to wild type ($p<0.001$). However, at 150 mM NaCl all transgenic plants showed significantly greater salt resistance with 2-3 times longer root growth compared to that of wild type plants ($p<0.001$) (Fig. 5.1B). Wild-type Col-0 plants grown in 175 mM NaCl showed severe inhibition of root growth although shoot growth did not appear to be greatly inhibited (Fig. 5.1A). The rate of transpiration in the plates is probably too low to cause a build-up of high concentrations of NaCl in the shoot (Verslues *et al.*, 2006). At 175 mM NaCl, root growth of transgenic lines was 5-7 times higher than that of wild-type Col-0 plants ($p<0.001$) with the transgenic lines F6bA and D5aA showing the greater resistance. At 200 mM NaCl, the root growth of transgenics was almost completely inhibited (data not shown).

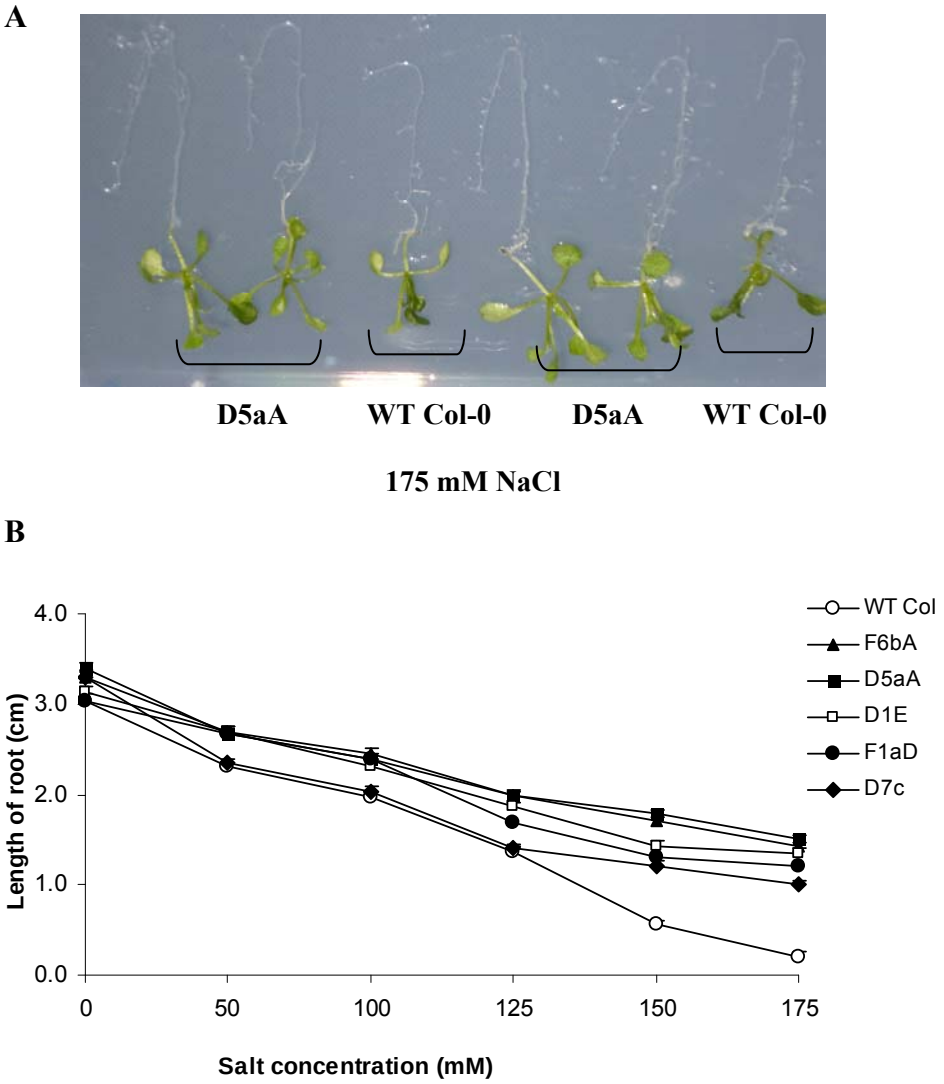


Fig 5.1 Effect of salt on the root growth of *SDG8i* and wild-type Col-0. **A)** Plants were grown upright for 4-6 days in MS media under normal condition and then transferred to media containing different concentrations of NaCl and the plates inverted. **B)** Root growth (cm) was measured after 7 days. Values are the means ± SE of 10 replicates

5.3.2 Freezing tolerance

The extent of freezing stress-induced damage of *SDG8i* transgenic and wild-type Col-0 plants was measured by testing the percentage of survival and recovery of plants after freezing stress. All transgenic and control wild-type Col-0 plants exposed to -4^{o}C freezing stress survived (Table 5.1). At -8^{o}C, all *SDG8i* transgenic lines survived, whereas the survival rate of wild-

type Col-0 plants was 83% ($p<0.01$) (Fig 5.2, Table 5.1). At -12°C, control wild-type Col-0 plants were greatly affected, with a survival rate of only 34% ($p<0.001$). However, most of the transgenic plants survived freezing at -12°C, with the survival rate ranging from 69-72% (Table 5.1, Fig 5.2).

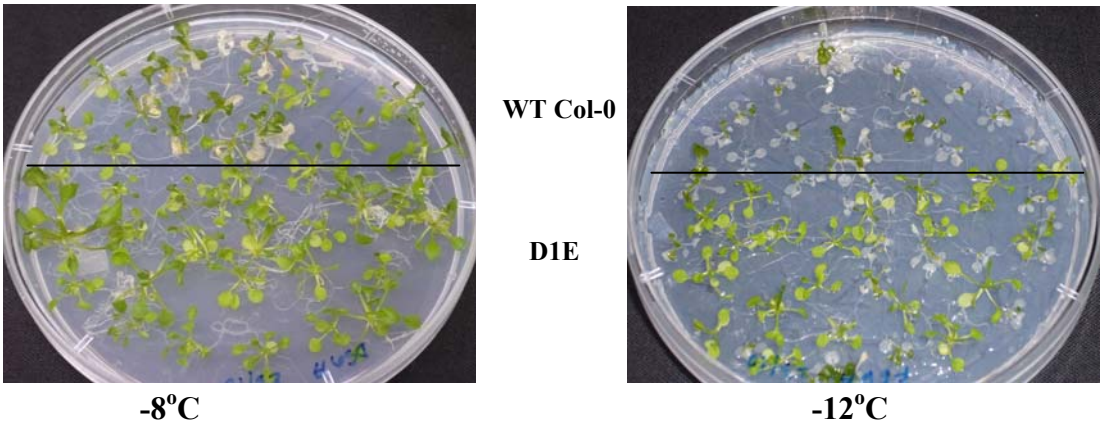


Fig 5.2 Freezing tolerance test of *SDG8i* transgenic and wild-type Col-0. Plants were exposed to freezing at -4°C, -8°C and -12°C after cold-acclimated at -1°C for 16 hours and then returned to normal conditions at 22°C. Survival was scored 7 days after plants were returned to normal conditions.

Table 5.1 Percent survival of different 8i transgenic lines and wild-type Col-0 in freezing tolerance test

Plants were exposed to freezing at -4°C, -8°C and -12°C after cold-acclimated at -1°C for 16 hours and then returned to normal conditions at 22°C. Survival was scored 7 days after plants were returned to normal conditions. * Asterix indicates significant difference in survival rate.

Name of plants	Plant survival (%) at -4°C	Plant survival (%) at -8°C	Plant survival (%) at -12°C
WT Col-0	100	83*	34*
F6bA	100	100	72
D5aA	100	100	70
D1E	100	100	72
F1aD	100	100	69

5.3.3 Drought tolerance

The drought tolerance of *SDG8i* transgenic plants was evaluated by water withholding and water vapor equilibration experiments and compared to that of wild-type control.

In the water withholding experiment, plants were kept fully watered by sub-irrigation until the 6-7 leaf pre-flowering stage and water was then withheld. Wild-type Col-0 plants showed their first signs of wilting just after 7 days without water, while all *SDG8i* transgenic plants retained a healthy appearance (Fig 5.3A).

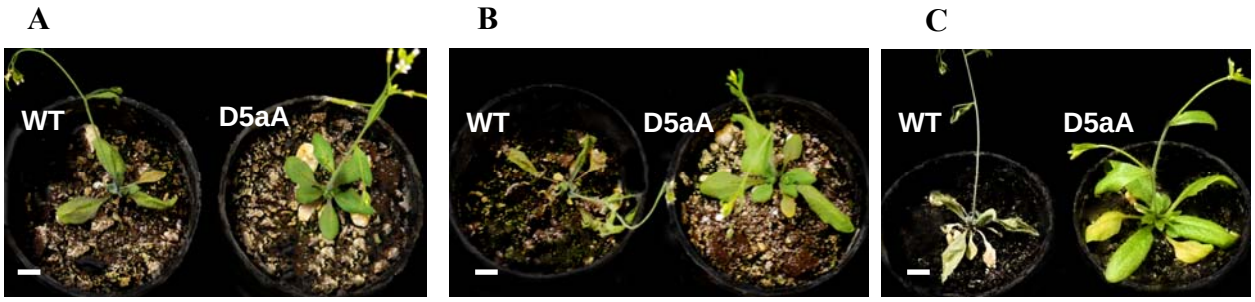


Fig 5.3 Drought stress of wild-type Col-0 and *SDG8i* transgenic plants. Plants were grown under a controlled water system for 2 weeks and then without water for 13 days. Plants were re-watered on day 14 and observed for recovery following day.
(A) 7 days without water, **(B)** 13 days without water, and **(C)** 1 day following re-watering. Size bar = 2cm.

After 13 days of drought, all wild-type Col-0 plants suffered clearly from water loss and were severely dehydrated; however, all *SDG8i* transgenic plants still appeared healthy (Fig 5.3B). On day 14 all plants were rewatered and the recovery was observed after 24 hours. All the *SDG8i* transgenic plants recovered, but none of the wild-type Col-0 plants showed any sign of recovery (Fig 5.3C).

The experiment was repeated over a longer period of time to determine how long transgenic plants could survive without water. The survival of wild-type Col-0 and *SDG8i* transgenic

plants was calculated following exposure to dehydration over a period of 3 weeks (Fig 5.4). A subset of plants was removed at various time intervals over the 3 week drought period, re-watered and the survival checked over the following days. The survival rate of wild-type Col-0 plants dropped below 50% after 11 days without water. This was significantly less than the 100% survival of transgenic plants ($P<0.001$). All wild-type Col-0 plants died after 13 days while all the transgenic plants looked healthy, with 100% survival rate during that period. From day 15 the transgenic plants started looking dehydrated and their survival rate reduced to 50% after 17 days. Transgenic plants looked severely dehydrated on day 18 and they did not recover after 19 days.

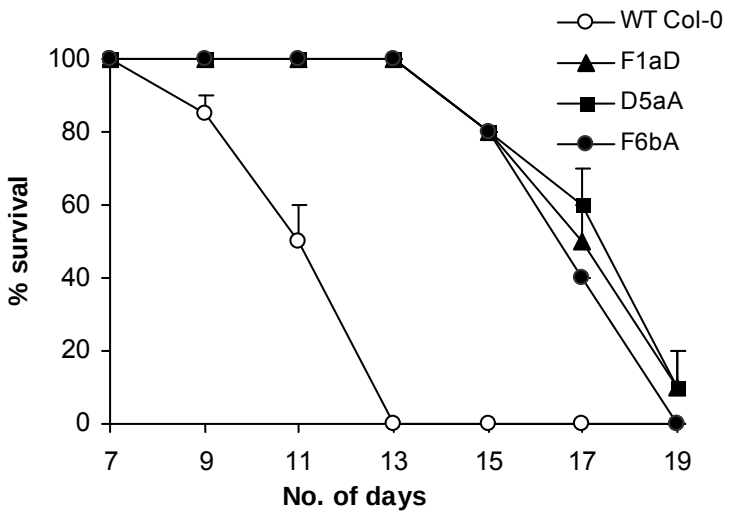


Fig 5.4 Percent survival of wild-type Col-0 and *SDG8i* transgenic plants at different time intervals during prolonged period of drought. Values are the means ± SE of 2 replicates.

To quantify the increase in drought tolerance in shoot tissue conferred by the expression of *SDG8i*, a measure of protoplasmic drought tolerance (PDT; determined as the percent relative humidity at which 50% of leaf cells survive) was obtained. The PDT of wild-type Col-0 plants, as determined by both the subjective method (Figure 5.5A) and the objective method (Figure 5.5B) was ~97-98% RH (~-2.8 to -4.2MPa).

The results indicate that *Arabidopsis* is very sensitive to water deficit, which is not unexpected in the thin tender leaves on well-watered greenhouse plants of an ephemeral species. The transgenic lines F1aD, D5aA and F6bA gave closely similar data to each other in both methods. The determinations of their PDT were ~94-95% RH (~-7.0 to -8.4MPa) – considerably better than the values obtained for wild-type Col-0 plants indicating a substantial improvement of PDT ($p<0.05$).

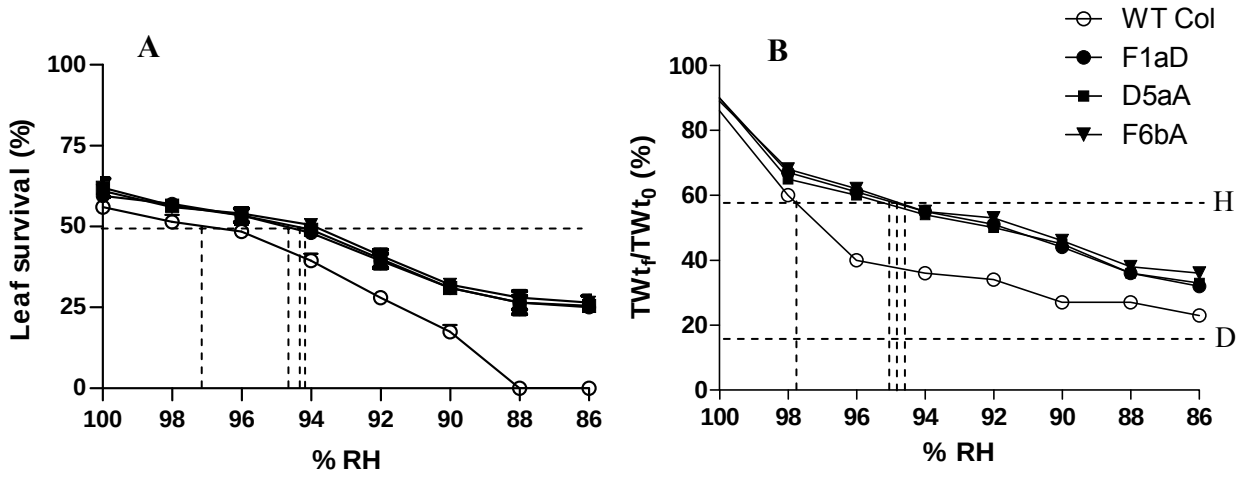


Fig 5.5 A) leaf survival, B) PDT survival curve [% of final saturation weight to the initial turgid weight ratio] showing the effect of decreasing relative humidity on cell survival of *SDG8i* and wild-type Col-0 shoots. PDT values are the water potential (expressed as the corresponding equilibrium relative humidity at 20°C) at which 50% of cells were dead after 3 days vapor equilibration over $CaCl_2$ osmotica. Line D represents the lowest value of TWt_f/TWt_0 reached by comparable shoots killed by chloroform vapor. Line H is the 50% survival point halfway between line D and 100% TWt_f/TWt_0.

The neutral red uptake test on hand sections of live leaves from wild-type Col-0 and *SDG8i* transgenic plants at 90% RH showed that a substantially higher number of damaged cells were present in the wild-type Col-0 plants compared to all the transgenic plants (Figure 5.6).

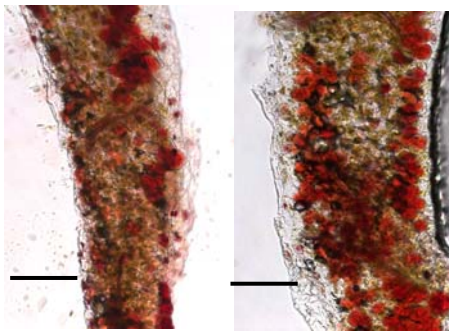


Fig 5.6 Neutral red stained sections of leaves performed at 90% RH of *SDG8i* and wild-type Col-0 plants. The scale bar is 100 μm.

5.4 Discussion

The widespread occurrence, diversity and complexity of glycosides throughout the plant kingdom indicate that the largest Family 1 GT has broad functionality with a wide range of potential substrates from plant secondary metabolism. Limited information exists on the biological role and molecular mechanisms of UGTs mainly in response to environmental stress. However, their chemical properties have been widely studied. In the present study, ectopic expression of UGTSDG8i in *Arabidopsis* plants results in a substantial improvement in tolerance to cold, salt and drought stress. These environmental factors, particularly drought and salt, impose major constraints on crop productivity.

Salinity affects growth by changing water relations and by affecting the nutrient balance in plant tissue (Pessarakli *et al.*, 1991). Physiological mechanisms by which plants respond to salt stress include, decreases in fresh mass, reductions in leaf growth and increases in root growth and length (Evers *et al.*, 1998; Khodary, 2004). If the plant is under salt or water stress, the below-ground structures expand to facilitate growth and supply nutrients and water to the above-ground structures for normal growth (Saab *et al.*, 1990). A study by (Meloni *et al.*, 2004) showed root growth was less affected than shoot growth in *Prosopis alba* at NaCl concentrations as high as 300 mmol L^{-1}. An increased root:shoot mass ratio is a common adaptation to salinity, resulting in longer roots with increased lateral root development to facilitate more efficient water and nutrient uptake under salt stress (Gorham *et al.*, 1985). In the present study *SDG8i* transgenic seedlings grown with 175 mM NaCl showed a resistance to salinity by a significant increase in root growth compared to wild type, which could enhance the uptake of nutrients from the root system under osmotic disturbances. An examination of the effect of NaCl on the root:shoot ratio of the transgenic plants could determine if the plants have an altered root:shoot ratio. As ectopic expression of *UGTSDG8i* can increase tolerance to salt stress in *Arabidopsis*, it is assumed that the metabolite/s being glycosylated by UGTSDG8i may be involved in a regulation pathway of *Arabidopsis* in response to environmental stress. Transgenic *Arabidopsis* overexpressing UGT74E2 also showed resistance towards drought and salt stress by its glycosylation activity on indole-3-butyric acid (IBA) (Tognetti *et al.*, 2010).

In the water withholding experiment of the present study, the drought stressed wild-type plants ceased their vegetative growth, the leaves senesced rapidly and finally died after 13 days without watering. On the other hand, all the *SDG8i* transgenic plants showed no sign of senescence even after 12 days without watering, and they still looked green and healthy. When protoplasmic drought tolerance (PDT) of *SDG8i* transgenic plants was evaluated, they showed a two-fold improvement compared to wild-type *Arabidopsis* plants. PDT is the water potential of tissue at 50% survival point during drying (Kramer, 1983), and the measure of desiccation tolerance is the extremity of PDT being the lowest water potential of the cell protoplast that the protoplast may survive (Gaff and Oliver, 2013). The PDT improvement of *SDG8i* plants is similar to the maximum drought hardening reported for a crop plant drought stressed for 3 weeks, but does not reach the PDT values of desiccation tolerant angiosperm plants which are in the range from 0-30% RH as reported by Levitt *et al.* (1960). However, this PDT improvement of *SDG8i* transgenic plants is significant compared with wild-type plants considering the difficulties of the technique used to impose low-ψ_w stress on the plants (via the air phase). This vapour equilibration technique can only be used in darkness because light produces tissue-to-air temperature gradients which disturb the desired water potential gradient. Therefore when tissues are equilibrated in 100% RH in darkness it causes injury, which is not found at lower RH. This type of phenomenon was also shown in a study by (Pruzsinsky, 1960). It is therefore possible that *SDG8i* plants growing in the light would exhibit higher PDT values. Moreover, due to experimental difficulties caused by contamination of root with the soil, only shoot tissues were used, instead of whole plants, in the PDT experiment. The disturbance of the root system during the drying period in *S. stapfianus* was shown to disrupt the acquisition of the desiccation-tolerant state (Gaff *et al.*, 1997). Hence, the use of whole plants may show further improvements on PDT because this would allow hormonal interchange between roots and shoots in the drought stressed plants.

In the present study, *SDG8i* transgenic plants also showed greater tolerance to cold compared with wild-type plants. At the molecular level, plants show similar response to both freezing and drought. Some of the genes induced by freezing are also upregulated by drought (Shinozaki and Yamaguchi-Shinozaki, 2000). The formation of ice crystals during freezing creates a water potential gradient between inside and outside the cell causing freeze-induced

dehydration (Pearce, 1999), which suggests that a major component of the acquired freezing tolerance by the *SDG8i* transgenic plants may be the tolerance to dehydration stress.

Stress adaptation responses involve growth retardation, reduced metabolism and reallocation of resources. As the formation of ROS may be induced by a wide range of stresses, they may play a role in modulating auxin sensitivity and controlling developmental processes (Potters *et al.*, 2007; Potters *et al.*, 2009). Similarly, to the example where overexpression of UGT74E2, an IBA glucosyltransferase may increases drought and salt tolerance with reduced growth (Tognetti *et al.*, 2010), overproduction of WES1, an auxin conjugating enzyme, also decreased growth and increased stress tolerance in *Arabidopsis* (Park *et al.*, 2007). On the other hand, *SDG8i* transgenic plants of the present study showed enhanced growth under both stress and non-stress conditions which suggest *SDG8i* activity may be directly controlling growth. In *S. stapfianus*, high levels of *SDG8i* transcript are induced in leaves by dehydration and could be related to leaf regeneration in the rehydrated plant which is more than twice as rapid as in a well-watered plant following a dehydration/rehydration cycle (Blomstedt *et al.*, 2010).

Chapter 6

General discussion and future directions

Although it is not proved conclusively that the *Sporobolus* drought gene, UGTSDG8i, glycosylates endogenous strigolactones *in planta*, the phenoytype of *Arabidopsis* transgenics plants overexpressing *SDG8i* exhibit hyperbranching and a reduced ability to stimulate strigolactone-dependent germination of *Orobanchaceae* (*Phelipanche ramosa*) seed. This suggests that the bioactivity of one or more strigolactones known to be produced by *Arabidopsis* is affected by SDG8i enzyme activity.

SDG8i transgenics show reduced auxin related branching inhibition, with greater bud outgrowth compared to wild-type plants when auxin is applied to the decapitated plants. This could occur by an alteration in auxin bioactivity directly or through an effect on auxin signaling via second messenger activity. SL is known to be required as second messenger for auxin to regulate branching (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Bud outgrowth in SL mutant plants is resistant to inhibition by apically supplied auxin (Beveridge *et al.*, 2000; Sorefan *et al.*, 2003; Bennett *et al.*, 2006). The level of increased branching in the *SDG8i* transgenic plants suggests a substantial reduction in the bioactivity of a branching inhibitory signal, which is likely to be SL or a related compound. Because auxin induces SL biosynthesis (Sorefan *et al.*, 2003; Foo *et al.*, 2005), it is also possible that reduced auxin bioactivity or signaling could lead to a lower production of SL. It is necessary to examine the endogenous hormone concentrations and identify the glycosylated metabolite differences in *SDG8i* transgenic plants compared with wild-type controls to confirm how *SDG8i* activity acts to reduce the repression of bud outgrowth. Moreover, some phenotypic changes of *SDG8i* are similar to SL-deficient mutants such as increased branching and some are not such as reduced height. Therefore, if *SDG8i* is decreasing bioactivity of SL, the observed phenotypic changes suggest that a major role of SL is to suppress cellular growth in response to developmental and environmental cues.

The increased intensity of *DR5-GUS* in the leaves of *SDG8i* transgenic plants suggests an increased auxin production. The *axr1-3* mutant showed increased auxin concentrations as feedback compensation for reduced auxin sensitivity (Romano *et al.*, 1995). This would therefore suggest that *SDG8i* activity is affecting auxin signaling possibly due to an effect on the bioactivity of SL as auxin second messenger.

SDG8i transgenic plants are not affected in exogenous cytokinin increased branching, suggesting that *SDG8i* activity does not negatively affect CK activity. This indicates that CK pathway is working in *SDG8i* transgenic plants. Auxin is known to antagonise CK activity (Chatfield *et al.*, 2000). If auxin signaling is decreased, CK biosynthesis is initiated and the synthesized CK is transported to axillary buds where it induces bud outgrowth (Tanaka *et al.*, 2006). If *SDG8i* activity is affecting SL-like compounds, then CK activity could also be increased via reduced auxin signaling. Increased CK activity could possibly explain the phenotypic changes of *SDG8i* transgenic plants with increased shoot growth under normal conditions. CK is known to control not only cell proliferation and shoot branching in plants, but also has a very strong anti-senescence effect (Richmond and Lang, 1957). Because overproduction of CKs may retard leaf senescence (Gan and Amasino, 1995), elevated CK concentrations may be responsible for the reduced senescence phenotype of *SDG8i*. Again an examination of the endogenous hormone concentrations in *SDG8i* transgenic and WT plants would add greatly to the mechanistic explanation of the observed results.

The increased expression of *DR5-GUS* in the root vasculature and root tips of the *SDG8i* transgenic plants suggests there may be elevated auxin concentrations in the roots. Because auxin (at moderate concentrations but not low concentrations) is known to inhibit cell expansion in the root elongation zone (Stepanova and Alonso, 2009), it would seem unlikely that the enhanced root growth in *SDG8i* transgenic plants is due to increased auxin concentrations. SLs have been shown to enhance primary root growth and exert inhibitory effects on lateral root formation under normal Pi sufficient conditions (Kapulnik *et al.*, 2011; Ruyter-Spira *et al.*, 2011). However, SL signaling has been linked to the induction of ethylene synthesis, which in turn may affect auxin synthesis and transport (Koltai, 2011). Auxin and ethylene may potentially inhibit primary root elongation either independently or together.

Stimulation of auxin biosynthesis by SL-mediated ethylene production in the root meristem may lead to auxin transport to the root elongation zone where it inhibits root growth by slowing down cell elongation (Stepanova and Alonso, 2009). This is consistent with the low-Pi condition where primary root growth is inhibited and lateral root growth is stimulated by increased SL (López-Ráez *et al.*, 2008; Jamil *et al.*, 2011; Kohlen *et al.*, 2011). Because auxin sensitivity in the roots may be increased under low Pi conditions (Pérez-Torres *et al.*, 2008) it has been suggested that increased SL production may reduce root auxin concentrations and generate an auxin optima leading to lateral root induction (Ruyter-Spira *et al.*, 2011). Therefore, the longer primary root and increased lateral root branching phenotype of *SDG8i* plants under normal growth condition cannot be easily explained by alteration in the effective concentrations of these hormones. It is possible that *SDG8i* activity may be affecting SL-mediated ethylene production and reducing the auxin inhibitory effect in the root elongation zone to allow primary root elongation. An examination of root growth of *SDG8i* transgenic plants on low Pi media may indicate if SLs are required to induce LR formation under Pi starvation.

The plant stress response mainly involves reduced growth and reallocation of resources and therefore introduction of constitutive stress tolerance often correlates with reduced growth (Potters *et al.*, 2007). However, *SDG8i* transgenic plants showed significant enhanced growth under both stress and non-stress condition. In contrast to the situation reported in desiccation-sensitive plants where water-deficit decreases the expression of growth regulated genes (Bray, 2004), the enhanced growth exhibited by *SDG8i* transgenic plants under stress suggests that *SDG8i* may impede the growth retardation response which would normally occur under these conditions. Under prolonged water stress, resurrection plants may accumulate high levels of the transcripts in dried tissues which encode gene products that positively regulate growth associated processes. The subsequent translation of the accumulated transcripts in rehydrated plants could allow rapid growth when water becomes available. In *S. stapfianus*, substantially more rapid leaf regeneration occurs following a dehydration/rehydration cycle compared to that which occurs in well-watered plants (Blomstedt *et al.*, 2010).

Target of rapamycin (*TOR*) plays a central role in regulation of cell growth and metabolism and is conserved in eukaryotic species. The growth-related processes in response to stress conditions such as changes in protein synthesis, cytoskeleton remodeling and nutrient uptake are mainly regulated by TOR (Deprost *et al.*, 2007). In *Arabidopsis*, overexpression of TOR kinase increased production of ribosomal components as well as enhanced translation capacity which has strong effects on maintaining growth and osmotic stress resistance. TOR mRNA appears to be present in all plant tissues, and may be post-transcriptionally regulated during cell proliferation to initiate premitotic cytoplasmic growth (Robaglia *et al.*, 2004). This suggests that it may be worthwhile investigating if the *SDG8i* gene plays a role in upregulation of the TOR pathway to increase growth and cell size of *SDG8i* transgenic plants in response to stress.

The two-fold improvement of the protoplasmic drought tolerance (PDT) in *SDG8i* transgenics compared with the wild-type plants is considerable and is similar to the maximum 'drought hardening' reported for a crop plant droughted for 3 weeks (Levitt *et al.*, 1960). PDT measurements at cellular level shows less cell death in *SDG8i* transgenic plants compared with wild-type plants, even at a low water-deficit level. The delayed senescence exhibited by *SDG8i* transgenic plants following ABA treatment suggests that *SDG8i* activity may be associated with inhibition of drought-induced senescence programs in *S. stapfianus*. Therefore, the increased PDT at the cellular level in *SDG8i* transgenic plants may reflect a reduced senescence effect mediated by *SDG8i* activity. Non-resurrection plants use leaf senescence as an efficient strategy for surviving water stress by reducing canopy size and transpiration (Chaves *et al.*, 2003). But *SDG8i* transgenic plants show very little signs of senescence in the water withholding experiment, remaining green and healthy after 12 days without water in contrast to wild-type plants. Some of the transgenics produced one or more extra rosette leaves and a reproductive meristem during the drying period and until re-watering did not form any additional floral branches. This suggests that *SDG8i* transgenic plants may undergo a drought-induced delay in reproductive development. Future experiments to analyse the response of the *SDG8i* transgenics to drought, could include high-throughput imaging to provide detailed physiological data that may answer this question. In normal growth conditions, the reduced senescence, in combination with the larger leaves, may be related to

the higher seed yield of the *SDG8i* transgenic plants. Considerable research effort has been put into increasing crop production utilizing the CK biosynthetic gene *IPT* by delaying drought-induced senescence (Jordi *et al.*, 2000; Sýkorová *et al.*, 2008). The use of the *SDG8i* gene presents an alternative approach to reducing drought- induced senescence with an additional benefit of increasing yield under non-stress conditions.

The response of *SDG8i* transgenic plants to different stress conditions suggests links between glycosylation of a SL-related compound/s and many growth-related and stress-related hormonal activities. During water stress, ABA plays important role in inducing stomatal closure, reducing transpiration efficiencies, inhibiting shoot growth and promoting root growth (Creelman *et al.*, 1990; Zimmermann and Sentenac, 1999). ABA is also a regulator of SL biosynthesis, as shown in the tomato ABA-deficient mutants which exhibit decreased SL production (López-Ráez *et al.*, 2010). There is considerable evidence indicating that SLs play a role in nutrient stress responses. SL is thought to act as a signal to communicate the nutrient status in the soil to the plant shoot, allowing for increased branching or overall shoot growth when nutrient levels are high and a decrease in growth when nutrients are limited (Umehara *et al.*, 2008). Therefore, the enhanced growth exhibited by *SDG8i* plants under stress conditions, suggests that SL or a related compound may act principally to retard growth as part of the morphogenic response to the stresses, as occurs in the shoots of plants under nutrient deficiency (Arnholdt-Schmitt, 2004). *SDG8i* activity affects ABA signaling with respect to senescence. If *SDG8i* is doing this through affecting the bioactivity of SL, this suggests that SL may be required to act downstream of ABA under stress conditions to mediate ABA stress/drought responses such as reduced photosynthesis & chlorophyll degradation as well as reduced shoot growth. This could explain the enhanced growth under stress conditions as the stress signaling pathway is not fully functional. Further analyses should be done on physiological parameters associated with ABA activity such as stomatal conductance and the efficiency of photosystem II, accompanied by an analysis of stress induced ABA levels, in *SDG8i* transgenics compared with control plants.

Some of the phenotypic characteristics of *SDG8i* transgenic plants, such as hyperbranching, reduced senescence and interaction with auxin and ABA, mimic those of the SL biosynthesis

and signaling mutants of *MAX*. On the other hand, the enhanced shoot and root growth of *SDG8i* transgenic plants are opposed to those of SL-deficient mutants, which are normally dwarfed. In addition, the reduced stimulation of *Orobanche* germination, which is an indication of a reduced SL level in *SDG8i* transgenic plants, is not as pronounced as that which occurs in the SL biosynthesis mutants MAX3 and MAX4. This suggests *SDG8i* activity is reducing but not eliminating bioactivity of SL in the *SDG8i* transgenics. Using an *SlCCD7* antisense construct Vogel *et al.* (2010) showed that a 90% reduction in SL levels was required to produce strong branching phenotype in tomato with bioassays for *O. ramose* germination. The i*n vitro* enzyme assay in the present study shows that *SDG8i* has moderate affinity for GR24, which could be expected given the artificial nature of substrate and assay condition. But the results strongly suggest that SDG8i enzyme activity in *Arabidopsis* is affecting the bioactivity of an endogenous branching-related and growth-related inhibitory compound/s.

MAX2, the F-box leucine-rich repeat signaling protein is expressed universally in the plant, not only at nodes, and therefore plays diverse roles in different parts of the plant (Stirnberg *et al.*, 2002). Some of the processes associated with MAX2 activity seem to be negatively affected in *SDG8i* transgenic plants. *MAX2*, previously identified as the *ORE9* gene (Stirnberg *et al.*, 2002), acts as a positive regulator of *Arabidopsis* leaf senescence (Woo *et al.*, 2001) and important mediator for hypocotyl elongation in the light (Stirnberg *et al.*, 2002). *MAX2* is also involved in oxidative stress (Woo *et al.*, 2004) and drought responses (Tang *et al.*, 2005). Mutations in ORE9/MAX2 suppress the enhanced response to drought caused by mutation of EDR1, which encodes a CTR1-like protein kinase (Tang *et al.*, 2005). The phenotype of *SDG8i* transgenic plants with elongated hypocotyls, reduced senescence and increased branching suggests a reduction in the bioactivity of the signal/s that activate MAX2. Therefore crossing *SDG8i* transgenic plants with plants overexpressing MAX2 may be informative. Genetic analyses with MAX2 affected plants and other SL mutants as well as examination of expression levels of MAX pathway genes could help elucidate the mechanism whereby *SDG8i* activity confers the phenotype observed. However, to confirm the actual function of the *SDG8i* gene, identification and structural analysis of the endogenous target metabolite/s is required.

It could also be worthwhile to try and identify an endogenous enzyme with similar activity to SDG8i in *Arabidopsis* and other plants. The transgenic plants over-expressing *SDG7y* and *SDG10y* displayed similar phenotypes to that of *SDG8i* transgenics with substantial increase in shoot growth and shoot branching therefore suggesting they are driving expression of an *Arabidopsis SDG8i* orthologue. Further analysis of the phenotypes of *SDG10y* and *SDG7y* transgenic plants is necessary under different abiotic stress conditions to see if they also show similarities in stress response observed in *SDG8i* plants. In *Arabidopsis* there are over 100 uncharacterised glucosyltransferases and database searches indicated that the three most homologous genes to *SDG8i* in *Arabidopsis* are At3g16520, which is fungus induced, At1g01390 of unknown function and At1g05680, which recognises IBA. Substrate specificity can't be determined based on sequence data alone and specificity would need to be determined by producing and screening each recombinant glucosyltransferase individually. Additional experiments to further investigate potential *SDG8i* orthologues would include the generation of transgenic plants overexpressing the candidate homologous genes, which may give a similar phenotype to that of *SDG8i* transgenic plants. However, the homologous gene that recognizes IBA is unlikely to be the *SDG8i* orthologue because overexpression does not give the same phenotype as *SDG8i* transgenics (Tognetti *et al.*, 2010). It may also be worthwhile analysing the spatial and stress related expression pattern of candidate *SDG8i* homologues in *Arabidopsis* plants. In addition, analysis of the phenotypes of *Arabidopsis* plants with knockouts of the homologous genes may also be useful. Crossing of such knockout mutants with *SDG8i* transgenic plants could also be informative. These experiments may help identify a UGT in *Arabidopsis* with a similar glycosylation activity to that of *SDG8i*.

Root exudates may be collected from *SDG8i* transgenic as well as wild-type *Arabidopsis* plants. Different concentrations of these root extracts may be used in the germination bioassay to detect differences in the level of SL metabolites and glycosylated SL-like compounds. These root extracts could be analysed by HPLC or mass spectrometry. It may also be important to look at SL biosynthesis intermediates such as carlactone and other carotenoid-derived compounds such as karrikin which are structurally similar to SL.

SDG8i expression in *S. stapfianus* is specific to desiccation tolerant tissue, such as leaves detached after the plant has dried below 60% RWC (Gaff and Loveys, 1993; Le *et al*., 2007). It is thought that some form of root to shoot signal is required to establish the adaptive desiccation-tolerance program but the molecular nature of this signal is unknown (Blomstedt *et al*., 2010). The finding that disturbance of the roots during the drying period prevents the establishment of desiccation tolerance, supports this theory of a root signal (Gaff *et al*., 1997). This signal may be mechanical or xylem borne, including hormones such as ABA or auxin (Schachtman and Goodger, 2008). The identification of this signal is an ongoing research question in the area of desiccation tolerance (Blomstedt *et al*., 2010; Mitra *et al.*, 2013). The substrate glycosylated by *SDG8i* may be a candidate for this signal and further studies are underway to answer this question.

It is likely that a reduced level of SL or SL-like compounds resulting from the overexpression of the *SDG8i* is driving the phenotypic changes observed in the *SDG8i* transgenics. The transgenic plants showed a large number of agronomically-desirable traits without any adverse effect on plant development and morphology. Use of the *SDG8i* gene confers protection against salt, drought and cold stress as well as a reduction in drought-induced senescence, with the additional benefit of increasing yield under non-stress condition. The use of the *SDG8i* also generates plants that have reduced stimulation of parasitic seed germination which may reduce yields in many monocot and dicot crop species world-wide. Therefore, the use of the *SDG8i* gene has considerable potential for improving productivity in crop plants. Introduction of *SDG8i* into canola and sorghum is underway to determine if the desirable traits observed in *Arabidopsis* can be reproduced into commercially important crop species.

References

Abe, H., Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Hosokawa, D., and Shinozaki, K. (1997). Role of *Arabidopsis* Myc and Myb homologs in drought- and abscisic acid-regulated gene expression. Plant Cell 9, 1859-1868.

Abe, H., Urao, T., Ito, T., Sekic, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15, 63-78.

Abeles, F., Dunn, L.J., Morgens, P., Callahans, A., Dinterman, R.E., and Schmidit, J. (1988). Induction of 33-kD and 60-kD peroxidases during ethylene-induced senescence of cucumber cotyledons. Plant Physiol. 87, 609-615.

Aguilar-Martinez, J.A., Poza-Carrion, C., and Cubas, P. (2007). Arabidopsis *BRANCHED1* acts as an integrator of branching signals within axillary buds. Plant Cell 19, 458-472.

Agusti, J., Herold, S., Schwarz, M., Sanchez, P., Ljung, K., Dun, E., Brewer, P., Beveridge, C., Sieberer, T., Sehr, E., and Greb, T. (2011). Strigolactone signaling is required for auxin-dependent stimulation of secondary growth in plants. Proceedings, National Academy of Science, USA 108, 20242-20247.

Akiyama, K., Matsuzaki, K., and Hayashi, H. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435, 824-827.

Akiyama, K., Ogasawara, S., Ito, S., and Hayashi, H. (2010). Structural requirements of strigolactones for hyphal branching in AM fungi. Plant and Cell Physiology 51, 1104-1117.

Albini, F.M., Murelli, C., Patritti, G., Rovati, M., Zienna, P., and Finzi, P.V. (1994). Low-molecular weight substances from the resurrection plant *Sporobolus stapfianus*. Phytochemistry 37, 137-142.

Alder, A., Jamil, M., Marzorati, M., Bruno, M., Vermathen, M., Bigler, P., Ghisla, S., Bouwmeester, H.J., Beyer, P., and Al-Babili, S. (2012). The path from β-carotene to carlactone, a strigolactone-like plant hormone. Science 335, 1348-1351.

Allison, S.D., Chang, B., Randolph, T.W., and Carpenter, J.F. (1999). Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. Archives of Biochemistry & Biophysics 365, 289-298.

Arite, T., Umehara, M., Ishikawa, S., Hanada, A., Maekawa, M., Yamaguchi, S., and Kyozuka, J. (2009). *d14*, a strigolactone-insensitive mutant of rice, shows an accelerated outgrowth of tillers. Plant and Cell Physiology 50, 1416-1424.

Arite, T., Iwata, H., Ohshima, K., Maekawa, M., Nakajima, M., Kojima, M., Sakakibara, H., and Kyozuka, J. (2007). *DWARF10*, an *RMS1/MAX4/DAD1* ortholog, controls lateral bud outgrowth in rice. Plant Journal 51, 1019-1029.

Arnholdt-Schmitt, B. (2004). Stress-Induced Cell Reprogramming. A Role for Global Genome Regulation? Plant Physiology 136, 2579-2586.

Aroca, R., Porcel, R., and Ruiz-Lozano, J.M. (2012). Regulation of root water uptake under abiotic stress conditions. Journal of Experimental Botany 63, 43-57.

Artus, N.N., Uemura, M., Steponkus, P.L., Gilmour, S.J., Lin, C.T., and Thomashow, M.F. (1996). Constitutive expression of the cold-regulated *Arabidopsis thaliana* cor15a gene affects both chloroplast and protoplast freezing tolerance. Proc. Natl. Acad. Sci. 93, 13404-13409.

Arvidson, I. (1951). Austrocknungs- und Dürreresistenzverhältnisse einiger Repräsentanten inländischer Planzenvereine nebst Bemerkungen über Wasserabsorption durch oberirdische Organe, Oikos. Acta Oecologica Scandinavica Suppl. 1, 1.

Ashraf, M., and Harris, P.J.C. (2004). Potential biochemical indicators of salinity tolerance in plants. Plant Sci. 166, 3-16.

Ashraf, M., and Foolad, M.R. (2007). Roles of glycin ebetaine and proline in improving plant abiotic stress tolerance. Environ. Exp. Bot. 59, 206-216.

Assmann, S.M., Snyder, J.A., and Lee, Y.R.J. (2000). ABA-deficient (aba1) and ABA-insensitive (abi1-1, abi2-1) mutants of Arabidopsis have a wild-type stomatal response to humidity. Plant, Cell and Environment 23, 387-395.

Bai, F., and DeMason, D.A. (2008). Hormone interactions and regulation of PsPK2::GUS compared with DR5::GUS and PID::GUS in *Arabidopsis thaliana*. American Journal of Botany 95, 133-145.

Bainbridge, K., Sorefan, K., Ward, S., and Leyser, O. (2005). Hormonally controlled expression of the *Arabidopsis MAX4* shoot branching regulatory gene. Plant Journal 44, 569-580.

Baker, S.S., Wilhelm, K.S., and Thomashow, M.F. (1994). The 5_-region of *Arabidopsis thaliana* cor15a has *cis*-acting elements that confer cold-, droughtand ABA-regulated gene expression Plant Mol. Biol. 24, 701-713.

Bangerth, F. (1994). Response of cytokinin concentration in the xylem exudates of bean (*Phaseolus vulgaris* L.) plants to decapitation and auxin treatment, and relationship to apical dominance. Planta 194, 439-442.

Bangerth, F., Li, C.J., and Gruber, J. (2000). Mutual interaction of auxin and cytokinins in regulating correlative dominance. Plant Growth Regul 32, 205-217.

Bartels, D., and Salamini, F. (2001). Desiccation tolerance in the resurrection plant *Craterostigma plantagineum*. A contribution to the study of drought tolerance at the molecular level. Plant Physiology 127, 1346-1353.

Bartels, D., and Salamini, F. (2001). Desiccation tolerance in the resurrection plant *Craterostigma plantagineum*. A contribution to the study of drought tolerance at the molecular level Plant Physiology 127, 1346-1353.

Bartels, D., and Sunkar, R. (2005). Drought and salt tolerance in plants. Critical Reviews in Plant Sciences 24, 23-58.

Bartels, D., Schneider, K., Terstappen, G., Piatowski, D., and Salamini, F. (1990). Molecular cloning of abscisic acid-modulated genes which are induced during desiccation of the resurrection plant *Craterostigma plantagineum*. Planta 181, 27-34.

Battaglia, M., Olvera-Carrillo, Y., Garciarrubio, A., Campos, F., Alejandra A., and Covarrubias, A. (2008). The Enigmatic LEA Proteins and Other Hydrophilins1. Plant Physiology 148, 16-24.

Benjamins, R., and Scheres, B. (2008). Auxin: the looping star in plant development. Annu Rev Plant Biol 59, 443-465.

Benková, E., Michniewicz, M.S.M., Teichmann, T., Seifertova, D., Jürgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115, 591-602.

Bennett, T., Sieberer, T., Willett, B., Booker, J., Luschnig, C., and Leyser, O. (2006). The Arabidopsis MAX pathway controls shoot branching by regulating auxin transport. Curr Opin Plant Biol 16 553-563.

Berger, S., Bell, E., and Mullet, J.E. (1996). Two methyl jasmonate-insensitive mutants show altered expression of AtVsp in response to methyl jasmonate and wounding. Plant Physiol. 111, 525-531.

Bernacchia, G., Salamini, F., and Bartels, D. (1996). Molecular characterization of the rehydration process in the resurrection plant *Craterostigma plantagineum*. Plant Physiolgy 111 1043-1050.

Besserer, A., Puech-Pagès, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., Portais, J.-C., Roux, C., Bécard, G., and Séjalon-Delmas, N. (2006). Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. PLoS Biology 4, 1239-1247.

Beveridge, C.A. (2006). Axillary bud outgrowth: sending a message. Curr Opin Plant Biol 9, 35-40.

Beveridge, C.A., and Kyozuka, J. (2010). New genes in the strigolactone-related shoot branching pathway. Current Opinion in Plant Biology 13, 34-39.

Beveridge, C.A., Symons, G.M., and Turnbull, C.G.N. (2000). Auxin inhibition of decapitation-induced branching is dependent on graft-transmissible signals regulated by genes *rms1* and *rms2*. Plant Physiology 123, 689-697.

Beveridge, C.A., Dun, E.A., and Rameau, C. (2009). Pea Has Its Tendrils in Branching Discoveries Spanning a Century from Auxin to Strigolactones. Plant Physiol 151, 985-990.

Beveridge, C.A., Symons, G.M., Murfet, I.C., Ross, J.J., and Rameau, C. (1997). The *rms1* mutant of pea has elevated indole-3-acetic acid levels and reduced root-sap zeatin riboside content but increased branching controlled by graft-transmissible signal(s). Plant Physiology 115, 1251-1258.

Bewley, J.D. (1979). Physiological aspects of desiccation tolerance. Annu Rev Plant Physiol 30, 195-238.

Bewley, J.D., and Krochko, J.E. (1982). Desiccation-tolerance. In Encyclopaedia of plant physiology.Physiological Ecology II, O.L. Lange, P.S. Nobel, C.B. Osmond, and H. Ziegler, eds (Berlin: Springer-Verlag), pp. 325-378.

Bewley, J.D., Halmer, P., Krochko, J.E., Winner, W.E.I.e., and . , pp (1978). Metabolism of a drought-tolerant and drought-sensitive moss: respiration, ATP synthesis and carbohydrate status. In Dry biological systems, J.H. Crowe and J.S. Clegg, eds (New York: Academic Press), pp. 185-203.

Bianchi, G., Gamba, A., Murelli, C., Salamini, F., and Bartels, D. (1991). Novel carbohydrate metabolism in the resurrection plant *Craterostigma plantagenium*. Plant Journal 1, 355-359.

Bishopp, A., Benková, E., and Helariutta, Y. (2011). Sending mixed messages: auxincytokinin crosstalk in roots. Curr. Opin. Plant Biol. 14, 10-16.

Blomstedt, C.K., Gianello, R.D., Hamill, J.D., Neale, A.D., and Gaff, D.F. (1998a). Drought-stimulated genes correlated with desiccation tolerance of the resurrection grass Sporobolus stapfianus. Plant Growth Regulation 24, 153-161.

Blomstedt, C.K., Gianello, R.D., Gaff, D.F., Hamill, J.D., and Neale, A.D. (1998b). Differential gene expression in desiccation-tolerant and desiccation-sensitive tissue of the resurrection grass, *Sporobolus stapfianus*. . Australian Journal of Plant Physiology 25, 937-946.

Blomstedt, C.K., Griffiths, C.A., Fredericks, D.P., Hamill, J.D., Gaff, D.F., and Neale, A.D. (2010). The resurrection plant *Sporobolus stapfianus*: An unlikely model for engineering enhanced plant biomass? Plant Growth Regulation 62, 217-232.

Blum, A. (2005). Drought resistance, water-use efficiency, and yield potential-are they compatible, dissonant, or mutually exclusive? Australian Journal of Agricultural Research 56, 1159-1168.

Bockel, C., Salamini, F., and Bartels, D. (1998). Isolation and characterization of genes expressed during early events of the dehydration process in the resurrection plant *Craterostigma plantagineum*. J. Plant Physiol. 152, 158-166.

Bohnert, H. (2000). What makes desiccation tolerable? Genome Biology 1, 1010.1011-1010.1014.

Booker, J., Chatfield, S., and Leyser, O. (2003). Auxin acts in xylem-associated or medullary cells to mediate apical dominance. Plant Cell 15, 495-507.

Booker, J., Sieberer, T., Wright, W., Williamson, L., Willett, B., Stirnberg, P., Turnbull, C., Srinivasan, M., Goddard, P., and Leyser, O. (2005). *MAX1* encodes a cytochrome P450 family member that acts downstream of *MAX3/4* to produce a carotenoid-derived branch-inhibiting hormone. Developmental Cell 8, 443-449.

Bouwmeester, H.J., Matusova, R., Zhongkui, S., and Beale, M.H. (2003). Secondary metabolite signalling in host-parasitic plant interactions. Current Opinion in Plant Biology 6, 358-364.

Bowles, D., and Lim, E.-K. (2010). Glycosyltransferases of small molecules: Their roles in plant biology. In Encyclopedia of Life Sciences (Chichester: John Wiley &Sons.

Bowles, D., Lim, E.K., Poppenberger, B., and Vaistij, F.E. (2006). Glycosyltransferases of lipophilic small molecules. Ann Rev Plant Biol 57, 567-597.

Braun, N., de Saint Germain, A., Pillot, J.-P., Boutet-Mercey, S.p., Dalmais, M., Antoniadi, I., Li, X., Maia-Grondard, A., Le Signor, C., Bouteiller, N., Luo, D., Bendahmane, A., Turnbull, C., and Rameau, C. (2012). The Pea TCP Transcription Factor PsBRC1 Acts Downstream of Strigolactones to Control Shoot Branching. Plant Physiology 158, 225-238.

Bray, E.A. (1997). Plant responses to water deficit. Trends in Plant Science 2, 48-54.

Bray, E.A. (2002). Abscisic acid regulation of gene expression during waterdeficit stress in the era of the Arabidopsis genome. Plant Cell Environ. 25, 153-161.

Bray, E.A. (2004). Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. J Exp Bot 55, 2331-2341.

Bray, E.A. (2007). Molecular and physiological responses to water-deficit stress. In Advances in molecular breeding toward drought and salt tolerant crops, M.A. Jenks, P.M. Hasegawa, and S.M. Jain, eds (New York: Springer.

Brewer, P.B., Dun, E.A., Ferguson, B.J., Rameau, C., and Beveridge, C.A. (2009). Strigolactone acts downstream of auxin to regulate bud outgrowth in pea and *Arabidopsis*. Plant Physiology 150, 482-493.

Buchanan-Wollaston, V., Page, T., Harrison, E., Breeze, E., and Lim, P.O., et al. . (2005). Comparative transcriptome analysis reveals significant differences in gene expression and signaling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant Journal 42, 567-585.

Buitink, J., and Leprince, O. (2004). Glass formation in plant anhydrobiotes: survival in the dry state. Cryobiology 48, 215-228.

Campillo, E., and Lewis, L. (1992). Occurrence of 9.5 cellulase and other hydrolases in the flower reproductive organ undergoing major cell wall disruption. Plant Physiol 99, 1015-1020.

Cao, W.H., Liu, J., He, X.J., Mu, R.L., and Zhou, H.L., et al. (2007). Modulation of ethylene responses affects plant salt-stress responses. Plant Physiol 143, 707-719.

Cazzonelli, C.I. (2011). Carotenoids in nature: insights from plants and beyond. Functional Plant Biology 38, 833-847.

Chakrabortee, S., Tripathi, R., Watson, M., Schierle, G.S., Kurniawan, D.P., Kaminski, C.F., Wise, M.J., and Tunnacliffe, A. (2012). Intrinsically disordered proteins as molecular shields. Mol. Biosyst. 8, 210-219.

Chandra Babu, R., Zhang, J., Blum, A., David Ho, T.-H., Wu, R., and Nguyen, H.T. (2004). HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection. Plant Science 166, 855-862.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENEINSENSITIVE3 and related proteins. Cell 89, 1133-1144.

Chatfield, S., Stirnberg, P., Forde, B.G., and Leyser, O. (2000). The hormonal regulation of axillary bud growth in *Arabidopsis*. Plant Journal 24, 159-169.

Chaves, M.M., Maroco, J.P., and Pereira, J.S. (2003). Understanding plant responses to drought - from genes to the whole plant. Functional Plant Biology 30, 239-264.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.H., Hong, X.H., Agarwal, M., and Zhu, J.K. (2003). ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. Genes & Development 17, 1043-1054.

Chiou, T.-J., and Lin, S.-I. (2011). Signaling network in sensing phosphate availability in plants. Annu Rev Plant Biol 62, 185-206.

Chong, J., Baltz, R., Schmitt, C., Beffa, R., Fritig, B., and Saindrenan, P. (2002). Downregulation of a pathogen-responsive tobacco UDP-Glc:phenylpropanoid glucosyltransferase reduces scopoletin glucoside accumulation, enhances oxidative stress, and weakens virus resistance. Plant Cell 14, 1093-1107.

Cline, M., Yoders, M., Desai, D., Harrington, C., and Carlson, W. (2006). Hormonal control of second flushing in Douglas-fir shoots. Tree Physiol 26, 1369-1375.

Close, T.J. (1996). Dehydrins - emergence of a biochemical role of a family of plant dehydration proteins. Physiologia Plantarum 97, 795-803.

Close, T.J. (1997). Dehydrins: A commonalty in the response of plants to dehydration and low temperature. Physiol. Plant. 100, 291-296.

Colcombet, J., and Hirt, H. (2009). Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes. Biochem. J. 413, 217-226.

Collett, C.E., Harberd, N.P., and Leyser, O. (2000). Hormonal interactions in the control of Arabidopsis hypocotyl elongation. Plant Physiol. 124, 553-562.

Collett, H., Butowt, R., Smith, J., Farrant, J., and Illing, N. (2003). Photosynthetic genes are differentially transcribed during the dehydration-rehydration cycle in the resurrection plant, *Xerophyta humilis*. Journal of Experimental Botany 54 2593-2595.

Cook, C.E., Whichard, L.P., Turner, B., Wall, M.E., and Egley, G.H. (1966). Germination of Witchweed (*Striga lutea* Lour.): Isolation and Properties of a Potent Stimulant. Science 154, 1189-1190.

Cook, C.E., Whichard, L.P., Wall, M.E., Egley, G.H., Coggon, P., Luhan, P.A., and McPhail, A.T. (1972). Germination stimulants. 2. The structure of strigol-a potent seed germination stimulant for witchweed (*Striga lutea* Lour.). Journal of the American Chemical Society 94, 6198-6199.

Cooper, K., and Farrant, J.M. (2002). Recovery of the resurrection plant *Craterostigma wilmsii* from desiccation: Protection versus repair. J. Exp. Bot. 53, 1805-1813.

Cramer, G.R., and Läuchli, A. (1986). Ion activities in solution in relation to Na^+ - Ca^{2+} interactions at the plasmalemma. J. Exp. Bot. 37, 321-330.

Cramer, G.R., Läuchli, A., and Polito, V.S. (1985). Displacement of Ca^{2+} from the plasmalemma of root cells. A primary response to salt stress? . Plant Physiol. 79, 297-211.

Crawford, S., Shinohara, S., Sieberer, T., Williamson, L., George, G., Hepworth, J., Müller, D., Domagalska, M.A., and Leyser, O. (2010). Strigolactones enhance competition between shoot branchesby dampening auxin transport. Development Development 137, 2905-2913

Creelman, R.A., Tierney, M.L., and Mullet, J.E. (1992). Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc. Natl. Acad. Sci. USA 89, 4938-4941.

Creelman, R.A., Mason, H.S., Bensen, R.J., Boyer, J.S., and Mullet, J.E. (1990). Water deficit and abscisic acid cause differential inhibition of shoot versus root growth in soybean seedlings. Analysis of growth, sugar accumulation, and gene expression. Plant Physiol. 92, 205-214.

Crowe, J.H., Oliver, A.E., Hoekstra, F.A., and Crowe, L.M. (1997). Stabilization of dry membranes by mixtures of hydroxyethyl starch and glucose: The role of vitrification. Cryobiology 35, 20-30.

Cuming, A.C. (1999). LEA proteins. In Seed Proteins, P.R. Shewry and R. Casey, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 753-780.

Curtis, M.D., and Grossniklaus, U. (2003). A Gateway Cloning Vector Set for High-Throughput Functional Analysis of Genes in Planta. Plant Physiology 133, 462-469.

Dace, H., Sherwin, H.W., Illing, N., and Farrant, J.M. (1998). Use of metabolic inhibitors to elucidate the mechanism of recovery from desiccation stress in the resurrection plant Xerophyta humilis. Plant Growth Reg. 24, 171-177.

Dai, Y., Wang, H., Li, B., Huang, J., Liu, X., Zhou, Y., Mou, Z., and Li, J. (2006). Increased expression of MAP KINASE KINASE7 causes deficiency in polar auxin transport and leads to plant architectural abnormality in *Arabidopsis*. Plant Cell 18, 308-320.

DallaVechia, F., El Asmar, T., Calamassi, R., Rascio, N., and Vazana, C. (1998). Morphological and ultrastructural aspects of dehydration and rehydration in leaves of *Sporobolus stapfianus* Plant Growth Regulation 24, 219-228.

Davies, W.J., and Zhang, J. (1991). Root signals and the regulation of growth and development of plants in drying soils. Annu Rev Plant Biol Physiol Plant Mol Biol 42, 55-76.

De Smet, I. (2012). Lateral root initiation: one step at a time. New Phytol. 193, 867-873.

Dean, J.V., and Delaney, S.P. (2008). Metabolism of salicylic acid in wild-type, *ugt74f1* and *ugt74f2* glucosyltransferase mutants of *Arabidopsis thaliana*. Physiol Plant 132, 417-425.

Delaux, P.M., Xie, X., Timme, R.E., Puech-Pages, V., Dunand, C., Lecompte, E., Delwiche, C.F., Yoneyama, K., Becard, G., and Sejalon-Delmas, N. (2012). Origin of strigolactones in the green lineage. New Phytol. 195, 857-871.

Deprost, D., Yao, L., Sormani, R., Moreau, M., Leterreux, G., Nicolai, M., Bedu, M., Robaglia, C., and Meyer, C. (2007). The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation. EMBO Reports 8, 864-870.

Di Blasi, S., Puliga, S., Losi, L., and Vazzana, C. (1998). *S. stapfianus* and *E. curvula* cv. Consol in vivo photosynthesis, PSII activity and ABA content during dehydration Plant Growth Regulation 25, 97-104.

Dinakar, C., and Bartels, D. (2013). Desiccation tolerance in resurrection plants: new insights from transcriptome, proteome and metabolome analysis. Frontiers in Plant Science 4, 1-14.

Divi, U., Rahman, T., and Krishna, P. (2010). Brassinosteroid-mediated stress tolerance in Arabidopsis shows interactions with abscisic acid, ethylene and salicylic acid pathways. BMC Plant Biology 10, 151.

Doebley, J., Stec, A., and Hubbard, L. (1997). The evolution of apical dominance in maize. Nature 386, 485-488.

Doherty, C.J., Van Buskirk, H.A., Myers, S.J., and Thomashow, M.F. (2009). Roles for *Arabidopsis* CAMTA transcription factors in cold-regulated gene expression and freezing tolerance Plant Cell 21, 972-984.

Domagalska, M.A., and Leyser, O. (2011). Signal integration in the control of shoot branching. Nature Reviews Molecular Cell Biology 12, 211-221.

Dong, C., Agarwal, M., Zhang, Y., Xie, Q., and Zhu, J.K. (2006). The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. Proc. Natl Acad. Sci. USA 103, 8281-8286.

Drummond, R.S.M., Martínez-Sánchez, N.M., Janssen, B.J., Templeton, K.R., Simons, J.L., Quinn, B.D., Karunairetnam, S., and Snowden, K.C. (2009). *Petunia hybrida CAROTENOID CLEAVAGE DIOXYGENASE7* is involved in the production of negative and positive branching signals in petunia. Plant Physiology 151, 1867-1877.

Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought, high-salt and cold-responsive gene expression. Plant J. 33, 751-763.

Dubrovsky, J.G., Soukup, A., Napsucialy-Mendivil, S., Jeknic´, Z., and Ivanchenko, M.G. (2009). The lateral root initiation index: an integrative measure of primordium formation. Annals of Botany 103, 807-817.

Dubrovsky, J.G., Sauer, M., Napsucialy-Mendivil, S., Ivanchenko, M.G., Friml, J., Shishkova, S., Celenza, J., and Benková, E. (2008). Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. In Proceedings of the National Academy of Sciences of the USA, pp. 8790-8794.

Dun, E.A., Ferguson, B.J., and Beveridge, C.A. (2006). Apical dominance and shoot branching. Divergent opinions or divergent mechanisms? Plant Physiology 142, 812-819.

Dun, E.A., Brewer, P.B., and Beveridge, C.A. (2009). Strigolactones: discovery of the elusive shoot branching hormone. Trends in Plant Science 14, 364-372.

Dun, E.A., de Saint Germain, A., Rameau, C., and Beveridge, C.A. (2012). Antagonistic action of strigolactone and cytokinin in bud outgrowth control. Plant Physiol. 158, 487-498.

Durbak, A., Yao, H., and McSteen, P. (2012). Hormone signaling in plant development. Current Opinion in Plant Biology 15, 92-96.

Dure, L., Crouch, M., Harada, J., Ho, D.T.-h., Mundy, J., and Quatrano, R. (1989). Common amino acid sequence domains among the LEA proteins of higher plants. Plant Molecular Biology 12, 475-486.

Evers, D., Hemmer, K., and Hausman, J.-F. (1998). Salt stress induced biometric and physiological changes in *Solanum tuberosum* L. cv. *Bintje* grown in vitro. ACTA Physiol. Plant. 20, 3-7.

Fang, J., Chai, C., Qian, Q., Li, C., Tang, J., Sun, L., Huang, Z., Guo, X., Sun, C., Liu, M., Zhang, Y., Lu, Q., Wang, Y., Lu, C., Han, B., Chen, F., Cheng, Z., and Chu, C. (2008). Mutations of genes in synthesis of the carotenoid precursors of ABA lead to pre-harvest sprouting and photo-oxidation in rice. Plant J. 54, 177-189.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., and Basra, S.M.A. (2009). Plant drought stress: effects, mechanisms and management. Agron. Sustain. Dev. 29, 185- 212.

Farrant, J.M. (2000). A comparison of mechanisms of desiccation-tolerance among three angiosperm resurrection plant species Plant Ecol. 151, 29-39.

Farrant, J.M., Brandt, W., and Lindsey, G.C. (2007). An overview of mechanisms of desiccation tolerance in selected angiosperm resurrection plants. Plant Stress 1, 72-84.

Farrant, J.M., Lehner, A., Cooper, K., and Wiswedel, S. (2009). Desiccation tolerance in the vegetative tissues of the fern *Mohria caffrorum* is seasonally regulated. Plant J. 57, 65-79.

Farrant, J.M., Vander, Willigen, C., Loffell, D.A., Bartsch, S., and Whittaker, A. (2003). An investigation into the role of light during desiccation of three angiosperm resurrection plants. Plant Cell Environ. 26, 1275-1286.

Feys, B., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell 6, 751-759.

Figueras, M.J., Pujal, A., Saleh, R., Save, M., Pages, M., and Goday, A. (2004). Maize RAB17 overexpression in *Arabidopsis* plants promotes osmotic stress tolerance. Ann Appl Biol 144, 251-257.

Finlayson, S.A. (2007). Arabidopsis TEOSINTE BRANCHED1-LIKE 1 regulates axillary bud outgrowth and is homologous to monocot TEOSINTE BRANCHED1. Plant Cell Physiol. 48, 667-677.

Foo, E., and Davies , N.W. (2011). Strigolactones promote nodulation in pea. Planta 234, 1073-1081.

Foo, E., Buillier, E., Goussot, M., Foucher, F., Rameau, C., and Beveridge, C.A. (2005). The branching gene *RAMOSUS1* mediates interactions among two novel signals 464 and auxin in pea. Plant Cell 17, 464-474.

Foo, E., Morris, S.E., Parmenter, K., Young, N., Wang, H.T., Jones, A., Rameau, C., Turnbull, C.G.N., and Beveridge, C.A. (2007). Feedback regulation of xylem cytokinin content is conserved in pea and *arabidopsis*. Plant Physiology 143, 1418-1428.

Fowler, S., and Thomashow, M.F. (2002). *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14, 1675-1690.

Fujioka, S., and Yokota, T. (2003). Biosynthesis and metabolism of brassinosteroids. Ann Rev Plant Biol 54, 137-164.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr. Opin. Plant Biol. 9, 436-442.

Fujita, M., Fujita, Y., Maruyama, K., Seki, M., Hiratsu, K., Ohme-Takagi, M., Tran, L.S.P., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2004). A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. The Plant Journal 39, 863-876.

Fukao, T., Yeung, E., and Bailey-Serres, J. (2011). The Submergence Tolerance Regulator SUB1A Mediates Crosstalk between Submergence and Drought Tolerance in Rice. The Plant Cell 23, 412-427.

Gaff, D., and Ellis, R. (1974). Southern African grasses with foliage that revives after dehydration. Bothalia 11, 305-308.

Gaff, D., Bartels, D., and Gaff, J.L. (1997). Changes in gene expression during drying in a desiccation tolerant grass *Sporobolus stapfianus* and a desiccation sensitive grass *Sporobolus pyramidalis*. Australian J. Plant Physiology 24, 617-622.

Gaff, D.F. (1971). Desiccation tolerant flowering plants in Southern Africa. Science 174, 1033-1034.

Gaff, D.F. (1977). Desiccation tolerant flowering plants in southern Africa. Oecologia 21, 95-109.

Gaff, D.F. (1980). Protoplasmic tolerance of extreme water stress. In Adaptation of Plants to Water and High Temperature Stress, N.C. Turner and P.J. Kramer, eds (Wiley-Interscience:New York), pp. 207-231.

Gaff, D.F. (1989). Responses of desiccation tolerant resurrection plants to water stress. In Structural and Functional Responses to Environmental Stresses, K.H. Kreeb, H. Richter, and T.M. Hinckley, eds (The Hague: SPB Academic Publishing), pp. 255-268.

Gaff, D.F., and Churchill, D.M. (1976). *Borya nitida* Labill-an Australian species in the Liliaceae with desiccation-tolerant leaves. Aust J Bot 24, 209-224.

Gaff, D.F., and Loveys, B.R. (1993). Abscisic acid levels in drying plants of a resurrection grass. Trans Malaysian Society. Plant Physiology 3, 286-287.

Gaff, D.F., and Oliver, M. (2013). The evolution of desiccation tolerance in angiosperm plants: a rare yet common phenomenon. Functional Plant Biology 40, 315-328.

Gaff, D.F., Blomstedt, C.K., Neale, A.D., Le, T.N., Hamill, J.D., and Ghasempour, H.R. (2009). *Sporobolus stapfianus*, a model desiccation-tolerant grass. Functional Plant Biology 36, 589-599.

Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. Science 282, 2226-2230.

Gan, S., and Amasino, R.M. (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270, 1986-1988.

Gao, Z., Qian, Q., Liu, X., Yan, M., Feng, Q., Dong, G., Liu, J., and Han, B. (2009). *Dwarf 88*, a novel putative esterase gene affecting architecture of rice plant. Plant Mol Biol 71, 265-276.

Garay-Arroyo, A., Colmenero-Flores, J.M., Garciarrubio, A., and Covarrubias, A.A. (2000). Highly hydrophylic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. J. Biol. Chem. 275, 5668-5674.

Garg, A.K., Kim, J.K., Owens, T.G., Ranwala, A.P., Choi, Y.D., Kochian, L.V., and Wu, R.J. (2002). Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. PNAS 99, 15898-15903.

Gazzarrini, S., and McCourt, P. (2003). Cross-talk in plant hormone signalling: What Arabidopsis mutants are telling us. Ann. Bot. 91, 605-612.

Gechev, T., Dinakar, C., Benina, M., Toneva, V., and Bartels, D. (2012). Molecular mechanisms of desiccation tolerance in resurrection plants. Cell. Mol. Life Sci. 69, 3175-3186.

Gechev, T.S., Benina, M., Obata, T., Tohge, T., Sujeeth, N., Minkov, I., Hille, J., Temanni, M.R., Marriott, A.S., Bergström, E., Thomas-Oates, J., Antonio, C., Mueller-Roeber, B., Schippers, J.H., Fernie, A.R., and Toneva, V. (2013). Molecular mechanisms of desiccation tolerance in the resurrection glacial relic *Haberlea rhodopensis*. Cell. Mol. Life Sci. 70, 689-709.

Gepstein, S., and Thimann, K.V. (1980). Changes in the abscisic acid content of oat leaves during senescence. Proc. Natl. Acad. Sci. USA 77, 2050-2053.

Ghasempour, H.R., Anderson, E.M., and Gaff, D.F. (2001). Effects of growth substances on the protoplasmic drought tolerance of leaf cells of the resurrection grass *Sporobolus stapfianus*. Australian Journal of Plant Physiology 28, 1115-1120.

Ghasempour, H.R., Anderson, E.M., Gianello, R.D., and Gaff, D.F. (1998a). Growth inhibitor effects on protoplasmic drought tolerance and protein synthesis in leaf cells of the resurrection grass, *Sporobolus stapfianus*. Plant Growth Regulation 24, 179-183.

Ghasempour, H.R., Gaff, D.F., Williams, R.P.W., and Gianello, R.D. (1998b). Contents of sugars in leaves of drying desiccation tolerant flowering plants, particularly grasses. Plant Growth Regulation 24, 185-191.

Gilmour, S.J., Fowler, S.G., and Thomashow, M.F. (2004). Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Mol. Biol. 54, 767-781.

Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D., and Thomashow, M.F. (2000). Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol. 124, 1854-1865.

Glauser, G., Boccard, J., Rudaz, S., and Wolfender, J.L. (2010). Mass Spectrometry-based Metabolomics Oriented by Correlation Analysis for Wound-induced Molecule Discovery: Identification of a Novel Jasmonate Glucoside. Phytochem Anal 21, 95-101

Goldwasser, Y., Yoneyama, K., Xie, X., and Yoneyama, K. (2008). Production of strigolactones by *Arabidopsis thaliana* responsible for *Orobanche aegyptiaca* seed germination. Plant Growth Regulation 55, 21-28.

Gomez-Roldan, V., Fermas, S., Brewer, P.B., Puech-Pages, V., Dun, E.A., Pillot, J.P., Letisse, F., Matusova, R., Danoun, S., Portais, J.-C., Bouwmeester, H., Bécard, G., Beveridge, C.A., Rameau, C., and Rochange, S.F. (2008). Strigolactone inhibition of shoot branching. Nature Biotechnology 455, 189-194.

Gorham, J., Wyn Jones, R.G., and McDonnel, E. (1985). Some mechanisms of salt tolerance in crop plants. Plant Soil 89, 15-40.

Goyal, K., Walton, L.J., Browne, J.A., Burnell, A.M., and Tunnacliffe, A. (2005). Molecular anhydrobiology: identifying molecules implicated in invertebrate anhydrobiosis. Integrative and Comparative Biology 45, 702-703.

Grant, M.R., and Jones, J.D.G. (2009). Hormone (dis)harmony molds plant health and disease. Science 324, 750-752.

Grelet, J., Benamar, A., Teyssier, E., Avelange-Macherel, M.H., Grunward, D., and Macherel, D. (2005). Identification of pea seed mitochondria of a late embryogenesis abundant protein able to protect enzymes from drying. Plant Physiol. 137, 157-167.

Grennan, A.K. (2008). A transcriptomic footprint of reactive oxygen species. Plant Physiology 148, 1187-1188.

Guiltinan, M.J., Marcotte, W.R., and Quatrano, R.S. (1990). A plant leucine zipper protein that recognizes an abscisic acid response element. Science 250, 267-271.

Gut, H., Ruts, C., Matile, P., and Thomas, H. (1987). Leaf senescence in a non-yellowing mutant of *Festuca pratensis*: Degradation of carotenoids. Physiol. Plant. 70, 659-663.

Haake, V., Cook, D., Riechmann, J.L., Pineda, O., Thomashow, M.F., and Zhang, J.Z. (2002). Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. Plant Physiology 130, 639-648.

Hagen, G., and Guilfoyle, T. (2002). Auxin-responsive gene expression:genes, promoters and regulatory factors. Plant Mol Biol 49, 373-385

Halle, F. (1999). Ecology of reiteration in tropical trees. In The evolution of plant architecture M.H. Kurmann and A.R. Hemsley, eds (Kew: Royal Botanic Gardens), pp. 93-107.

Hamiaux, C., Drummond, R.S.M., Bart, J., Janssen, B.J., Ledger, S.E., Cooney, J.M., Newcomb, R.D., and Snowden, K.C. (2012). *DAD2* Is an a/b Hydrolase likely to Be Involved in the Perception of the Plant Branching Hormone, Strigolactone. Current Biology 22, 1-5.

Hara, M., Fujinaga, M., and Kuboi, T. (2004). Radical scavenging activity and oxidative modification of citrus dehydrin. Plant Physiology and Biochemistry 42.

Hare, P.D., Cress, W.A., and Van Staden, J. (1998). Dissecting the role of osmolyte accumulation during stress. Plant, Cell and Environment 21, 535-553.

Harrison, M.J. (2005). Signaling in the arbuscular mycorrhizal symbiosis. Annual Review of Microbiology 59, 19-42.

Hasewaga, P.M., Bressan, R.A., Zhu, J.-K., and Bohnert, H.J. (2000). Plant cellular and molecular response to high salinity. Annual Review of Plant Physiology and Plant Molecular Biology 51, 263-499.

Hattori, T., Vasil, V., Rosenkras, L., Hannah, L.C., McCarty, D.R., and Vasil, I.K. (1992). The viviparous-1 gene and abscisic acid activate the C1 regulatory gene for anthocyanin biosynthesis during seed maturation in maize. Genes & Development 6, 609-618.

Hayward, A., Stirnberg, P., Beveridge, C.A., and Leyser, O. (2009). Interactions between auxin and strigolactone in shoot branching control. Plant Physiology 151, 400-412.

He, Y., Fukushige, H., Hildebrand, D.F., and Gan, S. (2002). Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. Plant Physiol. 128, 876-884.

Himanen, K., Boucheron, E., Vanneste, S., Almeida Engler, J., Inze´, D., and Beeckman, T. (2002). Auxin-mediated cell cycle activation during early lateral root initiation. The Plant Cell 14, 2339-2351.

Hirayama, T., and Shinozaki, K. (2010). Research on plant abiotic stress responses in the post-genome era: past, present and future. The Plant Journal 61, 1041-1052.

Hirt, H. (1997). Multiple roles of MAP kinases in plant signal transduction. Trends in Plant Science 2, 11-15.

Hjellstrom, M., Olsson, A.S.B., Engstrom, O., and Soderman, E.M. (2003). Constitutive expression of the water deficit-inducible homeobox gene ATHB7 in transgenic *Arabidopsis* causes a suppression of stem elongation growth. Plant Cell and Environment 26, 1127-1136.

Hobo, T., Kowyama, Y., and Hattori, T. (1999). A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. PNAS 96, 15348-15353.

Hou, B., Lim, E.K., Higgins, G.S., and Bowles, D.J. (2004). N-glucosylation of cytokinins by glycosyltransferases of *Arabidopsis thaliana*. J Biol Chem 279, 47822-47832.

Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q., and Xiong, L. (2006). Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc Natl Acad Sci USA 35, 12987-12992.

Hu, Z., Yan, H., Yang, J., Yamaguchi, S., Maekawa, M., Takamure, I., Tsutsumi, N., Kyozuka, J., and Nakazono, M. (2010). Strigolactones negatively regulate mesocotyl elongation in rice durinjg germination and growth in darkness. Plant Cell Physiology 51, 1136-1142.

Huang, S., Raman, A.S., Ream, J.E., Fujiwara, H., Cerny, R.E., and Brown, S.M. (1998). Overexpression of 20-Oxidase Confers a Gibberellin-Overproduction Phenotype in Arabidopsis. Plant Physiology 118, 773-781.

Hughes, J., and Hughes, M.A. (1994). Multiple secondary plant product UDP-glucose glucosyltransferase genes expressed in cassava. DNA Sequence 5, 41-49.

Hull, G., Gaubier, P., Delseny, M., and Casse-Delbart, F. (1993). Abscisic acid inducible genes and their regulations in higher plants. Current Top Mol Genet (Life Sci Adv) 1, 289-305.

Hundertmark, M., and Hincha, D.K. (2008). LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*. BMC Genomics 9, 118.

Hung, K.T., and Kao, C.H. (2004). Hydrogen peroxide is necessary for abscisic acid-induced senescence of rice leaves. J. Plant Physiol. 161, 1347-1357.

Illing, N., Denby, K.J., Collett, H., Shen, A., and Farrant, J.M. (2005). The signature of seeds in resurrection plants: a molecular and physiological comparison of desiccation tolerance in seeds and vegetative tissues. Integr. Comp. Biol. 45, 771-787.

Ingle, R., Schmidt, U., Farrant, J., Thomson, J., and Mundree, S. (2007). Proteomic analysis of leaf proteins during dehydration of the resurrection plant *Xerophyta viscosa*. Plant Cell Environ. 30, 435-446.

Ishikawa, S., Maekawa, M., Arite, T., Onishi, K., Takamure, I., and Kyozuka, J. (2005). Suppression of tiller bud activity in tillering dwarf mutants of rice. Plant and Cell Physiology 46, 79-86.

Ishitani, M., Xion, L.M., Stevenson, B., and Zhu, J.K. (1997). Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis - interactions and convergence of abscisic acid-dependent and abscisic acid-independent pathways. The Plant Cell 9, 1935-1949.

Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006). Functional analysis of rice DREB1/CBF-type transcription factors involved in coldresponsive gene expression in transgenic rice. Plant Cell Physiol. 47, 141-153.

Itturriaga, G., Cushman, M., and Cushman, J. (2006). An EST catalogue from the resurrection plant *Selaginella lepidophylla* reveals abiotic stress adaptive genes. Plant Sci 170, 1173-1184.

Iturriaga, G., Leyns, L., Villegas, A., Gharaibeh, R., Salamini, F., and Bartels, D. (1996). A family of novel myb-related genes from the resurrection plant *Craterostigma plantagineum* are specifically expressed in callus and roots in response to ABA or desiccation. Plant Mol Biol 32, 707-716.

Ivanchenko, M.G., Muday, G.K., and Dubrovsky, J.G. (2008). Ethylene-auxin interactions regulate lateral root initiation and emergence in *Arabidopsis thaliana*. The Plant Journal 55, 335-347.

Iwasaki, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1995). Identification of a cis-regulatory region of a gene in *Arabidopsis thaliana* whose induction by dehydration is mediated by abscisic acid and requires protein synthesis. Molecular & General Genetics 247, 391-398.

Izawa, T., Foster, R., and Chua, N.-H. (1993). Plant bZIP protein DNA binding specificity. Journal of Molecular Biology 230, 1131-1144.

Jackson, R.G., Kowalczyk, M., Li, Y., Higgins, G., Ross, J., Sandberg, G., and Bowles, D.J. (2002). Over-expression of an *Arabidopsis* gene encoding a glucosyltransferase of indole-3-acetic acid: phenotypic characterisation of transgenic lines. Plant Journal 32, 573-583.

Jackson, R.G., Lim, E.-K., Li, Y., Kowalczyk, M., Sandberg, G., Hoggett, J., Ashford, D.A., and Bowles, D.J. (2001). Identification and biochemical characterization of an Arabidopsis indole-3-acetic acid glucosyltransferase. Journal of Biological Chemistry 276, 4350-4356.

Jaglo, K.R., Kleff, S., Amundsen, K.L., Zhang, X., Haake, V., Zhang, J.Z., Deits, T., and Thomashow, M.F. (2001). Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiology 127, 910-917.

Jaillais, Y., and Chory, J. (2010). Unraveling the paradoxes of plant hormone signaling integration. Nat Struct Mol Biol 17, 642-645.

Jain, N., Kulkarni, M.G., and Van Staden, J. (2006). Butenolide, isolated from smoke, can overcome the detrimental effects of extreme temperatures during tomato seed germination. Plant Growth Regul. 49, 263-267.

Jamil, M., Charnikova, T., Cardoso, C., Jamil, T., Ueno, K., Verstappen, F., Asami, T., and Bouwmeester, H. (2011). Quantification of the relationship between strigolactones and *Striga hermonthica* infection in rice under varying levels of nitrogen and phosphorus. Weed Research 51, 373-385.

Jefferson, R.A. (1987). Assay for chimeric genes in plants: The GUS fusion system Plant Molecular Biology Reporter 5, 387-405.

Jensen, P.J., Hangarter, R.P., and Estelle, M. (1998). Auxin transport is required for hypocotyl elongation in light-grown but not dark-grown Arabidopsis. Plant Physiology 116, 455-462.

Jiang, G., Wang, Z., Shang, H., Yang, W., Hu, Z., Phillips, J., and Deng, X. (2007). Proteome analysis of leaves from the resurrection plant *Boea hygrometrica* in response to dehydration and rehydration. Planta 225, 1405-1420.

Johnson, X., Brcich, T., Dun, E.A., Goussot, M., Haurogne, K., Beveridge, C.A., and Rameau, C. (2006). Branching genes are conserved across species. Genes controlling a novel signal in pea are coregulated by other long-distance signals. Plant Physiology 142, 1014-1026.

Jones, P., and Vogt, T. (2001). Glycosyltransferases in secondary plant metabolism:tranquilizers and stimulant controllers. Planta 213, 164-174.

Jordi, W., Schapendonk, A., Davelaar, E., Stoopen, G.M., Pot, C.S., De Visser, R., Van Rhijn, J.A., Gan, S., and Amasino, R.M. (2000). Increased cytokinin levels in transgenic P-SAG12-IPT tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning. Plant Cell Environment 23, 279-289.

Kanaoka, M.M., Pillitteri, L.J., Fujii, H., Yoshida, Y., Bogenschutz, N.L., Takabayashi, J., Zhu, J.-K., and Toriiad, K.U. (2008). SCREAM/ICE1 and SCREAM2 specify three cell-state transitional steps leading to *Arabidopsis* stomatal differentiation. Plant Cell 20, 1775-1785.

Kapulnik, Y., Delaux, P.-M., Resnik, N., Mayzlish-Gati, E., Wininger, S., Bhattacharya, C., Séjalon-Delmas, N., Combier, J.-P., Bécard, G., Belausov, E., Beeckman, T., Dor, E., Hershenhorn, J., and Koltai, H. (2011). Strigolactones affect lateral root formation and root hair elongation in Arabidopsis Planta 233, 209-216.

Kasprzewska, A. (2003). Plant chitinases-regulation and function. Cell Mol Biol Lett 8, 809-824.

Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1999). Improving plant drought, salt and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature Biotechnology 17, 287-291.

Kempa, S., Krasensk, J., Dal Santo, S., Kopka, J., and Jonak, C. (2008). A central role of abscisic acid in stress-regulated carbohydrate metabolism. PLoS One 3, 3935.

Khodary, S.E.A. (2004). Effect of salicylic acid on the growth, photosynthesis and carbohydrate metabolism in salt stressed maize plants. Int. J. Agri. Biol. 6, 5-8.

Kim, J.-A., Agrawal, G.K., Rakwal, R., Han, K.-S., Kim, K.-N., Yun, C.-H., Heu, S., Park, S.-Y., Lee, Y.-H., and Jwa, N.-S. (2003). Molecular cloning and mRNA expression analysis of a novel rice (*Oryza sativa* L.) MAPK kinase kinase, OsEDR1, an ortholog of *Arabidopsis* AtEDR1, reveal its role in defense/stress signalling pathways and development. Biochemical and Biophysical Research Communications 300, 868-876.

Kim, S.Y., Chung, H.J., and Thomas, T.L. (1997). Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using a modified yeast one-hybrid system. Plant Journal 11, 1237-1251.

Kiyosue, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994). Cloning of cDNAs for genes that are early-responsive to dehydration stress (ERDs) in *Arabidopsis thaliana* L. - identification of three ERDs as HSP cognate genes. Plant Molecular Biology 25, 791-798.

Kneera, R., and Zenk, M.H. (1992). Phytochelatins protect plant enzymes from heavy metal poisoning. Phytochem. 31, 2663-2667.

Knight, H., Veale, E.L., Warren, G.J., and Knight, M.R. (1999). The sfr6 mutation in *Arabidopsis* suppresses low-temperature induction of genes dependent on the CRT/DRE sequence motif. Plant Cell 11, 875-886.

Kochian, L.V., and Lucas, W.J. (1988). Potassium transport in roots. Adv. Bot. Res. 15, 93-178.

Kohlen, W., Charnikhova, T., Liu, Q., Bours, R., Domagalska, M.A., Beguerie, S., Verstappen, F., Leyser, O., Boumeester, H.J., and Ruyter-spira, C. (2011). Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in non-AM host *Arabidopsis thaliana*. Plant Physiology 155, 974-987.

Koltai, H. (2011). Strigolactones are regulators of root development. The New phytologist 190, 545-549.

Koltai, H., Dor, E., Hershenhorn, J., Joel, D.M., Weininger, S., Lekalla, S., Shealtiel, H., Bhattacharya, C., Eliahu, E., Resnick, N., Barg, R., and Kapulnik, Y. (2010). Strigolactones' effect on root growth and root-hair elongation may be mediated by auxin-efflux carriers. Journal of Plant Growth Regulation 29, 129-136.

Kong, X., Sun, L., Zhou, Y., Zhang, M., and Liu, Y., et al. . (2011). ZmMKK4 regulates osmotic stress through reactive oxygen species scavenging in transgenic tobacco. Plant Cell Rep 30, 2097-2104.

Kramer, P.J. (1983). Water Relations of Plants. (California: Academic Press Inc.).

Kramer, P.J., and Boyer, J.S. (1995). Water Relations of Plants and Soils San Diego. Academic Press.

Kuang, J.K., Gaff, D.F., Gianello, R.D., Blomstedt, C.K., Neale, A.D., and Hamill, J.D. (1995). Changes in *in vivo* protein complements in drying leaves of the desiccation-tolerant grass *Sporobolus Stapfianus* and the desiccation sensitive grass *Sporobolus pyramidlis*. Australian Journal of Plant Physiology 22, 1027-1034.

Lally, D., Ingmire, P., Tong, H.-Y., and He, Z.-H. (2001). Antisense Expression of a Cell Wall-Associated Protein Kinase, WAK4, Inhibits Cell Elongation and Alters Morphology. Plant Cell 13, 1317-1332.

Lam, E., and Chua, N.-H. (1991). Tetramer of a 21-base pair synthetic element confers seed expression and transcriptional enhancement in response to water stress and abscisic acid. Journal of Biological Chemistry 266, 17131-17135.

Langlois-Meurinne, M., Gachon, C.M.M., and Saindrenan, P. (2005). Pathogen-responsive expression of glycosyltransferase genes *UGT73B3* and *UGT73B5* is necessary for resistance to *Pseudomonas syringae* pv tomato in *Arabidopsis*. Plant Physiology 139, 1890-1901.

Laplaze, L., Benkova´, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M.B., Offringa, R., Graham, N., Doumas, P., Friml, J., Bogusz, D., Beeckman, T., and M., B. (2007). Cytokinins act directly on lateral root founder cells to inhibit root initiation. The Plant Cell 19, 3889-3900.

Le, T.N. (2004). Genetics of desiccation tolerant plants in the resurrection plant *Sporobolus stapfianus*. PhD thesis (Melbourne, Australia: Monash University).

Le, T.N., Blomstedt, C.K., Kuang, J., Tenlen, J., Gaff, D.F., Hamill, J.D., and Neale, A.D. (2007). Desiccation-tolerance specific gene expression in leaf tissue of the resurrection plant *Sporobolus stapfianus* Functional Plant Biology 34, 589-600.

Ledger, S.E., Janssen, B.J., Karunairetnam, S., Wang, T., and Snowden, K.C. (2010). Modified CAROTENOID CLEAVAGE DIOXYGENASE8 expression correlates with altered branching in kiwifruit (*Actinidia chinensis*). New Phytology 188, 803-813.

Lee, H.J., Xiong, L.M., Gong, Z.Z., Ishitani, M., Stevenson, B., and Zhu, J.K. (2001). The *Arabidopsis HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. Genes & Development 15, 912-924.

Lee , K.H., Piao, H.L., Kim, H.Y., Choi, S.M., Jiang, F., Hartung, W., Hwang, I., Kwak, J.M., Lee, I.J., and Hwang, I. (2006). Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. Cell 126, 1109 - 1120.

León, P., and Sheen, J. (2003). Sugar and hormone connections. Trends Plant Sci. 8, 110-116.

Leung, J., and Giraudat, J. (1998). Abscisic acid signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 25, 199-221.

Leung, J., Merlot, S., and Giraudat, J. (1997). The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell 9, 759-771.

Levitt, J. (1972). Responses of Plants to Environmental Stresses. (New York: Academic Press).

Levitt, J. (1980). Responses of Plants to Environmental Stresses. In Chilling, Freezing and High Temperature Stresses (New York: Academic Press.

Levitt, J., Sullivan, C.Y., and Krull, E. (1960). Some problems in drought resistance. Bulletin of Research Council of Israel 8D, 177-179.

Leyser, H.M., Lincoln, C.A., Timpte, C., Lammer, D., Turne, r.J., and Estelle, M. (1993). *Arabidopsis* auxinresistance gene *AXR1* encodes a protein related to ubiquitin-activating enzyme E1. Nature 364, 161-164.

Leyser, O. (2006). Dynamic integration of auxin transport and signalling. Curr Biol 16, R424-R433 doi:410.1016/j.cub.2006.1005.1014.

Leyser, O. (2009). The control of shoot branching: an example of plant information processing. Plant, Cell & Environment 32, 694-703.

Li, C.J., Guevera, E., Herrera, J., and Bangerth, F. (1995). Effect of apex excision and replacement by 1-naphthylacetic acid on cytokinin concentration and apical dominance in pea plants. Physiol. Plant. 94, 465-469.

Li, F., Asami, T., Wu, X., Tsang, E.W.T., and Cutler, A.J. (2007). A putative hydroxysteroid dehydrogenase involved in regulating plant growth and development. Plant Physiology 145 87-97.

Li, Y., Baldauf, S., Lim, E.K., and Bowles, D.J. (2001). Phylogenetic analysis of the UDP-glycosyltransferase multigene family of *Arabidopsis thaliana*. Journal of Biological Chemistry 276, 4338-4343.

Li, Z., Peng, J., Wen, X., and Guo, H. (2012). Gene Network Analysis and Functional Studies of Senescence-associated Genes Reveal Novel Regulators of Arabidopsis Leaf Senescence. Journal of Integrative Plant Biology 54, 526-539.

Liang, C.Y., Xi, Y., Shu, J., Li, J., Yang, J.L., Che, K.P., Jin, D.M., Liu, X.L., Weng, M.L., He, Y.K., and Wang, B. (2004). Construction of a BAC library of *Physcomitrella patens* and isolation of a LEA gene. Plant Science 167, 491-498.

Liang, J., Zhao, L., Challis, R., and Leyser, O. (2010). Strigolactone regulation of shoot branching in chrysanthemum (*Dendranthema grandiflorum*). Journal of Experimental Botany 61, 3069-3078.

Licausi, F., Ohme-Takagi, M., and Perata, P. (2013). APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. New Phytologist 199, 639-649.

Lichtenthaler, H. (1987). Chlorophylls and carotenoids: pigments of photosynthetic membranes. . Methods Enzymology 148, 350-382.

Lim, C.E., Ahn, J.H., and Lim, J. (2006). Molecular genetic analysis of tandemly located glycosyltransferase genes, *UGT73B1*, *UGT73B2*, and *UGT73B3*, in *Arabidopsis thaliana*. J. Plant Biol. 49, 309-314.

Lim, C.E., Choi, J.N., Kim, I.A., Lee, S.A., Hwang, Y.S., Lee, C.H., and Lim, J. (2008). Improved resistance to oxidative stress by a loss-of-function mutation in the *Arabidopsis UGT71C1* gene. Mol Cells 25, 368-375.

Lim, E.K., and Bowles, D.J. (2004). A class of plant glycosyltransferases involved in cellular homeostasis. EMBO J 23, 2915-2922.

Lim, E.K., Doucet, C.J., Hou, B., Jackson, R.G., Abrams, S.R., and Bowles, D.J. (2005). Resolution of (+)-abscisic acid using an *Arabidopsis* glycosyltransferase. Tetrahedron-Asym 16, 143-147.

Lim, E.K., Doucet, C.J., Li, Y., Elias, L., Worrall, D., Spencer, S.P., Ross, J., and Bowles, D.J. (2002). The activity of *Arabidopsis* glycosyltransferases toward salicylic acid, 4-hydroxybenzoic acid, and other benzoates. J. Biol. Chem. 277, 586-592.

Lim, E.K., Baldauf, S., Elias, L., Worrall, D., Spencer, S.P., Jackson, R.G., Taguchi, G., Ross, J., and Bowles, D.J. (2003a). Evolution of substrate recognition across a multigene family of glycosyltransferases in *Arabidopsis*. Glycobiology 13, 139-145.

Lim, P.O., Woo, H.R., and Nam, H.G. (2003b). Molecular genetics of leaf senescence in *Arabidopsis*. Trends Plant Sci. 8, 272-278.

Lim, P.O., Kim, H.J., and Nam, H.G. (2007). Leaf senescence. Annu Rev Plant Biol 58, 115-136.

Lin, H., Wang, R., Qian, Q., Yan, M., Meng, X., Fu, Z., Yan, C., Jiang, B., Su, Z., Li, J., and Wang, Y. (2009). DWARF27, an iron-containing protein required for the biosynthesis of strigolactones, regulates rice tiller bud outgrowth. Plant Cell 21, 1512-1525.

Lincoln, C., Britton, J.H., and Estelle, M. (1990). Growth and development of the *axr1* mutants of *Arabidopsis*. Plant Cell 2, 1071-1080.

Liu, H., Wang, X., Wang, D., Zou, Z., and Liang, Z. (2011). Effect of drought stress on growth and accumulation of active constituents in *Salvia miltiorrhyza* Bunge. Ind. Crops. Prod. 33, 84-88.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA-binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10, 1391-1406.

Liu, W., Wu, C., Fu, Y., Hu, G., Si, H., Zhu, L., Luan, W., He, Z., and Sun, Z. (2009). Identification and characterization of *HTD2*: a novel gene negatively regulating tiller bud outgrowth in rice. Planta 230, 649-658.

López-Ráez, J.A., Charnikhova, T., Gómez-Roldán, V., Matusova, R., Kohlen, W., De Vos, R., Verstappen, F., Puech-Pages, V., Bécard, G., and Mulder, P. (2008). Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. New Phytologist 178, 863-874.

López-Ráez, J.A., Kohlen, W., Charnikhova, T., Mulder, P., Undas, A.K., Sergeant, M.J., Verstappen, F., Bugg, T.D.H., Thompson, A.J., Ruyter-Spira, C., and Bouwmeester, H. (2010). Does abscisic acid affect strigolactone biosynthesis. New Phytologist 187, 343-354.

Lopez, C.G., Banowetz, G.M., Peterson, C.J., and Kronstad, W.E. (2003). Dehydrin expression and drought tolerance in seven wheat cultivars. Crop Science 43, 577.

Luan, S. (2009). The CBL-CIPK network in plant calcium signaling. Trends Plant Sci. 14, 37-42.

Lucas, S., Durmaz, E., Akpınar, B.A., and Budak, H. (2011). The drought response displayed by a DRE-binding protein from *Triticum dicoccoides*. Plant Physiol. Biochem. 49, 346-351.

Mackenzie, P., Owens, I., Burchell, B., Bock, K., Bairoch, A., Belanger, A., Fournel-Gigleux, S., Green, M., Hum, D., Iyanagi, T., Lancet, D., Louisot, P., Magdalou, J., Chowdhury, J., Ritter, J., Schachter, H., Tephly, T., Tipton, K., and Nebert, D. (1997). The UDP glycosyltransferase gene superfamily: recommended nomenclature update based on evolutionary divergence. Pharmacogenetics 7, 255-269.

Mader, J.C., Turnbull, C.G.N., and Emery, R.J.N. (2003). Transport and metabolism of xylem cytokinins during lateral bud release in decapitated chickpea (*Cicer arietinum*) seedlings. Physiol Plant 117, 118-129.

Mahajan, S., Pandey, G.K., and Tuteja, N. (2008). Calcium- and salt-stress signaling in plants: shedding light on SOS pathway. Arch. Biochem. Biophys. 471, 146-158.

Marchant, A., Bhalerao, R., Casimiro, I., Eklof, J., Casero, P.J., Bennett, M., and Sandberg, G. (2002). AUX1 promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the *Arabidopsis* seedling. Plant Cell 14, 589-597.

Marcotte, W.R., Russell, S.H., and Quatrano, R.S. (1989). Abscisic acid-responsive sequences from the Em gene of wheat. Plant Cell 1, 969-976.

Marillonnet, S., Thoeringer, C., Kandzia, R., Klimyuk, V., and Gleba, Y. (2005). Systemic *Agrobacteriun tumifaciens*-mediated transfection of viral replicons for efficient transient expression in plants Nature Biotechnology 23, 718-723.

Martin, R.C., Mok, M.C., and Mok, D.W. (1999). A gene encoding the cytokinin enzyme zeatin O-xylosyltransferase of *Phaseolus vulgaris*. Plant Physiol 120, 553-558.

Martin, R.C., Mok, M.C., Habben, J.E., and Mok, D.W. (2001). A maize cytokinin gene encoding an O-glucosyltransferase specific to cis-zeatin. Proc Natl Acad Sci USA 98, 5922-5926.

Martinez-Trujillo, M., Limones-Briones, V., Cabrera-Ponce, J.L.C., and Herrera-Estrella, L. (2004). Improving transformation efficiency of *Arabidopsis thaliana* by modifying the floral dip method. Plant Molec~dar Biology Reporter 22, 63-70.

Masclaux-Daubresse, C., and Chardon, F. (2011). Exploring nitrogen remobilization for seed filling using natural variation in *Arabidopsis thaliana*. Journal of Experimental Botany 62, 2131-2142.

Mashiguchi, K., Sasaki, E., Shimada, Y., Nagae, M., Ueno, K., Nakano, T., Yoneyama, K., Suzuki, Y., Asami, T., . (2009). Feedback-Regulation of Strigolactone Biosynthetic Genes and Strigolactone-Regulated Genes in Arabidopsis. Bioscience, Biotechnology, and Biochemistry 73, 2460-2465.

Matros, A., and Mock, H.P. (2004). Ectopic expression of a UDP-glucose:phenylpropanoid glucosyltransferase leads to increased resistance of transgenic tobacco plants against infection with Potato Virus Y. Plant Cell Physiol. 45, 1185-1193.

Matusova, R., Rani, K., Verstappen, F., Franssen, M.C.R., Beale, M.H., and Bouwmeester, H.J. (2005). The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche spp.* are derived from the carotenoid pathway. Plant Physiology 139, 920-934.

Mazel, A., and Levine, A. (2002). Induction of glucosyltransferase transcription and activity during superoxide-dependent cell death in *Arabidopsis* plants. Plant Physiol. Biochem. 40, 133-140.

McKeon, T., and Yang, S.F. (1995). Biosynthesis and metabolism of ethylene. In Plant Hormones and Their Role in Plant Growth and Development, P.J. Davies, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 118-139.

McSteen, P. (2009). Hormonal regulation of branching in grasses. Plant Physiology 149, 46-55.

McSteen, P., and Leyser, O. (2005). Shoot branching. Annual Review of Plant Biology 56, 353-374.

Medford, J.I., Horgan, R., El-Sawi, Z., and Klee, H.J. (1989). Alterations of endogenous cytokinins in transgenic plants using a chimeric *isopentenyl transferase* gene. Plant Cell 1, 403-413.

Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D., and Koornneef, M. (1998). *Arabidopsis thaliana*: a model plant for genome analysis. Science 282, 662-682.

Meissner, D., Albert, A., Bottcher, C., Strack, D., and Milkowski, C. (2008). The role of UDP-glucose : hydroxycinnamate glucosyltransferases in phenylpropanoid metabolism and the response to UV-B radiation in *Arabidopsis thaliana*. Planta 228, 663-674

Meloni, D.A., Rosalía GulottaII, M., MartínezII, C.A., and Oliva, M.A. (2004). The effects of salt stress on growth, nitrate reduction and proline and glycinebetaine accumulation in *Prosopis alba*. Braz. J. Plant Physiol. 16, 39-46.

Miguel, L.C., Longnecker, N.E., Ma, Q., Osborne, L., and Atkins, C.A. (1998). Branch development in *Lupinus angustifolius* L. I. Not all branches have the same potential growth rate. J Exp Bot 49, 547-553.

Minakuchi, K., Kameoka, H., Yasuno, N., Umehara, M., Luo, L., Kobayashi, K., Hanada, A., Ueno, K., Asami, T., Yamaguchi, S., and Kyozuka, J. (2010). *FINE CULM1 (FC1)* works downstream of strigolactones to inhibit the outgrowth of axillary buds in rice. Plant Cell Physiol. 51, 1127-1135.

Mito, T., Seki, M., Shinozaki, K., Ohme-Takagi, M., and Matsui, K. (2011). Generation of chimeric repressors that confer salt tolerance in Arabidopsis and rice. Plant Biotechnology Journal 9, 736-746.

Mitra, J., Xu, G., Wang, B., Li, M., and Deng, X. (2013). Understanding desiccation tolerance using the resurrection plant *Boea hygrometrica* as a model system. Front. Plant Sci. 12, 1-10.

Miura, K., Jin, J.B., and Hasegawa, P.M. (2007). Sumoylation, a post-translational regulatory process in plants. Curr. Opin. Plant Biol. 10, 495-502.

Mizoi, J., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2011). AP2/ERF family transcription factors in plant abiotic stress responses. Biochim Biophys Acta 1819, 86-96.

Mizoi, J., Ohori, T., and Yamaguchi-Shinozaki, K. (2013). GmDREB2A;2, a Canonical DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN2-Type Transcription Factor in Soybean, Is Posttranslationally Regulated and Mediates Dehydration-Responsive Element-Dependent Gene Expression. Plant Physiology 161, 346-361.

Mok, D.W.S., and Mok, M.C. (2001). Cytokinin metabolism and action. Annual Review of Plant Physiol Plant Mol Biol 52, 89-118.

Morris, K., Mackerness, S.A., Page, T., John. C.F, and Murphy, A.M., et al. (2000). Salicylic acid has a role in regulating gene expression during senescence. Plant J. 23, 677-685.

Moubayidin, L., Di Mambro, R., and Sabatini, S. (2009). Cytokinin-auxin crosstalk. Trends Plant Sci. 14, 557-562.

Muller, D., and Leyser, O. (2011). Auxin, cytokinin and the control of shoot branching. Annals of Botany 107, 1203-1212.

Mundy, J., Yamaguchi-Shinozaki, K., and Chua, N.-H. (1990). Nuclear proteins bind conserved elements in the abscisic acid responsive promoter of a rice rab gene. Proc. Natl. Acad. Sci. USA 87, 1406-1410.

Munns, R. (2002). Comparative physiology of salt and water stress. Plant Cell Environment 25, 239-250.

Munns, R., and Tester, M. (2008). Mechanisms of salinity tolerance. Annual Review of Plant Biology 59, 651-681.

Murelli, C., Adamo, V., Finzi, P.V., Albini, F.M., Bochicchio, A., and Picco, A.M. (1996). Sugar biotransformations by fungi on leaves of the resurrection plant *Sporobolus stapfianus*. Phytochemistry 43, 741-745.

Nakano, T., Suzuki, K., Fujimura, T., and Shinshi, H. (2006). Genome-wide analysis of the *ERF* gene family in *arabidopsis* and rice. Plant Physiology 140, 411-432.

Nakashima, K., Kiyosue, T., Yamaguchishinozaki, K., and Shinozaki, K. (1997). A nuclear gene, erd1 encoding a chloroplast-targeted clp protease regulatory subunit homolog is not only induced by water stress but also developmentally up-regulated during senescence in Arabidopsis thaliana. Plant Journal 12, 851-861.

Nam, H.G. (1997). Molecular genetic analysis of leaf senescence. Curr. Opin. Biotechnol. 8, 200-207.

Narusaka, Y., Nakashima, K., Shinwari, Z.K., Sakuma, Y., Furihata, T., Abe, H., Narusaka, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). Interaction between two *cis*-acting elements, ABRE and DRE, in ABA-dependent expression of *Arabidopsis rd29A* gene in response to dehydration and high-salinity stresses. Plant J. 34, 137-148.

Neale, A., Waleithner, J.A., Lund, M., Bonnett, H.T., Kelly, A., Meekswagner, D.R., Dennis, E.S., and Peacock, W. (1990). Chitinase, ß-1, 3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell 2, 673-684.

Neale, A.D., Blomstedt, C.K., Bronson, P., Le, T.N., Guthridge, K., Evans, J., Gaff, D.F., and Hamill, J.D. (2000). The isolation of genes from the resurrection grass Sporobolus stapfianus which are induced during severe drought stress. Plant, Cell & Environment 23, 265-277.

Nelson, D.C., Scaffidi, A., Dun, E., Waters, M.T., Flematti, G.R., Dixon, K.W., Beveridge, C.A., Ghisalberti, E.L., and Smith, S.M. (2011). F-box protein MAX2 has dual roles in karrikin and strigolactone signaling in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences of the United States of America 108, 8897-8902.

Nelson, D.E., Repetti, P.P., Adams, T.R., Creelman, R.A., Wu, J., Warner, D.C., Anstrom, D.C., Bensen, R.J., Castiglioni, P.P., and Donnarummo, M.G., et al. (2007). Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. Proc. Natl Acad. Sci. USA 104, 16450-16455.

Nooden, L.D., Guiamet, J.J., and Jobn, I. (1997). Senescence mechanisms. Physiologia Plantarum 101, 746-753.

Noodén, L.D. (1988). The phenomena of senescence and aging. In Senescence and Aging in Plants, L.D. Noodén and A.C. Leopold, eds (San Diego: Academic), pp. 1-50.

O'Mahony, P., and Oliver, M. (1999). Characterization of a desiccation responsive small GTP-binding protein (Rab2) from the desiccation-tolerant grass *Sporobolus stapfianus*. Plant Mol Biol 39, 809-821.

Oeda, K., Salinas, J., and Chua, N.-H. (1991). A tobacco bZIP transcription activator (TAF-1) binds to a G-box like motif conserved in plant genes. EMBO J 10, 1793-1802.

Oh, S.A., Park, J.-H., Lee, G.I., Paek, K.H., Park, S.K., and Nam, H.G. (1997). Identification of three genetic loci controlling leaf senescence in *Arabidopsis thaliana*. Plant J. 12, 527-535.

Oh, S.J., Song, S.I., Kim, Y.S., Jang, H.J., Kim, S.Y., Kim, M.J., Kim, Y.K., Nahm, B.H., and Kim, J.K. (2005). Arabidopsis CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. Plant Physiol. 138, 341-351.

Okushima, Y., Mitina, I., Quach, H.L., and Theologis, A. (2005). AUXIN RESPONSE FACTOR 2 (ARF2): a pleiotropic developmental regulator. Plant J. 43, 29-46.

Oliver, M.J. (1996). Desiccation-tolerance in vegetative plant cells. Physiol. Plant. 97, 779-787.

Oliver, M.J., and Bewley, J.D. (1997). Desiccation-tolerance in plant tissues. A mechanistic overview. Hortic.Rev. 18, 171-214.

Oliver, M.J., Wood, A.J., and O' Mahony, P. (1998). To dryness and beyond - preparation for the dried state and rehydration in vegetative desiccation-tolerant plants. Plant Growth Regulation 24, 193-201.

Oliver, M.J., Tuba, Z., and Mishler, B.D. (2000). The evolution of vegetative desiccation tolerance in land plants. Plant Ecol. 151, 85-100.

Oliver, M.J., Velten, J., and Mishler, B.D. (2005). Desiccation Tolerance in Bryophytes: A Reflection of the Primitive Strategy for Plant Survival in Dehydrating Habitats? Integr. Comp. Biol. 45, 788-799.

Oliver, M.J., Hudgeons, J., Dowd, S.E., and Payton, P.R. (2009). A combined subtractive suppression hybridization and expression profiling strategy to identify novel desiccation response transcripts from *Tortula ruralis* gametophytes. Physiol Plant 136, 437-460.

Oliver, M.J., Dowd, S.E., Zaragoza, J., Mauget, S.A., and Payton, P.R. (2004). The rehydration transcriptome of the desiccation-tolerant bryophyte *Tortula ruralis*: transcript classification and analysis. BMC Genomics 5, 89.

Oliver, M.J., Jain, R., Balbuena, T.S., Agrawal, G., Gasulla, F., and Thelen, J.J. (2010). Proteome analysis of leaves of the desiccation-tolerant grass, *Sporobolus stapfianus*, in response to dehydration. Phytochemistry 72, 1273-1284.

Oliver, M.J., Jain, R., Balbuena, T.S., Agrawal, G., Gasulla, F., and Thelen, J.J. (2011a). Proteome analysis of leaves of the desiccation-tolerant grass, *Sporobolus stapfianus*, in response to dehydration. Phytochemistry 72, 1273-1284.

Oliver, M.J., Guo, L., Alexander, D.C., Ryals, J.A., Wone, B.W.M., and Cushman, J.C. (2011b). A sister group contrast using untargeted global metabolomic analysis delineates the biochemical regulation underlying desiccation tolerance in *Sporobolus stapfianus*. The Plant Cell 23, 1231-1248.

Owen, P.C. (1952). The relation of germination of wheat to water potential. Journal of Experimental Botany 3, 188-203.

Panigrahi, B.M., and Audus, L.J. (1966). Apical dominance in *Vicia faba*. Annals of Botany 30, 457 -473.

Park, J.-E., Park, J.-Y., Kim, Y.-S., Staswick, P.E., Jeon, J., Yun, J., Kim, S.-Y., Kim, J., Lee, Y.-H., and Park, C.-M. (2007). GH3-mediated Auxin Homeostasis Links Growth Regulation with Stress Adaptation Response in Arabidopsis. Journal of Biological Chemistry 282, 10036-10046.

Park, Y., Tominaga, R., Sugiyama, J., Furuta, Y., Tanimoto, E., Samejima, M., Sakai, F., and Hayashi, T. (2003). Enhancement of growth by expression of poplar cellulase in *Arabidopsis thaliana*. The Plant J. 33, 1099-1106.

Parker, C., and Riches, C.R. (1993). Parasitic Weeds of the World: Biology and Control. (Wallingford, UK: CAB International).

Parry, A.D., and Horgan, R. (1992). Abscisic acid biosynthesis in roots. 1. The identification of potential abscisic acid precursors, and other carotenoids. Planta 187, 185-191.

Patil, V., and Widholm, J. (1997). Possible correlation between increased vigour and chitinase activity expression in tobacco. Journal of Experimental Botany 48, 1943-1950.

Pearce, R.S. (1999). Molecular analysis of acclimation to cold. Plant Growth Regulation 29, 47-76.

Peleg, Z., Reguera, M., Tumimbang, E., Walia, H., and Blumwald, E. (2011). Cytokinin-mediated source/sink modifications improve drought tolerance and increases grain yield in rice under water-stress. Plant Biotechnology Journal 9, 747-758.

Pellegrineschi, A., Reynolds, M., Pachew, M., Breto, R.M., Almeraya, R., Yamaguchi-Shinozaki, K., and Hoisington, D. (2004). Stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1 gene delay water stress symptoms under greenhouse conditions. Genome 47, 493-500.

Péret, B., Clément, M., Nussaume, L., and Desnos, T. (2011). Root developmental adaptation to phosphate starvation: better safe than sorry. Trends Plant Sci 16, 442-450.

Pérez-Torres, C.-A., López-Bucio, J., Cruz-Ramírez, A., Ibarra-Laclette, E., Dharmasiri, S., Estelle, M., and Herrera-Estrella, L. (2008). Phosphate Availability Alters Lateral Root Development in Arabidopsis by Modulating Auxin Sensitivity via a Mechanism Involving the TIR1 Auxin Receptor. The Plant Cell Online 20, 3258-3272.

Pessarakli, M., Tucker, T.C., and Nakabayashi, K. (1991). Growth response of barley and wheat to salt stress. J. Plant Nutr. 14, 331-340.

Petersen, J., Eriksson, S.K., Harryson, P., Pierog1, S., Colby, T., Bartels, D., and Röhrig, H. (2012). The lysine-rich motif of intrinsically disordered stress protein CDeT11-24 from *Craterostigma plantagineum* is responsible for phosphatidic acid binding and protection of enzymes from damaging effects caused by desiccation. Journal of Experimental Botany 63, 4919-4929.

Phillips, J., and Bartels, D. (2000). Gene expression during dehydration in the resurrection plant *Craterostigma plantagineum*. In Plant Tolerance to Abiotic Stresses in Agriculture: Role in Genetic Engineering, J.H. Cherry, J.D. Locy, and A. Rychtes, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 195-199.

Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S., and Van Wees, S.C.M. (2009). Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. 5, 308-316.

Poppenberger, B., Berthiller, F., Lucyshyn, D., Sieberer, T., Schuhmacher, R., Krska, R., Kuchler, K., Glossl, J., Luschnig, C., and Adam, G. (2003). Detoxification of the Fusarium mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. J Biol Chem 278, 47905-47914.

Poppenberger, B., Fujioka, S., Soeno, K., George, G.L., Vaistij, F.E., Hiranuma, S., Seto, H., Takatsuto, S., Adam, G., Yoshida, S., and Bowles, D. (2005). The UGT73C5 of *Arabidopsis thaliana* glucosylates brassinosteroids. Proc Natl Acad Sci USA 102, 15253-15258.

Porcel, R., Aroca, R., and Ruiz-Lozano, J.M. (2012). Salinity stress alleviation using arbuscular mycorrhizal fungi. A review. Agron Sust Dev 32, 181-200.

Potters, G., Pasternak, T.P., Guisez, Y., and Jansen, M.A. (2009). Different stresses, similar morphogenic responses: integrating a plethora of pathways. Plant, Cell and Environment 32, 158-169.

Potters, G., Pasternak, T.P., Guisez, Y., Palme, K.J., and Jansen, M.A.K. (2007). Stress-induced morphogenic responses: growing out of trouble? Trends in Plant Science 12, 98-105.

Press, M.C., Scholes, J.D., and Riches, C.R. (2001). Current status and future prospects for management of parasitic weeds (*Striga* and *Orobanche*). In The World's Worst Weeds, C.R. Riches, ed (Brighton, UK: British Crop Protection Council), pp. 71-90.

Priest, D.M., Ambrose, S.J., and Vaistij, F.E., et al. (2006). Use of the glucosyltransferase UGT71B6 to disturb abscisic acid homeostasis in *Arabidopsis thaliana*. Plant Journal 46, 492-502.

Priest, D.M., Jackson, R.G., Ashford, D.A., Abrams, S.R., and Bowles, D.J. (2005). The use of abscisic acid analogues to analyse the substrate selectivity of UGT71B6, a UDP-glycosyltransferase of *Arabidopsis thaliana*. FEBS Lett 579, 4454-4458.

Proctor, M. (2001). Patterns of desiccation tolerance and recovery in bryophytes. Plant Growth Regulation 35, 147-156.

Proust, H., Hoffmann, B., Xie, X., Yoneyama, K., Schaefer, D.G., Nogue, F., and Rameau, C. (2011). Strigolactones regulate protonema branching and act as a quorum sensing-like signal in the moss *Physcomitrella patens*. Development 138, 1531-1539.

Prusinkiewicz, P., Crawford, S., Smith, R.S., Ljung, K., Bennett, T., Ongaro, V., and Leyser, O. (2009). Control of bud activation by an auxin transport switch. Proceedings of the National Academy of Sciences 106, 17431-17436.

Pruzsinsky, S. (1960). Über Trocken- und Feuchtluftresistenz des Pollen. Osterreichische Akademie der Wissenschaften: Mathematik.-natur Klass 1, 43-100.

Qin, F., Li, J., Zhang, G.-Y., Zhao, J., Chen, S.-Y., and Liu, Q. (2003). Isolation and structural analysis of DRE-binding transcription factor from maize (*Zea mays* L.). Acta Botanica Sinica 45, 331-339.

Quartacci, M.F., Forli, M., Rascio, N., Dallavecchia, F., Bochicchio, A., and Navariizzo, F. (1997). Desiccation-tolerant *Sporobolus stapfianus*: lipid composition and cellular ultrastructure during dehydration and rehydration. Journal of Experimental Botany 48, 1269-1279.

Rani, K., Zwanenburg, B., Sugimoto, Y., Yoneyama, K., and Bouwmeester, H.J. (2008). Biosynthetic considerations could assist the structure elucidation of host plant produced rhizosphere signalling compounds (strigolactones) for arbuscular mycorrhizal fungi and parasitic plants. Plant Physiol Biochem 46, 617-626.

Rascio, N., and Rocca, N.L. (2005). Resurrection Plants: The Puzzle of Surviving Extreme Vegetative Desiccation. Critical Reviews in Plant Sciences 24, 209-225.

Ratnakumar, S., and Tunnacliffe, A. (2006). Intracellular trehalose is neither necessary nor sufficient for desiccation tolerance in yeast. FEMS Yeast Res. 6, 903-913.

Reyes, J.L., Compos, F., Wei, H., Arora, R., Yang, Y., Karlson, D.T., and Cavarrubias, A.A. (2008). Functional dissection of hydrophillins during in vitro freeze protection. Plant Cell Environ. 31, 1781-1790.

Reynolds, T., and Bewley, J. (1993). Characterisation of protein synthetic changes in a desiccation tolerant fern Polypodium virginianum: comparison of the effects of drying, rehydration and abscisic acid. Journal of Experimental Botany 44, 921-928.

Richmond, A.E., and Lang, A. (1957). Effect of kinetin on protein content and survival of detached Xanthium leaves. Science 125, 650-651.

Riemann , M., Müller , A., Korte , A., Furuya , M., Weiler , E.W., and Nick , P. (2003). Impaired induction of the jasmonate pathway in the rice mutant *hebiba*. Plant Physiol. 133, 1820 - 1830.

Rivard, D., Girard, C., Anguenot, R., Vézina, L.-P., Trépanier, S., and Michaud, D. (2007). MsCYS1, a developmentally-regulated cystatin from alfalfa. Plant Physiol. Biochem. 45, 508-514.

Robaglia, C., Menand, B., Lei, Y., Sormani, R., Nicolai, M., Gery, C., Teoule, E., Deprost, D., and Meyer, C. (2004). Plant growth: the translational connection. Biochem Soc Trans 32, 581-584.

Roberts, J.K., DeSimone, N.A., Lingle, W.L., and Dure, L.I. (1993). Cellular concentrations and uniformity of cell-type accumulation of two Lea proteins in cotton embryos. Plant Cell 5, 769-780.

Rock, C.D., and Sun, X. (2005). Crosstalk between ABA and auxin signaling pathways in roots of *Arabidopsis thaliana* (L.) Heynh. Planta 222, 98-106.

Rodriguez, M.C., Petersen, M., and Mundy, J. (2010). Mitogen-activated plants. Annu Rev Plant Biol 61, 621-649.

Romano, C.P., Robson, P.R., Smith, H., Estelle, M., and Klee, H. (1995). Transgene-mediated auxin overproduction in Arabidopsis: hypocotyl elongation phenotype and interactions with the hy6-1 hypocotyl elongation and axr1 auxin-resistant mutants. Plant Mol. Biol. 27, 1071-1083.

Ross, J., Li, Y., Lim, E.-K., and Bowles, D.J. (2001). Higher plant glycosyltransferases. Genome Biology 2, 3004.3001-3004.3006.

Ruyter-Spira, C., Kohlen, W., Charnikhova, T., van Zeijl, A., van Bezouwen, L., de Ruijter, N., Cardoso, C., Lopez-Raez, J.A., Matusova, R., Bours, R., Verstappen, F., and Bouwmeester, H. (2011). Physiological Effects of the Synthetic Strigolactone Analog GR24 on Root System Architecture in Arabidopsis: Another Belowground Role for Strigolactones? Plant Physiology 155, 721-734.

Saab, I.N., Sharp, R.E., Pritchard, J., and Voetberg, G.S. (1990). Increased endogenous abscisic acid maintains primary root growth and inhibits shoot growth of maize seedlings at low water potentials. Plant Physiol. 93, 1329-1336.

Sachs, T., and Thimann, K. (1967). The role of auxins and cytokinins in the release of buds from dominance. Am. J. Bot. 54, 136-144.

Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2002). DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem. Biophys. Res. Commun. 290, 998-1009.

Sambrook, J., Fritsch, E.F., and Maniatus, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Santner, A., and Estelle, M. (2009). Recent advances and emerging trends in plant hormone signalling. Nature Biotechnology 459, 1071-1078.

Schachtman, D.P., and Goodger, J.Q.D. (2008). Chemical root to shoot signaling under drought Trends in Plant Science 13, 281-287.

Schnepf, E. (1961). Über Veränderungen der plasmatischen Feinstrukturen während des Welkens. Planta (Berlin) 57, 156-175.

Schwab, K.B., Schreiber, U., and Heber, U. (1989). Correction of Ba 87130664. Response of Photosynthesis and Respiration of Resurrection Plants to Desiccation and Rehydration. Correction of Volume Number and Issue Number from Vol. 95. Iss. 3. Planta (Heidelberg) 177, 217-227.

Schwartz , S.H., and Zeevaart , J.A.D. (2004). Abscisic acid biosynthesis and metabolism. In Plant Hormones, Biosynthesis, Signal Transduction, Action!, P.J. Davies ed (Dordrecht: Kluwer), pp. 137 - 155.

Schwartz, S.H., Qin, X., and Zeevaart, J.A.D. (2003). Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes and enzymes. Plant Physiology 131, 1591-1601.

Scott, P. (2000). Resurrection plants and the secrets of eternal leaf. Ann. Bot. 85, 159-166.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., and Shinozaki, K. (2001). Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell 13, 61-72.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., and Shinozaki, K. (2002). Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold, and high salinity stresses using a full-length cDNA microarray. Plant Journal 31, 279-292.

Seo, Y.J., Park, J.B., Cho, Y.J., Jung, C., Seo, H.S., Park, S.K., Nahm, B.H., and Song, J.T. (2010). Overexpression of the ethylene-responsive factor gene BrERF4 from *Brassica rapa* increases tolerance to salt and drought in Arabidopsis plants. Mol. Cells 30, 271-277.

Sgherri, C.L.M., Loggini, B., Puliga, S., and Navari-Izzo, F. (1994). Antioxidant system in *Sporobolus stapfianus* - changes in response to desiccation and rehydration. Phytochemistry 35, 561-565.

Shani, Z., Dekel, M., Tsabary, G., Goren, R., and Shoseyov, O. (2004). Growth enhancement of transgenic plants by overexpression of *Arabidopsis thaliana* endo1, 4-b-glucanase cel1. Plant Mol Biol 14, 321-330.

Sharoni, A.M., Nuruzzaman, M., Satoh, K., Shimizu, T., Kondoh, H., Sasaya, T., Choi, I.R., Omura, T., and Kikuchi, S. (2011). Gene structures, classification and expression models of the AP2/EREBP transcription factor family in rice. Plant Cell Physiol. 52, 344-360.

Shen, H., Luong, P., and Huq, E. (2007). The F-box protein MAX2 functions as a positive regulator of photomorphogenesis in Arabidopsis. Plant Physiol 145, 1471-1483.

Shen, H., Zhu, L., Bu, Q.Y., and Huq, E. (2012). MAX2 affects multiple hormones to promote photomorphogenesis. Mol Plant. 5, 750-762.

Shen, Q., and Ho, D.T.-H. (1995). Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel cis-acting element. Plant Cell 7, 295-307.

Shen, Q., Zhang, P., and Ho, D.T.-H. (1996). Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. Plant Cell 8, 1107-1119.

Sherwin, H.W., and Farrant, J.M. (1998). Protection mechanisms against excess light in the resurrection plants Craterostigma wilmsii and Xerophyta viscosa. Plant Growth Regulation 24, 203-210.

Shinozaki, K., and Yamaguchi-Shinozaki, K. (1997). Gene expression and signal transduction in water-stress response. Plant Physiology 115, 327-334.

Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signalling pathways. Current Opinion in Plant Biology 3, 217-223.

Shinozaki, K., and Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. J. Exp. Bot. 58, 221-227.

Shinozaki, K., Yamaguchi-Shinozaki, K., and Seki, M. (2003). Regulatory network of gene expression in the drought and cold stress responses. Current Opinion in Plant Biology 6, 410-417.

Shou, H., Bordallo, P., and Wang, K. (2004). Expression of the Nicotiana protein kinase (NPK1) enhanced drought tolerance in transgenic maize. Journal of Experimental Botany 55, 1013.

Shukla, R.K., Raha, S., Tripathi, V., and Chattopadhyay, D. (2006). Expression of CAP2, an APETALA2 Family Transcription Factor from Chickpea, Enhances Growth and Tolernace to Dehydration and Salt Stress in Transgenic Tobacco. Plant Physiology 142, 113-123.

Simpson, S.D., Nakashima, K., Narusaka, Y., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). Two different novel cis-acting elements of erd1, a clpA homologous Arabidopsis gene, function in induction by dehydration stress and dark-induced senescence. Plant J. 33, 259-270.

Sivamani, E., Bahieldin, A., Wraith, J.M., Al-Niemi, T., Dyer, W.E., Ho, D.T.-H., and Qu, R. (2000). Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene. Plant Science 155, 1-9.

Smalle, J., Haegman, M., Kurepa, J., VanMontagu, M., and VanderStraeten, D. (1997). Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc Natl Acad Sci USA 94, 2756-2761.

Smirnoff, N. (1992). The carbohydrates of bryophytes in relation to desiccation-tolerance. J Bryol 17, 185-191.

Snowden, K.C., Simkin, A.J., Janssen, B.J., Templeton, K.R., Loucas, H.M., Simons, J.L., Karunairetnam, S., Gleave, A.P., Clark, D.G., and Klee, H.J. (2005). The Decreased apical dominance1/*Petunia hybrida* CAROTENOID CLEAVAGE DIOXYGENASE8 Gene Affects Branch Production and Plays a Role in Leaf Senescence, Root Growth, and Flower Development. The Plant Cell Online 17, 746-759.

Soderman, E., Mattsson, J., and Engstrom, P. (1996). The *Arabidopsis* homeobox gene ATHB-7 is induced by water deficit and by abscisic acid. Plant Journal 10, 375-381.

Song, J.T. (2005). Biochemical characterization of an *Arabidopsis* glucosyltransferase with high activity toward jasmonic acid. J Plant Biol 48, 422-428.

Song, J.T., Koo, Y.J., Seo, H.S., Kim, M.C., Choi, Y.D., and Kim, J.H. (2008). Overexpression of AtSGT1, an *Arabidopsis* salicylic acid glucosyltransferase, leads to increased susceptibility to *Pseudomonas syringae*. Phytochemistry 69, 1128-1134.

Soós, V., Sebestyén, E., Juhász A, Pintér, J., Light, M.E., Van Staden, J., and Balázs, E. (2009). Stress-related genes define essential steps in the response of maize seedlings to smoke-water. Funct. Integr. Genomics 9, 231-242.

Sorefan, K., Booker, J., Haurogné, K., Goussot, M., Bainbridge, K., Foo, E., Chatfield, S., Ward, S., Beveridge, C., Rameau, C., and Leyser, O. (2003). *MAX4* and *RMS1* are orthologous dioxygenase-like genes that regulate shoot branching in *Arabidopsis* and pea. Genes Dev. 17, 1469-1474.

Staswick, P.E., Su, W., and Howell, S.H. (1992). Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc Natl Acad Sci USA 89, 6837-6840.

Staswick, P.E., Serban, B., Rowe, M., Tiryaki, I., Maldonado, M.T., Maldonado, M.C., and Suza, W. (2005). Characterization of an *Arabidopsis* Enzyme Family That Conjugates Amino Acids to Indole-3-Acetic Acid. Plant Cell 17, 616-627.

Stepanova, A.N., and Alonso, J.M. (2009). Ethylene signaling and response: where different regulatory modules meet. Current Opinion in Plant Biology 12, 548-555.

Steponkus, P.L. (1984). Role of the plasma membrane in freezing injury and cold acclimation. Annu. Rev. Plant Physiol. 35, 543-584.

Stirnberg, P., Chatfield, S., and Leyser, H.M. (1999). *AXR1* Acts after Lateral Bud Formation to Inhibit Lateral Bud Growth in Arabidopsis. Plant Phys. 121, 839–847.

Stirnberg, P., van De Sande, K., and Leyser, O. (2002). *MAX1* and *MAX2* control shoot lateral branching in *Arabidopsis*. Development 129, 1131-1141.

Stirnberg, P., Furner, I.J., and Leyser, O. (2007). MAX2 participates in an SCF complex which acts locally at the node to suppress shoot branching. Plant Journal 50, 80-94.

Stockinger, E.J., Gilmour, S.J., and Thomashow, M.F. (1997). *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl. Acad. Sci. 94, 1035-1040.

Sun, Y.-G., Wang, B., Jin, S.-H., Qu, X.-X., Li, Y.-J., and Hou, B.-K. (2013). Ectopic Expression of *Arabidopsis* Glycosyltransferase *UGT85A5* Enhances Salt Stress Tolerance in Tobacco. Plos One 8.

Sussex, I.M., and Kerk, N.M. (2001). The evolution of plant architecture. Curr. Opin. Plant Biol. 4, 33-37.

Suzuki, M., Kao, C.-Y., Cocciolone, S., and McCart, D.R. (2001). Maize VP1 complements Arabidopsis abi3 and confers a novel ABA/auxin interaction in roots. The Plant Journal 28, 409-418.

Sýkorová, B., Kurešová, G., Daskalova, S., Trčková, M., Hoyerová, K., Raimanová, I., Motyka, V., Trávníčková, A., Elliott, M.C., and Kamínek, M. (2008). Senescence-induced ectopic expression of the *A. tumefaciens* ipt gene in wheat delays leaf senescence, increases cytokinins content, nitrate influx, and nitrate reductase activity, but does not affect grain yield. J Exp Bot 59, 377-387.

Szerszen, J.B., Szczyglowski, K., and Bandurski, R.S. (1994). *iaglu*, a gene from *Zea mays* involved in conjugation of growth hormone indole-3-acetic acid. Science 265, 1699-1701.

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H., and Mori, H. (2006). Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. Plant Journal 45, 1028-1036.

Tang, D., Christiansen, K.M., and Innes, R.W. (2005). Regulation of plant disease resistance, stress responses, cell death, and ethylene signaling in Arabidopsis by the EDR1 protein kinase. Plant Physiol. 138, 1018-1026.

Taylor, I.B., Sonneveld, T., Bugg, T.D.H., and Thompson, A.J. (2005). Regulation and manipulation of the biosynthesis of abscisic acid, including the supply of xanthophyll precursors. Journal of Plant Growth Regulation 24, 253-273

Taylor, I.B., Linforth, R.S., Al-Naieb, R.J., Bowman, W.R., and Marples, B.A. (1988). The wilty tomato mutants flacca and sitiens are impaired in the oxidation of ABA-aldehyde to ABA. Plant, Cell & Environment 11, 739-745.

Teale, W., Paponov, I., and Palme, K. (2006). Auxin in action: signalling, transport and the control of plant growth and development. Nature Review Molecular Cell Biology 7, 847-859.

Thimann, K.V., and Skoog, F. (1933). Studies on the growth hormone of plants: III. The inhibiting action of the growth substance on bud development. Proc Natl Acad Sci USA 19, 714-716.

Thomashow, M.F. (1998). Role of cold-responsive genes in plant freezing tolerance Plant Physiol 118 1-7.

Thomashow, M.F. (2010). Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. Plant Physiol. 154, 571-577.

Thompson, A.J., Jackson, A.C., Parker, R.A., Morpeth, D.R., Burbidge, A., and Taylor, I.B. (2000). Abscisic acid biosynthesis in tomato: regulation of zeaxanthin epoxidase and *9-cis-epoxycarotenoid dioxygenase* mRNAs by light / dark cycles, water stress and abscisic acid. Plant Molecular Biology 42, 833-845.

Tognetti, V., B., , Van Aken, O., Morreel, K., Vandenbroucke, K., van de Cotte, B., De Clercq, I., Chiwocha, S., Fenske, R., Prinsen, E., Boerjan, W., Genty, B., Stubbs, K.A., Inzé, D., and Van Breusegem, F., . (2010). Perturbation of Indole-3-Butyric Acid Homeostasis by the UDP-Glucosyltransferase UGT74E2 Modulates Arabidopsis Architecture and Water Stress Tolerance. Plant Cell 22, 2660-2679.

Toh, S., Kamiya, Y., Kawakami, N., Nambara, E., McCourt, P., and Tsuchiya, Y. (2012). Thermoinhibition uncovers a role for strigolactones in Arabidopsis seed germination. Plant Cell Physiol. 53, 107-117.

Toldi, O., Tuba, Z., and Scott, P. (2009). Vegetative desiccation tolerance: Is it a goldmine for bioengineering crops? Plant Science 176, 187-199.

Tompa, P. (2012). Intrinsically disordered proteins: a 10-year recap. Trends in Biochemical Sciences 37, 509-516.

Tran, L.P., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K., Fujita, M., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004). Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early response to dehydration stress 1 promoter. Plant Cell 16, 2481-2498.

Tran, L.P., Nakashima, K., Sakuma, Y., Osakabe, Y., Qin, F., Simpson, S.D., Maruyama, K., Fujita, Y., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006). Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the ERD1 gene in Arabidopsis. Plant J. 49, 46-63.

Tsuchiya, Y., Vidaurre, D., Toh, S., Hanada, A., Nambara, E., Kamiya, Y., Yamaguchi, S., and McCourt, P. (2010). A small-molecule screen identifies new functions for the plant hormone strigolactone. Nat. Chem. Biol. 6, 741-749.

Tunnacliffe, A., and Wise, M.J. (2007). The continuing conundrum of the LEA proteins. Naturwissenschaften 94, 791-812.

Turner, N.C. (1986). Crop water deficits: a decade of progress. Advances in Agronomy 39, 1-51.

Ülker, B., and Somssich, I.E. (2004). WRKY transcription factors: from DNA binding towards biological function. Curr. Opin. Plant Biol. 7, 491-498.

Umehara, M., Hanada, A., Magome, H., Takeda-Kamiya, N., and Yamaguchi, S. (2010). Contribution of strigolactones to the inhibition of tiller bud outgrowth under phosphate deficiency in rice. Plant and Cell Physiology 51, 1118-1126.

Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., Arite, T., Takeda-Kamiya, N., Magome, H., Kamiya, Y., Shirasu, K., Yoneyama, K., Kyozuka, J., and Yamaguchi, S. (2008). Inhibition of shoot branching by new terpenoid plant hormones. Nature 455, 195-200.

Umezawa, T., Fujita, M., Fujita, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006). Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Curr. Opin. Biotechnol. 17, 113-122.

Urao, T., Yamaguchi-Shinozaki, K., Urao, S., and Shinozaki, K. (1993). An *Arabidopsis* Myb homolog is induced by dehydration stress and its gene product binds to the conserved Myb recognition sequence. Plant Cell 5, 1529-1539.

Uversky, V.N., Oldfield, C.J., Midic, U., Xie, H., Xue1, B., Vucetic, S., Iakoucheva, L.M., Obradovic, Z., and Dunker, A.K. (2009). Unfoldomics of human diseases: linking protein intrinsic disorder with diseases. BMC Genomics 10(Suppl 1):S7.

van der Graaff, E., Schwacke, R., Schneider, A., Desimone, M., Flügge, U.I., and Kunze, R. (2006). Transcription analysis of Arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. Plant Physiol. 141, 776-792.

Van Staden, J., Jäger, A.K., and Strydom, A. (1995). Interaction between a plant-derived smoke extract, light and phytohormones on the germination of light-sensitive lettuce seeds. Plant Growth Regul. 17, 213-218.

Veach, Y.K., Martin, R.C., Mok, D.W.S., Malbec, J., Vankova, R., and Mok, M.C. (2003). O-Glucosylation of cis-zeatin in maize. Characterization of genes, enzymes, and endogenous cytokinins. Plant Phys. 131, 1374-1380.

Verslues, P.E., and Bray, E.A. (2006). Role of abscisic acid (ABA) and Arabidopsis thaliana ABA-insensitive loci in low water potential-induced ABA and proline accumulation. Journal of Experimental Botany 57, 201-212.

Verslues, P.E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., and Zhu, J.K. (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J. 45, 523-539.

Verwoerd, T.C., Dekker, B.M.M., and Hoekema, A. (1989). A small scale procedure for the rapid isolation of plant RNAs. Nucl. Acids. Res. 17, 2362.

Vicre, M., Farrant, J.M., and Driouich, A. (2004). Insights into the cellular mechanisms of desiccation tolerance among angiosperm resurrection plant species. Plant Cell Environment 27, 1329-1340.

Villalobos, M.A., Bartels, D., and Iturriaga, G. (2004). Stress tolerance and glucose insensitive phenotypes in Arabidopsis overexpressing the CpMYB10 transcription factor gene. Plant Physiology 135, 309-324.

Vlot, A.C., Dempsey, D.A., and Klessig, D.F. (2009). Salicylic Acid, a multifaceted hormone to combat disease. Annu. Rev. Phytopathol. 47, 177-206.

Vogel, G., Aeschbacher, R.A., Muller, J., Boller, T., and Wiemken, A. (1998). Trehalose-6-phosphate phosphatases from *Arabidopsis thaliana*: identification by functional complementation of the yeast tps2 mutant. Plant Journal 13, 673-683.

Vogel, J.T., Zarka, D.G., Van Buskirk, H.A., Fowler, S.G., and Thomashow, M.F. (2005). Roles of the CBF2 and ZATI2 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. Plant J. 41, 195-211.

Vogel, J.T., Walter, M.H., Giavalisco, P., Lytovchenko, A., Kohlen, W., Charnikhova, T., Simkin, A.J., Goulet, C., Strack, D., Bouwmeester, H.J., Fernie, A.R., and Klee, H.J. (2010). SlCCD7 controls strigolactone biosynthesis, shoot branching and mycorrhiza-induced apocarotenoid formation in tomato. The Plant Journal 61, 300-311.

Vogt, T., and Jones, P. (2000). Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. Trends in Plant Science 5, 380-386.

Wagner, T.A., and Kohorn, B.D. (2001). Wall-Associated Kinases Are Expressed throughout Plant Development and Are Required for Cell Expansion. Plant Cell 13, 303-318.

Wakui, K., and Takahata, Y. (2002). Isolation and expression of Lea gene in desiccation-tolerant microspore-derived embryos in Brassica spp. Physiol. Plant. 116, 223-230.

Wang, J., and Hou, B. (2009). Glycosyltransferases: key players involved in the modification of plant secondary metabolites. Front. Biol. China 4, 39-46.

Wang, X., Chen, S., Zhang, H., Shi, L., Cao, F., Guo, L., Xie, Y., Wang, T., Yan, X., and Dai, S. (2010). Desiccation tolerance mechanism in resurrection fernally *Selaginella tamariscina* revealed by physiological and proteomic analysis. J. Proteome Res. 9, 6561-6577.

Wang, Y., Jiang, J., Zhao, X., Liu, G., Yang, C., and Zhan, L. (2006). A novel LEA gene from *Tamarix androssowii* confers drought tolerance in transgenic tobacco. Plant Science 171, 655-662.

Weaver, L.M., Gan, S., Quirino, B., and Amasino, R.M. (1998). A comparison of the expression patterns of several senescence associated genes in response to stress and hormone treatment. Plant Mol. Biol. 37, 455-469.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmülling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15, 2532-2550.

Wilkinson, S., and Davies, W.J. (2002). ABA-based chemical signalling: The coordination of responses to stress in plants. Plant Cell Environ. 25, 195-210.

Wilson, A.K., Pickett, F.B., Turner, J.C., and Estelle, M. (1990). A dominant mutatio in Arabidopsis confers resistance to auxin, ethylene and abscisic acid. Mol. Gen. Genet. 222, 377-383.

Wingler, A. (2002). The function of trehalose biosynthesis in plants. Phytochemistry 60, 437-440.

Wolkers, W.F., McCready, S., Brandt, W.F., Lindsey, G.G., and Hoekstra, F.A. (2001). Isolation and characterization of a D-7 LEA protein from pollen that stabilizes glasses *in vitro*. Biochem. Biophys. Acta 1544, 196-206.

Woo, H.R., Kim, J.H., Nam, H.G., and Lim, P.O. (2004). The delayed leaf senescence mutants of Arabidopsis, *ore1*, *ore3*, and *ore9* are tolerant to oxidative stress. Plant Cell Physiol. 45, 923-932.

Woo, H.R., Chung, K.M., Park, J.-H., Oh, S.A., Hong, S.H., Jang, S.K., and Nam, H.G. (2001). ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. The Plant Cell 13, 1779-1790.

Woodward, A.W., and Bartel, B. (2005). Auxin: Regulation, Action, and Interaction. Annals of Botany 95, 707-735.

Wrzaczek, M., and Hirt, H. (2001). Plant MAP kinase pathways: how many and what for? Biology of the Cell 93, 81-87.

Xiao, H., and Nassuth, A. (2006). Stress- and development induced expression of spliced and unspliced transcripts from two highly similar dehydrin1 genes in *V. riparia* and *V. vinifera*. Plant Cell Rep 25, 968-977.

Xiao, X., Huang, Y., Tang, N., and Xiong, L. (2007). Overexpression of a LEA gene in rice improves drought resistance under field conditions. Theor Appl Genet. 115, 35-46.

Xin, Z., and Browse, J. (1998). *eskimo1* mutants of *Arabidopsis* are constitutively freezing-tolerant. PNAS 95, 7799-7804.

Xin, Z., Mandaokar, A., Chen, J., Last, R.L., and Browse, J. (2007). Arabidopsis ESK1 encodes a novel regulator of freezing tolerance. The Plant Journal 49, 786-799.

Xiong, L., Ishitani, M., Lee, H., and Zhu, J.-K. (1999). HOS5-a negative regulator of osmotic stress-induced gene expression in *Arabidopsis thaliana*. Plant Journal 19, 569-578.

Xiong, L., Lee, B.-h., Ishitani, M., Lee, H., Zhang, C., and Zhu, J.-K. (2001). FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signalling in *Arabidopsis*. Genes Dev. 15, 1971-1984.

Xu, D., Duan, X., Wang, B., Hong, B., Ho, D.T.-H., and Wu, R. (1996). Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiology 110, 249-257.

Xue-Xuan, X., Hong-Bo, S., Yuan-Yuan, M., Gang, X., Jun-Na, S., Dong-Gang, G., and Cheng-Jiang, R. (2010). Biotechnological implications from abscisic acid (ABA) roles in cold stress and leaf senescence as an important signal for improving plant sustainable survival under abiotic-stressed conditions. Critical Reviews in Biotechnology 30, 222-230.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (1993). Characterisation of the expression of a desiccation-responsive rd29 gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Mol Gen Genet 236, 331-340.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994). A novel cis-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6, 251-264.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (2005). Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends in Plant Science 10, 88-94.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006). Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu. Rev. Plant Biol. 57, 781-803.

Yan, H., Saika, H., Maekawa, M., Takamure, I., Tsutsumi, N., Kyozuka, J., and Nakazono, M. (2007). Rice tillering dwarf mutant *dwarf3* has increased leaf longevity during darkness-induced senescence or hydrogen peroxide-induced cell death. Genes & Genetic Systems 82, 361-366.

Yang, J.C., Zhang, J.H., Wang, Z.Q., Zhu, Q.S., and Liu, L.J. (2003). Involvement of abscisic acid and cytokinins in the senescence and remobilization of carbon reserves in wheat subjected to water stress during grain filling. Plant Cell Environ. 26, 1621-1631.

Yi, S.Y., Kim, J.-H., Joung, Y.-H., Lee, S., Kim, W.-T., Yu, S.H., and Choi, D. (2004). The pepper transcription factor CaPF1 confers pathogen and freezing tolerance in Arabidopsis. Plant Physiology 136, 2862-2874.

Yobi, A., Wone, B.W.M., Xu, W., Alexander, D.C., Guo, L., Ryals, J.A., Oliver, M.J., and Cushman, J.C. (2012). Comparative metabolic profiling between desiccation-sensitive and desiccation-tolerant species of *Selaginella* reveals insights into the resurrection trait. Plant J. 72, 983-999.

Yobi, A., Wone, B.W.M., Xu, W., Alexander, D.C., Guo, L., Ryals, J.A., Oliver, M.J., and Cushman, J.C. (2013). Metabolic profiling in *Selaginella lepidophylla* at various hydration states provides new insights into the mechanistic basis of desiccation tolerance. Mol. Plant 6, 369-385.

Yokota, T., Sakai, H., Okuno, K., Yoneyama, K., and Takeuchi, Y. (1998). Alectrol and orobanchol, germination stimulants for *Orobanche minor*, from its host red clover. Phytochemistry 49, 1967-1973.

Yoneyama, K., Xie, X., Kusumoto, D., Sekimoto, H., Sugimoto, Y., Takeuchi, Y., and Yoneyama, K. (2007). Nitrogen deficiency as well as phosphorus deficiency in sorghum promotes the production and exudation of 5-deoxystrigol, the host recognition signal for arbuscular mycorrhizal fungi and root parasites. Planta 227, 125-132.

Yoneyama, K., Xie, X., Sekimoto, H., Takeuchi, Y., Ogasawara, S., Akiyama, K., Hayashi, H., and Yoneyama, K. (2008). Strigolactones, host recognition signals for root parasitic plants and arbuscular mycorrhizal fungi, from Fabaceae plants. New Phytol 179, 484-494.

Zažímalová, E., Murphy, A.S., Yang, H., Hoyerová, K., and Hošek, P. (2010). Auxin transporters-why so many? Cold Spring Harbor Perspect. Biol. 2, a001552.

Zeevaart, J.A.D., and Creelman, R.A. (1988). Metabolism and physiology of abscisic acid. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 439-473.

Zhang, L., Xi, D., Luo, L., Meng, F., Li, Y., Wu, C.A., and Guo, X. (2011). Cotton GhMPK2 is involved in multiple signaling pathways and mediates defense responses to pathogen infection and oxidative stress. FEBS J. 278, 1367-1378.

Zhang, S., Cai, Z., and Wang, X. (2009). The primary signaling outputs of brassinosteroids are regulated by abscisic acid signaling. In Proceedings of the National Academy of Sciences of the United States of America, pp. 4543-4548.

Zhu, J.K. (2002). Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53, 247-273.

Zhu, J.K., Liu, J., and Xiong, L. (1998). Genetic analysis of salt tolerance in *Arabidopsis thaliana*: evidence of a critical role for potassium nutrition. Plant Cell 10, 1181-1192.

Zimmermann, S., and Sentenac, H. (1999). Plant ion channels: from molecular structures to physiological functions. Curr. Opin. Plant Biol. 2, 477-482.

Zou, J.H., Zhang, S.Y., Zhang, W.P., Li, G., Chen, Z.X., Zhai, W.X., Zhao, X.F., Pan, X.B., Xie, Q., and Zhu, L.H. (2006). The rice *HIGH-TILLERING DWARF1* encoding an ortholog of *Arabidopsis MAX3* is required for negative regulation of the outgrowth of axillary buds. Plant Journal 48, 687-696.

Zwanenburg, B., Mwakaboko, A.S., Reizelman, A., Anilkumar, G., and Sethumadhavan, D. (2009). Structure and function of natural and synthetic signalling molecules in parasitic weed germination. Pest Management Science 65, 478-491.

Appendices

Appendix 1

Nucleotide and amino acid sequence of SDG10y from *S. stapfianus*

```
   1 CAAGCACGAGCTCCTCAGCGACGACGAGAACTCACTCACACAGGACATACTCCGATGACC
                                                         M  T    2
  61 AACCGCATCTTCTTTCCCCCATCCATGGCGACGGCGAACCAGGAGTACATAATCCGATTC
      N  R  I  F  F  P  P  S  M  A  T  A  N  Q  E  Y  I  I  R  F   22
 121 GACGGCCAGTTCGAGGACCCCTCGCCGAGCTCCTCGGGCGTCGAACCACAGCCGGTGCCT
      D  G  Q  F  E  D  P  S  P  S  S  S  G  V  E  P  Q  P  V  P   42
 181 GAGGCGGCGTCGTTCCCGTGGAGGACGATCAGTCCCGAGCAGGAGCACGCGGTCATTGTG
      E  A  A  S  F  P  W  R  T  I  S  P  E  Q  E  H  A  V  I  V   62
 241 TCCGCCCTGCTCCACGTCCTCTCCGGCTACACCACGCCGCCGCCCCAGGTCTTCCCGGCG
      S  A  L  L  H  V  L  S  G  Y  T  T  P  P  P  Q  V  F  P  A   82
 301 TCGGCGGCTGCGCGAGTGGAGGCGTGCCGGATGTGCGGGATGGAGCGGTGCCTCGGCTGC
      S  A  A  A  R  V  E  A  C  R  M  C  G  M  E  R  C  L  G  C  102
 361 GAGTTCTTCGCCGCGGACGCCGGAGTGGTCGCGTCGGATGGATCAGAGAAGGTTCCCGCG
      E  F  F  A  A  D  A  G  V  V  A  S  D  G  S  E  K  V  P  A  122
 421 GCAGCAGAGACGACGACAGCTCCGGCGGCTGCAGGAGGGCAGAGGCGGCGGCGGAAGAAG
      A  A  E  T  T  T  A  P  A  A  A  G  G  Q  R  R  R  R  K  K  142
 481 AAGAACAAGTACCGCGGCGTGAGGCAGCGTCCGTGGGGGAAGTGGGCGGCGGAGATCCGC
      K  N  K  Y  R  G  V  R  Q  R  P  W  G  K  W  A  A  E  I  R  162
 541 GACCCGCGCCGCGCCGTGCGCAAGTGGCTCGGCACATTCGACACCGCCGAGGAGGCCGCC
      D  P  R  R  A  V  R  K  W  L  G  T  F  D  T  A  E  E  A  A  182
 601 AGGGCGTACGACCGCGCCGCCGTCGAGTTCCGCGGCCCGCGCGCCAAGCTCAACTTCCCG
      R  A  Y  D  R  A  A  V  E  F  R  G  P  R  A  K  L  N  F  P  202
 661 TTCCCAGAGCAACTGGCAGACCACGAAGACGCCAGCGCGGCAGCCAAGTCCGACACATTG
      F  P  E  Q  L  A  D  H  E  D  A  S  A  A  A  K  S  D  T  L  222
 721 TCGCCGTCGCTGTGCAGCGGCAGCGCGGACGCCGAGGACCGTAGCGTTGGGAGGCAGGAG
      S  P  S  L  C  S  G  S  A  D  A  E  D  R  S  V  G  R  Q  E  242
 781 TGGCCGCACCAGCACGGGATGGAAGAAACAGGGGAGCAGCTCTGGGACGACCTCCAGGAC
      W  P  H  Q  H  G  M  E  E  T  G  E  Q  L  W  D  D  L  Q  D  262
 841 CTGATGAACCTGGACGAAGGCGAGATCTGGTTCCCGCCAACCTCGACAACTTGGAATTGA
      L  M  N  L  D  E  G  E  I  W  F  P  P  T  S  T  T  W  N  *  281
 901 AACGAGTCTTAATCAAATCACGGCCGTTAAAGTAGATTGAAATTGACAATCGTAGCCTTT
 961 CAGTTTTCTCCTTTTGACCTTTTTTTCTTCTTCTTTTTATAAGCTCTGTTGTACTATTTG
1021 ATCGAAGCAGAGTTTTGAGTCTTGAGGTCTGTACAGCATCTCCGCAAGGAACACGCGAGC
1081 TGACTGATCTGTTGTTAACCATTTTTGTAGTCACAAAAAAAAAAAAAAAAAAAAAAAAA 1140
```

Appendix 1. Nucleotide and amino acid sequence of SDG10y from *S. stapfianus*.
The amino acids of the longest ORF are given below the nucleotide sequence. The putative stop codon (*) is indicated. The poly-A tail is represented by a string of A residues at the end of the sequence. Numbers correspond to nucleotides (left) and amino acids (right) respectively.

Appendix 2

Nucleotide and amino acid sequence of SDG7y from *S. stapfianus*

```
   1 CAAGCTTCTTCTTCTTCACCCACTCACTCGAGGCTCAACTCAGTCAGGGCTCGGTCATGA
                                                                 M     1
  61 CCAAGAAGCTGGATCCCGGCATGGCAACGAAGAATAAGCAAGGTCGCGAGGCGCAGCTCG
     T  K  K  L  D  P  G  M  A  T  K  N  K  Q  G  R  E  A  Q  L    21
 121 ACAGTCAGGGGAGACAGGTTTCGAACTACGCGGATGGAGGAACTGGAAGGTCGACGATCT
     D  S  Q  G  R  Q  V  S  N  Y  A  D  G  G  T  G  R  S  T  I    41
 181 CTCAGGAGCAGGAGCACAGCATCATGGTCGCGGCGCTGCGGCACGTCCTGTCCGGGTACA
     S  Q  E  Q  E  H  S  I  M  V  A  A  L  R  H  V  L  S  G  Y    61
 241 GCACGCCGCCGCCGGAGGTGGTCACGGTGGCGGCGAGCGGCGAGGCGTGCGGGACCTGCG
     S  T  P  P  P  E  V  V  T  V  A  A  S  G  E  A  C  G  T  C    81
 301 GCATCGACGGCTGCCTCGGCTGCGACTTCTTCGCGGGGGAGGAGCCCCCTCAGCTGCTAC
     G  I  D  G  C  L  G  C  D  F  F  A  G  E  E  P  P  Q  L  L   101
 361 GACAGGGACTGAGCTATGGCACAGGCACCGCGGCGGTGGCGACGAGTACCGATGAGAAGA
     R  Q  G  L  S  Y  G  T  G  T  A  A  V  A  T  S  T  D  E  K   121
 421 GGAGGAAGCGGCGCAGGAAGAGGAACGCGTTCCGCGGCGTGCGGCAGCGGCCATGGGGCA
     R  R  K  R  R  R  K  R  N  A  F  R  G  V  R  Q  R  P  W  G   141
 481 AGTGGGCGGCGGAGATCCGCGACCCCAGGAAGGCGGCGCGGGTGTGGCTCGGCACGTTCG
     K  W  A  A  E  I  R  D  P  R  K  A  A  R  V  W  L  G  T  F   161
 541 ACACCGCCGAGGAGGCCGCCAGGGCGTACGACCGCGCCGCGCTCGAGTTCCGTGGCGCGC
     D  T  A  E  E  A  A  R  A  Y  D  R  A  A  L  E  F  R  G  A   181
 601 GCGCCAAGCTCAACTTCCCCTACCAGGAGGAGCCGGTCGTCCTGCTGGGCCATCACAATG
     R  A  K  L  N  F  P  Y  Q  E  E  P  V  V  L  L  G  H  H  N   201
 661 GCGATTCGGCGGCTGGGACGACGCTCACGCCGCCGTCGTCGTGCAGCGTCGACGCCGAGG
     G  D  S  A  A  G  T  T  L  T  P  P  S  S  C  S  V  D  A  E   221
 721 ATCAGAGCGCGGACGGACTTGGGGATGAACTGTGGGACGGATTGCAGGACCTGATGAAGA
     D  Q  S  A  D  G  L  G  D  E  L  W  D  G  L  Q  D  L  M  K   241
 781 TGGATGACGCGGACTTCTGGTCGTTCGCGCCGTTCTATGGTGCAGCATCGTCTGGTGTTT
     M  D  D  A  D  F  W  S  F  A  P  F  Y  G  A  A  S  S  G  V   261
 841 GAGCGGTGATGTTGTACACAATAACACTAGGCGTAGCTCTTGAACTAGACTGACGCTATA
     *
 901 ACTAGTTCTCCGTAAAAAGCTGGATTACTAGTAGTTCGATATAGTTGAGTGAACCATTCT
 961 TTTGTTGTGTACAGTACATAAGTTGGATTAATTCATATTATTTGATTGGATTGCCCCAAT
1021 TCAGTTGTCAATATGAGCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
1081 AAAAAAAA 1088
```

Appendix 2. Nucleotide and amino acid sequence of *SDG7y* from *S. stapfianus*.
The amino acid sequence of the longest ORF is given below the nucleotide sequence. The putative stop codon (*) is indicated. The poly-A tail is represented by a string of A residues at the end of the sequence. Numbers correspond to nucleotides (left) and amino acids (right) respectively.

Appendix 3

List of publication and conference presentations:

1. **Islam**, **S.**, Griffiths, C. A., Blomstedt, C. K., Le, T., Gaff, D. F., Hamill, J. D., Neale, A. D., Increased Biomass, Seed Yield and Stress Tolerance Is Conferred in Arabidopsis by a Novel Enzyme from the Resurrection Grass *Sporobolus stapfianus* That Glycosylates the Strigolactone Analogue GR24. (2013), PLoS One, 8(11): e80035. doi:10.1371/journal.pone.0080035

2. **Islam, S**, Hamill, JD, Gleadow, R, Blomstedt CK, Selby, J, Healey, J and Neale, AD. "An analysis of the role of ERF transcription factors SDG10y and SDG7y from the resurrection grass *Sporobolus stapfianus* in heterogeneous monocot and dicot expression systems". (2008) ComBio, Canberra, Australia.

3. Blomstedt, C. K., Griffiths, C. A., **Islam, S**, Hamill, J. D., Gaff, D. F. and Neale, A. D. "Growth-related gene expression in desiccated resurrection plants". (2011) International Botanical Conference, Melbourne, Australia.

Appendix 4

Increased Biomass, Seed Yield and Stress Tolerance Is Conferred in *Arabidopsis* by a Novel Enzyme from the Resurrection Grass *Sporobolus stapfianus* That Glycosylates the Strigolactone Analogue GR24.

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Increased Biomass, Seed Yield and Stress Tolerance Is Conferred in *Arabidopsis* by a Novel Enzyme from the Resurrection Grass *Sporobolus stapfianus* That Glycosylates the Strigolactone Analogue GR24

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Abstract

Isolation of gene transcripts from desiccated leaf tissues of the resurrection grass, *Sporobolus stapfianus*, resulted in the identification of a gene, *SDG8i*, encoding a Group 1 glycosyltransferase (UGT). Here, we examine the effects of introducing this gene, under control of the CaMV35S promoter, into the model plant *Arabidopsis thaliana*. Results show that *Arabidopsis* plants constitutively over-expressing *SDG8i* exhibit enhanced growth, reduced senescence, cold tolerance and a substantial improvement in protoplasmic drought tolerance. We hypothesise that expression of *SDG8i* in *Arabidopsis* negatively affects the bioactivity of metabolite/s that mediate/s environmentally-induced repression of cell division and expansion, both during normal development and in response to stress. The phenotype of transgenic plants over-expressing *SDG8i* suggests modulation in activities of both growth- and stress-related hormones. Plants overexpressing the UGT show evidence of elevated auxin levels, with the enzyme acting downstream of ABA to reduce drought-induced senescence. Analysis of the *in vitro* activity of the UGT recombinant protein product demonstrates that SDG8i can glycosylate the synthetic strigolactone analogue GR24, evoking a link with strigolactone-related processes *in vivo*. The large improvements observed in survival of transgenic *Arabidopsis* plants under cold-, salt- and drought-stress, as well as the substantial increases in growth rate and seed yield under non-stress conditions, indicates that overexpression of *SDG8i* in crop plants may provide a novel means of increasing plant productivity.

Citation: Islam S, Griffiths CA, Blomstedt CK, Le T-N, Gaff DF, et al. (2013) Increased Biomass, Seed Yield and Stress Tolerance Is Conferred in *Arabidopsis* by a Novel Enzyme from the Resurrection Grass *Sporobolus stapfianus* That Glycosylates the Strigolactone Analogue GR24. PLoS ONE 8(11): e80035. doi:10.1371/journal.pone.0080035

Editor: Els JM van Damme, Ghent University, Belgium

Received June 28, 2013; **Accepted** September 27, 2013; **Published** November 5, 2013

Funding: Sharmin Islam was supported by a Monash University Graduate Research Scholarship and Cara Griffiths by a Monash University Faculty of Science Dean's Postgraduate Research Scholarship; URL;www.monash.edu. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: Monash University has lodged an Australian Provisional Patent Application No 2012904356, entitled "Method for improving crop productivity", associated with this work. This does not alter our adherence to all PLOS ONE policies on sharing data and materials.

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Introduction

The desiccation tolerant grass *Sporobolus stapfianus* grows in shallow, nutrient poor soils in regions experiencing intense seasonal drought. For their persistence these plants rely on the ability of the protoplasm of their vegetative tissue to desiccate (loss of ≥ 95% total water content) and rehydrate rapidly. The rehydrated plant restores normal metabolism within 24 hours [1], grows very quickly following rain, and has proven useful for pinpointing genes for increased stress-tolerance [2,3] and enhanced growth rate [4]. Characterization of *Sporobolus* drought genes (*SDG*s) that are specifically expressed in desiccation-tolerant tissue [5], has the potential to reveal mechanisms for coping with stress which are peculiar to, or enhanced in, resurrection plants. Such coping mechanisms include the ability to adjust growth rapidly in response to changes in water availability, to inhibit dehydration-induced senescence programs, to protect cellular components during dehydration and to reinstitute photosynthetic capacity quickly following a severe dehydration event [1]. The mechanisms required for *S. stapfianus* to exhibit these characteristics may rely on coordinately regulated plant hormone activity linked to environmental cues.

The *Sporobolus SDG8i* gene encodes a Group 1 UDP-glycosyltransferase (UGT) whose transcript levels increase substantially under severe water deficit [5]. Plant genomes typically encode a large number of UGTs that collectively can conjugate sugars to a range of acceptor molecules including many plant hormones, secondary metabolites and xenobiotics [6]. UGTs have an important role in cellular metabolism since glycosylation can affect the solubility, transport and biological activity of these compounds [7]. Hence glycosylation can control the bioactivity of plant growth regulators crucial to enabling adaption of plants to changing environments [8]. The majority of the classical hormones occur as glycosides *in planta* and UGTs capable of glycosylating auxins, cytokinin, ABA, salicylic acid, jasmonic acid and brassinosteroids or their synthetic precursors have been identified [9-15]. The possibility that glycosylation of one or more growth regulators may play a role in promoting onset of desiccation tolerance in *S. stapfianus* was suggested by the study of Le et al. [5],, but as yet no experimental evidence for such a role has been reported.

As no protocol for transformation of resurrection grasses exists, functional analysis of the dehydration-induced UGT SDG8i was undertaken in *Arabidopsis*. Ectopic expression of SDG8i in *Arabidopsis* was found to have a profound effect on plant architecture and growth and confer a substantial improvement in protoplasmic drought tolerance. Here we report that *SDG8i* encodes a functional UGT that can glycosylate the synthetic strigolactone analogue GR24, and that ectopic expression of this UGT leads to a substantial enhancement of plant growth and stress resistance.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh, *Sporobolus stapfianus* Gandoger and *Sorghum bicolor* L. seed were obtained from laboratory stocks. Wild-type (WT) plants refer to *Arabidopsis* accession Columbia-0 (Col-0). *Orobanche* seeds were obtained from the South Australian Department of Water, Land and Biodiversity Conservation. *Arabidopsis* plants were stratified at 4°C for 3 days and grown at 22°C under continuous light unless stated otherwise. Under long day (LD) photoperiod conditions the plants were subjected to a 16 hour light and 8 hour dark cycle. Under a short day (SD) photoperiod, the cycle consisted of 8 hours light and 16 hours dark. Soil grown plants were placed in a growth cabinet at 22°C, 25% relative humidity and approximately 200 µmole/m^2/sec light intensity. For axenic culture, seeds were surface-sterilized in 70% (v/v) ethanol and rinsed with sterile water and cultured at 22°C with approximately 100 µmole/m^2/sec light intensity. Crossing of *Arabidopsis* plants was performed as described in Weigel and Glazebrook [16].

Generation of transgenic plants

The *SDG8i* coding sequence (EMBL/GenBank accession number AM268210) was amplified and inserted into the donor vector pDONR221 using the Gateway cloning system (Invitrogen) following the manufacturer's instructions.

5′attB1 Primer;
GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACGAAG ACCGTGGTTCTG
3′attB2 Primer;
GGGGACCACTTTGTACAAGAAAGGTGGGTCTCACGGAC GACCGACAGCCTCCA

The pDONR221:SDG8i construct was transferred to the expression vector pMDC32 containing the 2 × 35S promoter [17] and transformed into *Arabidopsis* Columbia-0 (Col-0) using *Agrobacterium tumerfaciens* (AGL-1strain) by the floral dip method [18]. Second generation (T2) transgenic plants homozygous for *SDG8i* were generated under hygromycin resistance.

Recombinant UGT production

The UGT was produced by transient transformation of *Nicotiana bethamiana* leaves using a viral MagnICON vector system (Icon Genetics GmbH, Germany). The *SDG8i* sequence was amplified by RT-PCR, using RNA from *S. stapfianus*, with the primers:
Forward:
5′GAGAGAATTCATGACGAAGACCGTGGTTCTGTAC3 ′
Reverse: 5′GAGAGGATCCTCACGGACGACCGAC3 ′.

The PCR product was ligated into pICH11599 to generate pICH11599-*SDG8i*. The three pro-vector fragments: (i) pICH12190 (containing a chloroplast signal sequence), (ii) pICH11599-*SDG8i* and (iii) pICH14011 (the integrase) were separately electroporated into *Agrobacterium tumerfaciens* GV3101. For the vector-only control pICH11599 was used with (i) and (iii). Equal amounts of the three *A. tumerfaciens* strains were infiltrated into aluminum foil-covered *N. benthamiana* leaves using the protocol described by Marillonet et al. [19]. After 5 days co-incubation, the leaf tissue was ground in liquid nitrogen and mixed with 500 µl cold protein extraction buffer (5mM sodium phosphate buffer pH 7.5, 10mM EDTA, 0.1% (v/v) Triton X-100). The extract was centrifuged (13,500 g) for 10 minutes at 4°C and the protein content was determined by Bradford assay and extracts analysed by SDS-PAGE [20].

Observation of palisade cells in fully expanded leaves

The second fully expanded rosette leaves from transgenic and control seedlings were immersed in 0.1% (v/v) Triton X-100, then centrifuged (10,000g) for 1 min to remove air from intercellular spaces and imaged using a light microscope. The leaf area was measured with Adobe Photoshop CS5.1 using the formula [(No. of pixels of leaves/No. of pixels of physical unit) × Area of physical unit]. Twenty sub-epidermal palisade cells aligned along the proximo–distal and medio–lateral axes were used to calculate the average cell area using ImageJ software. The total cell number per leaf was calculated by dividing the leaf area by the palisade cell area for each leaf.

Examination of root architecture

Plants were grown under SD (8h day/16h night) for 13 days in vertically-orientated petri dishes on MS media (pH 5.8) with 1% (w/v) sucrose and 0.8% (w/v) agar. Roots were then fixed in 4% (v/v) formaldehyde in 0.025 M phosphate buffer (pH 7.2)

overnight at 4°C. The fixative was replaced with 30% (v/v) glycerol containing 2% (v/v) DMSO and left for 30 min at room temperature. Roots were mounted in a clearing solution (4.2 M NaI, 8 mM $Na_2S_2O_3$, 2% (v/v) DMSO in 65% (v/v) glycerol) and root primordia and root cell-length were examined 1 h after the sample preparation. The number of primordia was determined within the lateral-root-formation zone between the most-distal initiated primordium and the most-distal emerged lateral root [21]. Lateral root primordium density (d) was calculated for each individual primary root as number of primordia per mm. l_{LRI}, (the number of lateral root primordia initiated within a portion of the root that corresponds to the length (*l*, mm) of 100 fully elongated cortical cells in a single file in the same parent root) was determined as 100dl, where *l* is the average cortical cell length in mm for each individual primary root.

Histochemical analysis of ß-glucuronidase (GUS) activity

Seedlings were GUS stained at 37°C for 4 h according to the protocol of Jefferson et al. [22] and then cleared with 70% (v/v) ethanol. Images were taken using a microscope (Leica MEFLIII).

Salt stress assays

Seedlings, grown for six days on vertically-orientated germination media plates (½ MS, 0.5% (w/v) sucrose, pH 5.7, 0.8% (w/v) agar), were transferred to fresh media containing salt (0, 50, 100, 125, 150 and 175 mM NaCl) and the position of root tips noted. The plates were inverted to allow roots to grow downward in the shape of a hook. Root elongation was measured after 7 days on salt media.

Freezing stress assay

Seedlings (25/plate) were grown for 3 weeks at 22°C on MS-agar (½ MS, 0.5% (w/v) sucrose, pH 5.7, 0.8% (w/v) agar) before acclimation at -1°C for 16 hours. The temperature was lowered by 2°C per hour. Seedlings were held at the desired temperature for one hour before a plate containing a subsample was removed, and then the temperature was lowered further. Following the one hour exposure to -4°C, -8°C and -12°C, subsampled plants were transferred to 4°C overnight for recovery then returned to 22°C and survival scored 7 days later. Plants that were bleached were scored as dead, while green plants were scored as having survived the freezing test.

Water withholding test

Plants in separate pots were kept fully and uniformly watered by sub-irrigation under optimal growth conditions in LD at 22°C until the 6-7 leaf pre-flowering stage. Water was then withheld, and the plants were observed over a period of 3 weeks. Subsamples of drought treated plants were re-watered at regular intervals and recovery was monitored after 24 hours. Five plants from each line were tested at each sample point and the experiment was performed twice.

Water vapor equilibration

Protoplasmic drought tolerance (PDT), the water potential at which 50% of plant tissue survives in equilibrium with the air, was assessed using $CaCl_2$ solutions. This permits water potentials (ψ_w), down to 30% relative humidity (RH) in equilibrium with a saturated solution, to be imposed on the plants via the air phase [23]. The RH in equilibrium with the $CaCl_2$ solutions is calculated from freezing point depression data [24]. Chambers containing $CaCl_2$ solutions ranging from 98% (-2.8 MPa) to 86% (-20.5 MPa) RH at 25°C were used. Pre-flowering plants were soaked in water until turgid and then blotted dry. Shoots were detached and the initial turgid weight (TWt_o) recorded. Shoots were enclosed in insulated chambers for 3 days until equilibration to differing RH was reached ($\psi_{leaf} = \psi_{solution}$). The shoots were then rehydrated for 24 hours and the final turgid weight (TWt_F) recorded. Survival of plants was determined by: Method A, the number of leaves alive per plant, judged subjectively by the recovery of the healthy coloration and crisp texture (dead leaves are readily distinguished in the thin fibre-poor tissues of *Arabidopsis*); Method B, an objective test, depends on the loss of semipermeability in dead cells which prevents osmotic absorption of water, resulting in an apparent loss in the "turgid" weight per dry weight of dead tissue compared with that of live tissue before vapor-equilibration. The ratio TWt_F/TWt_o was plotted against % RH and the PDT determined as the % RH at which 50% of the tissue is alive. Four shoots from each line were tested for each sample point and the experiment was performed twice.

Senescence tests

For dark-induced senescence, excised leaves from 12 day old soil-grown *Arabidopsis* plants were floated, adaxial side up, on deionized water. For ABA treatment, detached leaves were floated on 3 mM MES buffer (pH 5.8) solution under continuous light in the presence or absence of 50 µM abscisic acid.

Chlorophyll analysis

Leaf tissue (20 mg) was ground under liquid nitrogen and extracted twice with 1.5 ml ice-cold 80% (v/v) acetone, centrifuged (14,000 rpm) at 4°C for 3 min and supernatants stored in the dark. The chlorophyll concentration was calculated as described by Lichtenthaler [25] . The F_v/F_m ratio was measured after dark adaptation of the leaves for 15 min using a PAM-210 (Teaching-PAM) (Heinz Walz GmbH, Germany).

UGT enzyme assay

Recombinant UGT activity was measured using the coupled enzyme assay described by Jackson et al., [9]. The assay was conducted at pH 7.4 with a final substrate concentration of 1 mM using 25 µg of protein extract. GR24 was obtained from Chiralix B.V. Nijmegen, The Netherlands. All other hormones were obtained from Sigma St. Louis, MO. Activity in millikatals kg^{-1} was calculated using the extinction coefficient 6.22 x 10^{-3} M^{-1} cm^{-1} for NADH. Background activity of extracts, monitored by measuring the rate without substrate addition, was subtracted from the reaction rate.

Orobanche germination assay

Orobanche (*Phelipanche ramosa* (L.) Pomel) seeds were surface-sterilized with 2% sodium hypochlorite containing 0.02% (v/v) Tween-20, rinsed with sterile ddH_2O and dried in a laminar flow cabinet. Seed (~100) on a sterile glass microfiber disk were placed on ddH_2O soaked filter paper in Petri dishes and preconditioned by adding 1ml of GA_3 (30 mg/L) and incubation at 20°C in the dark for 7 days. Surface-sterilized seeds of host plants were added to the microfiber disks and placed on ¼ MS media with 0.5% (w/v) sugar and 0.8% (w/v) agar. For controls, 0.6 ml of GR24 (0.0001 mg/L) or ddH_2O was applied to the preconditioned *Orobanche* seeds. The plates were covered in foil and incubated at 20°C for 7 days. *Orobanche* germination was determined by counting the seeds with an emerged radicle.

Effect of GR24 on shoot branching

To determine the effect of GR24 on shoot branching, *Arabidopsis* plants were grown in soil for 23 days to pre-bolting stage. The plants were then treated with 50 µl per plant of 5 µM GR24 applied to the shoot meristem and axillary meristem region. Control plants were treated with 50 µl of water. The treatment was repeated every third day for 20 days when the number of rosette branches (>5 mm) was counted.

Statistical Analyses

All data were examined by analyses of variance using GraphPad Prism software version 5.0. Tukey's Multiple Comparison Test was used for comparison between means of wild-type and SDG8i transgenic plants at 5% level of significance.

Results

Ectopic expression of SDG8i affects the shoot architecture and the growth rate of *Arabidopsis*

Several *Arabidopsis* lines (F1aD, D1E, F6bA, D5aA, D2E, D4I, D7c) designed to express the SDG8i protein under the control of the 35S promoter (SDG8i transgenic plants) were taken to homozygosity and the presence of the *SDG8i* transcript was confirmed (Fig. S1). The developmental phenotype of these independent SDG8i transgenic lines was examined under long day (LD: 16h day/8h night) and short day (SD: 8h day/16h night) photoperiods (Figure 1). Flower morphology, seed development and seed weight were normal (Fig. S2). Transgenic plants were not affected in flowering time in LD, with production of a similar number of primary rosette leaves as formed by the wild-type Col-0 controls. In SD, both wild-type Col-0 and transgenic plants produced the first inflorescence 52 days after germination, with wild-type plants producing around 30-34 primary rosette leaves compared with approximately 40-42 primary rosette leaves produced by the transgenics.

When compared to wild-type plants the transgenic lines were taller, and had increased branching, shoot biomass and seed yield (p<0.05) (Figure 1 and Fig. S2 and S3). These phenotypic differences were more pronounced in SD. One month after

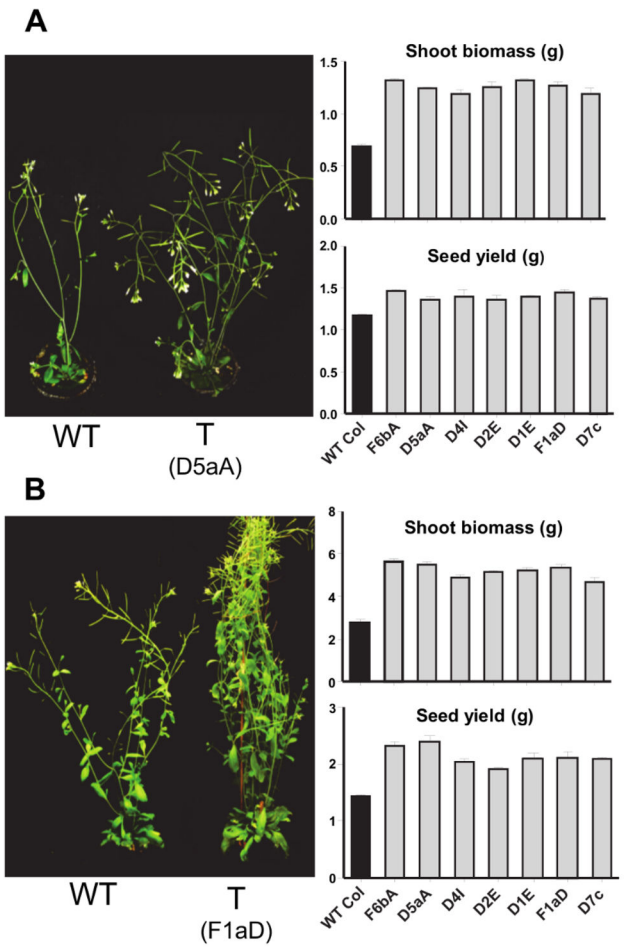

Figure 1. A phenotypic comparison of SDG8i transgenic (T) and wild-type Col-0 (WT) plants. Plants were grown at 21°C under (A) long day and (B) short day. The figure shows the increased height, branching, shoot biomass (FW) and seed yield typical of all the SDG8i transgenic lines. Dry seed was collected following senescence. The biomass was measured 16 days (LD) and 50 days (SD) after germination and before bolting. Values are the means ± SE of 5 replicates. The transgenic plant grown in short days has been tied with cotton thread.

doi: 10.1371/journal.pone.0080035.g001

flowering the total number of leaves, including primary rosette leaves and leaves formed in the rosette from axillary meristems by transgenic plants was 1.3 to 1.6 times more than wild-type plants (p<0.01) (Fig. S3A). The average size of the leaves of the transgenic plants was significantly larger than that of wild-type plants (p<0.05) (Fig. S3B) and the number of inflorescences produced was 1.4 to 1.6 times that of wild-type Col-0 (p<0.01) (Fig. S3C). Despite the increased branching, the height increases exhibited by SDG8i plants over wild-type ranged from 9% to 16% after 21 days of bolting (Fig. S3D). The rate of growth of the primary inflorescence of transgenic plants was approximately 20% greater (7.1 cm $week^{-1}$) than that of wild-type plants (5.4 cm $week^{-1}$). After 50 days in SD

Table 1. Leaf area, leaf cell size and leaf cell number in wild type (WT) and SDG8i plants.

Plant Type	Av. Leaf Area (mm^2)	Av. Cell Area (μm^2)	Av. Cell Number/leaf
WT	47.55 ± 1.98	3160 ± 30	15047 ± 586
D5aA	63.92 ± 0.84	3540 ± 159	18129 ± 845
D4I	62.0 ± 1.4	3575 ± 113	17392 ± 831
F6bA	59.97 ± 3.13	3592 ± 173	16858 ± 885

The leaf area and cell area were calculated in 25-day-old soil-grown plants in SD using imaging software. Values are the means ± SE of 3 replicates.

doi: 10.1371/journal.pone.0080035.t001

photoperiods the average fresh weight (FW) shoot biomass of all transgenic plants was almost twice that of wild-type (Figure 1) and the seed yield of SDG8i transgenic plants was 1.4-1.6 times the mass of seed from wild-type plants (p<0.001) (Figure 1).

The leaves of SDG8i plants have increased cell size and number

The rosette leaves of SDG8i plants are larger than wild-type Col-0 plants in both LD and SD. When the leaf cells of SD soil-grown plants were examined, it was found that the leaves of SDG8i transgenic plants contained more cells than comparable leaves from control plants and the cells were larger (p<0.01) (Table 1 and Fig. S4). The increase in the number and size of leaf palisade cells of transgenic plants indicates that SDG8i activity promotes both leaf cell expansion and cell division and may affect the number of meristematic cells allocated to the leaf primordium.

SDG8i expression increases root cell length and lateral root initiation

When grown in both LD and SD the average fresh weight of the root biomass of all SDG8i transgenic plants was almost twice that of wild-type (Figure 2A, B). A comparison of the root architecture in SDG8i transgenics and wild-type plants grown *in vitro* in SD was conducted using the method described in Dubrovsky et al., [21]. The primary root of all SDG8i plants was longer than wild-type under SD (p<0.001) (Figure 2C). The fully elongated cortical cells in all the transgenic plants were longer than wild-type (p<0.05) (Figure 2D). Calculation of the lateral root primordium density also showed a difference between SDG8i transgenic plants and wild-type plants (p<0.005) (Figure 2E). The higher estimation of I_{LRI} (root primordia/100 cortical cells) in transgenic plants indicates increased root branching (Figure 2F). These findings demonstrate that SDG8i activity promotes both primary root growth and lateral root initiation.

Overexpression of SDG8i affects auxin homeostasis

The spatial distribution of auxin controls many aspects of plant growth and development [26]. The synthetic auxin response reporter construct *DR5-GUS*, has been used in many studies to examine the patterns of auxin distribution [27]. To examine the effect of SDG8i activity on auxin levels and

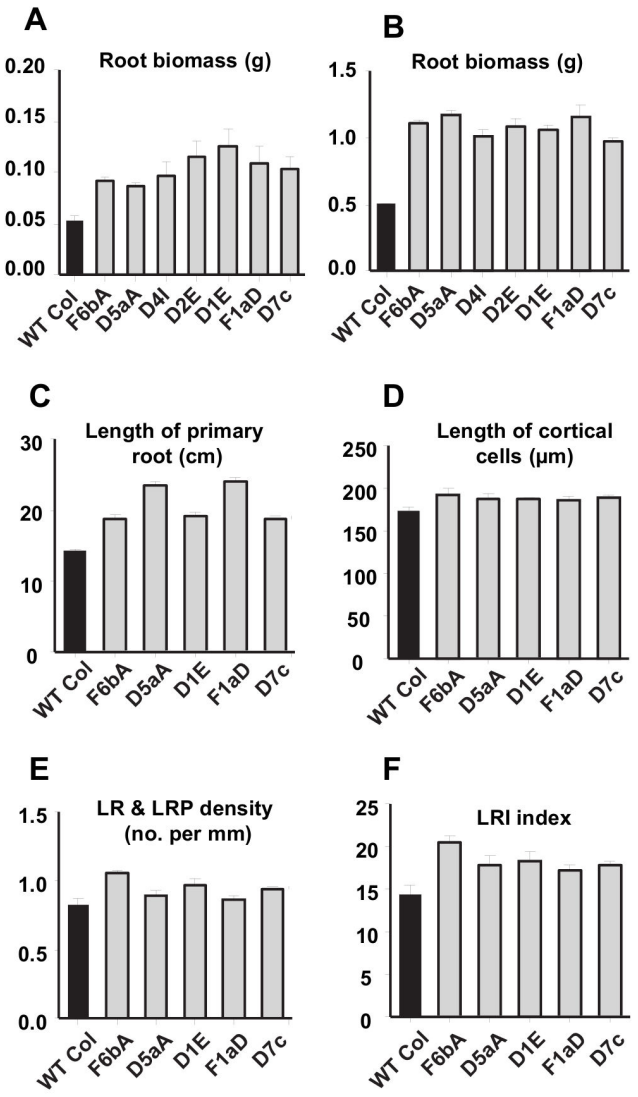

Figure 2. Root growth in SDG8i lines and wild-type Col-0 plants. Root biomass (FW) of pre-flowering plants growing in (A) long day at 21°C, measured 16 days after germination and (B) short day at 21°C, measured 50 days after germination. Values are the means ± SE of 4 replicates.

Root development in 13-d-old *Arabidopsis* plants grown on vertical plates at 21°C under SD showing; (C) length of primary root and (D) length of fully elongated cortical cells and (E) lateral root primordium density and (F) lateral root initiation index. Values are the means ± SE of 4 replicates.

doi: 10.1371/journal.pone.0080035.g002

distribution, the SDG8i transgenics were crossed with plants containing the *DR5-GUS* reporter construct [28] and the progeny analyzed for histochemical GUS activity [22]. While GUS activity appeared slightly elevated during early growth stages in SDG8i transgenics when compared with controls, the difference in staining was quite pronounced at the four leaf stage with transgenics showing much more GUS activity in the root tips and the vascular tissue of shoots and high level GUS

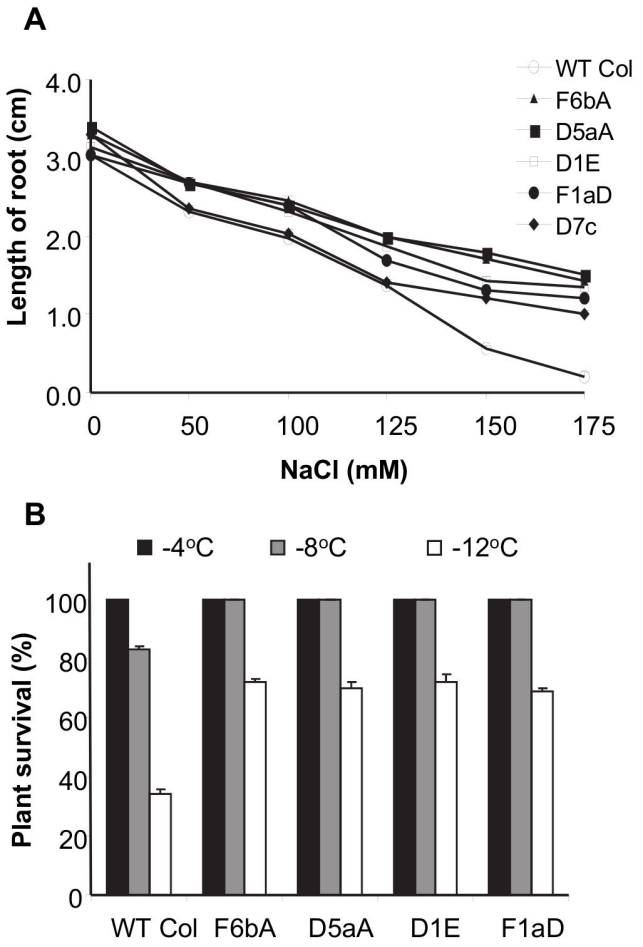

Figure 3. Salt and freezing stress tolerance of SDG8i lines. (A) Effect of salt on the root growth of SDG8i lines and wild-type Col-0. Root growth was measured after 7 days following transfer to NaCl. Values are the means ± SE of 10 replicates.

(B) Freezing tolerance of SDG8i lines and wild-type Col-0. Plants were exposed to freezing at -12°C after cold-acclimation at -1°C for 16 hours. Survival was scored 7 days after the plants were returned to normal growth conditions at 22°C. Values are the means ± SE of 3 replicates.

doi: 10.1371/journal.pone.0080035.g003

activity at the leaf margins (Fig. S5). These results suggest that endogenous auxin levels are elevated in SDG8i plants.

SDG8i plants exhibit salt and freezing tolerance

A comparison of the response of SDG8i transgenics and wild-type plants to growth on high salt media (Figure 3A) showed no substantial difference at salt concentrations below 100 mM after 7 days growth. However, at 150 mM NaCl all transgenic lines showed 2-3 times more primary root growth than that of wild-type plants (p<0.001). At 175 mM NaCl wild-type plants showed severe inhibition of root growth (Figure 3A and Fig. S6A) whereas the root growth of transgenic lines was 5-7 times that of wild-type plants (p<0.001).

A freezing tolerance test was also performed on the transgenic and wild-type control plants (Figure 3B and Fig. S6B). All transgenic and control wild-type Col-0 plants exposed to -4°C freezing stress survived. At -8°C, all SDG8i transgenic lines survived, whereas the survival rate of wild-type Col-0 plants was 83% (p<0.01). At -12°C, control wild-type Col-0 plants were affected greatly with a survival rate of only 34%, whereas the survival rate of the transgenic plants ranged from 69-72% (p<0.01).

SDG8i transgenics are drought tolerant

The response of SDG8i transgenics to water-deficit was compared to that of wild-type plants. Plants were kept fully watered by sub-irrigation until the 6-7 leaf pre-flowering stage. Water was then withheld over a period of 3 weeks, then the plants were re-watered and recovery monitored after 24 hours. In the wild-type Col-0 plants, wilting was visible after 7 days and after 13 days of drought all control plants were severely dehydrated and showed signs of senescence, whereas all SDG8i transgenic plants were still green and healthy (Figure 4A, B). The survival rate of wild-type plants was 50% after 11 days (p<0.001) and by the thirteenth day had dropped to zero. On the other hand, the survival rate of transgenic plants was 100% after 13 days and then reduced to 50% after 17 days. All the transgenic plants were severely dehydrated on day 18 and they did not recover after one further day of drought treatment.

To quantify the increase in drought tolerance conferred by SDG8i expression, a measure of protoplasmic drought tolerance (PDT; determined as the percent lowest relative humidity at which 50% of leaf cells survive) was obtained. The PDT of wild-type plants, as determined by both the subjective method (Figure 4C) and the objective method (Figure 4D) was ~97-98% RH (~-2.8 to -4.2MPa). This indicates that *Arabidopsis* is very sensitive to water deficit, which is not surprising in the thin tender leaves on well-watered greenhouse plants of an ephemeral species. The three transgenic lines gave closely similar data to each other in both methods. Their PDT value of ~94-95% RH (~-7.0 to -8.4MPa) is considerably better than the value obtained for wild-type plants, indicating a substantial improvement of PDT (p<0.05).

SDG8i activity delays dark-induced senescence and acts downstream of ABA

To test the effect of SDG8i activity on senescence, detached leaves from wild-type and transgenic plants were floated on ddH_2O in the dark and chlorophyll degradation monitored over several days.

After five days in darkness the chlorophyll content and the photochemical efficiency (F_v/F_m) values of wild-type plants decreased to around 20% (p<0.001) (Figure 5A, B) and the leaves turned completely yellow. In the SDG8i transgenic lines the leaves remained green after five days, retaining about 55-60% of their chlorophyll, with F_v/F_m values of about 52-53% of the original reading (p<0.05) (Figure 5A, B). These results indicate that SDG8i transgenic plants exhibit reduced dark-induced senescence.

To test the relationship between SDG8i activity and the senescence-promoting phytohormone ABA, we repeated the

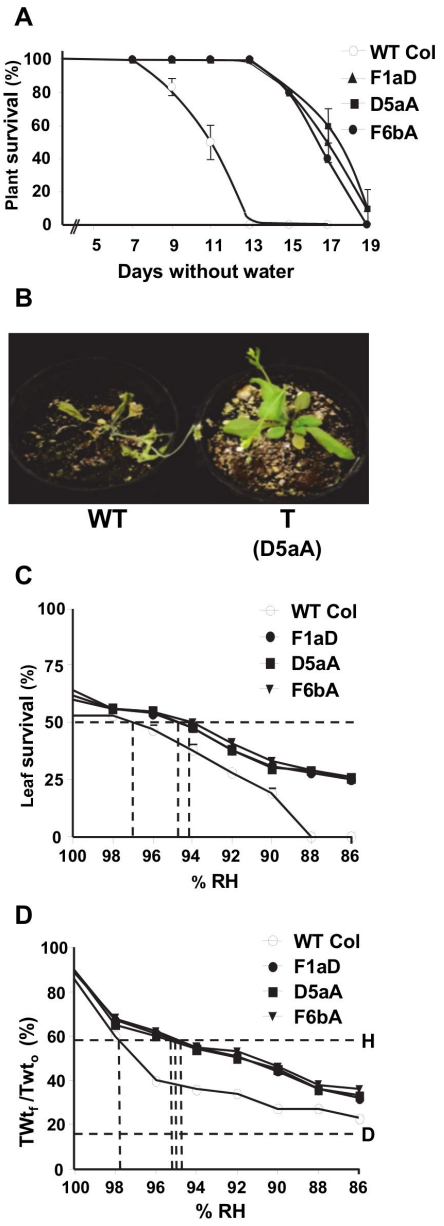

Figure 4. Drought tolerance of SDG8i plants. (A) Plants were deprived of water, re-watered at intervals and monitored for recovery as described in the text. Values are the means ± SE of 2 replicates.

(B) Wild-type Col-0 (WT) and a transgenic plant (T) after 13 days without water.

(C) Leaf survival and (D) PDT survival curve [% of final saturation weight to the initial turgid weight ratio] showing the effect of decreasing relative humidity on cell survival of SDG8i and WT shoots. PDT value is the water potential (expressed as the corresponding equilibrium relative humidity at 20°C) at which 50% of cells are dead after 3 days vapor equilibration over $CaCl_2$ osmotica. Line D represents the lowest value of TWt_f/TWt_0 reached by comparable shoots killed by chloroform vapor. Line H is the 50% survival point halfway between line D and 100% TWt_f/TWt_0.

doi: 10.1371/journal.pone.0080035.g004

assay under continuous light with, and without, the exogenous hormone (Figure 5C, D). Over the five days incubation in continuous light without ABA, the chlorophyll content of control wild-type leaves reduced to 54%, whereas SDG8i transgenic leaves retained around 71% of their chlorophyll content. The F_v/F_m values for both transgenic and control plants were not significantly different under these conditions. Upon treatment with ABA, the chlorophyll content of wild-type leaves decreased rapidly from day two down to 20% by day five ($p<0.001$) (Figure 5C). On the other hand, the SDG8i transgenic leaves retained about 75% of their chlorophyll up to day four and then reduced to around 58% by day five ($p<0.05$) (Figure 5C). The F_v/F_m value of SDG8i transgenics was also substantially higher than control plants on day five ($p<0.005$) (Figure 5D). These results show that SDG8i expression reduces ABA-induced senescence and suggests that it functions downstream of ABA.

SDG8i encodes a UGT that glycosylates GR24 *in vitro*

The phenotype of the transgenic plants suggests that SDG8i UGT activity is influencing hormone homeostasis. To investigate the catalytic function of SDG8i, the UGT activity of *Nicotiana benthamiana* leaf extracts infiltrated with an actin (*AtACT2*)-promoter driven *SDG8i* construct, using a viral-based system [19], was tested against a number of plant hormones as substrates and compared with UGT activity in extracts infiltrated with a vector-only control. The substrates used were chosen for their known ability to affect plant growth and stress responses. The SDG8i extract showed substantial glycosylation activity of the strigolactone analogue GR24 (Figure 6A) with a K_m of 0.349 mM and a V_{max} of 5.67 µmole/min/mg (Figure 6B). The activity observed with the other substrates showed very little increase over background endogenous NADH oxidase activity. Similarly, the vector-only control extract showed no substantial activity above background with GR24 or with any of the other substrates. The results indicate that *SDG8i* encodes a glucosyltransferase with *in vitro* activity against a strigolactone-like compound.

The stimulation of *Orobanche* germination is reduced in *Arabidopsis* expressing SDG8i

Root exudates from *Arabidopsis* can stimulate the germination of *Phelipanche ramosa* (*Orobanchaceae*) [29]. The *Orobanchaceae* germination bioassay has been used extensively to determine the level of strigolactone secreted from host plant roots [30]. To determine if SDG8i activity has an effect on the root secretion of these stimulus signals that are required by parasitic plants to germinate, four SDG8i transgenic *Arabidopsis* lines were compared with wild-type Col-0 *Arabidopsis* plants for the ability to stimulate *Orobanche* germination in axenic culture (Figure 6C and Fig. S7). GR24, *Sorghum bicolor* and *S. stapfianus* were included as controls. Germination of 95% was achieved by treatment with GR24, while 11% germination was observed in the water control. The levels of *Orobanche* seed germination stimulated by sorghum and *S. stapfianus* was 71% and 52% respectively. The 60% germination of *Orobanche* seeds by wild-type Col-0 plants was significantly higher than that of the all the transgenic lines tested ($p<0.001$) with SDG8i transgenic lines stimulating

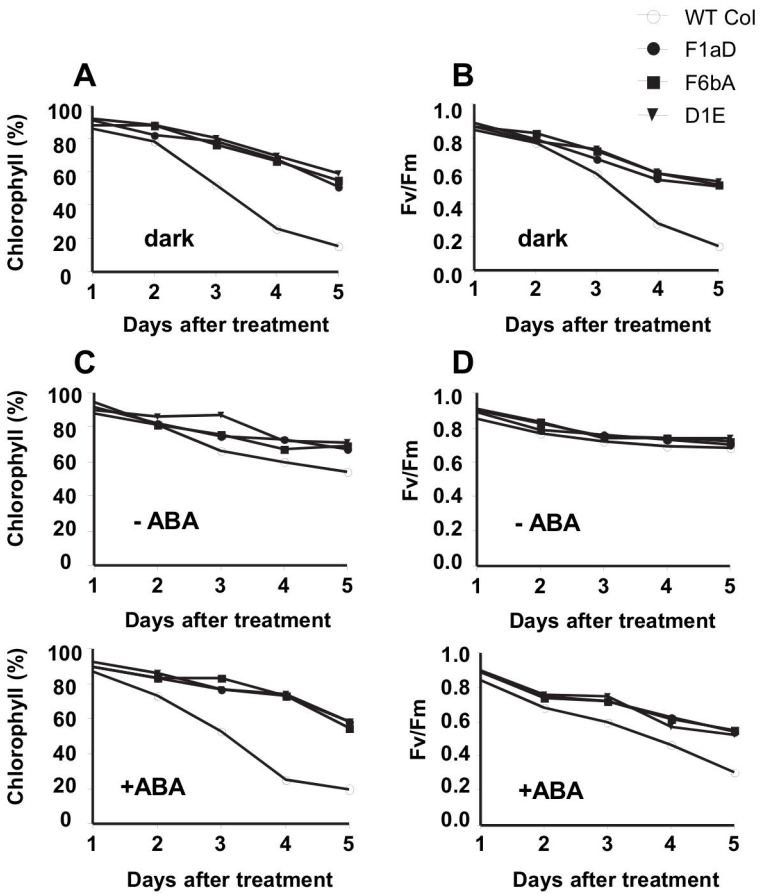

Figure 5. Dark- and ABA-induced senescence in wild-type and SDG8i leaves. (A) Chlorophyll content and (B) photochemical efficiency in dark-treated detached leaves of WT and SDG8i plants.
(C) Chlorophyll content and (D) photochemical efficiency in detached leaves of WT and SDG8i plants under continuous light in the absence or presence of 50 μM ABA.
doi: 10.1371/journal.pone.0080035.g005

42-47% germination. The transgenic line, F6bA, showed the highest reduction in *Orobanche* germination (30%) compared with the wild-type Col-0 *Arabidopsis* control. These results suggest that SDG8i activity may be reducing the level of strigolactone germination stimulants secreted from the roots of *Arabidopsis.*

GR24 inhibition of shoot branching is reduced in SDG8i transgenic plants

The branching response of wild-type Col-0, SDG8i transgenic and the branching mutant *max2 Arabidopsis* plants were tested by the application of GR24 to leaf axils and axillary buds before and during bolting as described by Gomez-Roldan et al. [31] in order to see whether they are able to respond to SL-mediated shoot branching inhibition (Figure 6D). In wild-type Col-0 plants, shoot branching decreased by 55% in response to GR24 compared to control plants ($p<0.001$). However, in all SDG8i transgenic plants and *max2* mutants, application of GR24 decreased shoot branching by only 10-15% compared to untreated plants ($p<0.001$) with branching remaining elevated over treated or untreated wild-type Col-0 plants.

Discussion

The phenotypic changes mediated by overexpression of *SDG8i* suggests the enzyme may affect strigolactone-related processes

Several UGTs, capable of influencing the biological activity of plant hormones via glycosylation, have been isolated [9-15]. The ability of SDG8i recombinant protein to glycosylate the synthetic strigolactone analogue GR24 *in vitro*, combined with the phenotypic changes observed in SDG8i overexpressing lines, suggests that SDG8i glucosyltransferase activity may be effecting the strigolactone signalling pathway in the transgenic plants. As well as mediating the interactions of host plants with symbiotic fungi or parasitic plants, the well-conserved strigolactone hormone signaling system contributes to environmental regulation of plant growth. Strigolactones have been shown to respond to environmental cues, such as low

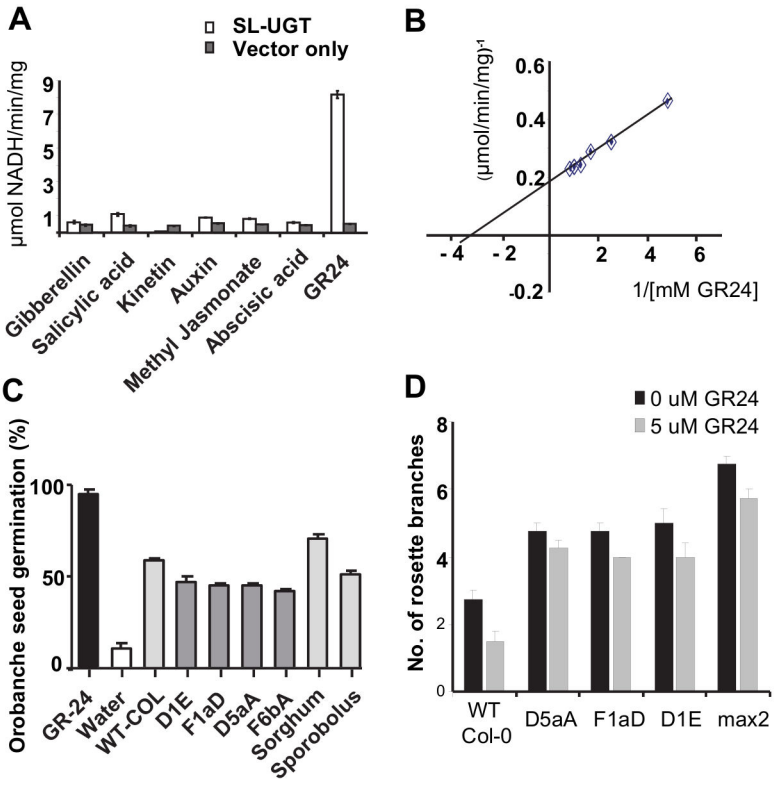

Figure 6. Analysis of the enzyme activity of *SDG8i in vitro*. (A) The glycosyltransferase activity of SDG8i recombinant protein extract using the plant hormones gibberillin (GA3), salicylic acid, kinetin, auxin (IAA), methyl jasmonate, ABA([±]-*cis*, trans-abscisic acid and the synthetic strigolactone analogue GR24 as substrate ($p < 0.05$). The coupled enzyme assay [9] was conducted at pH 7.4 using 25µg of protein extract with a final substrate concentration of 1mM. Rates were calculated per mg total protein extract. GR24 was obtained from Chiralix B.V. Nijmegen,The Netherlands. All other hormones were obtained from Sigma St. Louis, MO.
(B) A Lineweaver-Burke plot of SDG8i activity with varying concentrations of GR24 indicating a Km of 0.349mM and a Vmax of 5.67 µmole/min/mg. Rates were calculated per mg total protein extract.
(C) Level of stimulation of germination of *Orobanche* seeds in response to root induction by wildtype Col-O and SDG8i transgenic *Arabidopsis*, sorghum and *S. stapfianus* seedlings in vitro. The percent germination was calculated by counting the number of seeds having an emerged radicle. Values are the means ± SE of 5 replicates.
(D) Effect of GR24 (0 or 5 µM) on bud outgrowth of wild-type Col-0, SDG8i transgenic and max2 *Arabidopsis* plants. Plants were treated with GR24 on the rosette shoot meristem, axillary buds and leaf axils every third day for 20 days and the number of branches was counted after 43 days. Data are means ± 4 replicates.
doi: 10.1371/journal.pone.0080035.g006

nutrient conditions (Pi, N), to interact with abscisic acid (ABA), and to be involved in growth-related auxin and ethylene activity [32-35]. Strigolactones are carotenoid-derived terpenoid lactones that are synthesized mainly in the roots and can be transported in the xylem sap to the shoots [29]. In some plant species, mutations in strigolactone production or perception genes have been shown to delay flowering time, reduce senescence and decrease root mass [36,37]. Root exudates containing strigolactones can stimulate parasitic *Striga* and *Orobanche* plant germination and hyphal branching of symbiotic arbuscular mycorrhizal fungi [38]. Strigolactones have been shown to regulate root growth in response to phosphate and/or carbohydrate availability [39,40]. In the shoots, increased strigolactone levels inhibit tiller formation or lateral bud outgrowth [29,41] and mutations in the strigolactone biosynthesis and signaling pathway are often associated with a hyperbranched, dwarfed phenotype [32,33].

Several characteristics of the SDG8i ectopic expression lines mimic those of the strigolactone MAX mutants, including hyperbranching, reduced senescence and interactions with auxin and ABA [33,34,37]. However, the reduced stimulation by SDG8i plants of *Orobanche* germination, indicative of diminished strigolactone levels [30], is modest and the SDG8i enhanced shoot and root growth phenotypes are opposed to those of SL-deficient mutants which are usually dwarfed. While the moderate affinity of SDG8i for GR24 could be expected given the artificial nature of the substrate and assay conditions, it is clear that the SDG8i enzyme in *Arabidopsis* is affecting the bioactivity of an endogenous growth-related compound/s. MAX2/ORE9, the F-box leucine-rich repeat signaling protein is

expressed in the vasculature and plays multiple roles in different parts of the plant, not all of which are dependent on the activity of the SL branching signal generated by MAX3 and MAX4 [42]. Several of the processes associated with MAX2 activity appear to be negatively affected in SDG8i plants. In addition to auxin interactions and suppression of branching, MAX2 acts to promote leaf senescence [37] and repress hypocotyl elongation in the light [43] and is also involved in oxidative stress and drought responses [44,45]. Hence the phenotype of SDG8i transgenic plants (which includes elongated hypocotyls) appears to be consistent with a reduction in the activity of MAX2. The reduced branching inhibition in SDG8i plants by GR24 application also supports this contention. This would suggest that crossing SDG8i transgenics with MAX3 or MAX4 mutants would be unlikely to lead to suppression of the mutant phenotypes, however crossing SDG8i transgenics with plants overexpressing MAX2 may be informative in elucidating the mode of action of SDG8i. The genetic analyses with strigolactone mutants and an examination of expression levels of MAX pathway genes are underway, however, full comprehension of the mode of SDG8i action requires identification and structural analysis of the endogenous target metabolite/s. Identification of the metabolite/s could substantially enhance our knowledge of how environmental influences regulate plant growth. Since glycosylation is widely accepted as having a role in bioactivity, transport and stability of many growth regulators [7], an effort to identify an endogenous enzyme with similar SDG8i activity in *Arabidopsis* and other plants may be worthwhile, although substrate specificity of UGTs is not necessarily reflected in gene phylogeny and *in vitro* substrate screening of recombinant UGTs is usually required to identify functionally similar UGTs between species [8].

SDG8i activity may impede the stress-related growth retardation response

Plant productivity, even when plants are growing under near-ideal conditions, can be limited by the innate response of plants to seasonal influences or short-term stress events. These responses often involve reduced growth and/or reallocation of resources into less desirable growth patterns. The imposition of stress below toxic levels can elicit apoptosis or growth redistribution within minutes to diminish stress exposure [46]. The pathways integrating environmental stressors with inhibition of internal growth-related processes in plants is not well understood [47] and the introduction of stress-related genes to increase constitutive stress tolerance often correlates with diminished growth. In contrast, SDG8i activity promotes cell expansion and division in shoots and roots under both stress and non-stress conditions. In the resurrection grass *S. stapfianus*, SDG8i gene activity, following transcript accumulation in desiccated tissue, may be associated with leaf regeneration, which is more than twice as rapid following a dehydration/rehydration cycle as in a well-watered plant [4]. The enhanced growth exhibited by SDG8i transgenic *Arabidopsis* plants under stress, suggests that SDG8i activity may impede the growth retardation response which would normally occur under these conditions. A reduction in the ability of a plant to initiate morphogenic changes in response to environmental influences could be expected to compromise the survival of wild species over long-term stress events. However, substantial growth advantages may be conferred in the context of crop cultivation with ample mineral nutrition and shorter-term stress events. The phenotype of the SDG8i transgenic plants provide an important example of the potential of bioengineering to enhance shoot-root biomass and seed yield in plants, whilst simultaneously conferring substantial improvements in salt, cold and drought resistance. The ability to utilize SDG8i activity to control stress-related and seasonal morphogenic responses in crops could represent a substantial advance in domestication of food plants.

The *SDG8i*-mediated drought-tolerance improvement may be associated with inhibition of the drought-related senescence program

Considerable research effort has been put into increasing crop production utilizing the isopentenyltransferase (*IPT*) gene to increase cytokinin biosynthesis. Increased cytokinin levels can delay drought-induced senescence and allow retention of higher chlorophyll levels under water-deficit [48-50]. The problems associated with lower yields due to altered source sink distribution appears to have been overcome by utilizing a senescence associated receptor kinase (SARK) promoter to drive the *IPT* gene [51-53]. Use of the *SDG8i* gene presents an alternative approach to reducing drought-induced senescence and potentially has the additional benefit of increasing yield under non-stress conditions. The two-fold improvement in the water potential survived by the SDG8i transgenic lines, compared with the untransformed wild-type *Arabidopsis* plants, is considerable and is similar to the maximum 'drought hardening' reported for a crop plant droughted for 3 weeks [54]. The reduced senescence displayed by transgenic SDG8i plants suggests that SDG8i activity may also be associated with inhibition of dehydration-induced senescence programs, a phenomenon that occurs in the younger leaves of the desiccation-tolerant *S. stapfianus* plant. In non-resurrection plants, leaf senescence is thought to be an efficient strategy for surviving water-deficit by reducing canopy size and transpiration [55]. Interestingly, SDG8i transgenic plants showed no sign of senescence after 13 days without watering yet survived much better than wild-type plants. The droughted wild-type plants ceased vegetative growth, the leaves senesced rapidly, and the plants produced an inflorescence of 2-3 cm before dying. The transgenics on the other hand remained green and healthy. Some transgenics formed one or more extra rosette leaves and a reproductive meristem during the drying stage, but did not appear to accelerate reproductive development. The reduced senescence, in combination with the larger leaves, may be related to the higher seed yield of the SDG8i transgenic plants.

The use of SDG8i to generate a more robust productive plant with enhanced growth and stress resistance, combined with the benefit of reduced stimulation of parasitic seed germination, could herald a novel approach for increasing food production in agriculturally important crop plants.

Supporting Information

Figure S1. RNA gel-blot analysis showing the presence of *SDG8i* transcripts in SDG8i transgenic lines.
(TIFF)

Figure S2. Flower morphology, seed development and growth characteristics, of wild-type Col-0 plants (WT) and SDG8i plants (T) growing at 21°C in LD conditions.
(TIFF)

Figure S3. Growth characteristics of SDG8i plants growing in short days.
(TIFF)

Figure S4. Palisade cells in the second fully expanded rosette leaves of wild-type Col-0 and SDG8i transgenic lines.
(TIFF)

Figure S5. Histochemical staining showing differential GUS activity at various stages of development typical of wild-type Col-0 plants and SDG8i transgenic (D5aA) plants crossed with *DR5::GUS Arabidopsis* seedlings.
(TIFF)

Figure S6. Salt and freezing stress tests of wild-type Col-0 (WT) and SDG8i transgenic (T) *Arabidopsis* seedlings in vitro.
(TIFF)

Figure S7. Stimulation of germination of *Orobanche* seeds.
(TIFF)

Acknowledgements

We thank Dr John Matthews for assistance with *Orobanche*.

Author Contributions

Conceived and designed the experiments: ADN CKB JDH DFG. Performed the experiments: SI CAG TNL CKB ADN. Analyzed the data: ADN SI CAG CKB DFG. Contributed reagents/materials/analysis tools: ADN JDH. Wrote the manuscript: ADN SI CAG CKB TNL JDH.

References

1. Gaff DF, Blomstedt CK, Neale AD, Le TN, Hamill JD et al. (2009) *Sporobolus stapfianus*, a model desiccation-tolerant grass. Funct Plant Biol 36: 589-599. doi:10.1071/FP08166.
2. Neale AD, Blomstedt CK, Bronson P, Le TN, Guthridge K et al. (2000) The isolation of genes from the resurrection grass Sporobolus stapfianus which are induced during severe drought stress. Plant Cell Environ 23: 265-277. doi:10.1046/j.1365-3040.2000.00548.x.
3. Oliver MJ, Guo L, Alexander DC, Ryals JA, Wone BWM et al. (2011) A sister group contrast using untargeted global metabolomic analysis delineates the biochemical regulation underlying desiccation tolerance in Sporobolus stapfianus. Plant Cell 23: 1231-1248. doi:10.1105/tpc.110.082800. PubMed: 21467579.
4. Blomstedt CK, Griffiths CA, Fredericks DP, Hamill JD, Gaff DF et al. (2010) The resurrection plant *Sporobolus stapfianus*: An unlikely model for engineering enhanced plant biomass? Plant Growth Regul 62: 217-232. doi:10.1007/s10725-010-9485-6.
5. Le TN, Blomstedt CK, Kuang J, Tenlen J, Gaff DF et al. (2007) Desiccation-tolerance specific gene expression in leaf tissue of the resurrection plant *Sporobolus stapfianus*. Funct Plant Biol 34: 589-600. doi:10.1071/FP06231.
6. Ross J, Li Y, Lim E-K, Bowles DJ (2001) Higher plant glycosyltransferases. Genome Biol 2: 3004: 3001-3004.3006. PubMed: 11182895
7. Lim E-K, Bowles DJ (2004) A class of plant glycosyltransferases involved in cellular homeostasis. EMBO J 23: 2915-2922. doi:10.1038/sj.emboj.7600295. PubMed: 15241472.
8. Bowles D, Lim E-K (2010) Glycosyltransferases of small molecules: Their roles in plant biology. Encyclopedia of Life Sciences Chichester. John Wiley & Sons.
9. Jackson RG, Lim E-K, Li Y, Kowalczyk M, Sandberg G et al. (2001) Identification and biochemical characterization of an Arabidopsis indole-3-acetic acid glucosyltransferase. J Biol Chem 276: 4350-4356. doi:10.1074/jbc.M006185200. PubMed: 11042207.
10. Lim E-K, Doucet CJ, Li Y, Elias L, Worrall D et al. (2002) The activity of Arabidopsis glycosyltransferases toward salicylic acid, 4-hydroxybenzoic acid, and other benzoates. J Biol Chem 277: 586-592. PubMed: 11641410.
11. Lim E, Doucet CJ, Hou B, Jackson RG, Brams SR, et al. (2005) Resolution of (+)-abscisic acid using an *Arabidopsis* glycosyltransferase. Tetrahedron Asymmetry 16: 143-147. doi:10.1016/j.tetasy.2004.11.062.
12. Hou B, Lim E-K, Higgins GS, Bowles DJ (2004) N-glucosylation of cytokinins by glycosyltransferases of *Arabidopsis thaliana*. J Biol Chem 279: 47822-47832. doi:10.1074/jbc.M409569200. PubMed: 15342621.
13. Poppenberger B, Fujioka S, Soeno K, George GL, Vaistij FE et al. (2005) The UGT73C5 of *Arabidopsis thaliana* glucosylates brassinosteroids. Proc Natl Acad Sci U_S_A 102: 15253-15258. doi: 10.1073/pnas.0504279102. PubMed: 16214889.
14. Song J (2005) Biochemical characterization of an *Arabidopsis* glucosyltransferase with high activity toward jasmonic acid. J Plant Biol 48: 422-428. doi:10.1007/BF03030584.
15. Tognetti VB, Van Aken O, Morreel K, Vandenbroucke K, van de Cotte B et al. (2010) Perturbation of Indole-3-Butyric Acid Homeostasis by the UDP-Glucosyltransferase UGT74E2 Modulates Arabidopsis Architecture and Water Stress Tolerance. Plant Cell 22: 2660-2679. doi:10.1105/tpc.109.071316. PubMed: 20798329.
16. Weigel D, Glazebrook J (2002) *Arabidopsis*: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
17. Curtis MD, Grossniklaus U (2003) A Gateway Cloning Vector Set for High-Throughput Functional Analysis of Genes in Planta. Plant Physiol 133: 462-469. doi:10.1104/pp.103.027979. PubMed: 14555774.
18. Martinez-Trujillo M, Limones-Briones V, Cabrera-Ponce JLC, Herrera-Estrella L (2004) Improving transformation efficiency of *Arabidopsis thaliana* by modifying the floral dip method. Plant Mol Biol Rep 22: 63-70. doi:10.1007/BF02773350.
19. Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y (2005) Systemic *Agrobacteriun tumifaciens*-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol 23: 718-723. doi:10.1038/nbt1094. PubMed: 15883585.
20. Sambrook J, Fritsch EF, Maniatus T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
21. Dubrovsky JG, Soukup A, Napsucialy-Mendivil S, Jeknic´ Z, Ivanchenko MG (2009) The lateral root initiation index: an integrative measure of primordium formation. Ann Bot 103: 807-817. PubMed: 19151042.
22. Jefferson RA (1987) Assay for chimeric genes in plants: The GUS fusion system. Plant Mol Biol Rep 5: 387-405. doi:10.1007/BF02667740.
23. Ghasempour HR, Anderson EM, Gaff DF (2001) Effects of growth substances on the protoplasmic drought tolerance of leaf cells of the resurrection grass *Sporobolus stapfianus*. Aust J Plant Physiol 28: 1115-1120.

24. Owen P (1952) The relation of germination of wheat to water potential. J Exp Bot 3: 188-203. doi:10.1093/jxb/3.2.188.
25. Lichtenthaler H (1987) Chlorophylls and carotenoids: pigments of photosynthetic membranes. Methods Enzymol 148: 350-382. doi: 10.1016/0076-6879(87)48036-1.
26. Woodward AW, Bartel B (2005) Auxin: Regulation, Action, and Interaction. Ann Bot 95: 707-735. doi:10.1093/aob/mci083. PubMed: 15749753.
27. Bai F, DeMason DA (2008) Hormone interactions and regulation of PsPK2::GUS compared with DR5::GUS and PID::GUS in *Arabidopsis thaliana*. Am J Bot 95: 133-145. doi:10.3732/ajb.95.2.133. PubMed: 21632339.
28. Ulmasov T, Murfett JGH, Guilfoyle TJ (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9: 1963-1971. doi: 10.1105/tpc.9.11.1963. PubMed: 9401121.
29. Kohlen W, Charnikhova T, Liu Q, Bours R, Domagalska MA et al. (2011) Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in non-AM host Arabidopsis thaliana. Plant Physiol 155: 974-987. PubMed: 21119045.
30. López-Ráez JA, Charnikhova T, Gómez-Roldán V, Matusova R, Kohlen W et al. (2008) Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. New Phytol 178: 863-874. doi:10.1111/j.1469-8137.2008.02406.x. PubMed: 18346111.
31. Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pagès V, Dun EA et al. (2008) Strigolactone inhibition of shoot branching. Nature 455: 189-194. doi:10.1038/nature07271. PubMed: 18690209.
32. Dun EA, Brewer PB, Beveridge CA (2009) Strigolactones: discovery of the elusive shoot branching hormone. Trends Plant Sci 14: 364-372. doi:10.1016/j.tplants.2009.04.003. PubMed: 19540149.
33. Leyser O (2009) The control of shoot branching: an example of plant information processing. Plant Cell Environ 32: 694-703. doi:10.1111/j.1365-3040.2009.01930.x. PubMed: 19143993.
34. López-Ráez JA, Kohlen W, Charnikhova T, Mulder P, Undas AK et al. (2010) Does abscisic acid affect strigolactone biosynthesis. New Phytol 187: 343-354. doi:10.1111/j.1469-8137.2010.03291.x. PubMed: 20487312.
35. Koltai H (2011) Strigolactones are regulators of root development. New Phytol 190: 545-549. doi:10.1111/j.1469-8137.2011.03678.x. PubMed: 21638793.
36. Snowden KC, Simkin AJ, Janssen BJ, Templeton KR, Loucas HM et al. (2005) The Decreased apical dominance1/*Petunia hybrida* CAROTENOID CLEAVAGE DIOXYGENASE8 Gene Affects Branch Production and Plays a Role in Leaf Senescence, Root Growth, and Flower Development. Plant Cell 17: 746-759. doi:10.1105/tpc.104.027714. PubMed: 15705953.
37. Woo HR, Chung KM, Park J-H, Oh SA, Ahn T et al. (2001) ORE9, an F-box trotein that regulates leaf senescence in *Arabidopsis*. Plant Cell 13: 1779-1790. doi:10.2307/3871318. PubMed: 11487692.
38. Akiyama K, Hayashi H (2006) Strigolactones: chemical signals in fungal symbionts and parasitic weeds in plant roots. Ann Bot, 97: 925–931: 925–931 PubMed: 16574693.
39. Kapulnik Y, Delaux P-M, Resnick N, Mayzlish-Gati E, Wininger S et al. (2011) Strigolactones affect lateral root formation and root hair elongation in Arabidopsis. Planta 233: 209-216. doi:10.1007/s00425-010-1310-y. PubMed: 21080198.
40. Ruyter-Spira C, Kohlen W, Charnikhova T, van Zeijl A, van Bezouwen L et al. (2011) Physiological Effects of the Synthetic Strigolactone Analog GR24 on Root System Architecture in Arabidopsis: Another Belowground Role for Strigolactones? Plant Physiol 155: 721-734. PubMed: 21119044.
41. Umehara M, Hanada A, Magome H, Takeda-Kamiya N, Yamaguchi S (2010) Contribution of strigolactones to the inhibition of tiller bud outgrowth under phosphate deficiency in rice. Plant Cell Physiol 51: 1118-1126. doi:10.1093/pcp/pcq084. PubMed: 20542891.
42. Stirnberg P, Furner IJ, Leyser HMO (2007) MAX2 participates in an SCF complex which acts locally at the node to suppress shoot branching. Plant J 50: 80-94. doi:10.1111/j.1365-313X.2007.03032.x. PubMed: 17346265.
43. Stirnberg P, van de Sande K, Leyser HMO (2002) *MAX1* and *MAX2* control shoot lateral branching in *Arabidopsis*. Development 129: 1131-1141. PubMed: 11874909.
44. Woo HR, Kim JH, Nam HG, Lim PO (2004) The delayed leaf senescence mutants of Arabidopsis. Ore1: ore3, and ore9 are tolerant to oxidative stress. Plant Cell and Physiology 45: 923-932.
45. Tang D, Christiansen KM, Innes RW (2005) Regulation of plant disease resistance, stress responses, cell death, and ethylene signaling in *Arabidopsis* by the EDR1 protein kinase. Plant Physiol 138: 1018-1026. doi:10.1104/pp.105.060400. PubMed: 15894742.
46. Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MAK (2007) Stress-induced morphogenic responses: growing out of trouble? Trends Plant Sci 12: 98-105. doi:10.1016/j.tplants.2007.01.004. PubMed: 17287141.
47. Atkinson NJ, Urwin PE (2012) The interaction of plant biotic and abiotic stresses: from genes to the field. J Exp Bot 63: 3523-3543. doi: 10.1093/jxb/ers100. PubMed: 22467407.
48. Gan S, Amasino RM (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270: 1986-1988. doi: 10.1126/science.270.5244.1986. PubMed: 8592746.
49. Jordi W, Schapendonk CM, Davelaar E, Stoopen GM, Pot CS et al. (2000) Increased cytokinin levels in transgenic *PSAG12-IPT* tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning. Plant Cell Environ 23: 279-289. doi: 10.1046/j.1365-3040.2000.00544.x.
50. Sýkorová B, Kurešová G, Daskalova S, Trčková M, Hoyerová K et al. (2008) Senescence-induced ectopic expression of the *A. tumefaciens ipt* gene in wheat delays leaf senescence, increases cytokinin content, nitrate influx, and nitrate reductase activity, but does not affect grain yield. J Exp Bot 59: 377-387. doi:10.1093/jxb/erm319. PubMed: 18267946.
51. Hajouj T, Michelis R, Gepstein S (2000) Cloning and characterization of a receptor-like protein kinase gene associated with senescence. Plant Physiol 124: 1305-1314. doi:10.1104/pp.124.3.1305. PubMed: 11080306.
52. Rivero RM, Kojima M, Gepstein A, Sakakibara H, Mittler R et al. (2007) Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proc Natl Acad Sci U_S_A 104: 19631-19636. doi: 10.1073/pnas.0709453104. PubMed: 18048328.
53. Kuppu S, Mishra N, Hu R, Sun L, Zhu X et al. (2013) Water-deficit inducible expression of a cytokinin biosynthetic gene IPT improves drought tolerance in cotton. PLOS ONE 8: e64190. doi:10.1371/journal.pone.0064190. PubMed: 23675526.
54. Levitt J, Sullivan CY, Krull E (1960) Some problems in drought resistance. Bull Research Council Of Israel 8D: 177-179.
55. Chaves MM, Maroco JP, Pereira JS (2003) Understanding plant responses to drought - from genes to the whole plant. Funct Plant Biol 30: 239-264. doi:10.1071/FP02076.