Identification and characterization of three *Arabidopsis SUGAR INSENSITIVE* genes

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Dedicated in loving memory of my mother.

Abstract

Carbohydrates have signaling functions in regulating gene expression, metabolic pathways and developmental processes. Eukaryotic organisms have evolved conserved and novel mechanisms for sensing and responding to sugars. Plant sugar response pathways are complex and exhibit cross-talk with other response pathways.

Sugar responses and signaling pathways have been studied via physiological, biochemical and genetic approaches. Genetic screens have identified sugar response mutants with altered seedling growth phenotypes. The Gibson lab has isolated an array of *sugar insensitive* (*sis*) mutants by screening mutagenized *Arabidopsis* seeds on high concentrations of sugars. The identification and characterization of three of the *SIS* genes, *SIS7*, *SIS3* and *SIS8*, are presented here.

SIS7 is allelic to *NCED3/STO1*, an abscisic acid (ABA) biosynthetic gene, which is involved in drought and salt stress responses. Lateral root (LR) development of *sis7* mutants is resistant to the inhibitory effects of osmotica. Transcriptomic analysis revealed that a set of auxin-related genes are expressed at lower levels in *sis7* seeds than in wild-type seeds when incubated with glucose, suggesting that these genes may be involved in controlling LR development by both ABA and auxin.

SIS3 encodes a RING finger protein that functions as an E3 ligase in *in vitro* ubiquitination assays. The *sis3* seeds display wild-type germination responses to ABA and GA. However, the root growth of *sis3* mutants has slightly reduced sensitivity to ABA.

The *sis8* mutants have decreased sensitivity to high sugars and hyperosmolarity. Positional cloning of *sis8* revealed that the mutation is in a putative mitogen-activated protein kinase kinase kinase gene. Seed germination assays indicate that *sis8* mutants have wild-type sensitivity to ABA and GA, whereas overexpression of *SIS8* causes slight hypersensitive responses. Potential interaction partners of SIS8 have been identified via yeast two-hybrid screening. A T-DNA insertion in the gene encoding one potential SIS8-interacting protein, *UGT72E1*, causes a *sis* phenotype.

Further studies of the *SIS3* and *SIS8* genes will provide more insight into the mechanisms of sugar signaling in plants.

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Chapter I

Literature review

I. 1. Significance of the study

Autotrophic plants have the ability to convert light energy and carbon dioxide into carbohydrates through photosynthesis. The transport and metabolism of carbohydrates and the strength of source (net carbon exporting tissue) and sink (net carbon importing tissue) determine carbon allocation at the whole plant level. Thus, sugar metabolic pathways and mechanisms of sugar sensing and signaling have been subjected to extensive research and exciting advances in understanding how plants control carbon flow among organs and tissues have been made.

Changes in absolute sugar levels, sugar flux or sugar to nitrogen ratios have profound effects on plant metabolism, growth, development and stress responses. In general, sugar depletion promotes photosynthesis, remobilization of storage polysaccharides, proteins and lipids and nutrient export, whereas sugar abundance favors carbon import, storage and utilization (Koch, 1996). Sugars modulate various developmental processes throughout the entire life cycle of plants, including seed development and germination, seedling and vegetative growth, floral induction, senescence and responses to environmental stimuli (Gibson, 2005; Rolland et al., 2006). A number of genes involved in carbon and nitrogen metabolic processes have been shown to be sugar-responsive under sugar excess or starvation conditions (Graham, 1996; Koch, 1996; Fujiki et al., 2000).

The availability of full genome sequences of *Arabidopsis* (*Arabidopsis thaliana*) and rice (*Oryza sativa*) and the combination of genetic, biochemical and molecular approaches have greatly facilitated research on understanding sugar sensing and signaling mechanisms in plants. Multiple sugar signaling pathways including those for glucose, sucrose and trehalose have been characterized, and some sugar sensors and downstream components have been identified (Rolland et al., 2006). Knowledge of plant sugar signaling pathways has allowed genetic engineering of crop plants with agricultural benefits. For example, transgenic potatoes overexpressing a sucrose non-fermenting1-related protein kinase1 (SnRK1) gene, a central regulator of carbon metabolism, produced high-starch, low-glucose tubers (McKibbin et al., 2006).

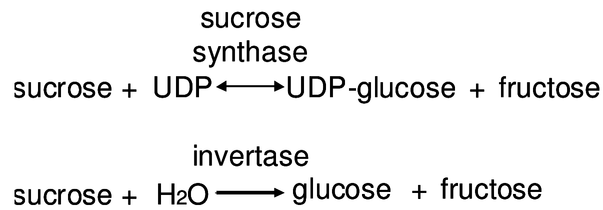
The advantages of using a model plant, *Arabidopsis,* for studying sugar signaling mechanisms are multifold. *Arabidopsis* plants are small in size, have a short life cycle and produce a large seed set from a single plant which all contribute to easy manipulation in the lab or greenhouse. The *Arabidopsis* genome is about 125 megabase pairs (Mbp) and is much smaller than those of crop plants such as wheat (16000 Mbp) or maize (2400 Mbp), which makes *Arabidopsis* genetically tractable. Extensive research efforts on *Arabidopsis* have yielded many useful knowledge sets and technical toolkits that often can be applied to other plant species as well. All these attributes make *Arabidopsis* an excellent start point for translational and basic research.

In the Gibson lab, *sugar insensitive* (*sis*) mutants have been isolated and characterized (Laby et al., 2000). These *sis* mutants are tolerant to high levels of exogenous sugars and develop green seedlings in the presence of concentrations of sugars that inhibit the early seeding development of wild-type plants. The *sis* mutants thus have defects in sugar sensing and/or response. Cloning and characterization of the *SIS* genes suggests cross-talk between sugar and other signaling pathways.

I. 2. Sugar metabolism and transport in plants

I. 2. 1. Sugar metabolism

Carbohydrate metabolism in plants has been well studied and includes complex and unique characteristics that differ from those of animals and microorganisms. Sucrose is the major product of photosynthesis and the main form of sugar used in long-distance transport in plants. Sucrose and its metabolic derivatives serve as raw materials for the synthesis of nucleic acids, proteins, lipids, starch and polysaccharides in the cell wall, as well as providing energy for plant growth and development. To be utilized by plant cells in the form of hexoses, sucrose is first cleaved by either invertases or sucrose synthases (SUS). These reactions are shown below.



Sucrose enters the sink cell either through plasmodesmata or across the plasma membrane. Plasmodesmata provide a symplastic pathway to channel sucrose directly from cytoplasm to cytoplasm within adjacent cells where it can be cleaved by cytoplasmic invertase (CIN) or SUS, or into the vacuole for cleavage by vacuolar invertase (VIN). Sucrose in the apoplastic space (cell wall) can be imported into cells by sucrose transporters, or cleaved by cell wall invertase (CIN) and imported by hexose transporters (Koch, 2004).

Invertase and SUS are often both present in the same tissue and their precise roles in sucrose degradation are not fully understood. One distinction between the two pathways is the energy status of their products. Adenosine triphosphate (ATP) and hexokinase (HXK) are needed to phosphorylate the glucose (glucose-6-phosphate) produced by the invertase reaction, whereas the phosphorylation of the UDP-glucose (glucose-1-phosphate) produced by SUS is ATP-independent with the help of UDP-glucose pyrophosphorylase. Glucose-1-P can be converted to glucose-6-P in the cytosol and enters the glycolytic pathway for energy production. In plastids, glucose-1-P is used by ADP-glucose pyrophosphorylase to produce ADP-glucose, the first committed step in starch synthesis. This starch reserve in plastids can be broken down to sucrose in the dark as an adaptation to the decreased concentration of hexose phosphates (Buchanan et al., 2000).

Sucrose oxidation involves three stages, glycolysis, the citric acid cycle and electron transfer/oxidative phosphorylation. Glycolysis converts sucrose to hexoses to pyruvate, a three-carbon compound which is transported into mitochondria and oxidized to CO_2 by the citric acid cycle. Electrons are transferred to the cofactors NAD^+ and FAD, yielding NADH and $FADH_2$. These reduced electron carriers ultimately donate electrons

to oxygen to produce H_2O. The free energy released during electron transfer is used in the process of oxidative phosphorylation to drive the formation from ADP to ATP. It is worthwhile to note that many intermediates produced from glycolysis and the citric acid cycle are used to provide carbon skeleton for other important cellular molecules, such as amino acids, nucleic acids and fatty acids (Buchanan et al., 2000).

In *Arabidopsis* germinating seeds, stored lipids are catabolized to produce sucrose for growth and development before the photosynthetic apparatus forms. This process occurs through coordination of enzymatic reactions in three subcellular compartments. In glyoxysomes, fatty acids are oxidized to acetyl-CoA which is converted to succinate via the glyoxylate cycle. The succinate is transported into the mitochondrial matrix where it is converted by the citric acid cycle to oxaloacetate, which enters the cytosol. Cytosolic oxaloacetate is then used as a starting material for gluconeogenesis to produce glucose-6-phosphate, the precursor of sucrose (Nelson and Cox, 2000).

Due to the central roles of sucrose in providing energy and building materials for plant growth and development, the metabolism of sucrose conceivably plays pivotal roles in sugar sensing and may be involved in self-regulatory mechanisms in plants (Koch, 2004). As sucrose is the main form of assimilated carbon transported in plants, sugar transport represents another layer of regulation by sugars.

I. 2. 2. Sugar transport and transporters

Long-distance transport of sucrose and other sugars between source (exporting) and sink (importing) organs is regulated by many factors, including monosaccharide and disaccharide transporters. Sugar transport in plants is realized by a specialized vascular structure, the phloem, which consists of two cell types, the sieve elements (SEs) and the companion cells (CCs), that are closely interconnected by plasmodesmata. In brief, sucrose released from mesophyll cells can move to the SE-CC complex via plasmodesmata (symplastic loading). Alternatively, sucrose is exported into the cell wall space (apoplastic loading) of mesophyll cells and actively imported into the SE/CC complex by membrane-localized proton/sucrose symporters. Sucrose moves in the SEs by bulk flow and is unloaded at the sink tissue. Similar to uploading, phloem and post-

phloem unloading can take place via plasmodesmata or sucrose transporters. Alternatively, sink cells can take up the hexoses produced by sucrose hydrolysis by cell wall invertase (Lalonde et al., 1999). Thus, sugar transporters play key roles in regulating carbon partitioning as well as in sugar signaling.

I. 2. 2. 1. Monosaccharide transporters (MSTs)

The MSTs have been isolated from various plant species and have a broad range of substrate specificities, including D-glucose, 3-O-methylglucose, 2-deoxyglucose, mannose, fructose and polyols. MSTs are believed to function in hexose uptake in sink cells and sub-cellular sugar compartmentation. A plant hexose carrier was first cloned from the unicellular green alga *Chlorella kessleri* as a H^+-cotransporter (Sauer and Tanner, 1989). Using a cDNA clone of this cotransporter as a hybridization probe, STP1 was identified from *Arabidopsis* as a hexose transporter with 12 putative transmembrane segments, which is structurally characteristic of both MSTs and disaccharide transporters (DSTs) (Sauer et al., 1990). Phylogenetic analysis revealed a monosacchride transporter (-like) gene family of 53 member from the *Arabidopsis* genome, which can be clustered into 7 sub-families, with the AtSTPs representing the best characterized sub-family among them (Büttner, 2007). The expression profiles of STPs in tissues such as guard cells, pollens, sepals and ovaries suggest they play important roles in transporting hexoses into sink cells (Scholz-Starke et al., 2003; Stadler et al., 2003). *Arabidopsis* MSTs are regulated in response to pathogen attack, wounding and starvation when reallocation of carbohydrates are necessary (Fotopoulos et al., 2003; Büttner, 2007). Interestingly, a grape protein VvMSA can bind to the promoter of a putative monosacchride transporter gene *VvHT1* which also contains two sugar boxes in its promoter. Expression of both genes are inducible by sucrose and glucose and sugar induction of *VvMSA* is strongly enhanced by abscisic acid (ABA), suggesting that VvMSA functions in a sugar and ABA signaling pathway (Çakir et al., 2003).

I. 2. 2. 2. Sucrose transporters

Plasma membrane H^+-sucrose symporters are responsible for the uploading of sucrose into the phloem, maintaining the mass flow of phloem sap and unloading of sucrose into the sink apoplast, which is reflected by the diverse locations of these transporters in source and sink tissues. For example, the potato sucrose transporter gene *StSUT1* was found to be expressed specifically in the phloem by RNA *in situ* hybridization, consistent with a role in phloem loading (Riesmeier et al., 1993). The *Arabidopsis* transporter gene *SUC2* is expressed in the phloem of leaves as well as in roots, suggesting that SUC2 is important for both phloem loading and unloading (Truernit and Sauer, 1995). *In vivo* evidence for SUT1 function comes from studies using transgenic potato plants harboring a *SUT1* antisense construct. These plants developed local bleaching of leaves and contained much higher concentrations of sugar and starch than wild-type plants (Riesmeier et al., 1994). Similarly, tobacco plants expressing an *NtSUT1* antisense construct exhibited leaf chlorosis, root system reduction and impaired ability to export recently fixed $^{14}CO_2$ (Bürkle et al., 1998). Roles of sucrose transporters in sink development have been studied. It was found that *AtSUC1* mRNA is expressed in floral tissue, such as anther connective tissue, funiculi and fully developed pollen grains and AtSUC1 functions in anther dehiscence and pollen tube growth (Stadler et al., 1999). Sivitz et al. (2008) have shown that AtSUC1 is expressed in roots and trichomes as well and SUC1 is important for sucrose-mediated anthocyanin accumulation and normal male gametophyte function (Sivitz et al., 2008) Another sink-localized sucrose transporter gene, *AtSUC5*, is involved in early seed development, based on its endosperm specific expression and high level of induction between 4 and 9 days after flowering (DAF). Analysis of *atsuc5* mutant seeds revealed a strong but transient reduction in fatty acid concentration 8 DAF (Baud et al., 2005). The *Arabidopsis* genome encodes nine putative sucrose transporters, all of which have been characterized to some extent. A recent study on *AtSUC9* indicates that it helps control flowering time, as *atsuc9* mutants flowered earlier than wild type under short day conditions (Sivitz et al., 2007).

I. 2. 2. 3. Regulation of sugar transporters

The activity of sugar transporters needs to be tightly regulated for plant cells to allocate fixed carbon between source and sink tissues correctly in response to developmental and environmental signals. There are multiple factors and levels where regulation might occur.

Regulation of proton-ATPase activity can influence H^+-sucrose symporter activity. Phytohormones can affect sucrose transport in a variety of plant species. Auxin inhibited active sucrose uptake in sugar beet roots (Saftner and Wyse, 1984). In another report, auxin and gibberellic acid enhanced sucrose uptake in isolated phloem tissue of celery and enhanced the export of ^{14}C assimilates from leaves of intact plants *in vivo* (Daie et al., 1986). The apparent discrepancies between different auxin effects on sucrose uptake may be due to the use of isolated plant tissues. In addition, environmental cues, such as wounding, pathogen elicitors and salt stress, have specific effects on expression of certain sugar transporter genes (Truernit et al., 1996; Noiraud et al., 2000). The expression of sucrose transporters is also developmentally regulated, as demonstrated by analysis of the *AtSUC2* promoter in young and older leaves (Truernit and Sauer, 1995).

Sugar regulation of transporter activity has been studied and has produced mixed results. A possible regulatory effect of glucose and sucrose on the activity of the *SUC2* promoter was studied and excluded, both in excised leaves and young seedlings of transgenic *Arabidopsis* plants (Truernit and Sauer, 1995). However, expression of a maize sucrose transporter gene, *ZmSUT1*, was positively regulated by the amount of soluble sugars in source leaves (Aoki et al., 1999).

The expression and activity of sugar transporters can be regulated at the transcriptional and post-transcriptional levels. A sucrose-specific response pathway has been suggested to down-regulate expression of a sugar beet H^+-sucrose symporter at the steady-state mRNA level (Chiou and Bush, 1998). Importantly, intragenic regulatory sequences have been shown to control *AtSUC9* expression (Sivitz et al., 2007). A protein phosphatase inhibitor experiment indicates phosphorylation may be involved in regulation of a sucrose transporter in sugar beet (Roblin et al., 1998).

I. 3. Sugar regulation of plant development and gene expression

I .3. 1. Sugar control of plant growth and development

I .3. 1. 1. Sugar control of seed development and germination

A number of plant developmental processes are postulated to be sugar regulated. The physiological and molecular mechanisms by which sugar regulation occurs are well studied for some of these processes. During embryogenesis, the fertilized egg cell undergoes multiple rounds of cell division before entering a phase of cell expansion, differentiation and reserve accumulation. Using large-seeded legume embryos as research material, a bioluminescence-based method has been applied to quantitatively measure local distributions of glucose and sucrose in differentiating cotyledon tissues. According to these studies, sugar concentration gradients are associated with a particular cell type. Higher glucose concentrations are found in non-differentiated premature regions with higher mitotic activity, whereas higher sucrose concentrations are present in actively elongating and starch-accumulating cells (Borisjuk et al., 1998; Borisjuk et al., 2002). The pattern of sugar gradients in cotyledon tissues can be partially accounted for by the differential expression of the hexose transporter VfSTP1 and the sucrose transporter VfSUT1. *VfSTP1* mRNA accumulates in epidermal cells covering the mitotically active parenchyma, whereas the *VfSUT1* transcript was restricted to outer epidermal cells showing transfer cell morphology and covering the storage parenchyma (Weber et al., 1997).

Exogenous sugars have been shown to control seed germination and early seedling development. Low concentrations (e.g. 10 mM) of the glucose analog mannose inhibit germination of *Arabidopsis* seeds and this effect involves a HEXOKINASE1 (HXK1)-mediated pathway (Pego et al., 1999). Exogenous ABA at micromolar concentrations also inhibits seed germination. Interestingly, the addition of metabolizable sugars (e.g. 60 mM glucose) can reverse the inhibition of seed germination by ABA or mannose (Pego et al., 1999; Finkelstein and Lynch, 2000). However, sugars themselves are inhibitors of germination at concentrations ranging from 30 mM to over 300 mM and this inhibition effect is not entirely due to osmotic stress (To et al., 2002; Price et al.,

2003). It has been suggested that the glucose-induced delay in germination is not caused by increased cellular ABA levels, but by a decreased decline in endogenous ABA (Price et al., 2003). Consistent with this hypothesis, addition of fluridone, an ABA biosynthesis inhibitor, does not suppress the glucose effect (Dekkers et al., 2004). Studies have shown that endogenous ABA levels may determine the severity of glucose repression because the ABA deficient mutants *aba2-1* and *aba3-1* show decreased sensitivity with respect to glucose inhibition of germination (Price et al., 2003; Dekkers et al., 2004). In contrast to mannose inhibition of germination, which is mediated through *HXK1*, the glucose response is independent of *HXK1* based on: (1) 3-O-methylglucose (e.g. 84 mM), a poor substrate for HXK1, delays germination; and (2) neither *gin2* (which carries a loss-of-function mutation in *HXK1*) nor *35S:AtHXK1* displayed altered sensitivity to 167 or 333 mM glucose (Price et al., 2003; Dekkers et al., 2004). Glucose-induced delay of seed germination also involves a regulatory pathway independent of *ABI2/ABI4/ABI5* (Price et al., 2003; Dekkers et al., 2004). Recently, it has been suggested that this glucose inhibition of germination is accomplished via the activation of the ABA signaling pathway through *ABI3* (Yuan and Wysocka-Diller, 2006). At the physiological level, germinating oilseeds such as *Arabidopsis* seeds convert stored carbon in the form of triacylglycerol into soluble carbohydrates. Exogenous sugars, such as glucose and mannose, have been shown to retard storage lipid mobilization in *Arabidopsis* germinating seeds and equimolar 3-O-methylglucose and sorbitol controls did not exert similar effects (To et al., 2002). The *sis5/abi4* mutant is resistant to glucose inhibition of storage lipid mobilization (To et al., 2002). Interestingly, the *abi4-1* mutant is also resistant to ABA inhibition of seed lipid breakdown (Penfield et al., 2006). These results suggest that ABI4 is involved in both glucose and ABA regulated seed lipid mobilization, or that mutations in *ABI4* make plants less sensitive to glucose-mediated delays in ABA breakdown.

In the presence of high levels of glucose or sucrose, *Arabidopsis* seed germination is delayed but will occur given sufficient time (Laby et al., 2000). Post-germination seedling establishment, however, is inhibited by high sugar levels and seedlings can resume their development once the sugars are removed. The arrested seedlings fail to

form expanded green cotyledons and true leaves (Zhou et al., 1998; Laby et al., 2000). Chloroplast biogenesis is also impaired by high glucose levels in *Arabidopsis* seedlings (To et al., 2003). Equimolar concentrations of mannitol or sorbitol do not have similar effects, suggesting sugar signaling and osmotic signaling are separable in regulating early seedling development. Forward genetic screens for sugar-response mutants that are hypersensitive or insensitive to the inhibitory effect of high sugar levels have yielded a number of mutants that are also defective in metabolism or response to phytohormones, such as ABA and ethylene (Zhou et al., 1998; Arenas-Huertero et al., 2000; Laby et al., 2000; Gibson et al., 2001; Cheng et al., 2002). The *prl1*, *ghs1* and *gin2/hxk1* mutants were also isolated from such screens (Németh et al., 1998; Moore et al., 2003; Morita-Yamamuro et al., 2004).

I .3. 1. 2. Sugar control of vegetative and reproductive development

Alterations in sugar transport, metabolism and concentration affect plant vegetative and reproductive growth and development. For example, transgenic plants over-expressing *ATHB13* cDNA, a homeodomain leucine zipper transcription factor, developed narrower cotyledons and leaves only on media containing metabolizable sugars. Over expression of *ATHB13* affects cotyledon shape by inhibiting lateral expansion of epidermal cells in sugar-treated seedlings (Hanson et al., 2001). High sucrose levels have been shown to be required for successful potato tuber induction (Fernie and Willmitzer, 2001). Manipulation of sucrose and starch metabolism affects tuber number, size and total yield. Expression of a yeast apoplasmic invertase in potato plants led to an increase in tuber size and a decrease in tuber number per plant (Sonnewald et al., 1997). Transgenic potatoes with reduced expression of ADP-glucose pyrophosphorylase displayed abolition of starch formation in tubers. The number of tubers increased and the size of individual tuber decreased (Müller-Röber et al., 1992). Exogenous sugars can also affect root development. *Arabidopsis* seedlings grown in a vertical orientation in the dark developed adventitious roots from elongated hypocotyls in a progressive manner with increased concentrations of sucrose (0.5%-2.0%), but 5.0% sucrose suppressed induction (Takahashi et al., 2003).

In addition to regulating vegetative development, sugars play important roles in floral induction and reproductive organ development. Several lines of evidence have indicated that sugar supply, whether endogenous or exogenous, to the shoot apical meristem (SAM) is essential for flowering in *Arabidopsis* (Bernier and Périlleux, 2005). A large, early and transient increase in carbohydrate export from leaves happens during floral induction (Corbesier et al., 1998). Supply of sucrose to the aerial parts of dark-grown plants promotes morphogenesis and flowering. Some late-flowering *Arabidopsis* ecotypes and mutants such as *fca*, *co* and *gi* exhibited accelerated flowering in darkness with sucrose (Roldán et al., 1999). Gibberellins promote *Arabidopsis* flowering by activating the promoter of *LFY*, a floral meristem identity gene. This activation by gibberellins requires sucrose (Blázquez et al., 1998). Application of exogenous sugars affects flowering time. *Arabidopsis* plants grown on 6% glucose-MS media exhibit delayed bolting compared with plants grown on 2% glucose (Zhou et al., 1998). Similar results were reported in another study. Wild-type *Arabidopsis* plants grown on 5% sucrose had more rosette leaves and flowered later than those on 2% sucrose. This delaying effect can not be reproduced with 3-O-methylglucose, a non-metabolizable glucose analog (Ohto et al., 2001). Sugar transport activity also affects flowering time as a sucrose transporter mutant, *atsuc9,* displayed early flowering under short day conditions (Sivitz et al., 2007). The above mentioned results indicate that the role of sugars on floral transition depends on their exact concentration, location and source.

There is also evidence that hexokinase is involved in plant senescence. When grown under high-light conditions, the *gin2/hxk1* mutant has tiny leaves that undergo delayed senescence compared to wild-type plants grown under the same conditions (Moore et al., 2003).

I .3. 1. 3. Sugar starvation responses

During the life cycle of a plant, many less favorable environmental factors can cause decreased photosynthetic activity in source tissues and negatively affect sink growth and development. For example, limited light, water and nutrient supply, or attacks by pathogens and herbivores may result in carbohydrate depletion in sink tissues. Under

such “famine” conditions, plant cells need to sense sugar deprivation and respond by altering their growth and development at the cellular, biochemical and genetic levels to ensure their survival (Yu, 1999). Most studies on sugar starvation have used cultured cells or excised plant tissues. Common cellular responses to sugar deprivation include rapid degradation of vacuolar sucrose, starch, proteins and lipids, increased activities of the fatty acid beta-oxidation enzymes, an initial accumulation of asparagine (a reliable marker of protein and amino acid degradation under stress conditions) and a progressive decrease in respiration rate (Journet et al., 1986; Brouquisse et al., 1991; Brouquisse et al., 1992; Dieuaide et al., 1992). These observations and a recent transcriptomic study using *Arabidopsis* suspension culture cells subjected to sucrose starvation suggest that sugar depletion forces cells to utilize the products from the degradation of proteins and fatty acids for maintaining respiration and key metabolic processes (Contento et al., 2004). Carbohydrate starvation can occur as a consequence of leaf senescence or exposure to darkness. A number of dark-inducible (*DIN*) genes have been identified and characterized from *Arabidopsis*. Expression of *DIN*s is induced by darkness, sucrose starvation and senescence and repressed by the addition of sucrose, glucose and 2-deoxyglucose, but not by mannitol and 3-O-methylglucose, suggesting that regulation of *DIN* genes requires phosphorylation of hexose and thus is HXK-dependent (Fujiki et al., 2000; Fujiki et al., 2001). Analyses using inhibitors of protein kinases and phosphatases have shown that signaling during sugar starvation involves protein phosphorylation and dephosphorylation events (Fujiki et al., 2000). Interestingly, *DIN6* expression can be specifically activated by *AKIN10* and *AKIN11*, *Arabidopsis* Snf1-related protein kinase1s (SnRK1s), and sugars inactivate the kinase activity of SnRK1s, confirming the key role of SnRK1s in sugar and stress signaling (Baena-González et al., 2007). In the same study, the repression of *DIN1* and *DIN6* by sugars was the same in the wild type as in the *HXK1*-null *gin2* mutant, indicating a HXK-independent pathway acts in *DIN1* and *DIN6* regulation. However, the involvement of other HXK genes in controlling *DIN* expression can not be ruled out since the *Arabidopsis* genome encodes a HXK gene family of six members and HXK2 and HXK3 have been shown to phosphorylate hexose as well (Karve et al., 2008).

I. 3. 2. Sugar regulation of gene expression

Due to the importance of sugars in regulating source-sink interactions and in plants' survival under stress conditions, extensive studies have focused on the carbohydrate control of gene expression and a variety of genes which encode proteins involved in diverse metabolic and biological processes have been found to be induced or repressed based on the abundance or depletion of carbon supply in plant cells. A number of excellent reviews on sugar control of gene expression have been published (Graham, 1996; Koch, 1996; Gibson, 2005; Rook et al., 2006). It is worth noting that many plant genes that respond to sugar signals are controlled by other signals as well, such as light, nitrogen, stress or phytohormones.

I. 3. 2. 1. Sugar induction of gene expression

Abundant supplies of sugars induce expression of genes involved in carbohydrate and protein storage, sucrose metabolism, defense and secondary metabolism. In Solanaceous species, such as potato, elevated sugar levels increase the expression of genes encoding class I patatin (major soluble proteins in potato tubers), ADP-glucose pyrophosphorylase (starch biosynthesis), granule-bound starch synthase (amylose biosynthesis) and sucrose synthase (Rocha-Sosa et al., 1989; Salanoubat and Belliard, 1989; Wenzler et al., 1989; Müller-Röber et al., 1990; Visser et al., 1991). In excised leaves and petioles of sweet potato, exogenous sugars induce the expression of two genes whose products are tuberous root storage proteins, sporamin and beta-amylase (Hattori et al., 1991; Nakamura et al., 1991). One study using excised soybean leaves and whole seedlings has shown that endogenous sugars and methyl jasmonate synergistically stimulate the accumulation of mRNAs of the vegetative storage protein genes *vspA* and *vspB* (Mason et al., 1992). Remarkably, the sugar-mediated induction of *vspB* was inhibited by the addition of phosphate and phosphate depletion stimulated *vspB* expression. Other sugar-responsive genes, including lipoxygenase A and chalcone synthase of soybean and proteinase inhibitor II and class I patatin of potato, were stimulated by phosphate depletion as well. These results suggest that the accumulation of

sugar-phosphate and reduction of cellular phosphate levels are in part responsible for sugar induction (Sadka et al., 1994).

Genes involved in sucrose metabolism, particularly sucrose synthase and invertase which catalyze the entry step for carbon use in sink tissues, are under delicate control by sugars. Sucrose synthase genes from a number of plant species are upregulated by sugars, including potato, rice, bean (*Vicia faba*) and *Chenopodium rubrum* (Salanoubat and Belliard, 1989; Karrer and Rodriguez, 1992; Heim et al., 1993; Godt et al., 1995). In Arabidopsis, *AtSUS1* mRNA levels increased in response to 250 mM sucrose and mannitol (Baud et al., 2004). However, two sucrose synthase genes in maize, *Sh1* and *Sus1*, show different responses to changing carbohydrate supply. High sugar levels induce expression of *Sus1* while repressing that of *Sh1* (Koch et al., 1992). Interestingly, the maize soluble invertase genes also show contrasting sugar responses, with *Ivr2* being induced and *Ivr1* repressed by sugars. Thus, the differential responses of two pathways involved in sucrose cleavage may have a profound influence on the import and use of sugars (Xu et al., 1996). The apoplasmic cell wall invertase provides the entry point for sucrose into the importing cells. In suspension culture cells of *Chenopodium rubrum* and maize, sugars increase the mRNA abundance of extracellular invertase *CIN1* and *Incw1*, respectively (Ehness et al., 1997; Cheng et al., 1999).

An array of genes encoding products involved in pigmentation and defense are upregulated by sugars. These products function during interactions with pollinators, pathogens or herbivores. In transgenic tobacco plants expressing vacuolar and apoplasmic yeast-derived invertases (vacInv and cwInv), the same threshold levels of hexoses are required for induction of pathogenesis-related (*PR*) protein transcripts and repression of chlorophyll a/b binding (*CAB*) protein transcripts (Herbers et al., 1996). The expression of two Arabidopsis *PR* genes, *PR1* and *PR5*, was induced by glucose and may be dependent on glycolysis (Xiao et al., 2000). Anthocyanins are one of the products of the phenylpropanoid pathway, which also produces lignins, flavonoids and tannins, among other secondary metabolites (Shirley et al., 1995). Anthocyanins are purple color pigments which accumulate in leaves as a response to stress (Winkel-Shirley, 2002). Chalcone synthase (CHS), a key enzyme in the phenylpropanoid pathway, has been

shown to be positively regulated by sugars (Tsukaya et al., 1991). Moreover, a transcript profiling study has shown that sucrose upregulates the mRNA levels of anthocyanin biosynthetic pathway genes, including phenylalanine ammonia lyase (*PAL*), *CHS*, chalcone isomerase (*CHI*) and dihydroflavonol reductase (*DFR*), as well as a transcription factor controlling the pathway, *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*). These results are consistent with *in vivo* sugar modulation of expression of anthocyanin biosynthetic genes, as is observed with the *pgm* mutant, which accumulates elevated levels of soluble sugars in leaves (Solfanelli et al., 2006).

High sugar levels can induce genes associated with nitrate reduction and uptake. In dark-adapted green *Arabidopsis*, the availability of sucrose can replace light to induce the accumulation of nitrate reductase (*NR1*) transcripts (Cheng et al., 1992). Two root nitrate transporter genes that are subjected to marked diurnal regulation can be induced by sugar supply at night (Lejay et al., 1999). These results suggest that carbon metabolites act on multiple regulatory steps of nitrate assimilation.

I. 3. 2. 2. Sugar repression of gene expression

In source cells, photosynthetic genes are normally downregulated when the supply of sucrose exceeds the demand of the sink cells, representing a feedback control mechanism in source-sink relations. A study using maize mesophyll protoplasts and a transient expression method has shown that the transcriptional activity of maize photosynthetic gene promoters, including those of *CAB* and ribulose bisphosphate carboxylase small subunit (*rbcS*), was specifically repressed by sugars (Sheen, 1990). The hypothesis that the accumulation of carbohydrate leads to decreased expression of photosynthetic genes has been verified in a range of plant species. Repression of *rbcS* transcripts was evident after feeding glucose to suspension cultures of *C. rubrum* cells or detached spinach leaves and in response to expressing invertase in the apoplast of tobacco leaves or cold-girdling of leaf petioles on intact tobacco or potato plants (Krapp et al., 1993). In *Arabidopsis*, sugars repress the mRNA levels of *rbcS* and plastocyanin (*PC*) independent of light (Cheng et al., 1992; Dijkwel et al., 1996).

Excess sugars down regulate genes involved in reserve remobilization, such as starch and lipid breakdown. In germinating oilseeds and during subsequent early seedling development, the stored lipids are converted to sucrose and transported to the embryo to provide energy and metabolic intermediates for growth. The glyoxylate cycle plays an important role in this process. The expression of two glyoxylate cycle enzyme genes, malate synthase (*MS*) and isocitrate lyase (*ICL*), are regulated by carbon metabolic status. The induction of *MS* and *ICL* expression in cucumber culture cells by sugar starvation can be reversed by sugar addition in the culture medium (Graham et al., 1994). *In vivo* evidence of sugar modulation of *MS* and *ICL* expression has been observed with whole cucumber plants. Shading or defoliation of the plants caused a marked decrease in glucose content in roots, with a concomitant increase in *MS* and *ICL* mRNAs (Ismail et al., 1997).

In cereal grains, the scutellum, aleurone and endosperm function as a nonphotosynthetic source of metabolites during seed germination and seedling growth. α-amylase (αAmy) secreted from the aleurone and scutellum help break down the starch in the endosperm into sugars which are transported and supplied to the embryonic sink tissues (Thomas and Rodriguez, 1994). The cereal α-amylase isozymes are encoded by multigene families. The expression of *αAmy* has been shown to be induced by gibberellic acid and sugar depletion and repressed by sugar addition in cultured rice cells (Yu et al., 1991). Similarly, metabolizable sugars repress the *αAmy* gene in rice and barley embryos (Perata et al., 1997; Umemura et al., 1998).

As nitrogen and carbon metabolism are interconnected, exogenous sugars repress the accumulation of the *Arabidopsis* glutamine-dependent asparagine synthetase (ASN1) gene transcripts in dark-grown plants. This sugar repression can be partially suppressed by supplies of asparagine or glutamine, indicating that a high nitrogen:carbon ratio directs the flow of nitrogen into asparagine, which is used for storage and long-distance transport of nitrogen in plants (Lam et al., 1994).

I. 3. 2. 3. Transcriptome profiling of gene expression by sugars

The availability of the complete *Arabidopsis* genome sequence and Affymetrix ATH1 whole genome GeneChips has enabled the study of gene expression response to carbon signals alone or in interaction with light, nitrogen or phytohormone signals in a high throughput manner.

Microarray analysis of the *pho3* mutant, which accumulates large amounts of soluble sugars and starch due to a mutation in the *SUC2* sucrose transporter gene, revealed altered responses of primary and secondary metabolism compared to the Ws wild type (Lloyd and Zakhleniuk, 2004). Not surprisingly, the elevated sugar levels repressed transcripts encoding enzymes of the Calvin cycle and induced those of starch synthesis and anthocyanin biosynthesis. Interestingly, mRNA levels for the gene for the glucose 6-phosphate/phosphate translocator (*GPT*) showed a marked increase in the mutant. The *GPT* gene is not normally expressed in the photosynthetic tissues. Its large upregulation by sugars suggests that the imported glucose-6-P is used as a substrate for starch synthesis in *pho3* source leaves (Lloyd and Zakhleniuk, 2004).

During the life cycle of plants, stress conditions and particular developmental stages may cause situations where decreases in carbohydrate supply to sink cells occur. In general, sugar starvation upregulates genes that are repressed by sugars, such as those associated with photosynthesis, reserve remobilization and sucrose metabolism. One well studied example of sugar starvation-mediated gene regulation is the induction of α-amylase gene in embryos of germinating rice seeds (Yu et al., 1996). Recently, a transcriptional profiling study was performed to examine the response of *Arabidopsis* suspension culture cells to sucrose starvation after 24 and 48 hours (Contento et al., 2004). More than 300 genes were found to have increased mRNA levels after starvation. Among them are genes involved in photosynthesis, carbohydrate metabolism and transport, protein and lipid degradation, trehalose metabolism and MYB and WRKY transcription factors. Genes that have decreased mRNA levels are related to protein translation and cell division. These observations suggest that plant cells initiate a highly regulated process of nutrient mobilization when sensing the deprivation of sugar nutrients (Contento et al., 2004).

It has been shown that the ratio of carbon and nitrogen can regulate *Arabidopsis* seedling growth and gene expression (Martin et al., 2002). Sugar and nitrogen signals interact to modulate gene expression and allow plants to adjust to metabolic and developmental cues. In one microarray study in which hydroponically grown *Arabidopsis* seedlings were treated with either 167 mM glucose, 60 mM nitrogen source or both glucose and nitrogen source, it was found that glucose is a more potent signal than inorganic nitrogen in modulating transcription (Price et al., 2004). Glucose and nitrogen can have synergistic or antagonistic effects on gene expression. Interestingly, glucose induction largely requires *de novo* protein synthesis, indicating glucose upregulation of gene expression is a multi-step event. A number of monosaccharide transporter genes were downregulated by glucose. The authors also found that glucose transcriptionally represses ethylene biosynthesis and signaling genes, providing a mechanism by which sugar response interacts with ethylene response during seedling development (Price et al., 2004). Another transcription profiling study using hydroponically grown plants treated with low levels of nitrate revealed induction of transcripts encoding enzymes in glycolysis and trehalose-6-phosphate metabolism, linking nitrogen signaling to carbon metabolism (Wang et al., 2003).

Carbon and light signals regulate plant growth and development interdependently. For example, photosynthetic genes (*CAB*, *PC*, *rbcS*) are induced by light but repressed by sugars (Koch, 1996). Sugar treatment can suppress the far-red light induced block of cotyledon greening (Barnes et al., 1996). Light and sugar treatment transcriptionally repress the asparagine synthetase (*ASN1*) gene in a synergistic fashion (Tsai and Coruzzi, 1991; Lam et al., 1998). Seeds of transgenic lines harboring an *ASN1::HPT2* construct were mutagenized and selected for putative carbon and light insensitive (*cli*) mutants which lose repression of the *ASN1::HPT2* transgene by light and carbon and show resistance to hygromycin (Thum et al., 2008). In one of the mutants, *cli186*, regulation in response to the interaction of light and carbon signals was significantly perturbed. Microarray analysis of wild type and *cli186* subjected to carbon-only, light-only or carbon-and-light treatment revealed that 966 genes are regulated by light and/or carbon in wild type and 216 genes are misregulated by light and/or carbon in *cli186*. Analysis of

the pattern of misregulation suggests that *cli186* is defective in a component that integrates light and carbon signaling. Genes involved in biological processes such as energy and metabolism are over-represented in both gene lists. One of the misregulated genes in *cli186*, *HAT22*, a homeobox leucine zipper transcription factor gene, appears to be a master regulator downstream of *CLI186* that integrates light and carbon regulation of genes involved in amino acid metabolism, glycolysis and gluconeogenesis (Thum et al., 2008).

Microarray analysis has been applied to study glucose and ABA interactions at the transcriptional level. There is evidence that ABA transcriptional regulators such as ABI4 can bind to promoters of sugar-responsive genes and modulate their expression (Rook et al., 2006). A recent study has established sugar and ABA responsiveness of an S-box in the conserved modular arrangement 5 (CMA5) unit of the *rbcS* promoter (Acevedo-Hernández et al., 2005). The transcriptional regulatory networks integrating carbohydrate and ABA signals in *Arabidopsis* have been characterized by microarray analysis and promoter classification (Li et al., 2006). Hydroponically grown seedlings were treated with glucose or ABA for a maximum of 6 hours and then collected for microarray experiments. Array analysis showed that 14% (95 genes) of the ABA-inducible genes were also induced by glucose. These genes are involved in diverse cellular and molecular functions including stress response, defense, fatty acid and lipid metabolism, secondary metabolism and cell wall metabolism, carbohydrate metabolism, transcript regulation and protein phosphorylation and dephosphorylation. Among genes corepressed by glucose and ABA are two genes encoding ACC oxidase, which is involved in ethylene biosynthesis, and a putative ethylene-responsive element binding factor, revealing the modulation of ethylene synthesis and signaling by both ABA and glucose. Promoter analysis identified the TELO motif as the strongest classifier of glucose upregulated gene expression and this motif was significantly enriched in the promoters of genes associated with protein and nucleotide synthesis (Li et al., 2006).

I. 4. Sugar sensing and signaling

Sugars control many aspects of plant growth and development and modulate gene expression extensively. Due to their dual roles as signals and metabolites, the exact sugar molecules being sensed as signals are difficult to identify. Sugar metabolism affects cellular energy status and phosphate pool levels, which may serve as signals as well. Thus, glucose or sucrose analogs, either metabolizable or nonmetabolizable, are used to address questions regarding which molecules and which steps of their transport and metabolism are sensed by plant cells. However, while useful, there are significant potential problems associated with the use of sugar analogs to characterize sugar-response pathways (Gibson, 2000). A range of studies provide evidence for the presence of sucrose- specific, hexokinase-dependent, hexokinase-independent and trehalose signaling pathways (Chiou and Bush, 1998; Wingler et al., 2000; Xiao et al., 2000).

I. 4. 1. Sucrose signals

The fact that sucrose synthase or invertase can catalyze the cleavage of sucrose to glucose and fructose makes it difficult to distinguish sucrose-specific signaling from hexose signaling. However, there is experimental evidence that sucrose is sensed by cells via a different mechanism from that used to sense hexose. Chiou and Bush have reported that exogenous sucrose downregulated the mRNA levels of a sugar beet proton-sucrose symporter. This sucrose effect can not be replicated with equimolar concentrations of sorbitol or hexoses. Mannoheptulose, a hexokinase inhibitor, did not block the sucrose effect. These results suggest that the repression of symporter transcript levels is sucrose-specific and hexokinase-independent (Chiou and Bush, 1998). The *Arabidopsis* bZIP transcription factor gene *ATB2* has been shown to be repressed specifically by physiological concentrations of sucrose at the translational level (Rook et al., 1998). Equimolar concentrations of glucose, fructose, maltose, lactose and raffinose did not have this repressive effect. The translational repression of *ATB2* involves the leader sequence (Rook et al., 1998). A follow-up study has found that a highly conserved upstream open reading frame (uORF) in the 5' untranslated region (5'UTR) of *ATB2* is essential for the repression (Wiese et al., 2004). Another *AtbZIP2* gene harboring the uORF is regulated similarly via sucrose-induced repression of translation, indicating a sucrose-sensing

pathway that controls translation of bZIP transcription factor genes through the uORF (Wiese et al., 2004). Another piece of evidence for a sucrose-specific signaling pathway comes from a study of the regulation of anthocyanin biosynthesis. Sucrose, but not glucose, fructose, glucose plus fructose, or the nonmetabolic sucrose analogs turanose and palatinose, specifically induced anthocyanin accumulation in *Arabidopsis* seedlings (Solfanelli et al., 2006). And *atsuc1* mutants were deficient in this sucrose signaling pathway, indicating that sucrose uptake is important (Sivitz et al., 2008).

I. 4. 2. Trehalose signaling

Trehalose is present in very low amounts in plants and its metabolism and signaling have drawn increased interest recently. The biosynthesis of the non-reducing disaccharide trehalose requires two key enzymes, trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). TPS catalyzes the formation of trehalose-6-phosphate (T6P) from UDP-glucose and glucose-6-phosphate, and TPP catalyzes the conversion of T6P to trehalose. Trehalose can be cleaved by trehalase to yield two glucose molecules (Ramon and Rolland, 2007). There are 11 putative TPSs and 10 putative TPPs in the *Arabidopsis* genome (Leyman et al., 2001). The large number of enzymes involved in T6P metabolism and current studies suggest that T6P, the precursor of trehalose, is a sugar signaling molecule that regulates carbon utilization and hence plant growth and development. It has been shown that yeast trehalose synthesis controls glucose influx into glycolysis and T6P might play a regulatory role in glycolysis by inhibiting hexokinase (Thevelein and Hohmann, 1995). In *Arabidopsis*, exogenous trehalose (25 mM) reduced root elongation and induced accumulation of starch in the shoots without changing soluble sugar levels. The amassment of starch was accompanied by an increased activity of AGPase and an induction of *ApL3* expression (Wingler et al., 2000). It has been shown that trehalose feeding causes T6P accumulation which inhibits growth in the absence of metabolizable sugars (Schluepmann et al., 2004). Further experiments have shown that transgenic plants, with elevated levels of T6P due to overexpression of the *TPS* gene and in intact isolated chloroplasts fed with T6P, exhibit increased redox activation of AGPase and increased starch accumulation. Overexpression

of *TPP* in transgenic plants caused decreased T6P levels and prevented AGPase activation in the presence of sucrose or trehalose (Kolbe et al., 2005). In addition, rising sugar levels in *Arabidopsis* plants are accompanied by increases in T6P levels and redox activation of AGPase. These results suggest T6P acts as a metabolic signal of sugar status in plant cells (Lunn et al., 2006). Consistent with the regulatory role of T6P in carbohydrate utilization and development, the *TPS1* gene is essential during embryo development, vegetative growth and the transition to flowering (Eastmond et al., 2002; Schluepmann et al., 2003; van Dijken et al., 2004). Interestingly, microarray analyses have revealed significant regulation of *TPS* and *TPP* genes by changes in sugar or nitrate availability (Wang et al., 2003; Contento et al., 2004; Price et al., 2004). Trehalose functions as stress protectant in some plant species and in yeast. In *Arabidopsis*, overexpression of the *AtTPS1* gene conferred drought tolerance as well as glucose-insensitive seedling development and ABA-insensitive seed germination phenotypes (Avonce et al., 2004). The *ABI4* and *CAB1* genes were upregulated in an *AtTPS1* overexpression line and *AtTPS1* might participate in the *HXK*-dependent signaling pathway (Avonce et al., 2004). Sucrose feeding rapidly induces high levels of *AtTPS5* expression, concomitant with T6P accumulation after sucrose feeding (Schluepmann et al., 2004). Remarkably, the T6P levels correlate with the expression of *AKIN11*, a central regulator of metabolism. *AKIN11* mRNA levels are higher in transgenic *TPS* seedlings with higher T6P levels than in wild type (Schluepmann et al., 2004). A recent biochemical analysis of 14-3-3 binding proteins has revealed that phosphorylation and subsequent 14-3-3 binding of TPS5 proteins are mediated by SnRK1s in response to 2-deoxyglucose (Harthill et al., 2006). These studies suggest that trehalose metabolism and T6P have important roles in primary carbon metabolism and plant development and stress defense.

I. 4. 3. Glucose sensing and signaling

Glucose acts as a signaling molecule in regulating gene expression and developmental processes. The mechanisms of glucose signaling in yeast cells have been well studied (Johnston, 1999; Gancedo, 2008). Recently, the identification and

characterization of a number of genes involved in glucose sensing and signaling, such as *HXK1*, *RGS1* and *SnRK1s,* have greatly advanced our understanding of sugar signaling mechanisms in plant cells (Rolland et al., 2006; Lu et al., 2007).

I. 4. 3. 1. Glucose sensing in yeast *Saccharomyces cerevisiae*

Due to similarities between yeast and plant cell sugar sensing mechanisms, yeast glucose signaling pathways are briefly introduced. Glucose, an essential nutrient to yeast cells, must be properly sensed and utilized by yeast cells to ensure their growth, development and survival. Yeast cells prefer to grow on glucose medium but also are capable to utilize other carbon sources such as sucrose, ethanol and glycerol. The presence of glucose transcriptionally induces genes associated with glycolysis and glucose transport, and represses a set of genes involved in utilization of alternative carbon sources, gluconeogenesis, the TCA cycle and respiration, a phenomenon known as the glucose repression pathway (Carlson, 1998). The repressed genes are derepressed upon glucose removal.

The main components of a major glucose repression pathway include Hxk2 (hexokinase2), Snf1 (sucrose non-fermenting1) and Mig1 (multicopy inhibitor of *GAL1* promoter). When glucose is abundant, it is transported into cells by yeast hexose transporter and phosphorylated by Hxk2. This generates a signal to inactivate the Snf1 kinase, which is a negative regulator of Mig1, a transcriptional repressor that can bind to the promoters of glucose-repressed genes and repress transcription with the help of other transcription factors. Removal of glucose activates Snf1, which subsequently phosphorylates Mig1 and the phosphorylated Mig1 translocates from the nucleus to the cytoplasm resulting in derepression of glucose-repressed genes (Johnston, 1999). The evidence that Hxk2 acts as a glucose sensor comes from the following studies. (1) The *hxk2* mutant is defective in glucose repression (Entian, 1980). (2) Glucose repression and hexokinase catalytic activity can be uncoupled in certain *hxk2* mutant alleles (Hohmann et al., 1999). (3) Hxk2 can enter the nucleus and interact with Mig1 to form a repressor complex, and the nuclear localization of Hxk2 is regulated by glucose (Ahuatzi et al., 2004; Ahuatzi et al., 2007). Snf1 is a serine/threonine protein kinase which is essential

for the derepression of glucose-repressed genes. The active kinase is comprised of Snf1, Snf4 and one of the members of the Sip family Sip1, Sip2 or Gal83 (Jiang and Carlson, 1997). The Snf1 protein complex is an ortholog of mammalian AMP-activated protein kinase (AMPK) that consists of the α, β and γ subunits (Halford and Hardie, 1998). Limited glucose supply activates Snf1 kinase with the assistance of the Snf4 subunit. The signal that activates Snf1 has been suggested to be the AMP:ATP ratio, based on the observation that AMPK activity is activated by high AMP:ATP ratios (Johnston, 1999).

S. cerevisiae prefers to ferment rather than oxidize glucose, even under conditions of sufficient oxygen. The limited ATP produced by fermentation of glucose molecules requires yeast cells to generate huge glucose influxes through the glycolytic pathway to obtain sufficient energy for life. Thus, glucose induces genes encoding glycolytic enzymes as well as glucose transporters. Yeast has some 20 hexose transporters (Hxt) that have either high affinity/low capacity (e.g. Hxt2) or low affinity/high capacity (e.g. Hxt1). Expression of these transporter genes is under transcriptional control by glucose availability to ensure optimal growth in a wide range of glucose concentrations. The glucose sensors for the glucose induction pathway are Snf3 and Rgt2, two transmembrane glucose transporter-like proteins that lack transporter activity. They differ from actual glucose transporters in that they have unusually long C-terminal tails that enhance glucose signaling (Johnston and Kim, 2005).

An additional pathway that involves transcriptional responses to glucose is the cyclic AMP (cAMP) pathway. Addition of glucose to glucose-deprived cells triggers an increase in cAMP levels, which requires the G-protein coupled receptor (Gpr1), the heterotrimeric G protein alpha subunit (Gpa2) and the regulator of G-protein signaling (Xue et al., 1998; Kraakman et al., 1999; Versele et al., 1999). Rolland *et al* (2000) have shown that besides the extracellular glucose detection GPCR system, a separable intracellular sugar sensing process dependent on hexose kinases is also essential (Rolland et al., 2000). Increased cAMP levels set off a protein kinase A (PKA)-mediated phosphorylation cascade, resulting in mobilization of trehalose and glycogen and rapid loss of stress resistance (Kraakman et al., 1999). A report has provided strong evidence that glucose and sucrose directly act as ligands of Gpr1, confirming Gpr1's role as the

sugar sensor in mediating the cAMP-PKA pathway (Lemaire et al., 2004). In addition, Gpr1 is a high-affinity sucrose, low-affinity glucose sensor (Lemaire et al., 2004).

I. 4. 3. 2. G-protein coupled receptor system in plant sugar sensing

In analogy to the role of Gpr1 in yeast sugar sensing, a plant G-protein coupled receptor (GPCR) system has been shown to regulate D-glucose sensing. In contrast to the mammalian GPCR system which has over 30 G-protein subunits and hundreds of GPCRs, *Arabidopsis* has only one canonical Gα subunit (AtGPA1), one Gβ subunit (AtAGB1) and two Gγ subunits (AtAGG1 and AtAGG2), one candidate GPCR (AtGCR1) and one regulator of G-protein signaling (AtRGS1) protein (Assmann, 2004). G-protein signaling has been implicated in a variety of processes including seed germination, root and shoot growth and development and stress responses based on G-protein mutant analyses (Perfus-Barbeoch et al., 2004). Interestingly, G-protein mutants exhibited altered responses to phytohormones such as gibberellic acid (GA), brassinosteroids (BR), ABA and auxin (Assmann, 2004). The evidence that G-protein mutants have altered sensitivity to sugars was first reported by Chen *et al* (2003), where the *rgs1* mutant was shown to be insensitive and the *gpa1* mutant to be hypersensitive to the inhibitory effects of 6% glucose on seedling greening when compared with wild-type plants (Chen et al., 2003). The *rgs1* mutant is also less sensitive to fructose and sucrose and sugar phosphorylation by hexokinase (HXK) is not required for RGS1-mediated sugar signaling (Chen and Jones, 2004). A recent study using biochemical, genetic and cellular imaging approaches has shown that RGS1 functions as a GTPase-accelerating protein (GAP) for GPA1 and its regulation of GPA1 GTP hydrolysis mediates D-glucose signal transduction during seedling development, suggesting that RGS1 is a glucose sensor (Johnston et al., 2007). Besides regulating seedling greening, *rgs1* mutants are insensitive and *gpa1* mutants are hypersensitive to the effect of sugars and ABA on seed germination (Chen et al., 2006). A downstream component of the RGS-coupled sugar signaling pathway has been identified as the plastid protein THYLAKOID FORMATION1 (THF1), which interacts with GPA1 (Huang et al., 2006). Root growth of the *thf1-1* mutant is hypersensitive to glucose and *THF1* overexpression lines have the opposite phenotype, indicating that

THF1 is involved in transmitting the glucose signal from GPA1 to root plastids (Huang et al., 2006). In summary, a GPCR system is mediating sugar signals in plant cells to regulate processes including cell proliferation and seed germination.

I. 4. 3. 3. Hexokinase as a cytosolic sugar sensor

Hexokinase (HXK) performs dual functions in glucose signaling and phosphorylation in plants. Early evidence for the signaling role of HXK comes from studies examining the effects of different sugars and sugar analogs on glucose-responsive gene expression. For example, mannose and 2-deoxyglucose (2-dG) can be transported into cells and phosphorylated by HXK, but the resulting sugar phosphate products are poorly metabolized. 6-deoxyglucose (6-dG) and 3-O-methylglucose (3-OMG) can be transported into cells but are very poor substrate for HXK and so are not phosphorylated or are phosphorylated at a very low rate (Cortès et al., 2003). In a study using a maize protoplast transient expression system and photosynthetic genes (e.g. *CAB*, *rbcS*) as marker genes, repression of gene expression has been observed with physiological levels (10 mM) of glucose, fructose, mannose and sucrose (Jang and Sheen, 1994). The glucose repression was not caused by depletion of cellular phosphate or ATP since addition of phosphate or ATP to the cells did not relieve the repression by glucose or mannose and mannose is often used to deplete phosphate and ATP *in vivo*. Both 6-dG and 3-OMG did not cause repression, suggesting sugar phosphorylation is required for repression. To test whether sugar phosphates or further metabolism of hexose phosphates is needed for repression, various sugar phosphates and metabolic intermediates of the glycolytic pathway downstream of glucose-6-phosphate were electroporated into cells and no repression was triggered. These results suggest that hexokinase is a potential glucose sensor (Jang and Sheen, 1994). Another study using cucumber cell cultures and different sugar analogs suggested the hexokinase reaction is required for initiation of sugar signals which repress the expression of the *MS* and *ICL* genes, which are involved in the glyoxylate cycle (Graham et al., 1994).

Genetic evidence for HXK acting as a sugar sensor has been shown by characterization of sugar responses in transgenic *Arabidopsis* plants or mutants with

altered expression of *HXK1* (Jang et al., 1997; Moore et al., 2003). Transgenic plants expressing antisense *HXK* are insensitive to seedling growth arrest induced by 6% glucose and less responsive to high glucose-mediated repression of photosynthetic gene expression, whereas transgenic plant expressing a sense *HXK* construct displayed the opposite phenotypes (Jang et al., 1997). Plants overexpressing the yeast glucose sensor *YHXK2* exhibited normal hexose phosphorylation activity but reduced sugar sensitivity, indicating that sugar signaling can be uncoupled from sugar metabolism by HXK (Jang et al., 1997). Furthermore, a loss-of-function HXK mutant, *glucose insensitive2* (*gin2*), was isolated from a genetic screen and characterization of *gin2* suggests that glucose phosphorylation activity is uncoupled from HXK1-dependent glucose signaling in this mutant (Moore et al., 2003). More direct evidence is provided by findings that the complementation of *gin2-1* with a catalytically inactive HXK1 protein restored glucose sensitivity of *gin2-1* at the physiological and transcriptional levels (Moore et al., 2003). Analysis of the *gin2* mutants revealed a broad spectrum of phenotypes including altered photosynthetic gene expression, reduced cell proliferation, smaller root and shoot systems, reduced reproduction, delayed senescence, decreased sensitivity to auxin and increased cytokinin response, indicating that HXK1 is a glucose sensor integrating signals from nutrient, light and phytohormones to regulate plant energy utilization, growth and development (Moore et al., 2003). Moreover, endogenous HXK1 performs nuclear functions with two partners to directly modulate the transcription of the glucose-repressible *CAB* gene (Cho et al., 2006). In the nucleus, HXK1 interacts with the vacuolar H^+-ATPase B1 (VHA-B1) and the 19S regulatory particle of proteasome subunit (RPT5B) to form a complex core that binds to the promoter region of *CAB2* and *CAB3* (Cho et al., 2006).

Based on the role of HXK1 in mediating glucose regulation of gene expression, three distinct glucose-response pathways have been proposed to exist in *Arabidopsis* (Xiao et al., 2000). Transgenic plants with altered *HXK* expression were used to monitor the mRNA levels of glucose-responsive genes. The *HXK1*-dependent pathway mediates glucose regulation of genes involved in photosynthesis (*CAB1*, *PC* and *rbcS*), nitrate assimilation (*NR1*), leaf senescence (*PLD*) and ABA signaling (*ERA*). The glycolysis-

dependent pathway regulates the expression of pathogenesis-related genes (*PR1* and *PR5*). The *HXK1*-independent pathway modulates transcript levels of genes associated with starch biosynthesis (*AGPase*), sucrose metabolism (*CIN1*), nitrogen metabolism (*AS1*) and anthocyanin biosynthesis (*CHS*, *PAL1* and *PAL3*) (Jang et al., 1997; Xiao et al., 2000).

I. 4. 4. Components of sugar signaling cascade

Besides the sugar sensor proteins HXK1 and RGS1, downstream components in the plant sugar signaling pathways are also being identified and characterized. Mechanisms common in other signal transduction pathways are present, such as phosphorylation cascades, calcium/calmodulin and SNF1-related protein kinases (Smeekens, 2000; Rolland et al., 2006).

I. 4. 4. 1. Protein phosphorylation and calcium signaling

Phosphor relay and calcium signaling are common elements in signal transduction pathways. There is evidence that these elements exist in plant sugar signaling. In excised mature leaves of sweet potato, treatment with specific inhibitors of protein phosphatases1 (PP1) and 2A (PP2A), such as okadaic acid, markedly inhibited sucrose-inducible increases in transcript levels for β-amylase, sporamin and the small subunit of AGPase, while the induction of sucrose synthase mRNA levels by sucrose was enhanced by okadaic acid (Takeda et al., 1994). The PP inhibitors inhibited sucrose induction of a *β-Amy:GUS* fusion gene in leaves of transgenic tobacco as well, demonstrating that dephosphorylation by PP is required for proper regulation of some sugar-responsive genes (Takeda et al., 1994). In another study the protein kinase (PK) inhibitor staurosporine enhanced glucose induction of *CIN1* and *PAL* expression in photoautotrophic culture cells of *C. rubrum* and D-glucose treatment resulted in a rapid and transient activation of myelin basic protein kinase activity (Ehness et al., 1997). Similarly, induction of *DIN* gene expression by sugar depletion was inhibited by K-252a, an inhibitor of serine/threonine protein kinases, in *Arabidopsis* suspension culture cells. Meanwhile, okadaic acid augmented the mRNA levels of *DIN* genes, supporting the

conclusion that sugar starvation signaling pathways also involve phosphorylation and dephosphorylation events (Fujiki et al., 2000).

Similar pharmacological studies have shown the involvement of Ca^{2+} signaling in sugar responses in sweet potato and tobacco (Ohto et al., 1995; Iwata et al., 1998). For example, in leaves of transgenic tobacco expression a *β-Amy:GUS* fusion gene, calmodulin inhibitors and inhibitors of Ca^{2+} channels prevented the sucrose-mediated induction of the fusion gene (Ohto et al., 1995). Furthermore, sugar solutions but not solutions of osmoticum caused an increase in cytosolic free Ca^{2+} in leaf discs of transgenic tobacco plants (Ohto et al., 1995). Combining these results, it suggests that elevated levels of cytosolic Ca^{2+} regulate downstream target proteins such as calmodulin or calcium-dependent kinases to mediate sugar signaling. Indeed, in tobacco leaves a calcium-dependent protein kinase (CDPK) that is associated with the plasma membrane is inducible by sucrose (Iwata et al., 1998). It is tempting to speculate that the Ca^{2+} signaling may regulate PP and PK activities to relay sugar signals to the nucleus, although other regulatory proteins may be involved.

I. 4. 4. 2. SNF1-related protein kinases

The plant SNF1-related protein kinases (SnRKs) have three subfamilies, SnRK1, SnRK2 and SnRK3. The SnRK1s share the most significant sequence identity and structural similarity to their counterparts in yeast and mammals, the sucrose nonfermenting1 (SNF1) and AMP-activated protein kinases (AMPK) (Halford and Hardie, 1998). SnRK1s from different plant species are able to complement the yeast *snf1* mutant phenotype by allowing the mutant to grow on non-glucose carbon sources (Muranaka et al., 1994; Takano et al., 1998; Bhalerao et al., 1999; Lovas et al., 2003; Thelander et al., 2004). The SNF1 and AMPK complexes have been considered to be global regulators of glucose metabolism and energy homeostasis and recent work on SnRK1s has suggested that they perform similar functions in plants in coordinating metabolic pathways to ensure optimal growth under constantly changing environmental conditions (Polge and Thomas, 2007).

Plant SnRK1s have been identified from a number of species (Halford and Hardie, 1998) and the *Arabidopsis* genome encodes three members of which only two are expressed and characterized, namely, AKIN10 and AKIN11 (Rolland et al., 2006). The SnRK1s regulate metabolism at multiple levels from the transcriptional to post-translational. Biochemical evidence suggests that SnRK1s phosphorylate and inactivate enzymes with important metabolic functions, including *Arabidopsis* 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR1), spinach nitrate reductase (NR) and sucrose phosphate synthase and *Arabidopsis* trehalose-6-phosphate synthase5 (TPS5) (Dale et al., 1995; Sugden et al., 1999; Harthill et al., 2006). Interestingly, 14-3-3 protein binding is coordinated with phosphorylation by SnRK1 to modulate the enzyme activities of NR and TPS5 (Sugden et al., 1999; Harthill et al., 2006). Post-translational redox regulation of AGPase is a new mechanism to coordinate starch synthesis with sucrose supply in potato tubers (Tiessen et al., 2002). One study has shown that potato SnRK1 is involved in a signaling pathway in which sucrose activates AGPase by redox modulation (Tiessen et al., 2002). Transcriptional regulation by SnRK1 has been shown for genes encoding sucrose synthase and α-amylase (Purcell et al., 1998; Laurie et al., 2003). For example, transgenic potatoes that have reduced SnRK1 activities due to expression of an antisense *SnRK1* sequence showed a decrease in sucrose synthase transcript levels in tubers and loss of sucrose-inducibility of sucrose synthase gene expression in excised leaves (Purcell et al., 1998).

A number of reports in which the expression of *SnRK1* was altered have identified interesting phenotypes associated with moss development, rice germination and seedling growth and potato tubers (Thelander et al., 2004; McKibbin et al., 2006; Lu et al., 2007). Transgenic potato lines overexpressing *SnRK1* showed an increase in starch content and a decrease in glucose levels in tubers. Transcript levels of genes encoding sucrose synthase and AGPase are higher in transgenic lines than in wild type, as are their enzymatic activities, emphasizing the important role of SnRK1 in control of starch accumulation (McKibbin et al., 2006). Similarly, knockout of the moss *Physcomitrella patens SnRK1* genes, *PpSNF1a* and *PpSNF1b*, leads to premature senescence, inability to grow in low light or in a day-night light cycle and deficiency in starch accumulation, confirming the

notion that SnRK1s function in low-energy conditions (Thelander et al., 2004). By making use of loss-of-function and gain-of-function mutant analysis, Lu *et al* (2007) have characterized the role of the rice *SnRK1A* gene in sugar signaling and seedling growth. Induction of SnRK1A expression by glucose starvation occurs at the post-transcriptional level in rice suspension cells and embryos. Using a rice embryo transient assay and an *snrk1a* mutant, it was found that *SnRK1A* acts upstream of, and relieves glucose repression of, *MYBS1* and *αAmy3* promoters. Partial loss-of-function of *snrk1a* seeds show delayed germination and seedling growth (Lu et al., 2007).

The essential functions of the Arabidopsis *SnRK1s*, *AKIN10* and *AKIN11*, in plant growth, development and survival under darkness, stress (hypoxia, DCMU) and sugar deprivation conditions have been demonstrated by a systems biology approach (Baena-González et al., 2007). *AKIN10/11* mediate induction of *dark-inducible* (*DIN*) gene expression by stress stimuli that deplete sugar supply. Sugar addition in the dark or expression of a catalytically inactive *AKIN10* abolish *DIN6:LUC* induction, indicating that sugars inactivate *SnRK1s* in the dark. Using a transient protoplast expression system and *Arabidopsis* ATH1 GeneChips, it was found that, after a stringent and multi-step filtering process, transient activation of AKIN10 triggers transcriptional reprogramming of a large array of genes, resulting in promotion of catabolism and suppression of anabolism. Double mutant *akin10 akin11* plants showed marked growth defects under short day conditions, including onset of early senescence and accumulation of leaf starch at the end of the night. In addition, the molecular switch-on of AKIN10 marker genes such as *DINs* by dark is abrogated in the double mutant, pointing out the role of AKINs in integrating stress, energy and developmental signals (Baena-González et al., 2007).

Regulatory mechanisms involved in controlling the activity and protein stability of SnRK1s have been studied and a number of upstream kinases and interacting partners have been identified for SnRK1s. Similar to the activation of AMPK and SNF1, plant SnRK1 is activated by phosphorylation on a conserved threonine residue (Sugden et al., 1999). Although SnRK1 is not allosterically activated by AMP, low concentrations of 5'-AMP can inhibit dephosphorylation of the threonine and consequent inactivation (Sugden et al., 1999). The upstream kinases of SnRK1 were identified recently as AtSnAK1 and

AtSnAK2, which complemented a yeast *elm1 sak1 tos3* triple mutant and phosphorylate the T-loop activation site of SnRK1 (Shen and Hanley-Bowdoin, 2006; Hey et al., 2007).

Of the proteins that interact with SnRK1s, PLEIOTROPIC REGULATORY LOCUS1 (PRL1) has been characterized particularly extensively (Bhalerao et al., 1999). The *prl1* mutant was identified through a mutant screen for increased sensitivity to 175 mM sucrose (Németh et al., 1998). The mutant also exhibited increased sensitivity to phytohormones including cytokinin, auxin, ethylene and ABA. The *prl1* mutation caused transcriptional derepression of glucose-repressible genes and accumulation of sugars and starch in leaves, which reflects an impaired ability to utilize carbon supply. These results indicate that PRL1 is a negative regulator of a function that mediates glucose repression (Németh et al., 1998). Interestingly, PRL1 can bind to the C-terminal domains of AKIN10 and AKIN11 and inhibit their kinase activity in light-grown *Arabidopsis* plants. This PRL1 function may explain the glucose derepression phenotype of the *prl1* mutant (Bhalerao et al., 1999). Furthermore, AKIN10/11 interact with the SCF ubiquitin ligase subunit SKP1/ASK1 and the 20S proteasome subunit α4/PAD1 in a yeast two-hybrid assay and *in vitro* pull-down assay (Farrás et al., 2001). In addition, PRL1 can recruit AKIN10 by disrupting its interaction with SKP1/ASK1. Co-immunoprecipitation and co-localization studies showed that AKIN10 is associated with SCF complex subunits SKP1/ASK1 and α4/PAD1 and is involved in mediating the binding of a SCF ubiquitin ligase complex to the 26S proteasome. However, AKIN10 itself is not likely to be ubiquitinated by the SCF complex, suggesting that AKIN10 may be involved in the phosphorylation event that is essential for SCF-mediated ubiquitination of substrate proteins (Farrás et al., 2001). More recently, in a study characterizing *Arabidopsis* and rice DWD (DDB1 binding WD40) proteins, the PRL1 protein, which contains two conserved DWD motifs, was shown to act as the substrate receptor of a CUL4-ROC1-DDB1-PRL1 E3 ligase involved in the proteolysis of AKIN10 (Lee et al., 2008). AKIN10 was degraded more slowly in *prl1* and *cul4* mutants than in wild type, suggesting factors other than CUL4-DDB1-PRL1 ligase are involved in controlling AKIN10 stability (Lee et al., 2008).

I. 5. Connections between sugar and other signaling pathways

A number of reviews have documented the extensive interactions between sugar signaling and phytohormone, light or nitrogen signaling pathways in plants (Smeekens, 2000; Coruzzi and Zhou, 2001; Gazzarrini and McCourt, 2001; Gibson, 2004; Rolland et al., 2006). Information about these interactions largely arises from sugar response mutant characterization. Selected cross-talk between different pathways is reviewed below.

I. 5. 1. Sugar and ABA signaling pathways

Genetic screens for sugar response mutants and identification of the corresponding loci have revealed cross-talk between sugar and ABA signaling pathways during *Arabidopsis* early seedling development (Rolland et al., 2002; Gibson, 2004). The high sugar insensitive mutants *glucose-insensitive1* (*gin1*), *impaired sucrose induction4* (*isi4*) and *sugar insensitive4* (*sis4*) are allelic to *aba2*, an ABA deficient mutant (Léon-Kloosterziel et al., 1996; Zhou et al., 1998; Laby et al., 2000; Rook et al., 2001; Cheng et al., 2002), whereas *gin6*, *isi3*, *sis5* and *sucrose-uncoupled6* (*sun6*) are allelic to *abi4*, an ABA insensitive mutant (Finkelstein, 1994; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000). High glucose levels are postulated to affect both ABA biosynthesis and signaling during seedling developmental arrest. Mutant alleles of *abi5*, *abi3* and *abi2* have *gin* phenotypes and overexpression of *ABI3*, *ABI4* and *ABI5* cause glucose hypersensitivity (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Finkelstein et al., 2002; Dekkers et al., 2008). The *abi8* mutant displays severely stunted growth that is alleviated by addition of glucose and is resistant to glucose levels that arrest wild-type seedling development (Brocard-Gifford et al., 2004). Interestingly, overexpression of *ABF2*, *ABF3* or *ABF4*, ABA-responsive elements (ABREs)-binding factor genes, induced hypersensitivity to 3% glucose, but only *abf2*, not *abf3* or *abf4*, mutants showed glucose insensitivity, suggesting that only specific components of the ABA signaling cascades are involved sugar response pathways (Kang et al., 2002; Kim et al., 2004).

Both glucose and ABA trigger early seedling developmental arrest. Seedling arrest by high sugar levels is characterized by the lack of cotyledon greening and

expansion and leaf formation (Zhou et al., 1998; Laby et al., 2000). ABA efficiently prevents vegetative growth by arresting development of mature germinated embryos (Lopez-Molina et al., 2001). The ABA-arrested seedlings are similar to those arrested by sugars with non-greening cotyledons and no leaves, but are smaller and exhibit little root growth (Dekkers et al., 2008). It has been shown that wild-type *Arabidopsis* seedlings lose sensitivity to the inhibitory effects of high sugar levels on early seedling development within 2 days after the start of imbibition (Gibson et al., 2001). ABA-mediated growth arrest is also triggered within two to three day period post-stratification and requires the functions of the *ABI3* and *ABI5* genes as *abi3* and *abi5* mutants do not exhibit growth arrest in the presence of ABA (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002). Wild-type seeds germinating in the absence of ABA have very low levels of ABI3 and ABI5. In contrast, stratified wild-type seeds germinating in the presence of ABA accumulate ABI3 and ABI5 proteins as well as higher transcript levels of the late embryogenesis genes *AtEm1*, *AtEm6* and *RAB18,* which are regulated by *ABI3* and *ABI5*, suggesting that late embryogenesis pathways are recruited *de novo* to desiccation tolerance on ABA (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002). Interestingly, stratified seeds germinating on high levels of glucose display increased expression levels of *ABI3*, *ABI5*, *AtEm1*, *AtEm6* and *RAB18* and this induction effect is confined to a similar time frame as observed for ABA (Dekkers et al., 2008). The glucose-induced expression of *ABI4*, *ABI5* and *RAB18* is reduced in *abi3* mutants, confirming that certain *abi3* mutations decrease the effects of sugar levels on gene expression (Dekkers et al., 2008).

The AP2-type transcription factor *ABI4* gene plays an important role in mediating sugar signals during early seedling development as well. Exogenous sugars retard *Arabidopsis* seed storage lipid mobilization and this inhibitory effect is relieved in the *abi4* mutant (To et al., 2002). It has been shown that *ABI4* expression is confined to the embryo and ABI4 is a repressor of lipid breakdown (Penfield et al., 2006). At the transcriptional level, maize ABI4 binds to the promoter of the sugar-regulated *ADH1* gene, and sugar- and ABA- responsive elements have been identified in the promoters of

ApL3, rbcS and possibly other photosynthetic genes, including *CAB* and *PC* (Niu et al., 2002; Acevedo-Hernández et al., 2005; Rook et al., 2006).

Although sugar and ABA signaling pathways exhibit extensive cross-talk in controlling post-germinative growth, other regulatory factors are involved as well. For example, dark-grown *high sugar response8* (*hsr8*) mutants showed glucose-hypersensitive development. The *hsr8* mutation lies in the *MURUS4* gene, which is involved in cell wall arabinose synthesis (Li et al., 2007). Also, the glucose insensitivity phenotypes are not the same for all developmental stages as *abi4* shows wild-type sensitivity to the inhibitory effects of high glucose level on seed germination but is tolerant to high sugar-induced seedling growth arrest (Price et al., 2003).

I. 5. 2. Sugar and ethylene signaling pathways

Another phytohormone signaling pathway that exhibits cross-talk with sugar-response pathways in controlling post-germinative growth is the ethylene response pathway. Repression of seedling shoot development by high sugar levels can be overcome by treating wild-type seeds with 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, indicating that ethylene may antagonize glucose signals to promote normal shoot development (Zhou et al., 1998). Consistent with this hypothesis, the ethylene *overproduction1* (*eto1-1*) and *constitutive triple response1* (*ctr1-1*) mutants showed glucose insensitive seedling growth, whereas *ethylene receptor1* (*etr1-1*) mutants displayed glucose hypersensitivity on 4% glucose. Epistatic analysis suggests that *ABA2/GIN1* acts downstream of the ethylene membrane receptor *ETR1* (Zhou et al., 1998). Moreover, genetic studies revealed that *sis1*/*gin4*/*ctr1* mutants are allelic and that the ethylene *insensitive4* (*ein4*) mutant showed hypersensitivity to sucrose-induce shoot development (Gibson et al., 2001; Rolland et al., 2002). As sugar inhibition is mediated at least partially through ABA biosynthesis and signaling, it can be argued that ethylene may decrease ABA biosynthesis or sensitivity to antagonize the effects of high sugar levels on seedling establishment (Gazzarrini and McCourt, 2001). One of the molecular mechanisms by which cross-talk between ethylene and sugar response pathways may occur is suggested by the observation that glucose signaling via

HXK1 enhances proteasome-mediated degradation of the ethylene transcriptional regulator EIN3, whereas ethylene enhances EIN3 stability (Yanagisawa et al., 2003). Sugars affect ethylene signaling at the transcriptional level as well. Transcriptional profiling of seedlings treated with 167 mM glucose revealed repression of *CTR1*, *EIN3* and genes associated with ACC metabolism (Price et al., 2004).

I. 5. 3. Sugar and GA signaling pathways

A good example to illustrate interactions between sugar and GA response pathways is the regulation of cereal α-amylase expression. During seed germination starch is degraded by α-amylase into sugars to provide energy and materials for embryo growth, and when sugar supply exceeds the demand of the sink cells α-amylase expression is repressed via a process involving sugar sensing. Sugar repression of α-amylase expression involves control of transcription and mRNA stability (Sheu et al., 1996; Chan and Yu, 1998; Lu et al., 1998). GA and sugar starvation activate α-amylase expression through coordinated regulation of GA-and sugar-responsive promoter elements. A sugar response sequence (SRS) in the promoter of the rice α-amylase gene *αAmy3* can confer sugar responsiveness to a minimal promoter. The SRS is comprised of three essential motifs: the GC box, the G box, and the TATCCA element (Lu et al., 2002). Three rice MYB transcription factors, OsMYBS1/2/3, with a single DNA binding domain, interact with the TATCCA element and differentially regulate α-amylase promoter activity. All OsMYBSs cooperate with a GA-regulated transcription factor, barley HvMYBGa, in high-level, GA-induced α-amylase gene expression (Lu et al., 2002). A recent study has shown that the GA response element (GARE) and rice MYBGA factor are required for high-level *αAmy8* expression in rice endosperm. MYBGA and MYBS may interact with each other to positively regulate *αAmy8* promoter and prevent sugar repression of *αAmy8* in endosperm (Chen et al., 2006)

I. 5. 4. Light, sugar and nitrogen signals

Light acts as a crucial signal and energy source for plant development. It induces the expression of photosynthesis-associated nuclear genes including *CAB*, *RBCS* and *PC*

(Quail, 2002). Analysis of *cis*-regulatory elements in the promoters of many light-regulated genes has revealed a conserved modular arrangements (CMAs) that constitute minimal light-responsive units (Argüello-Astorga and Herrera-Estrella, 1996). CMA5 responds positively not only to light signals but also negatively to ABA and sugars, reflecting a common regulatory mechanism for light and sugar signaling pathways (Acevedo-Hernández et al., 2005). Studies with *Arabidopsis* mutants that have carbon signaling defects suggest interactions between sucrose and phytochrome signals. For example, far-red light illumination prevents greening of *Arabidopsis* seedlings via a phytochrome A dependent pathway and sucrose can suppress this response (Barnes et al., 1996). However, sucrose repression of far-red light induced block of greening was perturbed in two *sucrose-uncoupled* (*sun*) mutants, *sun6* and *sun7,* which have reduced repression of the *PC* promoter by sucrose (Dijkwel et al., 1997).

Response to nitrogen, another essential nutrient for plant growth, exhibits cross-talk with response to carbon in many metabolic and developmental processes. Carbon metabolism is regulated to ensure efficient use of limiting nitrogen. Both high carbon and low nitrogen inhibit photosynthesis, suggesting C:N status may be more important than carbon status alone (Paul and Driscoll, 1997). Sugars can induce nitrate reductase (*NR*) expression and repress asparagine synthetase (*ASN*) expression, which possibly involves a mechanism for sensing C:N status in cells (Cheng et al., 1992; Lam et al., 1994). A study of *Arabidopsis* seedling development has shown that various parameters of plant growth, including morphology, storage lipid mobilization, chlorophyll levels and photosynthetic gene expression, are largely dependent on C:N ratios (Martin et al., 2002).

I. 6. Genetic dissection of sugar-response pathways

Genetic approaches to study sugar-response mutants have been successful in identifying components that are involved in sugar response/signaling pathways and/or in phytohormone signaling or metabolic pathways. In general, sugar-response mutants are defined by their altered sugar-responsive gene expression or varied sugar-induced biochemical/developmental phenomena (Rook and Bevan, 2003).

I. 6. 1. Mutants with altered expression of sugar-regulated genes

High levels of exogenous sugars induce the transcription of *Arabidopsis* β-amylase (*β-Amy*) (Mita et al., 1995). Based on this sugar response, mutants with either lower or higher expression of *β-Amy* in leaves compared with that of wild-type plants have been isolated. The *high-beta-amylase1* (*hba1*) mutant leaves have significantly higher expression of *β-Amy* than wild type when both are grown on media containing 2% sucrose (Mita et al., 1997). In contrast, the *low-beta-amylase1* (*lba1*) mutants have markedly lower levels of amylase activity than wild-type plants when their leaves are treated with 6% sucrose. The mutants also have reduced levels of sugar-inducible accumulation of anthocyanin (Mita et al., 1997). The *lba1* mutation was recently found to lie in the UPF1 RNA helicase gene, which is involved in nonsense-mediated mRNA decay (NMD) (Yoine et al., 2006).

The *sucrose-uncoupled* (*sun*) mutants show reduced sucrose-mediated repression of a reporter gene under the control of the plastocyanin reporter. In this study, luciferase was expressed under the control of the plastocyanin promoter and luciferase activity assayed in seedlings from a mutagenized population growing on media containing 3% sucrose (Dijkwel et al., 1996). The *sun6* mutant has reduced responsiveness to the negative effects of 2-deoxyglucose on whole plant photosynthesis and total Rubisco activity (Van Oosten et al., 1997). Both *sun6* and *sun7* have altered response to sucrose repression of the far-red light-inducible block of cotyledon greening (Dijkwel et al., 1997). The *SUN6* gene is allelic to *ABI4,* which is a component of the ABA signaling pathway (Huijser et al., 2000).

Patatin is the main storage proteins in potato tubers. Expression of patatin is regulated by carbohydrates and amino acids. In transgenic Arabidopsis plant carrying a construct consisting of a patatin class I promoter fused to the β-glucuronidase (GUS) reporter gene, the patatin promoter is mainly active in the root and is upregulated by sugars. The *reduced sugar response* (*rsr*) mutants were identified from a mutagenized population of the transgenic line (Martin et al., 1997). One of the mutants, *rsr1-1*, is also hypersensitive to low levels of proline and develops necroses. Addition of certain amounts of salt or glucose can ameliorate the toxicity of proline, demonstrating a link

between carbon/nitrogen metabolism and stress responses (Hellmann et al., 2000). The *rsr4-1* mutant has reduced leaf size, strongly reduced primary root length and other developmental abnormalities which can be fully complemented by provision of vitamin B6 (Wagner et al., 2006). The *rsr4-1* mutant carries a point mutation in a member of the pyridoxine biosynthesis protein1 (*PDX1*) family and consequently has reduced vitamin B6 content and altered amino and organic acid metabolism (Wagner et al., 2006).

The transcription of the ADP-glucose pyrophosphorylase large unit3 (*ApL3*) gene, which is involved in starch biosynthesis, is induced by sugars. Transgenic plants harboring the regulatory sequences of *ApL3* fused to a negative selection marker gene (bacterial cytochrome P_{450}, *ApL3::P450*) were generated and survived the presence of the R7402 proherbicide under non-inducing sugar conditions (Rook et al., 2001). Mutants derived from the parental transgenic line were selected for the ability to survive in the presence of R7402 and sucrose. These mutants showed reduced induction of the endogenous *ApL3* gene by sucrose and were named *impaired sucrose induction* (*isi*) mutants (Rook et al., 2001). The *isi3* mutant is allelic to *abi4* and *isi4* is allelic to *aba2*. ABA enhances *ApL3* induction by sugars although, ABA alone does not affect its expression, suggesting that ABA may increase the sensitivity of tissue response to sugar signals (Rook et al., 2001). The *ISI1* gene encodes a conserved plant-specific protein with structural similarities to Arm repeat proteins and is expressed mainly in the phloem in leaves following the sink to source transition (Rook et al., 2006). The *isi1* mutant displays reduced plant growth due to its inability to efficiently utilize available carbohydrates (Rook et al., 2006). Mutants with elevated *ApL3* expression on low levels of sugars have been isolated using a similar strategy where transgenic plants expressing the *ApL3::LUC* construct were mutagenized and their progeny screened on 1% sucrose (Baier et al., 2004). These *high sugar-response* (*hsr*) mutants show sugar hypersensitive growth in the light or in the dark, normal root elongation in response to ABA and normal hypocotyl lengths in the presence of ACC (Baier et al., 2004). The *HSR8* gene was recently cloned and is allelic to *MURUS4* (*MUR4*), which is involved in cell wall arabinose synthesis. Double mutant analysis showed that the *prl1* mutation suppresses the

sugar hypersensitive responses of *hsr8*, suggesting *PRL1* is linking the cell wall changes with sugar-responsive growth and development (Li et al., 2007).

I. 6. 2. Mutants with altered seedling growth response to sugars

High sugar levels have negative effects on *Arabidopsis* seedling establishment. High percentages of wild-type seedlings grown in the presence of elevated sugar levels (e.g. 300 mM) develop non-green cotyledons and do not form leaves and can only resume normal development when sugar levels are reduced. This sugar-induced growth arrest has been employed to screen for mutants with increased or decreased sensitivity to sugars.

The *prl1* mutant was isolated by its hypersensitive growth on 175 mM sucrose. In addition to increased sugar response, the mutant is hypersensitive to ABA, auxin, cytokinin and ethylene and accumulates sugar and starch in leaves (Németh et al., 1998). PRL1 negatively regulate AKIN10/11 activity and functions as the substrate receptor for AKIN10 in the CUL4-ROC1-DDB1-PRL1 E3 ligase complex (Bhalerao et al., 1999; Lee et al., 2008).

The *glucose hypersensitive1* (*ghs1*) mutant shows inhibition of chlorophyll synthesis and developmental arrest of leaves on 5% glucose. The *ghs1* mutation is caused by a T-DNA insertion in the *GHS1* gene, which encodes the plastid 30S ribosomal protein S21 involved in synthesis of proteins encoded by the chloroplast genome. Rubisco protein levels were reduced and abnormal plastid morphology was observed in the *ghs1* mutant, suggesting that signals from the plastid mediated by ribosomal proteins are important for proper sugar response in seedling development (Morita-Yamamuro et al., 2004). Interestingly, mutation in another plastid protein gene, *THF1,* also causes a glucose-hypersensitive root growth phenotype (Huang et al., 2006).

Interestingly, an *oversensitive to sugar1* (*osu1*) mutant shows hypersensitivity to imbalanced C/N conditions, high C/low N and low C/high N, with respect to anthocyanin accumulation in cotyledons and root growth inhibition. Under balanced C/N conditions, the *osu1* mutant has wild-type sensitivity to sugars (Gao et al., 2008). *OSU1* is allelic to *QUA2/TSD2*, which is involved in cell adhesion and localized to the Golgi. The findings that the *osu1/qua2/tsd2* mutants have defects in cell wall composition or structure and

hypersensitivity to C/N imbalance suggest a link between cell wall changes and proper C/N balance response (Gao et al., 2008), which is also demonstrated by the *hsr8* mutant (Li et al., 2007).

Mutants with decreased sensitivity to the inhibitory effects of high sugar levels on seedling growth have been identified. Examples include the *gin*, *mig* and *sis* mutants (Zhou et al., 1998; Laby et al., 2000; Pego et al., 2000). Mannose is able to suppress *Arabidopsis* seed germination at low millimolar concentrations in an HXK-dependent manner (Pego et al., 1999). Genetic screens thus were performed to isolate *mannose-insensitive germination* (*mig*) mutants. The *mig* screen showed a partial overlap with other screening strategies using high concentrations of sugars, indicating the existence of a common mechanism for regulating different sugar signaling pathways (Pego et al., 2000).

The *gin* mutants were isolated based on their resistance to the inhibitory effects of 7% glucose on seedling development. The molecular identities of several *GINs* have been established. *GIN1*, *GIN5* and *GIN6* are allelic to *ABA2*, *ABA3* and *ABI4* respectively, which are components of ABA metabolic and signaling pathways (Zhou et al., 1998; Arenas-Huertero et al., 2000; Cheng et al., 2002). *GIN4* is allelic to *CTR1*, which is involved in ethylene signaling (Rolland et al., 2002). The *gin2* mutant carries a mutation in the *HXK1* gene, which is a proposed sugar sensor (Moore et al., 2003; Cho et al., 2006).

The Gibson lab initiated a *sugar insensitive* (*sis*) screen for mutants that exhibit resistance to the inhibitory effects of 300 mM sucrose or glucose on early seedling development (Laby et al., 2000). The *sis4* and *sis5* mutants are allelic to the *aba2* and *abi4* mutants, respectively (Laby et al., 2000). *SIS1* is allelic to *CTR1* (Gibson et al., 2001). During research described in this dissertation, three *SIS* genes were cloned and characterized, namely *SIS7*, *SIS3* and *SIS8*.

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Chapter II

Identification, cloning and characterization of *sis7* and *sis10* sugar-insensitive mutants of *Arabidopsis*

Identification, cloning and characterization of *sis7* and *sis10* sugar-insensitive mutants of *Arabidopsis*

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Abstract

Background

The levels of soluble sugars, such as glucose and sucrose, help regulate many plant metabolic, physiological and developmental processes. Genetic screens are helping identify some of the loci involved in plant sugar response and reveal extensive cross-talk between sugar and phytohormone response pathways.

Results

A forward genetic screen was performed to identify mutants with increased resistance to the inhibitory effects of high levels of exogenous sugars on early *Arabidopsis* seedling development. The positional cloning and characterization of two of these *sugar insensitive* (*sis*) mutants, both of which are also involved in abscisic acid (ABA) biosynthesis or response, are reported. Plants carrying mutations in *SIS7/NCED3/STO1* or *SIS10/ABI3* are resistant to the inhibitory effects of high levels of exogenous Glc and Suc. Quantitative RT-PCR analyses indicate transcriptional upregulation of ABA biosynthesis genes by high concentrations of Glc in wild-type germinating seeds. Gene expression profiling revealed that a significant number of genes that are expressed at lower levels in germinating *sis7-1/nced3-4/sto1-4* seeds than in wild-type seeds are implicated in auxin biosynthesis or transport, suggesting cross-talk between ABA and auxin response pathways. The degree of sugar insensitivity of different *sis10/abi3* mutant seedlings shows a strong positive correlation with their level of ABA insensitivity during seed germination.

Conclusions

Mutations in the *SIS7/NCED3/STO1* gene, which is primarily required for ABA biosynthesis under drought conditions, confer a sugar-insensitive phenotype, indicating that a constitutive role in ABA biosynthesis is not necessary to confer sugar insensitivity. Findings presented here clearly demonstrate that mutations in *ABI3* can confer a sugar-insensitive phenotype and help explain previous, mixed reports on this topic by showing that ABA and sugar insensitivity exhibit a strong positive correlation in different *abi3*

mutants. Expression profiling revealed a potentially novel regulation of auxin metabolism and transport in an ABA deficient mutant, *sis7-1/nced3-4/sto1-4*.

Background

Plant growth and development are regulated by signal transduction pathways that incorporate environmental stimuli and internal signals such as metabolic status. The absolute levels of soluble sugars, and/or the rate of flux of soluble sugars through certain metabolic pathways, are important indicators of metabolic status. Recent studies have established the role of sugars in regulating, at least partially, a variety of developmental processes from seed development and germination, early seedling development, vegetative growth and senescence to abiotic stress response [1-10].

Genetic screens based on the phenomenon that early seedling development of wild-type *Arabidopsis* can be arrested by high concentrations of exogenous sucrose (Suc) or glucose (Glc) have yielded sugar-response mutants. Characterization of these mutants has revealed that many also have defects in phytohormone metabolism or response [11-15]. In particular, several of these sugar-response mutants are allelic to abscisic acid (ABA) biosynthesis or response mutants such as *aba2*, *aba3* or *abi4*. The sugar-resistant mutants *sis4* [16], *gin1* [15, 17] and *isi4* [18] are allelic to *aba2*, an ABA biosynthesis mutant [19]. The *gin5* mutant [6, 11] is allelic to another ABA deficient mutant, *aba3* [19, 20]. Similarly, mutations in *ABA1* have been shown to confer Glc insensitivity [13]. The sugar-resistant mutants *sis5* [16], *gin6* [11], *isi3* [18] and *sun6* [13] are allelic to *abi4*, an ABA-insensitive mutant [21]. The *ABI4* locus encodes an APETALA2 type transcription factor [22]. Mutations in *ABI5* have also been shown to confer a weak, but still significant, sugar-insensitive phenotype [11, 13, 16]. Interestingly, plants carrying mutations in *ABI1* or *ABI2* have been found to exhibit a wild-type [11, 16] or near wild-type [13], response to the inhibitory effects of high sugar concentrations on early seedling development. Previous research has given a mixed report on the role of ABI3 in sugar response. The *abi3-1* mutant was found to exhibit an almost wild-type response to high

levels of exogenous Glc [11, 16] but was also reported to show a less pronounced but significant Glc insensitive phenotype when compared to wild-type plants [13]. In addition, it has been observed that specific *abi3* alleles show decreased sensitivity to the inhibitory effects of a combination of ABA and Glc on seedling development [23]. Recently ABI3 has been reported to play an important role in inhibition of seed germination [24] and post-germinative growth [25] by Glc.

In screens of ethyl methane sulphonate (EMS)- or T-DNA mutagenized *Arabidopsis* populations for mutants with increased tolerance to the inhibitory effects of high levels of exogenous sugars on early seedling development, three *sugar insensitive* (*sis*) loci were identified. Map-based cloning experiments resulted in the identification of these loci. The *sis9-1* mutation was found to lie in *ABA1*, a gene previously shown to act in ABA biosynthesis [26], and was not further characterized. The *sis7* mutations lie in the same gene as the previously identified *nced3/sto1* [27] mutants. The *sis10* mutation lies in the same gene as the previously identified *abi3* mutants [28]. *NCED3/STO1* encodes a 9-cis-epoxycarotenoid dioxygenase [29]. Expression of *SIS7/NCED3/STO1* has been suggested to be rate-limiting in the positive feedback regulation of ABA biosynthesis by ABA under stress conditions [17, 26]. Expression profiling experiments identified 82 genes as being differentially regulated in the *sis7-1* mutant when compared to wild-type seeds upon treatment with 100 mM Glc. *ABI3* encodes a B3 domain transcription factor [30]. The sugar- and ABA-insensitivity of multiple *abi3* mutant alleles show a strong positive correlation in our assays.

Results

Isolation of sugar-insensitive *(*sis*) mutants* sis7 *and* sis10

Most wild-type *Arabidopsis* seeds sown on minimal media [31] supplemented with high levels of Suc or Glc (e.g., 300 mM) germinate but the resulting seedlings fail to develop green expanded cotyledons and true leaves [11-13, 15, 16, 32, 33]. Instead, the seedlings

develop small, white or purple cotyledons. In contrast, low concentrations (e.g., 30 mM) of exogenous Suc or Glc do not exert this inhibitory effect on normal shoot development. In addition, most wild-type seeds can develop into seedlings with expanded cotyledons and true leaves on media containing high concentrations (300 mM) of sorbitol, which acts as a non-metabolizable sugar analog in *Arabidopsis*. This observation indicates that the inhibitory effects of high sugar levels on early seedling development are not solely due to osmotic stress [16].

Genetic screens based on the inhibitory effects of high sugar levels on early seedling development led to the discovery of a number of sugar resistant mutants [11-13, 15, 16]. In one such screen performed previously by our lab, approximately 60,000 M2 seeds derived from an EMS-mutagenized *Arabidopsis thaliana* var. Col population were screened to identify seedlings that are resistant to 300 mM Suc. The mutants identified via this screen are also resistant to high concentrations of Glc and so were designated *sugar insensitive*, or *sis*, mutants [16]. Cloning and characterization of one of these mutants, the *sis7-2* mutant, is described below. A similar mutant screen was conducted as part of the work reported here. This new mutant screen was conducted using a T-DNA mutagenized *Arabidopsis thaliana* var. Col population. The 35SpBARN binary vector used to generate this population contains random *Arabidopsis* cDNAs driven by the Cauliflower Mosaic Virus 35S promoter [34]. Approximately 200,000 seeds from ~20,000 independent *Arabidopsis thaliana* var. Col transgenic lines were screened on media containing 340 mM Suc and ~1,700 seedlings that developed shoot systems with expanded cotyledons and true leaves were transferred to soil. This high rate of seedlings exhibiting an apparent sugar-resistant phenotype is due to the fact that some fraction (~1% in this experiment) of wild-type seedlings are able to escape the developmental arrest caused by high concentrations of exogenous sugars [16]. Therefore, it is necessary to screen seeds harvested from each putative mutant to identify the relatively small percentage that exhibit a heritable *sis* phenotype. Towards this end, the 1,700 seedlings were allowed to grow to maturity and seeds were harvested from each plant. Re-screening of seeds from each of the 1,700 putative mutants on media containing 340 mM

Suc revealed that 18 of the putative mutants exhibit a reproducible *sis* phenotype. Characterization of these 18 mutants revealed that they appear to represent nine independent mutagenic events. The cloning and characterization of three of these new mutants, *sis7-1, sis7-3* and *sis10-1*, as well as the *sis7-2* mutant, is described below. The results of these studies indicate that the *sis7* mutations lie in a gene previously shown to affect ABA metabolism, whereas the *sis10* mutations lie in *ABI3*, which acts in ABA response [28].

sis7 *mutants exhibit a sugar-insensitive phenotype*

A significantly higher percentage of *sis7* than of wild-type seeds develop into seedlings with relatively normal shoot systems on media containing 300 mM Suc or Glc (Figure 1). On media containing 300 mM Suc, more than 80% of all three *sis7* mutants form expanded cotyledons and true leaves, whereas less than 6% of wild-type seedlings develop expanded cotyledons and true leaves. The mutants were also assayed for osmotic tolerance using media containing high concentrations of sorbitol. When assayed on equimolar (i.e. 300 mM) sorbitol supplemented with a low (30 mM) amount of Suc, differences are hard to detect between wild-type and mutant lines due to the fact that development of wild-type seedlings is not strongly affected at this concentration of sorbitol. In contrast, when assayed on media containing 400 mM sorbitol and 30 mM Suc, all three mutant alleles confer significantly increased osmotic resistance. Mannose (Man) is a Glc analogue that inhibits seed germination and early seedling development at millimolar concentrations via a mechanism that has been postulated to involve hexokinase [35]. Therefore, the mutants were assayed for Man sensitivity. None of the three *sis7* mutant alleles tested cause a significant alteration in sensitivity to 1.5 mM Man compared to wild-type seedlings (Figure 1).

sis7-1 *exhibits resistance to the inhibition of seed germination by ABA and paclobutrazol*

Previous studies have shown that *sis4/aba2* mutants are resistant to the inhibitory effects of paclobutrazol, an inhibitor of gibberellin biosynthesis, on seed germination. Similarly, *sis5/abi4* mutants are resistant to the inhibitory effects of both paclobutrazol and ABA on seed germination [16]. To determine whether mutations in *SIS7* exert similar effects, the germination rates of *sis7-1* seeds on media containing ABA or paclobutrazol were determined. Seeds carrying the *sis7-1* mutation exhibit significantly faster germination rates than wild-type seeds on media containing 1 μM ABA and slightly faster germination rates on 2 μM ABA, but have wild-type germination rates on higher concentrations (e.g. 5 μM) of ABA (Figure 2). Germination rates of *sis7-1* and wild-type seeds were also assayed on media containing several different concentrations of paclobutrazol. Seeds carrying the *sis7-1* mutation display enhanced germination rates compared to wild-type seeds on media containing high levels (e.g. 60 or 240 μM) of paclobutrazol (Figure 3).

Cloning of SIS7 *and pharmacological complementation of the* sis7 *phenotype*

A map-based cloning approach was used to identify the *SIS7* gene. The *sis7-1* mutant, which is in the Col background, was crossed to wild-type Hi-O plants. F2 progeny of this cross were screened on media containing 340 mM Suc. Those seedlings that formed relatively normal shoot systems (i.e. displayed a *sis* mutant phenotype) were selected and used to form a mapping population of 940 plants. SSLP and CAPS markers were used to localize *sis7* to a 78 kb region between BAC clones MLN21 and MIE1 on chromosome 3. DNA spanning the entire 78 kb region was isolated from *sis7-1* by PCR and sequenced. Analysis of this sequencing data reveals that *sis7-1* carries a 21 nucleic acid deletion and a partial T-DNA insertion in the exon region of At3g14440. No other mutations were found in the region shown by mapping to contain the *sis7-1* mutation. Another *sis* mutation was independently mapped to chromosome 3 between BACs MLN21 and MSJ11, the region which contains the At3g14440 gene. Sequencing of the putative *SIS7* gene from this mutant, now named *sis7-2*, identified a point mutation in the exon region that changes amino acid codon 245 from Gly to Asp. Sequencing of the putative *SIS7*

gene from a third *sis* mutant revealed that this mutant, now named *sis7-3*, contains a large deletion in the *SIS7* gene (Figure 4A). The finding that three independent *sis* mutants all carry mutations in At3g14440 provides convincing evidence that the mutations in this gene are responsible for the *sis* phenotype observed in these lines. At3g14440 has previously been shown to encode 9-cis-epoxycarotenoid dioxygenase (NCED3), a key enzyme in the biosynthesis of ABA [29, 36]. Plants carrying a T-DNA insertion in *NCED3* are drought sensitive [29]. An additional mutant of *NCED3*, named *sto1*, was identified in a screen for salt-tolerant mutants. These *sto1* mutants exhibit resistance to the inhibitory effects of hyperosmotic stress on seed germination, but not on post-germinative growth [27]. A third mutant carrying a T-DNA insertion in *NCED3* has also been described [37]. The three mutant alleles identified in this study were accordingly re-designated *sis7-1/nced3-4/sto1-4, sis7-2/nced3-5/sto1-5* and *sis7-3/nced3-6/sto1-6*. To determine whether *SIS7* mRNA levels are altered in the *sis7* mutants, *SIS7* transcript abundance was assayed by RT-PCR in wild-type plants and in all three *sis7* mutants. *SIS7* transcript was not detected in *sis7-1* or *sis7-3*, whereas in *sis7-2 SIS7* transcript abundance is approximately the same as in wild-type plants (Figure 4B).

Consistent with other *aba* mutants, *sis7-1/nced3-4/sto1-4* exhibits a wilty phenotype when subject to water deprivation (data not shown). To determine if the sugar insensitivity of the *sis7/nced3/sto1* mutants is affected by ABA deficiency, pharmacological complementation of *sis7/nced3/sto1* mutant seedlings was conducted as described [11, 17]. Early seedling development of Col wild type, three *sis7* mutant alleles, the ABA deficient mutant *aba2-3* and an ABA insensitive mutant *abi4-103* were not affected by 220 mM Glc media. Addition of a noninhibitory level of 0.1 μM ABA to the media containing 220 mM (4%) Glc restored Glc sensitivity of *sis7-1/nced3-4/sto1-4, sis7-2/nced3-5/sto1-5* and *sis7-3/nced3-6/sto1-6*, as well as of the ABA deficient mutant *aba2-3*, but not that of the ABA insensitive mutant *abi4-103* (Figure 5). These results indicate that the *sis* phenotype of all three *sis7* mutant alleles is caused by ABA deficiency.

Glc regulation of ABA biosynthesis gene expression in germinating seeds

Previously it has been shown that 7% Glc causes ABA accumulation (or decreased metabolism) in wild-type seedlings and that an increase in ABA levels is required for Glc-dependent developmental arrest [11]. The expression levels of the ABA biosynthesis genes, *ABA1*, *ABA2*, *AAO3* and *ABA3* were found to be increased by 2 and 6% Glc. In contrast, *NCED3* expression was not activated by Glc [17]. However, as these studies were conducted using young seedlings, the mechanism of high Glc-induced ABA accumulation in germinating seeds remains unclear. Furthermore, it has been shown that high concentrations of exogenous sugars inhibit early seedling development only during a narrow temporal window (less than 40 hours after the start of imbibition) [12]. We therefore performed quantitative real-time PCR (qRT-PCR) analysis of ABA biosynthesis gene expression in both *sis7* and wild-type germinating seeds. In brief, two biologically independent batches of Col wild-type, *sis7-1* and *sis7-3* seeds were sown on minimal media for 20 h and transferred to media supplemented with 300 mM Glc or 300 mM sorbitol for 13 h under continuous light before harvest. The total time of treatment was within the temporal window during which exogenous sugars can arrest seedling development [12]. Total RNA was extracted for qRT-PCR analysis. As shown in Figure 6, *ABA1*, *SIS7/NCED3*, *ABA2* and *ABA3* exhibit higher steady-state mRNA levels in wild-type seeds germinating in the presence of 300 mM Glc than in the presence of 300 mM sorbitol. In contrast, *AAO3* steady-state mRNA levels are not significantly different in wild-type seeds germinating on Glc than on sorbitol. Glc induction of *SIS7/NCED3*, as well as *ABA1*, *ABA2* and *ABA3*, is abolished in the *sis7-1* and *sis7-3* null mutants, suggesting a certain level of endogenous ABA may be required for Glc induction of ABA biosynthesis gene expression in germinating seeds.

Transcriptional profiling identifies mis-regulated genes in the sis7/nced3/sto1 *mutant*

To identify genes that are differentially expressed in *sis7-1/nced3-4/sto1-4* versus wild type, *Arabidopsis* Affymetrix ATH1 GeneChips were used for transcriptional profiling of

germinating *sis7-1/nced3-4/sto1-4* and wild-type seeds. Col wild-type and *sis7-1/nced3-4/sto1-4* seeds were sown on minimal media for 20 h and transferred to 100 mM Glc media for 13 h under continuous light to allow induction or repression of Glc-responsive genes. At the end of this incubation period 0-4% of the seeds had germinated. The reason that seeds were collected after a total of 33 h is based on the observations that exogenous sugars inhibit seedling development during a narrow temporal window (less than 40 hours after start of imbibition) [12]. Data analysis using the Expressionist software package is as detailed in "Materials and methods". To identify genes that are expressed at significantly different levels in *sis7-1/nced3-4/sto1-4* versus wild-type germinating seeds, the results of three independent GeneChip experiments using mutant seeds were compared with the results of six independent biological replicates of wild-type samples. Significant differences in expression values were defined as those where the average expression levels between mutant and wild-type seeds differ by at least two fold and have a student's t-test p value of less than 0.05. Using these cutoffs, the levels of 82 transcripts were found to be altered in *sis7-1/nced3-4/sto1-4* versus wild-type seeds incubated in the presence of 100 mM Glc (Table 1). Of these genes, 20 had higher mRNA levels in the mutant than in the wild type, whereas 62 genes had lower mRNA levels in the mutant.

Functional categorization of these mis-regulated genes was performed using the Gene Ontology (GO) Annotations tool from TAIR [38] and the results are presented in Figure 7. Notably, a significant percentage of the mis-regulated genes are involved in response to abiotic or biotic stimulus and to stress (a combined 25.82%). Over-representation analysis (ORA) was also performed. ORA is a useful tool for analyzing gene expression data obtained from microarray experiments as it can determine whether a set of genes are statistically over-represented among the total genes expressed on the microarray. An online version of the GeneMerge program [39] was used for ORA to identify whether particular biological processes or molecular functions, as defined by GO annotations, are statistically enriched in the set of differentially expressed genes in the *sis7-1* mutant compared to the wild type [40]. Genes involved in response to stimulus (GO:0050896)

are significantly enriched (16/82; *p*=0.0095 after Bonferroni correction for multiple tests) and genes in response to abiotic stimulus (GO:0009628, a child term of GO:0050896) are also significantly over-represented (10/82; *p*=0.0167), both of which include *PIN1*, *PIN3* and *CYP83B1*. Manual inspection of the data identified two other genes involved in auxin metabolism: *CYP79B2* and At4g27260. Thus, interestingly, genes involved in auxin biosynthesis and transport are significantly over-represented amongst the genes that are expressed at lower levels in *sis7-1*. For example, *CYP79B2* is involved in Trp metabolism and converts Trp to indole-3-acetaldoxime (IAOx), a precursor to indole acetic acid (IAA) [41, 42]. Expression of *CYP79B2* is reduced more than five-fold in mutant seeds relative to wild-type seeds. At4g27260 encodes an IAA-amido synthase that conjugates Asp and other amino acids to auxin *in vitro*. An insertional mutation in this gene causes increased sensitivity to IAA in seedling roots [43]. *CYP83B1* is required for red light signal transduction and auxin homeostasis [44]. *PIN1* and *PIN3* are auxin efflux transporters and regulate root development [45]. Interplay between auxin and ABA signaling to regulate lateral root development has been suggested in previous studies [46-48]. The decreased mRNA levels of these auxin-related genes in Glc-treated *sis7-1/nced3-4/sto1-4* seeds provide further evidence of cross-talk between these pathways.

Quantitative real-time RT-PCR validation of microarray data

To re-test the results obtained through microarray experiments, qRT-PCR analysis was performed on selected genes, with an emphasis placed on genes related to auxin transport and metabolism. Two biologically independent batches of Col wild-type, *sis7-1* and *sis7-3* seeds were sown on minimal media, incubated for 20 h and then transferred to 100 mM Glc or 100 mM sorbitol media and incubated for an additional 13 h under continuous light before harvest. Total RNA was extracted for qRT-PCR analysis to monitor the relative expression levels of an array of genes, including *PIN1*, *PIN3*, *CYP83B1*, *CYP79B2* and *AT4G27260* (an IAA-amido synthase). On 100 mM sorbitol (osmotic control) media, the expression levels of these genes are similar in *sis7-1*, *sis7-3* and Col wild type. In contrast, on 100 mM Glc media, the transcript levels of each gene are lower

in the *sis7-1* and *sis7-3* mutants than in the Col wild type (Figure 8). These qRT-PCR results confirm the findings from microarray data that the steady-state mRNA levels of genes involved in auxin transport and metabolism are lower in the *sis7* mutants than in the wild type on 100 mM Glc.

sis7-1 *mutants exhibit increased lateral root systems*

The decreased mRNA levels of auxin-related genes observed in Glc-treated *sis7-1/nced3-4/sto1-4* seeds provide further evidence of cross-talk between these pathways. To test whether the osmotic regulation of lateral root development is altered in the *sis7* mutants, wild-type and *sis7-1/nced3-4/sto1-4* seeds were germinated and grown on sorbitol media. The *sis7-1* seedlings exhibit increased lateral root systems on sorbitol, when compared to the wild-type plants (Figure 9). These observations are consistent with previous findings that *aba2-1* and *aba3-1* display increased root system size on mannitol [47].

Evidence for an expanded role for ABI3 in sugar response through mapped-based cloning of SIS10

The *sis10-1* mutant was isolated during the same screen of a T-DNA mutagenized population, described above, that was used to isolate the *sis7-1* and *sis7-3* mutants. The *sis10-1* mutant has strong Glc and Suc insensitive phenotypes during early seedling development. A map-based approach was used to identify the *SIS10* gene. Towards this end, *sis10-1* (in the Col-0 ecotype) was crossed with a wild-type plant of the Hi-O ecotype. F2 seeds from this cross were selected using *Arabidopsis* media supplemented with 320 mM Suc to identify seedlings with a *sis* mutant phenotype. An F2 population of 320 *sis* plants was then analyzed using SSLP markers. The *sis10-1* mutation was mapped to chromosome 3, approximately 0.15 cM above marker 470509 on BAC MSD24. Examination of this region of the genome revealed that *ABI3* (At3g24650) is present in the region believed to contain the *sis10* locus. Sequencing of *ABI3* DNA isolated from *sis10-1* revealed that the *sis10-1* mutation results in deletion of 16 nucleotides between

base pairs 2715 and 2730 of the genomic DNA (with respect to the start codon) in the 6th exon of *ABI3*. Accordingly, the *sis10-1* mutant has been renamed *abi3-15*.

Previous work from several labs [11, 13, 16] characterizing the *abi3-1* mutant, which is in the Landsberg *erecta* background, found only slight to no effect of this mutation on sugar response. Therefore, the finding that the *sis10-1* mutation, which causes a strong *sis* phenotype, lies in the *ABI3* gene was unexpected. To test whether the mutation in *ABI3* is truly the cause of the *sis10-1* sugar insensitive phenotype, two additional *abi3* mutant lines were obtained and characterized. SALK_023411 carries a mutation, here designated as *abi3-16*, consisting of a T-DNA insertion in the first exon of the *ABI3* gene (Figure 10). SALK_003216 carries a mutation, here designated as *abi3-17*, consisting of a T-DNA insertion in the *ABI3* promoter region [49]. Sugar-insensitivity assays on plants homozygous for the *sis10-1/abi3-15*, *abi3-16* and *abi3-17* mutations reveal that all three mutations confer significant resistance to the inhibitory effects of 300 mM Suc on early seedling development, compared to wild-type plants (Figure 11). These results confirm that mutations in *ABI3* can lead to a strong sugar-insensitive phenotype.

Previous work indicated that *abi3-1* has a relatively strong ABA insensitive seed germination phenotype [28], but displays only a very subtle sugar insensitive phenotype during early seedling development [11, 13, 16]. The finding that other *abi3* mutants, such as *sis10-1/abi3-15*, *abi3-16* and *abi3-17*, can have strong sugar-insensitive phenotypes during early seedling development raised the possibility that different mutations in *ABI3* may predominantly affect either ABA or sugar response. To test this hypothesis a careful analysis was conducted to determine the relative degrees of sensitivity to the inhibitory effects of ABA on seed germination and of Suc on early seedling development of nine different *abi3* mutant lines (Figure 12). These nine *abi3* lines included the *sis10-1/abi3-15*, *abi3-16* and *abi3-17* mutants described above, as well as the *abi3-8, abi3-9, abi3-10, abi3-11, abi3-12* and *abi3-13* mutants [23]. All of these mutants are in the Col ecotype. The molecular lesions of the different *abi3* mutants are depicted in Figure 10. To measure sugar response, seeds of all nine *abi3* mutants plus

Col wild-type were sown on minimal *Arabidopsis* media [31] supplemented with 270 or 320 mM Suc and grown in continuous light for 14 d before scoring. These Suc concentrations were chosen to allow detection of differences in the degree of Suc insensitivity. The 270 mM Suc concentration is sufficient to allow detection of mutants with relatively weak sugar-insensitive phenotypes, as these mutants will still exhibit a significantly greater ability than wild-type plants to develop expanded cotyledons and true leaves on 270 mM Suc. However, a 270 mM concentration of Suc may not be sufficient to distinguish between mutations that confer moderate versus strong sugar-insensitive phenotypes. This distinction can be made using the 320 mM Suc concentration. Selection on media supplemented with 320 mM Suc revealed that five of the *abi3* mutations (*abi3-8, abi3-9, abi3-10, abi3-16* and *abi3-17*) confer strong sugar-insensitive phenotypes. The other *abi3* mutations tested conferred lesser degrees of sugar-insensitivity. The relative degree of sugar insensitivity of these other four mutants, from highest to lowest, is: *abi3-15, abi3-13, abi3-12* and *abi3-11* (Figure 12A). To assess the relative effects of the different *abi3* mutations on ABA sensitivity, seed germination was assayed at different time points and in the presence of different concentrations of ABA (Figure 12B). A regression analysis of the results indicates a strong positive correlation between sugar insensitivity and ABA insensitivity of tested *abi* mutants (Fig 12C). In other words, mutations that confer a high degree of insensitivity to ABA also confer a high degree of insensitivity to Suc. Conversely, mutations that confer only weak ABA insensitivity also confer weak insensitivity to Suc.

Discussion

Molecular genetic studies on *Arabidopsis* sugar response mutants have revealed extensive evidence for cross-talk between sugar and phytohormone response pathways [11-13, 15-18, 50, 51]. For example, exogenous Glc has been proposed to slow the decrease in ABA concentrations that occurs during seed germination [52]. Glc has also been shown to help regulate expression of a number of genes involved in ABA metabolism in seedlings. Several ABA biosynthetic genes, including *ABA1*, *AAO3* and *ABA3* are upregulated by

110 mM and 330 mM Glc via a mechanism that requires that a certain endogenous ABA level is maintained [17]. Interestingly, these same genes are downregulated by 330 mM mannitol, via a mechanism that does not appear to require wild-type levels of endogenous ABA. These results suggest that regulation of these three genes by Glc is distinct from their regulation by osmotic stress. Similarly to *ABA1, AAO3* and *ABA3*, the ABA biosynthetic gene *ABA2* is also upregulated by 110 mM and 330 mM Glc via a mechanism that requires that endogenous ABA levels be maintained above a certain level. However, unlike *ABA1, AAO3* and *ABA3*, expression of *ABA2* is also upregulated by 110 mM and 330 mM mannitol via a mechanism that does require maintenance of endogenous ABA levels [17]. So, although regulation of all four genes by Glc appears similar, *ABA2* exhibits significant differences in expression in response to osmotic stress.

The ABA biosynthetic gene *SIS7/NCED3/STO1* also exhibits an expression pattern that is distinct from that of the other ABA biosynthetic genes discussed above. In wild-type seedlings, the expression of *SIS7/NCED3/STO1* is not activated by Glc but is strongly induced by salt or osmotic stress [17, 26]. In fact, SIS7/NCED3/STO1 may act primarily in the biosynthesis of ABA during stress conditions [29]. Due to these regulatory and functional differences, it was not clear whether mutations in *SIS7/NCED3/STO1* would cause a sugar-insensitive phenotype, as had been previously shown for mutations in other ABA biosynthetic genes [11, 13, 16]. Results presented here, showing that the *sis7* mutations lie in *NCED3/STO1*, demonstrate that mutations in *SIS7/NCED3/STO1* do confer a strong sugar-insensitive phenotype.

Expression of *SIS7/NCED3/STO1* has been suggested to be rate-limiting in the positive feedback regulation of ABA biosynthesis by ABA under stress conditions [53, 54]. Transcriptional upregulation of *SIS7/NCED3/STO1* by ABA has been found in certain ABA-deficient mutants and ecotypes [17, 26]. However, the transcriptional regulation of *NCED3* as well as other ABA biosynthesis genes by Glc in germinating seeds is unclear. Results presented here (Figure 6) show that in wild-type germinating seeds high concentrations of exogenous Glc result in increased *SIS7/NCED3* steady-state mRNA

levels. Steady-state mRNA levels of *ABA1*, *ABA2* and *ABA3* in wild-type germinating seeds are also upregulated by high concentrations of exogenous Glc. In contrast, in germinating seeds of the *sis7-1* and *sis7-3* mutants, Glc induction of *ABA1*, *SIS7/NCED3*, *ABA2* and *ABA3* is abolished. Previously it has been shown that 333 mM Glc induces ABA accumulation (or decreases ABA metabolism) in imbibed seeds [52]. The results of experiments presented here, analyzing Glc regulation of ABA biosynthesis genes in *sis7* and wild-type germinating seeds, indicate that high Glc levels activate ABA biosynthesis by increasing the steady-state meRNA levels of ABA biosynthesis genes. These increases may, in turn, subsequently lead to ABA accumulation.

Besides transcriptional regulation, translational and post-translational mechanisms may also be involved in sugar-mediated regulation of ABA levels and/or signaling in germinating seeds. For example, loss-of-function of the *KEEP ON GOING* (*KEG*) gene, which is involved in ABI5 degradation, causes the mutant seedlings to exhibit a sugar hypersensitive phenotype [55]. In wild-type germinating seeds, the Glc-mediated increases in ABA levels may induce *SIS7/NCED3* expression, as well as the expression of other ABA biosynthetic genes, thus resulting in sustained increases in ABA levels [26, 54]. In the *sis7/nced3/sto1* mutant, this positive feedback control loop is likely broken as is evidenced by the loss of Glc induction of *ABA1*, *SIS7/NCED3*, *ABA2* and *ABA3* in *sis7-1* and *sis7-3* germinating seeds. Thus the failure to accumulate ABA under 'sugar stress' may render mutant seedlings unable to carry out a normal sugar response. Consistent with this possibility are findings that the *nced3/sto1* mutant accumulates only one-third as much ABA as wild type under sorbitol stress [27]. In addition, it has been reported that ABA levels in *sto1/nced3* plants are somewhat lower than those found in unstressed wild-type plants [27]. These results may explain the enhanced germination rate of *sis7-1* seeds on very low (1 μM), but not on higher (2-5 μM), levels of ABA. Also, addition of exogenous ABA to the 220 mM Glc media eliminated the sugar-insensitive phenotype of *sis7/nced3/sto1* seedlings, indicating that the *sis* phenotype is caused by a deficiency in endogenous ABA.

As some mutants that are resistant to Glc and Suc also exhibit resistance to Man, the ability of the *sis7-1* mutant to germinate on media containing 1.5 mM and higher concentrations of Man was tested. The results of these experiments indicate that *sis7-1* does not exhibit increased resistance to the inhibitory effects of exogenous Man on seed germination. This finding is consistent with the results of Man seed germination assays conducted on other *aba* mutants, where *aba1-1* and *aba3-2* were found to be unable to germinate in the presence of Man and *aba2-1* [13] and *aba2-3* and *aba2-4* [16] were found to be only very slightly resistant to Man. Man inhibition of seed germination has been postulated to act through a hexokinase-dependent pathway. This hypothesis is based on the fact that the hexokinase inhibitor mannoheptulose can reverse the inhibitory effects of Man on germination of wild-type seeds [35]. The *sis7-1* mutant has a strong *sis* phenotype but no significant Man-insensitive phenotype, suggesting that response to Man may not require ABA accumulation. Notably, *NCED3* belongs to a multigene family with nine members in *Arabidopsis*, five of which are postulated to be involved in ABA biosynthesis [36, 56]. Characterization of mutants in other members of the gene family may help distinguish their functions in sugar response.

Transcriptional profiling analyses identified 82 genes with altered mRNA levels in *sis7-1* germinating seeds. Functional categorization and over-representation analyses by GO annotations revealed significant enrichment of genes involved in response to stimulus, particularly abiotic stimulus. An interesting finding from these analyses is the decreased steady-state mRNA levels of auxin biosynthesis and transport genes in *sis7-1* treated with Glc when compared to the wild type. These findings were re-tested by qRT-PCR analyses. The results of the qRT-PCR experiments confirm that the steady-state mRNA levels of these auxin-related genes are indeed lower in *sis7-1* and *sis7-3* germinating seeds compared to wild-type seeds (Figure 8). Previous studies have suggested cross-talk between the ABA and auxin response pathways in regulating *Arabidopsis* root system development [46-48]. The *sis7-1* mutant exhibits increased lateral root development on sorbitol media when compared to the wild type (Figure 9). In addition, *AtNCED3::GUS* expression has been observed at lateral root initiation sites, suggesting control of lateral

root development by both ABA and auxin [36]. The findings reported here suggest that part of the molecular mechanism(s) by which ABA/auxin cross-talk occurs during regulation of root development may involve regulation of auxin biosynthesis and transport genes in response to ABA levels.

Different *abscisic acid insensitive* (*abi*) mutants display different responses to high levels of exogenous Glc and Suc. Previous studies showed that mutations in *ABI4* confer significant resistance to the inhibitory effects of high concentrations of exogenous Glc or Suc on early seedling development, whereas the *abi1-1*, *abi2-1*, *abi3-1* and *abi5-1* mutants exhibit only slight to no resistance to Glc or Suc [11, 13, 16, 18]. Interestingly, *abi3-6* mutants, which lack one third of the *ABI3* gene and are thus believed to be null mutants [57], have been shown to be resistant to the inhibitory effects of 330 mM Glc on early seedling development [25]. However, quantitative data indicating the degree to which *abi3-6* mutants are resistant to Glc are lacking. Therefore, the finding that the *sis10-1* mutant, which has a strong *sis* phenotype, lies in the *ABI3* gene was unexpected. In addition, the findings reported here that *sis10-1/abi3-15* has significant *sis* and *abi* phenotypes, whereas *abi3-1* has been reported to have a significant *abi* phenotype [28] but little to no *sis* phenotype [11, 13, 16], suggested that it might be possible to separate genetically the role of ABI3 in ABA and sugar response. To test this hypothesis, quantitative assays were conducted to determine the sensitivities of nine different *abi3* mutant lines, all in the Col background, at two Suc and two ABA concentrations. The results of these experiments indicate that the degree of Suc resistance shows a close, positive correlation with the degree of ABA resistance. These results therefore do not provide evidence for a genetically separable role for ABI3 in sugar and ABA resistance, although such a possibility can not be ruled out at this time. The previous findings that *abi3-1* has only a very slight effect on sugar response may be at least partially explained by the fact that the *abi3-1* mutation is in the Landsberg *erecta* rather than the Col background. The Landsberg *erecta* ecotype exhibits a significantly greater level of sugar sensitivity than that of the Col ecotype (data not shown and [16]). A role for ABI3 in both sugar and ABA response is also consistent with results indicating that mutants

carrying specific *abi3* alleles exhibit increased resistance to Glc in the presence of ABA [23] and that overexpression of *ABI3* confers hypersensitivity to Glc [58].

Conclusions

In this paper the map-based cloning and characterization of two genes that affect sugar response at the early seedling developmental stage are reported. These studies resulted in identification of new *sis* mutants which carry defects in the ABA biosynthesis gene *NCED3/STO1* or the ABA response gene *ABI3*. The *SIS7/NCED3/STO1* gene is rate-limiting in the positive feedback regulation of ABA biosynthesis by ABA under stress conditions [17, 26]. Transcriptional upregulation of ABA biosynthesis genes by 300 mM Glc has been shown to occur in germinating wild-type seeds, but not in germinating *sis7-1* or *sis7-3* seeds. Transcriptional profiling experiments resulted in identification of 82 transcripts with altered steady-state mRNA levels in *sis7-1* versus wild-type germinating seeds treated with 100 mM Glc. Over-representation analysis revealed that these 82 genes are significantly enriched for genes involved in response to abiotic stimulus. Of particular interest are findings that auxin metabolism and transport genes are significantly over-represented in genes with lower transcript levels in the *sis7-1/nced3-4/sto1-4* mutant. These results have been verified by qRT-PCR analyses. These findings suggest that part of the molecular mechanism(s) by which ABA and auxin interact during regulation of *Arabidopsis* root development may involve regulation of auxin homeostasis and/or transport by ABA levels. Previous reports that *abi3-1* has a significant *abi* phenotype but little to no *sis* phenotype, together with results presented here showing that *sis10-1/abi3-15* has significant *sis* and *abi* phenotypes, suggested that it may be possible to separate genetically the role of ABI3 in sugar and ABA response. However, quantitative analyses of the magnitudes of the defects in Suc and ABA response of nine different *abi3* mutants, all in the Col background, show that the strengths of these two phenotypes exhibit a strong positive correlation. The relatively weak *sis* phenotype shown by the *abi3-1* mutant may be due, at least in part, to the fact that it is in the Landsberg *erecta*

background, as different ecotypes have been shown to exhibit significantly different levels of endogenous sugar sensitivity (data not shown and [16]).

Methods

Plant materials, growth conditions and media

The *sis7-1*, *sis7-3* and *sis10-1* mutants were isolated from a mutant population generated by insertion of a T-DNA library carrying random *Arabidopsis* cDNAs into the genome of *Arabidopsis thaliana* of the Columbia (Col) ecotype [34]. The *sis7-2* mutant was obtained by screening ethyl methyl sulfonate (EMS)-mutagenized *Arabidopsis thaliana* var. Col from Lehle Seeds (Tucson, AZ, USA). The *gl1* mutation was removed from the *sis7-2* mutant line by backcrossing this line to wild-type plants of the Col ecotype. The *abi3-16* (SALK_023411) and *abi3-17* (SALK_003216) mutants were identified from a T-DNA mutagenized population of *Arabidopsis thaliana* var. Col [49]. Seeds that are homozygous for the *abi3-16* mutation were obtained from Drs. Laurie Host and Mauricio Bustos (University of Maryland, Baltimore County). Seeds segregating for the *abi3-17* mutation were obtained from the Arabidopsis Biological Resource Center. PCR was then used to screen for plants that are homozygous for the *abi3-17* mutation. The *abi3-8*, *abi3-9*, *abi3-10*, *abi3-11*, *abi3-12* and *abi3-13* mutants [23] of *Arabidopsis thaliana* var. Col were obtained from Dr. Eiji Nambara (RIKEN Plant Science Center, Japan). Isolation of *abi4-103*, which is in the Col background, has previously been described [16]. Seeds of *spy-3* [59], which is also in the Col background, were obtained from Dr. Neil Olszewski. Wild-type Hi-O (CS6736) and Col-0 (CS6000) seeds were obtained from the Arabidopsis Biological Resource Center at Ohio State University. Seeds to be sown on plates were surface-sterilized and stratified in the dark at 4°C for 3 d. Plants were grown on plates under continuous white fluorescent light at 22°C unless otherwise noted. Minimal *Arabidopsis* media was prepared as described [31].

Sugar-insensitive mutant screen and genetic analyses

To isolate sugar-insensitive mutants, M2 seeds derived from EMS-mutagenized Col seeds were sown on Petri plates containing minimal media supplemented with 300 mM Suc. Seeds derived from a T-DNA insertion library [34] were screened on minimal media supplemented with 340 mM Suc. Subsequent screening procedures were performed as described [16]. All *sis* mutations were caused by single locus recessive mutations (data not shown). The *sis7-1/nced3-4/sto1-4* mutant was backcrossed twice and the *sis7-2/nced3-5/sto1-5* mutant was backcrossed once to wild-type Col before physiological assays were conducted.

Sugar response assays

The sugar sensitivity of the mutants was assayed as described previously [16].

Abscisic acid and paclobutrazol response assays

To measure ABA sensitivity, seeds were sown on minimal media supplemented with 0, 1, 2 or 5 µM ABA. Plates were incubated at 25°C under continuous light. The percentages of seeds that had germinated were determined at time points of up to 9 d. Germination is defined as the emergence of the radicle from the seed coat. To measure paclobutrazol sensitivity, seeds were sown on minimal media supplemented with 0, 30, 60 or 240 µM paclobutrazol. Plates were incubated at 25°C under continuous light. The percentages of seeds that had germinated were determined until the eighth d.

Map-based cloning

Mapping populations for *sis7-1* and *sis10-1* were generated as described in the Results section. Each of these F2 mapping populations was screened on media containing 300-340 mM Suc to identify plants that are homozygous for the *sis* mutation carried within that population. Genomic DNA was prepared from leaves of these seedlings via alkaline

lysis [60]. Other mapping procedures were performed as described [61, 62]. The molecular markers used to identify recombinants in the mapping experiments were simple sequence length polymorphisms (SSLPs), cleaved amplified polymorphic sequences (CAPS) and single nucleotide polymorphisms (SNPs) that were obtained from the The Arabidopsis Information Resource (TAIR) and Monsanto SNP and Ler sequence collections [61] and tested for polymorphisms between Col and Hi-O wild-type plants.

Reverse Transcriptase (RT) PCR Assays

The Qiagen RNeasy plant mini kit (Qiagen, Valencia, CA) was used to isolate total RNA from leaves of 4-week-old wild-type Col and *sis7* mutant plants grow on soil under continuous light. First strand cDNA was synthesized from total RNA (0.5 µg) using the Promega Improm-II RT system kit (Promega, Madison, WI). Two µl of cDNA were used for PCR reactions. Primers used were: *SIS7*, 5'-TAAAAACCGTTGGTCGGTTC-3' and 5'-CGACGTCCGGTGATTTAGTT-3'; *ACTIN*, 5'-TGCTGACCGTATGAGCAAAG-3' and 5'-GATTGATCCTCCGATCCAGA-3'.

Quantitative Real-Time PCR

For Glc regulation of ABA biosynthesis gene expression, seeds of Col, *sis7-1* and *sis7-3* were stratified and sown on nytex screens on minimal media and incubated for 20 h under continuous light and then transferred to 300 mM Glc or sorbitol media for an additional 12 h under continuous light before harvesting. For validation of microarray data, seeds of Col, *sis7-1* and *sis7-3* were treated as described above, except that the seeds were transferred to 100 mM Glc or sorbitol media. Seeds from two biologically independent batches were used to minimize the variations in gene expression engendered by possible changes in plant growth conditions. Total RNA was isolated using the Spectrum™ Plant Total RNA Kit (Sigma). First strand cDNA was synthesized with the Promega Improm-II RT system kit (Promega) in a reaction volume of 20 µl. Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR Machine using the

SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis, MO). *UBQ6* (AT2G47110) was used as an internal reference. The following primers were used: *UBQ6*, 5'-CCATCGACAATGTCAAGGCC-3' and 5'-GGTACGTCCGTCTTCGAGCT-3'; *ABA1*, 5'-GAACGTACTATAAAGGGAGAATGG-3' and 5'-CTGAGACGAAGGGATCACAAT-3'; *SIS7/NCED3*, 5'-TAAAAACCGTTGGTCGGTTC-3' and 5'-CGACGTCCGGTGATTTAGTT-3'; *ABA2*, 5'-CTAAACTCGCTTTGGCTCATT-3' and 5'-CGCTACATCATCAACCGTCAG-3'; *ABA3*, 5'-TTTGCTGGGATGACAATGAT-3' and 5'-CCTTCCACTGACGACGGTTC-3'; *AAO3*, 5'-GTCAGCGAGGTGGAAGTGGA-3' and 5'-CAAATGCTCCTTCGGTCTGT-3'; *CYP83B1*, 5'-CCCTAACCGCCCTAAACAAGA-3' and 5'-CATACCACCACTGCAGCCG-3'; *CYP79B2*, 5'-GACAATCCATCAAACGCCGT-3' and 5'-GCGGAGGATAGCTTTGACGTA-3'; *AT4G27260*, 5'-TGTTGTCACCACTTACGCCG-3' and 5'-CGTTCTGAAGCTCAACCTCGT-3'; *PIN1*, 5'-CGGTGGGAACAACATAAGCA-3' and 5'-TGAAGGAAATGAGGGACCAGG-3' and *PIN3*, 5'-TGGAGATTTCGGAGGAGAACA-3' and 5'-TTCGTTGACTTGCTTCGGC-3'.

Gene expression analysis using Affymetrix ATH1 GeneChips

For transcriptional profiling experiments, Col wild-type and *sis7-1* seeds were sown on nytex screens on minimal media and incubated for 20 h under continuous light and then transferred to 100 mM Glc media for an additional 12 h under continuous light before harvesting. Seeds were pooled from different batches to minimize the variation in gene expression profiles engendered by possible changes in plant growth conditions. Total RNA extraction was performed as described [63]. For reproducibility, three independent seed tissue collections and RNA extractions were performed for *sis7-1* and six independent experiments were performed for Col wild-type. The generation of biotinylated cRNA from total RNA and subsequent hybridization to *Arabidopsis* ATH1 genome arrays and quantification of fluorescent signals were performed by the Molecular Genomics Core Facility at the University of Texas Medical Branch at Galveston. Data

analyses were performed using Expressionist (Genedata AG, Switzerland). Gene expression values and presence/absence calls were computed using the Affymetrix Statistical Analysis (MAS5) algorithm. Student's t-test values and fold changes of individual transcript signal intensities from *sis7-1* and WT replicates were determined. The mis-regulated gene list was generated by identifying transcripts that varied between mutant and wild-type samples with a *p* value of less than 0.05 and a fold change value of greater than 2.0. For over-representation analysis using GeneMerge , the population genes consist of 8791 genes called present on the ATH1 arrays by the Expressionist program. *P* value was calculated after Bonferroni correction.

Authors' contributions

YH identified the *SIS7/NCED3/STO1* gene, performed most of the characterization of the *sis7/nced3/sto1* mutants and drafted the major part of the manuscript. CL identified the *SIS9/ABA1* and *SIS10/ABI3* genes, performed the characterization of the *sis10/abi3* mutants and aided in drafting the manuscript. KB identified the *sis7-1, sis7-3* and *sis10-1* mutants and performed a preliminary characterization of those mutants. SG conceived of the study, assisted in its design and the interpretation of the data and aided in drafting the manuscript. All authors approved of the manuscript.

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Note

During submission of this manuscript, a manuscript appeared (Plant Mol Biol 67: 151) that also describes findings that mutations in *ABI3* can confer a sugar-insensitive phenotype.

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Figure Legends

Figure 1 - Mutations in *SIS7* confer a *sugar-insensitive* phenotype

Col wild-type and *sis7* seeds were stratified for 3 d and then sown on the indicated media and scored after 14 d at 22°C under continuous light. Data represent the means of three independent assays. The error bars indicate standard deviations. Asterisks indicate results where the mutant phenotype differed from the corresponding wild-type phenotype with a *p*-value of less than 0.05 according to a Student's t-test.

Figure 2 - The *sis7-1/sto1-4/nced1-4* mutation confers a subtle ABA-insensitive phenotype

Col wild-type and *sis7-1/sto1-4/nced3-4* seeds were stratified for 3 d and then sown on media containing 1, 2 or 5 µM ABA and incubated at 25°C under continuous light conditions. Germination rates on minimal media were also checked and found to be consistently high, indicating a high rate of viability in the seeds used for these experiments (data not shown). Seed germination was scored at the indicated time points. Germination is defined as the emergence of the radicle from the seed coat. Data represent the means of three independent assays. The error bars represent standard deviations. The *abi4-103/sis5-3* [16] seeds were included as a positive control.

Figure 3 - The *sis7-1/sto1-3/nced1-3* mutation confers a paclobutrazol-resistant phenotype

Col wild-type and *sis7-1/sto1-4/nced3-4* seeds were stratified for 3 d and then sown on media containing 30, 60 or 240 µM paclobutrazol and incubated at 25°C under continuous light conditions. Germination rates on minimal media were also checked and found to be consistently high, indicating a high rate of viability in the seeds used for these experiments (data not shown). Seed germination was scored at the indicated time points. Germination is defined as the emergence of the radicle from the seed coat. Data represent the means of three independent assays. The error bars represent standard deviations. The *spy-3* [59] seeds were included as a positive control.

Figure 4 - Characterization of the *sis7* molecular lesions

(A) Mutations in *SIS7/NCED3/STO1*. The molecular lesions associated with the three *sis7/nced3/sto1* mutants identified as part of this work are shown. Nucleotides are numbered with respect to the start codon.

(B) *SIS7* transcript levels in wild-type and *sis7* plants. RT-PCR was used to amplify *SIS7*/*NCED3*/*STO1* transcripts from 4-week old wild-type (Col) and *sis7* mutant plants. 2 µL cDNA was used as a template for the first PCR reaction (35 cycles). *ACTIN* transcripts were amplified as a positive control.

Figure 5 - Exogenous ABA causes *sis7* mutants to exhibit approximately wild-type sugar sensitivity

Wild-type (Col) and mutant seeds were cold treated for 3 d and then sown on minimal media supplemented with 220 mM Glc, with or without 0.1 µM ABA. Additional mutants deficient in ABA biosynthesis (*aba2-3*) or ABA response (*abi4-103*) were included as controls. Percent seed germination, cotyledon expansion and true leaf formation were scored after an additional 14 d incubation under continuous light at 22°C. Data represent the means of three independent assays. The error bars represent standard deviations. Asterisks indicate results where the phenotype of one of the mutants differed from that of the wild type with a *p*-value of less than 0.05 according to a Student's t-test.

Figure 6 - Quantitative RT-PCR analysis of ABA biosynthesis genes in *sis7* and wild-type germinating seeds

Relative expression levels were measured by qRT-PCR in Col, *sis7-1* and *sis7-3* germinating seeds treated with 300 mM Glc or 300 mM sorbitol. Data were obtained from two biologically independent experiments and relative mRNA levels were determined by using *UBQ6* as a reference. The relative mRNA level of each gene in sorbitol-treated Col was set to 100. Bars indicate standard deviations.

Figure 7 - Functional categorization of genes mis-regulated in *sis7-1*

Affymetrix ATH1 GeneChips were used for transcriptional profiling of germinating *sis7-1/nced3-4/sto1-4* and wild-type seeds. Col wild-type and *sis7-1/nced3-4/sto1-4* seeds were sown on minimal media for 20 h and transferred to 100 mM Glc media for 13 h under continuous light. The Expressionist software package was used to identify genes with transcript levels that differ by at least two fold and with a p value of less than 0.05 when comparing the results of mutant versus wild-type samples. Using these cutoffs, the levels of 82 transcripts were found to be altered in *sis7-1/nced3-4/sto1-4* versus wild-type seeds (Table 1). Functional categorization of these mis-regulated genes was performed using the GO (Gene Ontology) Annotations tool from TAIR [38]. Panel A shows the distribution of annotations for molecular function and panel B shows the distribution for annotations for biological process. Percentages were calculated as: (number of annotations to terms in this GOslim category/number of total annotations to terms in this ontology) X 100.

Figure 8 - Quantitative RT-PCR analysis of auxin-related genes

Genes involved in auxin metabolism or transport were chosen for analysis. Relative expression levels of these genes were measured by qRT-PCR in wild-type Col, *sis7-1* and *sis7-3* germinating seeds treated with 100 mM Glc or 100 mM sorbitol. Data were obtained from two biologically independent experiments and relative mRNA levels were determined by using *UBQ6* as a reference. The relative mRNA level of each gene in sorbitol-treated wild-type Col was set to 100. Bars indicate standard deviations.

Figure 9 - *sis7-1* exhibits increased root system size on sorbitol media

Wild-type Col and *sis7-1* seedlings were grown for 12 d on vertically-oriented Petri plates containing the indicated media. Root systems of *sis7-1* are less repressed by osmotica than are the root systems of wild-type seedlings.

Figure 10 - Molecular lesions present in different *abi3* mutants

Nucleotides are numbered with respect to the start codon. Exons are depicted with boxes and introns are depicted with single lines.

Figure 11 - Suc-response assays of three *abi3* mutants

Seeds were sown on media containing 300 mM Suc and incubated at 22°C for 14 d under 2200-3400 Lux continuous light prior to scoring. Seed germination, cotyledon expansion and true leaf formation were scored as previously described [16]. Error bars indicate standard deviations. Asterisks indicate results where the mutant phenotype differed from the corresponding wild-type phenotype with a *p*-value of less than 0.05 according to a Student's t-test.

Figure 12 - Quantification of Suc and ABA insensitivity in nine *abi3* mutants

(A) For Suc-response assays seeds were sown on media containing 270 or 320 mM Suc and incubated at 22°C for 14 d under 2200-3400 Lux continuous light prior to scoring. Seed germination, cotyledon expansion and true leaf formation were scored as previously described [16]. Error bars indicate standard deviations. Asterisks indicate results where the mutant phenotype differed from the corresponding wild-type phenotype with a *p*-value of less than 0.05 according to a Student's t-test.

(B) For ABA response assays seeds were imbibed on media with the indicated ABA concentrations and scored for germination after 24, 48, 82 and 106 h. Seed germination is defined as the emergence of the radicle from the seed coat. Error bars indicate standard deviations. Asterisks indicate results where the mutant phenotype differed from the corresponding wild-type phenotype with a *p*-value of less than 0.05 according to a Student's t-test.

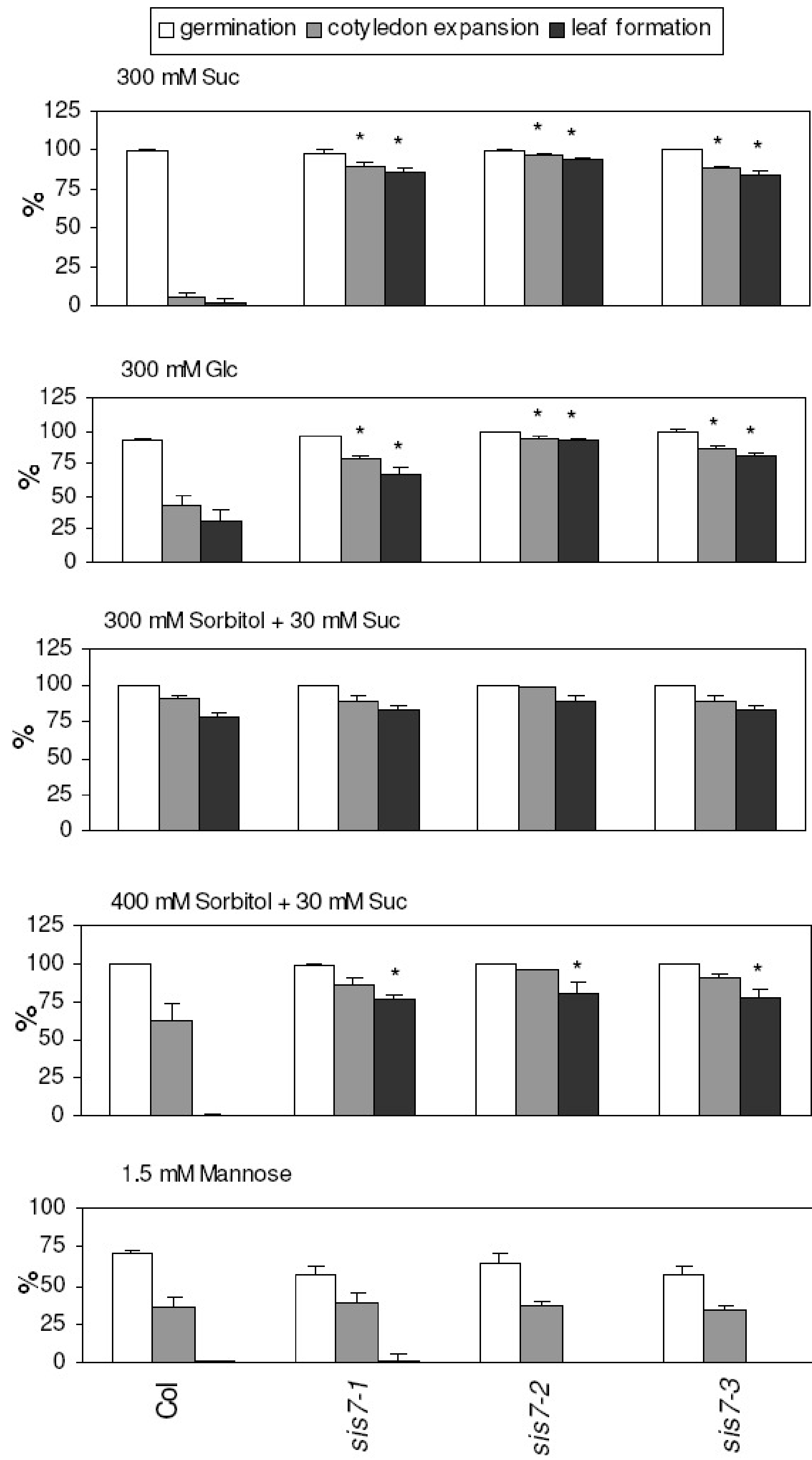
(C) Regression analysis of data on Suc and ABA response assays reveals strong positive correlation between sugar- and ABA- insensitivity. Each data point on the scatterplot represents the percentage of normal shoot development (defined as seedlings with green expanded cotyledons) on Suc media and the percentage of seeds that germinated after 106 h on ABA media for each genotype. Correlation coefficients were calculated for two assay conditions: 1 μM ABA and 270 mM Suc representing relatively non-stringent assay conditions and 2 μM ABA and 320 mM Suc representing more stringent assay conditions.

Table 1 - Genes with altered expression in *sis7-1* versus wild-type seeds incubated on 100 mM Glc media.

Description	AGI	*sis7-1*	WT	sis7-1/WT	*P*-Value
genes with higher transcript levels in *sis7-1*					
Expressed protein	AT5G58375	116965	3030	38.60	9.47E-09
2S seed storage protein 4	AT4G27170	15028	874.6	17.18	0.002
cytosolic small heat shock protein	AT5G12030	6968.2	977.5	7.13	0.020
protease inhibitor/seed storage family protein	AT5G54740	9474.8	1824	5.19	0.033
heat shock protein 70, putative	AT3G12580	36044	9615	3.75	0.016
expressed protein	AT2G32210	5544.3	1555	3.56	0.004
gibberellin-responsive protein 3	AT4G09600	2994.7	1066	2.81	0.008
alternative oxidase 1a, mitochondrial (AOX1A)	AT3G22370	4803.2	1982	2.42	0.024
low temperature and salt responsive protein	AT4G30650	4272.1	1769	2.42	0.029
putative GTP-binding protein	AT1G09180	1816.2	758.1	2.40	0.026
AP2 domain transcription factor	AT2G40350	1848	787.9	2.35	0.031
steroid 5alpha-reductase-like protein	AT5G16010	10002	4287	2.33	0.001
expressed protein	AT5G24570	3708.1	1594	2.33	0.028
arabinogalactan-protein	AT5G64310	33863	14633	2.31	0.011
CDC48 - like protein	AT3G53230	21244	9218	2.30	0.007
pathogenesis-related thaumatin family protein	AT4G36010	10479	4607	2.27	0.013
mitochondrial heat shock 22 kd protein-like	AT5G51440	3154.5	1492	2.11	0.041
expressed protein	AT5G64230	2056.4	982.4	2.09	0.026
glycine-rich protein / oleosin	AT3G18570	3872.5	1872	2.07	0.006
salt-tolerance zinc finger protein	AT1G27730	4462.6	2200	2.03	0.017
genes with lower transcript levels in *sis7-1*					
cytochrome P450 79B2, putative (CYP79B2)	AT4G39950	1388.8	7597	0.18	0.001
RuBisCO activase	AT2G39730	1489.5	4706	0.32	0.007
auxin-responsive GH3 family protein	AT4G27260	2323.6	6679	0.35	0.047
cytochrome P450 83B1 (CYP83B1)	AT4G31500	15681	44352	0.35	0.003
S1 RNA-binding domain-containing protein	AT1G12800	1258.8	3475	0.36	0.007
pectin methylesterase	AT1G11580	4379.7	11956	0.37	0.009
DegP2 protease	AT2G47940	1758.8	4776	0.37	0.007
AMP-binding protein	AT3G23790	877.64	2291	0.38	0.040
WPP-domain interacting protein 3	AT3G13360	626.53	1614	0.39	0.014
MATE efflux family protein	AT1G71870	1830.1	4621	0.40	0.016
glutathione S-transferase	AT2G30860	36776	92522	0.40	0.002
hydrolase family protein	AT2G25870	980.77	2438	0.40	0.000
endo-beta-1,4-glucanase	AT1G64390	2208.2	5381	0.41	0.011
heat shock protein 100, putative	AT5G15450	1817.2	4398	0.41	0.026
eIF-2 family protein	AT1G76810	748.36	1809	0.41	0.035
auxin transport protein, putative (PIN3)	AT1G70940	1147.3	2735	0.42	0.009
male sterility MS5 family protein	AT5G48850	1109	2638	0.42	0.032
phosphatidylinositolglycan class family protein	AT3G01380	1149.7	2691	0.43	0.006
epsilon-adaptin, putative	AT1G31730	1766.7	4134	0.43	0.016
PSII low MW protein	ATCG00220	11808	27598	0.43	0.026
protein kinase family protein	AT1G75640	393.21	897.5	0.44	0.021
guanylate-binding family protein	AT5G46070	606.9	1382	0.44	0.018

proteaseI (pfpI)-like protein (YLS5)	AT2G38860	4038.7	9166	0.44	0.008
sulfotransferase family protein	AT1G74100	11829	26477	0.45	0.002
chlorophyll A-B binding protein 4, chloroplast	AT3G47470	531.64	1175	0.45	0.042
senescence-associated protein-related	AT5G20700	633.48	1395	0.45	0.028
early dehydration-induced gene ERD13	AT2G30870	3683.9	8114	0.45	0.046
squamosa promoter-binding protein-like 1	AT2G47070	975.98	2147	0.45	0.011
heavy-metal-associated domain protein	AT5G19090	1140.7	2502	0.46	0.010
expressed protein	AT3G50370	1209.1	2652	0.46	0.004
methyltransferase MT-A70 family protein	AT4G09980	849.6	1840	0.46	0.005
SET domain-containing protein	AT2G19640	2023.8	4381	0.46	0.008
UV-damaged DNA-binding protein, putative	AT4G05420	2387	5151	0.46	0.000
expressed protein	AT4G27450	7572.7	16323	0.46	0.010
zinc finger protein-related	AT3G18290	1139.9	2444	0.47	0.005
uroporphyrinogen decarboxylase, putative	AT2G40490	1408.2	2997	0.47	0.017
molybdenum cofactor sulfurase family protein	AT5G44720	1618.5	3427	0.47	0.020
expressed protein	AT1G36990	1152.5	2437	0.47	0.008
kinesin motor family protein	AT5G66310	255.11	538.8	0.47	0.019
pentatricopeptide repeat-containing protein	AT1G71060	418.58	883	0.47	0.035
protein kinase-related	AT3G03930	441.29	927.2	0.48	0.008
exportin1 (XPO1)	AT5G17020	2876.7	6035	0.48	0.010
auxin efflux carrier protein, putative (PIN1)	AT1G73590	2462.7	5154	0.48	0.021
pentatricopeptide repeat-containing protein	AT5G46580	3046.1	6365	0.48	0.007
pentatricopeptide repeat-containing protein	AT5G28370	3099.1	6453	0.48	0.019
expressed protein	AT5G63135	902.49	1875	0.48	0.011
male sterility MS5 family protein	AT1G04770	2548	5292	0.48	0.004
MAPK, putative (MPK8)	AT1G18150	749.12	1541	0.49	0.046
expressed protein	AT5G64460	768.54	1580	0.49	0.043
importin beta-2 subunit family protein	AT2G31660	2429.2	4987	0.49	0.007
defense-related protein, putative	AT4G30530	3242.1	6650	0.49	0.003
calcium-transporting ATPase 4	AT2G41560	6059	12419	0.49	0.001
ubiquitin-transferase family protein	AT4G38600	5326.7	10886	0.49	0.011
sulfite reductase / ferredoxin (SIR)	AT5G04590	4299.1	8781	0.49	0.020
VARIEGATED 3	AT5G17790	646.42	1317	0.49	0.005
heat shock protein, putative	AT1G11660	1119.9	2280	0.49	0.030
pentatricopeptide repeat-containing protein	AT2G17033	715.5	1452	0.49	0.013
nuclease family protein	AT5G61780	8109.9	16406	0.49	0.027
pentatricopeptide repeat-containing protein	AT2G31400	3807.3	7676	0.50	0.040
patched family protein	AT4G38350	2103.6	4230	0.50	0.015
Involved in the biosynthesis of brassinosteroids	AT2G07050	3755.4	7538	0.50	0.022
ATP phosphoribosyl transferase	AT1G09795	1402.3	2805	0.50	0.000

Figure 1



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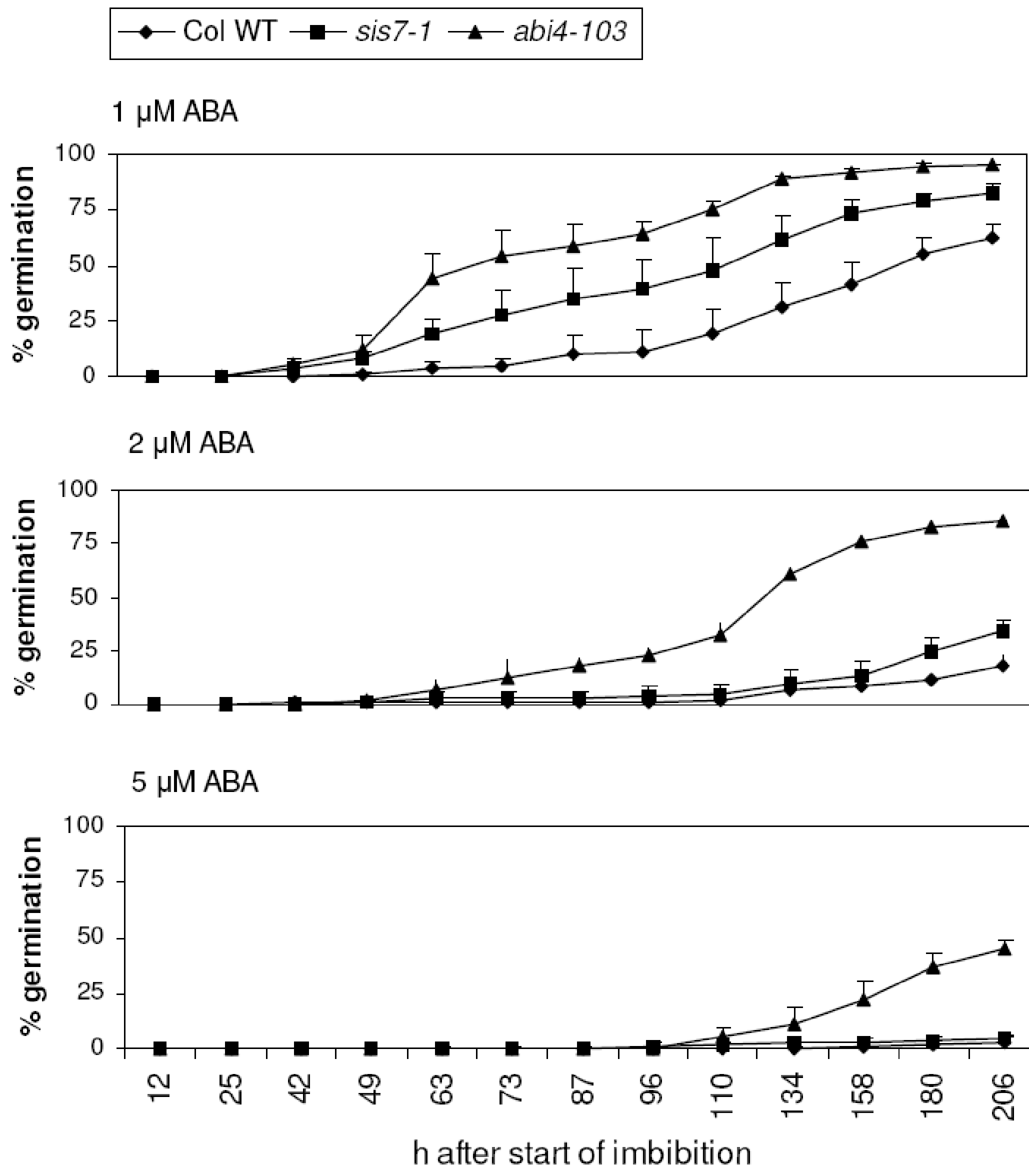

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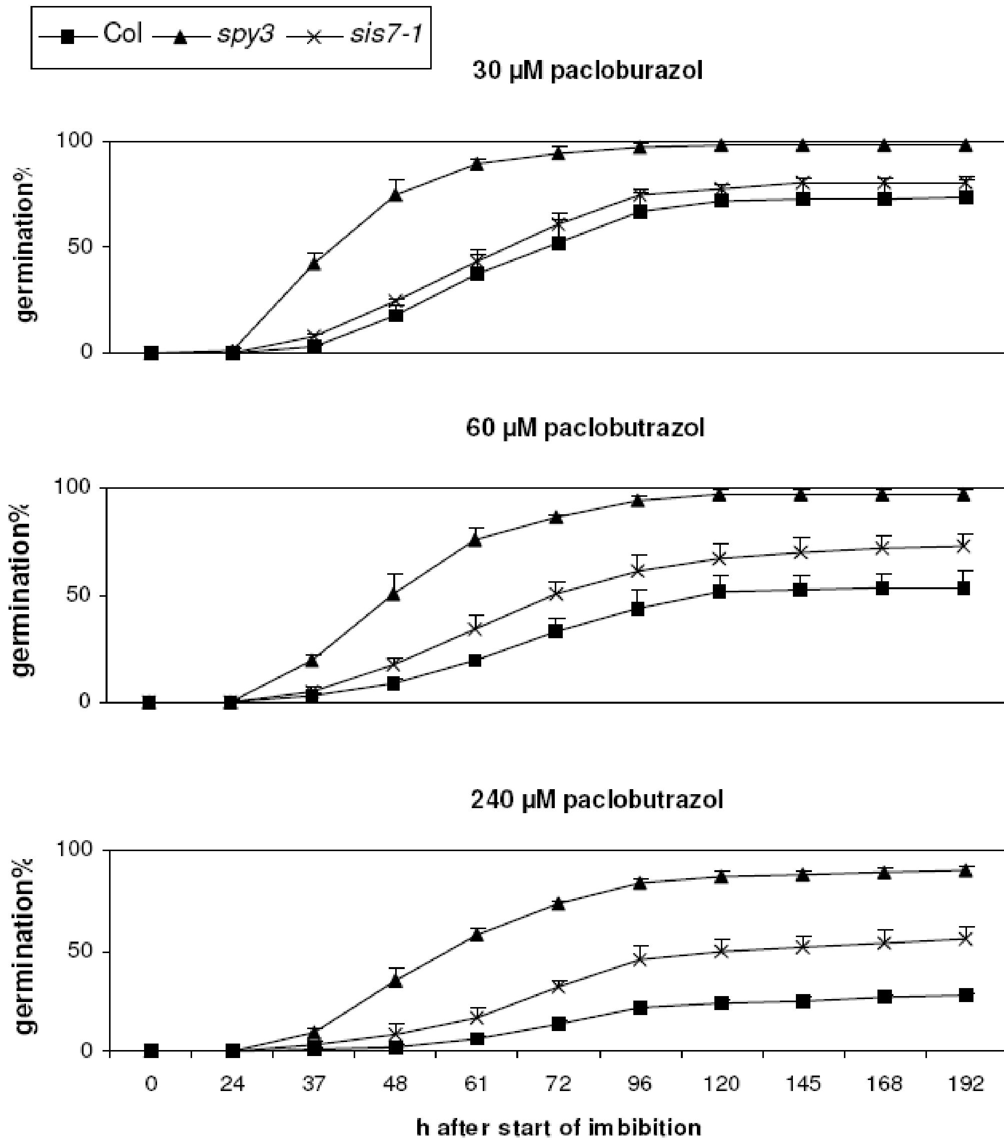

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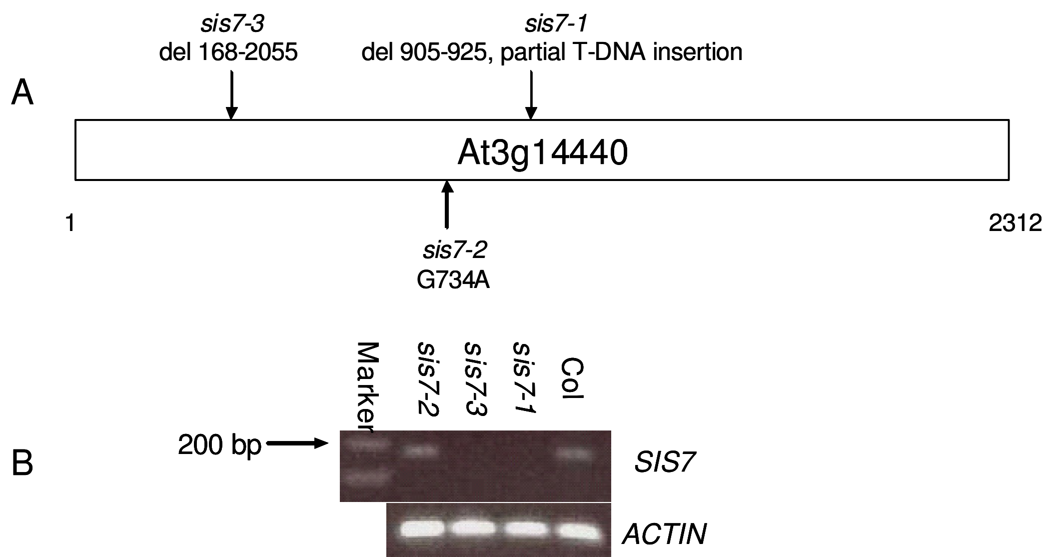

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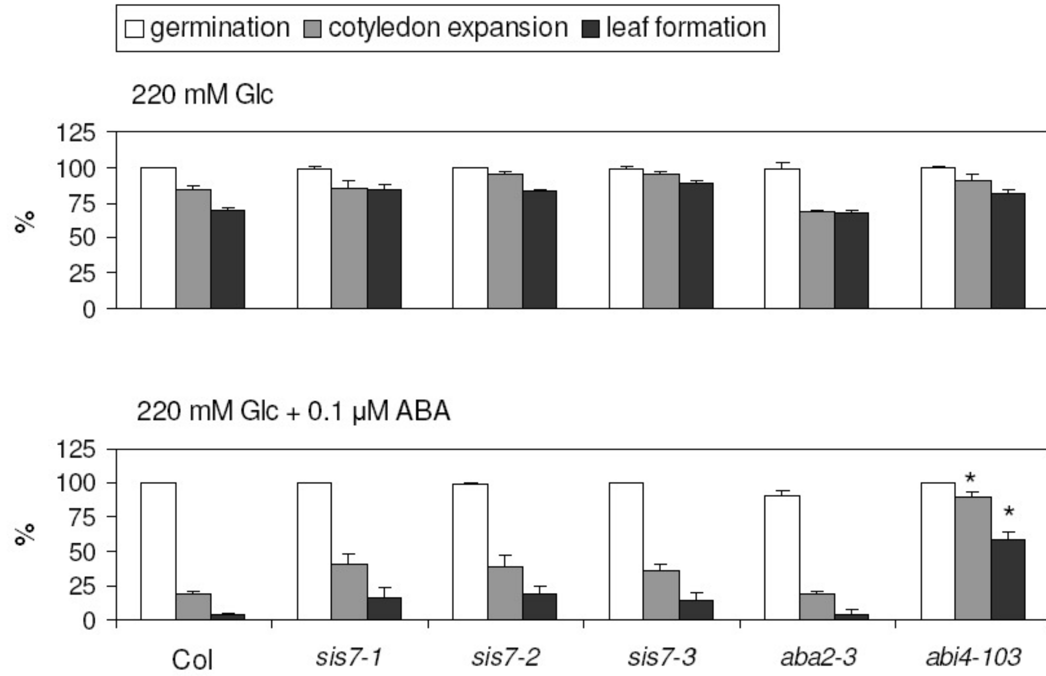

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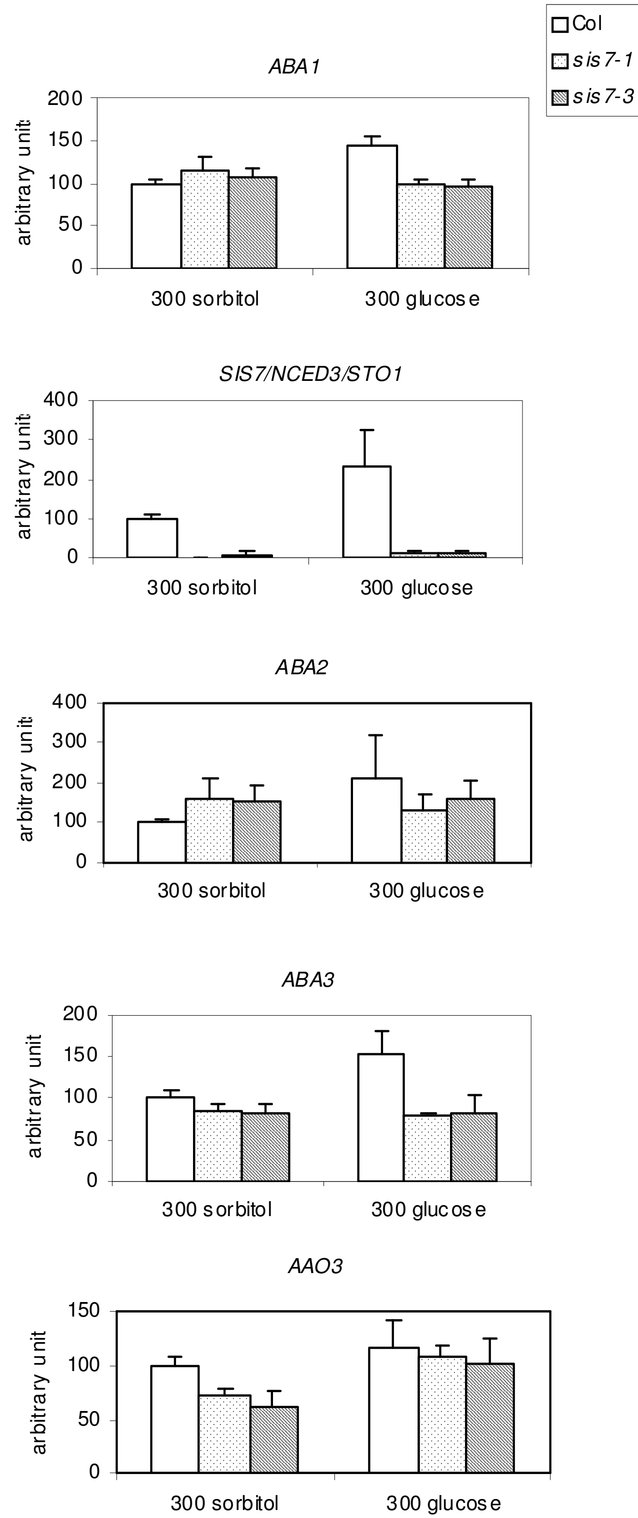

Figure 7

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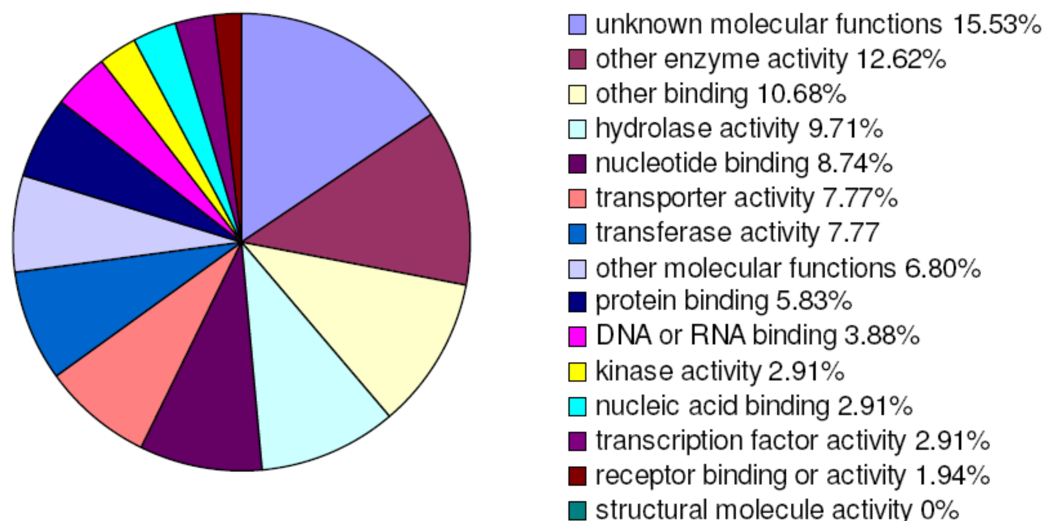

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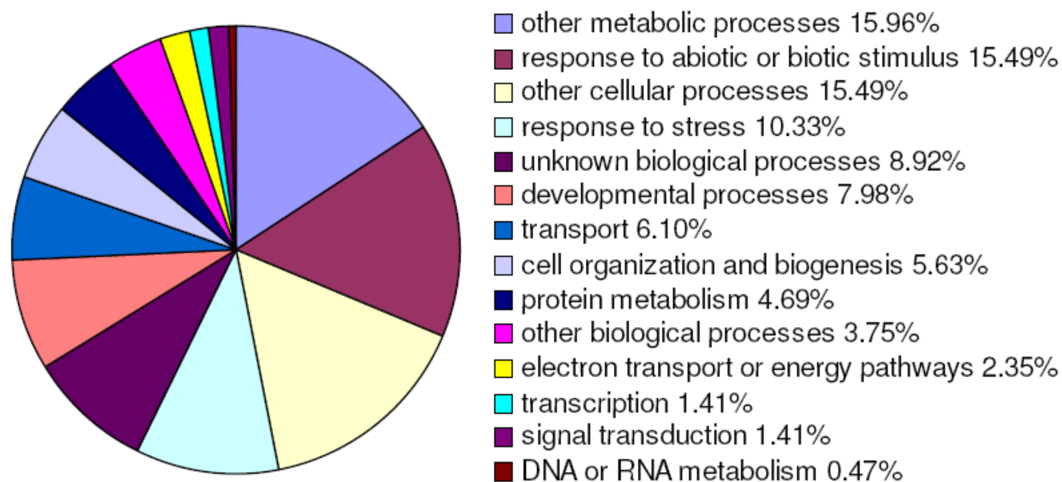

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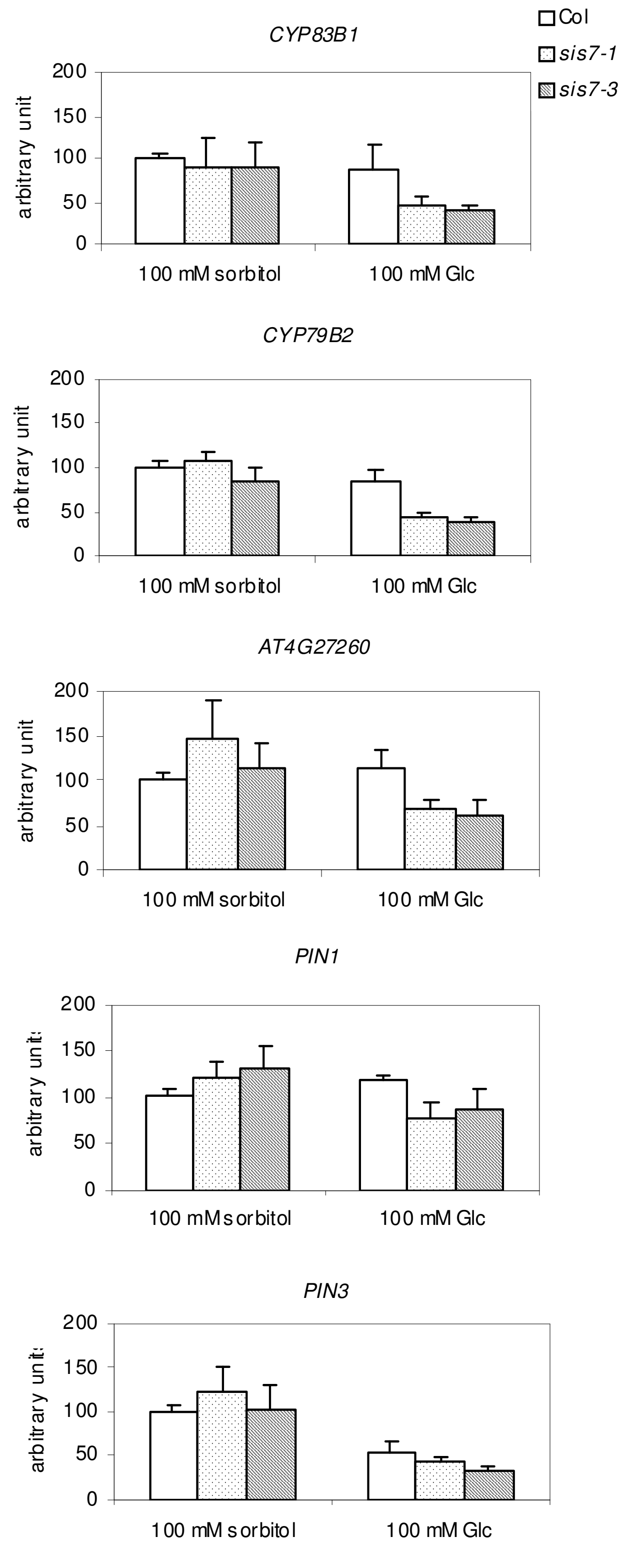

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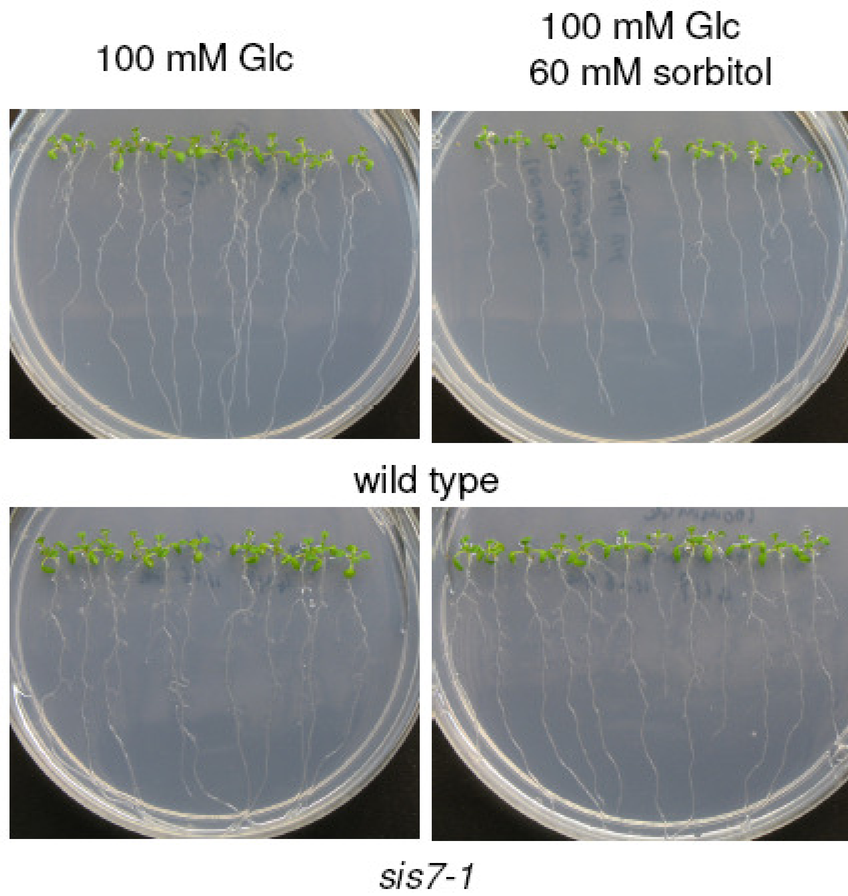

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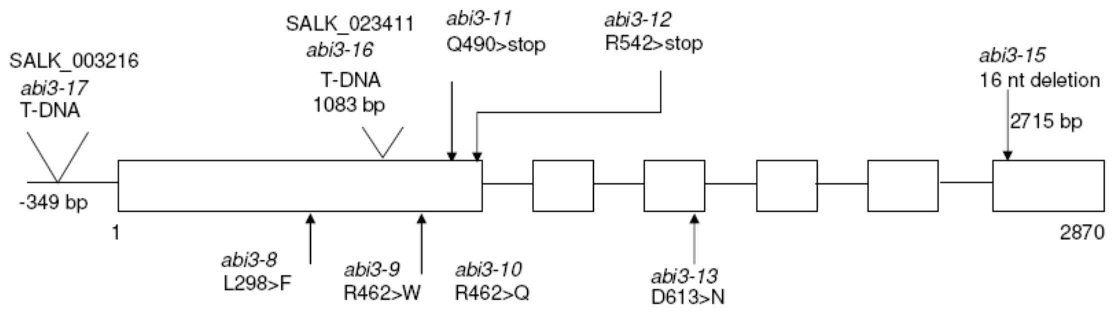

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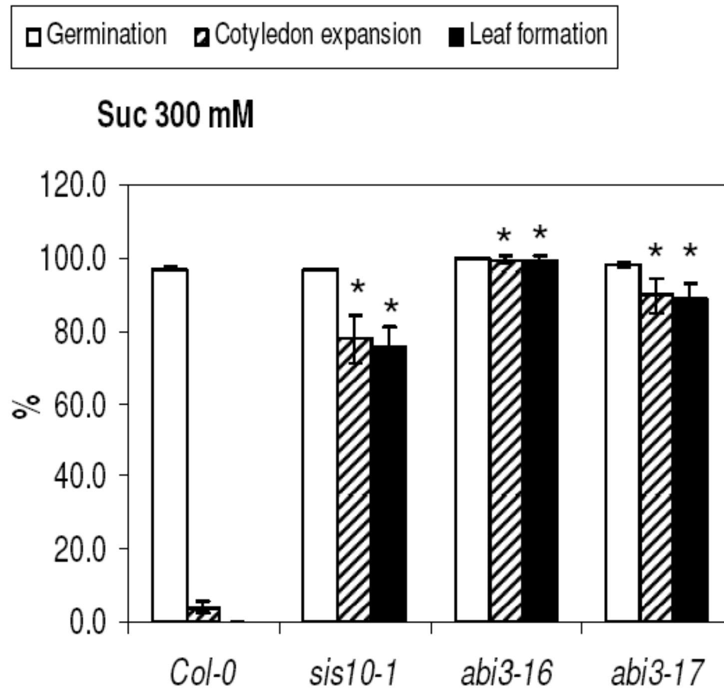
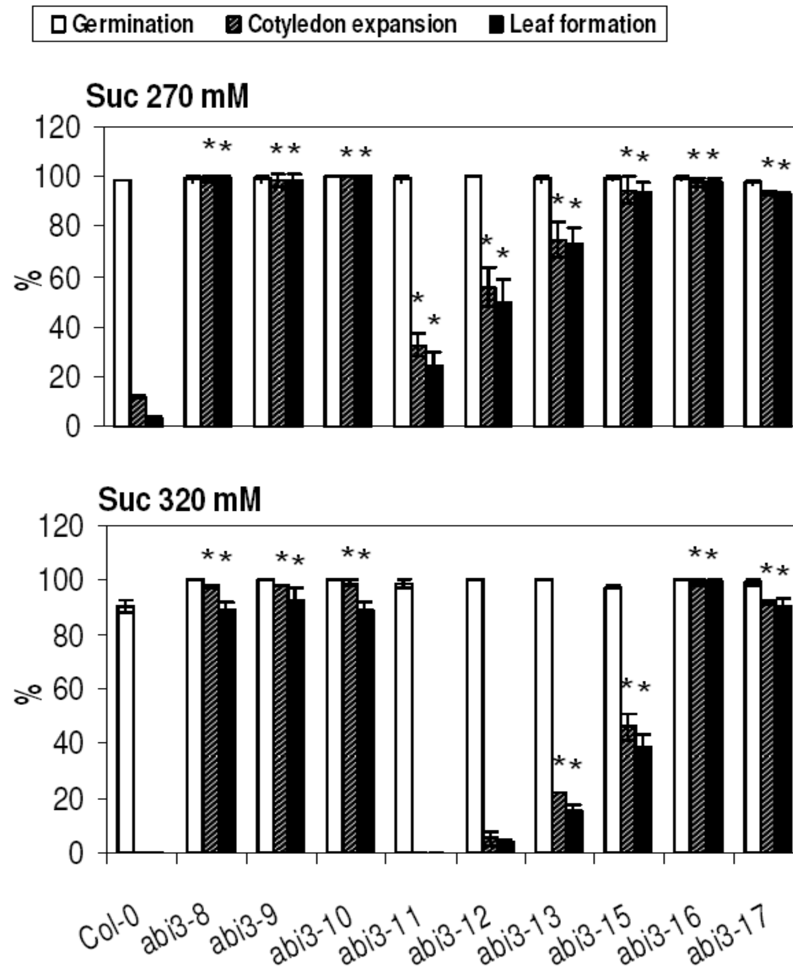
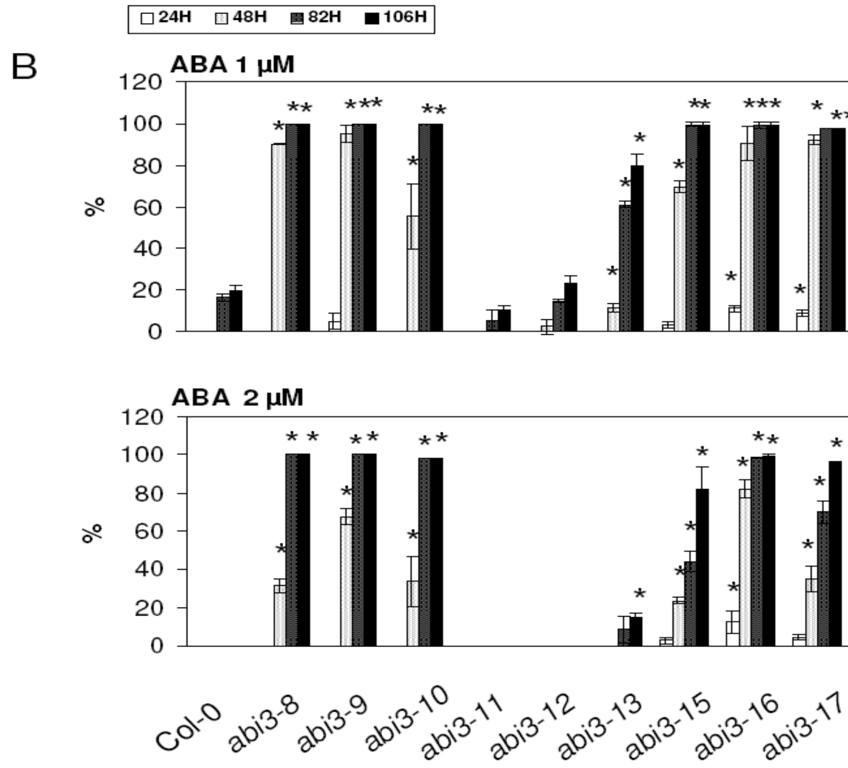


Figure 12




24H
48H
82H
106H
B
ABA 1 µM
ABA 2 µM
%
Col-0
abi3-8
abi3-9
abi3-10
abi3-11
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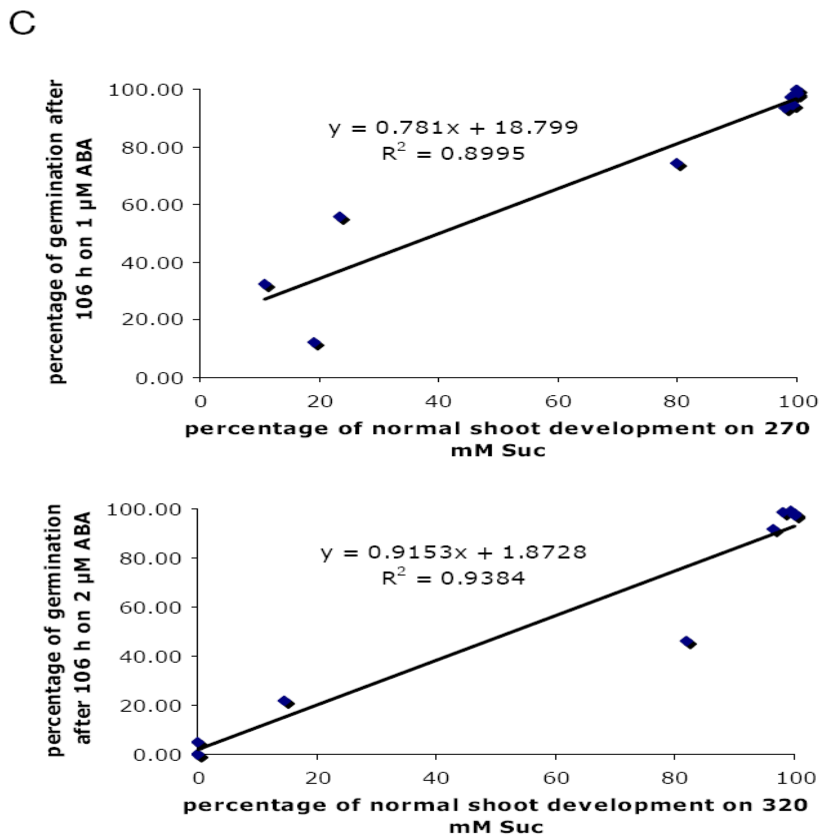
abi3-13
abi3-15
abi3-16
abi3-17


C
y = 0.781x + 18.799
R2 = 0.8995
percentage of germination after 106 h on 1 µM ABA
percentage of normal shoot development on 270 mM Suc
y = 0.9153x + 1.8728
R2 = 0.9384
percentage of germination after 106 h on 2 µM ABA
percentage of normal shoot development on 320 mM Suc

Chapter III

SUGAR INSENSITIVE3, a RING E3 ligase, is a new player in plant sugar response

SUGAR INSENSITIVE3, a RING E3 ligase, is a new player in plant sugar response

Yadong Huang [*], Chun-Yao Li [*], Donna L. Pattison[*], William M. Gray, Sungjin Park and Susan I. Gibson

*These authors contributed equally to this work.

Abstract

Sugars, such as sucrose and glucose, have been implicated in the regulation of diverse developmental events in plants and other organisms. We isolated an *Arabidopsis thaliana* mutant, *sugar-insensitive3 (sis3*), that is resistant to the inhibitory effects of high concentrations of exogenous glucose and sucrose on early seedling development. In contrast to wild-type plants, *sis3* mutants develop green, expanded cotyledons and true leaves when sown on media containing high concentrations (e.g. 270 mM) of sucrose. Unlike some other sugar-response mutants, *sis3* exhibits wild-type responses to the inhibitory effects of abscisic acid and paclobutrazol, a gibberellic acid biosynthesis inhibitor, on seed germination. Map-based cloning revealed that *SIS3* encodes a RING finger protein. Complementation of the *sis3-2* mutant with a genomic *SIS3* clone restored sugar sensitivity of *sis3-2*, confirming the identity of the *SIS3* gene. Biochemical analyses demonstrated that SIS3 is functional in an *in vitro* ubiquitination assay and that the RING motif is sufficient for its activity. Our results indicate that *SIS3* encodes a ubiquitin E3 ligase that is a positive regulator of sugar signaling during early seedling development.

Introduction

Almost all living organisms rely on the products of plant photosynthesis for sustenance, either directly or indirectly. Carbohydrates, the major photosynthates, provide both energy and carbon skeletons for fungi, plants and animals. In addition, sugars, such as sucrose (Suc) and glucose (Glc), function as signaling molecules to regulate plant growth, development, gene expression and metabolic processes. Sugar response pathways are integrated with other signaling pathways, such as those for light,

phytohormones, stress and nitrogen (Dijkwel et al., 1997; Zhou et al., 1998; Roitsch, 1999; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Coruzzi and Zhou, 2001; Rook et al., 2001; Rolland et al., 2006).

Several components of plant sugar response pathways have been identified based on the conservation of sugar sensing mechanisms among eukaryotic cells (Rolland et al., 2001; Rolland et al., 2006) or by mutant screens. Yeast HEXOKINASE2 functions in the glucose-mediated catabolite repression pathway (Entian, 1980). In *Arabidopsis thaliana*, mutations in *HEXOKINASE1* (*HXK1*) cause a glucose insensitive phenotype and HXK1 demonstrates dual functions in glucose sensing and metabolism (Moore et al., 2003; Cho et al., 2006). Recent studies reveal the involvement of G-protein coupled receptor (GPCR) systems in sugar response in yeast and Arabidopsis (Chen et al., 2003; Lemaire et al., 2004). Arabidopsis *regulator of G-protein signaling1* (*rgs1*) mutant seedlings are insensitive to 6% Glc (Chen and Jones, 2004), whereas *G-protein α subunit* (*gpa1*) null mutant seedlings are hypersensitive to Glc (Chen et al., 2003). The SNF1/AMPK/SnRK1 protein kinases are postulated to be global regulators of energy control (Polge and Thomas, 2007). Studies conducted on two members of the *Arabidopsis* SnRK1 (SNF1-Related Protein Kinases1) family, AKIN10 and AKIN11, have revealed their pivotal roles in stress and sugar signaling (Baena-González et al., 2007). A genetic screen for reduced seedling growth on 175 mM Suc identified the *pleiotropic regulatory locus1* (*prl1*) mutant, which encodes a nuclear WD protein. Further analyses revealed that PRL1 functions in glucose and phytohormone responses (Németh et al., 1998). Interestingly, PRL1 negatively regulates the *Arabidopsis* SnRK1s AKIN10 and AKIN11 *in vitro* (Bhalerao et al., 1999).

Isolation of additional mutants defective in sugar response has revealed cross-talk between sugar and phytohormone response pathways. For example, abscisic acid (ABA) biosynthesis and signaling mutants have been isolated by several genetic screens for seedlings with reduced responses to the inhibitory effects of high levels of Suc or Glc on seedling development. These mutants include *abscisic acid deficient2* (*aba2*), *abscisic acid deficient3* (*aba3*) and *abscisic acid insensitive4* (*abi4*) (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001; Cheng et al., 2002; Rolland et

al., 2002), indicating interplay between ABA- and sugar-mediated signaling. Ethylene also exhibits interactions with sugars in controlling seedling development. Both the ethylene overproduction mutant *eto1* and the constitutive ethylene response mutant *ctr1* exhibit Glc (Zhou et al., 1998) and Suc (Gibson et al., 2001) insensitivity, whereas the ethylene insensitive mutants *etr1, ein2* and *ein4* show sugar hypersensitivity (Zhou et al., 1998; Gibson et al., 2001; Cheng et al., 2002).

Further characterization of sugar-response factors has suggested that ubiquitin-mediated protein degradation may play a role in sugar response. In particular, the PRL1-binding domains of SnRK1s have been shown to recruit SKP1/ASK1, a conserved SCF ubiquitin ligase subunit, as well as the α4/PAD1 proteasomal subunit, indicating a role for SnRK1s in mediating proteasomal binding of SCF ubiquitin ligases (Farrás et al., 2001). The ubiquitin/26S proteasome pathway plays important roles in many cellular processes and signal transduction pathways in yeast, animals and plants (Hochstrasser, 1996; Hershko and Ciechanover, 1998; Smalle and Vierstra, 2004). The key task of the pathway is to selectively ubiquitinate substrate proteins and target them for degradation by the 26S proteasome. In short, the multi-step ubiquitination process starts with the formation of a thiol-ester linkage between ubiquitin and a ubiquitin-activating enzyme (E1). The activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), and a ubiquitin protein ligase (E3) then mediates the covalent attachment of ubiquitin to the substrate protein. The specificity of the pathway is largely realized by the E3s, which recognize the substrates that should be ubiquitinated. In *Arabidopsis* more than 1300 genes encode putative E3 subunits and the E3 ligases can be grouped into defined families based upon the presence of HECT (Homology to E6-AP C Terminus) or RING/U-box (Really Interesting New Gene) domains (Smalle and Vierstra, 2004). The RING type E3s can be subdivided into single subunit E3s which contain the substrate recognition and the RING finger domains on the same protein, and multi-subunit E3s which include the SCF (Skp-Cullin-F-box), CUL3-BTB (Broad-complex, Tramtrack, Bric-a-Brac) and APC (Anaphase Promoting Complex) complexes (Weissman, 2001; Moon et al., 2004).

The cysteine-rich RING finger was first described in the early 1990s (Freemont et al., 1991). It is defined as a linear series of conserved cysteine and histidine residues (C3HC/HC3) that bind two zinc atoms in a cross-brace arrangement. RING fingers can be divided into two types, C3HC4 (RING-HC) or C3H2C3 (RING-H2), depending on the presence of either a Cys or His residue in the fifth position of the motif (Lovering et al., 1993; Freemont, 2000). A recent study of the RING finger ubiquitin ligase family encoded by the Arabidopsis genome resulted in identification of 469 predicted proteins containing one or more of the eight classes of RING domains (Stone et al., 2005). However, the *in vivo* biological functions of all but a few of the RING proteins remain unknown. Recent studies have implicated several Arabidopsis RING proteins in a variety biological processes, including COP1 and CIP8 (photomorphogenesis) (Hardtke et al., 2002; Seo et al., 2004), SINAT5 (auxin signaling) (Xie et al., 2002), ATL2 (defense signaling) (Serrano and Guzman, 2004), BRH1 (brassinosteroid response) (Molnár et al., 2002), RIE1 (seed development) (Xu and Li, 2003), NLA (nitrogen limitation adaptation) (Peng et al., 2007), HOS1 (cold response) (Dong et al., 2006), AIP2 (ABA signaling) (Zhang et al., 2005), KEG (ABA signaling) (Stone et al., 2006) and SDIR1 (ABA signaling) (Zhang et al., 2007).

Here we report the isolation, identification and characterization of an *Arabidopsis* mutant, *sugar-insensitive3* (*sis3*), which is resistant to the early seedling developmental arrest caused by high exogenous sugar levels. The responsible locus, *SIS3*, was identified through a map-based cloning approach and confirmed with additional T-DNA insertional mutants and complementation tests. The *SIS3* gene encodes a protein with a RING-H2 domain and three putative transmembrane domains. GST-SIS3 recombinant proteins exhibit *in vitro* ubiquitin E3 ligase activity. Together these results indicate that a ubiquitination pathway involving the SIS3 RING protein is required to mediate sugar response during early seedling development.

Results

Isolation of the *sis3* mutant

Most wild-type *Arabidopsis* seeds sown on minimal media supplemented with high levels of Suc or Glc (e.g., 300 mM) germinate but the resulting seedlings fail to develop green expanded cotyledons and true leaves (Zhou et al., 1998; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Gibson et al., 2001). Instead, the seedlings develop small, white or purple cotyledons. In contrast, low concentrations (e.g., 30 mM) of exogenous Suc or Glc do not exert this inhibitory effect on normal shoot development. In addition, most wild-type seeds can develop into seedlings with expanded cotyledons and true leaves on media containing high concentrations (300 mM) of sorbitol, which acts as a non-metabolizable sugar analog in *Arabidopsis*. This observation indicates that the inhibitory effects of high sugar levels on early seedling development are not solely due to osmotic stress (Laby et al., 2000).

A screen was performed to identify mutants that are resistant to the inhibitory effects of high concentrations of exogenous sugars on early seedling development. The mutant collection used for this screen was generously provided by the DuPont Company. This mutant collection is comprised of Arabidopsis ecotype Wassilewskija (Ws-2) containing random T-DNA insertions. Mutant seeds were sown on minimal Arabidopsis media (Kranz and Kirchheim, 1987) supplemented with 270 mM Suc and left in continuous light at room temperature for 12-14 days. Seedlings that developed green cotyledons and true leaves were transferred to soil and grown to maturity to produce seeds. The progeny were re-assayed to eliminate false positives. Further analyses indicated that the mutants identified via this screen are also resistant to high concentrations of Glc, and so were designated *sugar insensitive*, or *sis*, mutants.

Cloning and characterization of one of these mutants, *sis3*, is described here. A significantly higher percentage of *sis3-1* than of wild-type seeds develop into seedlings with relatively normal shoot systems on media containing 270 mM Suc (Fig. 1) More than 20% of the *sis3-1* mutants form expanded cotyledons and true leaves, whereas only 8% of Ws-2 wild-type seedlings develop expanded cotyledons and true leaves when grown on this media. As an osmotic control, the mutants were also assayed on equimolar (i.e. 270 mM) sorbitol supplemented with a low (10 mM) amount of Suc. At this

concentration of sorbitol no significant growth differences are detected between the wild-type and *sis3-1* lines. These results indicate that the *sis3-1* mutation confers resistance to high concentrations of sugars and is not simply osmo-tolerant.

Map-based cloning of the *SIS3* gene

The *sis3-1* mutant was isolated from a population carrying random T-DNA insertions in the Ws-2 background. However, linkage analysis indicated that the kanamycin-resistant phenotype conferred by the T-DNA insertion does not co-segregate with the sugar-insensitive phenotype of the *sis3-1* mutant. Therefore the *SIS3* gene is not disrupted by a T-DNA insertion. Thus, a map-based cloning approach was initiated. The s*is3-1* mutant was crossed to wild-type plants of the Columbia ecotype. F2 seeds from this cross were selected on minimal *Arabidopsis* media (Kranz and Kirchheim, 1987) supplemented with 300 mM Suc to identify those seedlings with a *sis* phenotype. A mapping population of 1,200 plants displaying a *sis* phenotype (i.e. seedlings that formed relatively normal shoot systems) was identified and characterized to allow fine mapping of the *sis3* locus. SSLP and CAPS markers were used to map the *sis3-1* locus to a 72-kb region from 17702-17774 kb on BAC clone T17F15 on chromosome 3 (Fig. 2A). Primers were designed to amplify DNA fragments spanning this entire 72-kb region. These primers were then used to amplify DNA from *sis3-1* by PCR.

Sequencing of the *sis3-1* PCR products revealed the presence of only one mutation in the 72-kb region shown to contain the *sis3-1* mutation. This mutation consists of a large, 7.5-kb deletion from 17,711,128 bp to 17,718,664 bp. This 7.5-kb region contains the full sequences of At3g47980 and At3g47990 and the 3’ end sequence of At3g48000. To identify the gene responsible for the *sis3-1* phenotype, T-DNA insertional lines (Col-0 background) carrying mutations in each of these three genes were obtained from the Arabidopsis Biological Resource Center. Homozygous mutant lines were identified and assayed on media containing 280 mM Glc. Lines that are homozygous for T-DNA insertions in At3g47980 (SALK_010350) and At3g48000 (SALK_143632; SALK_080982) do not show a *sis* phenotype (data not shown), whereas

two out of the three homozygous T-DNA mutants of At3g47990 are sugar insensitive. These lines were thus named *sis3-2* (SALK_135584) and *sis3-3* (SALK_023817) (Fig. 2B). To determine why the third line carrying a T-DNA insertion in the putative *SIS3* gene does not exhibit a *sis* phenotype, reverse-transcriptase (RT) PCR was used to measure *SIS3* transcript levels in all three T-DNA tagged lines carrying insertions in At3g47990 (Fig. 2B). The *sis3-2* (SALK_135584) mutant failed to give an RT-PCR product when a primer pair (pair B) spanning the *sis3-2* insertion site was used. Similarly, the *sis3-3* (SALK_023817) mutant failed to produce an RT-PCR product when a primer pair (pair A) spanning the *sis3-3* T-DNA insertion site was used. In contrast, the *sis3-4* (SALK_076566) mutant produced RT-PCR products with both primer pairs A and B, despite the fact that primer pair B spans the *sis3-4* T-DNA insertion site. This result indicates that the intron in which the T-DNA inserted in *sis3-4* is correctly excised a significant percentage of the time, despite the presence of the T-DNA insert. Thus, the *sis3-4* mutant is able to produce wild-type *SIS3* mRNA, explaining its lack of a *sis* phenotype. In contrast, both *sis3-2* and *sis3-3* have *sis* phenotypes that are similar to that of the original *sis3-1* mutant (Fig. 3A), indicating that the mRNAs produced by *sis3-2* and *sis3-2* are not sufficient to confer a wild-type phenotype.

To further test whether At3g47990 corresponds to the *SIS3* locus and whether the *sis3* phenotype is the result of a mutation in a single gene, a complementation test was performed. A 3.5-kb At3g47990 genomic clone, which includes the predicted 836-bp promoter region, was cloned into the binary plant transformation vector pEarleyGate302 (Earley et al., 2006) and used to transform the *sis3-2* mutant via Agrobacterium-mediated transformation (Clough and Bent, 1998). Seeds from the T3 homozygous transgenic lines were collected and subjected to further characterization. Using semi-quantitative RT-PCR, *SIS3* transcript levels in one of the complementation transgenic lines were found to be comparable to those of wild type (Fig. 3B, *sis3 com*). Characterization of this complementation test line revealed that the genomic *SIS3* construct restores *sis3-2* to a wild-type response to both 300 mM Suc and 300 mM Glc (Fig. 3A). These data demonstrate that At3g47990 is the *SIS3* gene. The growth phenotypes of the *sis3* mutants

are visually similar to that of the wild type when grown on soil under long day or continuous light conditions.

As disruption of *SIS3* causes sugar insensitivity, it was of interest to test whether overexpressing *SIS3* can cause sugar hypersensitivity. The *SIS3* coding sequence driven by the constitutive cauliflower mosaic virus 35S promoter in the pEarleyGate100 binary vector (Earley et al., 2006) was introduced into Columbia-0 wild-type plants. One homozygous T3 transgenic line overexpressing *SIS3* (Fig. 3B) was used for sugar hypersensitivity assays on 220 mM Suc and Glc. At these sugar concentrations, wild-type seedlings are able to grow and develop relatively normally. The seedling development of *35S-SIS3* plants is similar to that of wild type (Fig. 3C), indicating that overexpression of *SIS3* mRNA does not confer sugar hypersensitivity.

sis3 **and** ***35S-SIS3*** **lines exhibit wild-type responses to ABA and paclobutrazol**

The plant hormones ABA and gibberellins (GAs) are important regulators of seed germination, with ABA inhibiting germination and GAs promoting it. To test if SIS3 plays a role in response to ABA or GAs during seed germination, *sis3-2*, *sis3-3, 35S-SIS3* and wild-type seeds were sterilized, stratified and sown on minimal media containing the indicated concentrations of ABA or paclobutrazol (a GA biosynthesis inhibitor). The seeds were scored for percent germination after 9 days. In the presence of 0.5 or 1 μM ABA, the percentages of *sis3-2*, *sis3-3* and *35S-SIS3* seeds that germinate are similar to that of wild type (Fig. 4A). In the presence of 30 μM paclobutrazol, the mutant and wild-type seeds also exhibit similar germination percentages, with approximately 30% of the seeds from each line germinating (Fig. 4B).

sis3 **and** ***35S-SIS3*** **lines exhibit altered root growth responses**

Although *sis3* and *35S-SIS3* seeds exhibit wild-type germination percentages on media containing ABA, their root growth in the presence of ABA is altered relative to that of wild-type seedlings. On media without ABA, the primary root lengths of the *35S-*

SIS3 seedlings are significantly shorter than that of the wild type, whereas the *sis3-2* and *sis3-3* seedlings have primary root lengths that are similar to those of the wild type (Fig. 5). When assayed on 0.5 μM ABA, the primary root growth of the wild-type and *35S-SIS3* seedlings is strongly inhibited. However, the *sis3-2* and *sis3-3* seedlings display a slight, but detectable, resistance to the inhibitory effects of ABA by developing short primary roots. When grown on 1 μM ABA, all of the seedlings tested fail to develop primary roots. These results indicate that loss of function mutations in the *SIS3* gene confer a slight degree of resistance to ABA-mediated inhibition of primary root growth.

Characterization of *SIS3* mRNA levels

To determine the relative steady-state mRNA levels of *SIS3* in different Arabidopsis organs, semi-quantitative RT-PCR was performed. In these experiments, *SIS3* transcripts were detected at a consistent level among different organs of Arabidopsis, including roots, stems, leaves, siliques and flowers (Fig. 6A). These data were compared with those compiled by Genevestigator (Zimmermann et al., 2004) (https://www.genevestigator.ethz.ch). According to Genevestigator, *SIS3* is ubiquitously expressed in all major organ and tissue types except in pollen, where mRNA levels are very low. The developmental expression profile reveals that *SIS3* is expressed at a slightly higher level in germinated seeds (1140) than in seedlings (879). Analysis of stimuli response data by Genevestigator (https://www.genevestigator.ethz.ch) indicates that *SIS3* steady-state mRNA levels are increased 2.5-fold by syringolin, a cell death-inducing chemical, and 1.5-fold by cold and anoxia treatment, but are decreased nearly 2-fold by heat, suggesting *SIS3* may affect pathogen and abiotic stress responses. To investigate whether high concentrations of exogenous Glc affect *SIS3* steady-state mRNA levels in germinating seeds, RT-PCR and quantitative real-time PCR were conducted as described in the materials and methods section. The Glc-repressed gene *CAB4* served as a positive control for Glc treatment. The results of these experiments suggest that the transcript levels of *SIS3* are not significantly regulated by high concentrations of Glc in

germinating seeds (Fig. 6B and 6C). These results suggest that any effects of Glc on SIS3 activity are at the post-transcriptional level.

SIS3 exhibits *in vitro* ubiquitin E3 ligase activity

The *SIS3* gene contains ten exons and nine introns and encodes a protein with a predicted length of 358 amino acids, a molecular weight of 40,686 daltons and an isoelectric focusing point of 6.34. The SIS3 protein contains a 27 amino acid signal peptide at the N terminus, three transmembrane domains near the N terminus and one RING domain, according to predictions by the SMART program (http://smart.embl-heidelberg.de/). The RING domain is between amino acids 235 and 275, near the C terminus of the protein (Fig. 7A). RING domain proteins are one type of E3 ligases, which are involved in the ubiquitination/26S proteasome pathway (Lorick et al., 1999; Smalle and Vierstra, 2004). According to published reports, the SIS3 protein is a member of the RING-H2 type protein and has not previously been tested for E3 ligase activity (Stone et al., 2005). Thus, it was of interest to test the ability of SIS3 to function as an E3 ligase in the ubiquitination process. Towards this end, SIS3 was tested for E3 ligase activity using *in vitro* assays (Fig. 7B). Recombinant Glutathione S-Transferase (GST)-SIS3 FL (full length SIS3) and GST-SIS3 RING (contains only the RING domain from SIS3) proteins were produced in *E. coli* and affinity-purified using glutathione agarose. In the presence of E1 and E2 (UBC8), both GST-SIS3 FL and GST-SIS3 RING stimulate E2-dependent ubiquitination. In the absence of either E1 or E2, the ubiquitination activity is not observed with GST-SIS3 RING (the ubiquitination activity of GST-SIS3 FL was not tested in the absence of E1 or E2). These results indicate that SIS3 is an E3 ligase and that the RING domain is sufficient for autoubiquitination.

Discussion

Sugar sensing and response plays an important role in plant growth, development and metabolism. Development of wild-type *Arabidopsis* seedlings is arrested in the

presence of elevated levels of Glc or Suc (e.g. 300 mM), and a number of mutants that are resistant to the effects of high sugar levels have been isolated from forward genetic screens (Zhou et al., 1998; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Gibson et al., 2001). Here we report the isolation of a new *sugar-insensitive* mutant, *sis3*, and the identification of the corresponding *SIS3* gene, At3g47990, which encodes a RING finger protein with E3 ligase activity.

The *sis3-1* mutant was isolated based on its ability to develop green, expanded cotyledons and true leaves in the presence of 270 mM Suc. Positional cloning was used to narrow the site of the *sis3-1* mutation to a 72-kb region on BAC clone T17F15 on chromosome 3. Sequencing of genomic DNA from *sis3-1* spanning this entire region resulted in the identification of a single mutation, which consists of a large, 7.5-kb deletion. This deletion spans the full sequences of At3g47980 and At3g47990 and the 3' end sequence of At3g48000. *Arabidopsis* lines that are homozygous for T-DNA insertions in all three of these genes were identified. Only those lines that are homozygous for T-DNA insertions in At3g47990 are *sugar insensitive*. A complementation test was performed by introducing the wild-type At3g47990 genomic sequence into the *sis3-2* mutant, which restored the sugar sensitivity of *sis3-2* on 300 mM Suc or Glc. These results indicate that At3g47990 is the *SIS3* gene.

SIS3 is annotated as a RING finger protein. As some RING finger proteins have been shown to play key roles in the ubiquitination/proteasome process by acting as ubiquitin E3 ligases (Hershko and Ciechanover, 1998; Smalle and Vierstra, 2004), SIS3 was tested for E3 ubiquitin ligase activity using an *in vitro* assay. The results from this assay indicate that SIS3 has ubiquitin E3 ligase activity. An array of RING finger proteins have been shown to possess ubiquitin E3 ligase activity and to help regulate turnover of proteins involved in diverse developmental and response pathways, including seed development, photomorphogenesis, defense response, nitrogen limitation adaptation, cold response, auxin signaling and ABA signaling (Xie et al., 2002; Xu and Li, 2003; Seo et al., 2004; Serrano and Guzman, 2004; Zhang et al., 2005; Dong et al., 2006; Stone et al., 2006; Peng et al., 2007; Zhang et al., 2007). For example, ABI3 and ABI5, which are components of the ABA-response pathway, have been identified as targets of the RING

proteins AIP2 and KEG (Zhang et al., 2005; Stone et al., 2006). Interestingly, *abi3* (Huijser et al., 2000; Zeng and Kermode, 2004) and *abi5* (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000) mutants have been shown to be sugar resistant in previous studies. In addition, root growth of *keg* mutants is hypersensitive to ABA and Glc and the *aip2-1* null mutant is hypersensitive to ABA (Zhang et al., 2005; Stone et al., 2006).

Based on these previous studies showing that mutations in genes encoding components of the ABA-response pathway can confer sugar insensitive phenotypes, it was of interest to test if mutations in *SIS3* affect ABA response. Towards this end, the seed germination responses of *sis3* and *35S-SIS3* seeds were examined on ABA-containing media. Almost identical percentages of mutant and wild-type seeds germinate on media containing 0, 0.5 or 1 µM ABA, indicating that mutations in *SIS3* do not affect ABA-mediated inhibition of seed germination.

To determine if mutations in *SIS3* affect other ABA responses, the root lengths of wild-type, *sis3* and *35S-SIS3* seedlings growing on different concentrations of ABA were examined. On media without ABA, no statistically significant differences were found between the primary root lengths of wild-type, *sis3-2* and *sis3-3* seedlings. The primary roots produced by *35S-SIS3* seedlings on media lacking ABA are significantly shorter than those produced by wild-type seedlings. However, the shoot systems produced by *35S-SIS3* seedlings on media lacking ABA are also smaller than those produced by wild-type seedlings, suggesting that overexpression of SIS3 may have a general, deleterious effect on seedling growth. When grown on media containing 0.5 µM ABA, *sis3-2* and *sis3-3* seedlings produce roots that are noticeably longer than those produced by wild-type or *35S-SIS3* seedlings. However, when grown on media containing 1 µM ABA, the *sis3-2*, *sis3-3*, *35S-SIS3* and wild-type seedlings all fail to produce significant root systems. Thus, the *sis3-2* and *sis3-3* mutations confer a very weak resistance to the inhibitory effects of ABA on seedling root growth.

The molecular basis for this slight effect of the sis3-2 and *sis3-3* mutations on ABA-mediated inhibition of root growth is unclear, due to the fact that several response pathways are known to exhibit “cross-talk” in regulation of root growth. For example,

several ethylene-insensitive mutants, including *ein2-44*, *ein3-1*, *etr1-2* and *etr1-3*, exhibit a high degree of resistance to the inhibitory effects of ABA on seedling root growth (Ghassemian et al., 2000). Thus, the slight effect of the *sis3-2* and *sis3-3* mutations on ABA-mediated inhibition of root growth may be due to cross-talk and relatively indirect. Alternatively, the *sis3-2* and *sis3-3* mutations may have subtle effects on ABA response or metabolism. For example, wild-type Arabidopsis seedlings grown on high concentrations (e.g. 7%) of Glc have ABA levels that are approximately six-fold higher than those of wild-type seedlings grown on media lacking Glc (Arenas-Huertero et al., 2000). The *sis3-2* and *sis3-3* mutants may be slightly insensitive to this Glc-mediated increase in endogenous ABA levels, leading to a subtle decrease in the sensitivity of these mutants to the inhibitory effects of exogenous ABA on root growth.

As GA is known to antagonize the effect of ABA on seed germination, and some mutants that exhibit a *sis* phenotype are also resistant to the inhibitory effects of paclobutrazol (an inhibitor of GA biosynthesis) on seed germination (Laby et al., 2000; Gibson et al., 2001), the ability of *sis3* seeds to germinate in the presence of paclobutrazol was tested. The *sis3* mutant, *35S-SIS3* overexpression and wild-type seeds germinate at almost identical percentages on media containing 30 µM paclobutrazol, suggesting that *SIS3* is not involved in GA response during seed germination. The stimuli response data compiled by Genevestigator (https://www.genevestigator.ethz.ch) indicate that paclobutrazol treatment does not change *SIS3* expression. Together these results suggest that SIS3 does not play a significant role in GA response during seed germination.

In recent years, a number of genes have been identified as encoding components of different sugar-response pathways in *Arabidopsis*, including *HXK1* (Jang et al., 1997; Moore et al., 2003), *RGS1* and *GPA1* (Chen et al., 2003), *PRL1* and *SnRK1s* (Németh et al., 1998; Bhalerao et al., 1999; Baena-González et al., 2007). In this study, we present evidence that a functional ubiquitin E3 ligase, SIS3, is involved in sugar-regulated postgerminative growth and acts as a positive regulator of sugar response. In the future, double-mutant and transcriptional profiling analyses may help determine how SIS3 exerts its function. Particularly interesting challenges are to identify the target(s) of SIS3 and to

determine its position in relation to other components of the known sugar-response pathways.

Materials and Methods

Plant materials, growth conditions and isolation of *sis3* mutants

A mutant population comprised of Arabidopsis ecotype Wassilewskija (Ws-2) plants containing random T-DNA insertions was obtained from the DuPont Company and screened on 270 mM Suc. The *sis3-1* mutant was isolated based on its resistance to the postgermination arrest of development by media containing high levels of sucrose. Seed segregating for the *sis3-2* (SALK_135584), *sis3-3* (SALK_023817) and *sis3-4* (SALK_076566) mutations were obtained from the Arabidopsis Biological Resource Center (ABRC) in the Col-0 (CS6000) background. PCR was then used to screen for plants that are homozygous for the *sis3-2*, *sis3-3* and *sis3-4* mutations. The *sis3-2* and *sis3-3* seeds used for the physiological assays and real-time RT-PCR analysis were backcrossed twice to wild-type plants of the Col-0 background. Isolation of *abi4-103*, which is in the Columbia background, has previously been described (Laby et al., 2000). Seeds of *spy-3* (Jacobsen and Olszewski, 1993), which is also in the Columbia background, were obtained from Dr. Neil Olszewski. Wild-type Hi-O (CS6736) and Col-0 (CS6000) seeds were obtained from the ABRC at Ohio State University. Seeds (typically 50-100) to be sown on plates were surface-sterilized and stratified in the dark at 4°C for 3 days. Plants were grown on plates under continuous white fluorescent light at 22°C for an additional 12-14 days prior to scoring. Minimal *Arabidopsis* media was prepared as described (Kranz and Kirchheim, 1987).

Expression analysis by RT-PCR

For *SIS3* transcript level analysis in Col-0, *sis3-2*, *sis3-3* and *sis3-4* plants, the Qiagen RNeasy plant mini kit (Qiagen, Valencia, CA) was used to isolate total RNA

from aerial parts of 4-week old plants grow on soil under continuous light. For *SIS3* transcript level analysis in Col-0, *sis3 com*, *35S-SIS3* and different organs of Col-0, the Sigma Spectrum Plant Total RNA kit (Sigma, St. Louis, MO) was used to isolate total RNA from aerial parts of indicated organs of 32-day old plants grow on soil under continuous light. After DNase I treatment (Promega, Madison, WI), first strand cDNA was synthesized from total RNA (1 μg) using the Promega Improm-II RT system kit (Promega, Madison, WI) in a reaction volume of 20 μl. One μl of cDNA was used for PCR reactions. The expression of *ACTIN2* was used as an internal control. Primers used were: *SIS3* A 5'-TTGTTGACAATGGTCTTGCTTC-3' and 5'-AAGCCCACAGAAATGGATACAG-3', *SIS3* B 5'-AGGCACTCATTCAAGAACTTCC-3' and 5'-GTCTATGCACTCCACATGGAAA-3', *ACTIN2* 5'-TGCTGACCGTATGAGCAAAG-3' and 5'-GATTGATCCTCCGATCCAGA-3'.

Glc regulation of *SIS3* expression in wild-type germinating seeds

Col-0 wild-type seeds were stratified and sown on nytex screens on solid minimal media (Kranz and Kirchheim, 1987) and incubated for 20 h under continuous light and then transferred to minimal media supplemented with 300 mM Glc or sorbitol for an additional 12 h under continuous light before harvesting. The total time of treatment was within the temporal window during which exogenous sugars can arrest seedling development (Gibson et al., 2001). Seeds from two biologically independent batches were used to minimize the variation in gene expression engendered by possible variations in plant growth conditions. Total RNA was isolated using the Spectrum™ Plant Total RNA Kit (Sigma, St. Louis, MO). First strand cDNA was synthesized using the Promega Improm-II RT system kit (Promega, Madison, WI) in a reaction volume of 20 μl. Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR Machine using the SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis, MO). *UBQ6* (AT2G47110) was used as an internal reference. Primers used were: *UBQ6*, 5'-CCATCGACAATGTCAAGGCC-3' and 5'-GGTACGTCCGTCTTCGAGCT-3'; *CAB4*,

5'-GCTAACGGACGATGGGCTAT-3' and 5'-CCAGCATCGTACCACTCAGGA-3'; *SIS3*, 5'- TTGTTGACAATGGTCTTGCTTC -3' and 5'-AAGCCCACAGAAATGGATACAG -3'.

Map-based cloning of the *SIS3* gene

Mapping populations for *sis3-1* were generated as described in the Results section. Each of these F2 mapping populations was screened on media containing 300 mM Suc to identify plants that are homozygous for the *sis* mutation carried within that population. Genomic DNA was prepared from leaves of these seedlings via alkaline lysis (Klimyuk et al., 1993). Other mapping procedures were performed as described (Lukowitz et al., 2000; Jander et al., 2002). The molecular markers used to identify recombinants in the mapping experiments were simple sequence length polymorphisms (SSLPs), cleaved amplified polymorphic sequences (CAPS) and single nucleotide polymorphisms (SNPs) that were obtained from the TAIR and Monsanto SNP and Ler sequence collections (Jander et al., 2002) and tested for polymorphisms between Columbia and WS-2 wild-type plants.

Plasmid constructionss and transformation of *Arabidopsis thaliana*

All plasmids were generated using the Gateway system (Invitrogen, Carlsbad, CA). The *SIS3* genomic clone (3518 bp), ranging from 836 bp before the start codon until the stop codon, was amplified by PCR, confirmed by DNA sequencing to be free of mutations and cloned into the destination vector pEarleyGate 302 (Earley et al., 2006). The *SIS3* full length coding sequence (1077 bp) was amplified by PCR, confirmed by DNA sequencing to be free of mutations and cloned into the destination vector pEarleyGate 100 (Earley et al., 2006), in which transgene expression is driven by the constitutive CaMV 35S promoter. Both binary vectors were then introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Transformation of *sis3-2* mutant and Col-0 wild-type plants was performed using the floral dip method (Clough

and Bent, 1998). T1 transgenic plants showing resistance to media containing BASTA (7 μg/mL) were further verified by PCR analysis to contain the appropriate construct and then transferred to soil to obtain T2 seeds. T3 and T4 homozygous lines were used for complementation tests, seed germination assays and gene expression analyses.

Sugar, abscisic acid and paclobutrazol assays

To assay sugar response, 50-100 seeds were sterilized, stratified and sown on minimal media (Kranz and Kirchheim, 1987) containing the indicated sugars and scored for seed germination, cotyledon expansion and true leaf formation as described (Laby et al., 2000). To measure ABA sensitivity, seeds were sown on minimal media supplemented with 0, 0.5 or 1 μM ABA. Plates were incubated at 22°C under continuous light. The percentages of seeds that had germinated were determined on the ninth day. Germination is defined as the emergence of the radicle from the seed coat. For root growth assays, MS medium containing 1.5% Suc was supplemented with 0, 0.5 or 1 μM ABA. Seeds were grown on vertically-oriented Petri plates for 8 days under constant light. To measure paclobutrazol sensitivity, seeds were sown on minimal media supplemented with 0 or 30 μM paclobutrazol. Plates were incubated at 22°C under continuous light. The percentages of seeds that had germinated were determined on the ninth day.

E3 ubiquitin ligase assay

The *SIS3* full length coding sequence and partial regions coding for the RING domain (corresponding to amino acid 181-358) were amplified, verified by DNA sequencing to be free of mutations and cloned into the pDEST 15 vector using the Gateway system (Invitrogen, Carlsbad, CA) to produce in-frame fusions with the glutathione S-transferase (GST) tag. The vector was then transformed into *Escherichia coli* strain BL21 by electroporation. Production of fusion proteins was induced by L-arabinose and the fusion proteins purified using glutathione agarose. The E3 ubiquitin ligase assays were performed as previously described with modifications (Hardtke et al.,

2002). In brief, reactions (40 μl) containing 25 mM Tris (pH 7.5), 0.25 M $MgCl_2$, 2 mM $ZnCl_2$, 10 mM DTT, 0.1 M ATP, pyrophosphate, ubiquitin (Sigma, St. Louis, MO), E1 lysate, UBC8 (E2) lysate, purified GST-SIS3 and GST-RING were incubated at room temperature for 90 minutes. Reactions were stopped and then analyzed by SDS-PAGE followed by western blotting using anti-ubiquitin antibodies. Results were visualized using enhanced chemiluminescence as instructed by the manufacturer (Pierce Biotech, Rockford, IL).

Authors' contributions

CL and YH identified the *SIS3* gene. DP performed preliminary mapping of the *SIS3* gene and characterization of the *sis3-1* mutant. YH performed most of the characterization of the *sis3-2*, *sis3-3* and *35S-SIS3* mutants and drafted the manuscript. CL performed the quantitative real-time PCR analysis. SP aided in plasmid construction. WG and YH performed the SIS3 ubiquitin ligase assay. SG conceived of the study, assisted in its design and the interpretation of the data and aided in drafting the manuscript.

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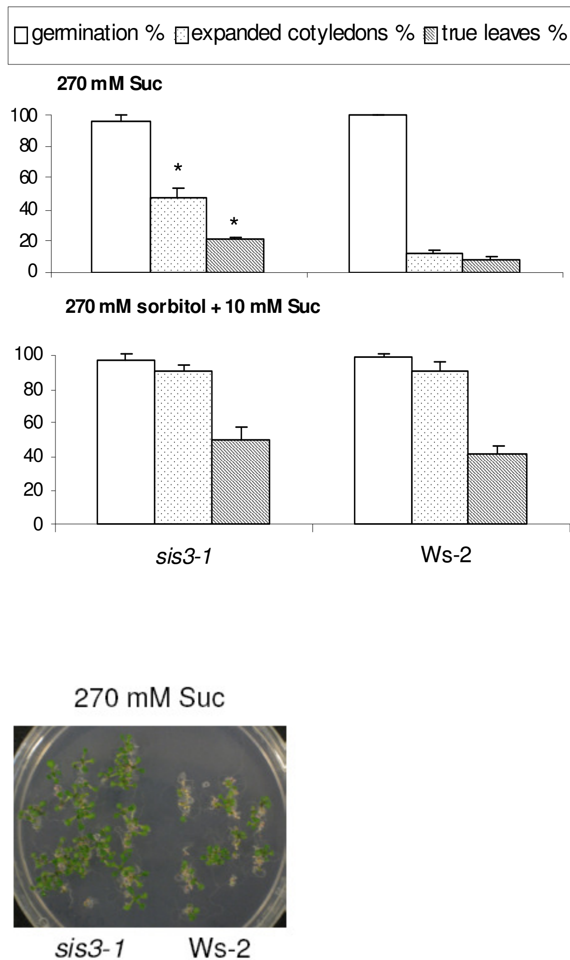


Figure 1. Sucrose-insensitive phenotype of *sis3-1*. Seeds of *sis3-1* and Ws-2 wild type were sown on minimal media containing the indicated sugars and grown under continuous light at 22°C for two weeks. The percentages of seeds that germinated and developed into seedlings with green expanded cotyledons and true leaves were scored. Data represent the mean of three independent experiments. Error bars represent standard deviations (SD). Asterisks indicate the phenotype of the *sis3-1* mutant differed from that of the wild type by $p < 0.05$, according to a Student's t-test. Sorbitol-containing media was included as an osmotic control. This assay was repeated with similar results. The inset shows the growth phenotypes of *sis3-1* and Ws-2 WT on 270 mM Suc after 12 days.

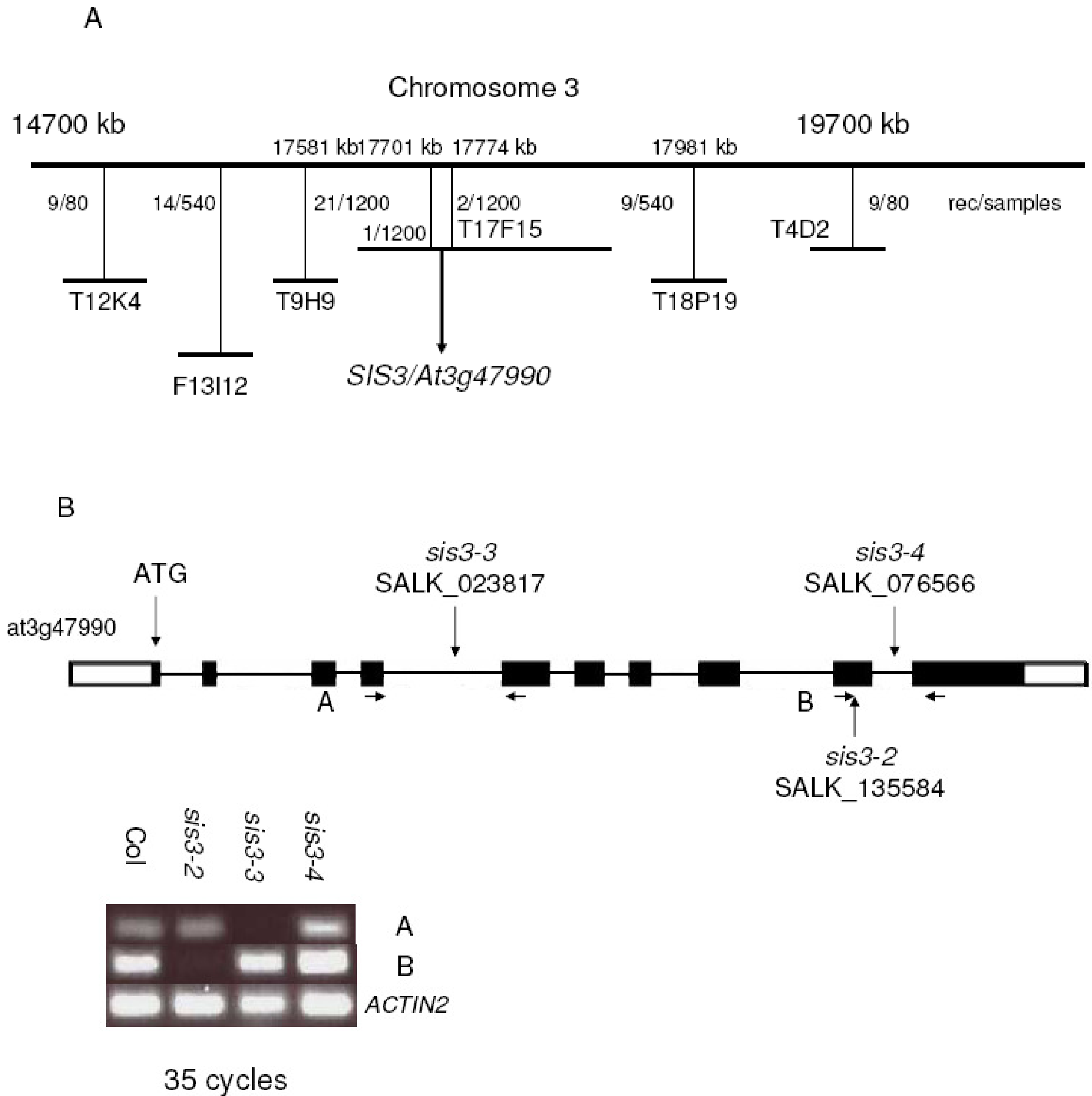
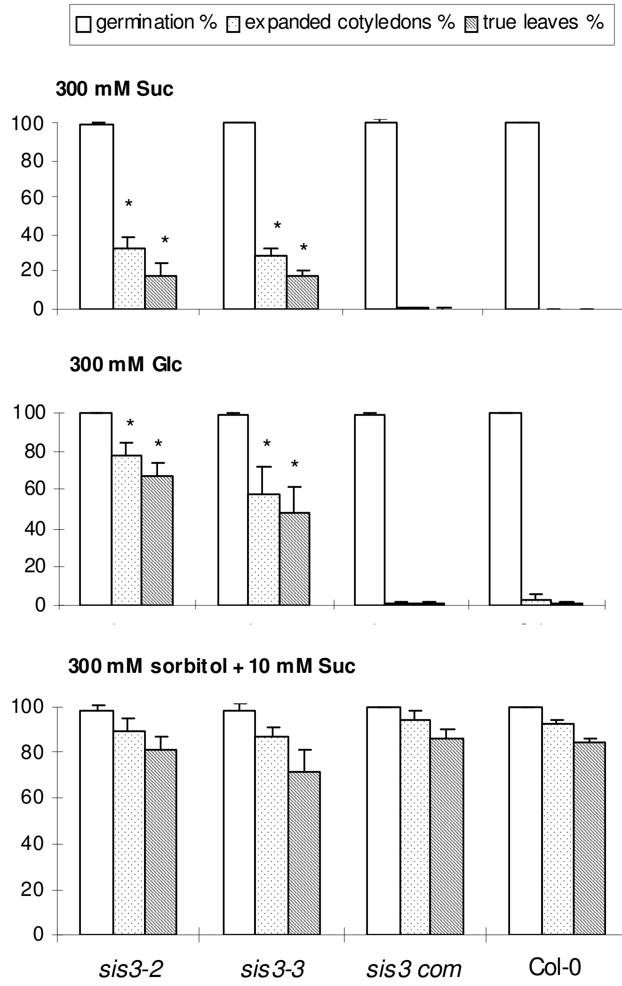
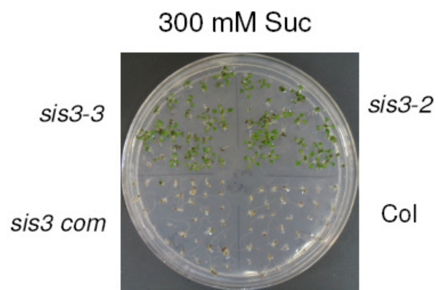
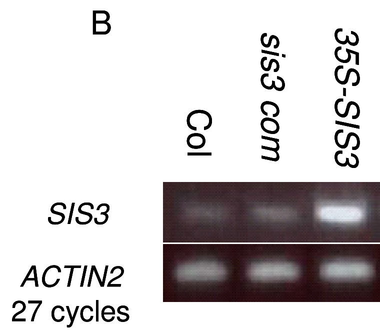


Figure 2. Map-based cloning of SIS3. (A) Map of chromosome 3 surrounding *SIS3*. The number of recombinants found in 1200 DNA samples is shown. *SIS3* is located between 17701 kb and 17774 kb on BAC T17F15. (B) Structure of *SIS3* genomic sequence displaying positions of T-DNA insertions. Boxes represent exons and lines represent introns. Arrows indicate positions of A and B primer pairs used for the RT-PCR reactions. Total RNA was extracted from aerial parts of 4-week old plants for use in the RT-PCR reactions. *ACTIN2* was included as a positive control.

A






B
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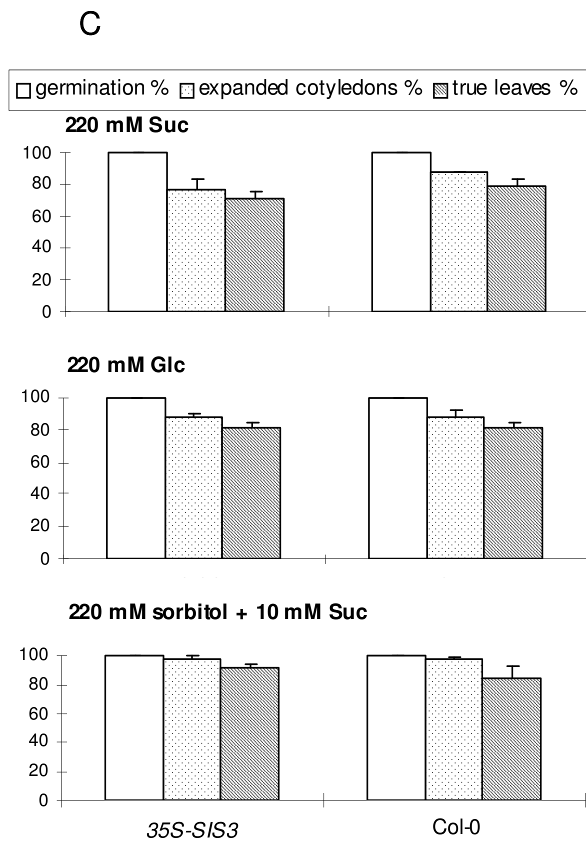
C
germination %
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220 mM Suc
220 mM Glc
220 mM sorbitol + 10 mM Suc
100
80
60
40
20
0
35S-SIS3
Col-0

Figure 3. *SIS3* mediates sugar response during early seedling development.

(A) Seedlings homozygous for the *sis3-2* and *sis3-3* mutations are more resistant to 300 mM Suc and Glc than are wild-type Col-0 seedlings. Complementation of *sis3-2* with a genomic clone of At3g47990 (*sis3 com*) restores the mutant to a wild-type level of sugar sensitivity. Sorbitol-containing media was included as an osmotic control. Mutant, transgenic and wild-type seedlings exhibit similar phenotypes on media containing 300 mM sorbitol. Data represent the mean of three independent assays ± SD. Asterisks indicate results where the phenotypes of *sis3-2* and *sis3-3* differ from that of the wild type by $p<0.05$, according to a Student's t-test. The results of the *sis3-2* line transformed with a genomic clone of At3g47990 (*sis3 com*) did not differ significantly from those of wild type. The inset shows the growth phenotypes of *sis3-2*, *sis3-3*, *sis3 com* and Col WT on 300 mM Suc after 12 days.

(B) Transcript levels of the *sis3* complementation line (*sis3 com*) and overexpression line (*35S-SIS3*). Total RNA was extracted from aerial parts of 32-day old plants grown on soil. RT-PCR was performed with *SIS3* specific or *ACTIN2* specific primers.

(C) Seedlings overexpressing *SIS3* in Col-0 background exhibit similar responses to 220 mM Suc or Glc compared to the wild type. Sorbitol-containing media was included as an osmotic control. Data represent mean of three independent assays ± SD.

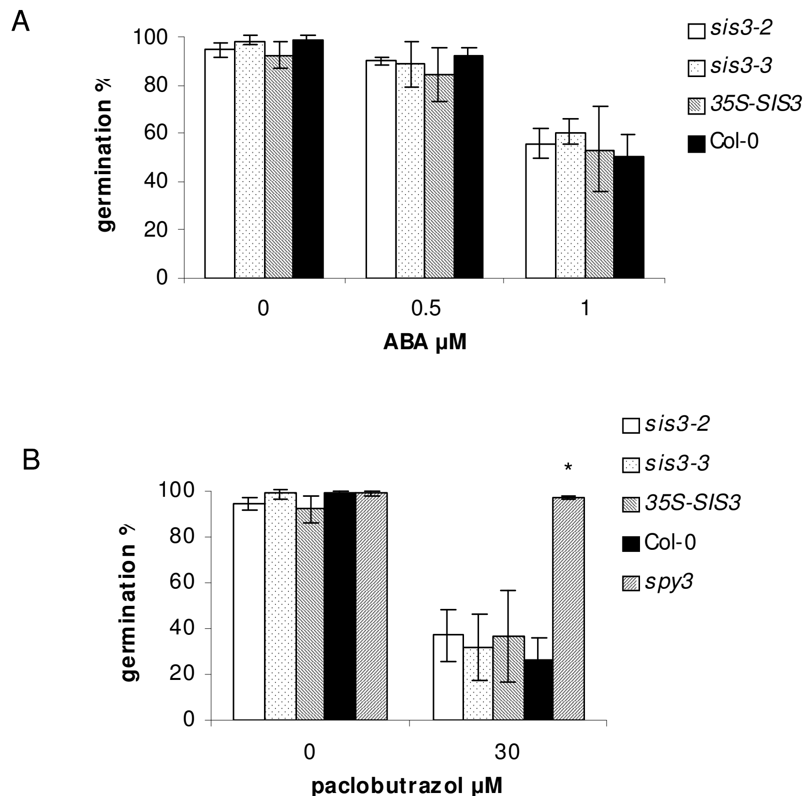
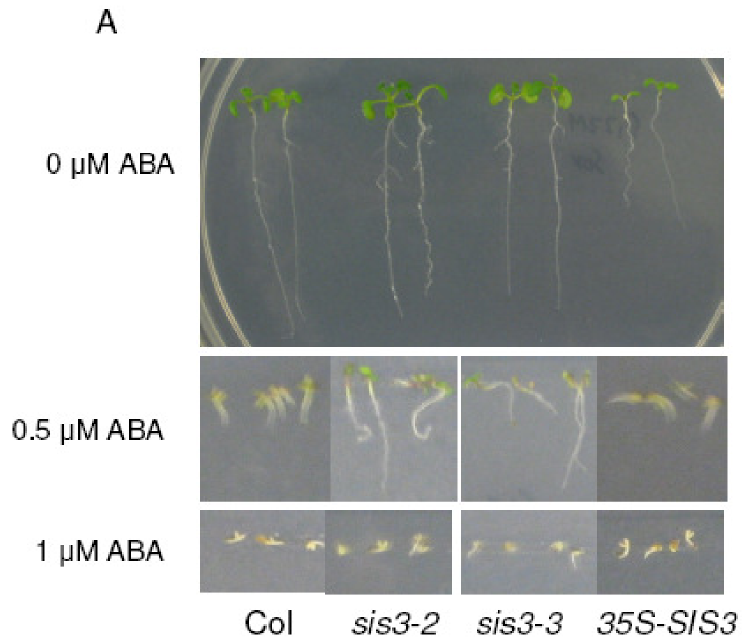


Figure 4. The *sis3* mutant and overexpression lines display wild-type responses to ABA and GAs during seed germination. (A) Seeds were sown on minimal media (Kranz and Kirchheim, 1987) supplemented with 0, 0.5 or 1 µM ABA and incubated at 22°C under continuous light for 9 days prior to scoring. Germination is defined as the emergence of the radicle from the seed coat. The *sis3-2*, *sis3-3* and *35S-SIS3* seeds exhibit wild-type germination rates on media containing 0, 0.5 or 1 µM ABA. Data represent the mean of three independent assays ± SD. (B) The *sis3-2*, *sis3-3* and *35S-SIS3* seeds exhibit wild-type responses to media containing 30 µM paclobutrazol. The *spy3* seeds were used as a positive control. Data represent the mean of three independent assays ± SD. Asterisks indicate the phenotype of *spy3* differs from that of *sis3-2*, *sis3-3*, *35S-SIS3* and the wild type by $p<0.05$.

A



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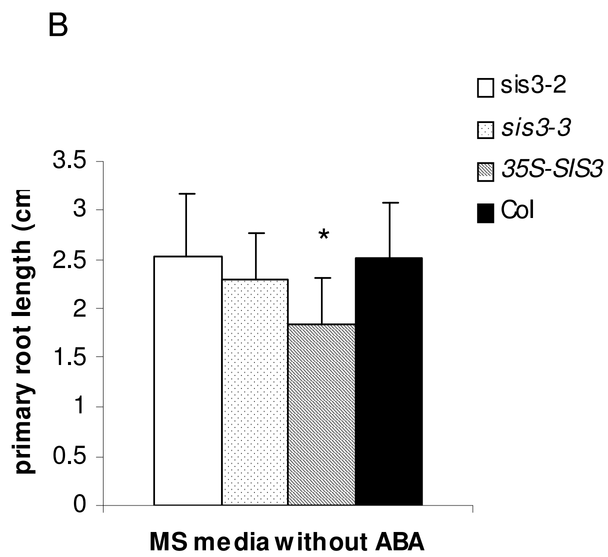

Figure 5. Root growth phenotypes of Col wild type, *sis3-2*, *sis3-3* and *35S-SIS3* plants. (A) Growth of wild-type Col, *sis3-2*, *sis3-3* and *35S-SIS3* plants on vertical MS plates supplemented with different concentrations (0, 0.5 and 1 μM) of ABA. Plants were grown for 8 days and representative seedlings are shown. (B) Statistical analysis of primary root length of wild-type Col, *sis3-2*, *sis3-3* and *35S-SIS3* on MS media without ABA. Data represent mean ± SD (n = 12-16). Asterisk indicates the phenotype of *35S-SIS3* differs from that of the wild type by $p<0.01$, according to a Student's t-test. The results were reproducible with another set of biologically independent samples.

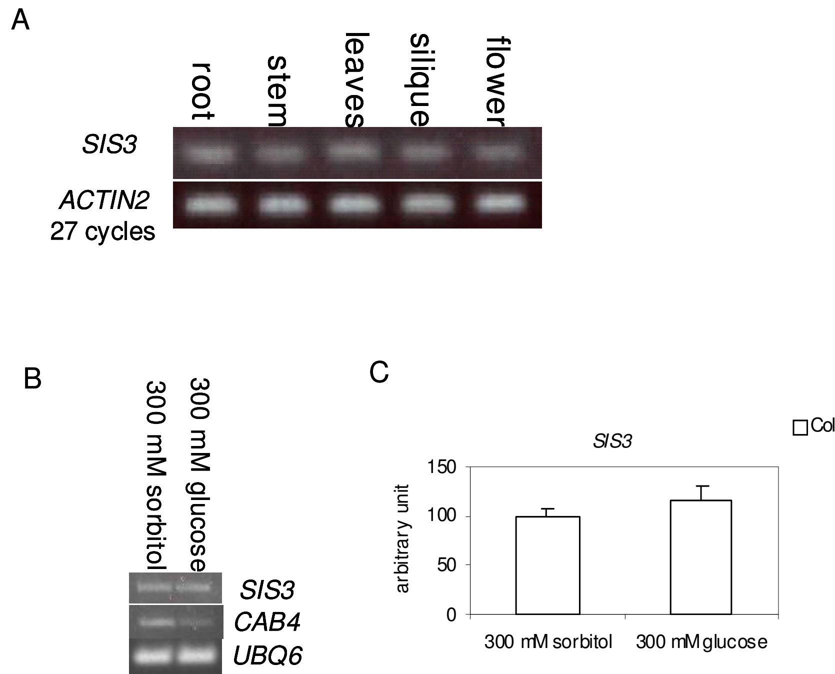

Figure 6. Characterization of *SIS3* mRNA levels. (A) Organ-specific expression of the *SIS3* gene. Total RNA was extracted from aerial parts of indicated organs of 32-day old plants grown on soil under continuous light. RT-PCR was performed with *SIS3* or *ACTIN2* specific primers. (B) Expression of *SIS3* in germinating seeds treated with 300 mM Glc or sorbitol. RT-PCR analysis was conducted as described in Materials and methods. The Glc-repressed *CAB4* gene used as a positive control for Glc treatment. *UBQ6* used as an internal control. (C) Quantitative real-time PCR analysis of *SIS3* transcript levels in germinating seeds. Data were obtained from two biologically independent experiments and relative mRNA levels were determined by using *UBQ6* as a reference. The *SIS3* mRNA levels in sorbitol-treated Col were set to 100. Data represent mean ± SD.

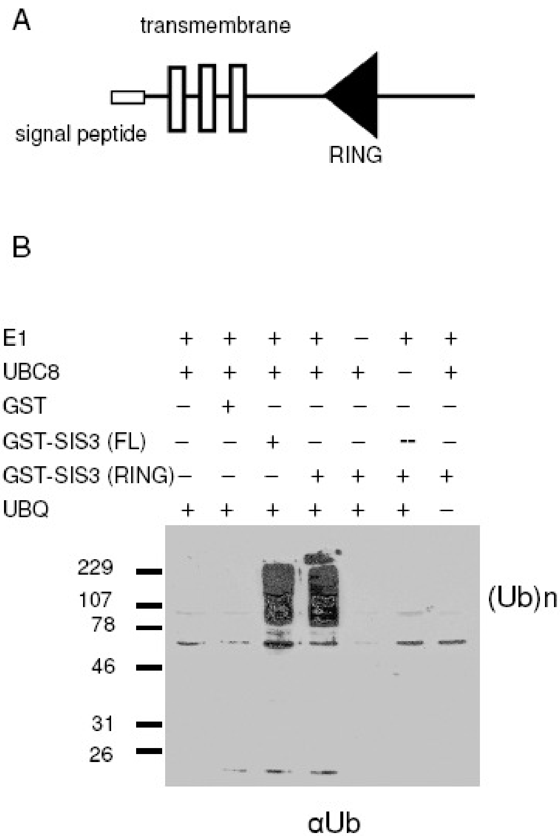

Figure 7. SIS3 has ubiquitin E3 ligase activity. (A) Schematic depiction of the SIS3 protein domains predicted by the SMART program. (B) E3 ubiquitin ligase activity of SIS3 *in vitro*. GST-SIS3 fusion proteins containing the full-length SIS3 protein (FL) or only the RING domain of SIS3 (RING) were incubated with E1, E2 (UBC8) and ubiquitin (UBQ). Polyubiquitin chains were visualized with anti-ubiquitin antibodies. Omission of E1, E2 or ubiquitin resulted in a loss of ubiquitination. GST by itself served as a negative control. Numbers on the left indicate the molecular masses of marker proteins in KDa.

Chapter IV

SIS8, a Putative Mitogen-Activated Protein Kinase Kinase Kinase, Regulates Sugar Resistant Seedling Development in *Arabidopsis*

SIS8, a Putative Mitogen-Activated Protein Kinase Kinase Kinase, Regulates Sugar Resistant Seedling Development in Arabidopsis

Yadong Huang, Chun-Yao Li, Sungjin Park and Susan I. Gibson

Abstract

Sugar signaling pathways have been evolutionarily conserved among eukaryotes and are postulated to regulate plant growth, development and responses to environmental cues. Forward genetic screens have identified sugar signaling or response mutants. Here we report the identification and characterization of *Arabidopsis thaliana sugar insensitive 8* (*sis8*) mutants, which display a sugar resistant seedling development phenotype. Unlike many other *sugar insensitive* mutants, *sis8* mutants exhibit wild-type responses to the inhibitory effects of abscisic acid and paclobutrazol (an inhibitor of gibberellin biosynthesis) on seed germination. Positional cloning of the *SIS8* gene revealed that it encodes a putative mitogen-activated protein kinase kinase kinase (MAPKKK). An *Arabidopsis* transgenic line overexpressing *SIS8* exhibits hypersensitivity to sugars and sorbitol, an osmoticum. The *SIS8* overexpressor line also exhibits hypersensitivity to ABA and paclobutrazol. *SIS8* mRNA is expressed ubiquitously among *Arabidopsis* organs. A SIS8-YFP fusion protein localizes to the cytosol when transiently expressed in tobacco leaf cells. A yeast two-hybrid screen was performed to identify possible interacting partners of SIS8. A T-DNA insertion in one of the genes encoding a potential SIS8-interacting protein causes sugar insensitivity. This gene (At3g50740) is predicted to encode a UDP-glycosyltransferase. These results indicate that SIS8, a putative MAPKKK, is a negative regulator of sugar tolerance in *Arabidopsis* that may play a broad role in regulating metabolic stress response.

Introduction

Sugars, such as sucrose (Suc) or glucose (Glc), not only function as substances to provide energy and building materials to plant cells, but also act like signaling molecules to modulate gene expression. An abundance of sugars induces expression of genes for storage and utilization and represses expression of genes for photosynthesis and reserve mobilization, while sugar depletion exerts the opposite effects (Koch, 1996; Roitsch, 1999). Plant responses to sugars help integrate metabolism, growth, development and response to environmental stresses, and exhibit extensive interactions with other signaling pathways, such as those involved in response to light, nitrogen and phytohormones (Koch, 1996; Smeekens, 2000; Coruzzi and Zhou, 2001; Gibson, 2005; Rolland et al., 2006).

The mechanisms underlying sugar sensing in plants have been intensively studies in recent years and multiple signal transduction pathways and their components have been identified and investigated. Studies on *Arabidopsis* HEXOKINASE1 (HXK1) have suggested that it acts as a glucose sensor and that a major effect of the HXK1-mediated signaling pathway is the repression of photosynthetic gene expression (Jang et al., 1997; Moore et al., 2003; Cho et al., 2006). A cell surface D-glucose receptor function has recently been suggested for the *Arabidopsis* heterotrimeric G-protein α subunit (GPA1) and the regulator of G-protein signaling (RGS) proteins (Chen et al., 2003; Chen and Jones, 2004; Chen et al., 2006). Protein phosphorylation and dephosphorylation are important regulatory events controlling protein activity. It has been suggested that SNF1-related protein kinase1 (SnRK1) plays a key role in plant sugar signaling and global carbon metabolism (Halford et al., 2003; Tiessen et al., 2003; Baena-González et al., 2007). Interestingly, a nuclear WD protein, pleiotropic regulatory locus1 (PRL1), has been shown to inhibit AKIN 10 and AKIN11 (*Arabidopsis* SnRK1s) activity *in vitro* and the *prl1* mutant exhibits transcriptional derepression of glucose responsive genes as well as hypersensitivity to sugars and multiple phytohormones (Németh et al., 1998; Bhalerao et al., 1999). Moreover, it has been shown that AKIN10 and AKIN11 interact with SKP1/ASK1, a conserved SCF ubiquitin ligase complex subunit (Farrás et al., 2001) and AKIN10 stability may be regulated by a CUL4-based E3 ligase complex that use PRL1 as the substrate receptor (Lee et al., 2008).

One aspect of plant development regulated by sugars is early seedling establishment. High levels of exogenous sugars (e.g. 300 mM) prevent the majority of *Arabidopsis* Columbia wild-type seedling from developing green expanded cotyledons and true leaves. Instead, seedlings form white or pink unexpanded cotyledons. Genetic screens for mutants resistant to the inhibitory effects of sugars on seedling development have identified extensive interactions between abscisic acid (ABA), ethylene and sugar signaling pathways (Gazzarrini and McCourt, 2001; Finkelstein and Gibson, 2002; Rook and Bevan, 2003). For example, the *SUN6*/*SIS5*/*GIN6*/*ISI3* gene is allelic to *ABI4*, which encodes an AP2-domain transcription factor involved in ABA-mediated germination response (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001). The *GIN1*/*SIS4*/*ISI4* gene is allelic to *ABA2*, which encodes a short-chain dehydrogenase/reductase required for ABA biosynthesis (Zhou et al., 1998; Arenas-Huertero et al., 2000; Laby et al., 2000; Rook et al., 2001; Cheng et al., 2002). In addition, alleles of *CTR1*, a Raf-like MAPKKK that negatively regulates ethylene responses, have been isolated from sugar-response screens (Gibson et al., 2001; Rolland et al., 2002).

Mitogen-activated protein kinase (MAPK) cascades are conserved signaling pathways found in yeast, humans and plants that link extracellular stimuli to cellular responses. Plant MAPK pathways are known to regulate the cell cycle, plant growth and death and responses to abiotic stress and pathogen attack (Tena et al., 2001; Zhang and Klessig, 2001; Jonak et al., 2002). A minimal MAPK pathway is composed of three kinases, a MAPK, a MAPK kinase (MAPKK) and a MAPK kinase kinase (MAPKKK). Sequential phosphorylation of a MAPKK and a MAPK connects upstream receptors/signals to downstream targets. The *Arabidopsis* genome is predicted to encode 20 MAPKs, 10 MAPKKs and 60 MAPKKKs. The MAPKKKs can be divided into two main classes, the MEKKs and Raf-like MAPKKKs (Ichimura et al., 2002). Recent years have witnessed the identification of MAPKKKs involved in a wide range of developmental, hormonal and stress responses, such as cell division, stomatal development, ethylene and auxin response, oxidative stress and resistance to powdery mildew (Kieber et al., 1993; Kovtun et al., 1998; Frye et al., 2001; Krysan et al., 2002; Bergmann et al., 2004; Nakagami et al., 2006; Yoo et al., 2008). Two *Arabidopsis* Raf-

like MAPKKKs, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) and ENHANCED DISEASE RESISTENCE1 (EDR1), are negative regulators of ethylene response and disease resistance respectively. The *ctr1* mutant was identified more than a decade ago (Kieber et al., 1993) and evidence suggests that a MAPKK-MAPK module acts downstream of CTR1 (Ouaked et al., 2003; Yoo et al., 2008). The *edr1* mutant displays enhanced resistance to powdery mildew (Frye and Innes, 1998) and the *EDR1* gene was found to encode a putative MAPKKK similar to CTR1 (Frye et al., 2001). Interestingly, the *edr1* mutant also displays an enhanced ethylene-induced senescence phenotype that is suppressed by the *ein2* mutation (Tang et al., 2005). Recently an *Arabidopsis* mutant, *at6*, was isolated based on its improved salt tolerance and the responsible locus was identified as At1g73660. At1g73660 encodes a Raf-like MAPKKK that is closely related to CTR1 and EDR1. Mutations in At1g73660 have no significant effects on response to ABA, gibberellin (GA) or ethylene (Gao and Xiang, 2008).

In this study, the *SUGAR INSENSITIVE 8* (*SIS8*) gene was isolated by map-based cloning and found to be At1g73660. Overexpression of SIS8 causes hypersensitivity to sugars, as well as a slight degree of hypersensitivity to ABA and to paclobutrazol, a GA biosynthesis inhibitor. Possible interacting partners of SIS8 have been identified through a yeast two-hybrid screen. Interestingly, a mutation in the gene encoding one of these potential SIS8-interacting proteins confers a *sugar insensitive* phenotype. Together, our results indicate that *SIS8* is a negative regulator of seedling sugar tolerance in *Arabidopsis*.

Results

Isolation of the *sis8* mutant

High levels of exogenous Suc or Glc (e.g., 300 mM) prevent the majority of wild-type *Arabidopsis* seedlings from developing green expanded cotyledons and true leaves. Instead, the seedlings develop small, white or purple cotyledons (Zhou et al., 1998; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000). In contrast, low (e.g., 30 mM) or moderate (e.g., 200 mM) concentrations of exogenous Suc or Glc do not exert

this inhibitory effect on normal shoot development. In addition, most wild-type seeds can develop into seedlings with expanded cotyledons and true leaves on media containing high concentrations (300 mM) of sorbitol, which acts as a non-metabolizable sugar analog in *Arabidopsis*. This observation indicates that the inhibitory effects of high sugar levels on early seedling development are not solely due to osmotic stress (Laby et al., 2000).

The ability of high levels of sugars to inhibit early seedling development has been used to identify mutations that render the seedlings more tolerant to sugars. Analysis of such mutants has led to the discovery of genes involved in sugar signaling pathways (Jang et al., 1997; Zhou et al., 1998; Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Gibson et al., 2001). In one such screen performed by our lab, approximately 60,000 M2 seeds derived from an ethylmethane sulphonate (EMS)-mutagenized *Arabidopsis thaliana* var. Columbia population were screened to identify seedlings that are resistant to 300 mM Suc. The mutants identified via this screen are also resistant to high concentrations of Glc and so were designated *sugar insensitive*, or *sis*, mutants (Laby *et al*., 2000). Cloning and characterization of one of these mutants, the *sis8* mutant, is described below.

Genetic characterization of the *sis8* mutant and positional cloning of the *SIS8* gene

In order to characterize the inheritance of the sugar tolerant seedling phenotype in the *sis8* mutant, the mutant was backcrossed to the Columbia wild type. The F1 seedlings show the same phenotype as wild-type plants when grown on 300 mM Suc, indicating that the mutant's sugar-insensitive phenotype is recessive. The F2 seedlings resulting from the cross between *sis8* and wild-type plants display a 3:1 segregation of wild-type and *sis* phenotypes (data not shown), also suggesting that the *sis8* phenotype is due to a single, recessive nuclear mutation. As the *sis8-1* mutation was caused by EMS mutagenesis, a positional cloning approach was employed to identify the *SIS8* gene (Lukowitz et al., 2000; Jander et al., 2002). The *sis8-1* mutant, which is in the Col background, was crossed to wild-type plants of the Hi-O background. F2 progeny of this cross were screened on media containing 300 or 320 mM Suc. Those seedlings that

formed relatively normal shoot systems (i.e. displayed a *sis* mutant phenotype) were selected and used to form a mapping population of 1,964 plants. First-pass mapping using a few hundred F2 plants established close linkage of *SIS8* with two simple sequence length polymorphism (SSLP) markers, NGA111 and NGA692, on the bottom arm of chromosome 1. Fine-scale mapping utilizing SSLP and single nucleotide polymorphism (SNP) markers localized *SIS8* to a 13 kb region on BAC clone F25P22 that contains four genes (Fig. 1A). DNA spanning the entire 13 kb region was isolated from *sis8-1* by PCR and fully sequenced. This sequencing revealed the presence of only a single point mutation within the 13 kb region shown to contain the *SIS8* gene. This point mutation causes a C to T transition at position 4,653 (according to TAIR sequence accession 1009091393) in the genomic DNA of At1g73660. At1g73660 is predicted to encode a putative plant raf family MAPKKK involved in *Arabidopsis* salt tolerance (Gao and Xiang, 2008). The *sis8-1* mutation causes an amino acid change of serine to proline at position 968 in exon 12, which is within the predicted kinase domain.

To further test whether At1g73660 is the *SIS8* gene, three independent lines carrying T-DNA insertions in the At1g73660 gene were obtained from the Arabidopsis Biological Resource Center (ABRC). Plants that are homozygous for each of these T-DNA insertions were identified using PCR. The positions of the T-DNA insertions are shown in Fig. 1B. Reverse-Transcriptase (RT) PCR was used to check *SIS8* transcript levels in these lines (Fig. 1B). For the SALK_059205 line, mRNA was detected with primer pair A, which amplifies a region upstream of the T-DNA insertion site, but no product was detected with primer pair B, which spans the insertion site. Similarly, *SIS8* mRNA was detected in line SALK_001982 with primer pair A, which amplifies a region upstream of the insertion site, but not with primer pair B, which is downstream of the insertion site. For the SALK_004541 line, no mRNA was detected with primer pair A, which spans the insertion site, but transcript was detected with primer pair B, which is downstream of the insertion site. These mutants were renamed as *sis8-1* (the original *sis8* point mutation mutant), *sis8-2* (SALK_059205), *sis8-3* (SALK_001982) and *sis8-4* (SALK_004541). The sugar insensitivity phenotypes of the *sis8* mutants were assayed on 270 mM Suc and 270 mM Glc (Fig. 2A). All of the *sis8* mutants display significant

resistance to sugars compared with the wild type. In contrast, no statistically significant differences were found between the *sis8* mutants and wild-type seedlings grown on media containing equi-molar concentrations of sorbitol. However, all of the *sis8* mutations cause a statistically significant increase in the percentage of seedlings that form true leaves on media containing 400 mM sorbitol, suggesting that the *sis8* mutations affect tolerance to osmotic stress. Given that all four *sis8* alleles have similar *sis* phenotypes (Fig. 2A), it can be concluded that At1g73660 does correspond to *SIS8* and that none of the *sis8* mutants produce wild-type levels of fully functional SIS8 protein.

Overexpression of the *SIS8* gene cause sugar and osmotic hypersensitivity

As loss-of-function of *SIS8* renders plants more tolerant to high levels of exogenous sugars, it was of interest to investigate whether gain-of-function (increased expression) of *SIS8* makes plants more sensitive to moderate levels of sugars. The coding sequence of *SIS8* was cloned into a pEarleyGate100 binary vector under the control of constitutive 35S promoter of cauliflower mosaic virus (Earley et al., 2006) and introduced into *Agrobacterium tumefaciens*. *A. tumefaciens*-mediated transformation of *Arabidopsis* Columbia-0 wild-type plants was performed using the floral dip method (Clough and Bent, 1998). One homozygous T3 transgenic line overexpressing *SIS8* (Fig. 2B) was used further analysis. When grown in soil under constant light conditions, transgenic plants overexpressing *SIS8* show no obvious growth differences from wild-type plants. However, when grown on plates supplemented with 220 mM Suc or Glc, *35S-SIS8* plants display a significant decrease in the percentage of seedlings that form expanded cotyledons and true leaves, as compared to wild-type seedlings grown on the same media. For example, less than 20% of *35S-SIS8* seedlings develop green cotyledons and true leaves when grown on media with 220 mM Suc, as compared with 80% of wild-type seedlings do. Thus, the seedling development of *35S-SIS8* plants is hypersensitive to both Suc and Glc (Fig. 2C). As an osmotic control, the shoot development of *35S-SIS8* seedlings was characterized on equi-molar (i.e. 220 mM) sorbitol and found to be quite similar to that of wild-type seedlings grown on the same media. However, when grown on greater than equi-molar (270 mM) sorbitol, 30% of *35S-SIS8* seedlings developed true

leaves, as opposed to 80% of wild-type seedlings do. When grown on 400 mM sorbitol, *35S-SIS8* seedlings exhibited a significant decrease in the percentage of seedlings that formed expanded cotyledons when compared to wild-type seedlings grown on the same media (Fig. 2D). These data suggest that overexpression of *SIS8* confers a slight degree of osmotic hypersensitivity compared to the wild type. However, this effect is only observed at concentrations of the osmoticum (sorbitol) that are higher than those needed to detect statistically significant levels of hypersensitivity to Glc and Suc.

Seed germination response of *sis8* and *35S-SIS8* seeds to ABA and GA

The phase transition from seed dormancy to germination is mediated by both external environmental factors and internal plant hormones, such as ABA and gibberellin (GA). ABA and GA have antagonistic effects on seed germination, with ABA inhibiting the process while GA promoting it (Koornneef et al., 2002). To test if SIS8 plays a role in seed germination, seeds of Col-0 wild-type, *sis8-1*, *sis8-2*, *sis8-3*, *sis8-4* and *35S-SIS8* plants were sterilized, stratified and sown on minimal media containing the indicated concentrations of ABA or paclobutrazol (a GA biosynthesis inhibitor) and scored for percent germination after 9 days. When assayed on media lacking both ABA and paclobutrazol, high percentages of seeds from all of the mutant, transgenic and wild-type lines germinate. Similarly, when sown on media supplemented with 1 µM ABA, seeds from *sis8-1*, *sis8-2*, *sis8-3* and *sis8-4* plants exhibit germination percentages that are not significantly different from seeds from wild-type plants. In contrast, *35S-SIS8* seeds show slightly increased sensitivity to ABA, with approximately 30% of *35S-SIS8* seeds germinating on 1 µM ABA, as opposed to approximately 50% of wild-type seeds (Fig. 3A). The results of the seed germination assays on paclobutrazol are similar to those of the assays on ABA. As with the ABA germination assays, seeds from *sis8-1*, *sis8-2*, *sis8-3* and *sis8-4* plants exhibit germination rates that are similar to those of seeds from wild-type plants when the seeds are assayed in the presence of 30 µM paclobutrazol (Fig. 3B). In contrast, only 5% of *35S-SIS8* seeds germinate on media containing 30 µM paclobutrazol, as compared with over 20% of wild-type seeds. The germination responses of the *sis8* seeds to ABA and GA are in agreement with the observations of Gao and

Xiang (2008). The results presented here also suggest that although loss-of-function of SIS8 has no obvious effects on germination responses to ABA and paclobutrazol, gain-of-function of SIS8 confers a slight degree of hypersensitivity to both germination regulators.

Expression of *SIS8* mRNA in *Arabidopsis* organs and in response to Glc

Semi-quantitative RT-PCR was performed to determine the relative expression levels of *SIS8* in different Arabidopsis organs. The *SIS8* transcript was detected in diverse organs of *Arabidopsis* including roots, stems, leaves, siliques and flowers, with higher expression in leaves (Fig. 4A). Our data were compared with microarray data compiled by Genevestigator (Zimmermann et al., 2004) (https://www.genevestigator.ethz.ch). According to Genevestigator, *SIS8* is expressed ubiquitously in all major organ and tissue types tested, with higher expression levels found in leaves. A developmental expression profile reveals that *SIS8* mRNA is present at slightly higher levels at seedling stage (760) than in germinated seeds (513), with mature siliques having the lowest expression levels (423). Analysis of stimuli response data compiled by Genevestigator indicates that *SIS8* mRNA levels are 2- to 3-fold lower on ABA and 2-fold lower on NAA, a synthetic auxin analog, but are increased nearly 3-fold by heat stress and 3.5-fold by brassinolide (BL) application, suggesting *SIS8* may affect plant hormone signaling and abiotic stress responses. Interestingly, salt treatment caused a decrease in *SIS8* mRNA levels in seedlings (Gao and Xiang, 2008). To investigate whether high Glc affects *SIS8* expression in germinating seeds, RT-PCR and quantitative real-time PCR were conducted as described in the materials and methods section. The Glc-repressed gene *CAB4* served as a positive control for Glc treatment. Our results suggest that the transcript levels of *SIS8* are not significantly regulated by high Glc treatment in germinating seeds (Fig. 4B and 4C).

A yeast two-hybrid screen identifies putative SIS8-interacting proteins

The yeast two-hybrid system (Y2H) has been used extensively for detecting novel protein-protein interactions. Y2H screening was conducted by the Molecular Interaction

Facility at the University of Wisconsin-Madison using the SIS8 protein as bait and proteins generated from random Arabidopsis cDNAs as prey. Six possible SIS8 interacting proteins were identified (Table. 1). The interactions of each of the six prey constructs and the SIS8 bait construct in yeast were re-tested *in vivo* under high stringency conditions and via β-galactosidase assays (Fig. 5). The results of these assays indicate that each of the prey constructs produces a fusion protein that gives a positive interaction with the fusion protein produced by the SIS8 bait construct. Furthermore, none of the prey constructs gives a positive result with the empty bait construct, indicating that a positive interaction is dependent on production of a SIS8 fusion protein (Fig. 5).

The identities of the genes encoding the six putative SIS8-interacting proteins are listed in Table 1.The protein that shows the strongest interaction with SIS8 in the Y2H assays is UGT72E1 (At3g50740), an UDP-glucose:glycosyltransferase that specifically conjugates lignin monomers such as coniferyl aldehyde and sinapyl aldehyde (Lim et al., 2005). Other genes encoding putative SIS8-interacting proteins include the *FASS/TON2* gene (At5g18580), which encodes a protein phosphatase 2A regulatory subunit that helps control the organization of cortical microtubules (Camilleri et al., 2002). At4g17250 encodes a protein of unknown biochemical function which, based on its expression profile, has been suggested to play a role in heat acclimation (Lim et al., 2006). At4g31670 encodes an ubiquitin carboxyl-terminal hydrolase family protein that has been designated as ubiquitin-specific protease 18, or UBP18. At4g08850 is predicted to encode a leucine-rich repeat family protein/protein kinase family protein. At1g61520 encodes a PSI type III chlorophyll a/b binding protein.

A mutation in a gene encoding a putative SIS8-interacting protein causes a *sugar-insensitive (sis)* phenotype

The yeast two-hybrid assays described above resulted in identification of six genes encoding putative SIS8-interacting proteins. It was therefore of interest to determine whether mutations in any of these six genes cause defects in sugar response. Towards this end, T-DNA insertion lines predicted to carry inserts in each of these six

genes were obtained from the ABRC. Seeds segregating for each of the predicted inserts were screened by PCR to identify lines that are homozygous for each T-DNA insertion. Seeds from these homozygous lines were then screened on 230 mM Suc to test for a hypersensitive response and on 270 mM Glc to test for a Glc-resistant phenotype. Interestingly, the T-DNA mutant (SALK_078702) of *UGT72E1* displays sugar insensitivity in the presence of 270 mM Glc (Fig. 6). When grown on media supplemented with 270 mM Glc, the SALK_078702 homozygous mutant line displays percentages of both cotyledon expansion and true leaf formation that are significantly higher than those of wild-type seedlings grown on the same media. In contrast, when grown on media supplemented with 270 mM sorbitol as an osmotic control, the SALK_078702 homozygous mutant line does not display statistically significant differences in percentages of cotyledon expansion or true leaf formation from the wild-type line grown on the same media. These results indicate that the SALK_078702 homozygous mutant has a sugar-insensitive phenotype that does not appear to be the result of an osmo-tolerant phenotype.

The SIS8 protein localizes primarily to the cytosol

The *SIS8* gene is predicted to contain thirteen exons and twelve introns and to encode a protein with a length of 1,030 amino acids, a molecular weight of 112, 000 daltons and an isoelectric point of 6.08. According to SMART (Schultz et al., 1998), a domain architectures prediction program, the SIS8 protein contains a ubiquitin-interacting motif (UIM) between amino acids 139 and 158 near the N terminus and a protein kinase domain (STYKc) between amino acids 748 to 1,000 with a possible dual specificity to phosphorylate serine/threonine and tyrosine residues (Fig. 7A). To determine the sub-cellular location of SIS8, the *SIS8* full length coding sequence without the stop codon was cloned into the pEarleyGate 101 vector (Earley et al., 2006) to form a translational fusion construct with the Yellow Fluorescent Protein (YFP) driven by the constitutive CaMV 35S promoter. This construct was then introduced into *Agrobacterium tumefaciens* strain GV3101, which was used to inoculate 3-week old tobacco *Nicotiana benthamiana* leaf epidermal cells as described (Sparkes et al., 2006). Transient

expression of the SIS8-YFP protein was found primarily in the cytosol 44 hours after inoculation (Fig. 7B). For comparison, the plasma membrane localized SUC9-YFP fusion protein was used as a control (Sivitz et al., 2007).

Discussion

Sugars, such as glucose and sucrose, regulate a wide variety of plant developmental processes from embryogenesis and seed germination to flowering and senescence. The available evidence indicates that multiple sugar signaling pathways exist in plants. Adding to the complexity of plant sugar response are findings that sugar-response pathways exhibit cross-talk with response pathways for other nutrients, such as nitrogen, as well as with response pathways for environmental signals, such as light, and with response pathways for different phytohormones (Coruzzi and Zhou, 2001; Gazzarrini and McCourt, 2001; Brocard-Gifford et al., 2003; Karthikeyan et al., 2006; Radchuk et al., 2006; Rook et al., 2006; Baena-González et al., 2007; Banás and Gabry´s, 2007; Muller et al., 2007).

Despite the importance of sugar-response pathways to a wide variety of plant developmental and metabolic processes, only a fraction of the factors likely involved in sugar-response pathways have been identified. In this work, a forward genetic screen was used to identify an *Arabidopsis thaliana* mutant, *sis8-1*, with enhanced tolerance to the inhibitory effects of high (e.g. 300 mM) concentrations of exogenous Suc. The *sis8-1* locus was identified by map-based cloning and found to lie in At1g73660. To further test whether At1g73660 corresponds to *SIS8*, three independent T-DNA lines that carry insertions in At1g73660 were identified and tested for a *sugar-insensitive* phenotype. All three of these lines have *sugar-insensitive* phenotypes, confirming that At1g73660 does correspond to *SIS8*.

The *SIS8* gene encodes a putative Raf-like MAPKKK. According to Ichimura et al. (2002), SIS8 belongs to subgroup B3 MAPKKKs, which also includes CTR1 and EDR1. The *ctr1* mutant was originally isolated as a negative regulator of the ethylene response pathway (Kieber et al., 1993). New mutant alleles of *CTR1* were later identified

through sugar response screens, such as *SIS1* (Gibson et al., 2001) and *GIN4* (Rolland et al., 2002). The *ctr1* and *ethylene overproducing1* (*eto1*) mutants are resistant to sugars, whereas the ethylene insensitive mutants, *etr1* and *ein2*, exhibit sugar hypersensitivity (Zhou et al., 1998; Gibson et al., 2001; Cheng et al., 2002). EDR1 functions as a negative regulator of disease resistance and ethylene-induced senescence (Frye et al., 2001; Tang et al., 2005). In our study, the *edr1* mutant (Frye et al., 2001) did not display sugar tolerant seedling growth (data not shown). More recently, a mutant designated as *at6* was found to exhibit improved salt tolerance and the *AT6* gene was shown to correspond to *At1g73660* (Gao and Xiang, 2008). Testing of multiple *at6* mutants showed that all of them exhibit a wild-type response to ACC, a precursor of ethylene, in the dark (Gao and Xiang, 2008). Thus, although *CTR1*, *EDR1* and *SIS8/AT6* are phylogenetically closely related, they appear to play distinct roles in different signaling pathways.

Contrary to the sugar insensitive phenotype of *sis8* mutants, plants overexpressing the *SIS8* gene exhibit a sugar-hypersensitive phenotype. When grown on media supplemented with 220 mM Suc or Glc, the *35S-SIS8* line exhibits significantly lower rates of cotyledon expansion and true leaf formation than wild-type plants grown on the same media. In contrast, when grown on media supplemented with an equi-molar concentration of sorbitol as an osmotic control, *35S-SIS8* plants exhibit wild-type seedling development. Together, these results suggest that SIS8 acts as a negative regulator of high Suc and Glc tolerance. Although loss-of-function mutations in *SIS8* can confer a slight degree of resistance to the inhibitory effects of very high (e.g. 400 mM) concentrations of sorbitol on early seedling development, however, the effects of mutations in *SIS8* on sugar response cannot be explained solely by alterations in response to high concentrations of osmoticum, as all of the *sis8* mutants tested exhibit a wild-type response to concentrations of sorbitol that are the same as those sufficient to reveal alterations in response to Glc and Suc.

The expression of *AT6/SIS8* is downregulated by salt treatment in seedlings (Gao and Xiang, 2008). As high concentrations of exogenous sugars exert inhibitory effects on seedling development only during a narrow temporal window, which is within 40 hours after the start of seed imbibition (Gibson et al., 2001), we tested whether the expression

of *SIS8* is regulated by high concentrations of exogenous Glc in germinating seeds and found that high Glc does not significantly affect *SIS8* transcript levels. However, it remains possible that high Glc regulates *SIS8* at translational or posttranslational levels. For example, Glc has been shown to promote the degradation of EIN3, a transcriptional regulator in ethylene signaling (Yanagisawa et al., 2003).

Several previously identified sugar-response mutants have been found also to exhibit defects in response to phytohormones, particularly, ethylene, ABA and GA, In a recent study, testing of multiple *at6/sis8* mutants revealed that all of them exhibit a wild-type response to ACC, a precursor of ethylene, in the dark (Gao and Xiang, 2008). In this study, the seed germination responses of *sis8* loss-of-function mutants to ABA and paclobutrazol (a GA synthesis inhibitor) were tested and found to be similar to those of wild-type seeds, which is consistent with the findings of Gao and Xiang (2008). However, transgenic plants overexpressing *SIS8* exhibit a slight, but statistically significant, hypersensitive seed germination response to low concentrations(1 µM) of ABA and to 30 µM paclobutrazol.

The observations that *sis8* mutants are tolerant to high sugars and hyperosmolarity and *35S-SIS8* transgenic plants are hypersensitive to moderate levels of sugars and osmotica, as well as to ABA and paclobutrazol, suggest that SIS8 plays a role in multiple metabolic stress responses. This model is consistent with findings from a number of systems. For example, the AMP-activated protein kinases (AMPK) have long been postulated to act in response to stresses that cause depletion of ATP levels in mammals. The yeast homolog of AMP-activated protein kinases, the SNF1 protein kinase, has been shown to be required for response to glucose starvation. These results indicate that AMPK/SNF1 may act to mediate environmental stresses that cause nutritional stresses (Corton et al., 1994; Hardie et al., 1994; Hardie and Carling, 1997; Hardie et al., 1998; Hardie, 1999). Similar results have been reported for SNF1-related protein kinase1s (SnRK1s), the plant homolog of AMPK/SNF1 (Halford and Hardie, 1998; Sugden et al., 1999; Halford et al., 2000; Halford et al., 2003; Baena-González et al., 2007). The Glc sensor, HXK1, has been postulated to act in both sugar and phytohormone response,

helping to integrate environmental response with plant growth and metabolic response (Moore et al., 2003).

To gain insight into the molecular mechanism by which SIS8 acts in sugar response, a yeast two-hybrid screen was conducted and six potential interacting partners were identified (Table 1). Interestingly, an *Arabidopsis* line (SALK_078702) carrying a T-DNA insertion in the gene encoding one of these putative SIS8-interacting proteins, At3g50740, displays a sugar tolerant seedling growth phenotype. The At3g50740 gene encodes an UDP-glucose:glycosyltransferase, UGT72E1, which is capable of glucosylation of lignin monomers (Lim et al., 2005). Alterations in the composition and properties of the plant cell wall have been linked to plant cellular growth and development and are capable of triggering signaling pathways (Pilling and Höfte, 2003). Furthermore, a recent study showed that loss-of-function mutations in the *HSR8/MUR4* gene, which is involved in arabinose synthesis, lead to a Glc-hypersensitive seedling development in the dark. Genetic analysis using *mur4-1 prl1* double mutant indicate that PRL1, which have previously been shown to be involved in plant sugar response, is required for the increased sugar responses seen in *hsr8/mur4* mutants (Li et al., 2007). Our findings that SIS8 interacts with UGT72E1 in yeast and that mutations in *SIS8* and *UGT72E1* cause sugar tolerant seedling growth phenotypes suggest that SIS8 may help link changes in the cell wall to cellular sugar response.

In summary, we have identified *SIS8*/*AT6*, which is predicted to encode a putative MAPKKK, as a negative regulator of high sugar tolerance during *Arabidopsis* early seedling development. Overexpression of *SIS8* causes hypersensitive seed germination response to ABA and paclobutrazol and mutations in *SIS8* also confer a slight degree of tolerance to high concentrations of osmoticum, suggesting that SIS8 may play a broad role in regulating metabolic stress response, as has been previously postulated for SnRK1s and HXK1. Although SIS8 is annotated as a MAPKKK based on primary sequence and domain structure, whether a MAPKK-MAPK cascade acts downstream of SIS8 remains to be tested. Further investigation of the SIS8 downstream targets will help elucidate the mechanism by which SIS8 functions.

Materials and methods

Plant materials, growth conditions and isolation of *sis8* mutants

A mutant population comprised of 60,000 M2 seeds derived from ethylmethane sulphonate (EMS)-mutagenized *Arabidopsis thaliana* var. Columbia was screened on 300 mM Suc. The *sis8-1* mutant was isolated based on its sugar tolerant seedling growth phenotype on media containing high levels of Suc. Seed segregating for the *sis8-2* (SALK_059205), *sis8-3* (SALK_001982) and *sis8-4* (SALK_004541) mutations were obtained from the ABRC in the Col-0 (CS6000) background. PCR was then used to screen for plants that are homozygous for the *sis8-2*, *sis8-3* and *sis8-4* mutations. The *sis8-1*, *sis8-2* and *sis8-4* seeds used for the physiological assays and real-time RT-PCR analyses were from mutant plants backcrossed to wild-type plants of the same ecotype three times, twice and once, respectively. Seeds of *spy-3* (Jacobsen and Olszewski, 1993), which is also in the Columbia background, were generously provided by Dr. Neil Olszewski. Wild-type Col-0 (CS6000) and Hi-O (CS6736) seeds were obtained from the ABRC at the Ohio State University. Seeds to be sown on plates were surface-sterilized and stratified for 3 d in the dark at 4°C. Typically 50-100 seeds were sown per plate. Plants were grown on Petri plates at 22°C under continuous white fluorescent light for an additional 12-14 d prior to scoring. Minimal *Arabidopsis* media was prepared as described (Kranz and Kirchheim, 1987).

Expression analysis by RT-PCR

For analysis of *SIS8* mRNA levels in wild-type Col-0, *sis8-1*, *sis8-2*, *sis8-3* and *sis8-4* plants, the Qiagen RNeasy plant mini kit (Qiagen, Valencia, CA) was used to isolate total RNA from the aerial parts of 4-week old plants grown in soil under continuous light conditions. For analysis of *SIS8* mRNA levels analysis in wild-type Col-0, *35S-SIS8* and different organs of wild-type Col-0, the Sigma Spectrum Plant Total RNA kit (Sigma, St. Louis, MO) was used to isolate total RNA from aerial parts of indicated organs of plants grown for 32 d on soil under continuous light. For analysis of *AT3G50740* mRNA levels, the Sigma Spectrum Plant Total RNA kit (Sigma, St. Louis,

MO) was used to isolate total RNA from two-week-old wild type and homozygous SALK_078702 seedlings grown under continuous light on Petri plates containing solid minimal Arabidopsis media supplemented with 270 mM Glc or with 270 mM sorbitol + 10 mM Suc as an osmotic control. After DNase I treatment (Promega, Madison, WI), first strand cDNA was synthesized from 1 μg total RNA using the Promega Improm-II RT system kit (Promega, Madison, WI) in a reaction volume of 20 μl. One μl of cDNA was used for PCR reactions. *ACTIN2* mRNA levels were used as an internal control. Primers used were: *SIS8* A 5'-TCAACGTGATAATGGCAAAGTT-3' and 5'-ATGGGGGAGGAGTCTGAGTT-3', *SIS8* B 5'-TCCCGACTTTGTAGATCCAG-3' and 5'-AAGAGGAACTGGGGACTGGT-3', *ACTIN2* 5'-TGCTGACCGTATGAGCAAAG-3' and 5'-GATTGATCCTCCGATCCAGA-3', *AT3G50740* 5'-CAGATGATGAACGCGACACT-3' and 5'- CCTGACACGCTCCAAAAGAT-3'.

For analysis of *SIS8* mRNA levels in response to Glc treatment, Col-0 wild type seeds were surface sterilized and stratified for 3 d. The seeds were then sown on nytex screens on *Arabidopsis* minimal media and incubated for 20 h under continuous light and then transferred to minimal media supplemented with 300 mM Glc or 300 mM sorbitol and incubated for an additional 12 h under continuous light before harvesting. The total time of treatment was within the temporal window during which high concentrations of exogenous sugars can arrest seedling development (Gibson et al., 2001). Seeds from two biologically independent batches were used to minimize any variation in gene expression that might be caused by any variations in plant growth conditions. Total RNA was isolated using the Spectrum™ Plant Total RNA Kit (Sigma, St. Louis, MO). First strand cDNA was synthesized with the Promega Improm-II RT system kit (Promega, Madison, WI) in a reaction volume of 20 μl. Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR Machine and the SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis, MO). *UBQ6* (AT2G47110) was used as an internal reference. Primers used were: *UBQ6*, 5'-CCATCGACAATGTCAAGGCC-3' and 5'-GGTACGTCCGTCTTCGAGCT-3'; *CAB4*, 5'-GCTAACGGACGATGGGCTAT-3' and 5'-CCAGCATCGTACCACTCAGGA-3'; *SIS8*, 5'- TCCCGACTTTGTAGATCCAG-3' and 5'- AAGAGGAACTGGGGACTGGT-3'.

Map-based cloning of the *SIS8* gene

To generate the *sis8-1* mapping population, F2 seedlings from a cross between *sis8-1* (which is in the Columbia background) and wild-type plants of the Hi-0 background were screened on media containing 300 or 320 mM Suc to identify plants that are homozygous for the *sis8-1* mutation. Genomic DNA was prepared from leaves of these seedlings using the alkaline lysis method (Klimyuk et al., 1993). Other mapping procedures were performed as described (Lukowitz et al., 2000; Jander et al., 2002). The molecular markers used to identify recombinants included simple sequence length polymorphisms (SSLPs), cleaved amplified polymorphic sequences (CAPS) and single nucleotide polymorphisms (SNPs) from the TAIR and Monsanto SNP and Ler sequence collections (Jander et al., 2002). These markers were tested for polymorphisms between Columbia and Hi-O wild-type plants prior to use in the mapping experiments.

Plasmid constructions and generation of transgenic *Arabidopsis thaliana*

All constructs were generated using the Gateway system (Invitrogen, Carlsbad, CA). The *SIS8* full length coding sequence (3,093 bp) was amplified by PCR, confirmed by sequencing to be free of mutations and then cloned into the pEarleyGate 100 destination vector (Earley et al., 2006). Transgene expression is driven by the constitutive CaMV 35S promoter in this vector. The resulting binary vector was then transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Transformation of wild-type Col-0 was performed using the floral dip method (Clough and Bent, 1998). T1 transgenic plants exhibiting resistance to media containing 7 μg/ml BASTA were further tested by PCR analysis and confirmed to contain the appropriate plant transformation construct. These T1 plants were then transferred to soil to obtain T2 seeds. T3 and T4 homozygous lines were used for sugar response tests, germination assays and gene expression analysis.

Sugar, abscisic acid and paclobutrazol assays

For sugar-response assays, 50-100 seeds were surface sterilized, stratified at 4°C in the dark for 3 d and then sown on minimal *Arabidopsis* media (Kranz and Kirchheim,

1987) supplemented with the indicated sugars and scored for seed germination, cotyledon expansion and true leaf formation as described (Laby et al., 2000). For ABA-response assays, seeds were sown on *Arabidopsis* minimal media supplemented with 0 or 1 μM ABA and incubated at 22°C under continuous light for 9 d prior to scoring for percent seed germination. Germination is defined as the emergence of the radicle from the seed coat. For paclobutrazol-response assays, seeds were sown on minimal media supplemented with 0 or 30 μM paclobutrazol and incubated at 22°C under continuous light for 9 d prior to scoring for percent seed germination. All experiments were performed a minimum of two times, with similar results each time.

Yeast two-hybrid experiments

The full length coding sequence of *SIS8* minus the stop codon (TGA) was amplified by PCR, verified by sequencing to be free of mutations and cloned into the pCR8/GW/TOPO vector (Invitrogen, Carlsbad, CA). The resulting construct was sent to the Molecular Interaction Facility at the University of Wisconsin-Madison where the yeast two-hybrid screen was performed. The *SIS8* gene was cloned into the pBUTE vector to produce a hybrid gene encoding a SIS8-GAL4 DNA binding domain (BD) fusion protein. The prey constructs were generated using the pGADT7-Rec vector, into which *Arabidopsis* cDNAs were inserted to create a library that produces fusion proteins consisting of random *Arabidopsis* proteins fused to the GAL4 activation domain (AD). The yeast strain PJ69-4A (Ura^-, His^-, Leu^-, Ade^-), which carries three chromosomally integrated reporter constructs (*GAL1-HIS3*, *GAL2-ADE2* and *GAL7-lacZ*), was used as the host (James et al., 1996). Screening of the *Arabidopsis* cDNA library in pGADT7-Rec with pBUTE-SIS8 as a bait resulted in identification of 14 genes that gave a positive interaction signal. Re-testing of these 14 genes revealed that six of them give a reproducible positive interaction signal in the presence of the construct encoding the SIS8-GAL4 DBD fusion protein, but not in the presence of the empty vector construct, which encodes only the GAL4 DBD. The plasmids containing these six genes were rescued into *E. coli* and sequenced to identify the *Arabidopsis* gene present on each

construct.. The 6 *E. coli* cultures containing the prey plasmids and the yeast strains containing pBUTE with or without the *SIS8* insert (numbered 340 and 341 respectively) were obtained from the facility and used in the validation assay conducted in our lab. Each prey plasmid was transformed into yeast strain 340 or 341 using the LiAc method (Gietz and Schiestl, 2007). Single transformants were picked and grown on SC-Leu-Ura-Ade plates to detect protein-protein interactions, which result in growth of white colonies. Red colonies form when no interaction occurs. β-galactosidase activity assays were done to quantify the relative strengths of the interactions between SIS8 and each prey (Ausubel et al., 2002).

Subcellular localization of SIS8-YFP fusion proteins in tobacco epidermal cells

The *SIS8* full length coding sequence lacking the stop codon was amplified by PCR, verified by sequencing to be free of mutations and cloned into the pEarleyGate 101 vector (Earley et al., 2006) to create the *35S::SIS3:YFP* construct. The *35S::SUC9:YFP* construct (in the pEarleyGate 101 vector) was obtained from Dr. John Ward. The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101. Tobacco plants (*Nicotiana benthamiana*) were grown for three weeks at 22°C with a light regime of 16 h light/8 h dark and a relative humidity of 75%. The agroinfiltration of the tobacco epidermal cells and transient expression of the SIS8-YFP protein were performed as described (Sparkes et al., 2006). Leaf epidermal tissues were examined for fluorescence using a Nikon C1 spectral imaging confocal microscope with a 514nm YFP filter 44 h after infiltration. Images were obtained with the accompanying EZ-C1 acquisition and analysis software.

Authors' contributions

YH identified the *SIS8/AT6* gene, performed most of the characterization of the *sis8* and *35S-SIS8* mutants and prepared the first draft of the manuscript. CL performed the quantitative real-time PCR analysis. YH and SP performed the SIS8 localization experiment. SG conceived of the study, assisted in its design and the interpretation of the data and aided in drafting the manuscript. All authors approved of the manuscript.

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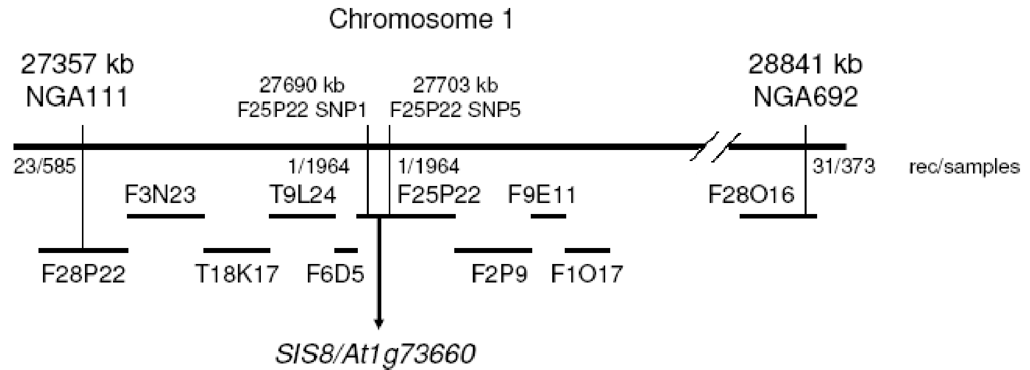
Figure 1A



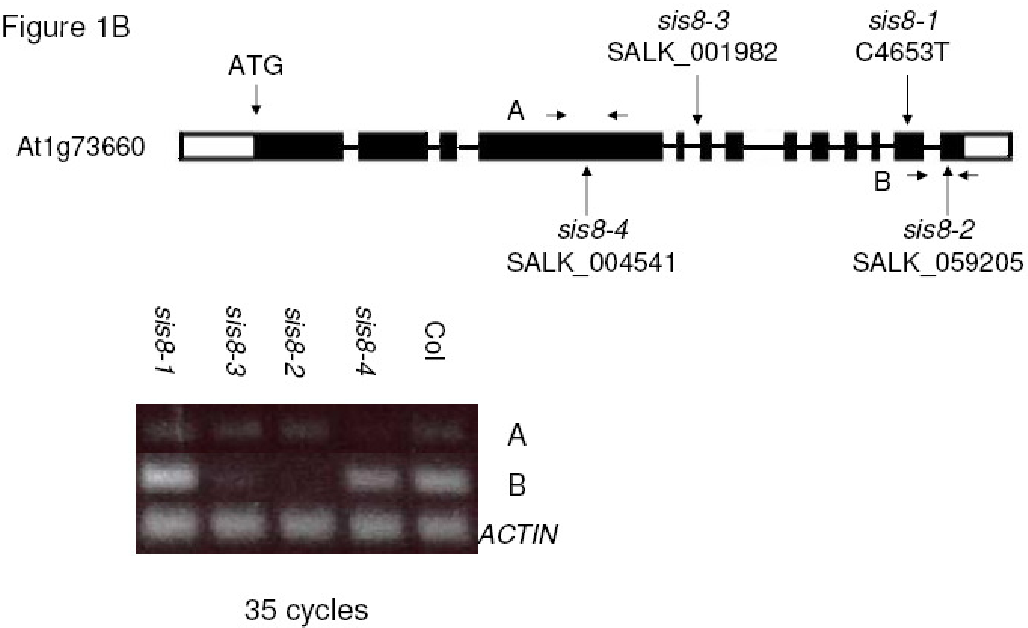
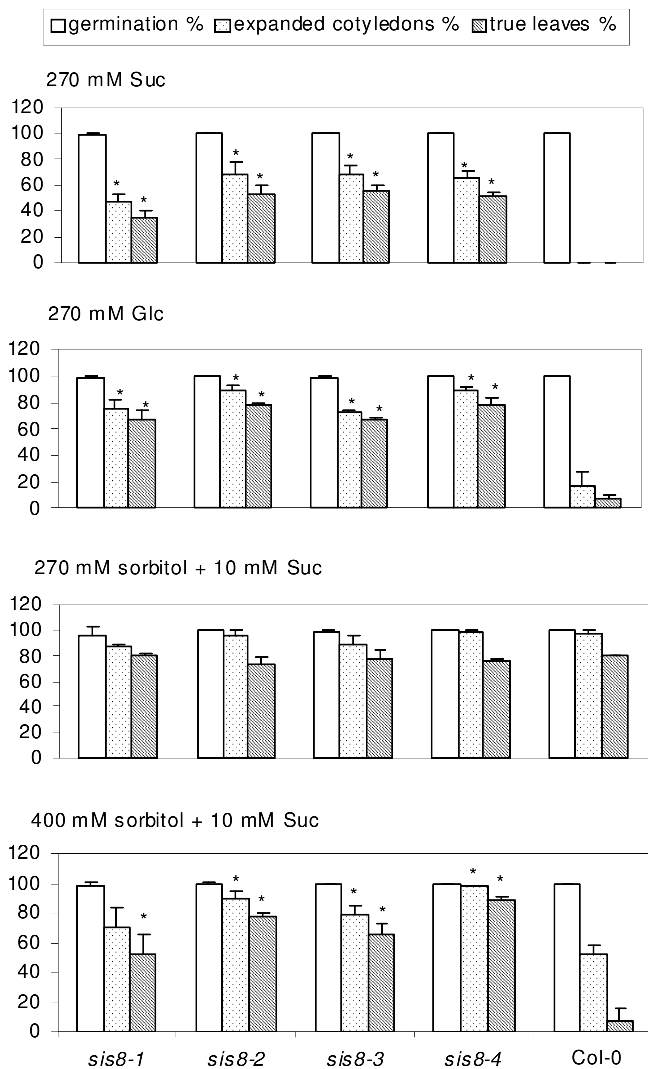
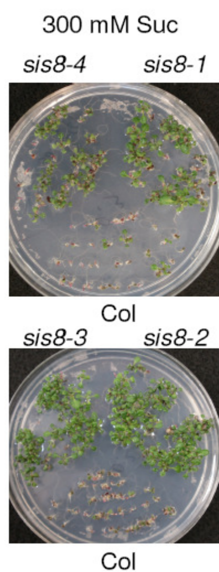


Figure 1. Positional cloning of *SIS8*. (A) Schematic representation of the physical map of chromosome 1 surrounding the *SIS8* region. The number of recombinants found in 1,964 DNA samples is shown. *SIS8* is located between 27,690 kb and 27,703 kb on BAC F25P22.

(B) Schematic depiction of the *SIS8* genomic DNA sequence displaying the positions of the T-DNA insertions. Boxes indicate exons. Lines indicate introns. Arrows indicate positions of primers. Letters represent primer pairs used for the RT-PCR reactions. Total RNA was extracted from aerial parts of 4-week-old plants. Col refers to wild-type plants of the Columbia background. *ACTIN2* was used as a control.

Figure 2A





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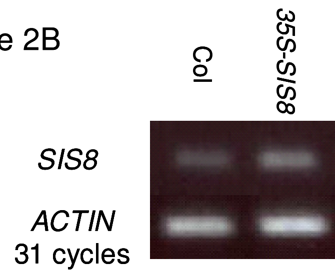

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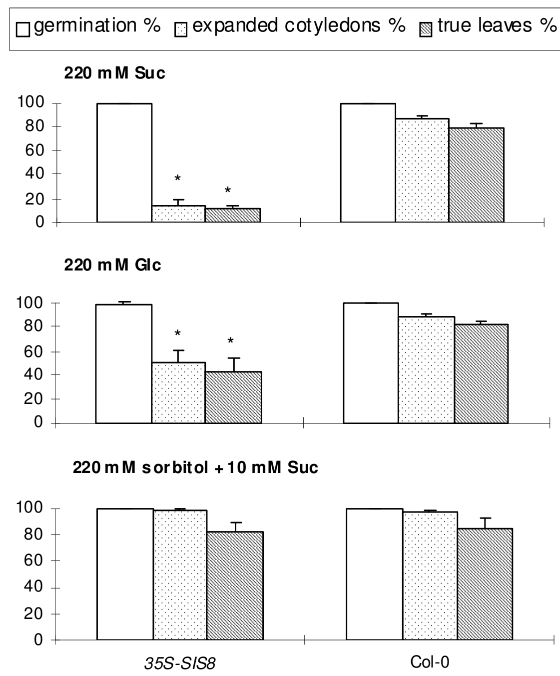

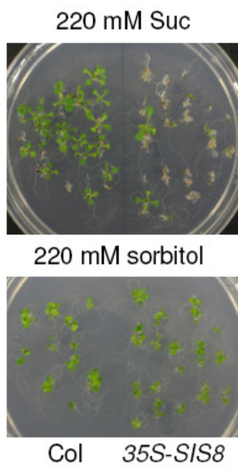

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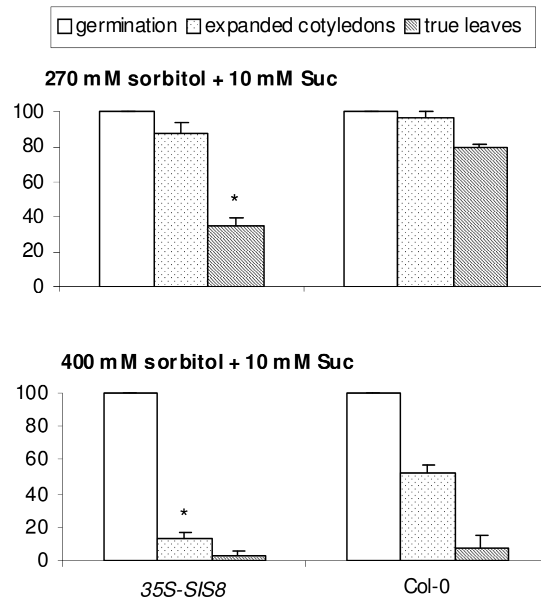


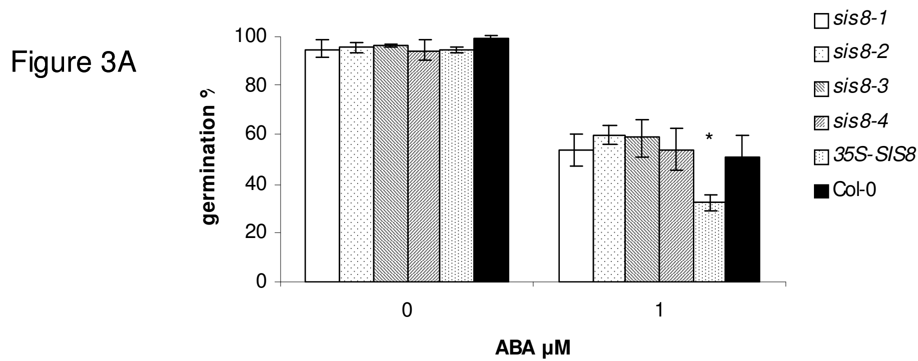
Figure 2. *SIS8* mediates sugar response during early seedling development.

(A) Wild-type, *sis8-1*, *sis8-2*, *sis8-3* and *sis8-4* seeds were sown on minimal Arabidopsis media supplemented with the indicated sugars and then grown under continuous light at 22°C for two weeks prior to scoring. Mutant seedlings are significantly more tolerant than wild-type seedlings to 270 mM Suc and Glc. Media supplemented with 270 mM sorbitol was included as an equi-molar osmotic control. When grown on control media, no statistically significant differences were found between the mutant and wild-type seedlings for percentages of seed germination, cotyledon expansion or true leaf formation. In contrast, all four *sis8* mutants exhibit significant resistance to 400 mM sorbitol compared to the wild type. Data represent the mean of three independent assays ± SD. Asterisks indicate results where the phenotype of a *sis8* mutant differed from that of the wild type by $p<0.05$. The inset shows the seedling growth phenotypes of *sis8* mutants and wild-type Col on 300 mM Suc after 12 days.

(B) Transcript levels of the SIS8 overexpression line (*35S-SIS8*). Total RNA was extracted from aerial parts of 32-day old plants grown on soil under continuous light. RT-PCR was performed with *SIS8*-specific or *ACTIN2*-specific primers.

(C) Seedlings overexpressing *SIS8* in the Col-0 background exhibit a hypersensitive response to 220 mM Suc or Glc, but not to equi-molar concentrations of sorbitol. Media supplemented with equi-molar sorbitol was included as an osmotic control. Data represent the mean of three independent assays ± SD. Asterisks indicate results where the phenotype of the *35S-SIS8* transgenic plants differed from that of the wild type by $p<0.05$. The inset shows the seedling growth phenotypes of the *SIS8* overexpression line and wild-type Col seedlings on 220 mM Suc or sorbitol after 12 days.

(D) Seedlings overexpressing *SIS8* in the Col-0 background exhibit a hypersensitive response to 270 and 400 mM sorbitol compared to the wild type. Although seedlings overexpressing *SIS8* are not hypersensitive to 220 mM sorbitol, they do exhibit statistically significant increases in the percentage of seedlings that form true leaves on 270 mM sorbitol and in the percentage of seedlings that form expanded cotyledons on 400 mM sorbitol. Data represent the mean of three independent assays ± SD. Asterisks indicate results where the phenotype of *35S-SIS8* differed from that of the wild type by $p<0.05$.



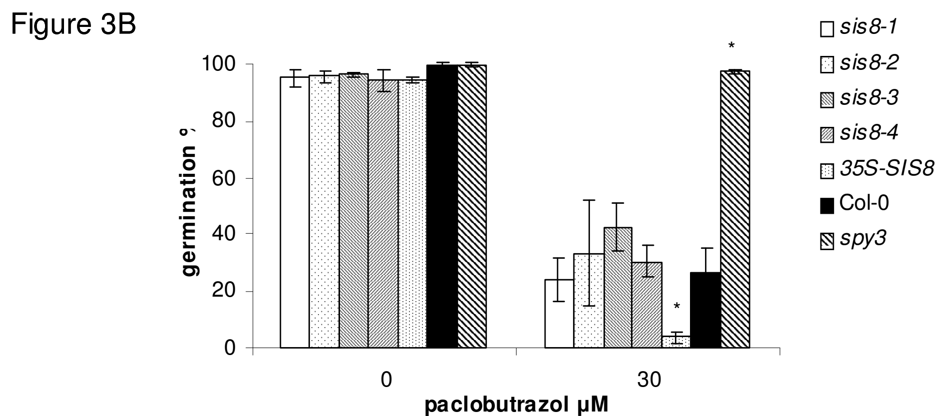


Figure 3. Germination response of *sis8* mutants and overexpression line to ABA and GA.

(A) Seeds of *sis8-1*, *sis8-2*, *sis8-3* and *sis8-4* plants exhibit wild-type seed germination responses on media containing 1 µM ABA, whereas *35S-SIS8* seeds exhibit a hypersensitive response. Data represent the mean of three independent assays ± SD. The asterisk indicates that the phenotype of *35S-SIS8 seeds* differs from that of the wild type by $p<0.05$.

(B) Seeds of *sis8-1*, *sis8-2*, *sis8-3* and *sis8-4* plants exhibit wild-type seed germination responses on media containing 30 µM paclobutrazol, whereas *35S-SIS8* seeds exhibit a hypersensitive response. The *spy3* (Jacobsen and Olszewski, 1993) seeds were used as a positive control. Data represent the mean of three independent assays ± SD. Asterisks indicate that the phenotypes of the *35S-SIS8* and *spy3* mutants differ from that of the wild type by $p<0.05$.

Figure 4A

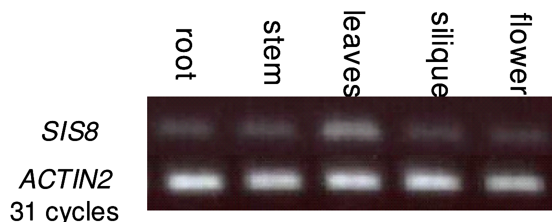


Figure 4B

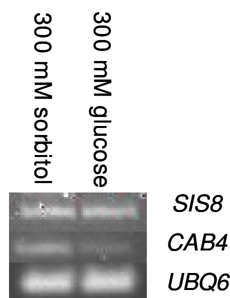


Figure 4C

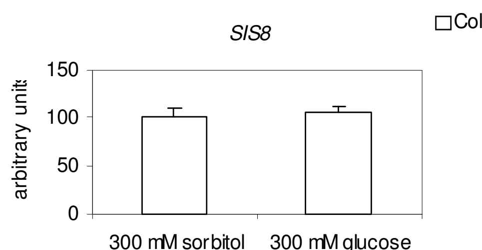


Figure 4. *SIS8* expression analyses. (A) Organ-specific expression of the *SIS8* gene. Total RNA was extracted from aerial parts of indicated organs of 32-day old plants grown on soil under continuous light. RT-PCR was performed with *SIS8*-specific or *ACTIN2*-specific primers.

(B) Expression of *SIS8* in germinating seeds treated with 300 mM Glc or sorbitol (osmotic control). RT-PCR analysis was conducted as described in Materials and methods. The Glc-repressible *CAB4* gene used as a positive control for high Glc treatment. *UBQ6* was used as an internal control.

(C) Quantitative real-time PCR analysis of *SIS8* transcript levels in germinating seeds. Data were obtained from two biologically independent experiments and relative mRNA levels were determined by using *UBQ6* as a reference. The relative level of *SIS8* mRNA in sorbitol-treated wild-type Col was set to 100. Data indicate means ± SD.

Figure 5A

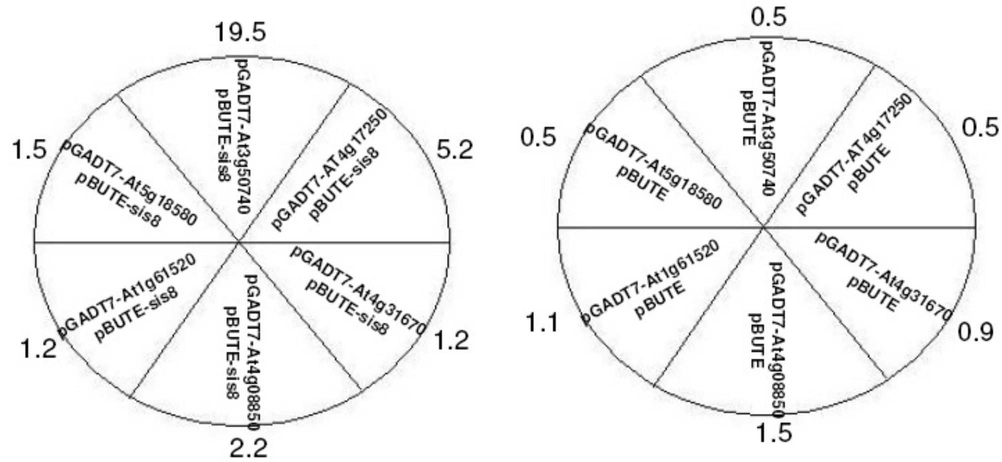


Figure 5B

Figure 5. Identification of putative SIS8-interacting proteins.

(A) The yeast two hybrid (Y2H) bait and prey constructs transformed into yeast strain PJ69-4A are indicated in each sector. The numbers outside of the circle represent the levels of β-galactosidase activity produced by each plasmid combination. pGADT7 is the prey vector, which carries the GAL4 activation domain, and pBUTE is the bait vector, which carries the GAL4 DNA-binding domain.

(B) Effects of each bait-prey plasmid combination on yeast colony growth on SC-Leu-Ura-Ade plates. White colonies indicate an interaction between the two fusion proteins produced by the bait and prey constructs, whereas red colonies indicate no detectable interaction.

Figure 6A

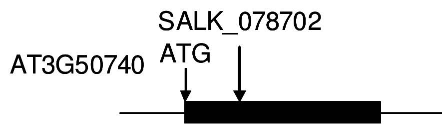


Figure 6C

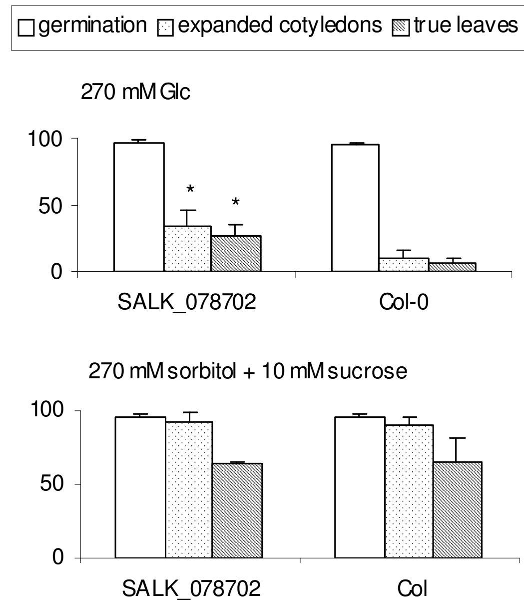


Figure 6B

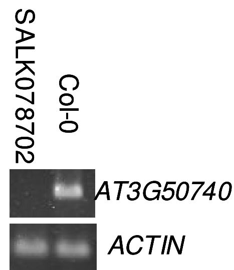


Figure 6. A mutation in a gene encoding a putative SIS8-interacting protein confers a *sugar-insensitive* (*sis*) phenotype

(A) Gene structure of At3g50740 and relative position of the T-DNA insert present in *Arabidopsis* line SALK_078702. Box indicates exon.

(B) *At3g50740* transcript levels in wild-type Col-0 and homozygous SALK_078702 analyzed by RT-PCR from aerial parts of 2-week-old soil grown seedlings . *ACTIN2* was used as a control.

(C) Statistical analysis of seedling development of wild-type Col-0 and SALK_078702 on 270 mM Glc media. Media supplemented with an equi-molar concentration of sorbitol was included as an osmotic control. Data represent the means of three independent assays ± SD. Asterisks indicate results where the phenotype of SALK_078702 differed from that of the wild type by $p<0.05$.

Figure 7A

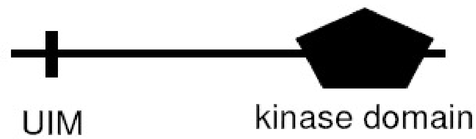


Figure 7B

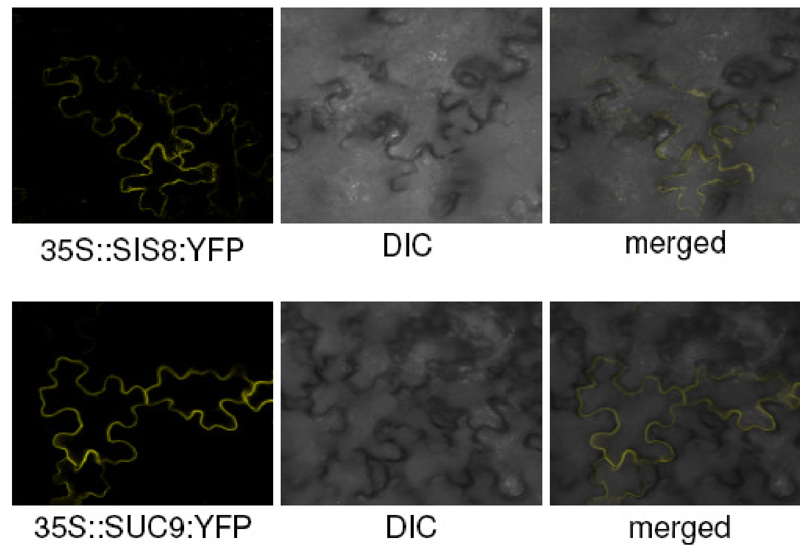


Figure 7. Subcellular localization of the SIS8 protein

(A) Schematic depiction of the SIS8 protein domains predicted by the SMART domain architectures prediction program (Schultz et al., 1998). UIM, ubiquitin interaction motif. (B) Cytosolic localization of SIS8-YFP in tobacco leaf epidermal cells. Tobacco leaf cells were inoculated with *Agrobacterium tumefaciens* carrying a plant transformation vector expressing a SIS8-YFP fusion protein under the control of the constitutive CaMV 35S promoter. Fluorescence in cells was examined using a confocal microscope 44 hours after infiltration of the leaves. Plasma membrane localized SUC9-YFP (Sivitz et al., 2007) was used as a control. DIC, differential interference contrast.

Table 1. Putative SIS8-interacting proteins identified using a yeast two-hybrid screen.

AGI	Gene product
At3g50740	UGT72E1, UDP-glycosyltransferase
At4g17250	Unknown protein
At4g31670	ubiquitin carboxyl-terminal hydrolase family protein
At4g08850	leucine-rich repeat family protein / protein kinase family protein
At1g61520	LHCA3, chlorophyll a/b-binding protein
At5g18580	FASS/TON2, protein phosphatase 2A regulatory subunit

Chapter V

Conclusions and future directions

V.1. Sugar response, ABA biosynthesis and lateral root development-*SIS7/NCED3/STO1*

The *sis7-1* and *sis7-3* mutants were isolated from an Arabidopsis population that was mutagenized by the introduction of T-DNAs carrying random *Arabidopsis* cDNAs under the control of the constitutive cauliflower mosaic virus 35S promoter (LeClere and Bartel, 2001). The *sis7-2* mutant was isolated from ethylmethane sulphonate (EMS)-mutagenized seeds. All three *sis7* mutant alleles are in the Columbia (Col) background and exhibit resistance to high levels of exogenous sugars (e.g. 300 mM) by developing green expanded cotyledons and true leaves, whereas the wild-type Col plants show arrested growth with white cotyledons and no leaf formation on the same media. The *SIS7* gene was identified by positional cloning and found to be allelic to the *NCED3/STO1* (At3g14440) gene, which encodes 9-cis-epoxycarotenoid dioxygenase (NCED), a key enzyme in ABA biosynthesis. Mutations in the *NCED3* gene have been reported to cause hypersensitivity to drought and enhanced germination on salt and nonionic sorbitol media (Iuchi et al., 2001; Ruggiero et al., 2004).

The finding that the *sis7/nced3/sto1* mutants have decreased sensitivity to high sugar-induced seedling growth arrest is consistent with previous reports that ABA-deficient mutants have *gin* (glucose insensitive) or *sis* (sugar insensitive) phenotypes (Arenas-Huertero et al., 2000; Laby et al., 2000; Cheng et al., 2002). High sugar levels have been show to increase endogenous ABA levels in seedlings and germinating seeds and to induce expression of ABA biosynthetic genes, such as *ABA1*, *ABA2*, *ABA3* and *AAO3* but not *NCED3*, in seedlings (Arenas-Huertero et al., 2000; Cheng et al., 2002; Price et al., 2003). It has been observed that the sensitivity of wild-type seeds to the inhibitory effect of high sugar levels on seedling development is defined by a narrow time window, which is within approximately 2 days after stratification (Gibson et al., 2001). Thus, it is important to study the effects of high sugar levels on regulation of ABA biosynthetic genes in germinating seeds during this time window. Quantitative real-time PCR (qRT-PCR) analysis has been performed to study the expression of ABA biosynthesis genes in the wild-type, *sis7-1* and *sis7-3* germinating seeds. The stratified seeds were germinated on minimal media for 20 hours and transferred to 300 mM

glucose or sorbitol media for another 13 hours before harvest for gene expression analysis. The transcript levels of *ABA1*, *SIS7/NCED3*, *ABA2* and *ABA3* were higher in wild-type seeds germinated on media containing glucose than on media supplemented with an equimolar concentration of sorbitol, but remained the same in the *sis7-1* and *sis7-3* seeds germinated in the presence of either glucose or sorbitol. These results indicate that the induction of ABA biosynthetic genes by high concentrations of glucose requires a certain level of endogenous ABA in germinating seeds, and that the ABA deficiency in the *sis7/nced3* mutants arises from reduced expression of other ABA biosynthetic genes in addition to the primary lesion in *SIS7/NCED3*.

Transcriptional profiling analyses were conducted to examine SIS7/NCED3-mediated signaling in Col wild-type and *sis7-1* germinating seeds treated with 100 mM glucose. 20 genes are expressed at higher levels in *sis7-1* than in wild-type seeds, suggesting that SIS7 negatively affects the transcript levels of these genes. 62 genes are expressed at lower levels in *sis7-1* than in wild-type seeds, suggesting SIS7 positively affects the transcript levels of these genes. Over-representation analysis has revealed that a set of genes associated with auxin metabolism or transport are over-represented among the genes with lower transcript levels in *sis7-1*, including *PIN1*, *PIN3*, *CYP83B1*, *CYP79B2* and *AT4G27260* (an IAA-amido synthase), which has been verified with qRT-PCR analysis. The lateral root (LR) development of the *sis7-1* mutants showed a reduced response to the inhibitory effects of osmotica (e.g. sorbitol), consistent with a previous report that the ABA deficient mutants *aba2-1* and *aba3-1* have decreased responses to mannitol (Deak and Malamy, 2005). Although auxin has long been known to regulate LR development, the role of ABA in controlling LR development has emerged only recently and appears to exhibit cross-talk with auxin, ethylene and sugars (Brady et al., 2003; De Smet et al., 2003; De Smet et al., 2006). Our finding that the transcripts of several auxin-related genes are expressed at lower levels in the *sis7/nced3* mutant seeds incubated with glucose, together with the observation that the *abi3*, *aba2-1* and *aba3-1* mutants have reduced responses to polar auxin transport inhibitors (Brady et al., 2003; Deak and Malamy, 2005), indicate that these auxin-related genes might be part of the links for the

ABA, auxin and sugar response pathways in controlling LR development. Further experiments are needed to dissect the molecular mechanism of these interactions.

V.2. A RING E3 ligase-SIS3

The *sis3-1* mutant was isolated from a T-DNA insertional library of *Arabidopsis* ecotype Wassilewskija (Ws-2), but the T-DNA insertion does not show close linkage with the *sis* phenotype. Map-based cloning of *sis3-1* revealed a deletion that spans three genes. Based on seedling sugar response assays of additional T-DNA insertional lines from ABRC and a complementation line, the *SIS3* gene was identified as *At3g47990.* This gene encodes a RING motif protein.

The *sis3-2* and *sis3-3* mutants exhibit wild-type germination responses on media containing ABA and paclobutrazol, a GA biosynthesis inhibitor. In contrast, when assayed for root elongation on media containing 0.5 μM ABA, both mutants show reduced sensitivity to ABA repression of primary root growth. However, this reduction in ABA sensitivity is slight, as it disappears when the mutants are grown on 1 μM ABA. The mechanism by which this decreased response to the inhibitory effects of ABA on root elongation occurs is unknown. It can be speculated that the effect of *SIS3* mutation on ABA-mediated root growth response might be indirect. Firstly, the *sis3* mutants have a normal response to ABA-mediated inhibition of seed germination. Secondly, cross-talk between the ABA response pathway and other response pathways, such as that of ethylene, has been shown to regulate root growth. For example, the ethylene insensitive mutants *ein2-44* and *ein3-1* have reduced sensitivity to ABA-mediated repression of root elongation (Ghassemian et al., 2000).

The RING finger proteins have been shown to function as ubiquitin protein ligases (E3) in the ubiquitin/26S proteasome pathway. Genes encoding a total of 469 predicted RING finger ubiquitin ligases have been identified in the *Arabidopsis* genome, but few have been assigned functions in specific biological processes (Stone et al., 2005). For example, the KEEP ON GOING (KEG) RING E3 ligase has been shown to maintain low levels of ABI5 in the absence of stress and is essential for postgerminative growth and development. Light-grown *keg* seedlings are hypersensitive to 4% glucose and their

root growth is hypersensitive to exogenous ABA (Stone et al., 2006). The E3 ligase activity of SIS3 has been tested by *in vitro* ubiquitination assays. Both the full length SIS3 and the RING finger motif show E2-dependent polyubiquitination.

The transcript levels of *SIS3* are not significantly regulated by high glucose levels in germinating seeds, indicating high sugar levels may control SIS3 activity at post-transcriptional levels. Although SIS3 target proteins have not yet been identified, it can be hypothesized that SIS3 is a negative regulator of a function that promotes early seedling establishment in the presence of high sugar levels. Another interesting aspect of the SIS3 protein is that it is predicted to be membrane localized. However, in a preliminary study using transient expression of a *35S::SIS3:YFP* construct in tobacco leaves, the subcellular localization of SIS3 appeared to be to the cytoplasm and nucleus. However, this result needs to be repeated with proper controls and substantiated with cell fractionation assays before any conclusions can be drawn.

V.3. A putative MAPK kinase kinase-SIS8

M2 seeds derived from an EMS-mutagenized *Arabidopsis thaliana* ecotype Columbia population were screened to identify seedlings that are resistant to 300 mM Suc. One of the mutants, *sis8-1*, is tolerant to high sugar-mediated inhibition of young seedling establishment. Map-based cloning revealed that the *sis8* locus corresponds to At1g73660. Three independent *Arabidopsis* lines carrying T-DNA insertions in At1g73660 show the same *sis* phenotypes as that of *sis8-1*, confirming that At1g73660 corresponds to the *SIS8* gene. *SIS8* encodes a putative plant Raf-related mitogen-activated protein kinase (MAPK) kinase kinase with an extended N-terminal domain and a central alanine-rich region (Ichimura et al., 2002). It has recently been shown that *At1g73660* negatively regulates salt tolerance in *Arabidopsis* (Gao and Xiang, 2008).

Besides being tolerant to high levels of exogenous sugars, all *sis8* mutant alleles display hyperosmolarity tolerance. Similarly, overexpression of *SIS8* in the wild-type background causes hypersensitivity to both 220 mM sugars and 270 mM sorbitol. In germination response assays on ABA and paclobutrazol, the *sis8* mutants demonstrate similar germination percentages as that of wild-type seeds, which is consistent with the

observations by Gao and Xiang (2008). However, a *SIS8* overexpression line exhibits increased sensitivity to both ABA and paclobutrazol, as seeds of this overexpression line germinate at significantly lower percentages than wild-type seeds. Although *At1g73660* is phylogenetically closely related to *CTR1*, a negative regulator of ethylene-inducible genes, mutations in *At1g73660* do not alter the sensitivity to ACC, the ethylene precursor (Gao and Xiang, 2008).

SIS8 transcripts were detected among various organs of *Arabidopsis* including roots, stems, leaves, siliques and flowers. RT-PCR analysis suggests that the transcript levels of *SIS8* were not significantly regulated by high Glc levels in germinating seeds. However, salt stress has been shown to down-regulate *At1g73660* expression in wild-type seedlings (Gao and Xiang, 2008). These results indicate that the regulation of *At1g73660* may be at different molecular levels and/or depends on developmental stage.

A number of putative SIS8-interacting partners have been identified through a yeast two-hybrid screen. These candidates include proteins involved in lignin metabolism, photosynthesis, ubiquitin removal, protein phosphorylation and dephosphorylation. Interestingly, an Arabidopsis line carrying a T-DNA insertion in the gene, *UGT72E1*, encoding one of the putative SIS8-interacting proteins displays a sugar tolerant seedling growth phenotype in the presence of 270 mM glucose. The *UGT72E1*gene is involved in glucosylating lignin monomers such as coniferyl aldehyde and sinapyl aldehyde (Lim et al., 2005). Cells have the ability to perceive changes in cell wall composition and to integrate these with cellular signaling pathways (Pilling and Höfte, 2003). Remarkably, two recently identified and characterized mutants defective in proper sugar signaling, *high sugar response8* (*hsr8/mur4*) and *oversensitive to sugar1* (*osu1/qua2/tsd2*), have been shown to have defects in cell wall composition and/or structure as well, suggesting links from cell wall modifications to altered sugar-responsive gene expression, growth and development (Li et al., 2007; Gao et al., 2008). The findings that SIS8 interacts with UGT72E1 in yeast and that mutations in the *SIS8* and *UGT72E1* genes cause sugar tolerant seedling growth further corroborate the above mentioned concept and suggest that SIS8 is involved in connecting changes in the cell wall with cellular sugar response.

Phylogenetic analysis of MAPKKKs reveals that SIS8 is closely related to two other Raf proteins, CTR1 and EDR1, that function as negative regulators of ethylene signaling and defense response, respectively (Kieber et al., 1993; Frye et al., 2001; Champion et al., 2004). Recently, downstream components of CTR1 have been identified as MKK9-MPK3/MPK6. Ethylene inactivates the negative regulator CTR1 to activate the positive MKK9-MPK3/MPK6 cascade to promote EIN3-mediated ethylene signaling (Yoo et al., 2008). Gao and Xiang (2008) hypothesize that At1g73660 might serve as a negative regulator of salt tolerance. The *sis* phenotype of *sis8* and sugar hypersensitivity of the *SIS8* overexpressor line suggest that SIS8 is activated by high sugar levels and plays a negative role in seedling development in the presence of high sugar levels. Whether a MAPKK-MAPK cascade operates downstream of SIS8 is open to debate. T-DNA insertional lines of *MAPKK*s have been obtained from the ABRC and further experiments testing their seedling growth in response to sugars may yield insight into the above question.

V.4. Summary

The cloning and characterization of the *sis7*, *sis3* and *sis8* mutants have revealed both old friends and new players in the sugar-signaling pathways. The findings that a RING E3 ligase and a putative MAPKKK function in high sugar-mediated seedling developmental arrest are intriguing and invite further studies to elucidate the molecular mechanisms of their functions. Genetic and biochemical approaches, such as double mutant analysis and co-immunoprecipitation assays, will be particularly useful in integrating *SIS3* and *SIS8* into the sugar signaling network.

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