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SIS8, a putative mitogen-activated protein kinase kinase kinase, regulates sugar-resistant seedling development in Arabidopsis

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SUMMARY

Sugar signaling pathways have been evolutionarily conserved among eukaryotes and are postulated to help 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 insensitive8* (*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; At1g73660). *SIS8* mRNA is expressed ubiquitously among Arabidopsis organs. A UDP-glucosyltransferase, UGT72E1 (At3g50740), was identified as an interacting partner of SIS8 based on a yeast two-hybrid screen and *in planta* bimolecular fluorescence complementation. Both SIS8–yellow fluorescent protein (YFP) and UGT72E1–YFP fusion proteins localize to the nucleus when transiently expressed in tobacco leaf cells. T-DNA insertions in At3g50740 cause a sugar-insensitive phenotype. These results indicate that SIS8, a putative MAPKKK, is a regulator of sugar response in Arabidopsis and interacts with a UDP-glucosyltransferase in the nucleus.

Keywords: sugar response, sugar signaling, glucose response, sucrose response, MAP3K, SIS8, glucosyltransferase, UGT72E1, *Arabidopsis thaliana*.

INTRODUCTION

Sugars, such as sucrose (Suc) or glucose (Glc), function not only as substances providing energy and building materials to plant cells, but also act like signaling molecules to modulate gene expression. An abundance of sugar induces the expression of genes for storage and utilization and represses the expression of genes for photosynthesis and reserve mobilization, whereas sugar depletion has the opposite effects (Koch, 1996; Roitsch, 1999). The responses of plants 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 responses to light, nitrogen and phytohormones (Koch, 1996; Smeekens, 2000; Coruzzi and Zhou, 2001; Gibson, 2005; Rolland *et al.*, 2006; Sheen, 2010).

The mechanisms underlying sugar sensing in plants have been the focus of increasing numbers of studies. Multiple signal transduction pathways and some of their components have been identified. Studies on Arabidopsis HEXOKINASE1 (HXK1) have suggested that it acts as a Glc sensor (Jang *et al.*, 1997; Moore *et al.*, 2003; Cho *et al.*, 2006; Granot *et al.*, 2013). A cell surface D-Glc receptor function has been suggested for the Arabidopsis heterotrimeric G-protein α subunit (GPA1) and the regulator of G-protein signaling (RGS) proteins (Chen *et al.*, 2003, 2006; Chen and Jones, 2004; Grigston *et al.*, 2008). It has also 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; Jossier *et al.*, 2009; Delatte *et al.*,

2011). Mutations in *PLEIOTROPIC REGULATORY LOCUS1* (*PRL1*), which encodes a nuclear WD protein, confer a sugar-hypersensitive phenotype and cause transcriptional derepression of Glc-responsive genes. Interestingly, PRL1 has been shown to inhibit AKIN10 and AKIN11 (Arabidopsis SnRK1s) activity *in vitro* and *in vivo* (Németh *et al.*, 1998; Lee *et al.*, 2008). TARGET OF RAPAMYCIN (TOR) has been implicated in the regulation of carbon and nitrogen metabolism in plants and overall plant growth (Ren *et al.*, 2012; Dobrenel *et al.*, 2013), and also plays an important role in linking Glc derived from photosynthesis with meristem function (Xiong *et al.*, 2013).

One aspect of plant development regulated by sugars is early seedling development. Exposure to high levels of exogenous sugars (e.g. 0.3 M) during approximately the first 40 h after the start of imbibition prevents the majority of Arabidopsis Columbia (Col-0) wild-type seedlings from developing green expanded cotyledons and true leaves (Laby *et al.*, 2000). Genetic screens for mutants resistant or hypersensitive to the inhibitory effects of high sugar levels on seedling development have identified several genes involved in sugar response and have also revealed extensive interactions between abscisic acid (ABA), ethylene and sugar signaling pathways (reviewed in Gibson, 2005; Sheen, 2010; Eveland and Jackson, 2012). For example, alleles of *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*), a Raf-like mitogen-activated protein kinase kinase kinase (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 (Rodriguez *et al.*, 2010). A minimal MAPK pathway is composed of three kinases, a MAPK, a MAPK kinase (MAPKK) and a MAPKKK. Sequential phosphorylation of a MAPKK and a MAPK connects upstream receptors/signals to downstream targets. The Arabidopsis genome is predicted to encode 20–23 MAPKs, 10 MAPKKs and 60–80 MAPKKKs. The MAPKKKs can be divided into two main classes, the MEKKs and Raf-like MAPKKKs (Ichimura *et al.*, 2002; Jonak *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; Kim *et al.*, 2012). Two Arabidopsis Raf-like MAPKKKs, CTR1 (Kieber *et al.*, 1993) and ENHANCED DISEASE RESISTENCE1 (EDR1) (Frye and Innes, 1998), are negative regulators of ethylene response and disease resistance, respectively. Mutations in *CTR1* also cause defects in sugar response (Zhou *et al.*, 1998; Gibson *et al.*, 2001; Rolland *et al.*, 2002). Recently an Arabidopsis mutant, *at6*, was isolated based on its improved salt tolerance and the responsible locus was identified as *At1g73660* (Gao and Xiang, 2008). *At1g73660* encodes a predicted Raf-like MAPKKK that is closely related to CTR1 and EDR1.

In this study, the *SUGAR INSENSITIVE8* (*SIS8*) gene was isolated by map-based cloning and found to correspond to At1g73660, a putative MAPKKK. A glucosyltransferase, UGT72E1 (At3g50740), was found to interact with SIS8 through yeast two-hybrid (Y2H) screening and bimolecular fluorescence complementation (BiFC) in the nuclei of tobacco leaf cells. In addition, mutations in *UGT72E1* confer a *sugar insensitive* phenotype. Taken together, our results indicate that SIS8 is a regulator of seedling resistance to high sugar in Arabidopsis and interacts with a UDP-glucosyltransferase.

RESULTS

Isolation of the *sis8-1* mutant

High levels of exogenous Suc or Glc (e.g. 0.3 M) prevent the majority of wild-type Arabidopsis seedlings from developing green expanded cotyledons and true leaves (Zhou *et al.*, 1998; Arenas-Huertero *et al.*, 2000; Huijser *et al.*, 2000; Laby *et al.*, 2000). In contrast, equimolar concentrations of sorbitol (a non-metabolizable sugar analog in Arabidopsis) do not exert this effect, indicating 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 sugar levels to inhibit early seedling development has been used to identify mutations that alter seedling sensitivity to sugars (Zhou *et al.*, 1998; Arenas-Huertero *et al.*, 2000; Huijser *et al.*, 2000; Laby *et al.*, 2000; Gibson *et al.*, 2001; Huang *et al.*, 2008, 2010). In one such screen performed by our lab, approximately 60 000 M2 seeds derived from an ethyl methanesulfonate (EMS)-mutagenized *Arabidopsis thaliana* var. Col-0 population were screened to identify seedlings that are resistant to 0.3 M 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 *sis8-1* mutant, is described below.

Genetic characterization of the *sis8-1* mutant and positional cloning of the *SIS8* gene

The *sis8-1* mutant, which is in the Col-0 background, was backcrossed to the Col-0 wild type. The resulting F_1 seedlings exhibit a wild-type response to 0.3 M Suc, indicating that the mutant's *sugar insensitive* phenotype is recessive. To initiate map-based cloning of *SIS8*, *sis8-1* was crossed

to wild-type plants of the Hi-O background. F_2 progeny of this cross were screened on media containing 0.3 or 0.32 M Suc. Those seedlings that displayed a *sis* mutant phenotype were used to form a mapping population of 1964 plants. Fine-scale mapping localized *SIS8* to a 13 kb region on bacterial artificial chromosome (BAC) clone F25P22 that contains four genes. 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 4653 (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 salt tolerance (Gao and Xiang, 2008). The *sis8-1* mutation causes an amino acid change of proline to serine (Pro→Ser) at position 968 in exon 12, which lies within the predicted kinase domain.

To test whether *At1g73660* is the *SIS8* gene, three independent lines carrying T-DNA insertions in At1g73660 (Figure 1a) were obtained from the Arabidopsis Biological Resource Center (ABRC). Quantitative RT-PCR (qRT-PCR) analyses revealed that each of these T-DNA insertions disrupts production of full-length *SIS8* mRNA (Figure 1b). 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). Testing revealed that all of these mutants display significant resistance to high concentrations of Suc and Glc. In contrast, *sis8* and wild-type seedlings exhibit similar morphologies when grown on media containing equimolar concentrations of sorbitol (Figure 2a and Figure S1 in Supporting Information). A complementation test was performed to test further whether *At1g73660* corresponds to the *SIS8* gene. A 5.9-kb genomic DNA fragment from *At1g73660*, including the predicted 1.5-kb promoter, was cloned into the Gateway destination vector pEarleyGate302 (Earley *et al.*, 2006) and used to transform *sis8-1* via the floral dip method (Clough and Bent, 1998). Seeds from a T_3 homozygous transgenic line were used in qRT-PCR and sugar response assays. The expression level of *SIS8* in the complementation line is approximately twice that of Col-0 wild type (Figure 1b). The complementation line is not resistant to 0.27 M Suc or Glc (Figure 2a), further indicating that *At1g73660* is the *SIS8* gene. The function of SIS8 in sugar response was further investigated in *SIS8* overexpression lines. The *SIS8* coding region was placed under the control of the CaMV 35S promoter and introduced into Col-0 wild-type plants. Three independent T_3 homozygous transgenic lines were recovered and qRT-PCR analysis revealed overexpression of the *SIS8* transcript in all three lines (Figure 2b). The *SIS8* overexpression lines exhibit a hypersensitive response to the inhibitory effects of 0.22 M Glc or Suc on early seedling development when compared with wild-type plants (Figure 2c). The *SIS8* overexpression lines also exhibit slightly lower rates of seed germination, cotyledon expansion and true leaf formation than wild-type plants when grown on medium supplemented with 0.22 M sorbitol, but these effects are substantially smaller than the effects of 0.22 M Glc or Suc.

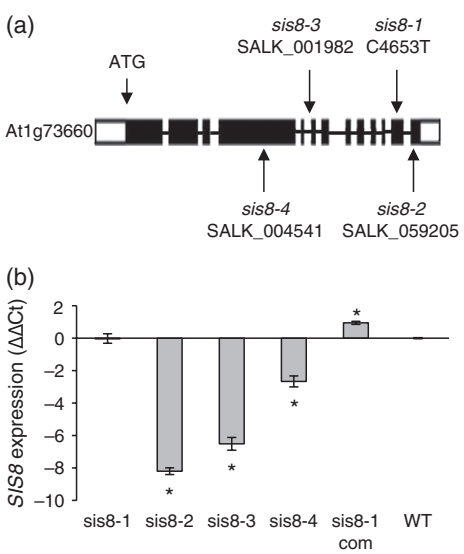

Figure 1. Characterization of *sis8* mutations.
(a) Schematic depiction of the *SIS8* genomic DNA sequence displaying the positions of the T-DNA insertions. Boxes indicate exons and lines indicate introns.
(b) *SIS8* transcript levels were assayed by quantitative RT-PCR in leaves from 21-day-old *sis8* mutants, the *sis8-1* complementation line (sis8-1 com) and Col-0 wild-type (WT) plants. Relative *SIS8* transcript levels are presented as ΔΔCt. $\Delta\Delta Ct = \Delta Ct_{WT} - \Delta Ct_{\text{indicated plant line}}$, where $\Delta Ct = Ct_{SIS8} - Ct_{UBQ10}$. Positive ΔΔCt values indicate that *SIS8* transcript levels are higher in the indicated line than in the wild type whereas negative ΔΔCt values indicate that *SIS8* transcript levels are lower in the indicated line than in the wild type. Data represent the means of three independent assays ± SD. *SIS8* expression differed between the wild type and other lines with $^*P < 0.05$, according to a Student's *t*-test.

Seed germination response of *sis8* seeds to ABA and paclobutrazol

As some previously characterized sugar-response mutants also exhibit defects in ABA or gibberellin (GA) response, the *sis8* mutants were tested for aberrant response to ABA and GA. Seeds of mutant and wild-type plants were sterilized, stratified and sown on minimal medium containing the indicated concentrations of ABA or paclobutrazol (an inhibitor of GA biosynthesis) and scored for percentage germination after 9 days. When assayed on media lacking both ABA and paclobutrazol, high percentages of seeds from all of the lines germinated. When sown on media supplemented with 1 μM ABA, *sis8-1*, *sis8-2*, *sis8-3* and *sis8-4* seeds exhibit germination percentages that are not significantly different from wild-type seeds (Figure 3a). Similarly, when assayed on 30 μM paclobutrazol, *sis8-1*,

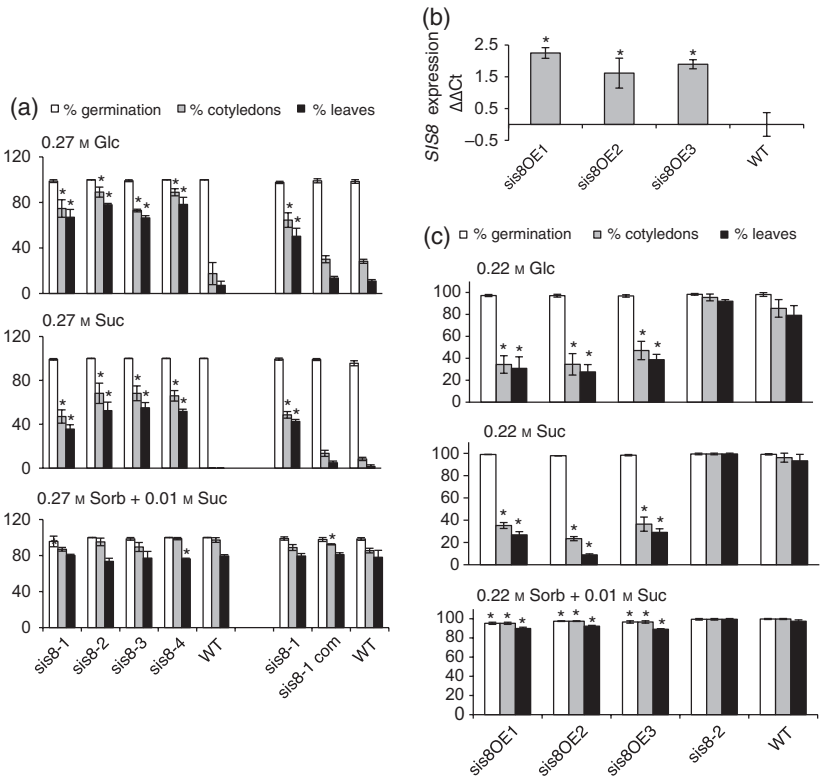

Figure 2. *SIS8* mediates sugar response during early seedling development.
(a) Wild-type (WT), mutant and complementation line (sis8-1 com) seeds were sown on minimal Arabidopsis medium supplemented with 0.27 M glucose (Glc), sucrose (Suc) or sorbitol (Sorb) and grown under continuous light at 22°C for 2 weeks prior to determining the percentages of seeds of each line on each medium that germinated, developed expanded cotyledons and formed true leaves. Data represent the means of three independent assays ± SD. The phenotypes of the wild type and other lines differed with $*P < 0.05$, according to a Student's *t*-test.
(b) Transcript levels of the *SIS8* overexpression lines (sis8OE). Total RNA was extracted from aerial parts of 5-day-old plants grown on soil under continuous light. Relative *SIS8* transcript levels are presented as ΔΔCt. $\Delta\Delta Ct = \Delta Ct_{WT} - \Delta Ct_{\text{indicated plant line}}$, where $\Delta Ct = Ct_{SIS8} - Ct_{ACTIN7/UBQ6}$. Data represent the means of three independent assays ± SD. *SIS8* expression differed between the wild-type and *SIS8* overexpression lines with $*P < 0.05$, according to a Student's *t*-test.
(c) Seedlings overexpressing *SIS8* in the Col-0 background exhibit a hypersensitive response to 0.22 M Suc or Glc. Data represent the means of two (mutant and overexpression lines) or four (wild-type line) independent assays ± SD. The phenotypes of the wild-type and *SIS8* overexpression lines differed with $*P < 0.05$, according to a Student's *t*-test.

sis8-2, *sis8-3* and *sis8-4* seeds exhibit germination rates that are similar to those of wild-type seeds (Figure 3b). The wild-type germination responses of the *sis8* seeds to ABA and GA are in agreement with the observations of Gao and Xiang (2008).

Gene expression analyses

Semi-quantitative RT-PCR was performed to determine the relative expression levels of *SIS8* in different Arabidopsis organs. *SIS8* transcript was detected in diverse organs of Arabidopsis including roots, stems, leaves, siliques and flowers, with higher expression in leaves (Figure 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 the seedling stage than in germinated seeds, with mature siliques having the lowest levels of expression. Analysis of stimulus response data compiled by Genevestigator indicates that *SIS8* mRNA levels are two- to three-fold lower on ABA and two-fold lower on NAA, a synthetic auxin analog, but are increased nearly three-fold by heat stress and 3.5-fold by brassinolide application, suggesting that *SIS8* is affected by plant hormone signaling and abiotic stress responses. Interestingly, salt treatment caused a decrease in levels of *SIS8* mRNA in seedlings (Gao and Xiang, 2008). To investigate whether Glc levels affect *SIS8* expression in germinating seeds, qRT-PCR experiments were conducted. The results of these experiments indicate that *SIS8* transcript levels are not substantially affected by Glc treatment in germinating seeds (Figure 4b).

To determine whether SIS8 regulates the expression of genes involved in different sugar response pathways, germinating seeds of *sis8-3* and wild-type Col-0 were treated with 0.1 M Suc or sorbitol and transcript levels determined

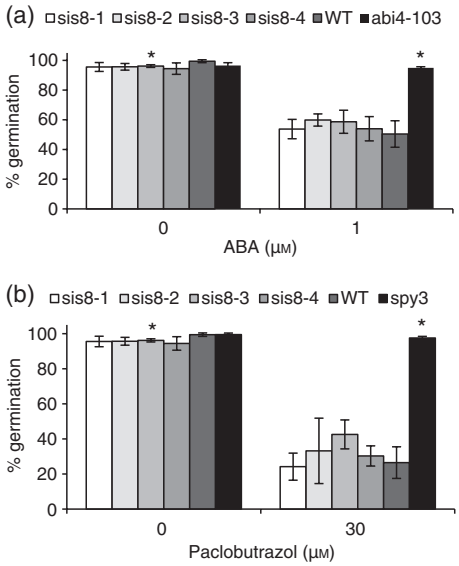

Figure 3. Germination responses of *sis8* mutants to ABA and paclobutrazol.
(a) Seeds were sown on media containing 0 or 1 μM ABA and percentage germination scored after 9 days. The ABA-insensitive mutant, *abi4-103*, was used as a positive control. Data represent the means of three independent assays ± SD. Asterisks indicate results where the phenotype of a mutant line differed from that of the wild type by $P < 0.05$.
(b) Seeds were sown on media containing 0 or 30 μM paclobutrazol and germination scored after 9 days. The *spy3* seeds, which are known to be resistant to paclobutrazol, were used as a positive control. Data represent the means of three independent assays ± SD. Asterisks indicate results where the phenotype of a mutant line differed from that of the wild type by $P < 0.05$.

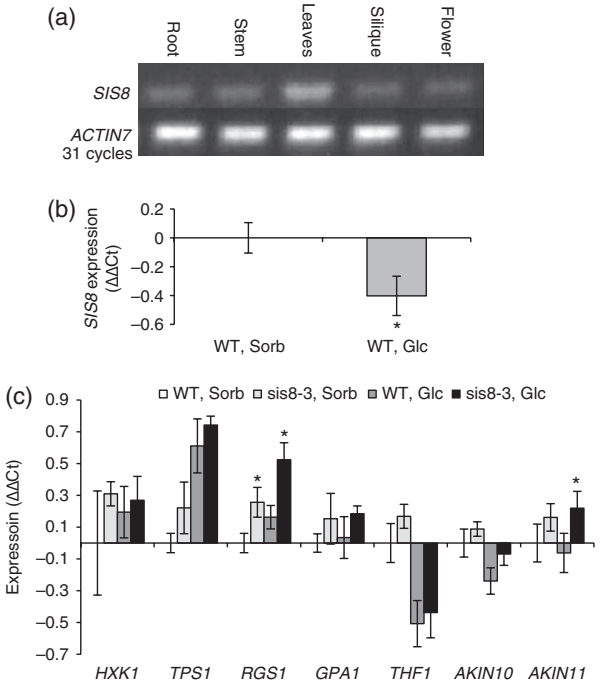

Figure 4. Gene expression analyses.
(a) Organ-specific expression of the *SIS8* gene. Total RNA was extracted from indicated organs of 32-day-old plants grown on soil under continuous light. Reverse transcriptase-PCR was performed with *SIS8*-specific or *ACTIN7*-specific primers.
(b) Expression of *SIS8* in wild-type (WT) seeds germinating on media supplemented with 0.1 M glucose (Glc) or sorbitol (Sorb) was analyzed by quantitative RT-PCR. Relative *SIS8* transcript levels are presented as ΔΔCt. $\Delta\Delta Ct = \Delta Ct_{sorbitol} - \Delta Ct_{indicated\ media}$, where $\Delta Ct = Ct_{SIS8} - Ct_{ACTIN7/UBQ6}$. Data represent the means of three independent assays ± SD. *SIS8* expression differed between seeds germinated on 0.1 M Glc or sorbitol with $^*P < 0.05$, according to a Student's *t*-test.
(c) Quantitative RT-PCR analysis of gene expression in *sis8-3* germinating seeds. Mutant and wild-type seeds were sown on Arabidopsis minimal medium and then transferred after 20 h to medium supplemented with either 0.1 M sorbitol (Sorb) or Glc and then harvested after an additional 13 h. Relative transcript levels are presented as ΔΔCt. $\Delta\Delta Ct = \Delta Ct_{WT\ on\ sorbitol} - \Delta Ct_{indicated\ plant\ line\ on\ indicated\ media}$, where $\Delta Ct = Ct_{indicated\ gene} - Ct_{ACTIN7/UBQ6}$. Positive ΔΔCt values indicate that transcript levels of that gene are higher in *sis8-3* on the indicated medium than in the wild type on the same medium. Conversely, negative ΔΔCt values indicate that transcript levels of that gene are lower in *sis8-3* on the indicated medium than in the wild type on the same medium. Data represent the means of three independent assays ± SD. Gene expression differed between mutant and wild-type seeds germinated on the same medium with $^*P < 0.05$, according to a Student's *t*-test.

by qRT-PCR. The genes analyzed include components of the hexokinase-dependent pathway (*HXK1* and *TPS1*) (Jang *et al.*, 1997; Avonce *et al.*, 2004), the RGS1 pathway (*RGS1*, *GPA1* and *THF1*) (Chen *et al.*, 2003; Huang *et al.*, 2006) and the SnRK1 pathway (*AKIN10* and *AKIN11*) (Bhalerao *et al.*, 1999). Levels of *RGS1* transcript are higher in *sis8-3* seeds germinating on 0.1 M Glc or sorbitol than in wild-type seeds germinating on the same media and *AKIN11* transcript levels are higher in *sis8-3* seeds germinating on 0.1 M Glc than in wild-type seeds germinating on 0.1 M Glc (Figure 4c). However, the effects of the *sis8-3* mutation on expression of these genes are quite small, with a maximum difference in transcript levels between the mutant and wild type of less than 30%.

SIS8 interacts and co-localizes with a UDP-glucosyltransferase, UGT72E1

The Y2H system has been used extensively for detecting protein–protein interactions. Yeast two-hybrid screening was conducted at 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 retested *in vivo* (Figure 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 (Figure 5).

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

AGI no.	Gene product	*Sugar insensitive* phenotype[a]
At3g50740	UGT72E1, UDP-glucosyltransferase	Yes
At4g17250	Unknown protein	Not determined
At4g31670	Ubiquitin carboxyl-terminal hydrolase family protein	No
At4g08850	Leucine-rich repeat family protein/protein kinase family protein	No
At1g61520	LHCA3, chlorophyll *a*/*b*-binding protein	Not determined
At5g18580	FASS/TON2, protein phosphatase 2A regulatory subunit	No

AGI, Arabidopsis Genome Initiative.
[a]The *sugar insensitive* phenotype was characterized by assaying at least one homozygous T-DNA insertion line for each gene.

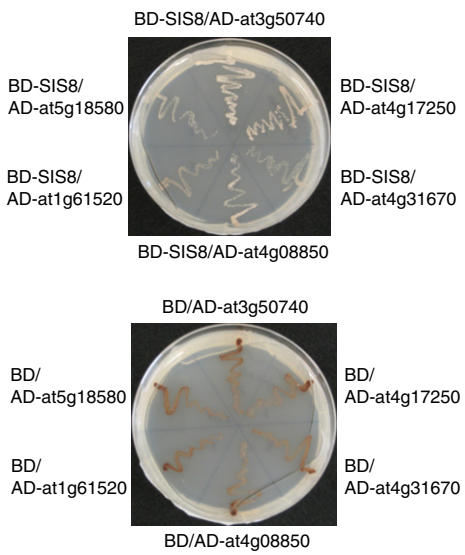

Figure 5. Yeast two-hybrid assay identifies SIS8-interacting proteins. Effects of each bait–prey plasmid combination on yeast colony growth on synthetic complete (SC)–Leu–Ura–Ade plates. White colonies indicate an interaction between the two fusion proteins produced by the bait and prey constructs, whereas red colonies indicate no detectable interaction. The prey constructs have the GAL4 activation domain (AD) fused to a gene encoding a potential SIS8-interacting partner. The bait constructs have the GAL4 DNA-binding domain (BD) fused to SIS8 (top photo) or to nothing, as a negative control (bottom photo).

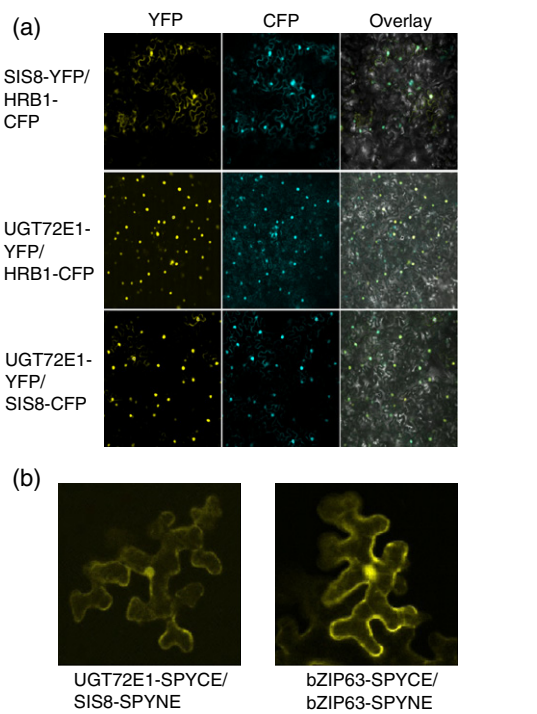

Figure 6. SIS8 and UGT72E1 co-localize and interact in the nucleus.
(a) Transient co-expression of SIS8–yellow fluorescent protein (YFP)/HRB1–cyan fluorescent protein (CFP), UGT72E1–YFP/HRB1–CFP and UGT72E1–CFP/SIS8–YFP in tobacco epidermal cells. Left panels, YFP images; middle panels, CFP images; right panels, merged images of YFP and CFP.
(b) Bimolecular fluorescence complementation analysis of the interaction between SIS8 and UGT72E1. Left image, SIS8-SPYNE co-expressed with UGT72E1-SPYCE. Right image, nuclear bZIP63 used as a positive control.

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), a UDP-glucose:glycosyltransferase that specifically conjugates lignin monomers such as coniferyl aldehyde and sinapyl aldehyde (Lim *et al.*, 2005). To test the interaction between SIS8 and UGT72E1, a co-localization study was performed. SIS8 and UGT72E1 were fused with fluorescent proteins and transiently expressed in tobacco leaves (*Nicotiana benthamiana*) (Sparkes *et al.*, 2006). A previously reported nuclear protein, HRB1, was used as a positive control (Kang *et al.*, 2005). Signals from fluorescent proteins clearly demonstrate that SIS8 and UGT72E1 are targeted to the nucleus (Figure 6a). To visualize the SIS8–UGT72E1 interaction in living plant cells, the BiFC technique, which has been used successfully for visualization of protein–protein interactions (Walter *et al.*, 2004), was adopted. *SIS8* was cloned into the split YFP vector *pUC-SPYNE* (N-terminal fragment), and *UGT72E1* was cloned into *pUC-SPYCE* (C-terminal fragment). Mixtures of agrobacteria carrying the SIS8–SPYNE and UGT72E1–SPYCE constructs were co-transformed into tobacco leaves. Epidermal cells expressing both constructs show yellow fluorescence in the nucleus, confirming the SIS8–UGT72E1 protein–protein interaction and its nuclear localization (Figure 6b). The

bZIP63 protein was used as a positive control (Walter *et al.*, 2004) and exhibits a yellow fluorescence signal in the nucleus.

Mutations in UGT72E1 cause a *sugar insensitive* (*sis*) phenotype

To determine whether mutations in the genes encoding potential SIS8-interacting proteins cause a *sugar insensitive* phenotype, T-DNA insertion lines predicted to carry inserts in four of the six genes (Table 1) were obtained from the ABRC. Seeds from lines homozygous for each insertion were screened on 0.23 M Suc to test for a hypersensitive response and on 0.27 M Glc to test for a Glc-resistant phenotype. Interestingly, SALK_078702 (*ugt72e1-1*) and GABI_340H02 (*ugt72e1-2*), which carry T-DNA inserts in *UGT72E1* (Figure 7a), display *sugar insensitive* phenotypes. When grown on medium supplemented with 0.26 M Suc, both homozygous *ugt72e1* mutant lines display rates of cotyledon expansion and true leaf formation that are significantly higher than those of wild-type seedlings grown on the same medium. In contrast, when grown on minimal medium with no sugar supplement, the *ugt72e1* lines develop expanded cotyledons and true leaves at rates similar to wild-type plants grown on the same medium (Figures 7b and S1). The *ugt72e1* lines also exhibit wild-type sensitivity to the effects of 0.26 M sorbitol and 0.15 M NaCl on early seedling development (Figure S2). *UGT72E1* transcript levels are not regulated by 0.1 M Glc in germinating seeds. Similarly, the *sis8-3* mutation does not significantly affect *UGT72E1* transcript levels in germinating seeds (Figure 7c).

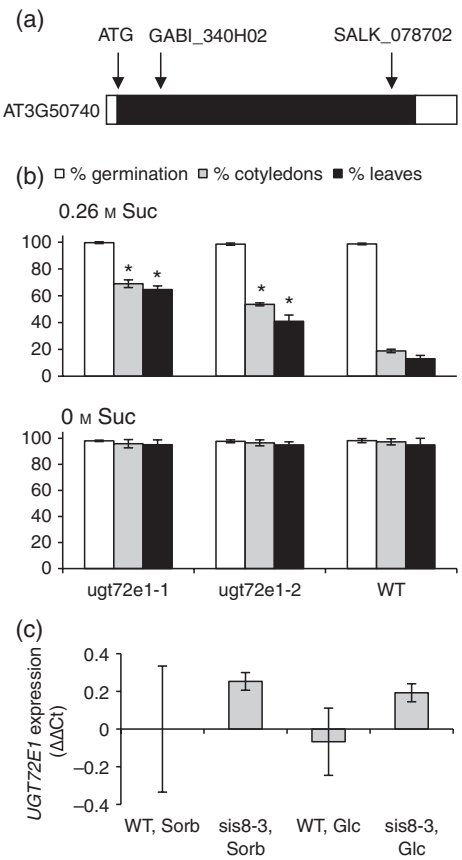

Figure 7. Mutations in *UGT72E1* (*At3g50740*) confer a *sugar insensitive* (*sis*) phenotype.
(a) Gene structure of *At3g50740* and approximate positions of the T-DNA inserts in Arabidopsis lines SALK_078702 and GABI_340H02. The black box indicates the coding region and the white boxes indicate the 5′ and 3′ untranslated regions. *At3g50740* has only a single exon.
(b) Seedling development of SALK_078702 (*ugt72e1-1*), GABI_340H02 (*ugt72e1-2*) and wild-type Col-0 (WT) on 0 and 0.26 M sucrose (Suc). The *sis* assays were conducted as described. Data represent the means of three independent assays ± SD. The phenotypes of the wild type and other lines differed with $*P < 0.05$, according to a Student's *t*-test.
(c) Expression of *UGT72E1* in *sis8-3* and wild-type Col-0 (WT) seeds germinating on media supplemented with 0.1 M Glc or sorbitol (Sorb) was analyzed by quantitative RT-PCR. Relative *UGT72E1* transcript levels are presented as ΔΔCt. $\Delta\Delta Ct = \Delta Ct_{\text{wild type on sorbitol}} - \Delta Ct_{\text{indicated line on indicated medium}}$, where $\Delta Ct = Ct_{UGT72E1} - Ct_{ACTIN7/UBQ6}$. Data represent the means of three independent assays ± SD. *UGT72E1* expression levels did not differ significantly between the samples analyzed.

DISCUSSION

Sugars, such as Glc and Suc, regulate a wide variety of developmental and metabolic processes in plants. The available evidence indicates that multiple sugar signaling pathways exist in plants and that these pathways interact 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; Radchuk *et al.*, 2006; Rook *et al.*, 2006; Baena-González *et al.*, 2007; Banás and Gabryś, 2007; Karthikeyan *et al.*, 2007; Müller *et al.*, 2007). Despite the importance of sugar response, only a fraction of the factors that are likely to be involved in sugar-response pathways have been identified.

In this work, a forward genetic screen was used to identify an *A. thaliana* mutant, *sis8-1*, with enhanced resistance to the inhibitory effects of high (e.g. 0.27 M) concentrations of exogenous Suc and Glc. The effects of mutations in *SIS8* on sugar response cannot be explained solely by alterations in osmotolerance, as all of the *sis8* loss-of-function mutants tested exhibit a wild-type response to equimolar concentrations of sorbitol. In contrast to plants carrying loss-of-function mutations in *SIS8*, plants overexpressing *SIS8* exhibit a sugar-hypersensitive phenotype. When grown on media supplemented with 0.22 M Suc or Glc, the *SIS8* overexpression lines exhibit significantly lower rates of cotyledon expansion and true leaf formation than wild-type plants grown on the same media. These results suggest that SIS8 plays an important role in response to both Suc and Glc.

The *sis8-1* locus was identified by map-based cloning and found to lie in *At1g73660*. Three independent lines that carry T-DNA insertions in *At1g73660* were identified and found to have *sugar insensitive* phenotypes. Complementation analysis confirmed that *At1g73660* corresponds to *SIS8*. The *SIS8* gene encodes a putative Raf-like MAPKKK. According to Ichimura *et al.* (2002), SIS8 belongs to the 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 screens for *sugar insensitive* mutants, such as *SIS1* (Gibson *et al.*, 2001) and *GIN4* (Rolland *et al.*, 2002). EDR1 functions as a negative regulator of disease resistance and ethylene-induced senescence (Frye *et al.*, 2001; Tang *et al.*, 2005). 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 1-aminocyclopropane-1-carboxylic acid (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.

The expression of *AT6*/*SIS8* in seedlings is downregulated by salt treatment (Gao and Xiang, 2008). As high concentrations of exogenous sugars only exert inhibitory effects on seedling development during a narrow temporal window (within 40 h after the start of seed imbibition; Gibson *et al.*, 2001), we tested whether the expression of *SIS8* is regulated by exogenous Glc in germinating seeds and found that 0.1 M Glc has only a very minor effect on *SIS8* transcript levels. However, it remains possible that Glc regulates *SIS8* at the translational or post-translational levels. For example, Glc has been shown to promote the degradation of ethylene insensitive 3 (EIN3), a transcriptional regulator in ethylene signaling (Yanagisawa *et al.*, 2003).

As several previously identified sugar-response mutants have also been found to exhibit defects in response to phytohormones, particularly ethylene, ABA and GA, it was of interest to test the *sis8* mutants for altered response to these phytohormones. 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 the present study, *sis8* loss-of-function mutants were found to exhibit a wild-type response to the inhibitory effects of ABA and paclobutrazol (an inhibitor of GA synthesis) on seed germination, results that are consistent with the findings of Gao and Xiang (2008). These results suggest that SIS8 does not affect the ABA and/or GA response at the seed germination stage.

To gain insight into the molecular mechanism by which SIS8 acts in sugar response, a Y2H screen was conducted and six potential interacting partners were identified (Table 1). Interestingly, two Arabidopsis mutants (SALK_078702 and GABI_340H02) carrying T-DNA insertions in the gene encoding one of these putative SIS8-interacting proteins, UGT72E1 (At3g50740), display a sugar-resistant seedling growth phenotype. Through co-localization and BiFC analyses, SIS8 and UGT72E1 were found to interact in the nucleus. *At3g50740* encodes a UDP-Glc:glycosyltransferase 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 the growth and development of plant cells and are capable of triggering signaling pathways (Pilling and Höfte, 2003). Furthermore, studies have shown that loss-of-function mutations in the *HSR8*/*MUR4* gene, which is involved in arabinose synthesis, lead to Glc-hypersensitive seedling development in the dark. Genetic analysis using a *mur4-1 prl1* double mutant indicates that PRL1, which has previously been shown to be involved in sugar response in plants, is required for the increased sugar responses seen in *hsr8*/*mur4* mutants (Li *et al.*, 2007). Our findings that SIS8 interacts with UGT72E1, and that mutations in *SIS8* and *UGT72E1* both cause sugar-resistant seedling growth phenotypes, suggest that SIS8 may help link changes in the cell wall to the cellular sugar response. Alternatively, UGT72E1 may function in other processes in addition to cell wall metabolism. Based on computational analyses, UGT72E1 has been inferred to play possible roles in the nitrate response, nitrate transport, the tryptophan catabolic process and/or indoleacetic acid biosynthesis (Heyndrickx and Vandepoele, 2012). As carbon (e.g. sugar) and nitrogen response pathways can interact, the involvement of UGT72E1 in nitrate transport or nitrate response could explain some or all of the effects of mutations in *UGT72E1* on sugar response. Similarly, as phytohormone and sugar response pathways can interact, involvement of UGT72E1 in the biosynthesis of indoleacetic acid could explain some or all of the effects of *ugt72e1* mutations on sugar response. The findings reported here that UGT72E1 is present in the nucleus appear consistent with UGT72E1 acting in processes in addition to cell wall metabolism. However, direct experimental evidence confirming the involvement of UGT72E1 in these other processes is currently not available.

In summary, we have identified *SIS8*/*AT6*, which is predicted to encode a putative MAPKKK, as a regulator of resistance to high sugar levels during early seedling development in Arabidopsis. In addition, SIS8 has been shown to interact with a UDP-glucosyltransferase UGT72E1 in the nucleus and mutations in *UGT72E1* have been shown to confer a *sis* phenotype. Although SIS8 is annotated as a MAPKKK based on its predicted 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 may help elucidate the mechanism by which SIS8 functions.

EXPERIMENTAL PROCEDURES

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

A mutant population comprised of 60 000 M2 seeds derived from EMS-mutagenized *A. thaliana* var. Col-0 was screened on 0.3 M Suc. The *sis8-1* mutant was isolated based on its sugar-resistant seedling growth phenotype. Seed segregating for the *sis8-2* (SALK_059205), *sis8-3* (SALK_001982), *sis8-4* (SALK_004541) and *ugt72e1-1* (SALK_078702) mutations were obtained from the ABRC in the Col-0 (CS60000) background. GABI_340H02 seeds that are homozygous for the *ugt72e1-2* mutation were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (Kleinboelting *et al.*, 2012). Polymerase chain reaction was used to identify plants that are homozygous for the *sis8-2*, *sis8-3*, *sis8-4* and *ugt72e1-1* mutations. The *sis8-1*, *sis8-2* and *sis8-4* seeds used for the physiological assays and qRT-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 Col-0 background, were provided by Dr Neil Olszewski (University of Minnesota). Wild-type Col-0 (CS6000) and Hi-O (CS6736) seeds were obtained from the ABRC. Seeds to be sown on plates were surface sterilized and stratified for 3 days 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 days prior to scoring. Minimal Arabidopsis medium was prepared as described (Kranz and Kirchheim, 1987).

Expression analysis by RT-PCR

Gene expression was assayed in the tissues indicated for each experiment. To assay gene expression in germinating seeds, seeds were stratified and sown on nytex screens on solid minimal Arabidopsis medium and incubated for 20 h under continuous light and then transferred to minimal medium supplemented with the indicated additive for another 12–13 h under continuous light before harvesting. Isolation of RNA was done using either the Qiagen RNeasy plant mini kit (Qiagen, http://www.qiagen.com/) or the Sigma Spectrum Plant Total RNA kit (Sigma, http://www.sigmaaldrich.com/). After DNaseI treatment (Promega, http://www.promega.com/), first-strand cDNA was synthesized from 1 μg total RNA using the Promega Improm-II RT system kit (Promega). Quantitative RT-PCR was performed using a Roche Lightcycler 480 II PCR Machine and Roche Lightcycler 480 SYBR Green I Master reagent (Roche Diagnostics, http://www.roche.com/). Primers used were: *UBQ10* (*At4g05320*), 5′-GGCCTTGTATAATCCCTGATGAATAAG-3′ and 5′-AAAGAGATAACAGGAACGGAAACATAGT-3′; *UBQ6* (*At2g47110*), 5′-CCATCGACAATGTCAAGGCC-3′ and 5′-GGTACGTCCGTCTTCGAGCT-3′; *ACTIN7* (*At5g09810*), 5′-TGCTGACCGTATGAGCAAAG-3′ and 5′-GATTGATCCTCCGATCCAGA-3′; *SIS8* A 5′-CAACGTGATAATGGCAAAGTT-3′ and 5′-ATGGGGGAGGAGTCTGAGTT-3′; *SIS8* B 5′-TCCCGACTTTGTAGATCCAG-3′ and 5′-AAGAGGAACTGGGGACTGGT-3′; *UGT72E1* (*At3g50740*), 5′-CAGATGATGAACGCGACACT-3′ and 5′-CCTGACACGCTCCAAAAGAT-3′; *HXK1* (*At4g29130*), 5′-GCAACCGCGATCCCTAAAT-3′ and 5′-CCAGCGTGTGATCAAACTCG-3′; *TPS1* (*At1g78580*), 5′-GGTGTCACAAAGGGAGCTGC-3′ and 5′-CATCTTCGTCCTTCCCCAAG-3′; *RGS1* (*At3g26090*), 5′-TTTCCTCCCCCTTGTTTTGTT-3′ and 5′-ATGAAGGCCTGCAACTGGG-3′; *GPA1* (*At2g26300*), 5′-TGAACGTTTGCGAGTGGTTC-3′ and 5′-GGCGCCGTGTTCTGGTAATA-3′; *AKIN10* (*At3g01090*), 5′-TCCCCGTGAAATAATGACGG-3′ and 5′-CATACCATCTGCGCTGCTGT-3′; *AKIN11* (*At3g29160*), 5′-TCCTATGCGCACACCTGAAG-3′ and 5′-TCCAAGAGCCCATTTTCGAT-3′; *THF1* (*At2g20890*) 5′-CAGCAGGTTCTTCGCTGTTG-3′ and 5′-GTCCCGATCCACGCTTTTC-3′. For qRT-PCR experiments two technical replicates were performed for each biological replicate and data were normalized by comparison with *UBQ10* or the geometric average (Vandesompele *et al.*, 2002) of *ACTIN7* and *UBQ6*, as indicated for each experiment.

Map-based cloning of *SIS8*

To generate the *sis8-1* mapping population, F_2 seedlings from a cross between *sis8-1* and wild-type plants of the Hi-0 background were screened on media containing 0.3 or 0.32 M 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). Molecular markers from the TAIR and Monsanto SNP and L*er* sequence collections (Jander *et al.*, 2002) were tested for polymorphisms between Col-0 and Hi-O wild-type plants prior to use in the mapping experiments.

Plasmid constructions and generation of transgenic *A. thaliana*

All constructs were generated using the Gateway system (Invitrogen, http://www.invitrogen.com/). For *SIS8* overexpression lines, the *SIS8* full length coding sequence (3093 bp) was amplified from Col-0 leaf total RNA by PCR, confirmed by sequencing to be free of mutations and then cloned into the *pEarleyGate100* destination vector behind the CaMV 35S promoter (Earley *et al.*, 2006). For the complementation tests, a *SIS8* genomic DNA fragment (including 1500 bp upstream of the ATG start codon, all exons and introns, and ending before the stop codon) was amplified from wild-type Col-0 DNA and cloned into *pEarleyGate302*. The resulting binary vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Transformation of *sis8-1* or wild-type Col-0 was performed using the floral dip method (Clough and Bent, 1998). T_1 transgenic plants exhibiting resistance to media containing 7 μg ml^{-1} Basta were further tested by PCR analysis and confirmed to contain the appropriate plant transformation construct. These T_1 plants were then transferred to soil to obtain T_2 seeds. T_3 and T_4 homozygous lines were used for sugar response assays, germination assays and gene expression analysis.

Sugar, NaCl, ABA and paclobutrazol assays

For sugar-response assays, 50–100 seeds were surface sterilized, stratified at 4°C in the dark for 3 days, sown on minimal Arabidopsis medium supplemented with the indicated sugars, incubated at 22°C in the light for 14 days and then scored for seed germination, cotyledon expansion and true leaf formation as described (Laby *et al.*, 2000). For NaCl response assays, 50–100 seeds were surface sterilized, stratified at 4°C in the dark for 3 days, sown on MS medium supplemented with NaCl, incubated at 22°C in the light for 14 days and then scored for cotyledon expansion as described (Gao and Xiang, 2008). For ABA and paclobutrazol response assays, seeds were surface sterilized, stratified at 4°C in the dark for 3 days and then sown on Arabidopsis minimal medium supplemented with the indicated chemicals and incubated at 22°C under continuous light for 9 days prior to scoring for percentage seed germination. Germination is defined as

the emergence of the radicle from the seed coat. 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). The resulting construct was sent to the Molecular Interaction Facility at the University of Wisconsin-Madison where the Y2H 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). The Y2H screen resulted in identification of six genes that give a reproducible positive interaction signal in the presence of the construct encoding the SIS8–GAL4 BD fusion protein, but not in the presence of the empty vector construct, which encodes only the GAL4 BD. To retest the results of the Y2H screen, each positive prey plasmid was introduced into yeast strains carrying pBUTE with or without the *SIS8* insert using the lithium acetate method (Gietz and Schiestl, 2007). Single transformants were picked and grown on synthetic complete (SC)–Leu–Ura–Ade plates to detect protein–protein interactions, which result in growth of white colonies.

Subcellular localization and BiFC studies

To analyze subcellular localization, 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 and 102 vectors to create the *35S::SIS8:YFP* and *35S::SIS8:CFP* constructs, respectively. Similarly, the *UGT72E1* full-length coding sequence without the stop codon was used to create *35S::UGT72E1:YFP* (in pEarleyGate 101). The *35S::HRB1:CFP* construct (in the pEarleyGate 102 vector) was obtained from Dr Xiaodong Sun (University of Minnesota). The constructs were introduced into *A. tumefaciens* strain GV3101 (pMP90). Agrobacteria carrying the corresponding constructs were cultured at 28°C to approximately OD_{600} = 1. The bacterial cells were spun down and resuspended with MES buffer (10 mM MES, 10 mM $MgCl_2$, 150 μM acetosyringone, pH 5.6) to OD_{600} = 0.4. In cases where two bacterial cultures were used, both bacterial cultures were mixed at a 1:1 ratio with OD_{600} = 0.4 for each. Then the bacterial suspension was incubated at 28°C with mild agitation for 1–2 h. Tobacco plants (*N. benthamiana*) were grown for 3–4 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 leaf epidermal cells and transient expression of the fusion proteins were performed as described (Sparkes *et al.*, 2006). Two days later, the leaf samples were collected and the fluorescent proteins expressed in the abaxial epidermal cells were observed using a Nikon C1 Spectral Imaging Confocal Microscope (http://www.nikon.com/) with a 20 × objective lens. Images were obtained with the accompanying EZ-C1 acquisition and analysis software. For BiFC studies, the *UGT72E1* and *SIS8* full-length coding sequences were cloned into pUC-SPYCE^G and pUC-SPYNE^G (Walter *et al.*, 2004) to make constructs pUC-SPYCE–UGT72E1 and pUC-SPYNE–SIS8, respectively. Both constructs were co-transformed into tobacco leaf cells as described above for the subcellular localization experiment. Co-transformation of pUC-SPYCE–bZIP63 and pUC-SPYNE–bZIP63 into tobacco leaves was conducted as a positive control.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Effects of different sucrose and sorbitol concentrations on early seedling development of *sis8*, *ugt72e1* and wild-type lines.

Figure S2. The *ugt72e1* mutants exhibit wild-type sensitivity to sorbitol and NaCl.

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