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Isolation and characterization of *shs1*, a sugar-hypersensitive and ABA-insensitive mutant with multiple stress responses

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Abstract To identify salt tolerance determinants, we screened for double mutants from a T-DNA tagged *sos3-1* mutant population in the *Arabidopsis* Col-0 *gl1* background. The *shs1-1* (sodium hypersensitive) *sos3-1* mutant was isolated as more sensitive to NaCl than *sos3-1* plants. TAIL-PCR revealed that the introduced T-DNA was located 62 bp upstream of the initiation codon of an adenylate translocator-like protein gene on chromosome IV. *SHS1* mRNA did not accumulate in *shs1-1 sos3-1* plants although it accumulated in shoots of both *sos3-1* and the wild type plants, indicating that this gene is inactive in the mutant. Genetic co-linkage analysis revealed that the mutation causing the phenotype segregated as a recessive, single gene mutation. This mutant showed altered sensitive responses to salt as well as to cold stress. It also demonstrated sugar sensitive and ABA insensitive phenotypes including enhanced germination, reduced growth, altered leaf morphology, and necrosis on leaves at an early growth stage. Sensitivity of *sos3-1 shs1-1* root growth to LiCl, KCl, and mannitol was not significantly different from growth of *sos3-1* roots. Further, expression of *35S::SHS1* in *sos3-1 shs1-1* plants complemented NaCl and sugar sensitivity and partially restored the leaf morphology.

Keywords *Arabidopsis* · Saline stress · Sugar sensitivity · Stress response · Adaptation

G. Inan and F. Goto contributed equally in this work.

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Introduction

Excess salts are harmful for plants and reduce agricultural production and proper concentration of salt is essential for their growth. To cope with the detrimental effects of salt stress on growth and development, plants must coordinate salt stress responses at the molecular level by complex connections between various signal transduction systems and ion transport systems (Zhu 2002).

Salt and drought stress signaling can be divided into three functional categories including ionic and osmotic stress signaling for the reestablishment of cellular

homeostasis under stress conditions, detoxification signaling to control and repair stress damages, and signaling to coordinate cell division and expansion to levels suitable for the particular stress conditions (Zhu 2001). For the ionic aspect of salt stress, a signaling pathway based on the *SOS* (salt overly sensitive) genes has been established as a requirement for plant salt tolerance. The SOS pathway is the main route for regulation of Na^+ tolerance involving Ca^{2+} dependent salt stress signaling and ion homeostasis (Zhu 2000). Salt stress elicits a cytosolic calcium signal (Knight et al. 1997). A myristoylated calcium-binding protein encoded by *SOS3* presumably senses the salt-elicited calcium signal and translates it to downstream responses (Ishitani et al. 2000; Liu and Zhu 1998). SOS3 physically interacts with and activates SOS2, a serine/threonine protein kinase (Halfter et al. 2000; Liu et al. 2000). The SOS2 and SOS3 complex regulates the expression level of *SOS1*, a salt tolerance effector gene encoding a plasma membrane Na^+/H^+ antiporter (Shi et al. 2000, 2002). More importantly, SOS2 and SOS3 are required for the activation of SOS1 transport activity (Qiu et al. 2002). Mutation in *SOS1* causes hypersensitive root growth when seedlings are placed on medium containing less than 25 mM NaCl (Wu et al. 1996). Transgenic plants overexpressing SOS1 have shown enhanced salt tolerance, indicating that Na^+ efflux is essential for salt tolerance (Shi et al. 2003, Ward et al. 2003).

Hormonal control is also critical in salt stress adaptation. Abscisic acid (ABA) regulates many important events during the growth of plants, and plays a major role in late seed development and adaptation to environmental stresses (Gazzarini and McCourt 2001). Genes affecting ABA response have been found to encode proteins that affect many processes including transcription, protein phosphorylation or farnesylation, RNA processing, and phosphoinositide metabolism (Finkelstein et al. 2002; Himmelbach et al. 2003; Kuhn and Schroeder 2003). Screens for mutations with decreased sensitivity to ABA have identified 8 ABA-insensitive genes (ABI1-ABI8) and many mutants with enhanced response to ABA at the level of germination (*era* mutants) have also been identified including a protein farnesyl transferase (ERA1) as an attenuator of both seed and vegetative ABA mediated responses. In addition, there is substantial evidence for cross talk between signaling pathways regulating response to ABA and abiotic stresses (e.g., drought, salinity, and cold) (Ishitani et al. 1997). Possible mechanisms of cross talk, particularly among ABA, sugar, and ethylene signaling, are discussed in many reviews (Gazzarini and McCourt 2001; Finkelstein and Gibson 2002; Rolland and Sheen 2005). The loci shown to be associated with both ABA and sugar responses have been limited so far to those encoding ABA biosynthetic enzymes (ABA1, ABA2/GIN1/ISI1/SIS4, and ABA3/GIN5/LOS5), the transcription factors (ABI3, ABI4/GIN6/ISI3/SIS5/SUN6, and ABI5) and related proteins (ABF3 and ABF4), and EIN2/ERA3 and CTR1 (Brochard-Gifford et al. 2004). Among these, the ABI transcription factors appear to function in the same signaling pathway mediating ABA response (Finkelstein et al. 2002). Expression of all three is induced by glucose in a hexokinase-dependent manner (Leon and Sheen 2003). The EIN2/ERA3 and CTR1 loci also have been shown to function in mediating ethylene response via effects on EIN3 and resulting ethylene-dependent gene expression (Wang et al. 2002). This pathway was shown to be modulated by hexokinase-dependent degradation of EIN3 (Yanagisawa et al. 2003). The *ctr1* alleles have been isolated as enhancers of ABA resistance in *abi1-1* mutant (Ghassemian et al. 2000).

To isolate salt tolerance determinants that would increase the sensitivity of *sos* mutants to NaCl, we screened a T-DNA population (> than 65,000 independent T_2 lines) in the genetic background of *Arabidopsis* Col-0 *gl1 sos3-1* (Rus et al. 2001). One of the resulting mutants isolated from this screen was characterized as *shs1-1* (for *salt hypersensitive 1*). *shs1-1* has a T-DNA insertion in the promoter of the gene At4g32400. This is annotated as a putative adenine nucleotide translocator (ANT), *BRITTLE-1* (*BT1*) like, included in the mitochondrial carrier protein family. Homologous of BT1 protein have been described in both mono- and dicotyledonous plants like maize, barley and potato as plastidic ANTs, which are involved in starch biosynthetic processes (Sullivan et al. 1991; Patron et al. 2004; Leroch et al. 2005). The *shs1* mutant was characterized and further studied as a single mutant and as a double mutant with the *sos3-1* background to investigate its role in abiotic stress tolerance.

Results

Identification of *shs1-1* mutation

T_2 seedlings from 65,000 individual T-DNA insertion lines generated in the *sos3-1* genetic background were previously screened for suppressors of *sos3-1* and resulted in the isolation of *Athkt1* mutants (Rus et al. 2001). The same population was used to screen for mutants, which would enhance the sensitivity of *sos3-1* at 75 mM NaCl. One mutation *shs1-1* (salt hypersensitive) was identified having reduced root growth and increased anthocyanin accumulation, leaf cupping and chlorosis relative to the *sos3-1* mutant on medium supplemented with NaCl (Fig. 1). On MS nutrient medium, aerial parts of the mutant plants were indistinguishable from the wild type, but the roots of the mutants grew more slowly than the wild type (Fig. 1A).

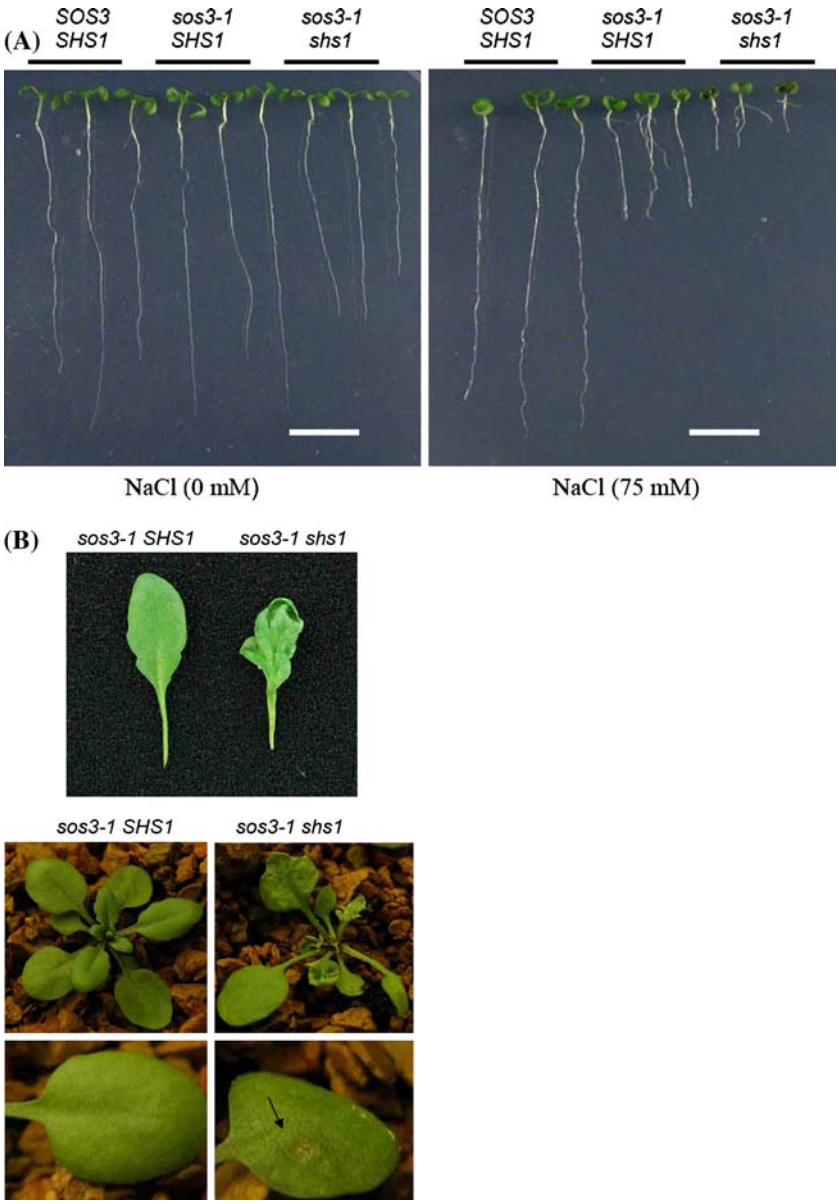

Fig. 1 The *shs1-1* mutation enhances the NaCl sensitive phenotype of *sos3-1*. (**A**) Four-day-old T_3 seedlings grown on MS agar medium were transferred on MS medium containing 0 (left) and 75 (right) mM NaCl. The photographs were taken 5 days after transferring to NaCl. (**B**) *shs1* mutation affects plant development. The wild-type (*sos3-1 SHS1*) and mutant (*sos3-1 shs1-1*) plants were grown in a greenhouse in soil for 30 days without any treatment. An arrow head indicates lesion spots

Upon transfer to the medium supplemented with 75 mM NaCl, *sos3-1* mutant plants carrying the *shs-1* mutation exhibited under greenhouse conditions reduced shoot mass and modified leaf shape with lesions (Fig. 1B). However, when we applied salt to soil-grown plants, we observed that *sos3-1 shs1-1* plants displayed no difference in sensitivity to salt in soil (data not shown).

The *sos3-1 shs1-1* mutant plants were backcrossed with the *sos3-1 SHS1* plants and F_1 progeny (45 of 45) were Basta® resistant and all exhibited *sos3-1* like phenotype in the presence of 45 mM NaCl. F_2 progeny ($n = 688$) from 27 F_1 hybrids segregated with 3:1 ratio for *sos3-1* phenotype when the seedlings were grown on MS medium containing 45 mM NaCl indicating that the *shs1* phenotype is the result of a recessive mutation in a nuclear gene.

We crossed *sos3-1 shs1-1* plants with *sos2-1 SHS1* and *sos1-1 SHS1* separately. The progeny of these crosses showed no root growth reduction in media supplemented with NaCl (Fig. 2) indicating that *sos2* and *sos1* mutations are not allelic to *shs1-1*.

The *shs1-1* mutation in *sos3-1* plants causes Na^+ specific hypersensitivity

Root elongation has been used as an indicator of *Arabidopsis* seedling growth (Wu et al. 1996). Measurement of root elongation showed quantitatively that *sos3-1 shs1-1* mutant was hypersensitive to NaCl in a dose-dependant manner compared with *sos3-1 SHS1* mutants. The root growth of 9–10-day-old *sos3-1 shs1-1* was approximately

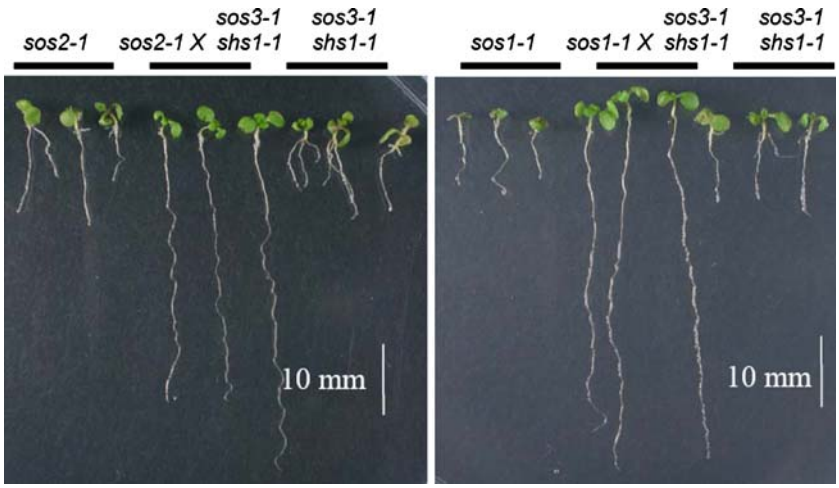

Fig. 2 The mutation of *sos3-1 shs1-1* is not allelic of *sos1* or *sos2*. *sos2-1* (left) and *sos1-1* (right) plants were crossed with *sos3-1 shs1-1* (♂). F_1 seedlings were transferred onto MS medium supplemented with 75 mM NaCl. The picture was taken 6 days after transferring

68% of that of *sos3-1 SHS1* and the wild type Col seedlings at 0 mM NaCl (Fig. 1A). However, root growth of *sos3-1 shs1-1* was dramatically reduced when 4-day-old seedlings were exposed to the media containing various concentrations of NaCl and compared with the *sos3-1* mutants (Fig. 3A). At 75 mM NaCl, the relative growth of *sos3-1 shs1-1* mutants was 54% of that of the *sos3-1* seedlings within a 6-day treatment period. The enhanced salt sensitivity of the double mutant was much clearer at a lower concentration (40 mM) of NaCl, where the relative growth of the *sos3-1 shs1-1* was only 46% of the single mutant (Fig. 3A). To determine whether the *sos3-1 shs1-1* mutant is hypersensitive to osmotic stress or to specific ions, the seedlings were also treated on media supplemented with KCl, LiCl, or mannitol. As shown in Fig. 3, no significant difference in growth and development was observed between *sos3-1 SHS1* and *sos3-1 shs1-1* in LiCl, KCl and mannitol treatments. The results suggest that the *shs1* mutation does not result in hypersensitivity to general osmotic stress but is restricted to Na^+ hypersensitivity.

SHS1 encodes a putative adenylate translocator

To identify the T-DNA tagged gene, Thermal asymmetric interlaced PCR (TAIL-PCR) analysis was used (Rus et al.

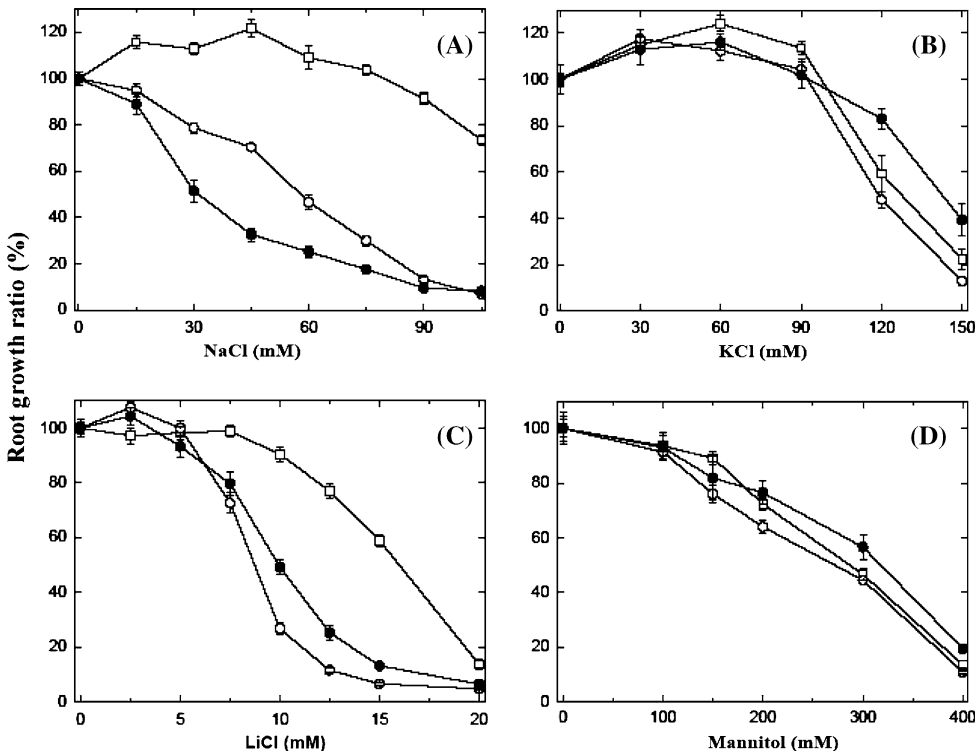

Fig. 3 The *shs1* mutation enhances *sos3* Na^+ sensitivity but not Li^+, K^+ and mannitol. *SOS3 SHS1* (open squares), *sos3-1 SHS1* (open circles) and *sos3-1 shs1-1* (closed circles) were grown 4 days on agar plates containing MS nutrients and then transferred to agar plates containing MS supplemented with NaCl, KCl, LiCl, or mannitol. Root growth was measured 6 days after transfer. Growth on MS nutrient was considered as 100%. Values are the mean ± SE, n = 12–15

2001). The results showed that T-DNA insertion was located 62 bp upstream from the putative start codon of a gene annotated as a BRITTLE-1 (BT1) like protein, a putative adenylate translocator (At4g32400 in MIPS *Arabidopsis thaliana* Database (MAtDB) (Millar and Heazlewood 2003) (Fig. 4A). The open reading frame of At4g32400 is 1,552 bp containing 3 exons and 2 introns. PCR analysis determined T_3 progeny of *shs1-1 sos3-1* are homozygous for the *shs1-1* allele. Reverse transcription PCR (RT-PCR) indicated that *SHS1* transcript accumulated predominantly in the shoot of 12-day-old *SOS3 SHS1* and *sos3-1 SHS1* seedlings whereas it was not detectable in the shoot or root of *shs1-1 sos3-1* seedlings. The results indicate that *SHS1* is expressed normally in shoots and is inactivated in *shs1-1 sos3-1* plants. *SHS1* expression is not altered in *sos3-1 SHS1* seedlings, indicating that the SOS pathway does not regulate *SHS1* at the transcriptional level (Fig. 4B).

SHS1 encodes a 392 amino acid polypeptide with a calculated molecular mass of 42.6 kD. TopPred (Topology prediction of membrane proteins) suggested that SHS1 has 4 membrane spanning domains (202–221), (261–280), (302–322), and (360–378) (von Heijne 1992. http://www.bioweb.pasteur.fr/seqanal/interfaces/toppred.html). A PFAM search (Protein Families Database of Alignment) resulted in matching of three regions of the SHS1 sequence (109–196, 203–291, and 297–389) with a mitochondrial carrier protein functional domain (pfam00153) and two copies of the amino acid sequence motif known as "the mitochondrial energy transfer signature" (Bateman et al. 2000 http://www.sanger.ac.uk/Software/Pfam/search.shtml). It was previously shown that SHS1 has a strong homology to the mitochondrial carrier family (MCF) proteins, StBT1 from *Solanum tuberosum* ZmBT1 from *Zea mays* and HvBT1 (HvNST1) from *Hordeum vulgare*. Phylogenetic analyses classify SHS1 (AtBT1) as a member of MCF in *Arabidopsis* (Leroch et al. 2005).

A current search of the Plants T (Functional Genomics of Plant Transporters) database gives 218 proteins in the mitochondrial carrier family. In databases, MCF members are often annotated as putative or hypothetical mitochondrial carriers, peroxisomal Ca^{2+}-dependent carriers or simply unknown proteins. A variety of substrate carrier proteins that are involved in energy transfer are found in the inner mitochondrial membrane or are integral to the membrane of other eukaryotic organelles such as the peroxisome of animals and amyloplast of plants. Some are proposed to transport adenine nucleotides, phosphate, protons or dicarboxylates, based on amino acid sequence (Millar and Heazlewood 2003).

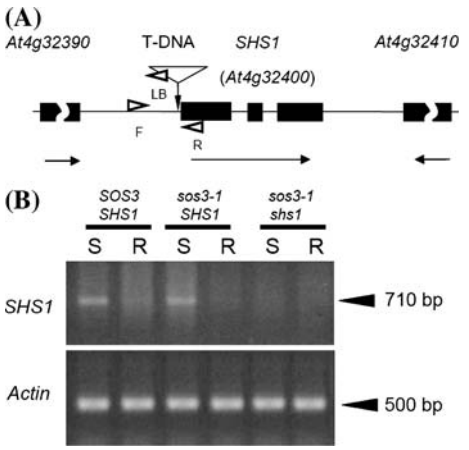

Fig. 4 *SHS1* locus was determined using TAIL-PCR and T-DNA was co-inherited with Na^+ sensitivity phenotype of *sos3-1 shs1-1*. (**A**) Schematic illustration indicates the location of the T-DNA insertion in *SHS1* (At4g32400) on chromosome #4. Closed boxes and arrows under the closed boxes indicate the coding region of a gene and direction of the predicted transcription, respectively. Open arrowheads indicate primer position (F, R and LB) and direction for diagnostic PCR. (**B**) Abundance of *SHS1* transcript was observed in the shoots of wild-type (*SOS3 SHS1*) and *sos3* (*sos3-1 SHS1*) but not in *sos3-1 shs1-1*. 2 µg of total RNA extracted from 12-day-old seedlings was used as template for synthesis of first-strand cDNA. 1 µl of the cDNA product was used for PCR (26 cycles). Primer set was designed to amplify the fragment, predicted size is 710 bp, from the middle of the first exon to the middle of the third exon in order to detect the fragment derived from cDNA or from genomic DNA. Actin gene was used as a positive control. S: shoots. R: roots

Expression of *SHS1* complements salt sensitivity, growth defect and leaf shape

To demonstrate that the mutation in At4g32400 is responsible for the *sos3-1 shs1-1* mutant phenotype, a 1.6 kb genomic fragment, including the coding region of At4g32400, under the control of CaMV 35S promoter was transferred into *sos3-1 shs1-1* plants. PCR analysis confirmed 12 out of 13 T_1 plants contained the transgene after hygromycin selection. T_2 progeny of six T_1 lines (#2, 3, 8, 10, 11 and 15) segregated at a ratio of 3:1 for *sos3-1 SHS1: sos3-1 shs1-1* phenotypes on MS medium containing 45 mM NaCl ($\chi^2 = 1.588$, $P < 0.1$). The progeny of three lines (1, 7 and 9) either did not have any or had a low number of *sos3-1 shs1-1* segregants, presumably because there were multiple insertions (Table 1). No transgene was detected in the *sos3-1 shs1-1* phenotype plants and PCR analysis showed the transgene co-segregated with the *sos3-1* phenotype. Furthermore, expression of *SHS1* in *sos3-1 shs1-1* seedlings suppressed the growth defect on media with and without NaCl (Fig. 5), and the transgenic plants expressing *SHS1* showed the *sos3-1* phenotype in leaf shape and no necrotic lesions on leaves were observed when grown in a greenhouse (picture not shown). The complementation results demonstrate that the mutation

Table 1 Overexpression of *SHS1* complemented the Na^+ sensitivity phenotype of *shs1*

Genotype	Number			Root length (mm ± SD)
	Total	*sos3-1 SHS1*[a]	*sos3-1 shs1-1*[a]	
sos3-1 SHS1	12	12		20.0 ± 4.3
sos3-1 shs1-1	12	0	12	9.0 ± 4.6
sos3-1 shs1-1 (vector)	12	0	12	9.5 ± 3.7
sos3-1 shs1-1 (35S: *SHS1*) #1	24	24	0	22.05 ± 5.9[b]
sos3-1 shs1-1 (35S: *SHS1*) #2	13	10	3	18.8 ± 4.6[b]
sos3-1 shs1-1 (35S: *SHS1*) #3	23	16	7	19.1 ± 4.9[b]
sos3-1 shs1-1 (35S: *SHS1*) #7	22	21	1	19.4 ± 4.5[b]
sos3-1 shs1-1 (35S: *SHS1*) #8	21	18	3	22.00 ± 5.0[b]
sos3-1 shs1-1 (35S: *SHS1*) #9	15	15	0	19.0 ± 5.6[b]
sos3-1 shs1-1 (35S: *SHS1*) #10	13	11	2	18.3 ± 4.7[b]
sos3-1 shs1-1 (35S: *SHS1*) #11	15	12	3	21.5 ± 2.7[b]
sos3-1 shs1-1 (35S: *SHS1*) #15	26	22	4	18.9 ± 3.5[b]

[a] The number of plants that show *sos3-1 SHS1 or sos3-1 shs1-1* phenotype

[b] Average was calculated without *sos3-1 shs1-1* phenotype seedlings

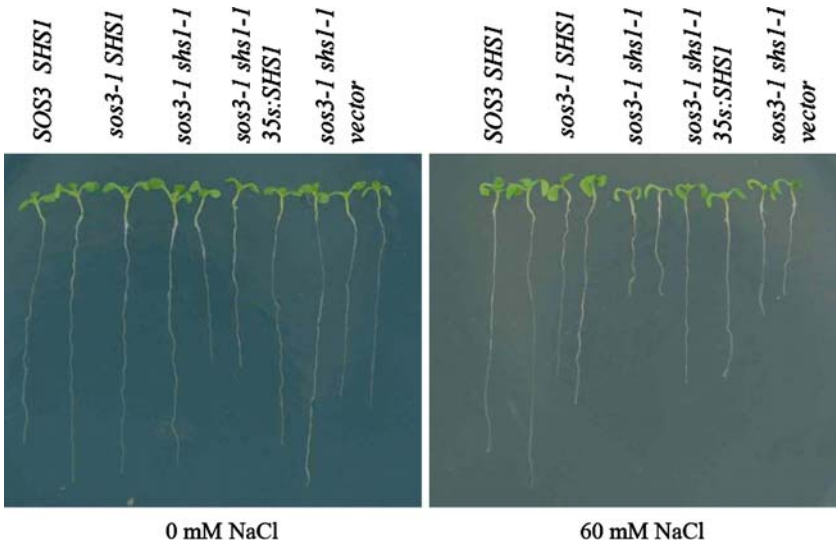

Fig. 5 Constitutive expression of *SHS1* gene complemented the *sos3-1 shs1-1* mutant. Four-day-old seedlings of *sos3-1 SHS1*, *sos3-1 shs1-1* and *sos3-1 shs1-1* with the wild-type of *SHS1* gene under the control of 35S promoter (T_2) were transferred onto MS medium supplemented with 60 mM NaCl. Photo was taken 5 days after transferring

(*shs1-1*) in *SHS1* is responsible for Na^+ sensitivity of *shs1-1 sos3-1*.

shs1-1 Single mutation shows growth defect and altered leaf shape but not salt sensitivity

We isolated the *shs1-1* single mutant (*SOS3 shs1-1*) from F_2 progeny resulting from a cross between *Col-0 gl1* (*SHS1 SOS3)* x *shs1-1 sos3-1*. The genotype of the single mutant was confirmed with diagnostic PCR using 2 primer sets: one is for detecting the *shs1-1* allele and the second is for the *SOS3* allele (data not shown). *shs1-1* Seedlings showed no notable growth reduction on MS medium containing up to 125 mM NaCl 6 days after transfer compared with the wild type *Col-0 gl1* (Fig. 6B). Relative growth in root elongation of *shs1-1* mutants was also similar to that of the wild type when treated with various concentrations of NaCl. However, *shs1-1* plants showed an apparent growth defect on the medium without NaCl (Fig. 6A). Similar results were obtained from plants grown in Turface. Two-week-old plants was transferred into Turface® and grown for 22 days. The *shs1-1* plants were smaller than *Col-0 gl1* with less average fresh and dry weight (data not shown). Furthermore, deformity in leaf morphology like that seen in *sos3-1 shs1-1* plants was also observed in *shs1-1* (Fig. 6C). These results indicate that the *shs1-1* mutation causes not only NaCl sensitivity but also reduced root growth and altered leaf shape. However, when the seedlings were treated with salt on MS media, the contribution of the *shs1-1* mutation to salt hypersensitivity was limited. The fact that growth reduction and leaf morphology phenotypes were observed in both the *sos3-1 shs1-1* and *shs1-1* mutants suggests that these phenotypes are *shs1* specific.

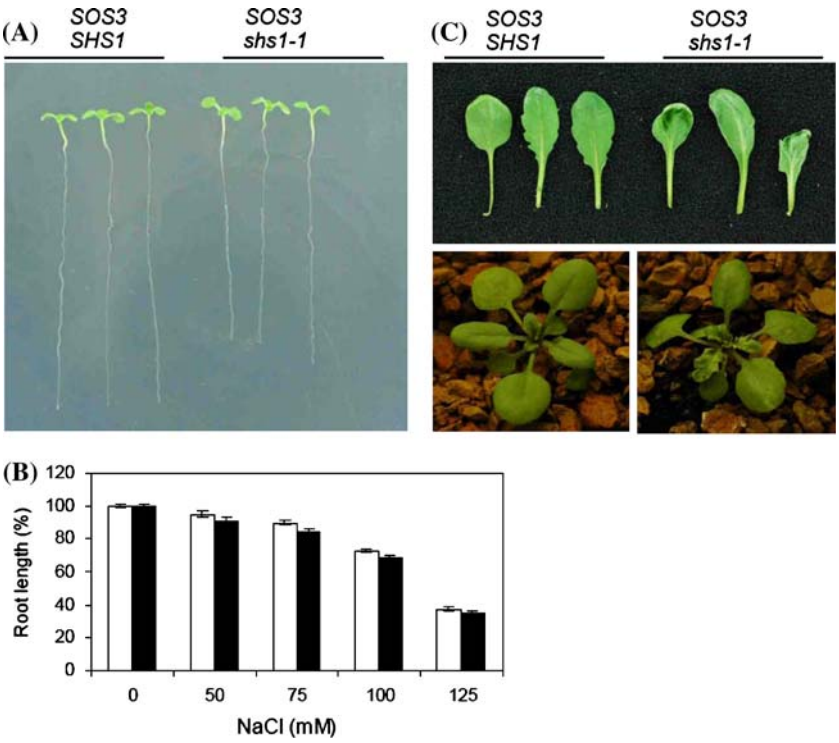

Fig. 6 Salt treatment on *SOS3 shs1-1* seedling (**A**) Growth of *SOS3 SHS1* and *SOS3 shs1-1* seedlings on MS medium for 5 days. (**B**) *SOS3 SHS1* and *SOS3 shs1-1* plants were grown in soil for 30 days without any treatment. (**C**) Root growth of plants grown on MS medium supplemented with NaCl for 2 weeks. White bar, *SOS3 SHS1*; black bar, *SOS3 shs1-1*. Bars represents mean ± SE, $n = 15$

The *shs1-1* mutant plants show a sugar hypersensitive phenotype during early seedling development

High concentrations of exogenous sugars are known to inhibit early seedling development of *Arabidopsis* (Leon and Sheen 2003). When we sowed *shs1-1* mutant seeds onto regular sucrose media (3%), all *shs1-1* seeds germinated. However, unlike the wild type, the geminating *shs1-1* seedlings were bleached and purple and unable to form green cotyledons until the 4th day after germination (Fig. 7A). When placed onto sugar free medium, *shs1*-1 mutant seedlings exhibited a phenotype similar to wild type with open cotyledons and green leaves (Fig. 7B-1). Furthermore, **e**xpression of *SHS1* under control of 35S promoter complemented the sugar hypersensitivity phenotype (data not shown).

To evaluate whether the sugar sensitivity observed in *shs1-1* was specific to sucrose, we replaced sucrose in the media with glucose (2% and 3%), maltose (4%) and the glucose analog 3-0-methylglucose (3%). The *shs1-1* mutants were hypersensitive to glucose (Figs. 7B, 8A), and the effect was gradually reversed during prolonged growth after germination. In addition, the response of the mutant to maltose was more severe (Fig. 8B). In maltose media, the mutant plants were unable to form open and fully expanded cotyledons in the first 3 days after germination, and then beyond this time, formed smaller leaves with anthocyanin accumulation (Fig. 8C). In contrast, the mutant plants showed no alteration in sensitivity when germinated on 3-0-methylglucose (3%) (data not shown). The *shs1-1* mutant was also examined for resistance to mannose (Fig. 8D). Mannose is a glucose analog that has been postulated to affect sugar-regulated gene expression (Jang and Sheen 1994) and seed germination (Pego et al. 1999) through a hexokinase-mediated signaling pathway. The *shs1* mutation confers significant sensitivity to mannose. Because the concentration of mannose used in this experiment (2.7%) is quite low, the mannose-sensitive phenotype of mutant is not due to osmotic stress as demonstrated by Jang and Sheen (1994).

To further determine whether the increased sensitivity of *shs1-1* mutants to sugars was caused by the osmotic strength of the medium, we analyzed seedling development of the *shs1-1* mutants on the media supplemented with mannitol (3%) or sorbitol (3%). The greening of the germinating seedlings of mutants or wild-type was not affected, indicating that osmotic stress alone could not cause the same defects in seedling development induced by sucrose and glucose (data not shown) (Lu and Hills 2002). *shs1-1* Mutant plants did not show altered sensitivity to either mannitol or sorbitol during later seedling growth and development compared to wild-type (data not shown). Taken together, these results suggest that the *shs1-1* mutation specifically affects responses to sugars but not osmotic stress.

shs1-1 shows less sensitivity to ABA during germination and early growth

It is known that the plant hormone ABA affects seed germination and that soluble sugar levels dramatically alter the response of *Arabidopsis* seeds to ABA (Finkelstein and

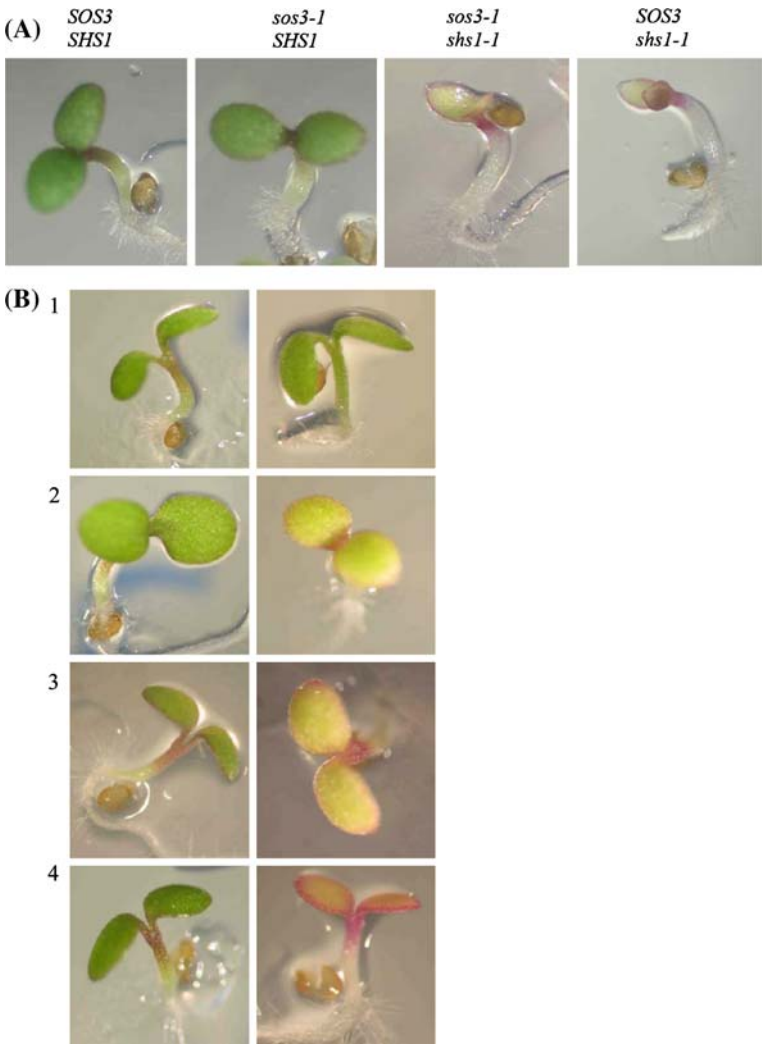

Fig. 7 The *shs-1* mutant plants showed sucrose hypersensitive phenotype. (**A**) The wild type and mutant plants are grown on MS media with 3% sucrose for 3 days. (**B**) The sugar hypersensitivity of *shs-1* mutant plants is dosage dependant. Wild type (left) and *shs1-1* (right). (1) MS medium with 0% glucose for 4 days. (2) MS medium with 2 % glucose for 4 days. (3) MS medium with 3% glucose for 4 days (4) MS medium with 4% glucose for 4 days

Gibson 2002). Therefore, we tested the response of the mutant and wild-type seeds on media containing a range of ABA concentrations with glucose. As shown in Fig. 9, the germination (radicle emergence) of *shs1-1* was significantly less sensitive to ABA than that of the wild type (Fig. 9A). *shs1-1* showed no reduction in germination rate when the wild type seeds had approximately 50% seeds germinated at 0.2 μM ABA. Moreover, post-germination growth of *shs1-1* mutants also exhibited reduced sensitivity to ABA inhibition (Fig. 9B–E).

Cold sensitivity of the *shs1-1* mutant

The microarray data from TAIR Microarray Expression Search and NASC Arrays website showed changes in *SHS1* expression in Col-0 plants grown in vitro in response to low temperature over time (http://www.arabidopsis.org/). We then examined the response of *shs1-1* mutant seedlings to cold treatment (4°C). We found that the mutant plants were also sensitive to cold. The treated *shs1-1* plants displayed more severe lesions on the leaves compared to control plants (Fig. 10).

SHS1 is localized to the endoplasmic reticulum

Leroch et al. (2005) found that StBT1 from *Solanum* to be localized in plastids similar to other BT1 proteins. Subcellular localization can provide insights for possible functions of SHS1. To determine the subcellular localization of SHS1, the Green Fluorescence Protein (GFP) cDNA was fused in frame to the C-terminal coding sequence *AtSHS1* and the resulting *AtSHS1::GFP* was transiently expressed in *Arabidopsis* mesophyll protoplasts (Jin et al. 2001). Green fluorescent signals revealed tubular structures in the chloroplast free cytosolic region of the cell (Fig. 11A), which is similar to the network patterns of the

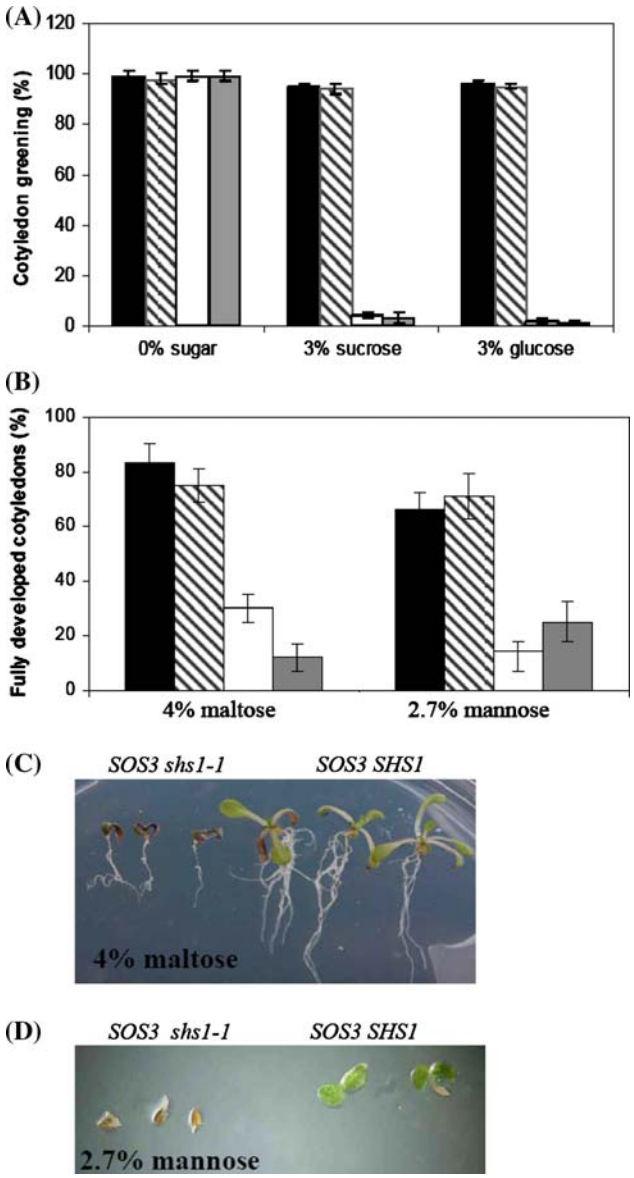

Fig. 8 Seedling development of *shs1-1* mutant plants with different sugars. Seedlings were germinated on the media supplemented with (**A**) 0% sugar, 3% sucrose and 3% glucose. (**B**) 4% maltose and 2.7% mannose. Data were taken at the 3rd day. (**C**) Mutant and wild-type plants at 4% maltose and (**D**) 2.7% mannose at day 12. *SOS3 SHS1* (black bar), *sos3-1 SHS1* (shaded bar), *SOS3 shs1-1* (white bar), *sos3-1 shs1-1* (gray bar). Cotyledon greening rate was expressed as the percentage of greening seedlings based on germinated seeds. Error bars indicate mean ± SE, $n = 100$

ER marker, BiP:RFP (Jin et al. 2001). To elucidate possible ER localization of SHS1, *SHS1:GFP* and *BiP:RFP* were transiently co-expressed in *Arabidopsis* mesophyll protoplasts. Red fluorescent signals represent the tubular structure of ER network, which overlapped (merged) with the majority of green fluorescent signals (Fig. 11). These results suggest that SHS1:GFP fusion proteins are associated with the ER membrane but not the chloroplast.

Discussion

Salt tolerance determinants can be identified using the *sos3-1* background

The SOS pathway exerts a powerful influence on the salt tolerance mechanisms of glycophytes and the expression of its components are tightly regulated depending on different environmental conditions. Therefore modulators of this pathway can be considered to be involved with the extent of salt tolerance, although their individual contribution to salt tolerance could be minor. The original *sos* mutants were identified by selection in *Arabidopsis* wild type using a root-bending assay (Wu et al. 1996). Using wild type to screen for extreme salt sensitivity is difficult and may lead to other SOS pathway components. For these reasons, we screened, in the genetic background of *Arabidopsis* Col-0 *gl1 sos3-1* (Rus et al. 2001) which is already salt sensitive. One of the resulting mutants isolated from this screen was characterized as *shs1-1* (for *salt hypersensitive 1*).

SHS1 is not a component of the SOS signal pathway

The *shs1* mutation enhances the Na^+ sensitivity of the *sos3-1* mutant but not to Li^+, K^+, Cl^- or mannitol (Fig. 3). All *sos* mutants display LiCl hypersensitivity (Zhu et al. 1998; Wu et al. 1996; Liu and Zhu 1997). However, the *shs1* mutation does not enhance, yet slightly increased, Li^+ sensitivity of *sos3-1*. The double mutant (*sos3-1 shs1-1*) also showed greater sensitivity to NaCl than did the single mutants (*sos3-1 SHS1* and *SOS3 shs1-1*), indicating that additive effects of the mutation exist. Furthermore, the sensitivity of *SOS3 shs1-1* to LiCl, KCl and mannitol was also examined and no difference between *SOS3 shs1-1* and controls was observed (data not shown). Therefore, SHS1 regulation of NaCl tolerance is genetically independent from the SOS pathway.

shs1-1 shows sugar hypersensitivity

Sugar-dependant responses, such as inhibition of chlorophyll synthesis (inability to form green cotyledons) and the accumulation of anthocyanin in the early stages of development were observed in the *shs1-1* mutant plants (Figs. 7 and 8). The *shs1* mutation causes hypersensitivity to exogenous levels of sucrose and glucose during germination and early growth (Fig. 7). Five days after germination the mutants resembled the wild-type. The primary structure of At4g32400 is highly similar to a group of proteins called BRITTLE-1 like proteins, which are suggested to be amyloplast ADP-Glucose (ADP-Glc) transporters (Shannon

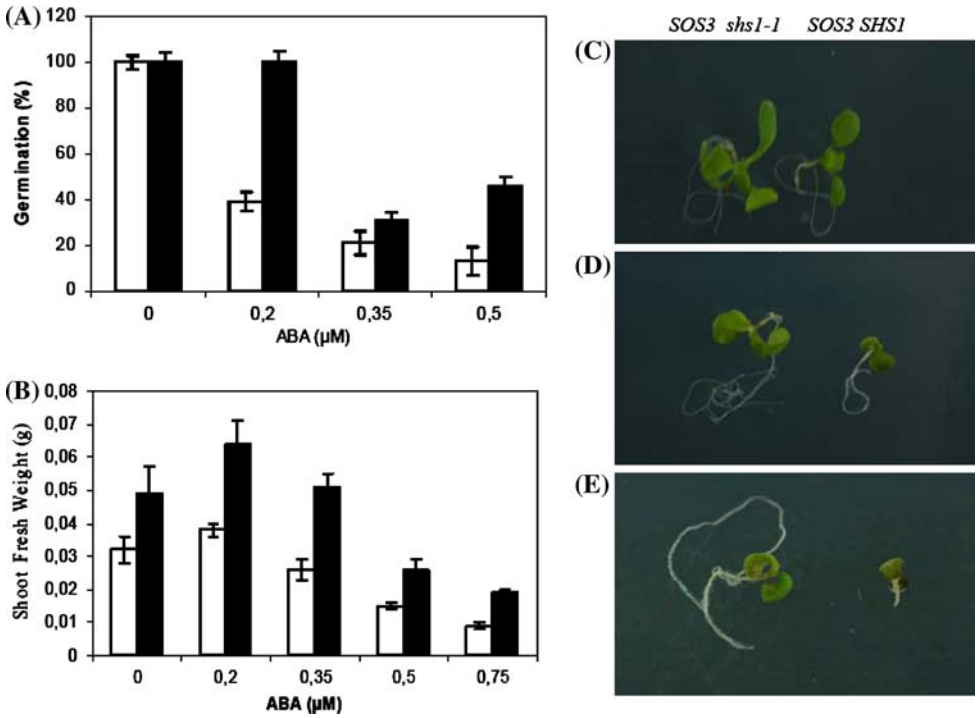

Fig. 9 *shs1-1* shows insensitivity to ABA during germination and early growth. Seedlings were germinated on the media for 10 days (with 2% Glc) supplemented with various amounts of ABA. (**A**) Germination data was taken after 5 days. (**B**) Shoot fresh weight was measured after 10 days after radicle emergence. Error bars indicate SE, $n = 100$ (**C**) MS medium with 0 μM ABA and 2% Glc. (**D**) MS medium with 0.5 μM ABA and 2% Glc. (**E**) MS medium with 0.75 μM ABA and 2% Glc. *SOS3 SHS1* (white bar), *SOS3 shs1-1* (black bar)

Fig. 10 Chilling sensitivity of *shs-1* mutant. Picture was taken after treatment for 4 weeks in 4°C

et al. 1998; Patron et al. 2004) important in starch metabolism of cereal endosperms. Furthermore, an ADP-Glc binding motif was identified in the sequence of *SHS1* at a similar position as other starch synthesizing enzymes (data not shown). This motif has been suggested to be common among ADP-Glc binding proteins of starch metabolism (Furukawa et al. 1993). ADP-Glc uptake has been shown for amyloplasts from wheat and maize. The BT1 protein probably acts as an ADP-Glc translocator. Although BT1 shows significant structural homology to all members of the mitochondrial carrier family, it is localized to the chloroplast inner envelope membrane. Mutations at the *BT1* locus result in a reduction of starch accumulation and in an increase of the ADP-Glc content of immature endosperm. In maize and barley *bt1* mutant plants, accumulation of ADP-Glc in endosperm tissue leads to changes in *bt1* mutant seeds. The reduced starch syhtesis in kernels results in a collapsed angular appearance at maturity (Sullivan et al. 1991). Recent research showed that StBT1 may function as a plastidic adenine nucleotide uniporter to transport the adenine nucleotide from plastids to other compartments (Leroch et al. 2005). However, SHS1 protein is located in the ER and the shrunken phenotype of *bt1* mutant seeds could not be observed in *shs-1* seeds by a visual observation (data not shown). This discredits the possibility of a similar function for SHS1 in the ADP-Glc transport across the chloroplast leading to sugar sensitivity due to the accumulation of ADP-Glc in the cytoplasm.

The *sugar-insensitive*, or *sis* (Laby et al. 2000) and *glucose-insensitive*, or *gin* (Arenas-Huertero et al. 2000), mutants exhibit reduced sensitivity to high sugar concentrations. There have been no reports regarding the response of *gin* mutants to sucrose (Suc). However, the *sis* mutants have altered responses to both sugars (Laby et al. 2000). These results suggest that sucrose inhibits wild-type seedling development by being metabolized to glucose (Glc), thereby triggering a hexose-response pathway. Besides being insensitive to high concentrations of Glc and Suc,

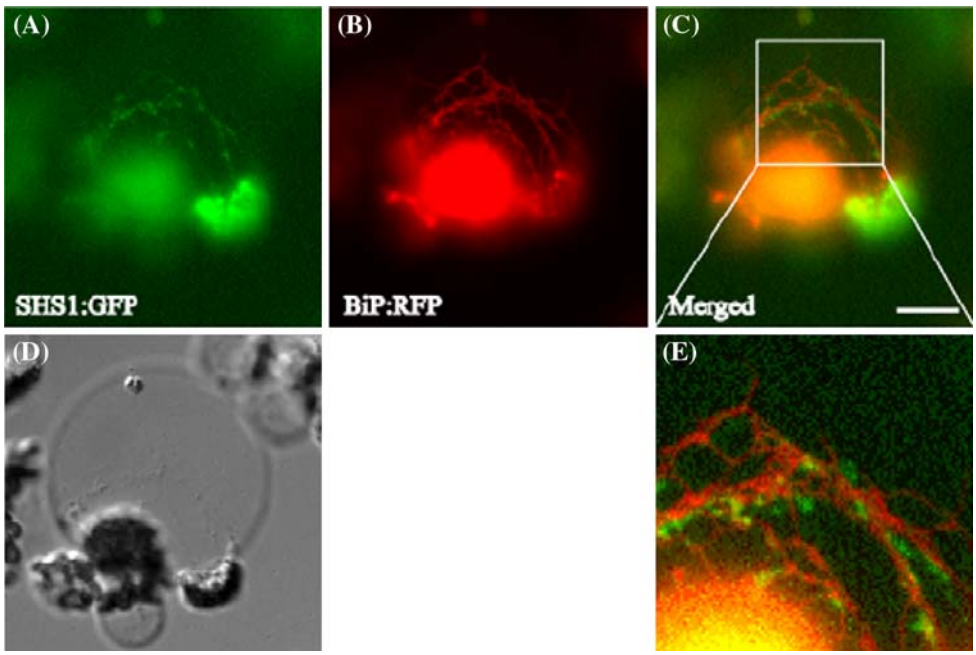

Fig. 11 AtSHS1:GFP closely associates with ER network. SHS1:GFP and BiP:RFP were transiently co-expressed in protoplasts and green and red fluorescent signals were analyzed. Merge, merging of GFP and RFP images; **D**, transmitted light image. **E**, enlargement of image box in **C**

most of the *sis* and *gin* mutants also exhibit decreased sensitivity to the inhibitory effects of exogenous mannose (Man) at concentrations too low to exert an osmotic stress (Zhou et al. 1998; Laby et al. 2000). Similarly, *shs1-1* mutants also displayed Suc and Glc as well as Man hypersensitivity (Fig. 7). Man is a Glc analog that has been postulated to inhibit seed germination via a hexokinase-mediated sugar-response pathway (Pego et al. 1999). However, Man metabolism has yet to be analyzed in *Arabidopsis*. Therefore, the possibility remains that hexokinase-mediated inhibition of *Arabidopsis* seed germination by Man is the result of Man-6-P being metabolized to form a toxic product, rather than of induction of a sugar-response pathway. As a result, the possibility that some sugar-response mutants may have relatively broad defects in stress responses, rather than being specifically defective in sugar responses, cannot be ruled out at this time.

Lu and Hills (2002) showed that high concentrations of mannitol did not affect greening of the seedlings in wild-type or sugar sensitive plants indicating that osmotic stress alone could not induce the seedling development defect that was induced by high glucose. *Arabidopsis* seeds sown on media containing high (e.g. 0.3 M) concentrations of Suc germinate, but the majority of seedlings fail to develop green, expanded cotyledons or true leaves. High concentrations of exogenous Glc exerted a similar effect. It is interesting that germinating seeds/seedlings are only sensitive to high sugar concentrations during approximately the first 48 h after the start of imbibition. We observed that *shs1-1* mutant also shows greatest sensitivity to sugar during this time period.

shs1-1 is insensitive to ABA

The lack of greening at early development stages can be explained on the basis of altered ABA responses of the *shs1-1* mutant. Abscisic acid and sugars similarly affect seedling growth. Both promote growth at low doses, but inhibit it at high concentrations. In addition, ABA and sugars both suppress the expression of photosynthetic genes. Previous studies showed that mutants for biosynthesis and signaling of ABA show a sugar insensitive phenotype during seedling development (Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000). Recent genetic studies show that all ABA-deficient mutants and some ABA signaling mutants (i.e. *abi4* and *abi5*) are insensitive to sugars, indicating that ABA and part of its signaling cascades are integral components of the sugar response pathway. The glucose insensitivity of the *abf2* mutant seedlings demonstrates that ABF2 is also a positive component of the glucose signal transduction (Kim et al. 2004). The *shs1-1* mutant showed a higher germination rate then the wild-type under all ABA conditions tested suggesting that the glucose hypersensitivity of the mutant might be caused by ABA insensitivity (Fig. 9).

A number of genes that determine the plant response to ethylene and abscisic acid have also been shown to be involved in early seedling sugar sensing (Gazzarini and McCourt 2001). Although results suggest that ABA signaling and carbon homeostasis are tightly coupled, these interactions are complex since only a subset of ABA response mutants alter the response of plants to high sugar. ABA synthesis and loss-of-function mutations in ABI4 or ABI5 confer a sugar-insensitive phenotype, but ABI1-1,

ABI2-1, and ABI3-1 (mutations that reduce ABA sensitivity) do not show altered sugar sensitivity. ABI3 has an allelic specific role in sugar-ABA interactions and has been suggested to perceive a sugar signal with more than one domain (Nambara 2002).

The results presented here are consistent with an indirect role for SHS1 as a nucleotide-transporter in the ER that can affect the ABA responsive transcription factors ABI3 and ABI5 which are involved in ABA-sugar signaling interactions. This possibility should be further investigated.

SHS1 affects multiple stress tolerance

Although not all stress responses are mediated by ABA (Shinozaki and Yamaguchi-Shinozaki 2000), ABA is essential for full protection against most of the common abiotic stresses: drought, cold, high salinity, high temperature, and oxidative stress (Thomashow 1999; Schroeder et al. 2001; Xiong et al. 2002). Our results indicate that *SHS1* is likely involved in the adaptation to multiple stresses, including salt and cold stress.

SHS1 is localized in the endoplasmic reticulum

SHS1 is located in the endoplasmic reticulum (ER) and, together with a nucleotide-sugar binding site motif in its sequence and the existence of a mitochondrial carrier family domain, suggest that SHS1 might be a nucleotide sugar transporter of the ER. To investigate the possible function of SHS1, the sequence was compared with other known transporters in the ER. *SHS1* has no obvious sequence similarities at the amino acid level to nucleotide sugar transporters (NSTs) that are found in the Golgi apparatus and ER of eukaryotes. NSTs belong to the drug/metabolite transporter superfamily of transporters. Considering the ER location, it is unlikely that the function of SHS1 is in starch metabolism similar to its homologs ZmBT1, HvBT1 or StBT1. However, sequence data suggests that SHS1 might be a nucleotide-Glc transporter. Further, combined with its localization in the ER, these data lend support to a role for SHS1 in the glycosylation of proteins in ER as a potentially novel member of NST protein family in the ER.

Glycosylation of proteins, proteoglycans and sphingolipids occur in the lumen of the Golgi apparatus. The nucleotide substrates of these reactions (nucleotide sugars) are generally sythesized in the cytosol and must be translocated into the lumen of the Golgi before they can be used in the post translational modification. NSTs establish a functional link between membrane transport of the nucleotide sugars synthesized in the cytoplasm and the glycosylation processes that takes place in the ER and Golgi apparatus. The transport of nucleotide sugars has been detected in vitro using Golgi vesicles from mammalian tisues and cell lines of yeast, protozoa and plants.

NST have mainly been characterized in the Golgi apparatus. However, NST located in the ER have also been described. A subfamily of NSTs, consisting of *A. thaliana* sequences have not been identified but functionally catagorized. They contain an ATP/GTP-binding site motif that was detected by PROSITE (Database of protein domains, families and functional sites) (http://www.expasy.ch/prosite/) and fits the pattern of "[AG]xxxxGK[ST]". This particular site (AVYLLGKS) is located in the predicted cytosolic loop 2. It is a conserved motif among numerous proteins that bind ATP and GTP, and it permits the formation of a loop between a beta strand and an alpha helix. This loop may interact with the phosphate group of the nucleotide and suggests that it could be of relevance to the cytosolic nucleotide recognition of this subfamily.

Recently, the first two plant Golgi NSTs were characterised from *Arabidopsis thaliana:* GONST1 is capable of transporting GDP-Man (Baldwin et al. 2001), whereas AtUTR1 transports UDP-galactose and UDP-glucose (Norambuena et al. 2002). Analyses of mutants that are defective in nucleotidesugar transport into the Golgi have demonstrated the physiological relevance of NSTs in yeast, nematodes, protozoa, Drosophila, mammalian cell lines and humans. In each of these cases, mutations in NSTs result in a loss of the corresponding sugar in glycoconjugates, which can in turn produce dramatic phenotypes (Segawa et al. 2002). Similar studies in plants have not yet been reported.

The relationship between the sugar responsive phenotype and NaCl sensitivity of SHS1 is difficult to explain because the ER localization of SHS1 reduces the likelihood that starch synthesis, and therefore reduced availability of sugar-based osmoprotectants, are responsible for the phenotypes observed in *shs1-1*. Moreover, the *shs1* mutant was not responsive to osmotic stress applied by sorbitol and mannitol. There are other mutants that are sugar responsive but independent of an osmotic component. Several lines of evidence suggest that these sugar-response phenotypes of the mutants are not simply a result of osmotic-stress tolerance. Osmo-tolerant and sugar-response phenotypes are genetically separable, as some mutants, such as the *abi2-1* mutant, that are osmo-tolerant exhibit normal sugar responses (Laby et al. 2000). In contrast to the *sis* and *gin* mutants, the *pleiotropic regulatory locus 1* (*prl1*) mutant shows increased sensitivity to Glc and Suc (Németh et al. 1998). The *PRL1* gene encodes a protein with sequences characteristic of WD-40 repeat proteins (Németh et al. 1998). It is interesting that the PRL1 protein interacts with the yeast SNF1 protein, as well as with two SNF1-related

proteins from *Arabidopsis*, in a yeast two-hybrid system (Bhalerao et al. 1999). These results suggest that PRL1 may play an important role in one or more sugar-response pathways. However, as SNF1 and related proteins may act in stress responses, as well as in sugar responses, the possibility that PRL1 primarily functions in stress responses must also be considered (Gibson and Graham 1999). A small clue for a possibility of such mechanism for the SHS1 protein comes from a result of COG (Clusters of Orthologous Groups) database search (data not shown). One of the top five matched COGs is a WD-40 repeat family protein of yeast with 30% similarity. This family of highly conserved proteins has been shown to play a role in numerous cellular functions including signal transduction, mRNA processing, gene regulation, vesicular trafficking and regulation of the cell cycle and chromatin remodeling.

Materials and methods

Isolation of the *sos3-1 shs-1* mutant plants

Arabidopsis thaliana plants (Col-0 *gl1 sos3-1*) were mutagenized with an *Agrobacterium tumefaciens*-mediated (strain GV3101) T-DNA transformation using the activation tagging vector pSKI015 provided by Dr. Weigel, The Salk Institute, La Jolla, CA. 66750 T_2 plants resistant to bialaphos (30 mg/l) were screened for mutants that exhibited increased NaCl sensitivity compared to *sos3-1*. Seeds were surface sterilized and sown onto cellophone mebrane (BIO-RAD, no. 1650963, Hercules, CA) that was placed over germination medium [1X of the Murashige and Skoog (MS) salt formulation (Murashige and Skoog 1962), B5 vitamins, 3% sucrose and 1.2% agar, pH5.7]. Four-day-old seedlings were inoculated onto NaCl containing medium (basal constituents and 75 mM NaCl) by transferring the entire cellophane membrane onto the fresh medium. After 15 days, hypersensitive plants were rescued and the progeny (T_3) of the plants were obtained.

PCR analyses for identification of *S0S3* and *sos3-1* alleles

DNA was isolated from T_3 mutant progeny by using a modified rapid DNA mini-preparation procedure (Liu et al. 1995) and used as a template to confirm the *sos3-1* genetic background. The forward primer for *SOS3* was 5′-ATGTGCT TTCAAGTTGTACG-3′ and for *sos3-1* was 5′-GCT GTGCTTTCAAGTTCAAG TTACG-3′. The same reverse primer was used to detect either allele: 5′-TTTA TCTTTCCTTGCATGGC-3′.

Thermal asymmetric interlaced (TAIL)-PCR for identification T-DNA flanking region

To determine the genomic sequence flanking the T-DNA left border, thermal asymmetric interlaced-PCR analysis (Rus et al. 2001) was done. A nested set of three primers (oriented outward) corresponding to the sequence in the T-DNA left border was used in the successive PCR reactions: Left border primer 1: 5′-ATACGACGGATC GTAATTTGTC-3′, Left border primer 2: 5′-TAATAACGCT GCGGACATCTAC-3′, Left border primer 3: 5′-TTGACCATCATACTCATTGCTG-3′, Degenerate primer 1: 5′-WGCNAGTNAGWANAAG-3′, Degenerate primer 2: 5′-AWGCANGNCWGA NATA-3′, W = A/T and N = A/T/G/C. The nucleotide sequence of the PCR product was determined and subjected to BLASTN analysis.

Detection of *SHS1* and *shs1-1* allele

The *SHS1* wild-type allele was identified by PCR analysis by using forward (5′-CTCGTCGGCAATTATTTCGTGG-3′) and reverse (5′-TGACACTTCAGTGCTGGGTT-3′) primers corresponding to genome regions flanking both sides of the T-DNA insertion in the *sos3-1 shs-1* mutant. Detection of the mutant *shs1-1* allele was based on reactions using the forward primer for amplification of *SHS1* sequence and as a reverse primer, the T-DNA LB primer 3 (5′-TTGACCATCATACTCATTGCTG -3′). The inheritance of the T-DNA insertion in the F_2 plants of *sos3-1 SHS1* x *sos3-1 shs1-1* was confirmed by PCR.

Reverse transcription (RT)-PCR analysis of *SHS1* expression

Total RNA extracted from shoots and roots of 12-day-old seedlings using Trizol Reagent® and 2 μg of total RNA was used for first-strand cDNA synthesis with thermoscript™ RT-PCR system (Invitrogen, Carlsbad, CA). Gene specific primers for *SHS1* were as follows: Forward primer 5′-AGTAGTTAATGGCGAGAAGAGG-3′ Reverse primer, 5′-ACTTCAAGGGAAAGTTGCAG -3′. One micro liter of the cDNA product was used for PCR (26 cycles). The primer set was designed to amplify the fragment with a predicted size of 710 bp, from the middle of the first exon to the middle of the third exon in order to detect the fragment derived from cDNA or from genomic DNA. Primers specific to the actin gene were used as a positive control.

Germination and growth measurements

For root growth measurements, 4-day-old seedlings grown on vertical MS medium were transferred to fresh MS medium with varied concentration of NaCl, KCl, LiCl and mannitol. Root growth was measured after 5 days. For sugar and ABA sensitivity germination assays, seeds were sown to half strength MS containing various sugar and ABA concentrations. All plates were incubated at 4°C for 4 days and then transferred to a growth chamber with a light/dark cycle of 16/8 h at 21°C.

Complementation test

To clone the *SHS1* from Col-0 g*l1* genomic DNA, the set of primers were used for PCR as following: 5′-TAACCCGGGATTGGTCATGGGGAAAAC-3′ and 5′-GCCTCTAGACCACGAATCCATACAA-3′. The PCR-amplified fragment of *SHS1* digested with SmaI and XbaI was cloned into the region down stream of the 35S promoter in pTEX plasmid. The sequence of *SHS1* was confirmed. A fragment between Hind III and Xba I site was cut off from pTEX and sub-cloned into binary vector pBIB:HYG. The new vector of the pBIB:HYG was transferred into *Agrobacterium tumefacience* (strain GV3101). The resulting transformants were transferred into *sos3 shs1* mutant plants using the floral dip transformation protocol (Clough and Bent 1988). GV3101 containing PBIB:HYG without *SHS1* was used for transforming *sos3-1* as a control.

Plasmid construction and subcellular localization of SHS1:GFP

The *AtSHS1* coding region was in-frame fused to the N terminus of the GFP, which was constructed into a CaMV 35S promoter controlled transient expression vector (Jin et al. 2001). The plasmids were introduced into *Arabidopsis* protoplasts prepared from whole seedlings by polyethylene glycol-mediated transformation (Jin et al. 2001). Images were acquired using a SPOT RT-slider digital camera (Diagnostic Instruments, Inc.) on a NIKON E800 (Nikon, Tokyo, Japan) fluorescence microscope. GFP fluorescence emitted between 500 nm and 530 nm was detected. RFP fluorescence emission between 570 and 605 was detected. A transmitted light image was also detected as a reference.

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