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Increased tolerance to salt stress in the phosphate-accumulating *Arabidopsis* mutants *siz1* and *pho2*

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Abstract

High salinity is an environmental factor that inhibits plant growth and development, leading to large losses in crop yields. We report here that mutations in *SIZ1* or *PHO2*, which cause more accumulation of phosphate compared to the wild type, enhance tolerance to salt stress. The *siz1* and *pho2* mutations reduce the uptake and accumulation of Na^+. These mutations are also able to suppress the Na^+ hypersensitivity of the *sos3-1* mutant, and genetic analyses suggest that *SIZ1* and *SOS3* or *PHO2* and *SOS3* have an additive effect on the response to salt stress. Furthermore, the *siz1* mutation cannot suppress the Li^+ hypersensitivity of the *sos3-1* mutant. These results indicate that the phosphate accumulating mutants *siz1* and *pho2* reduce the uptake and accumulation of Na^+, leading to enhanced salt tolerance, and that, genetically, *SIZ1* and *PHO2* are likely independent of *SOS3*-dependent salt signaling.

Keywords

phosphate, salt signaling, salt tolerance

Abbreviations

ICP-AES inductively coupled plasma- Atomic Emission Spectrometry

MS Murashige and Skoog

NaPi Na^+-coupled phosphate transporter

Pi phosphate

ROS reactive oxygen species

SA salicylic acid

SUMO small ubiquitin-related modifier

Introduction

High salinity affects plant growth and development, as do specific toxic ions (Binzel and Reuveni 1994; Zhu 2002). A living cell must maintain intracellular ionic homeostasis (Mahajan et al. 2008). Na^+ is the major toxic ion in most saline soils. As the soil dries, the concentration of salt in the soil increases. Glycophytes, including most crop plants, are sensitive to saline conditions because these plants are sensitive to the accumulation of Na^+ in shoot tissues (Tester and Davenport 2003). The maintenance of K^+ and Na^+ homeostasis becomes crucial under salt stress (Mahajan et al. 2008). Salt stress causes the disruption of ion homeostasis in plants, the excess accumulation of toxic Na^+ and deficiency of essential ions, including K^+ (Hasegawa et al. 2000). This stress inhibits the activity of many essential enzymes, cell division and expansion, and membrane disorganization, leading to growth inhibition (Zhu 2002).

Several ion transporters play roles in pumping ions out of plant cells. Na^+ is also transported into vacuoles through tonoplast-localized Na^+/H^+ antiporters (Apse et al. 1999). Na^+ efflux is mediated by the plasma membrane Na^+/H^+ antiporter SOS1 (Qiu et al. 2002; Shi et al. 2000; Shi et al. 2002). SOS1 also controls the loading and unloading of Na^+ in the xylem and phloem to mediate long-distance Na^+ transport (Shi et al. 2002). SOS1 is activated by the SOS2 and SOS3 proteins under high salt conditions (Qiu et al. 2002; Quintero et al. 2002). SOS3 is a myristoylated calcium-binding protein that is capable of sensing cytosolic calcium changes elicited by salt stress (Liu and Zhu 1998; Ishitani et al. 2000). SOS3 binds to the 21-amino acid FISL motif in SOS2 (Guo et al. 2001; Sánchez-Barrena et al. 2007).

HKT1 is a high-affinity K^+/Na^+ co-transporter and Na^+ influx transporter (Schachtman and Schroeder 1994; Gassman et al. 1996). At high Na^+ concentrations, K^+ transport via HKT1

is blocked (Rubio et al. 1995; Gassmann et al. 1996; Rubio et al. 1999). The *Arabidopsis* genome encodes a single *HKT1* gene (Uozumi et al. 2000). Expression of *AtHKT1* in *Xenopus laevis* oocytes and *Saccharomyces cerevisiae* demonstrated that AtHKT1 has a relatively Na^+-selective transport activity in these heterologous expression systems (Uozumi et al. 2000; Mäser et al. 2002). AtHKT1 is localized to the plasma membrane of xylem parenchyma cells in the shoot (Sunarpi et al. 2005). Loss-of-function mutations in AtHKT1 ameliorate sos3 Na^+-hypersensitivity (Rus et al. 2001; Rus et al. 2004). AtHKT1 is thought to function in retrieval of Na^+ from the xylem (Mäser et al., 2002; Sunarpi et al., 2005; Davenport et al., 2007).

Screening of second-site mutations that enhance or reduce NaCl tolerance led to the identification of one suppressor, *hkt1*, and two enhancers, *enh1* and *shs1* (Rus et al. 2001; Miura et al. 2005; Inan et al. 2007; Zhu et al. 2007). *ENH1* encodes a chloroplast-localized protein with a PDZ domain at the N-terminal region and a rubredoxin domain in the C-terminal part and functions in the detoxification of reactive oxygen species produced in response to salt stress (Zhu et al. 2007). *SHS1* encodes a BRITTLE-1 like protein, a putative adenylate translocator, and may function in response to multiple stresses including salt, cold, and sugar (Inan et al. 2007). Herein, we demonstrate that the *siz1-1* mutation suppressed *sos3-1* NaCl hypersensitivity.

Na^+-dependent transport systems have been identified in several fungi, cyanobacteria, and algae. Na^+-dependent uptake of glucose, amino acids, and nitrate has been demonstrated in marine diatoms (Hellebust 1978; Rees et al. 1980). Na^+-coupled phosphate (Pi) uptakes been reported in fungal species (Versaw and Metzenberg 1995; Martínez and Persson 1998; Zvyagilskaya et al. 2001). Na^+ was found to stimulate Pi uptake in the green alga *Ankistrodesmus* (Ullrich and Glaser 1982). There are few reports about Na^+-coupled transport

systems in vascular plants, but the existence of a Na^+-dependent Pi uptake system cannot be excluded, as previously suggested (Rausch and Bucher 2002).

We have already demonstrated that SIZ1 is a SUMO (small ubiquitin-related modifier) E3 ligase that regulates the responses to Pi deficiency, drought, and cold and heat stress; innate immunity; flowering time; and development (Miura et al. 2005; Yoo et al. 2006; Lee et al. 2007; Miura et al. 2007a; Catala et al. 2007; Jin et al. 2008; Miura et al. 2010; Miura et al. 2011). The conjugation of SUMO to substrates (sumoylation) plays an important role in the regulation of diverse biological processes such as the regulation of transcription factors, DNA repair, and the compartmentalization of the substrates in yeast and metazoans (Geiss-Friendlander and Melchior 2007; Liu and Shuai 2008). Sumoylation occurs in a series of biochemical steps that are catalyzed sequentially by SUMO-activating, conjugating, and ligating enzyme (Geiss-Friendlander and Melchior, 2007; Miura et al., 2007b; Miura and Hasegawa, 2010). Biochemical and genetic evidence has established that the sumoylation process is conserved in plants (Miura et al. 2007b; Miura and Hasegawa 2010).

In this study, we demonstrated that mutations in *SIZ1* or *PHO2* enhance tolerance to NaCl in *Arabidopsis*. These mutants accumulated Pi as described (Delhaize and Randall 1995; Miura et al., 2005). The *pho2* mutant has been isolated as Pi over-accumulator from ethyl methylsulfonate-treated mutant population (Delhaize and Randall, 1995). When grown under high transpiration conditions, *pho2* accumulated high level of Pi and exhibited growth retardation. *PHO2* encodes ubiquitin-conjugating E2 enzyme (UBC24) (Aung et al., 2006). *PHO2* is a target of microRNA399 (Lin et al., 2008), which is regulated by a MYB transcription factor PHR1 (Bari et al., 2006). The *siz1* and *pho2* had decreased uptake and accumulation of Na.

Genetic analysis demonstrated that *SIZ1* and *PHO2* are likely to be independent of the *SOS3*-dependent signaling pathway.

Materials and Methods

Plant materials and growth conditions

The *Arabidopsis* T-DNA insertion mutant (*sos3-1 siz1-1*) was generated by mutagenizing *salt overly sensitive 3* (*sos3-1*; Liu and Zhu 1997) mutant plants (ecotype Col-*gl1*) with *Agrobacterium tumefaciens*-mediated T-DNA transformation (pSKI015) (Miura et al. 2005). The T-DNA insertion mutants (ecotype Col-0), *siz1-2* and *siz1-3*, were obtained as described (Miura et al. 2005). Unless indicated otherwise, 4-day-old seedlings were transferred to basal medium containing 1x Murashige and Skoog (MS) mineral salts, 3% sucrose, and 1.2% agar without and with various amounts of NaCl, KCl, LiCl, and CsCl. Root growth is the difference in root length between the beginning and the end of the growth evaluation period and was measured using ImageJ version 1.36b (http://rbs.info.nih.gov/ij). Three-week-old plants grown in soil were treated with various concentrations of NaCl twice per week. Sixteen days after treatment, fresh weight was measured (n = 15). Three independent experiments were performed. Significance was calculated by Student's t-test.

Ion content measurements

Three-week-old plants grown in soil under short day conditions (8 h light and 16 h dark or 10 h light and 14 h dark) were transferred to hydroponic culture containing 0.2 x Hoagland's solution and grown for another month. These plants were incubated with 0.2 x Hoagland's solution with or without 50 mM NaCl for 2 weeks. The shoots of plants were then harvested, rinsed with a large amount of deionized H_2O, dried in an oven at 65ºC for 72 h, and weighed. Tissues were ground, extracted with 0.1 M nitric acid for 2 h, and filtered through filter papers (Whatman grade 307). The Na, K, or P content was determined (n = 3) using an ICP Atomic Emission Spectrometer ICPS-8100 (Shimadzu, Japan). Significance was calculated by Student's t-test.

Assay of Na uptake

Three-week-old plants grown in soil under short day conditions (8 h light and 16 h dark or 10 h light and 14 h dark) were transferred to hydroponic culture containing 0.2 x Hoagland's solution and grown for another month. Plant roots were rinsed with 0.2 x Hoagland's solution, and the plants were transferred to 50 mL of 0.2 x Hoagland's solution containing ^{22}NaCl (0.04 MBq, PerkinElmer). Plants were grown in this solution for an additional 12 h and then the radioactivities of ^{22}Na in the plants were detected with the Bio-Imaging Analyzer BAS-1800 II (Fuji Film, Japan). The pixel value was obtained with ImageJ software (Abramoff et al., 2004).

RNA isolation and real-time quantitative PCR analysis

Seedlings were grown on quarter-strength MS (Murashige-Skoog) media supplemented with 2% sucrose and 0.7% agar for 5 days and, then, were transferred to vertical agar plates containing

100 mM NaCl for 8h as described (Oh et al., 2010). Isolation of total RNA,first-strand cDNA synthesis, and real-time PCR analysis were performed as described (Miura and Ohta, 2010; Miura et al., 2011). For amplification, gene specific primers, *ACT2* (Miura et al., 2009), *SIZ1* (5'-ATAGCGCCTCTGGGAATCAT-3' and 5'-GCCTTGTCTTGTCTACTGTCATTCATAC-3'), and *PHO2* (5'-CAGAGAGTGACTACAGTGGCGC-3' and 5'-TGCCTTCCGAACTTACAGTG-3'). The relative expression level was calculated with comparative C_T methods (n = 3) (Miura et al., 2007a). Significance was calculated by Student's t-test. The *pho2* mutant harbors a point mutation, thus *pho2* mutant still has *PHO2* transcript (Fig. 5c).

Results

The *siz1* mutation suppresses the Na^+ hyper-sensitivity of *sos3*

Among the 65,000 individual T-DNA insertion lines generated in the *sos3-1* genetic background (Rus et al. 2001), the *siz1-1* mutation suppressed the NaCl sensitivity of *sos3-1* seedlings (Miura et al. 2005; Fig. 1). Root growth comparison (Fig. 1a) and visual observation of shoot anthocyanin pigment accumulation (Fig. 1b) indicated that the *siz1-1* mutation partially suppressed *sos3-1* salt sensitivity. The *sos3-1* mutant is hypersensitive to NaCl and LiCl but not KCl, CsCl, or mannitol (Liu and Zhu 1997). The root growth of *sos3-1 siz1-1* seedlings was greater than that of *sos3-1* seedlings on medium containing 25-100 mM NaCl and less than that of wild-type seedlings (Fig. 1c), but this suppression of *sos3-1* Na+ hypersensitivity was not as great as the suppression caused by the *hkt1* alleles (Rus et al. 2001). In contrast, the root growth

of *sos3-1 siz1-1* seedlings was similar to that of *sos3-1* seedlings on medium containing LiCl (Fig. 2a). Furthermore, both *sos3-1* and *sos3-1 siz1-1* plants exhibited growth similar to wild-type seedlings on medium with KCl (Fig. 2b) and CsCl (Fig. 2c). These results suggest that *SIZ1* is involved in the response to Na^+, but not in the ionic stress response.

The *siz1* mutation enhances tolerance to salt stress

We investigated whether soil grown *siz1* plants were resistant to Na^+. In addition to *sos3-1 siz1-1*, the single mutants *siz1-2* and *siz1-3* were used for this experiment. As described, the *siz1* mutation increases the accumulation of salicylic acid (SA) without any treatment, leading to a dwarf-like phenotype (Fig. 3a, upper panel; Miura et al. 2010). When these plants were supplied with a nutrient solution containing 100 mM NaCl, the growth of wild-type plants and *sos3-1* plants was inhibited (Fig. 3a). In particular, the *sos3-1* plants treated with NaCl accumulated anthocyanin (Fig. 3a), as described above (Fig. 1b). In contrast, the *sos3-1 siz1-1* mutant did not accumulate anthocyanin compared to *sos3-1* (Fig. 3a). The single mutants *siz1-2* and *siz1-3* grown under high salt conditions were larger than those grown under normal conditions (Fig. 3a). To quantitate these results, the shoot fresh weights of Col-*gl1*, *sos3-1*, *sos3-1 siz1-1*, Col-0, *siz1-2*, and *siz1-3* plants were measured when these plants were grown on various concentrations of NaCl (Fig. 3b). The double mutant *sos3-1 siz1-1* plants were able to grow better than *sos3-1* plants, and the *siz1-2* and *siz1-3* single mutant plants were also more resistant to salt stress than the wild-type Col-0 plants (Fig. 3b). These results suggest that the *siz1* mutation enhances salt tolerance and that *SIZ1* and *SOS3* have a genetically additive effect with respect to the response

to salt stress. The resistant phenotype of *siz1-2* plants was complemented by the introduction of *Pro$_{SIZ1}$::SIZ1:GFP* (Fig. 3c).

The *siz1* mutant accumulated SA under normal conditions, as described (Yoo et al., 2006; Lee et al., 2007) and several articles reported that salt tolerance is improved by application of SA (Shakirova et al., 2003; Stevens et al., 2006). Thus, we tested salt sensitivity of *siz1-2* and *nahG siz1-2* (Fig. 3d). Introduction of *nahG* reduces SA level in *siz1* (Lee et al., 2007). *nahG siz1-2* exhibited significant reduction of salt tolerance of *siz1-2*, indicating that high level of SA improves salt tolerance. But *nahG siz1-2* was slightly different from Col-0, suggesting that another factor may be involved in salt tolerance caused by the *siz1-2* mutation.

Uptake and accumulation of Na^+ were decreased in *siz1*

To investigate how the *siz1* mutation affects the response to Na^+ concentration, radiolabeled ^{22}Na was incorporated into *siz1-3*. Compared to the accumulation in Col-0 plants, the accumulation of ^{22}Na in the shoots of *siz1-3* was decreased (Fig. 4a, b). Because mild salt treatment enhanced growth of *siz1* (Fig. 3), the ion contents of Na (sodium), P (phosphorus), and K (potassium) were determined in wild-type and *siz1-3* plants treated with or without 50 mM NaCl. After induction of salt stress, the accumulation of Na in *siz1-3* was less than wild type (Fig. 4c). In contrast, accumulation of P was increased in *siz1-3* plants (Fig. 4c), as described (Miura et al. 2005). These results suggest that increased P accumulation is correlated with decreased Na accumulation. The K content in salt-treated wild-type and non-stressed *siz1-3* was decreased compared to non-stressed wild type and salt-treated *siz1-3*, respectively. Thus, the

greater size of salt-treated *siz1-3* plants relative to untreated *siz1-3* plants might be due, at least in part, to enhanced K accumulation.

Pi accumulation mutant *pho2* enhances salt tolerance

The *siz1* plants accumulated more Pi than wild-type plants (Fig. 4c; Miura et al. 2005),as well as SA (Lee et al., 2007). In mammals, sodium-Pi cotransporters play an important role in the regulation of Pi homeostasis (Marks et al. 2010). It is possible that sodium homeostasis is regulated by Pi accumulation. Thus, the Pi accumulation mutant *pho2* (Delhaize and Randall 1995) was subjected to salt stress. Mild salt stress (50 or 75 mM NaCl) promoted the growth of *pho2* (Fig. 5). The double mutant *sos3-1 pho2* was prepared and subjected to salt stress. The *sos3-1 pho2* double mutant exhibited more tolerance to salt stress than *sos3-1* did, but less than the *pho2* single mutant did (Fig. 5). These genetic data suggest that *PHO2* and *SOS3* have a genetically additive effect with respect to the response to salt stress, i.e., *PHO2* is genetically independent of *SOS3*-dependent signaling.

Radiolabeled ^{22}Na was incorporated into *pho2* to investigate how the Pi accumulating *pho2* mutation is involved in Na^+ homeostasis. The uptake of ^{22}Na in the shoots of *pho2* was decreased (Fig. 4a, b), as it was in the *siz1* mutant. The ion content of Na was decreased, whereas P was hyper-accumulated (Fig. 4c). These results suggest that accumulation of Pi is involved in the regulation of Na homeostasis.

To elucidate relationship between *SIZ1* and *PHO2*, both of which regulate Pi accumulation (Delhaize and Randall 1995; Miura et al., 2005). expression of *SIZ1* and *PHO2* was investigated in *siz1-2* and *pho2* mutants. *PHO2* was down-regulated in *siz1-2* (Fig. 5c),

suggesting that *SIZ1* may regulate Pi accumulation through down-regulation of *PHO2*. After treatment with salt, *PHO2* expression was also decreased in Col-0, suggesting that plants may keep Pi homeostasis. On the other hand, no significant alternation was observed about *SIZ1* expression in Col-0 and *pho2*.

Discussion

Salt concentration is a major environmental factor influencing growth and development. The research reported herein was undertaken to improve our understanding of the physiological processes that determine salt sensitivity and the relationship between Na^+ homeostasis and Pi accumulation. The Pi-accumulating mutants *siz1* and *pho2* exhibited tolerance to salt stress, probably because of decreased in Na^+ uptake and accumulation in leaves. *SIZ1*- or *PHO2*-dependent salt signaling is apparently not genetically dependent on *SOS3*-dependent signaling.

Pi accumulation alleviates/reduces Na^+ uptake and contents in leaves.

Both Pi accumulating mutants, *siz1* and *pho2*, exhibited salt tolerance (Fig. 3 and 5). This suggests that higher concentration of Pi might suppress uptake and accumulation of Na^+. For example, the dry matter and chlorophyll content of cucumber and pepper were maintained during salt treatment when supplied with high Pi (Kaya et al. 2001). In *Anabaena doliolum,* the chlorophyll concentration and the protein content decreased with increasing salt concentration, and the effects of NaCl were greater in Pi-deficient medium (Rai and Sharma 2006). Pi supply and Pi accumulation may affect salt sensitivity. High accumulation of Na^+ is toxic to cells. Thus, Na^+ is transported into vacuoles (Gaxiola et al., 2002). Na^+/H^+ antiporters mediate the exchange

of cytosolic Na^+ for vacuolar H^+ (Apse et al., 1999; Yokoi et al., 2002; He et al., 2005). This exchange causes cytoplasmic acidification and vacuolar alkalization (Katsuhara et al., 1989). To energize this transport system, proton electrochemical gradient is required. Therefore, proton is moved from cytosol to vacuole by H^+ pumps; vacuolar H^+-ATPase and H^+-PPase (Silva and Gerós, 2009). Interestingly, ^{31}P-nuclear magnetic resonance study revealed that Na^+ uptake into the cell is also accompanied by a rapid increase in vacuolar Pi (Fan et al., 1989; Gruwel et al., 2001). The transport mechanism for the tonoplast Pi transporter has been unclear, but vacuoles apparently play the dual role of sink and source for Pi in plant cells. It is possible that H^+-Pi coupled transporter and/or Na^+-dependent Pi symport system are present at tonoplast membrane. If H^+-Pi coupled transporter exist at vacuolar membrane, high accumulation of Pi in *siz1* and *pho2* may enhance influx of H^+ into vacuole, leading to generation of proton electrochemical gradient for energizing Na^+/H^+ antiporters. In mammals, Na^+-coupled Pi transporters (NaPi) play a role in the Pi reabsorption process and the regulation of Pi homeostasis (Segawa et al. 2009). Recently, a few NaPi have been identified in green algae and vascular plants (Mimura et al. 2002; Rubio et al. 2005; Pavón et al. 2008). Biochemical and physiological studies have suggested an interaction between salt stress and Pi metabolism in plants. In *Chara corallina*, external Na^+ is required for NaPi cotransport activity to uptake (Mimura et al. 2002). If NaPi exists at vacuolar membrane, highly accumulated Pi in *siz1* or *pho2* may drive more Na^+ movement into vacuole.

As demonstrated, hyper-accumulation of Pi is also toxic; retardation of plant growth and enhancement of chlorosis and necrosis (Delhaize and Randall 1995). As indicated (Fig. 3 and 5), mild salt concentrations promoted plant growth of *siz1* and *pho2*. It is possible that these salt

stresses improve Pi homeostasis, leading to better growth in these mutants. That may be a reason why we could isolate the *siz1* mutation as a suppressor of *sos3-1* Na^+ hypersensitivity.

SA contributes to alleviation of salt sensitivity in *siz1*

The *siz1* mutant accumulated SA under normal conditions, as described (Yoo et al. 2006; Lee et al. 2007). Several articles that application of SA increases tolerance to salt stress. The application of SA improved plant growth under salt stress and caused the accumulation of ABA and prolines (Shakirova et al. 2003). Root drenching with SA protected tomato plants against 200 mM NaCl stress (Stevens et al. 2006). SA also alleviated high-salinity-inhibited photosynthesis and yield through a decrease in Na^+ and Cl^- and an increase in nitrogen, phosphate, and potassium levels and in the activity of antioxidant enzymes in mung bean (Nazar et al. 2010; Khan et al. 2010). High salinity also increased the endogenous SA levels and the activity of the SA biosynthesis enzyme, benzoic acid 2-hydroxylase, in rice seedlings (Sawada et al., 2006). Because SA treatment is accompanied by a transient increase in the levels of H_2O_2, which plays a role as a signal molecule to induce the antioxidant defense system (Gill and Tuteja 2010), the activation of the antioxidant system, i.e., ROS scavenging systems, may contribute to the equilibration of ROS homeostasis to enhance salt stress tolerance. In addition to enhanced phosphate uptake as described above, the accumulation of SA contributes to salt tolerance in the *siz1* mutant. But SA is not involved in response to Pi deficiency, because *nahG siz1-2* exhibited similar phenotype as did *siz1-2* (Miura et al., 2010). It is plausible that SA and Pi homeostasis independently regulate salt tolerance.

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Figure legends

Fig. 1 The *siz1* mutation suppresses *sos3-1* NaCl hypersensitivity Photographs show representative Col-*gl1* wild type *(SOS3 SIZ1), sos3-1 (sos3-1 SIZ1),* and *sos3-1 siz1-1* seedlings 6 days after transfer into 1x MS medium without (0) or with 50 mM NaCl (**a**) and anthocyanin accumulation on medium with 50 mM NaCl after 18 days (**b**). Root growth of wild-type (Col-*gl1*, Col-0), *sos3-1*, *sos3-1 siz1-1*, *siz1-2* (*SOS3 siz1-2*), and *siz1-3* (*SOS3 siz1-3*) seedlings was evaluated 6 days after transfer of the plants into 1x MS medium with various amounts of NaCl. The *siz1* single mutants (*siz1-2* and *siz1-3*) exhibited a phenotype similar to Col-0. The mean values ± SE, $n = 15$, are presented. * indicates significant different ($p < 0.05$) from value of *sos3-1*.

Fig. 2 Root growth of the *siz1* seedlings was similar to that of wild-type seedlings in response to LiCl, KCl, and CsCl. Four-day-old seedlings were transferred onto 1x MS-agar plates supplemented with various concentrations of LiCl (**a**), KCl (**b**), and CsCl (**c**) and allowed to

grow for 6 additional days. The length of primary root growth after transfer was measured. Error bars represent standard errors (n = 15).

Fig. 3 The *siz1* mutation confers tolerance to NaCl. (**a**) Three-week-old plants were treated with or without treatment of NaCl twice per week. Photographs were taken 18 days after the salt treatments. (**b**) Shoot fresh weight at the end of salt treatment. Three-week-old plants were treated with various concentrations of NaCl twice per week. Shoot fresh weight was measured 16 days after treatment. Data are shown as a percentage of shoot fresh weight in the absence of NaCl. Error bars represent the standard error (n = 9). * and † indicate significant different (p < 0.05) from Col-0 and *sos3-1*, respectively. (**c**) The NaCl tolerance of *siz1* plants was complemented with wild-type *SIZ1*. *SIZ1:GFP* was transformed into the *siz1* mutant (Jin et al., 2008). Three-week-old plants were treated with various concentrations of NaCl twice per week. Shoot fresh weight was measured 16 days after treatment. Illustrated data are the mean values ± SE, n = 8. * indicates significant different (p < 0.05) from Col-0. (**d**) The NaCl tolerance of *nahG siz1-2* was reduced compared to *siz1-2* plants.* indicates significant different from Col-0 (p < 0.05).

Fig. 4 Autoradiogram showing the distribution of ^{22}Na radioactivity of wild-type, *siz1-3,* and *pho2* (**a**). Plants hydroponically grown in 0.2 x Hoagland's solution were moved to the same solution containing 0.04 MBq ^{22}NaCl and incubated for 24 h. (**b**) Relative radioactivities of ^{22}Na in the plants were calculated. The value of radioactivity in Col-0 was set to 100%. (**c**) Ion contents in wild-type, *siz1-3,* and *pho2* mutant plants. The Na, P, and K contents in wild-type, *siz1-3,* and *pho2* shoots grown with or without 50 mM NaCl were measured by ICP-AES. Error

bars represent standard error (n = 3). Bars with different letters were significant different, p < 0.05.

Fig. 5 The *pho2* mutation enhances resistance to salt stress. (**a**) Shoot fresh weight after treatment with salt for 16 days was measured. Three-week-old wild-type, *pho2*, *sos3-1*, and *sos3-1 pho2* plants were treated with various concentrations of NaCl twice per week. Data are shown as a percentage of shoot fresh weight in the absence of NaCl. Error bars represent the standard error (n = 9). * and † indicate significant different (p < 0.05) from Col-0 and *sos3-1*, respectively. (**b**) Photographs were taken 28 days after the salt treatments. (**c**) Relative mRNA levels of *SIZ1* and *PHO2* in Col-0, *siz1-2*, and *pho2* grown on media without (mock) or with 100 mM NaCl were determined by quantitative RT-PCR analysis. Data are means ± SD (n = 3).* indicates significant different (p < 0.05). Expression levels are expressed relative to transcript abundance in Col-0 seedlings without NaCl treatment.

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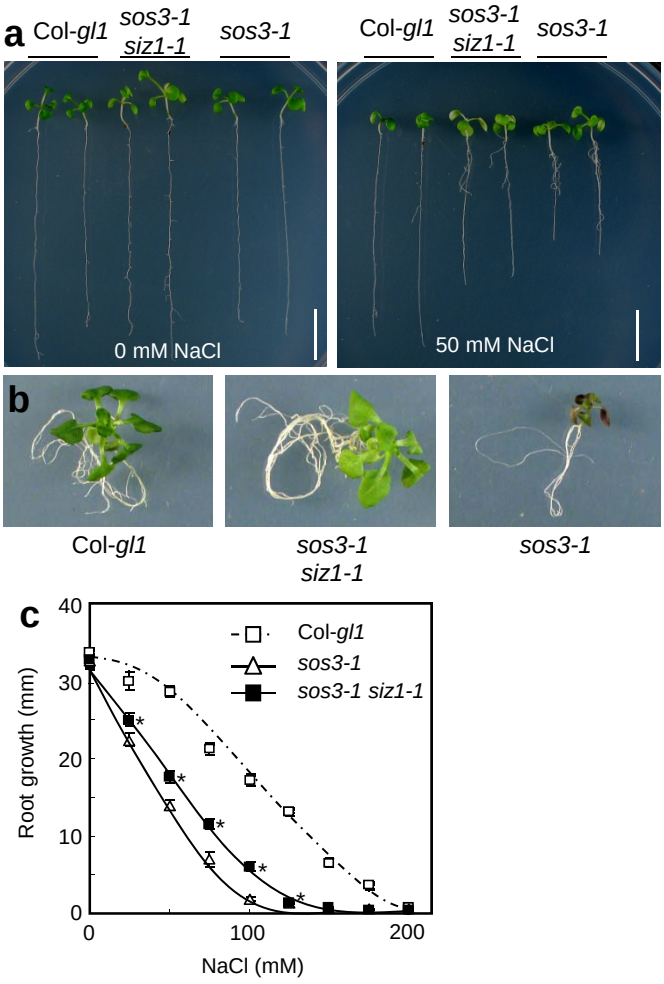
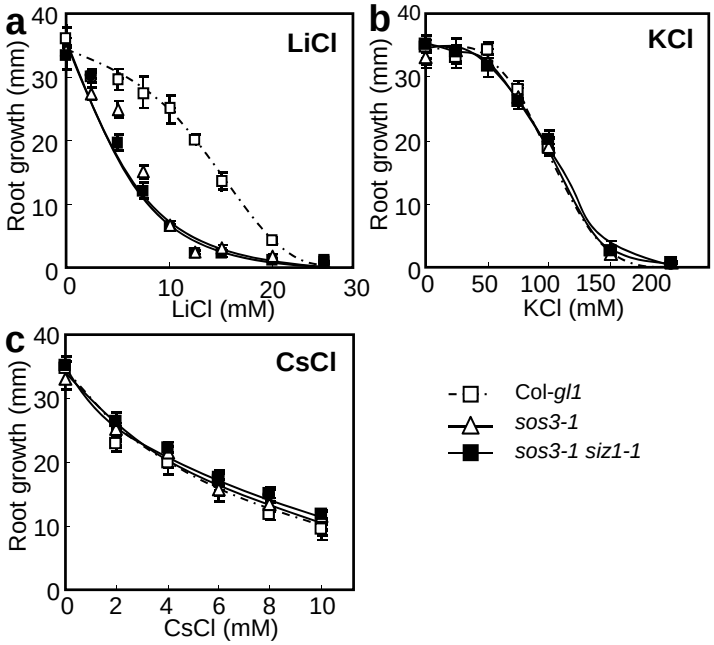
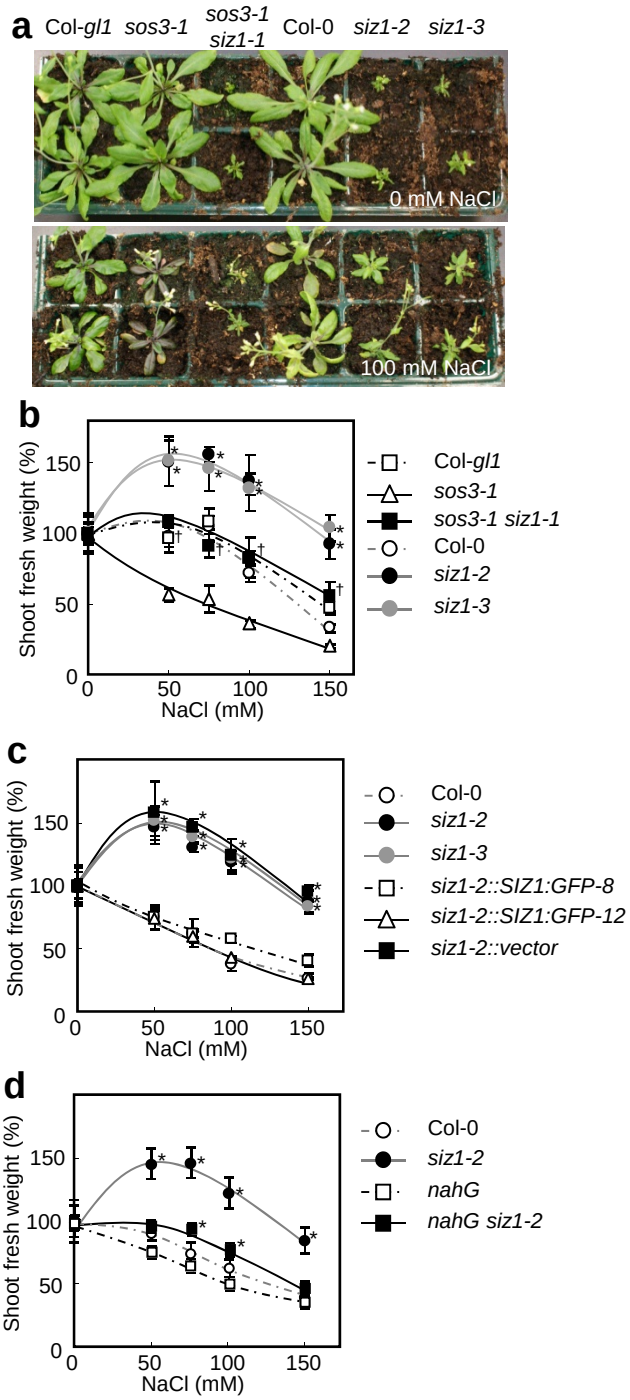
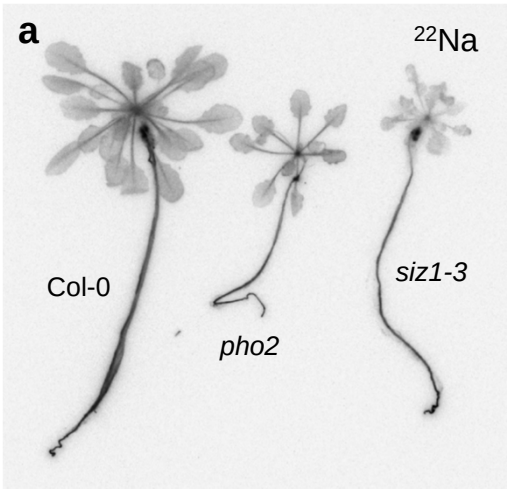
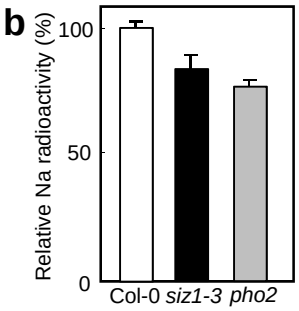


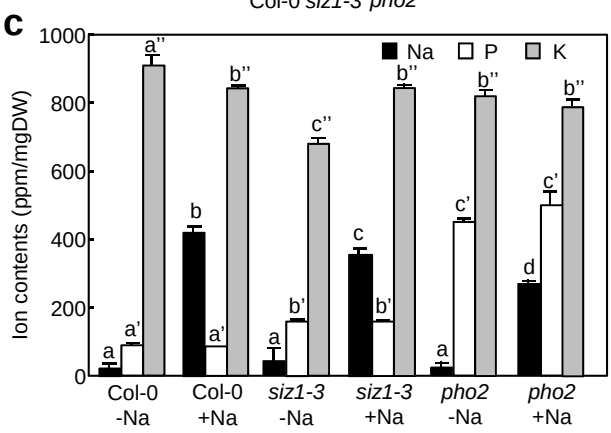
Figure 1. Miura et al.

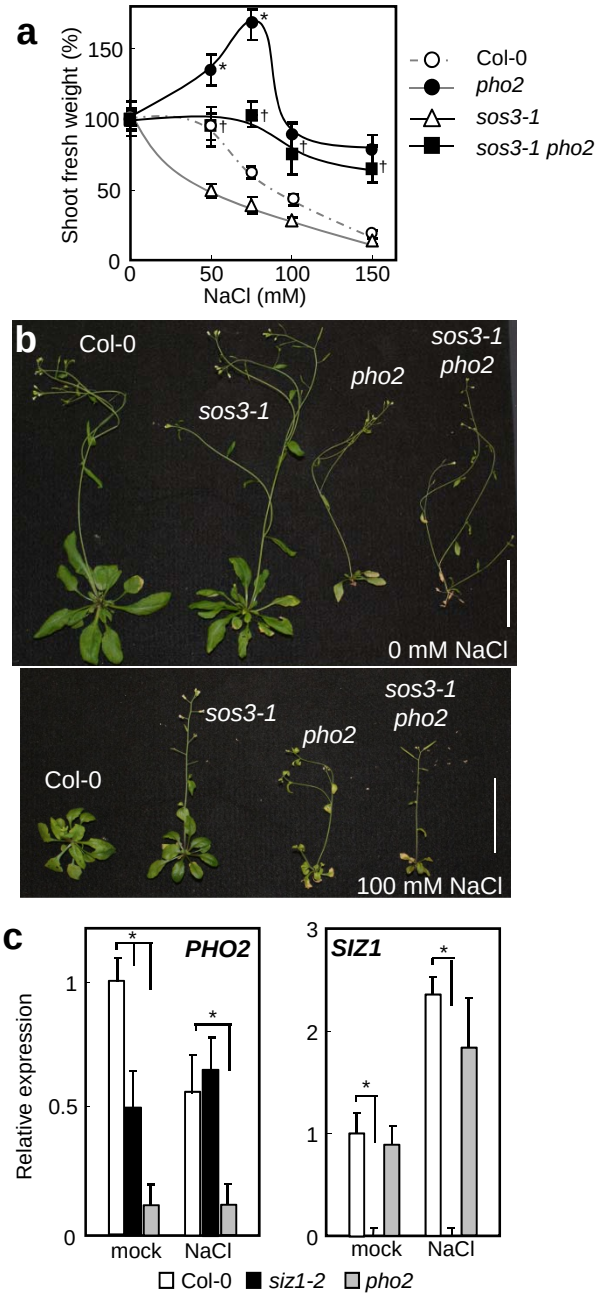

a
LiCl
Root growth (mm)
LiCl (mM)
b
KCl
KCl (mM)
c
CsCl
CsCl (mM)
Col-gl1
sos3-1
sos3-1 siz1-1


a
Col-gl1
sos3-1
sos3-1 siz1-1
Col-0
siz1-2
siz1-3
0 mM NaCl
100 mM NaCl
b
Shoot fresh weight (%)
NaCl (mM)
Col-gl1
sos3-1
sos3-1 siz1-1
Col-0
siz1-2
siz1-3
c
Shoot fresh weight (%)
NaCl (mM)
Col-0
siz1-2
siz1-3
siz1-2::SIZ1:GFP-8
siz1-2::SIZ1:GFP-12
siz1-2::vector
d
Shoot fresh weight (%)
NaCl (mM)
Col-0
siz1-2
nahG
nahG siz1-2


a
^{22}Na
Col-0
pho2
siz1-3


b
Relative Na radioactivity (%)
100
50
0
Col-0 siz1-3 pho2


c
Ion contents (ppm/mgDW)
1000
800
600
400
200
0
Na
P
K
a
a'
a''
b
a'
b''
a
b'
c''
c
b'
b''
a
c'
b''
d
c'
b''
Col-0 -Na
Col-0 +Na
siz1-3 -Na
siz1-3 +Na
pho2 -Na
pho2 +Na


a
Shoot fresh weight (%)
150
100
50
0
0
50
100
150
NaCl (mM)
Col-0
pho2
sos3-1
sos3-1 pho2
b
Col-0
sos3-1
pho2
sos3-1
pho2
0 mM NaCl
sos3-1
pho2
sos3-1
pho2
Col-0
100 mM NaCl
c
PHO2
SIZ1
Relative expression
1
0.5
0
3
2
1
0
mock
NaCl
mock
NaCl
Col-0
siz1-2
pho2