Plant Biotechnology 31, 167–172 (2014)
DOI: 10.5511/plantbiotechnology.14.0109a

Short Communication

Overexpression of *SIZ1* enhances tolerance to cold and salt stresses and attenuates response to abscisic acid in *Arabidopsis thaliana*

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Received October 24, 2013; accepted January 9, 2014 (Edited by N. Sasaki)

Abstract Sumoylation is a post-translational modification to control several cellular functions, including responses to environmental stresses and hormones in plants, and SIZ1 is a SUMO (small ubiquitin-related modifier) E3 ligase that plays an important role in sumoylation. In this study, we produced transgenic *Arabidopsis thaliana* plants with a moderately higher expression of *SIZ1*. Compared to wild type, the transgenic plants, with or without cold acclimation, were tolerant to freezing stress when subjected to freezing temperatures. The transgenic plants exhibited enhanced performance associated with robust shoot and root growth under salinity stress, whereas the wild type exhibited necrosis in the leaves with salt treatment. Furthermore, *SIZ1* overexpression attenuated the root growth inhibition caused by abscisic acid. These results suggest that SIZ1 has the potential to genetically improve plant responses to abiotic stresses.

Key words: ABA response, cold tolerance, salt tolerance, SUMO E3 ligase.

Protein modification and modulation are essential for living organisms. The modifications are important for such processes as responses to environmental changes and development. Phosphorylation, methylation, acetylation, lipidation, glycosylation, etc. are important protein modifications. In addition to chemical modifications, small peptides also act as post-translational modifiers, with ubiquitin being the most notable example. However, there are several types of ubiquitin-like modifiers that function in post-translational protein modification, including SUMO (small ubiquitin-related modifier) (Miura et al. 2007a; Xu and Yang 2013). Sumoylation is the conjugation of SUMO to proteins, and a series of biochemical steps catalyzed by SUMO-specific E1, E2, and E3 ligases are required for this conjugation. Unlike ubiquitylation, sumoylation is not associated with protein degradation, but rather, to the control of the target protein's function, such as the interference of protein–protein interactions, recruitment of other proteins recognizing SUMO, and conformation changes (Miura and Hasegawa 2010).

Eight putative SUMO isoforms exist in *Arabidopsis*, although the expression of only four of the genes (*SUM1*, *SUM2*, *SUM3*, and *SUM5*) has been confirmed. SUM1 and SUM2 are functionally equivalent and are capable of forming poly-SUMO chains, whereas SUM3 does not perform this function. According to SUMO profiles, SUM1/2 and SUM3 have different specificities and possibly different targets (Castano-Miquel et al. 2011; Kurepa et al. 2003). SUMO is covalently conjugated to a target protein through a three-step cascade via E1, E2, and E3. SUMO is typically conjugated to a lysine residue of the target protein within a canonical sumoylation consensus motif ΨKxE (Ψ, a large hydrophobic residue; K, lysine; x, any amino acid; E, glutamic acid).

Recently, several alternative SUMO-conjugation sites have been described, including the inverted consensus motif ExKΨ, phosphorylation-dependent SUMO motif ΨKxExxSP, negatively charged amino acid-dependent SUMO motif ΨKxExxEEEE, and hydrophobic cluster SUMO motif ΨΨΨKxE (Da Silva-Ferrada et al. 2012). The sumoylation machinery is essential for embryogenesis, as mutations in SUM1/2, SAE2 (subunit of E1), or SCE1 (E2) are embryonic lethal (Saracco et al. 2007). Conversely, the overexpression of SUMO isoforms results in growth-defective plants (Budhiraja et al. 2009; van den Burg et al. 2010). Although there are several SUMO proteins, only two SUMO E3 ligases have been characterized in *Arabidopsis*–SIZ1 and MMS21/HPY2 (Huang et al. 2009; Ishida et al. 2009; Miura et al. 2005). The *siz1* and *mms21*/*hpy2* mutants exhibit pleiotropic phenotypes that involve ABA (abscisic acid), biotic, and

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Abbreviations: ABA, abscisic acid; SUMO, small ubiquitin-related modifier.

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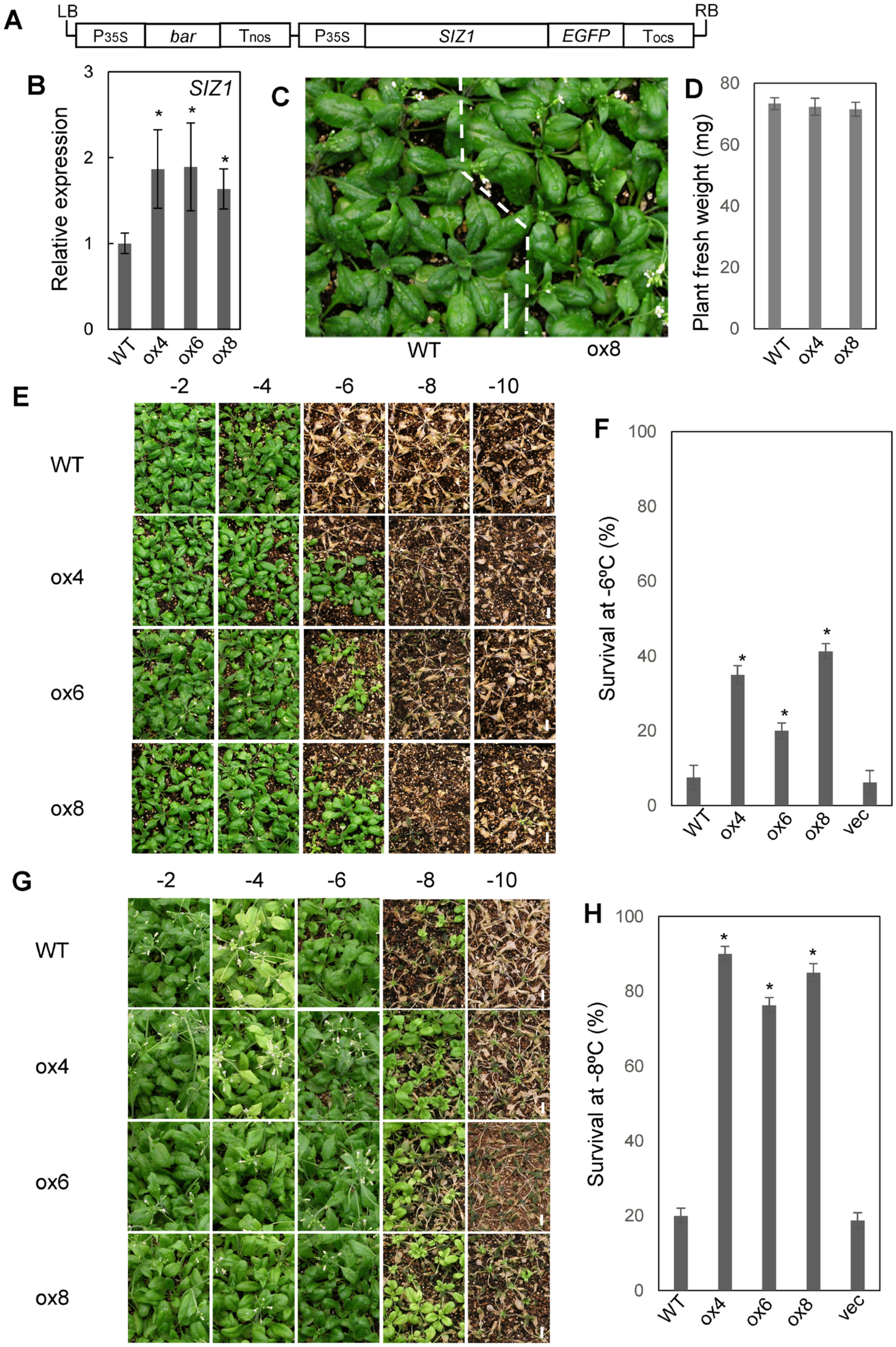

Figure 1. Overexpression of *SIZ1* enhances freezing tolerance. (A) Schematic diagram of a vector. LB, left border; P_{35S}, 35S promoter; bar, bialaphos resistance gene; T_{nos}, *nos* terminator; EGFP, enhanced green fluorescent protein; T_{ocs}, *ocs* terminator. (B) Quantitative RT-PCR to determine the transcript abundance of *SIZ1* in wild type (WT) and the lines transformed with P_{35S}::*SIZ1*:*GFP*. *Actin2* was used for normalization. Data are means±S.E. calculated from data of three independent experiments. Asterisks indicate statistical difference ($p<0.05$ by Student *t*-test). (C) Photographs are of representative 3-week-old WT and *SIZ1* overexpressing plants. (D) The fresh weight of 3-week-old wild-type and *SIZ1* overexpressing plants was measured. (E) Non-acclimated or (G) cold-acclimated plants were exposed for 2 h to the temperature indicated. After treatment of freezing temperatures, plants were incubated at 23°C for 1 week. Photographs are of representative WT and *SIZ1*-overexpressing plants (ox4, ox6, and ox8). (F, H) Survival was determined for 20 plants after freezing treatment at the indicated temperature (at −6°C for non-acclimated plants and at −8°C for cold-acclimated plants). Data are means±S.E. calculated from data of three independent experiments. Asterisks indicate statistical difference ($p<0.05$ by Student *t*-test). Bars=1 cm.

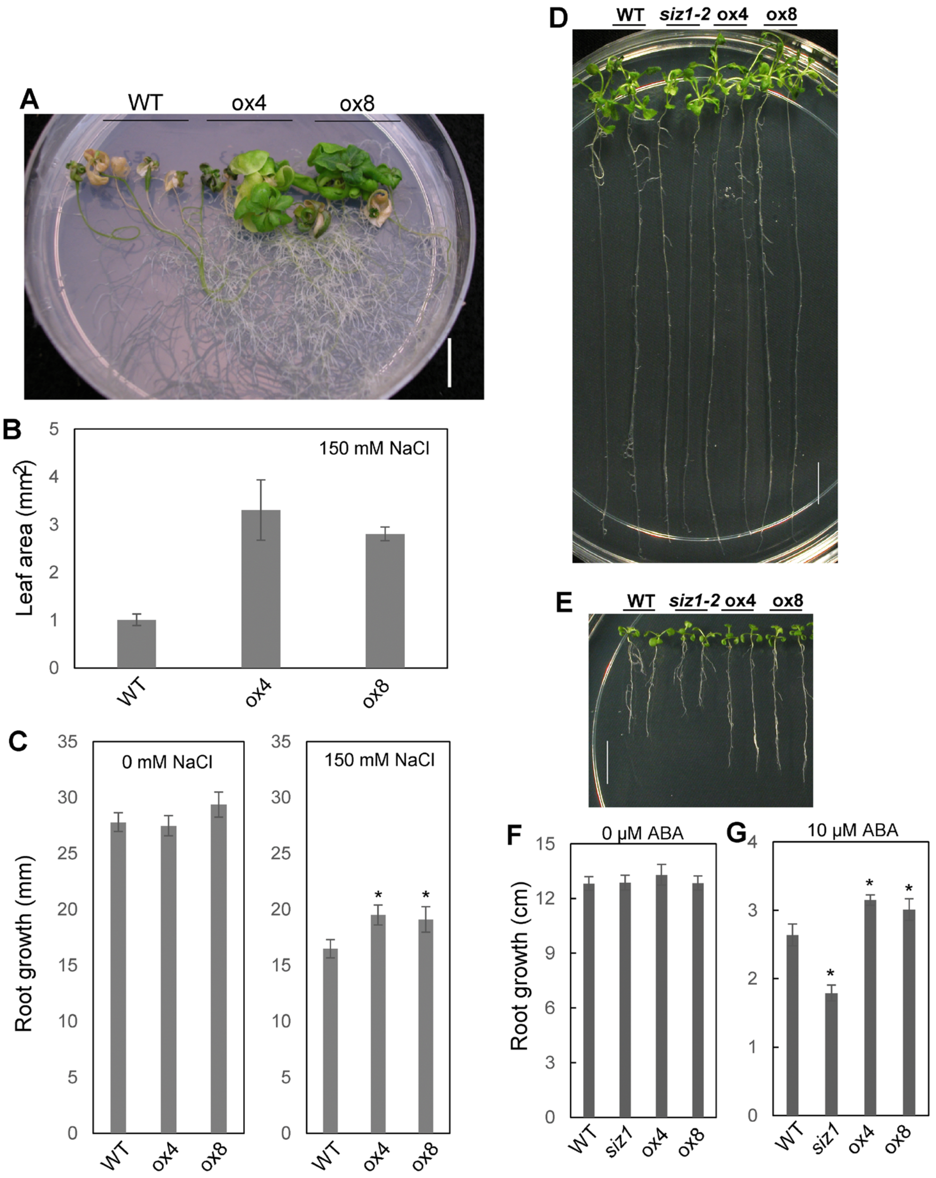

Figure 2. *SIZ1* enhances salt tolerance and attenuates ABA-induced growth inhibition. (A) Three-day-old seedlings were transferred onto the media containing 150 mM NaCl and incubated for one and half month. (B) Leaf area of wild type and overexpression lines treated with 150 mM NaCl for 1.5 month was calculated. (C) After treatment with 150 mM NaCl for 7 days, root growth was measured. Asterisks indicate statistical difference ($p<0.05$ by Student *t*-test). A bar=1 cm. (D, E) Three-day-old seedlings were transferred onto MS media containing 0 μM or 10 μM ABA. Photographs are of representative WT, *siz1-2*, and overexpressing lines on the media without (D) or with ABA (E) for 18 days. (F, G) Root growth was measured and the values are expressed as means ± S.E. ($n=10$). Asterisks indicate statistical difference, compared to wild type ($p<0.05$ by Student *t*-test). Bars=1 cm.

abiotic stress (heat, cold, salt, and drought) responses, nutrient availability, plant development, and flowering (Catala et al. 2007; Chen et al. 2011; Jin et al. 2008; Lee et al. 2007; Miura et al. 2007b; Miura et al. 2011a; Miura et al. 2009; Miura et al. 2010; Miura and Ohta 2010; Miura et al. 2013; Miura et al. 2011b; Park et al. 2011; Yoo et al. 2006). These findings indicate the importance of SUMO E3 ligases in plants. Neither the *siz1* nor *mms21*/*hpy2* mutation is embryonic lethal, but double mutations do result in embryonic lethality (Ishida et al. 2012), indicating that the functions of SIZ1 and MMS21/HPY2 may be partially overlapping and that both are indispensable for embryogenesis.

Sumoylation and desumoylation may be precisely regulated because the overexpression of SUM1 causes growth retardation (van den Burg et al. 2010) and the expression of *35Spro:SCE1* causes co-suppression in *Arabidopsis* (Lois et al. 2003). These data suggest that a high level of sumoylation causes abnormality in plants. Additionally, the overexpression of *Spartina alterniflora SaSce9*, which shows high similarity (approximately 85–93%) with plant SUMO conjugating enzymes, in *Arabidopsis* enhances salt and drought tolerance (Karan and Subudhi 2012). In the present study, we generated plants that moderately overexpress *SIZ1*; indeed, obtaining transgenic plants that strongly express the gene is difficult. We observed that the overexpression of *SIZ1* conferred cold tolerance and that the transgenic plants exhibited an attenuated response to ABA. These data suggest that engineering the sumoylation status modulates plant responses to abiotic stresses.

To evaluate whether overexpression of *SIZ1* enhance abiotic stress tolerance, the vector was constructed (Figure 1A). The PCR product of *SIZ1* was inserted into pEZT-NL (https://deepgreen.dpb.carnegiescience.edu/). The resulting vector was transformed into *Arabidopsis* by using *Agrobacterium*-mediated floral dip method. The transformants were selected by the herbicide Basta. The expression of *SIZ1* in P_{35S}::*SIZ1* transgenic plants was measured, with three lines showing a moderately higher expression of *SIZ1* compared to the expression in wild type (Figure 1B). The plant growth of wild-type and *SIZ1* overexpressing plants under normal condition was similar (Figure 1C, 1D). The transgenic plants exhibited a higher tolerance to freezing stress than wild type when subjected to freezing temperatures, both without (Figure 1E) and with cold acclimation (Figure 1G). The survival of the transgenic plants subjected to −6°C without cold acclimation or subjected to −8°C with cold acclimation was 20–41% or 76–90%, respectively, whereas that of wild type was 7% or 20%, respectively (Figure 1F, 1H). These results indicate that *SIZ1* overexpression enhances cold tolerance. The transgenic plants also exhibited salt tolerance (Figure 2A): almost all the wild-type plants exhibited leaf necrosis, though the transgenic plants did not (Figure 2A); furthermore, the shoot and biomass was completely different between wild-type and transgenic plants (Figure 2B). The root growth of the transgenic plants was longer than that of wild type under 150 mM NaCl treatment (Figure 2C). These data suggest that the overexpression of *SIZ1* enhances tolerance to abiotic stresses.

The exogenous application of ABA results in primary root growth inhibition in seedlings. At three days after sowing seeds on basal media without ABA, the seedlings were transferred onto a medium with or without ABA and incubated for 18 days. Without ABA, the root growth of all seedlings was similar (Figure 2D, 2E). In contrast, the roots of the transgenic seedlings were longer than wild type with ABA (Figure 2F, 2G), whereas the opposite was observed for the *siz1* mutant (Figure 2D–2G). These results indicate that the overexpression of *SIZ1* attenuates ABA-induced primary root growth inhibition.

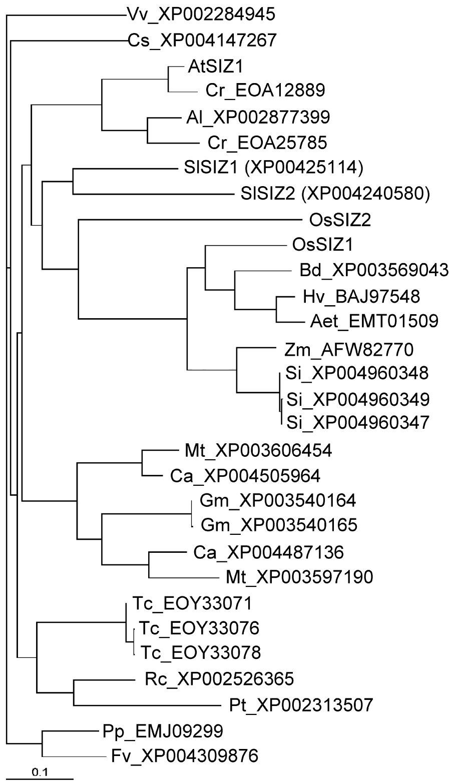

Figure 3. Phylogenic tree of plant SIZ proteins and their homologs. Phylogram of these proteins was constructed by TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) after alignment with CLUSTALW program (http://clustalw.ddbj.nig.ac.jp). The sequence data for the plant SIZ1 homologs were obtained from the NCBI protein database. The NCBI accession number is described with a species acronym. Abbreviations: Aet, *Aegilops tauschii*; Al, *Arabidopsis lyrata*; At, *Arabidopsis thaliana*; Bd, *Brachypodium distachyon*; Ca, *Cicer arietinum*; Cr, *Capsella rubella*; Cs, *Cucumis sativus*; Fv, *Fragaria vesca*; Gm, *Glycine max*; Mt, *Medicago truncatula*; Os, *Oryza sativa*; Pp, *Prunus persica*; Pt, *Populus trichocarpa*; Rc, *Ricinus communis*; Si, *Setaria italica*; Sl, *Solanum lycopersicum*; Tc, *Theobroma cacao*; Vv, *Vitis vinifera*; Zm, *Zea mays*.

As described previously, SIZ1 mediates the sumoylation of transcription factors ICE1 and ABI5 in the control of cold signaling (Miura and Hasegawa 2008; Miura et al. 2007b) and ABA signaling (Miura and Hasegawa 2009; Miura et al. 2009), respectively (Miura and Hasegawa 2010). In *Arabidopsis*, the SIZ1-mediated sumoylation (at K393) of the MYC-type transcription factor ICE1 is required to activate cold signaling and freezing tolerance (Miura et al. 2007b), and the introduction of an ICE1(K393R) variant protein represses the expression of cold-responsive genes and reduces freezing tolerance. ICE1-dependent cold signaling for cold acclimation is highly conserved among plants (Miura and Furumoto 2013). For example, the overexpression of tomato *ICE1* enhances chilling tolerance and the accumulation of antioxidants, several amino acids, and sugars in red tomato fruits (Miura et al. 2012a; Miura et al. 2012b). As SIZ proteins are also conserved in plants (Figure 3), it is likely that the heterologous overexpression of SIZ enhances cold tolerance in plants. Recently, it was reported that the expression of rice OsSIZ1 resulted in tolerance to drought and heat stresses and phosphate starvation in creeping bentgrass (Li et al. 2013). Although cold tolerance was not investigated in that study, it is likely that OsSIZ1 also enhances cold tolerance, as we observed for SIZ1(Figure 1).

In addition to cold tolerance, the transgenic plants exhibited tolerance to salt (Figure 2A–2C). Because the abundance of SUMO1/2 conjugates was observed to increase in a salt concentration-dependent manner (Conti et al. 2008), sumoylation may be involved in the regulation of salt tolerance. The *ots1 ots2* double mutant, which is impaired in SUMO protease activity, exhibits salt sensitivity (Conti et al. 2008), and the amount of SUMO1/2 conjugates under salt stress are reduced in *ots1 ots2* mutants than in wild type. Thus, the overexpression of *SIZ1* may enhance SUMO1/2 conjugates under salt stress, most likely leading to the augmentation of the tolerance to salt stress. However, the sumoylation target to control salt tolerance has not yet been identified. Indeed, the identification of the target would help to elucidate the detailed mechanism by which sumoylation controls responses to salinity.

SIZ1 overexpression attenuated the ABA-mediated inhibition of root growth (Figure 2D–2G). Similarly, the overexpression of *AtSUMO1* or *AtSUMO2* was also found to repress this inhibition (Lois et al. 2003). Biochemical and genetic evidence have demonstrated that SIZ1 facilitates the sumoylation of a bZIP-type transcription factor, ABI5, at K391 to stabilize the ABI5 protein (Miura et al. 2009). Together, the data suggest that sumoylation negatively regulates ABA signaling.

In summary, our results demonstrate that the overexpression of SIZ1 SUMO E3 ligase enhances plant tolerance to cold and salt stresses and attenuates ABA-inhibited root growth. According to the SUMO1/2-conjugation profile, the sumoylation of many proteins is stimulated by various environmental stresses (Catala et al. 2007; Elrouby and Coupland 2010; Kurepa et al. 2003; Miller et al. 2010; Miura et al. 2007b), resulting in the modification of the expression of many genes. SIZ1 is an important SUMO E3 ligase in plants (Miura and Hasegawa 2010) and functions to modulate diverse signaling pathways at various levels. Our results suggest that the modification of SUMO E3 ligase expression is a useful strategy to genetically engineer plants with enhanced resistance to abiotic stresses.

Acknowledgements

The laboratory is supported by a Grant for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (MEXT) on 'Environmental Sensing of Plants: Signal Perception, Processing Cellular Responses'.

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