The plasma-membrane polyamine transporter PUT3 is regulated by the Na⁺/H⁺ antiporter SOS1 and protein kinase SOS2

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Summary

- In Arabidopsis, the plasma membrane transporter PUT3 is important to maintain the cellular homeostasis of polyamines and plays a role in stabilizing mRNAs of some heat-inducible genes. The plasma membrane Na\(^+\)/H\(^+\) transporter SOS1 and the protein kinase SOS2 are two salt-tolerance determinants crucial for maintaining the intracellular Na\(^+\) and K\(^+\) homeostasis.
- Here, we report that PUT3 genetically and physically interacts with SOS1 and SOS2, and these interactions modulate PUT3 transport activity.
- Overexpression of PUT3 (PUT3OE) results in hypersensitivity of the transgenic plants to polyamine and paraquat. The hypersensitivity of PUT3OE is inhibited by the sos1 and sos2 mutations, which indicates that SOS1 and SOS2 are required for PUT3 transport activity. Protein interaction assay revealed that PUT3 physically interacts with SOS1 and SOS2 in yeast and plant cells. SOS2 phosphorylates PUT3 both in vitro and in vivo. SOS1 and SOS2 synergistically activate the polyamine transport activity of PUT3, and PUT3 also modulates SOS1 activity by activating SOS2 in yeast cells.
- Overall, our findings suggest that both plasma-membrane proteins PUT3 and SOS1 could form a complex with the protein kinase SOS2 in response to stress conditions and modulate the transport activity of each other through protein interactions and phosphorylation.

Keywords: Polyamine, Membrane transporter, Phosphorylation, PUT3, SOS1, SOS2, Salt tolerance
**Introduction**

Unlike animals, plants cannot avoid harsh environments and thus must respond in order to survive. Because of their sessile nature, plants have evolutionarily developed an array of mechanisms in coping with different environmental stresses, among which is the accumulation of protective metabolites for cells and organisms. Polyamines (PAs), including putrescine (Put), spermidine (Spd), and spermine (Spm), are positively charged metabolites and are considered as essential growth regulators modulating a broad spectrum of physiological processes, e.g., embryogenesis, cell proliferation, cell division, morphogenesis and development, in all organisms (Liu et al., 2015).

In plants, PAs have also been implicated in growth, development and stress response (Chen et al., 2019). The role of PAs in abiotic stress tolerance is supported by the fact that the biosynthesis of PAs is enhanced under different stress conditions, and exogenously applied PAs elevate plant stress tolerance (Liu et al., 2015). Several PA-biosynthetic genes, including the gene ADC encoding for the key enzyme arginine decarboxylase in the PA biosynthesis pathway, are upregulated by abiotic stresses, and consequently, the accumulation of PAs is elevated under these stress conditions. Overexpression of PA-biosynthetic genes confers resistance to abiotic stresses in plants (Liu et al., 2015).

In Arabidopsis, five genes encode L-type amino acid transporter (LAT) family proteins (LAT1-LAT5). LAT3/PUT1, LAT4/PUT2, and LAT1/PUT3 display high sequence similarity to each other (68–76%) and exhibit polyamine transport activity (Fujita and Shinozaki, 2014). PUT3 is localized in the plasma membrane with 12 predicted transmembrane domains and cytoplasmic N- and C-terminal (Fujita et al., 2012, Shen et al., 2016). PUT3 was first reported to transport PAs as well as their structural analog paraquat (PQ) into the plant cells (Fujita et al., 2012), and was later found to be involved in mediating the long-distance transport of both PAs and thiamine (vitamin B1) in the phloem from shoot to root (Martinis et al., 2016). The rmv1/put3 mutant displays impaired root growth and delayed flowering time (Martinis et al., 2016), which could be attributed to the altered levels of polyamines since alteration in polyamine levels has been implicated in root development (Watson et al., 1998) and flowering (Applewhite et al., 2000; Havelange et al., 1996). Under heat stress, PUT3 is responsible for maintaining the stability of the mRNAs of several heat-responsive
genes. Exogenous application of spermine reduces the degradation of the heat-induced transcripts with a relatively short half-life, such as those from *HSFA2*, *HSP101*, and *HSP70*, but has little effect on the transcripts with a relatively long half-life (Shen et al. 2016). Despite its import role in polyamine homeostasis and heat stress response, the regulation of transport activity of PUT3 is largely unknown.

Soil salinity is among the major abiotic stresses affecting agriculture and causes ion toxicity, hyperosmotic stress, and oxidative damage in plants (Zhu, 2016). In Arabidopsis, a well-studied calcium-dependent SOS pathway for salt stress signaling and tolerance has been identified (Zhu, 2002). In root, the myristoylated calcium-binding protein SOS3 perceives the salt-elicited calcium signal and the calcium-bound SOS3 interacts with and activates the serine/threonine protein kinase SOS2. The SOS3-SOS2 complex is necessary for activating the plasma membrane Na\(^+\)/H\(^+\) antiporter SOS1 (Quintero et al., 2002). In shoot, SOS3-like calcium-binding protein 8 (SCaBP8) recruits SOS2 to the plasma membrane under salt stress condition. The salt-induced phosphorylation of SCaBP8 by SOS2 further stabilizes the SCaBP8-SOS2 complex at the plasma membrane, and SOS2 then phosphorylates and thus enhances the Na\(^+\)/H\(^+\) exchange activity of SOS1 (Lin et al., 2009).

SOS1 is a plasma membrane protein with predicted 12 transmembrane domains and a large cytoplasmic C-terminal tail (Shi et al., 2000). Both loss- and gain-of-function analyses of *SOS1* have confirmed its crucial role in salt tolerance (Shi et al., 2000; Shi et al., 2003). The C-terminal of SOS1 interacts with RCD1 (Katiyar-Agarwal et al., 2006), a protein implicated in stress response including oxidative stress response. Both *sos1* and *rcd1* mutant show resistance to the broad-spectrum herbicide paraquat (Katiyar-Agarwal et al., 2006; Chung et al., 2008), suggesting that SOS1 might play a role in oxidative stress response. SOS2 belongs to the SNF-1 related kinases 3 (SnRK3) subfamily and is comprised of an N-terminal catalytic domain and a regulatory C-terminal domain. The FISL motif located in the C-terminal regulatory domain of SOS2 can bind with its N-terminal catalytic kinase domain, and thus autoinhibits the activity of SOS2, while its binding with SOS3 releases the autoinhibition and activates SOS2 (Guo et al., 2001). In addition to SOS1, several other proteins have been identified as SOS2-interacting or SOS2-phosphorylaing proteins. 14-3-3 proteins interact with and repress the activity of SOS2, which is promoted by the phosphorylation of SOS2 by the SOS2-LIKE PROTEIN KINASE5 (PKS5) under non-stressed conditions but suppressed by the interaction
between the 14-3-3 proteins with PKS5 under salt stress condition (Zhou et al., 2014; Yang et al., 2019). SOS2 also interacts with ABI2, a type-2C protein phosphatase important in ABA signaling (Ohta et al., 2003). Recently, SOS2 was found to interact with and phosphorylate the calcium-permeable transporter AtANN4 under salt stress, and the phosphorylated AtANN4 altered the calcium transients and results in a salt-specific calcium signal (Ma et al., 2019).

Previous studies have identified several Arabidopsis mutants showing resistance to paraquat, including sos1, sos2 and put3 (Katiyar-Agarwal et al., 2006; Chung et al., 2008; Shen et al., 2016, Chai et al., 2017b). We thus reasoned that these three genes may function in the same pathway to modulate paraquat resistance. Both genetic and biochemical evidence showing in this study strongly indicated that PUT3, SOS1 and SOS2 could form a complex to regulate the transport activity of PUT3 and SOS1, and phosphorylation of PUT3 by SOS2 enhances PUT3 function. Our findings suggest that different transmembrane transporters, together with intracellular components, could form a complex to regulate their transport activities in response to environmental cues.

Materials and Methods

Plant materials and growth conditions

The PUT3OE transgenic line (Shen et al., 2016), sos1 (Shi et al., 2000), sos2 (Liu et al., 2000), and Col-0 wild type were used in this study. The PUT3OE transgenic line was crossed with sos1-1 and sos2-2, respectively, and the homozygous lines were identified in the F3 generation (Supporting information Fig. S1). For the primary root growth assay, surface-sterilized seeds were grown in 1/2 MS medium with 1.2% agar for 5 days and then transferred to the 1/2 MS medium with 1.2% agar containing different concentrations of chemicals for an additional 6 days’ growth. For phenotype assay in the presence of H2O2, four 7-day-old seedlings per treatment condition were transferred to the liquid 1/2 MS medium supplemented with different concentrations of H2O2, and fresh weights were measured after 7 days. Plants were grown in a growth room at 22 °C under a long-day cycle condition (16 h light / 8 h dark).

Split-ubiquitin yeast two-hybrid assay
The bait and prey vectors were obtained from the manufacturer (DUALsystems Biotech, Schlieren, Switzerland). *PUT3*, *SOS1*, and *SOS2* coding sequences were fused with Cub and NubG, respectively (Supporting information Table S1). Yeast growth on synthetic complete (SC) medium without leucine, tryptophan, histidine supplemented with/without 3-Aminotriazole (3-AT) was tested to detect a direct interaction between two proteins. Pictures were taken after 5 days of yeast growth.

**Split-luciferase (LUC) complementation assay**

The coding sequences of *PUT3*, *SOS1*, and *SOS2* were cloned into pCAMBIA-nLUC and pCAMBIA-cLUC vectors (Chen et al., 2008) (Table S2). The *Agrobacterium tumefaciens* GV3101(pSoup-p19) strain with the silencing suppressor P19 and carrying different constructs was cultured overnight at 28 °C and centrifuged at 3,500 ×g for 10 min. Then the pellet was resuspended to an OD600 of 1.0 in injection buffer (10 mM MES, pH 5.6, 10 mM MgCl2, and 100 μM acetosyringone). Equal amounts of culture were mixed in different combinations and kept at room temperature for 2 - 4 h. The resulting mixtures were then infiltrated into *N. benthamiana* leaves. After 2 days, luciferase activity was detected with a NightShade LB 985 in vivo Plant Imaging System (Berthold Technologies, Oak Ridge, USA).

**Biomolecular fluorescence complementation (BiFC) assay**

BiFC assays were performed as previously described (Gehl et al., 2009). The *PUT3*, *SOS1*, and *SOS2* coding sequences were recombined into the Gateway destination vectors pDEST-GWVYNE and pDEST-GWVYCE from pENTR-PUT3, pENTR-SOS1, and pENTR-SOS2 (Table S3). The *Agrobacterium tumefaciens* GV3101(pSoup-p19) strain carrying plasmids to express PUT3-cYFP and SOS2-nYFP was injected into *N. benthamiana* leaves as described in Split-LUC complementation assay. After 2 days of co-culture, the fluorescence of infiltrated areas was examined using a Leica TCS-SP8 microscope and the preset settings for YFP with Ex: 514 nm, Em: 525 - 575 nm.

**Co-Immuno precipitation (Co-IP) assay**

The *Agrobacterium tumefaciens* GV3101 carrying binary plasmids with PUT3-nLUC (pCAMBIA-nLUC) and SOS2-FLAG (pGWB511) was used to infiltrate the leaves of *N.
The leaves infiltrated with GV3101 carrying GUS-FLAG (pGWB511) and PUT3-nLUC (pCAMBIA-nLUC) were used as negative controls. Two days after infiltration, total proteins were extracted by using extraction buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% Glycerol, 5 mM EDTA, 5 mM DTT, 1% Protease inhibitor cocktail, 2 mM PMSF, 1% NP40, 10 mM Na$_2$MoO$_4$, 10 mM NaF, 2 mM Na$_3$VO$_4$) and were subsequently precipitated by anti-FLAG M2 affinity gel (Sigma-Aldrich, F2426). PUT3-nLUC, SOS2-FLAG, and GUS-FLAG were detected by Western blotting using anti-luciferase (Sigma-Aldrich, L0159, St. Louis, USA), anti-FLAG (Sigma-Aldrich, F7425), anti-Rabbit IgG (Sigma-Aldrich A0545).

Membrane protein extraction and digestion

Twelve-day-old seedlings of PUT3OE and PUT3OE/sos2 were treated with or without 38 °C treatment for 1 h. Membrane proteins were extracted by using a Minute Plasma Membrane Protein Isolation Kit for Plants (Invent Biotechnologies, Plymouth, USA) with EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitor cocktail (Sigma-Aldrich). Proteins were then digested as previously described (Zhao et al., 2017; Hsu et al., 2018). In brief, proteins were suspended in digestion buffer (12 mM SDC (sodium deoxycholate), 12 mM SLS (sodium lauroyl sarcosinate), 100 mM Tris-HCl (pH = 8.5)) with 10 mM TECP and 40 mM CAA. Samples were diluted 5-fold using 50 mM TEAB (triethylammonium bicarbonate), and Lys-C was added onto the filter at a 1:100 (w/w) ratio for 3 h at 37 °C, and trypsin was subsequently added to a final ratio of 1:100 (w/w) and incubated overnight. The digested products were acidified with 10% trifluoroacetic acid (TFA) to pH ~3 and desalted using a 50 mg Sep-Pak C18 column.

Phosphopeptide enrichment and LC-MS/MS analysis

Phosphopeptides enrichment was performed by using the metal-directed immobilized metal ion affinity chromatography as previously described (Tsai et al., 2014; Wang et al., 2018). Briefly, tryptic peptides (200 µg) were reconstituted in 6% (v/v) acetic acid (pH 3.0) and loaded onto the IMAC StageTip. After successive washes, bound phosphopeptides were eluted with 150 µL of 200 mM NH$_4$H$_2$PO$_4$. For LC-MS/MS analysis, the phosphopeptides were dissolved in 5 µL of 0.3% formic acid (FA) with 3% ACN and injected into an ACQUITY UPLC (Waters, Milford, USA). Peptides
were separated on a 45 cm in-house packed column (360 µm OD × 75 µm ID) containing C18 resin (2.2 µm, 100Å, Michrom Bioresources, Auburn, USA). The mobile phase buffer consisted of 0.1% FA in ultra-pure water (buffer A) with an eluting buffer of 0.1% FA in 80% ACN (buffer B) run over a linear 60 mins gradient of 6% - 30% buffer B at the flow rate of 250 nL/min. The Easy-nLC 1000 was coupled online with a Q-EXACTIVE plus mass spectrometer (Thermo Fisher, Waltham, USA). The mass spectrometer was operated in the data-dependent mode with a full-scan MS (from m/z 350-1500 with the resolution of 60,000 at m/z 200). The 10 most intense ions were subjected to high-energy collision dissociation (HCD) fragmentation (normalized collision energy (NCE) 27, AGC 4e5, max injection time 120 ms). The raw files were searched directly against the *Arabidopsis thaliana* database (TAIR10) with no redundant entries using Proteome Discover 2.2. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.02 Da. Search criteria included a static carbamidomethylation of cysteines (+57.0214 Da) and variable modifications of oxidation (+15.9949 Da) on methionine residues, and phosphorylation (+79.996 Da) on serine, threonine or tyrosine residues were searched. Search was performed with full tryptic digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence database. Relaxed and strict false discovery rates (FDR) were set to 0.05 and 0.01, respectively. GO term enrichment analysis was performed with DAVID (Database for Annotation, Visualization, and Integrated Discovery) (Huang et al., 2009).

### Protein expression and purification

The sequences corresponding to the soluble portions of PUT3 predicted by THMMM (Krogh et al., 2001) were cloned into the pGEX-4T-1 vector with a GST tag (Table S4). To generate PUT3 fragments with different point mutations, the DNA fragments with required mutations were synthesized and cloned into a pGEX-4T-1 vector by homologous recombination (Table S5). The constructs were transformed into *E. coli* strain BL21 (DE3). The expression of recombinant proteins was induced with 0.1 mM IPTG at 37 °C for 3 h. The cells were then collected by centrifuged at 7,700 ×g for 10 mins at 4 °C and lysed by sonication. The recombinant proteins were purified with glutathione-Sepharose 4B (GE Healthcare, Princeton, USA) according to the manufacturer’s protocol.
**In vitro phosphorylation assay**

In a 20 µL reaction system, GST-SOS2 and GST-PUT3 fragments were added into the kinase reaction buffer (20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 10 µM ATP, and 1 mM DTT). Enzymatic reactions were initiated with the addition of 0.5 µL [γ-32P] ATP (5 µCi) (Perkin Elmer, Waltham, USA). The reaction mixtures were then incubated at 30 °C for 30 mins. Reactions were terminated by adding Laemmli’s sample buffer and incubated at 95 °C for 5 mins. Proteins were separated by SDS-PAGE and the gel was first stained with Coomassie brilliant blue R250 followed by exposing to a phosphor screen overnight. Radioactivity was detected with Typhoon FLA 9500 (GE Healthcare).

**Transport assay**

Transport assay was performed as described in Chai et al. (2017a). Briefly, twenty 2-week-old seedlings of each line with entire roots were saturated with liquid 1/2 MS medium and incubated 30 mins at the designed temperatures for pretreatment (Table S6). Pretreated seedlings were then incubated with 1/2 MS liquid medium containing the designed amount of ³H-labeled spermidine (Spd) (American Radiolabeled Chemicals, ART 1749, Saint Louis, USA) at 25°C for 1 h. Roots of seedlings were then removed by scissor and collected into a centrifuge tube and washed with pre-cold liquid 1/2 MS medium 5 times. Intracellular contents in roots were extracted in 500 µL cold extraction buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM β-mercaptoethanol). After centrifugation at 13,000 ×g for 20 mins at 4 °C, the supernatant was collected and measured for radioactivity using a Tri-Carb® 2910TR Liquid Scintillation Analyzer (Perkin Elmer). Three biological replicates were used at the same time.

**Yeast growth**

The yeast strain △agp2 harboring PUT3 gene was described previously (Shen et al., 2016). The coding region of SOS1 was cloned into the pESC-LEU vector (Supplemental Table S7). The full length of wild type SOS2 (SOS2 WT) and constitute active SOS2 (SOS2 CA) coding sequences were cloned into the pESC-HIS vector, respectively (Table S7). The yeast strain △agp2 harboring different combinations of constructs were grown in SC liquid medium without histidine, leucine, uracil, and with 2% galactose at 30 °C overnight with 250 RPM shaking. The OD₆₀₀ of the overnight culture was

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adjusted to 1.0, and 5 µL diluted cell culture and their 5-fold series dilutions were spotted on synthetic complete (SC) medium without histidine, leucine, uracil medium with 2% galactose and supplemented with different concentrations of spermine. Plates were incubated at 30 °C and images were taken after 3 days.

The yeast strains YP890 and AXT3K were described previously (Guo et al., 2004; Quintero et al., 2002). The growth of yeast cells in the presence of NaCl was tested on the AP medium, which is deficient in alkali cations (Guo et al., 2004). The coding region of PUT3 was cloned into the p424GPD vector, and the coding sequences of both SOS2 WT and SOS2 CA were cloned into the p416GPD vector, respectively. Yeast cells were cultured overnight in liquid AP medium containing 1 mM KCl, the OD$_{600}$ of the overnight culture was adjusted to 0.8 and 5 µL of series dilutions were spotted onto AP medium plus 1 mM KCl and 100 mM NaCl and allowed to grow at 28°C for 4 days.

Results

Genetic interaction between PUT3, SOS1, and SOS2

Since sos1, sos2 and put3 mutants show the same paraquat resistant phenotype, we tested the genetic interactions among these three genes to determine whether SOS1, SOS2 and PUT3 function in the same pathway. Overexpression of PUT3 (PUT3OE) results in hypersensitivity to polyamines due to increased uptake transport of polyamines into the transgenic plants (Shen et al., 2016). We thus utilized the PUT3 gain-of-function phenotype and tested whether sos1 and sos2 mutations affect the PUT3 function. The sos1-1 and sos2-2 mutations were introgressed into the PUT3OE background, respectively, by genetic crossing, and the homozygous lines of PUT3OE/sos1 and PUT3OE/sos2 were identified (Supporting information Fig. S1). As expected, the PUT3OE transgenic line was hypersensitive to polyamines as indicated by severely inhibited primary root growth in the presence of Spd, Spm, or paraquat (Fig. 1; Figs. S2, S3). However, the sos1 mutation clearly suppressed the hypersensitivity of PUT3OE (Fig. 1; Figs. S2, S3), and the primary root growth of PUT3OE/sos1 showed 26% reduction comparing with 89% reduction in PUT3OE in the presence of 35 µM Spd (Fig.
Similarly, the sos2 mutation also significantly decreased the sensitivity of PUT3OE seedlings to Spd (Fig. 1b, d).

In addition to the hypersensitivity to polyamines and paraquat, PUT3OE also displayed hypersensitive phenotype to H2O2, which was indicated by bleached leaves and remarkably reduced fresh weight of PUT3OE when compared with Col-0 (Fig. 2a, c). The sos1, and sos2 to less extend, also attenuated the hypersensitivity of PUT3OE to H2O2, as indicated by greener leaves and increased fresh weight in PUT3OE/sos1 and PUT3OE/sos2 in the presence of H2O2 (Fig. 2a, c). We also investigated the sensitivity of sos1, sos2, and put3 mutants to H2O2, and the results showed that both sos1 and sos2 mutants were more resistant to H2O2 than Col-0 (Fig. 2b, d). Together, the genetic data clearly indicates that SOS1 and SOS2 are required for PUT3 function, and the attenuation of PUT3OE sensitivity to polyamines and H2O2 by sos1 and sos2 mutations is likely due to a decrease in polyamine uptake transport through PUT3.

PUT3 physically interacts with SOS1 and SOS2

Since both PUT3 and SOS1 are transmembrane proteins in plasma membrane, we therefore used the split-ubiquitin membrane-based yeast two-hybrid assay, and the results showed that PUT3 could interact with both SOS1 and SOS2 in yeast (Fig. 3a). The interactions were further verified by using split-luciferase complementation assay in plants. The interactions of cLUC-PUT3 with nLUC-SOS1 and nLUC-PUT3 with cLUC-SOS2 were clearly evidenced by the activity of reconstituted luciferase enzyme in N. benthamiana leaves (Fig. 3b). We also subjected the N. benthamiana leaves transiently co-expressing SOS2-FLAG with PUT3-nLUC to co-immunoprecipitation (Co-IP) assay, and the result showed that, when FLAG-tagged SOS2 was precipitated by anti-FLAG antibody, PUT3-nLUC was co-precipitated with SOS2-FLAG (Fig. 3c). Moreover, bimolecular fluorescence complementation (BiFC) assay revealed that PUT3 was associated with SOS2 on the plasma membrane in N. benthamiana leaves, but not with the membrane-associated receptor-like kinase BAM1, which was used as a control in the assay (Fig. 3d). Both Co-IP and BiFC assays failed to detect the interaction between PUT3 and SOS1 in N. benthamiana leaves or Arabidopsis protoplasts, which is likely due to a low level of expression of the SOS1 transgene.
PUT3 phosphorylation is attenuated in the sos2 mutant

We used phosphoproteomics approach to determine whether PUT3 can be phosphorylated by SOS2 in planta. Total membrane proteins were isolated from PUT3OE and PUT3OE/sos2 plants with or without 38 °C 1 h treatment, and phosphopeptides were enriched for LC-MS/MS analysis. A total of 11751 phosphopeptides detected, which were originated from 4735 proteins (Table S8). Among these 4735 phosphoproteins, 1748 proteins showed at least 2-fold increase in the abundance of phosphopeptides after 38 °C treatment for 1 h (Fig. S4), and 996 out of these 1748 proteins are either localized in the plasma membrane or membranes of different cellular organelles including membrane proteins and membrane-interacting proteins.

Two phosphopeptides from PUT3 proteins were identified in both PUT3OE and PUT3OE/sos2. These two phosphopeptides are adjacent to each other at the predicted N-terminal soluble portion of PUT3. The first identified phosphopeptide, ranging from amino acid 1 to 17 in PUT3, showed slightly higher phosphorylation in PUT3OE/sos2 mutant than in PUT3OE with or without heat stress treatment (Fig. 4a), suggesting that this phosphopeptide may be phosphorylated by other kinases rather than SOS2. However, the abundance of another identified phosphopeptide (aa 18 to 45) was about 2-fold higher in PUT3OE than PUT3OE/sos2 after heat stress treatment (Fig. 4b). Interestingly, phosphorylation of this peptide was clearly increased in response to heat stress, while this increase was abolished in the sos2 mutant background (Fig. 4b). These results indicate that heat stress induces the phosphorylation of PUT3 by SOS2.

SOS2 phosphorylates PUT3 in vitro.

To verify the phosphorylation of PUT3 by SOS2, we performed in vitro protein phosphorylation assay. Eight PUT3 fragments that are predicted to be soluble (Fig. 5a) with possible phosphorylation sites were expressed in E. coli and purified for phosphorylation assay. The constitutively active form of SOS2 (T168D S228D/Δ308) was used in this assay due to its much higher protein kinase activity than the wild type SOS2 and its independence of other proteins for activation (Fujii and Zhu, 2009).
In agreement with the phosphoproteomics analysis, the glutathione S-transferase (GST)-fused N-terminal fragment (GST-frag A) of PUT3 could indeed be phosphorylated by SOS2 in vitro (Fig. 5b, c). Moreover, GST-fragment H (GST-frag H), which was not detected by the phosphoproteomics analysis, could also be phosphorylated by SOS2 in vitro (Fig. 5c). Fragment B, C, D, E, F and G fusion proteins did not show any phosphorylation signals, which may be explained by their relatively limited spatial confirmations that could restrict the binding of a protein kinase. On the contrary, fragment A and H are located at the soluble N-terminal and C-terminal tails, respectively, which possess enough space for interaction with other proteins.

To determine the SOS2 phosphorylation sites in PUT3, we generated multiple point mutations in both fragment A and fragment H. For the fragment A, when all the 13 serine and threonine residues were mutated to alanine (M13), the GST-frag A M13 no longer showed phosphorylation by SOS2 in vitro (Fig. 5d). Since only nine out of thirteen S/T in the fragment A were predicted to be potential phosphorylation sites from our phosphoproteomics analysis (Table S8), we thus mutated all the nine potential phosphorylation sites (M9) (S5A, S6A, S19A, T20A, S31A, T35A, T36A, T41A, S42A). Fig. 5d shows that, although the mutations of M9 did not completely abolish phosphorylation, the phosphorylation signal of M9 is much lower than wild type GST-frag A, which indicates that the possible phosphorylation site is among these nine residues. We therefore further generated individual point mutations within the nine potential sites and found that S42A showed a similar decreased phosphorylation as M9 (Fig. 5d), which suggests that S42 may be a major phosphorylation site by SOS2. In addition, we also generated individual mutations for the four sites (T2A, S11A, S13A, S46A) that were not predicted to be the phosphorylation sites in the fragment A, and the results showed that the phosphorylation levels of these mutants were comparable to or higher than wild type GST-frag A (Fig. 5d). Fragment H includes four sequentially connected serine and threonine residues, STSS from aa 480 to 483 (Fig. 5b). When all four residues were mutated to alanine, the phosphorylation signal disappeared (Fig. 5e). Individual point mutations (S480A, T481A, S482A, S483A) did not result in reduced phosphorylation signal (data not shown), indicating that there could be more than one phosphorylation sites in this fragment. We then mutated wild type fragment H with amino acid sequence STSS to SAAA, ATAA, AASA, AAAS, ATAS, and SASA. GST-fusion proteins with amino acid SAAA and AASA displayed lower phosphorylation signal than wild type GST-frag H,
whereas ATAA, AAAS, and ATAS showed the signals comparable to wild type GST-frag H (Fig. 5e). Furthermore, SASA showed a decreased signal similar as SAAA and AASA (Fig. 5e). Together, these results indicate that all four residues in the fragment H could be phosphorylated by SOS2 in vitro but T481 and S483 are likely the major phosphorylation sites in PUT3 by SOS2 kinase.

**SOS1 and SOS2 regulate the polyamine uptake activity of PUT3**

Whether SOS1 and SOS2 can directly modulate the activity of PUT3 was assessed by transport assay as described in Shen et al. (2016). The Spd uptake transport activities were compared among the seedlings of Col-0, sos1, sos2, PUT3OE, PUT3OE/sos1, and PUT3OE/sos2. As shown in Fig. 6, PUT3OE displayed 3.6-fold increase in Spd transport activity comparing with Col-0 wild type, while PUT3OE/sos1 and PUT3OE/sos2 showed reduced Spd transport activity when compared to PUT3OE. PUT3OE/sos1 and PUT3OE/sos2 exhibited 85% and 66% transport capacity of PUT3OE. Moreover, the sos1 and sos2 mutants showed lower basal polyamine uptake transport activity than Col-0, with 57% and 61% Spd transport capacity compared to Col-0 wild type, respectively (Fig. 6). These results suggest that SOS1 and SOS2 directly affect the polyamine uptake transport activity of PUT3.

**Co-expression of SOS1 and SOS2 activates PUT3 in yeast**

The function of SOS pathway proteins can be reconstituted in a yeast strain that lacks the major sodium transporters NHA1, NHX1, and ENA1-4 (Quintero et al., 2002, 2011). Yeast strain △agp2 lacks the polyamine influx transporter AGP2 and exhibits tolerance to polyamines (Aouida et al., 2005), and the introduction of PUT3 increases the sensitivity of the mutant to polyamines (Mulangi et al., 2012; Shen et al., 2016). To test whether SOS1 and SOS2 regulate PUT3 synergistically or independently, we co-expressed PUT3 with SOS1 and SOS2 in yeast cells and determined the ability of the yeast cells to grow on the medium supplemented with spermine. As shown in Fig. 7a, the concurrent expression of SOS1, SOS2, and PUT3 inhibited the yeast growth compared to the expression of PUT3 alone. However, the yeast cells lacking SOS1 or SOS2 did not show the inhibited growth, and PUT3 + SOS1 and PUT3 + wild type SOS2 (SOS2 WT) or + constitutively active SOS2 (SOS2 CA) exhibited the same growth phenotype as PUT3 alone. Notably, compared with the SOS2 WT, SOS2 CA co-expressed with PUT3 and SOS1 showed greater inhibition of yeast growth by
spermine. Taken together, these results indicate that both Na⁺/H⁺ antiporter SOS1 and protein kinase SOS2 are necessary for the in vivo activation of PUT3.

**SOS2 and PUT3 increase the SOS1-confered salt tolerance in yeast**

We also tested whether PUT3 can affect the salt tolerance rendered by SOS1. SOS2 WT and SOS2 CA were introduced into *S. cerevisiae* strain YP890, in which the major endogenous sodium transporters (plasma membrane NHA1, vacuolar Na⁺/H⁺ exchanger NHX1, and Na⁺ efflux proteins ENA1-4) were knocked out, and the Arabidopsis *SOS1* gene was inserted into the chromosome and constitutively expressed (Guo et al., 2004). As shown in Fig. 7b, salt tolerance was not increased either by the expression of PUT3 or the SOS2 WT alone, while expression of the SOS2 CA increased the salt tolerance of YP890. The concurrent expression of PUT3 and SOS2 displayed a greater increase in salt tolerance than expression of the SOS2 CA, and co-expression of PUT3 and SOS2 CA showed the highest capability to improve salt tolerance of the yeast cells (Fig. 7b). To exclude the possibility that PUT3 can increase salt tolerance in a SOS1-independent manner, we also transformed the same combinations to the AXT3K yeast strain, in which ENA1-4 and NHA1 were knocked out but lacked the *SOS1* chromosomal integration (Quintero et al., 2002). The yeast cells expressing these combinations did not grow in the presence of 100 mM NaCl (Fig. S5), the same concentration used for the yeast strain YP890 showing in Fig. 7b. This result indicates that the increased salt tolerance of the SOS1-expressing yeast strain YP890 by PUT3 and SOS2 was SOS1-dependent and was not resulted from PUT3 or SOS2 alone. Taken together, our results demonstrate that PUT3 alone could not render salt tolerance, but PUT3 can activate SOS2 and thus enhance the salt tolerance rendered by SOS1. The mechanism of SOS2 activation by PUT3 is still unclear and awaits further investigation.

**Discussion**

Numerous membrane integral proteins and membrane associated proteins play essential roles in many cellular processes including cell signaling, nutrient uptake, efflux of ions or exclusion compounds, etc. Although membrane proteins could potentially flow freely in the membrane due the fluidity of the lipid bilayer, many membrane proteins form complexes in the plasma membrane or
other membranes to carry out their functions. This has been demonstrated by numerous examples such as the membrane complexes of endocytosis and exocytosis (Doherty and McMahon, 2009; Heider and Munson, 2012), the ATP synthase (Junge and Nelson, 2015), etc. It is also well known that the membrane receptors dimerize upon ligand binding, which is a typical early signaling event in eukaryotic cells. However, how different transmembrane transporters form complexes to regulate their transport activity, especially in response to stress conditions, is largely unknown. In mammalian cells, the mitochondrial solute carrier SLC25A23 is a Mg/ATP-Pi transporter located in the inner membrane and contains functional Ca\(^{2+}\)-binding EF-hands (Bassi et al., 2005). The mitochondrial inner membrane localized Ca\(^{2+}\) uniporter (MCU) controls the influx of Ca\(^{2+}\) from the cytosol to mitochondria (Rizzuto, et al., 2012). SLC25A23 participates in mitochondrial Ca\(^{2+}\) uptake through interacting with MCU and its regulator MICU1 (Hoffman et al., 2014). Functionally, SLC25A23 senses Ca\(^{2+}\) by its EF-hands, which provides a response to enhance MCU-mediated mitochondrial Ca\(^{2+}\) uptake through such interactions in the mitochondrial inner membrane. In this study, we identified the interaction between two plasma membrane transporters PUT3 and SOS1 with different transport properties. PUT3 is a polyamine uptake transporter (Shen et al., 2016), while SOS1 is a Na\(^{+}/H^{+}\) antiporter (Shi et al., 2000). The functional interaction and regulation of the transport activities require the cytosolic protein kinase SOS2. We show that the sos1 and sos2 mutations reduce the hypersensitivity of PUT3OE to polyamines (Fig. 1), strong genetic evidence indicating the involvement of SOS1 and SOS2 in the regulation of PUT3. PUT3 physically interacts with SOS1 and SOS2 (Fig. 3), and SOS2 can phosphorylate PUT3 both in vitro and in vivo (Figs. 4 and 5). Therefore, the regulation of polyamine uptake transport activity of PUT3 by SOS1 and SOS2 (Fig. 6) is likely through its interaction with SOS1 and phosphorylation by SOS2. The paraquat resistant phenotype of sos1 and sos2 is likely due to impaired activity of PUT3 and thus reduced uptake of this toxic herbicide. The sos1 and sos2 mutations only partially rescued the PUT3OE phenotypes, indicating that PUT3 has a basal activity when it is not complexed with SOS1/SOS2. Alternatively, SOS1 and SOS2 could be the major regulators of PUT3, but other membrane transporters and protein kinases may also play roles in modulating PUT3 transport activity.

SOS2 is a well-known protein kinase crucial for salt tolerance by phosphorylating the plasma membrane Na\(^{+}/H^{+}\) antiporter SOS1 (Quintero et al., 2002). In this study, we identified PUT3 as one of
the SOS2 phosphorylation substrates. Phosphorylation of PUT3 is induced by heat stress, while this heat-induced phosphorylation is abolished in the sos2 mutant (Fig. 4). This strongly suggests that the SOS2-mediated PUT3 phosphorylation is elicited by heat stress and the association of PUT3, SOS1 and SOS2 is likely to be heat-responsive. In yeast, four plasma membrane transporters, DUR3, SAM3, GAP1, and AGP2, and two protein kinases, PTK1 and PTK2, are crucial for polyamine uptake (Igarashi and Kashiwagi, 2010). PTK2 phosphorylates T250, S251, and T684 of DUR3 and thus regulates the polyamine uptake transport activity of DUR3 (Igarashi and Kashiwagi, 2010). Sequence analysis shows that SOS2 (CIPK24) and other CIPKs in Arabidopsis share significant similarity with the yeast PTK2 (Chai et al., 2017b), which suggests that phosphorylation of polyamine transporters by SOS2-type protein kinases could be a conserved mechanism to regulate polyamine uptake in eukaryotes.

Interestingly, among the 1748 heat-induced phosphoproteins, 908 phosphoproteins are SOS2-related (Fig. S4), i.e. these proteins could be phosphorylated either by SOS2 directly or by the SOS2 downstream kinases. However, only 16 phosphoproteins are SOS2-related under room temperature. These results, together with previous findings, suggest that SOS2 is activated mainly under stress conditions. Gene Ontology (GO) analysis of the 1748 heat-induced phosphoproteins (Fig. S6) showed the enrichment of 173 protein kinases including membrane-anchored kinases (e.g., CIPK23, etc.) and receptor-like kinases (e.g., Leucine-rich receptor-like protein kinase family proteins, etc.), and 66 phosphoproteins in the “response to ABA”, including calcium-dependent protein kinase family protein (CPK6) (Brandt et al., 2015), SNF1-related kinases (SnRK2.1, 2.2, 2.4, 2.10) (Boudsocq et al., 2004; Zhu, 2016), SLAC1 homologs (SLAH2 and SLAH3) (Geiger et al., 2009), the receptor-like kinase GHR1 (Hua et al., 2012), the respiratory burst oxidase homolog D (RbohD) (Zhang et al., 2009), and the plasma membrane H^+-ATPase AHA1, AHA2, and AHA11 (Merlot et al., 2007). Notably, the heat stress enhanced phosphorylation of RbohD, GHR1, CPK6, AHA1, AHA2, and AHA11 are SOS2-related. Our phosphoproteomics analysis revealed that, in addition to PUT3 and SOS1, SOS2 also phosphorylates many other proteins directly or indirectly in response to heat stress (Table S8). This provides valuable information for future study of the complex network of SOS2 functions in plant stress response.
In our study, we found that PUT3 could also enhance the activity of SOS1 by activating SOS2 in yeast (Fig. 7B). Determining whether this regulatory mechanism occurs in Arabidopsis requires further study. However, it is possible that PUT3 is associated with SOS1 and SOS2 under salt stress condition and enhances SOS1 activity by recruiting and activating SOS2 at the plasma membrane. Since put3 mutant is not hypersensitive to salt stress, we speculate that the PUT3 homologs such as PUT1 and PUT2 may have redundant function with PUT3 in the regulation of SOS1 by activating SOS2.

Both sos1 and sos2 mutants showed increased resistance to H$_2$O$_2$, while PUT3OE displayed hypersensitivity to H$_2$O$_2$ (Fig. 2). These results indicate that these three genes are involved in oxidative stress response. It has been well-known that abiotic stresses cause the accumulation of reactive oxygen species (ROS), which result in oxidative stress as a common second layer of stress under different stress conditions (Zhu 2016). Therefore, the association of these three proteins could be a mechanism to mitigate oxidative stress under heat or salt stress conditions. Polyamines have been well-known to protect organisms from ROS damage, while polyamine catabolism generates ROS (Murry Stewart et al., 2018). Thus, polyamine homeostasis is crucial for oxidative stress response. We propose that under heat stress, SOS2 is activated by a yet to be identified mechanism, and PUT3, SOS1, and SOS2 form a complex; SOS2 phosphorylates PUT3 and enhances PUT3 transport activity, which increases the cytosolic concentration of polyamines. The accumulated polyamines in cytosol could function as anti-ROS molecules to reduce oxidative stress as well as protecting molecules to stabilize cellular components such as mRNAs as we have shown in our previous report (Shen et al., 2016). By forming this complex under heat stress, the activity of SOS1 could also be enhanced, and increased SOS1 activity could create a microenvironment of H$^+$ gradient that favors PUT3 transporter. Indeed, PUT3 activity has been shown to be pH-sensitive (Fujita et al., 2012). Under salt stress, SOS2 is activated, and by forming a complex of PUT3-SOS1-SOS2, SOS1 and PUT3 are activated. Activated SOS1 confers salt tolerance by transporting Na$^+$ out and thus reducing cytosolic Na$^+$ accumulation, while activated PUT3 promotes cytosolic polyamine accumulation and protects cells from oxidative stress.

In conclusion, both genetic and biochemical evidence strongly supports that the polyamine transport activity of PUT3 is positively regulated by SOS1 and SOS2 through protein-protein
interaction and SOS2-mediated phosphorylation. In addition, PUT3 also enhances the activity of SOS1 by activating SOS2 through an unclear mechanism. This reciprocal regulatory phenomenon between two transmembrane transporters involving a protein phosphorylation event represents a novel mechanism that modulates transport activities across the plasma membrane in response to different stress conditions.

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Author Contributions
H.C, J-K.Z. and H.S conceived the project. H.C., J.G., Y.Z., C-C. H. designed and carried out the experiments. H.C., C.Z., P.W., H.S analyzed the data. H.C. H.S. drafted and revised the manuscript.

References


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Legends for Supplemental Figures

Fig. S1. Phenotypical verification of PUT3OE/sos1 and PUT3OE/sos2 lines.
Fig. S2. The sos1 and sos2 mutations attenuate the hypersensitivity of PUT3OE to spermine.
Fig. S3. The sos1 and sos2 mutations attenuate the hypersensitivity of PUT3OE to paraquat.
Fig. S4. Numbers of proteins with at least 2-fold increase in phosphorylation level.
Fig. S5. Salt tolerance assay of yeast cells expressing PUT3 and SOS2.
Fig. S6. Gene ontology enrichment analysis of heat-induced phosphorylated proteins.

Legends for Supplemental Tables

Table S1. Primers used for Split-Ubiquitin yeast two-hybrid.
Table S2. Primers used for Split-luciferase (LUC) complementation assay.
Table S3. Primers used for TOPO cloning
Table S4. Primers used for recombinant proteins expression for in vitro phosphorylation assay.
Table S5. Synthesized double stranded DNA fragments used for site mutations.
Table S6. Designed temperatures and Spd concentrations used in transport assay of plant seedlings.
Table S7. Primers used for yeast reconstitution assay.
Table S8. Phosphoproteomics data showing phosphopeptides and phosphoproteins detected in PUT3OE and PUT3OE/sos2 with or without heat stress.

Figure Legends

Fig. 1. The genetic interactions of PUT3 with SOS1 and SOS2.
(a) and (b) The Arabidopsis sos1 (a) and sos2 (b) mutations decreased the hypersensitivity of PUT3OE to spermidine (Spd). Five-day-old seedlings were transferred to 1/2 MS medium supplemented with Spd and allowed to grow for another 6 days.
(c) and (d) Quantitative measurement of relative primary root length of Arabidopsis Col-0, PUT3OE, PUT3OE/sos1, PUT3OE/sos2 and sos1, sos2. The root lengths were measured by using the software ImageJ. Values are means ± standard deviation (***P < 0.001; n = 15 (two-tailed, type 2 t test)).

Fig. 2. The sos1 and sos2 mutations reduce the hypersensitivity of PUT3OE to H2O2.
(a) Growth phenotype of Arabidopsis Col-0, PUT3OE, PUT3OE/sos1, and PUT3OE/sos2 seedlings in the presence of H2O2.
(b) Growth phenotype of Arabidopsis Col-0, sos1, sos2, and put3 seedlings in the presence of H2O2.
(c) Quantitative measurement of the relative fresh weight of Arabidopsis Col-0, PUT3OE, PUT3OE/sos1, PUT3OE/sos2. Seven-day-old seedlings were treated with H2O2 and allowed to grow for another 7 days. Values are means ± standard deviation (*P < 0.05, **P < 0.01; n = 3 (two-tailed, type 2 t test)).
(d) Quantitative measurement of the relative fresh weight of Arabidopsis wild type, sos1, sos2, and put3. Seven-day-old seedlings were treated with $\text{H}_2\text{O}_2$ and allowed to grow for another 7 days. Values are means ± standard deviation (**P < 0.01; n = 3 (two-tailed, type 2 t test)).

**Fig. 3. PUT3 physically interacts with SOS1 and SOS2.**

(a) Split-ubiquitin assay showing the interactions between PUT3, SOS1 and SOS2 in *Saccharomyces cerevisiae*. Full-length PUT3 was fused with the Cub and NubG, respectively. Full-length SOS2 was fused with the Cub. Wild type NubI with cub-fused PUT3 or SOS2 were served as positive controls. Yeast cells grown on the SC-WL (synthetic complete medium without tryptophan and leucine) medium were shown as growth controls. Protein interaction was indicated by yeast cells growing on the SC-HWL (synthetic complete medium without histidine, tryptophan, and leucine). Plates were incubated at 30°C and photographed after 5 days of yeast growth.

(b) Split-luciferase assay showing interactions of PUT3 with SOS1 and SOS2 in *Nicotiana benthamiana* leaves. PUT3 was fused with nLUC and cLUC, respectively. SOS1 was fused with nLUC and SOS2 was fused with cLUC. Luciferase activities were detected at 48 h after infiltration. Dotted-line circles indicate leaf areas that were infiltrated with Agrobacterium strains containing the indicated constructs.

(c) Co-immunoprecipitation of PUT3-nLUC and SOS2-FLAG proteins transiently co-expressed in *N. benthamiana* leaves. After 48 h inoculation, total proteins were extracted and used for immunoprecipitation (IP) with anti-FLAG M2 affinity gel, and the eluted fractions were detected by immunoblot analysis (IB) with anti-luciferase antibodies. Total proteins (INPUT) were detected with anti-Flag and anti-luciferase antibodies. GUS-FLAG was served as a negative control. Numbers on the left indicate molecular weight.

(d) BiFC assay of PUT3 and SOS2 in *N. benthamiana* leaves. PUT3 was fused with cYFP and SOS2 was fused with nYFP. The membrane associated receptor-like kinase BAM1 was used as a negative control. The constructs were co-injected into *N. benthamiana* leaves, and YFP signals were observed after 48 - 72 h. Scale bars, 25 μm.

**Fig. 4. Detection of PUT3 phosphorylation in vivo.**

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(a) and (b) The abundance of two phosphopeptides from PUT3 in Arabidopsis wild type and sos2 mutant treated with or without 38 °C for 1 h. The phosphopeptides were determined by phosphoproteomics. Values are means ± standard deviation (*P < 0.05, **P < 0.01, ***P < 0.001, NS, not significant, n = 3 (two-tailed, type 2 t test))

Fig. 5. SOS2 phosphorylates PUT3 in vitro.
(a) A schematic diagram showing the topology of PUT3, as predicted by TMHMM.
(b) Amino acid sequences of the fragment A and H. Serine and Threonine residues are in grey.
(c) Phosphorylation of PUT3 fragments by SOS2 in vitro. Phosphorylated GST-fragment A and H phosphorylation by SOS2 (bands at the bottom) and GST-SOS2 autophosphorylated GST-SOS2 (bands at the top) are shown by the arrows. Gel image showing Coomassie blue staining was used as a loading control.
(d, e) In vitro phosphorylation of PUT3 fragments with point mutations in fragment A (d) and fragment H (e). Phosphorylated GST-fragments (the bottom bands) and autophosphorylated GST-SOS2 (the top bands) are indicated by arrows. Gel images showing Coomassie blue staining were used as loading controls.

Fig. 6. SOS1 and SOS2 regulate spermidine uptake activity of PUT3 in Arabidopsis seedlings.
The values were normalized with the value of Col-0 wild type. Values are means ± standard deviation (*P < 0.05, **P < 0.01; n = 3 (two-tailed, type 2 t test)).

Fig. 7. Regulation of PUT3 and SOS1 activities through SOS2 in yeast.
(a) SOS1 and SOS2 enhance the function of PUT3 in yeast. Yeast mutant strain △agp2 transformed with empty vectors (control) or expressing the indicated combination of Arabidopsis genes were grown overnight in liquid SC -His -Leu -Ura medium (synthetic complete medium without histidine, leucine, and uracil) with 2% galactose. Five microliters of series dilutions were spotted onto the solid medium supplemented with or without 1 or 1.5 mM Spm. The yeast cells were incubated at 28°C and photographed after 2.5 days of yeast growth.
(b) PUT3 and SOS2 increase salt tolerance rendered by SOS1. The yeast strain YP890 constitutively expressing the Arabidopsis SOS1 was transformed with the indicated combinations of Arabidopsis genes. Yeast cells were first grown overnight in liquid AP medium with 1 mM KCl. Five microliters of series dilutions were spotted onto the agar medium supplemented with or without 100 mM NaCl. Plates were incubated at 28°C and photographed after 4 days of yeast growth.
Figure 1

The figure shows the relative root growth of different genotypes under various conditions. The graphs illustrate the effects of PUT3OE and PUT3OE/sos on root growth in comparison to Col-0 and sos1/sos2 genotypes in 1/2 MS and 35 μM SPD treatments. The data indicates a significant increase in root growth with PUT3OE expression, especially under 35 μM SPD conditions. The asterisks signify statistical significance: **p < 0.01, ***p < 0.001.
Figure 2

(a) Comparison of plant growth under different H$_2$O$_2$ concentrations for Col-0, PUT3OE, PUT3OE/sos1, and PUT3OE/sos2.

(b) Comparison of plant growth under different H$_2$O$_2$ concentrations for Col-0, sos1, sos2, and put3.

(c) Graph showing relative fresh weight (%) of plants under various H$_2$O$_2$ concentrations. Col-0, PUT3OE, PUT3OE/sos1, and PUT3OE/sos2 are compared.

(d) Graph showing relative fresh weight (%) of plants under various H$_2$O$_2$ concentrations. WT, sos1, sos2, and put3 are compared.
Figure 3


(b) SOS2-FLAG +, GUS-FLAG +, PUT3-nLUC + +

(c) YFP, BF, Merge

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Figure 4

(a) [-.METSSPNLDASQKR.] (AA 1 - 17)

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<td>RT</td>
<td>6.5</td>
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(b) [R].ISTENPPPPPPPHISIGVTGPDATSPAR.[T] (AA 18 - 45)

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<td>38 °C 1h</td>
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Figure 5

Fragment A (1-53): MTLESSLPMDSASQKPRISTENPPPPPHISIGVTTGDPATSPARTVNOI\[KKI
Fragment H (465-490): QCQKLQVEKKGWLKFSSTSSHLPMLE

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Figure 7

(a) SC -His -Leu -Ura, +1 mM Spm, +1.5 mM Spm

(b) AP 1mM KCl, +100 mM NaCl

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