AtPFA-DSP5 interacts with MPK3/MPK6 and negatively regulates plant salt responses

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ABSTRACT
Protein tyrosine phosphatases play essential roles in plant growth and development and in plant responses to biotic or abiotic stresses. We recently demonstrated that an atypical dual-specificity protein tyrosine phosphatase in plants, AtPFA-DSP5 (DSP3), negatively regulates plant salt tolerance. Here, we report that a homolog of DSP3, AtPFA-DSP5 (DSP5), affects the response of plants to high-salt conditions. A loss-of-function mutant of DSP5 showed reduced sensitivity to salt treatment at the seed germination and vegetative stages of development while a gain-of-function mutant of DSP5 showed increased sensitivity to salt stress. The salt responses of dsp3dsp5 double-mutant plants were similar to those of dsp3 and dsp5 single-mutant plants. Gel overlay and firefly luciferase complementation assays showed that DSP5 interacts with MPK3 and MPK6 in vitro and in vivo. These results indicate that DSP5 is a novel negative regulator of salt responses in Arabidopsis that interacts directly with MPK3 and MPK6.

Salinity, a major abiotic stress, affects plants throughout their development – from seed germination to plant growth. Excessive soil salinity affects the absorption of water and nutrients and reduces photosynthesis, energy metabolism, protein synthesis, and lipid metabolism in plants, thus affecting crop growth and yields. Therefore, research on how to improve the salt resistance of plants has become increasingly important.

Protein phosphorylation/dephosphorylation is essential for mediating many biological processes. The protein phosphorylation level in cells is tightly regulated by protein kinases and protein phosphatases. Based on their substrate specificity, protein phosphatases are divided into serine/threonine phosphatases and tyrosine phosphatases (PTPs). PTPs are further classified into tyrosine-specific PTPs and dual-specificity protein phosphatases (DsPTPs), which target both phosphorylated tyrosine and serine/threonine residues. In Arabidopsis, there is only one classical PTP, named AtPTP1. The DsPTPs in Arabidopsis include five well-known mitogen-activated protein kinase phosphatases (MKPs), five plant and fungi atypical dual-specificity phosphatases (PFA-DSPs), three phosphatase and tensin homolog (PTEN) phosphatases, and several other DsPTPs. PTPs play crucial roles in plant growth and development, as well as in plant responses to external stimuli. For example, plants carrying a mutation in IBR5, an MKP gene, displayed increased leaf serration and aberrant vascular patterning in Arabidopsis. Another MKP gene, PHSI, plays a role in regulating the floral transition in Arabidopsis. Silencing of AtPTEN1 causes pollen cell death after mitosis, suggesting that AtPTEN1 is essential for pollen development. In addition, PTPs were shown to be involved in plant abiotic and biotic stress responses. For example, plants with a loss of function in AtMKP1 display elevated resistance to the bacterium Pseudomonas syringae pv. tomato DC3000. AtPFA-DSP4 and its homolog OsPFA-DSP2 also negatively regulate the pathogen response in plants. OsPFA-DSP1, which is induced by drought stress, negatively regulates drought stress responses in rice. Detailed mechanistic studies showed that several PTPs function through the MAPK cascade, which is one of the most important signaling pathways mediating stress responses in plants.

In Arabidopsis, AtPTP1 and MKP1 influence microtubule stability through interactions with MPK3, MPK4, and MPK6 under conditions of salt stress. In wheat, TMKP1, the homolog of Arabidopsis MKP1, interacts directly with MPK3/MPK6. When TMKP1 is overexpressed in Arabidopsis, the transgenic plants are more tolerant to salt stress and especially lithium chloride stress, through an increase in antioxidant enzyme activity. Recently, we reported that AtPFA-DSP3 (DSP3) encodes a nuclear-localized protein tyrosine phosphatase that negatively regulates salt stress by directly interacting with MPK3/MPK6 and regulating their protein phosphorylation levels. However, our knowledge of the biological functions and regulatory mechanisms of PTPs remains limited.

DSP3 has four homologs in Arabidopsis, among them, AtPFA-DSP5 (DSP5) shares the highest degree of similarity with DSP3 (around 80% in amino acid sequence). DSP5 and DSP3 have similar three-dimensional structures and they both contain the conserved and characteristic motifs found in other members of the PFA-DSP family. However, DSP3 displays a high level of phosphatase activity toward P(3,5)P2 and pTyr, while DSP5 displays phosphatase activity only...
toward PI(3.5)P2 (not pTyr) in vitro. In addition, DSP3 was shown to be predominantly expressed in roots, while DSP5 was shown to be most highly expressed in flowers. To date, the biological function of DSP5 is unclear in plants and whether DSP5 is involved in salt stress responses is unknown. Here, we demonstrate that DSP5 plays a negative role in the salt stress responses of Arabidopsis plants.

**AtPFA-DSP5 plays a role in salinity-related phenotypes at the seed germination stage in Arabidopsis**

To investigate the function of PFA-DSP genes in Arabidopsis, the gene with the highest degree of homology to DSP3, AtPFA-DSP5 (DSP5), was chosen for further study. A mutant, *dsp5*, with a T-DNA insertion in the second intron of DSP5 was ordered from the Arabidopsis Biological Resource Center (ABRC; Figure 1a). Gene expression analyses by semi-quantitative RT-PCR showed that the transcript level of DSP5 was almost undetectable in the mutant compared to wild-type plants, indicating that *dsp5* is a loss-of-function mutant (Figure 1b). To analyze the involvement of DSP5 in salt stress responses during seed germination, three batches of harvested seeds were tested with about 100 seeds per genotype in each test. Under normal conditions, the germination rate of *dsp5* was not significantly different from that of wild type. However, when 175 mM sodium chloride (NaCl) was added to half-strength solid Murashige and Skoog (MS) medium, the wild-type seed germination rate was only about 10%, while *dsp5* exhibited a higher germination rate of ~80% (*dsp3* was used as a positive control; Figure 1c). To determine the biological function of DSP5 in plant salt responses, we produced DSP5-overexpressing (DSP5OX) lines in which the 35S promoter-driven DSP5 coding sequence was transformed into Columbia (Col) plants. The overexpression of DSP5 resulted in plants with a small stature and early flowering phenotype that correlated with the DSP5 expression level (similar to DSP3-overexpressing plants; Supplemental Figure S1a,b). The seeds of the plants were harvested and compared with wild-type seeds in terms of their response to salt. Under normal conditions, the germination rate of DSP5OX was not significantly different from that of wild type. However, when 175 mM NaCl was added to half-strength solid MS medium, the wild-type seed germination rate was ~60%, while the DSP5OX seed germination rate was only ~40% (DSP3OX was used as a positive control; Supplemental Figure S1c) indicating that DSP5OX induces poor salt resistance in Arabidopsis.

High salinity causes ionic stress and osmotic stress in plants. To determine which type of stress DSP5 is involved in, seeds from Col and dsp5 mutant plants were germinated on half-strength MS medium containing various concentrations of potassium chloride (KCl) or mannitol. The *dsp5* seeds displayed reduced sensitivity to both KCl and mannitol during germination compared to wild-type seeds (Figure 2), indicating that DSP5 is involved in both ionic stress and osmotic stress. This is different from TMKP1; its overexpression in Arabidopsis improves tolerance to NaCl but not to mannitol. These data suggest that the mechanisms whereby different protein phosphatases regulate salt tolerance are different.

**The mutation of DSP5 produces salinity-related phenotypes at the vegetative stage in Arabidopsis**

We recently reported that DSP3 plays a negative role in the salt responses of plants. Our data show that DSP5, a homolog of DSP3, plays a negative role in salt responses at the seed germination stage (Figures 1 and 2). To further investigate the function of these two genes, a *dsp3dsp5* double mutant was generated by crossing *dsp3* and *dsp5* single mutant plants. Homozygous mutant lines were separated by DNA genotyping (Figure 3a), and quantitative RT-PCR (RT-qPCR) confirmed that *dsp3dsp5* was a double mutant (Figure 3b). To analyze the salt stress responses of mature *dsp5* and *dsp3dsp5* plants, 3-week-old *dsp3*, *dsp5*, *dsp3dsp5*, and wild-type soil-grown plants were treated with 0 (mock) or 200 mM NaCl water solution. Under normal conditions, the *dsp5* and *dsp3dsp5* plants were not much different from wild-type or *dsp3* plants. However, following treatment with 200 mM NaCl, the *dsp5*, *dsp3*, and *dsp3dsp5* plants had fewer withered leaves than did wild-type plants (Figure 3c), and the survival rates of *dsp5* and *dsp3dsp5* were comparable to that of *dsp3* and higher than that of wild type (Figure 3d). The *dsp3dsp5* plants showed little difference compared with *dsp5* and *dsp3* in terms of salt sensitivity.
(Figure 3d). DSP5 and DSP3 are homologous genes, and our data show that both of them play negative roles in plant salt responses. Moreover, the double mutant did not exhibit an aggravated phenotype. Like DSP3, DSP5 was mainly localized to the nucleus in tobacco epidermal cells (Supplemental Figure S2). DSP5 is mainly expressed in flowers, while DSP3 is mainly expressed in roots. We thus speculate that DSP3 and DSP5 function in different tissues or at different developmental stages under conditions of salt stress. Another possibility is that some other DSP homolog plays a redundant role with DSP3 and DSP5 in plant salt responses; this requires further investigation.

**DSP5 interacts with MPK3/MPK6**

We next investigated the interaction between DSP5 and MPK3/MPK6. First, the interaction between DSP5 and MPK6 was examined in an overlay assay. Purified His-MPK6 (used as the primary antibody) was incubated with a nitrocellulose membrane filter containing GST, GST-DSP3, and GST-DSP5; His-HRP antibodies were used as the secondary antibody. His-MPK6 was detected at positions corresponding to the sizes of GST-DSP3 and GST-DSP5, but no band was detected at the position of the GST tag, suggesting a direct interaction between MPK6 and DSP3/DSP5 in vitro (Figure 4a). Furthermore, their interaction was tested using firefly luciferase complementation assays. DSP5 and MPK3 or MPK6 were fused with the N-terminal half and C-terminal half of firefly luciferase to generate DSP5-nLuc and cLuc-MPKs, respectively, and co-infiltrated into *Nicotiana benthamiana* leaves. As shown in Figure 4b, co-expression of cLuc-MPK3/6 with DSP5-nLuc resulted in a strong luminescence signal, as did co-expression of cLuc-MPK3/6 with DSP3-nLuc. The two negative controls (cLuc+DSP5-nLuc and cLuc-MPK3/6-nLuc) produced almost no signal, demonstrating a direct interaction of DSP5 with MPK3/MPK6 in vivo.

In conclusion, we found that DSP5, an *Arabidopsis* PFA-DSP, is involved in plant salinity responses. DSP5 negatively regulates salt sensitivity at the seed germination and vegetative stages of development, similar to DSP3. DSP5 interacted with MPK3/MPK6 in vitro and in vivo, implying that DSP5 regulates salt responses via the MAPK signaling pathway. So far, it is unclear whether DSP5 functions as a tyrosine protein phosphatase in vivo; this is worthy of further exploration. Detection of the phosphorylation levels of MPK3/MPK6 in *dsp5* mutant plants and a genetic phenotype analysis of DSP5 with MPK3/MPK6 should be performed. To further understand the role of DSP5 in the response of plants to salt stress, it will be crucial to identify new upstream and downstream effectors of DSP5-related signaling pathways. The negative mechanism triggered by DSP5 helps to fine-tune the physiological and biochemical processes of plants under conditions of salt stress, thereby promoting survival.

**Materials and methods**

**Plant materials and growth conditions**

In this study, *Arabidopsis thaliana* ecotype Col was used as the wild type. The T-DNA insertion mutant *dsp5* (SALKseq_083083) was obtained from the ABRC (https://abrc.osu.edu/). Seedlings were grown either in a growth
chamber under continuous light (100 μmol m⁻² s⁻¹ intensity) at 22°C or in a culture room under 16 h of light/8 h of darkness (90 μmol m⁻² s⁻¹ intensity) at 22°C and 55% relative humidity.

To identify the dsp3dsp5 double mutant, DNA was extracted from 7-day-old dsp3dsp5 and Col plants. The gDNA band of DSP5 was amplified using DSP5-F: GGAATTCATGGGCTTAATTGTGGATGATGA and DSP5-R: GGGGTACCTCCTTTGGTGGCTTGAGGTTTTTG, and the tDNA band of DSP5 was amplified using DSP5-R: GGGGTACCTCCTTTGGTGGCTTGAGGTTTTTG and LBB1.3: ATTTTGCCGATTTCGGAAC. The primers used to genotype DSP3 were described previously.13

To check the expression level of DSP5 or DSP3 in a different background, total RNA from 7-day-old seedlings was isolated using TRIzol reagent (Invitrogen), and cDNA was prepared using a PrimeScript RT Reagent Kit (Takara). The relative expression of DSP5 in dsp5 and wild-type plants was assessed by semi-quantitative RT-PCR using the primers DSP5-RT-F: GGAATTCATGGGCTTAATTGTGGATGATGA and DSP5-RT-R: GGGGTACCTCCTTTGGTGGCTTGAGGTTTTTG. UBQ5 was used as an internal control.13 The relative expression of DSP5 in dsp3dsp5 and wild-type plants was assessed by RT-qPCR using the primers DSP5-QF: GTGCTCTTAGAGTACTAGTTGATGTTCGGGGTACCTCCTTTGGTGGCTTGAGGTTTTTG and DSP5-QR: CCAGAAGTGTGCGGGTTTGAAATGG. The RT-qPCR primers used for DSP3 and the internal control (PP2AA3) were described previously.13

To produce 35S promoter-driven untagged DSP5, the coding sequence of DSP5 without the stop codon was cloned into the vector pENTR™/SD/D-TOPO™ (Invitrogen) to create the donor vector pENTR/DPS5, and then into the binary vector pEarleyGate100. Then, the binary expression constructs were introduced into Agrobacterium tumefaciens strain GV3101 and transformed into Arabidopsis thaliana using the floral dip method for stable transformation.

**Stress treatment conditions**

For the germination assays, seeds were surface-sterilized in 75% ethanol for 8 min, and then rinsed twice with 95% ethanol, air-dried, and spread on half-strength MS medium containing 0.5% sucrose and 0.8% Gelzan™ CM supplemented without or
with 175 mM NaCl or with 175/200 mM KCl or with 350/400 mM mannitol. After stratification at 4°C for 3 days in darkness, the seeds were cultured in a growth chamber, and after 3 or 4 days of light exposure the germination rate was calculated.

To analyze the salt sensitivity of vegetative stage plants, seeds stratified for 3 days were sown in soil and grown in a culture room for 3 weeks, then treated with or without 200 mM NaCl. The survival rate was analyzed 12 days after salt treatment. Seedlings that were still green and continuing to produce new leaves were registered as survivors. All experiments were repeated at least three times.

**Protein–protein interaction analysis**

For the overlay assays, the coding sequence of DSP5 was transferred from the donor vector pENTR/DPS5 into gw-pGEX4T-1 by the LR reaction to produce GST-DSP5. The production of GST-DSP3 and His-MPK6 was described previously. Affinity-purified GST-DSP3, GST-DSP5, and GST were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Next, the gel blots containing GST-DSP3, GST-DSP5, and GST were incubated with ~2 μg of His-MPK6 (used as the primary antibody) followed by anti-His-HRP antibodies (CoWin Biosciences).

For the firefly luciferase complementation assays, the coding sequence of DSP5 was transferred into gw-pCAMBIA-nLuc by the LR reaction. Production of the MPK3-cLuc and MPK6-cLuc constructs was described previously. The above-mentioned binary expression constructs were introduced into *A. tumefaciens* strain GV3101 and then co-infiltrated into 4-week-old *N. benthamiana* leaves for transient assays. *Nicotiana benthamiana* leaves co-expressing cLuc-MPKs (3/6) and DSP5-nLuc were sprayed with the luciferase substrate D-luciferin after 2 days of co-infiltration. The leaves were kept in the dark for 5 min before the luminescence signal was captured with a CCD camera.

**Statistical analysis**

A one-way analysis of variance (ANOVA) (Figure 3d) or two-way ANOVA (Figure 1c, Figure 2b and Figure 1d, and Figure 3b) with Tukey's honestly significant difference test was performed to evaluate the differences across sample groups or
treatments. Different lowercase letters indicate statistically significant differences ($p < .05$).

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This work was supported by the National Science Foundation of China (Grant Nos. 31501151 and 31872830) and the Department of Education of Hebei Province (Grant No. BJ2019025).

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