# Inositol Polyphosphate 5-Phosphatase7 Regulates the Production of Reactive Oxygen Species and Salt Tolerance in Arabidopsis[1][C][W][OA]

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Plants possess remarkable ability to adapt to adverse environmental conditions. The adaptation process involves the removal of many molecules from organelles, especially membranes, and replacing them with new ones. The process is mediated by an intracellular vesicle-trafficking system regulated by phosphatidylinositol (PtdIns) kinases and phosphatases. Although PtdIns comprise a fraction of membrane lipids, they function as major regulators of stress signaling. We analyzed the role of PtdIns 5-phosphatases (5PTases) in plant salt tolerance. The Arabidopsis (*Arabidopsis thaliana*) genome contains 15 At5PTases. We analyzed salt sensitivity in nine *At5ptase* mutants and identified one (*At5ptase7*) that showed increased sensitivity, which was improved by overexpression. *At5ptase7* mutants demonstrated reduced production of reactive oxygen species (ROS). Supplementation of mutants with exogenous PtdIns dephosphorylated at the D5′ position restored ROS production, while PtdIns(4,5)$P_2$, PtdIns(3,5)$P_2$, or PtdIns(3,4,5)$P_3$ were ineffective. Compromised salt tolerance was also observed in mutant *NADPH Oxidase*, in agreement with the low ROS production and salt sensitivity of *PtdIns 3-kinase* mutants and with the inhibition of NADPH oxidase activity in wild-type plants. Localization of green fluorescent protein-labeled At5PTase7 occurred in the plasma membrane and nucleus, places that coincided with ROS production. Analysis of salt-responsive gene expression showed that mutants failed to induce the *RD29A* and *RD22* genes, which contain several ROS-dependent elements in their promoters. Inhibition of ROS production by diphenylene iodonium suppressed gene induction. In summary, our results show a nonredundant function of At5PTase7 in salt stress response by regulating ROS production and gene expression.

Phosphatidylinositol (PtdIns) and its phosphorylated derivatives (PtdInsPs) are implicated in a broad range of signaling processes, from organization of the actin cytoskeleton to the regulation of intracellular signaling and vesicle trafficking (Baluska et al., 2003; Samaj et al., 2005). The PtdInsPs constitute a family of eight molecules that undergo phosphorylation and dephosphorylation in the hydroxyl group of the inositol ring by specific kinases and phosphatases (Meijer and Munnik, 2003; van Leeuwen et al., 2004). Several extracellular signals were shown to induce the production of inositol 1,4,5-trisphosphate ($InsP_3$), PtdIns(3,4)$P_2$, and PtdIns(3,4,5)$P_3$ (van Leeuwen et al., 2004).

Abiotic stresses, such as osmotic or salt stress, induced the production of PtdIns(4,5)$P_2$ and $InsP_3$ in Arabidopsis (*Arabidopsis thaliana*; Heilmann et al., 2001; DeWald et al., 2005; Van Leeuwen et al., 2007), although it is not known whether Ins(1,4,5)$P_3$ is involved in plant signaling (Munnik and Vermeer, 2010). Among the kinases that function in plant phosphatidylinositide metabolism, only the PtdIns 3-kinase (PI3K) was shown to act in stress signaling (Leshem et al., 2007). Recently, the PtdIns 5-phosphatases (5PTases) were shown to be involved in the regulation of plant stress responses (Ercetin and Gillaspy, 2004; Williams et al., 2005).

In humans, there are eight characterized 5PTases, which have been divided into four groups, based on sequence homology and in vitro substrate specificity. Yeast contain four 5PTases that are homologous to the human synaptojanin and other group I or II enzymes (Majerus and York, 2009). The plant kingdom possesses increased amounts of *5PTase* genes: the Arabidopsis genome contains 15 *5PTase* genes, while rice (*Oryza sativa*) has more than 20 genes. Functional analysis of the four 5PTases from *Saccharomyces cerevisiae* showed that although they are not essential for viability under normal conditions, they have an im-

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portant role in osmotic stress tolerance (Stolz et al., 1998; Ooms et al., 2000).

Mutations in the 5PTases in humans have been associated with several diseases, including cancer (Loovers et al., 2003). A crucial role of 5PTase activity was also shown in the immune response in neutrophils, in the intracellular activation of the NADPH oxidase complex in the phagosomes (Simonsen and Stenmark, 2001; Babior, 2004). In plants, although not all of the components involved in the activation of NADPH oxidase have been identified, several factors that function in animals, such as cytosolic calcium, phosphatidic acid, and ras-related C3 small GTP binding protein were shown to have crucial roles in reactive oxygen species (ROS) production in plants (Potikha et al., 1999; Wong et al., 2007; Monshausen et al., 2009; Zhang et al., 2009). Moreover, recently, several Arabidopsis proteins with domain structure similar to mammalian $p67^{phox}$ have been described (van Leeuwen et al., 2004; Sumimoto et al., 2007).

Biochemically, the 5PTases act as phosphoric monoester hydrolases. The 5PTases are characterized by a conserved catalytic domain of approximately 350 amino acid residues. Two conserved catalytic motifs were identified as essential for 5PTase activity and have been grouped into four types according to their substrate specificity (Majerus et al., 1999). There are four known substrates for 5PTases: Ins(1,4,5)$P_3$, Ins(1,3,4,5)$P_4$, and the lipids PtdIns(4,5)$P_2$, and PtdIns(3,4,5)$P_3$. Type I enzymes hydrolyze the water-soluble inositol phosphate substrates, namely Ins(1,4,5)$P_3$ and Ins(1,3,4,5)$P_4$. The type II 5PTases hydrolyze all four 5PTase substrates, although with varying catalytic efficiency. The type III 5PTases hydrolyze phosphate from substrates phosphorylated in the D3′ position, such as PtdIns(3,4,5)$P_3$ and Ins(1,3,4,5)$P_4$. The type IV 5PTases dephosphorylate only phosphoinositols that are part of the membrane lipids, such as PtdIns-3,4,5-$P_3$, which forms a complex with PI3K (Majerus et al., 1999).

In yeast and animals, the type II 5PTases were shown to function in PtdIns metabolism, affecting diverse cellular functions. For example, mutation *Inp51p* in yeast increased PtdIns(4,5)$P_2$ concentration, resulting in improved cold tolerance (Stolz et al., 1998). Mutation in the human *OCRL1* gene, which is a PtdIns(4,5)$P_2$, and PtdIns(3,4,5)$P_3$5-phosphatase that causes Lowe syndrome was shown to increase PtdIns(4,5)$P_2$, which affected protein trafficking between endosomes and the trans-Golgi and resulted in a redistribution of clathrin (Zhang et al., 1998).

To explore the role of 5PTases in the plant response to stress, we analyzed Arabidopsis mutants for salt stress tolerance. A common consequence of salt stress in plants is increased generation of ROS, resulting in a secondary oxidative stress, which affects plant growth (Smirnoff, 1998). Previous work showed that ROS production was mediated by NADPH oxidase (Mazel et al., 2004). We showed later that the activation of NADPH oxidase during salt stress was mediated by PI3K (Leshem et al., 2007).

Recently, in animal cells, it was found that the activation of NADPH oxidase involved sequential phosphorylation of position D3′ by PI3K, followed by dephosphorylation of position D5′ by PtdIns-5PTase (Simonsen and Stenmark, 2001). Here, we show decreased ROS production during salt stress in *At5ptase7* (At2g32010) mutants and reduced expression of stress-responsive genes, resulting in increased salt sensitivity. The above responses were specific for *At5PTase7*, but not for other *At5ptase* mutants, indicating a nonredundant function of individual members of the 5PTase family in Arabidopsis. In summary, we show that At5PTase7 functions in the plant response to salt stress by regulating ROS production and gene expression, which suggests that At5PTase7 coordinates the above responses during salt stress, leading to salt tolerance.

## RESULTS

### The 5PTases from the Endonuclease/Exonuclease/Phosphatase Family in Arabidopsis

The Arabidopsis genome contains 15 members of the 5PTase enzyme family, composed of variable size proteins, from 300 to 1,305 amino acids. All of the proteins contain the conserved endonuclease/exonuclease/phosphatase and the inositol polyphosphate phosphatase catalytic domains, but some enzymes contain additional domains, such as WD40 (Chen et al., 2008) or Motile Sperm Protein domains that function in interaction with other proteins (Allam and Marshall, 2005; Supplemental Fig. S1). Most of the At5PTases contain distinct consensus domains I and II, originally described in yeast and *Caenorhabditis elegans* (Majerus et al., 1999). The arrangement of the domains in Arabidopsis is characteristic of the mammalian type II 5PTases. The Arabidopsis proteins show extreme sequence conservation of both domains. In fact, several At5PTases show 100% homology with the consensus sequence of the mammalian type II 5PTases (Majerus et al., 1999).

Analysis of *5PTase* gene expression during different abiotic stresses, using the University of Toronto Bio Array Resource database (Toufighi et al., 2005), showed rapid transcriptional changes (less than 0.5 h) in many of the *At5PTase* genes, suggesting possible functioning in regulation and signaling of the stress responses (Supplemental Fig. S2A). Interestingly, while some of the *At5PTase* genes were induced, others were suppressed by the same stress.

To analyze *At5PTase* gene expression in mature plants and to test different growth conditions, plants that grew in soil under normal conditions for 4 weeks were watered with 250 mM NaCl and RNA was extracted 6 h later. The expression of *At5PTase2*, *-3*, *-7*, and *-11* genes was analyzed by quantitative real-time reverse transcription (RT)-PCR. The transcriptional response of the *At5PTase7* gene in the mature plants grown in soil was similar to that of the seedlings

grown on agar plates, but *At5PTase2* and *-3* in mature plants were not induced (Supplemental Fig. S2B). Moreover, the transcription of the *At5PTase11* gene in the roots of seedlings was up-regulated, while in mature plants the response was reversed, highlighting the dynamic functioning of At5PTases.

### Involvement of the At5PTases in Arabidopsis Salt Tolerance

To analyze the physiological role of At5PTases in salt stress, we assayed the salt tolerance of several *At5ptase* mutants. Under normal (nonsaline) conditions, the phenotypes of all mutants were similar to the wild-type plants. After 7 d, the seedlings were transferred to plates containing 150 mM NaCl. Such an abrupt increase in salinity, shortly after germination, is common in arid areas that necessitate switching to irrigation with poor water quality. The majority (four out of five) of homozygous mutant lines (*At5ptase1*, *-2*, *-3*, and *-11*) looked similar to the wild type also during the salt stress, except for *At5ptase7*, which exhibited a salt-overly-sensitive phenotype (Fig. 1A).

The first symptom of salt stress in *At5ptase7* mutants was bleaching of cotyledons, which occurred within 2 d, spreading to true leaves after another 1 to 2 d, and resulting in death of the whole plant. In soil-grown 4-week-old plants, the stress symptoms developed slower, causing severe growth retardation, but not death of the whole plant (Fig. 1C). The mature wild-type plants also performed better than the *At5ptase7* mutants. The salt sensitivity of the *At5ptase7* mutants was reproduced in three different *At5ptase7* mutant lines (Salk_038828, Salk_038842, and Salk_040226). We isolated homozygous mutants from the Salk_038828 and Salk_040226 lines (Supplemental Fig. S3).

We also analyzed five heterozygous *At5ptase* mutants that at the time of analysis homozygous lines were not available: *At5ptase2*, *-4*, *-5*, and *-8* mutants exhibited salt tolerance similar to the wild type, while *At5ptase12* mutants showed a slightly reduced tolerance (Fig. 1B). Importantly, all of the mutants, including *At5ptase7* and *At5ptase12*, grew normally in control conditions, suggesting a specific function of these genes in response to salt stress.

To test whether the *At5PTase7* gene expression level influenced salt tolerance, we cloned it behind a constitutive 35S cauliflower mosaic virus promoter and introduced it into wild-type Arabidopsis. Two transgenic lines with the highest expression level as determined by RT-PCR (OE-6 and OE-9) were selected (Fig. 2A), and their salt tolerance was tested by germination and growth on 150 mM NaCl. More than 50% of the transgenic plants germinated and grew on salt, while none of the wild-type plants survived after 9 d (Fig. 2B).

### ROS Production in *At5ptase* Mutants during Salt Stress

The development of oxidative stress is an established consequence of salt stress (Smirnoff, 1998). We have shown previously that ROS production during salt stress is caused by NADPH oxidase (Mazel et al., 2004). Recently, it was shown that activation of NADPH oxidase in neutrophils is mediated by phosphorylation of the D3′ position by PI3K, followed by dephosphorylation of the D5′ position by SHIP1 5PTase (Ellson et al., 2001; Kawahara and Lambeth, 2008). The phosphorylation of D3′ and dephosphorylation of D5′ positions were also required for the

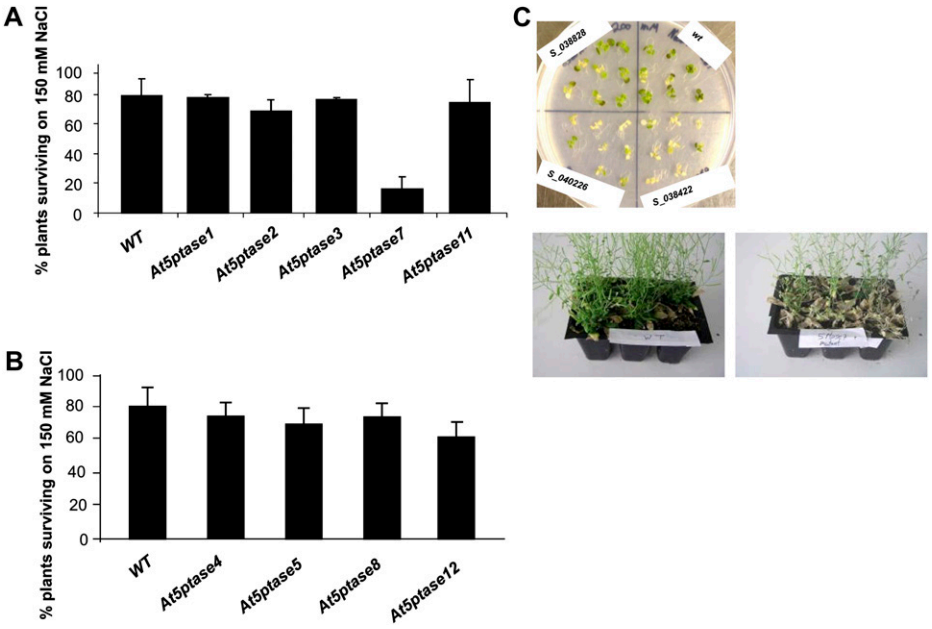


**Figure 1.** Analysis of salt tolerance in Arabidopsis *At5ptase* mutants. A, Survival of homozygous Arabidopsis *At5ptase* mutants grown on 150 mM NaCl. Seedlings of *At5ptase* mutants were grown for 7 d on one-half-strength MS medium and then transferred to 150 mM NaCl for 5 d. There was no difference in the appearance and growth of the mutants as compared with the wild-type (WT) plants when not stressed with NaCl. B, Survival of heterozygous mutant seedlings treated as in A. The T4 heterogeneous Salk mutant lines were selected on kanamycin. C, Mutants of *At5ptase7* (Salk_038828 heterozyg, Salk_038842 homozyg, and Salk_040226 homozyg) were treated with 200 mM NaCl (top). Four-week-old plants grown in soil were irrigated with 200 mM NaCl for 10 d and then irrigated with fresh water for 1 week (bottom).

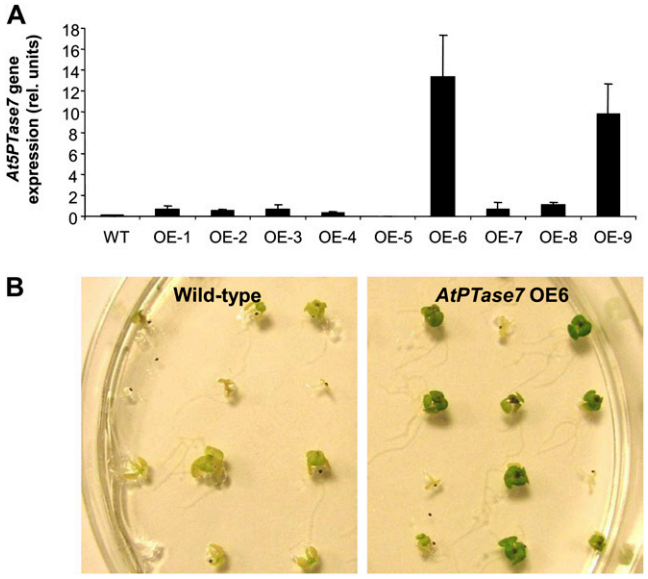


**Figure 2.** Salt tolerance in transgenic plants overexpressing the *At5PTase7 gene*. A, Expression of the *At5PTase7* gene in transgenic plants (OE, overexpressors). WT, Wild type. B, Wild-type (left) and *At5PTase7*-overexpressing plants (right). Transgenic *At5PTase7* plants were germinated and grown on plates supplemented with 150 mM NaCl for 1 week. There was no difference in the appearance and growth of the transgenic plants as compared with the wild-type plants when not stressed with NaCl.

restoration of salt-induced ROS production in Arabidopsis *pi3K* mutants that were supplemented with differentially phosphorylated exogenous phosphatidylinositides (Leshem et al., 2007).

ROS accumulation was measured in root tip and transition zone in *At5ptase3*, *-7*, and *-11* mutants 15 min after the beginning of salt stress. Normal ROS production was seen in *At5ptase11* mutants, and a slightly lower ROS level was observed in the root tip but not in the elongation zone of *At5ptase3*. However, very little ROS was detected in both regions of the *At5ptase7* mutants (Fig. 3, A and B). Therefore, we tested whether ROS production in *At5ptase7* mutants depended on phosphorylation of the D5′ position. Mutant seedlings were supplemented with differentially phosphorylated exogenous PtdInsPs, and the production of ROS was measured by confocal microscopy. The addition of exogenous PtdInsPs was shown to be taken up and delivered to correct subcellular locations in both animal and plant cells, including Arabidopsis seedlings (Ozaki et al., 2000; Lee et al., 2007; Leshem et al., 2007). The *At5ptase7* mutants were preloaded with PtdIns(3,4)$P_2$, PtdIns(3,5)$P_2$, PtdIns(4,5)$P_2$, or PtdIns(3,4,5)$P_3$ and stimulated with 200 mM NaCl. The production of ROS was restored in seedlings supplemented with PtdIns(3,4)$P_2$ but not with PtdIns(4,5)$P_2$, PtdIns(3,5)$P_2$, or PtdIns(3,4,5)$P_3$ (Fig. 3C), in line with similar results in animal cells (Kamen et al., 2008) and in *pi3K* Arabidopsis mutants (Leshem et al., 2007). Hence, our results show that the activation of NADPH oxidase in Arabidopsis also requires conserved PtdIns phosphorylation sites, namely phosphorylation of the D3′ position and dephosphorylation of D5′.

Recently, it was shown that ROS localization plays an important role in downstream signaling in plants as well as in animals (Bailey-Serres and Mittler, 2006; Leshem et al., 2010; Toledano et al., 2010). Therefore, we analyzed the subcellular distribution of ROS by confocal microscopy. ROS were detected throughout the cytoplasm as speckles that were primarily concentrated close to the plasma membrane (Fig. 4B, inset). Interestingly, a very definite ROS accumulation was detected in the nuclei in the roots of wild-type plants (Fig. 4A). Nuclear ROS production in wild-type plants was very rapid, becoming visible in less than 10 min after exposure to salt. However, very little ROS were measured in the nuclei of *At5ptase7* mutants (Fig. 4B).

### Salt Tolerance in *AtRboh* Mutants

Previous work showed that salt-induced ROS was inhibited by the NADPH oxidase inhibitor diphenylene iodonium (DPI; Jones et al., 2000; Mazel et al., 2004), suggesting that it was caused by gp91phox (phagocyte oxidase, also called respiratory burst oxidase [Rboh]). The Arabidopsis genome contains 10 members of the *Rboh* gene family, designated RbohA through RbohJ (Torres et al., 2002; Sagi and Fluhr, 2006). Reduced ROS production was observed in *AtrbohD* and *AtrbohF* mutants in response to salt and abscisic acid stresses (Kwak et al., 2003). Analysis of *AtRboh* gene expression during salt stress using the Bio Array Resource database (Toufighi et al., 2005) showed strong early induction of *AtRbohJ* and weak induction of *AtRbohD* in the wild-type plants (Supplemental Fig. S4). We confirmed these data by RT-PCR (Fig. 5). Interestingly, we found that expression of the *AtRbohJ* gene was lower in the *At5ptase7* mutants compared with the wild-type plants.

We also tested ROS production in the *Rboh* mutants to find out which isozyme is required for the salt-dependent oxidative burst by measuring ROS accumulation in the *AtRboh* mutants during the first 3 h of salt stress by 2′,7′-dichlorofluorescein ($H_2$DCFDA), which reports hydrogen peroxide-dependent oxidation, but we were unable to identify the specific gene, possibly due to a different type of ROS.

To test the effect of the Arabidopsis ROS-producing enzymes on seedling salt tolerance, we analyzed several mutants of the gp91 NADPH oxidase (*AtRboh*) family. Seeds of the wild type and *AtRboh* mutants were germinated on plates containing 100 to 150 mM NaCl, and their growth was observed daily. Significantly decreased salt tolerance was observed only in the *AtRbohJ* mutants (Fig. 6). The decreased salt tolerance in the *RbohJ* mutants is in agreement with

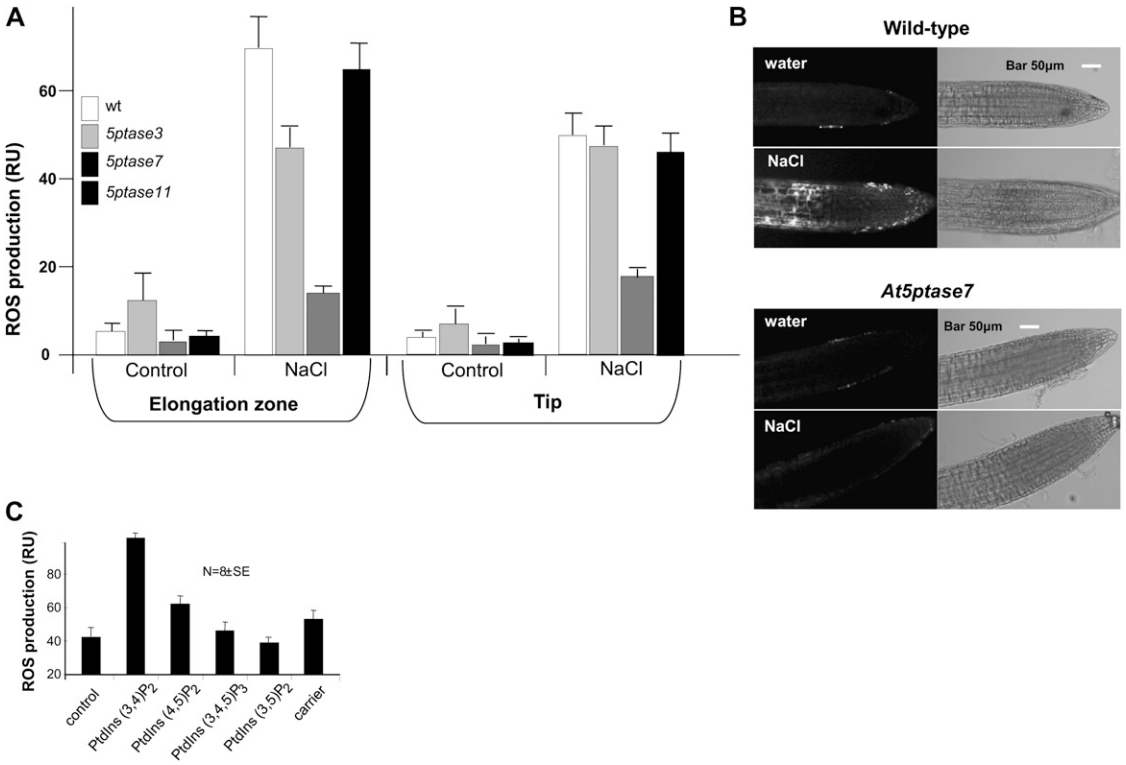


**Figure 3.** Salt-induced ROS production in wild-type and *At5ptase* mutant plants. A, Arabidopsis seedlings were grown on agar plates supplemented with one-half-strength MS nutrient medium. After 7 d, the seedlings were removed from the agar plates, washed, and transferred into medium with 200 mM NaCl. ROS generation was measured in the root tip and transition zone by confocal microscopy with $H_2$DCFDA in *At5Ptase3*, *-7*, and *-11* roots at 15 min after stimulation with 200 mM NaCl. wt, Wild type. B, Representative confocal images of wild-type and *At5ptase7* mutant plants treated with one-half-strength MS or 200 mM NaCl (magnification, ×200). Seedlings were grown and treated as in A. Bars = 50 $\mu$m. C, Restoration of ROS production in the *At5ptase7* mutants during salt stress. Seedlings were grown and treated as in A. The exogenous PtdIns-phosphates (indicated) were incubated with the seedlings for 1 h before treatment with NaCl. RU, Relative units.

the reduced ROS production and salt tolerance described in *pi3K* mutants and also with the result of treatment of wild-type plants with DPI (Leshem et al., 2007).

### Localization of the At5PTase7 Protein in Arabidopsis

The localization of protein is an important attribute of its function, especially for proteins involved in intracellular vesicle trafficking. Hence, to better characterize the cellular function of At5PTase7, we fused it to GFP and examined its localization in protoplasts and transgenic plants by epifluorescence and confocal microscopy. The At5PTase7-GFP protein was detected in the proximity of the plasma membrane and in the nuclei of transgenic plants in both roots and leaves (Fig. 7) as well as in protoplasts (data not shown). Strikingly, At5PTase7-GFP appeared as distinct nuclear speckles, which are thought to constitute subnuclear structures enriched in pre-mRNA splicing factors of the nucleoplasm (Lamond and Spector, 2003). The At5PTase7-GFP-containing speckles were observed in transiently transfected protoplasts as well as in the nucleoplasm of the transgenic plants (Fig. 7, B and C). The nuclear localization of At5PTase7-GFP is in agreement with the SubLoc program for protein subcellular location based on the Simple Object Access Protocol (Heazlewood et al., 2005), which is associated with The Arabidopsis Information Resource database (http://www.arabidopsis.org/portals/proteome/).

### Regulation of Stress-Related Gene Expression in *At5ptase7* Mutants

A central component of the a plant's adaptation to stress is the alteration of gene expression patterns (Chinnusamy et al., 2004). Two sets of genes that belong to early- or late-induced changes following exposure to salt stress have been described (Shinozaki and Yamaguchi-Shinozaki, 2000; Ma et al., 2006). We analyzed the transcription of two genes, *RD29A* and *RD22*, that were shown to be induced by salt and several other abiotic stresses in wild-type plants by quantitative real-time RT-PCR. The expression of both genes was strongly suppressed in the *At5ptase7* mutants, especially in the roots (Fig. 8A).

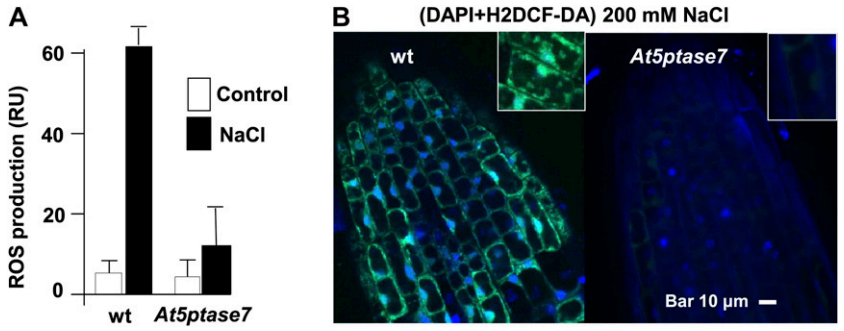


**Figure 4.** Subcellular localization of salt-induced ROS production. A, Measurements of ROS production localized to the nucleus in the root tip zone of wild-type and *At5ptase7* seedlings after 20 min of stimulation with 200 mM NaCl. The seedlings were treated as in Figure 3A. Shown are averages of 10 nuclei measurements in five plant replicates. RU, Relative units. B, Representative images of wild-type and *At5ptase7* root tips. High-magnification images of ROS accumulation (green) in the cytoplasm and the nucleus (blue) of root tip cells by confocal microscopy (magnification, ×630) are shown. The seedlings were treated as in Figure 3A for 20 min with 200 mM NaCl with the supplement of the DAPI fluorescent stain. Shown is a representative layer of a 1-$\mu$m optical section along the *z* axis. Insets show a higher magnification of one representative cell. Bar = 10 $\mu$m.

Given the differential ROS production in nuclei of wild-type and mutant plants during salt stress, we were interested to see whether ROS had an effect on the salt-responsive gene expression. Analysis of the promoters of *RD29A* and *RD22* genes revealed the presence of several ROS-responsive elements, such as AS-1, ABRE, and NRXe-2 (Supplemental Fig. S5), suggesting possible transcriptional regulation by redox (Garretón et al., 2002). To test the involvement of ROS in the regulation of *RD29A* gene expression, we inhibited the production of ROS in wild-type plants by DPI (Jones et al., 2000; Mazel et al., 2004) and analyzed the gene expression by quantitative real-time RT-PCR. DPI treatment strongly suppressed the induction of the *RD29A* gene to the level seen in the *At5ptase7* mutants (Fig. 8B). The expression of the *RD22* gene was not altered by DPI, suggesting redox-dependent regulation by ABRE and NRXe-2 elements.

### At5PTase7 Substrate Preference

In general, the 5PTase enzymes are capable of hydrolyzing both membrane-bound PtdInsPs and/or soluble InsPs. To understand the molecular function of individual At5PTases, it is important to determine which InsP and PtdInsP substrates they hydrolyze. The substrate preference of *At5PTase7* was compared with the previously characterized enzymatic activity of *At5PTase1*, *At5PTase2*, and *At5PTase3* (Berdy et al., 2001; Ercetin, 2005). Proteins were expressed as recombinant proteins in a *Drosophila* S2 cell system that has been previously shown to yield active 5PTase enzymes (Ercetin and Gillaspy, 2004).

Recombinant enzymes were immunoprecipitated and incubated in activity assay with fluorescent substrates including PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$. At5PTase3, known to hydrolyze PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$ (Ercetin, 2005), was used as a positive control (Fig. 9). At5PTase7 hydrolyzed both PtdIns(4,5)$P_2$ and PtdIns(3,4,5)$P_3$, albeit not with the same efficiency (Fig. 9). Note that the low-abundance band in PtdIns(3,4)$P_2$ and PtdIns(3,4,5)$P_3$ standards is most likely due to PtdIns$P_3$ contamination of the commercial standards. We also tested whether At5PTase7 would hydrolyze Ins(1,4,5)$P_3$ and found no hydrolysis of this substrate (data not shown). These results indicate that At5PTase7 has a substrate preference similar to the type IV 5PTases that hydrolyze only membrane-bound substrates (Ooms et al., 2000). These data are also similar to a recently published analysis using a different assay (Carland and Nelson, 2009).

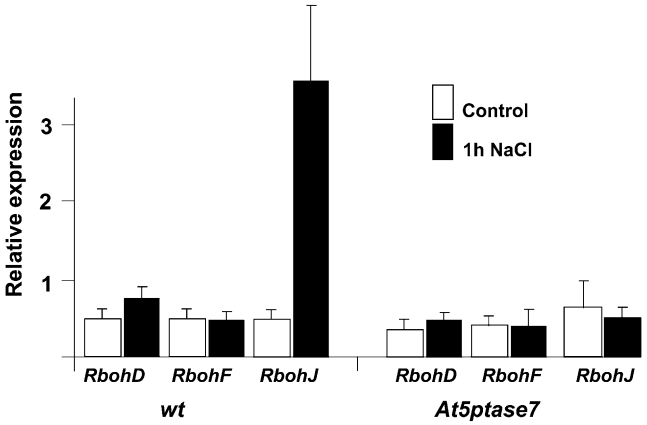


**Figure 5.** Expression of the *Rboh* gene in wild-type (wt) and *At5ptase7* mutant plants by salt stress. The transcription of *RbohJ*, *RbohD*, and *RbohF* in the roots of wild-type and *At5ptase7* mutant plants during salt stress was measured by semiquantitative RT-PCR. Plants were grown in one-half-strength MS medium for 7 d and then transferred to 0.2 M NaCl, and RNA was prepared from the roots of 20 individuals in three independent replicates.

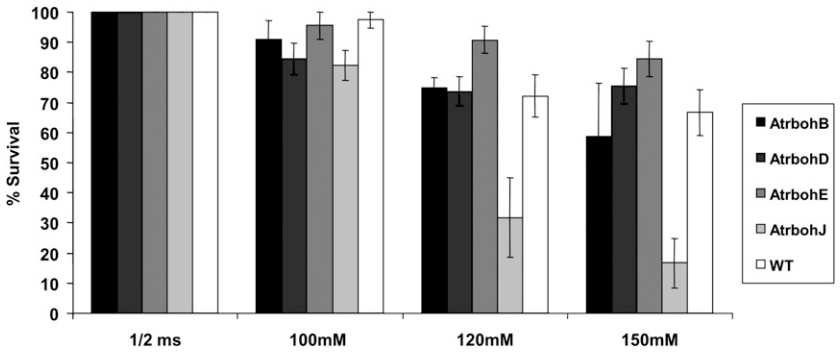


**Figure 6.** Analysis of *Atrboh* mutant salt tolerance. Wild-type (WT) and *AtRboh* mutant seeds were germinated and grown on plates that contained 100 to 150 mM NaCl. The seedlings were grown for 1 week and monitored for survival. There was no difference in the appearance and growth of the mutants as compared with the wild-type plants when not stressed with NaCl.

## DISCUSSION

### *At5PTase* Gene Expression during Abiotic Stress

Although phosphatidylinositides were described in plants some 50 years ago (Aylward and Showler, 1962), their involvement in different metabolic processes became recognized only recently (Ercetin and Gillaspy, 2004). Today, PtdIns are thought to function in intracellular vesicle trafficking and cytoskeleton organization (Leshem et al., 2007; Thole and Nielsen, 2008; Munnik, 2010). PtdIns signaling is mediated by differential phosphorylation of the inositol ring, which is carried out by specific kinases and phosphatases (van Leeuwen et al., 2004). The plant genomes possess more

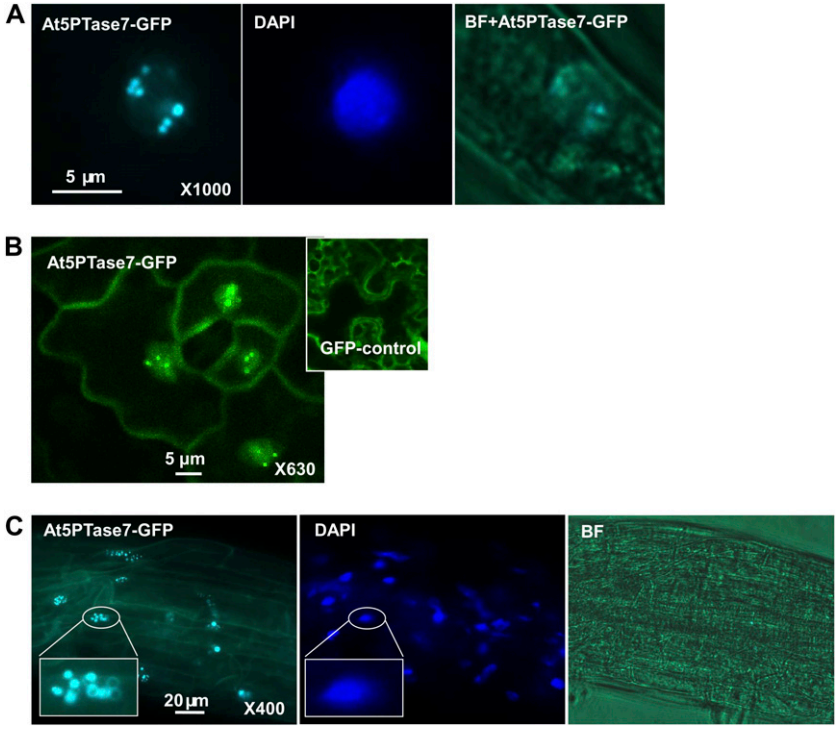


**Figure 7.** Subcellular localization of At5PTase7. A, Analysis of stably transformed Arabidopsis plants with At5PTase7-GFP. Localization of At5PTase7-GFP was in the nucleus of root hair cells (green). Nuclei of 7-d-old transgenic seedlings were stained for 10 min with DAPI fluorescent dye (blue). Image analysis was done using the Olympus IX70 epifluorescence microscope (100× oil-immersion objective) equipped with a Coolpix 950 camera (Nikon). GFP speckles can be seen in the nucleus. BF, Bright field. Bar = 5 μm. B, Localization of At5PTase7-GFP in Arabidopsis epidermal leaf cells. Image analysis was done using a Zeiss LSM 510 Laser Scanning Microscope with a 63× oil-immersion objective. The inset shows GFP expressed from the 35S promoter in epidermal leaf cells (control). Bar = 5 μm. C, Localization of At5PTase7-GFP in the root cells of Arabidopsis (green). Seven-day-old transformed seedlings were incubated for 10 min with DAPI fluorescent stain, which stains the nucleus (blue). Image analysis was done using the Olympus IX70 epifluorescence microscope (40× oil-immersion objective) equipped with a Coolpix 950 camera (Nikon). The insets show the magnification of one representative root cell nucleus. Speckles of GFP can be seen in the nucleus. Bar = 10 μm.

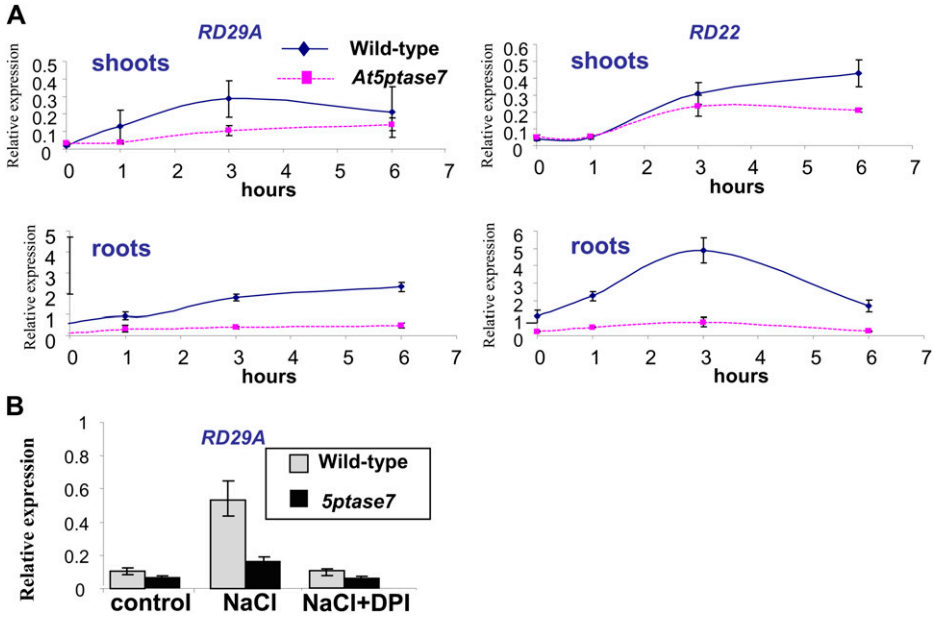


**Figure 8.** Induction of gene expression in wild-type and *At5ptase7* mutant plants by salt. A, Expression of *RD29A* (left panel) and *RD22* (right panel) genes was analyzed in the roots and shoots by quantitative real-time RT-PCR. Wild-type and *At5ptase7* mutant seeds were germinated on agar plates, grown for 2 weeks, and then treated with 0.2 M NaCl for 1, 3, and 6 h, as described in the Bio Array Resource for Arabidopsis Functional Genomics Web site (http://bbc.botany.utoronto.ca). RNA was extracted from the plants using the Tri-Reagent Kit (Molecular Research Center), and cDNA was prepared with the reverse transcriptase SuperScript III kit (Invitrogen). B, Inhibition of RD29A expression by DPI in roots treated with 200 mM NaCl for 3 h. Wild-type and *At5ptase7* mutant seedlings were grown as in A. For the DPI treatment, seedlings were preincubated with the DPI inhibitor for 1 h and then exposed to 200 mM NaCl. [See online article for color version of this figure.]

genes encoding *5PTases* than yeast or animals (Ercetin et al., 2008). In yeast, the *5PTase* genes are expressed constitutively (Padrón et al., 2003) and mutations of any single *5PTase* gene was not lethal, suggesting some redundancy, although some of the double mutants showed decreased growth (Stolz et al., 1998).

Analysis of the gene expression of individual *At5PTases* during various environmental stresses showed highly variable expression patterns, indicating that each gene responds to different conditions and suggesting individual roles for the stress management (Supplemental Fig. S2A). In some cases, the expression is altered more than 3-fold in less than 1 h.

### Localization of At5PTase7

The subcellular localization of proteins is of critical importance for their function, which is often associated with specific cellular regions, or organelles. PtdIns were shown to play an important role in organelle specificity (Meijer and Munnik, 2003; Behnia and Munro, 2005). Both animal and plant 5PTases were detected in the Golgi network, in phagosomes, in the endoplasmic reticulum, and in vacuoles (Thole and Nielsen, 2008). 5PTases were also detected in animal and plant nuclei (Ananieva et al., 2008; Barlow et al., 2010). Also, several enzymes manipulating PtdIns were identified in plant nuclei (Bunney et al., 2000), but their specific role is not yet known. A critical role for the subcellular localization of At5PTases was demonstrated in *fra7* mutants of *At5PTase15*, which while retaining their phosphatase activity showed altered protein localization that resulted in impaired cell wall biosynthesis (Zhong et al., 2005).

Also, in both plant and animal cells, nuclear PtdIns have been associated with nuclear speckles and mRNA processing (Lorković, 2009; Barlow et al., 2010). There are several other At5PTases, such as At5PTase13 and At5PTase6 (CVP2), besides At5PTase7 that are localized in the nucleus. Interestingly, the nuclear localization and the speckled pattern of At5PTase7 are similar to its closest mammalian homolog, SHIP2, which plays an important role in ROS-dependent signaling (Deleris et al., 2003; Zhang et al., 2007). The nuclear localization suggests a broad impact of PtdIns in plant gene expression (Carland and Nelson, 2004). Interestingly, At5PTase13 protein was detected only in the nuclei of some cells in Arabidopsis roots. It has been suggested that At5PTase13 distribution is linked to its activity in developmental signaling during growth under different nutrient conditions (Ananieva et al., 2008).

### ROS Production and Salt Tolerance in *At5ptase7* Mutants

We have shown previously that the salt-induced oxidative burst was mediated by NADPH oxidase

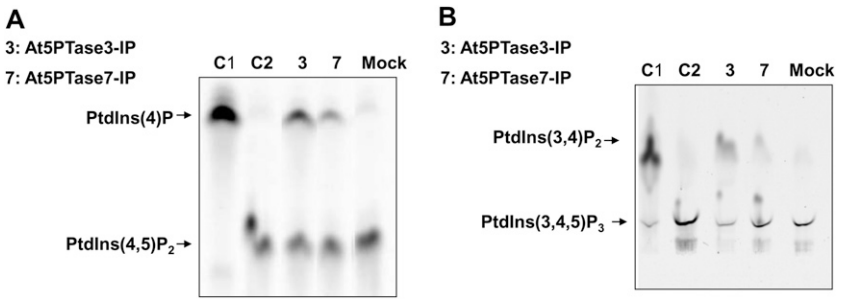


**Figure 9.** Substrate specificity of At5PTase3 and At5PTase7. A, Activity of At5PTase using fluorescent PtdIns(4,5)$P_2$ substrate with immunoprecipitated 5PTases. The reactions containing 1.5 $\mu$g of the fluorescent PtdIns(4,5)$P_2$ substrate were incubated for 1 h at room temperature. Lane 1, PtdIns(4)P substrate incubated with reaction buffer only; lane 2, PtdIns(4,5)$P_2$ substrate incubated with reaction buffer only. Other lanes contain reactions with the following immunoprecipitants (IPs): lane 3, At5PTase3-IP; lane 4, At5PTase7-IP; lane 5, control IP from mock-transfected S2 cells. B, Activity of At5PTase using fluorescent PtdIns(3,4,5)$P_3$ substrate with immunoprecipitated 5PTases. Phosphatase reactions (lanes 3 and 4) containing 1.5 $\mu$g of the fluorescent PtdIns(3,4,5)$P_3$ substrate were incubated for 1 h at room temperature. Lane 1, PtdIns(3,4)$P_2$ substrate incubated with reaction buffer only; lane 2, PtdIns(3,4,5)$P_3$ substrate incubated with reaction buffer only. Other lanes contain reactions with the following IPs: lane 3, At5PTase3-IP; lane 4, At5PTase7-IP; lane 5, control IP from mock-transfected S2 cells. The migration of standards is indicated. Note that the low-abundance band in the PtdIns(3,4)$P_2$ standard is most likely due to PtdIns$P_3$ contamination of the commercial substrate.

(Mazel et al., 2004). Moreover, treatment of Arabidopsis seedlings with DPI decreased the plant's salt tolerance, and an analogous effect was also seen in *pi3K* (Leshem et al., 2007). We show that a similar effect also occurs by reduced ROS production in *RbohJ* mutants (Fig. 6).

Here, we show that activation of the main subunit of the plant NADPH oxidase, gp91phox, in Arabidopsis cells requires dephosphorylation of the D5′ position of the inositol ring, as observed in animal cells (Simonsen and Stenmark, 2001; Leshem et al., 2007). The exact mechanism of NADPH oxidase activation in plants is not clear yet, but several proteins that bind to PtdIns-containing domains and that have significant homology to the cytosolic animal proteins have been identified (Karathanassis et al., 2002; van Leeuwen et al., 2004).

In recent years, protein interaction with phospholipids, including phosphatidic acid and a variety of phosphoinositides, has been described, and in several cases they were linked to the production of ROS (Sang et al., 2001; Zhang et al., 2003; Leshem et al., 2007). Here, we show that the *At5ptase7* mutant failed to induce ROS production in root tips when stimulated by salt stress, as opposed to other *At5ptase* mutants that were tested (*At5ptase3* and *-11*; Fig. 3; data not shown). The precise reason for this lack of oxidative burst in the *At5ptase7* mutants is not known, but it could stem from incorrect membrane-protein associations. For example, three members of the Arabidopsis proteins that contain a PX domain were shown to interact with PtdIns(3)P or PtdIns(3,4)$P_2$ but not with PtdIns that are phosphorylated on the D5′ residue (van Leeuwen et al., 2004). Moreover, plants that were supplemented with exogenous PtdIns and treated with wortmannin or mutated in *PI3K* confirmed the importance of phosphorylation of D3′ but dephosphorylation of D5′ for ROS production (Mazel et al., 2004; Leshem et al., 2007).

### The Role of At5PTase7 in Stress Gene Expression

Changes in gene expression play a central role in the plant stress response (Kawasaki et al., 2001; Bray, 2002). The localization of At5PTase7 in the nucleus suggests involvement in the regulation of stress-induced gene expression. Recently, PtdIns phosphorylated at the D5′ position have been detected in plant nuclei, although how the nuclear PtdIns5Ps regulate gene expression is not known (Jones et al., 2006; Munnik and Vermeer, 2010). One possible mechanism by which PtdIns may regulate gene expression involves nuclear ROS generation, which is absent from the nuclei of the *At5ptase7* mutant and thus may regulate redox-responsive transcription factors, such as the AP2 family, and other redox-responsive transcription factors (Fedoroff, 2006; Khandelwal et al., 2008; Van Breusegem et al., 2008). Indeed, the PtdIns-binding domains, such as PH, FYVE, and PHOX, were shown to interact with nuclear proteins, leading to the activation of transcription (Lemmon, 2008).

### At5PTase7 Substrate Specificity

An important difference for 5PTase function is its substrate preference. At5PTase1, At5PTase2, and At5PTase3 can hydrolyze all four potential substrates, including both soluble and membrane-bound molecules, such as Ins(1,4,5)$P_3$, Ins(1,3,4,5)$P_4$, PtdIns(4,5)$P_2$, and PtdIns(3,4,5)$P_3$ (Ercetin, 2005; Gillaspy, 2010). In contrast, At5PTase7 has substrate preference for hydrolyzing only the phospholipids, such as PtdIns(4,5)$P_2$

and PtdIns(3,4,5)$P_3$, classifying it as a group IV enzyme (Burnette et al., 2004; Fig. 9). The substrate specificity of membrane-bound PtdIns and its membrane localization are in agreement with its function in membrane trafficking and suggest an involvement in stress signaling.

## CONCLUSION

In summary, we show a specific function of At5PTase7 in plant salt tolerance: gene knockout reduced the production of ROS, resulting in reduced salt tolerance (Figs. 1 and 3). Interestingly, these results are highly similar to Arabidopsis *pi3K* mutants, which also showed reduced ROS production and salt sensitivity and were also reproduced by inhibition of the NADPH oxidase with DPI. The *At5ptase7* mutants also failed to induce the *RbohJ* gene that is responsible for ROS production during salt stress (Fig. 5), resulting in reduced induction of the salt-responsive genes *RD29* and *RD22* (Fig. 9).

Overexpression of *At5PTase7* improved the salt tolerance in transgenic Arabidopsis beyond the wild-type level. We propose that At5PTase7 acts in coordinating plant responses to salt stress by modulating interactions in the cellular protein-lipid network.

## MATERIALS AND METHODS

### Plant Material

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia plants were used as the wild type and as the background for the mutations and transgenic lines. The mutants analyzed here were from the Arabidopsis Biological Resource Center (Alonso et al., 2003), and *At5PTase1* and *At5PTase2* mutants were described by Gunesekera et al. (2007). Seeds were surface sterilized and germinated on one-half-strength Murashige and Skoog (MS) 0.8% (w/v) phytagar medium. Prior to germination, seeds were stratified at 4°C for 2 d and grown at 24°C under constant or long-day-regime (16 h of light) fluorescent light (120 $\mu$E). At5PTase7 (At2g32010) mutant lines Salk_040226, Salk_038842, and Salk_038828 were verified by PCR as described at the Salk Institute Genomic Analysis Laboratory Web site (http://signal.salk.edu/tdnaprimers.2.html).

### Bioinformatics

A phylogenetic unrooted neighbor-joining tree of the Arabidopsis 5PTases was constructed using the Kyoto University ClustalW multiple sequence alignment Web site (http://align.genome.jp/). Protein sequences of the At5PTases were downloaded from The Arabidopsis Information Resource Web site (http://www.arabidopsis.org/) and were uploaded to the Kyoto University ClustalW Web site. The conserved At5PTase consensus domains I and II were aligned to the domains of the human synaptojanin and type II 5PTase, as described by Majerus et al. (1999). The alignment was constructed using the European Bioinformatics Institute ClustalW2 multiple sequence alignment Web site (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

### Plant Treatments

Seedlings were germinated on 150 mM NaCl or on one-half-strength MS agar plates and replanted onto plates supplemented with salt as described by Leshem et al. (2007).

### Confocal and Epifluorescence Microscopy

Confocal imaging was performed with an inverted Zeiss LSM 510 laser scanning microscope with a 20× air objective and a 40× or 63× oil-immersion objective. For imaging $H_2$DCFDA (ROS) alone or GFP together with 4′,6-diamidino-2-phenylindole (DAPI), the single-track and multiple-track facilities of the confocal microscope were used, respectively. For imaging GFP and ROS, the 488-nm excitation line was used. For the DAPI stain, the 405-nm excitation laser was used. Fluorescence was detected using a 505- to 550-nm band-pass filter for GFP and ROS stains. A 420- to 480-nm band-pass filter was used for the DAPI stain, and a 650-nm long-pass filter was used for the chlorophyll autofluorescence. Postacquisition image processing was by LSM 5 Image Browser (Zeiss). In some experiments, we used an epifluorescence microscope (Olympus IX70) equipped with a narrow-band filter cube (excitation/emission, 485DF22/535DF35) from Omega. Images were taken with a Coolpix 950 digital camera (Nikon) using identical exposure settings for each set of images, as described (Mazel et al., 2004).

### Cloning of At5PTases

Cloning of At5PTases was performed as described previously (Ercetin and Gillaspy, 2004). Briefly, full-length cDNAs were generated by RT-PCR using 1 $\mu$g of Arabidopsis seedling mRNA and the following primer combination: At5PTase7-Nterm (5′-GCCATGGTGGTGATTCTTGAGAAC-3′) and At5PTase7-Cterm (5′-GAAAAATGTTAGCTCGGTGTATC-3′). The cDNA products were gel purified and cloned into the pMT/V5-His-TOPO vector (Invitrogen).

### Cloning of At5PTase7-GFP

RNA was extracted, and the coding region of 1,785 bp was converted to cDNA by the reverse transcriptase SuperScript III kit (Invitrogen). cDNA was amplified with the following primers: forward (5′-CACCATGAGAGACGATAAAACCAAGAAA-3′) and reverse (5′-TTAGAAAAATGTTAGCTCGGTGTA-3′). PCR was done with PrimeSTAR HS DNA Polymerase (Takara), and cloned into the pENTR/D-TOPO vector using the pENTR directional TOPO Cloning Kit (Invitrogen K2400-20) and later into the binary Gateway 35S overexpression vector pK7WG2 or GFP vector pK7WGF2 using Gateway technology (Karimi et al., 2002). The resulting plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 (Koncz et al., 1989) and transformed by the floral dip procedure (Clough and Bent, 1998). Transgenic lines expressing high levels of *At5PTase7* were propagated for three generations on kanamycin.

### Transient Protoplast Transfection

Arabidopsis ecotype Columbia plants were grown in short-day conditions at 20°C. Leaves were collected 4 to 6 weeks after germination and used for the isolation and transformation of protoplasts as described (Yoo et al., 2007). Fluorescence was detected 24 h after transfection using the Zeiss LSM 510.

### Analysis of Gene Expression

Roots were harvested separately and immediately frozen in liquid nitrogen. RNA was extracted from 200 mg of tissue using the Tri-Reagent Kit (Molecular Research Center) and analyzed by RT-PCR as described (Leshem et al., 2007). RNA samples were treated with DNase (DNA-free from Ambion) according to the manufacturer's instructions, followed by the RevertAid First Strand cDNA Synthesis Kit (Fermentas), using a 300-ng RNA sample. Expression was assayed in the linear phase (30–34 cycles) in all RT-PCR experiments. The *RD29A* transcription was tested with the following primers: forward (5′-CCCGGATCCTTTTCTGATATGGTTGCC-3′) and reverse (5′-GCCCTCGAGCCGAACAATTTATTAACC-3′). The *Actin2* gene was used as a loading control for all experiments: forward (5′-TAACCCAAAGGCCAACAGAG - 3′) and reverse (5′-CTTGGTGCAAGTGCTGTGAT-3′).

### Real-Time RT-PCR

RNA was extracted and analyzed by RT-PCR as described (Leshem et al., 2007). The real-time PCRs contained 6 $\mu$L of cDNA, 1.5 $\mu$L of 3 mM primers,

and 7.5 μL of DyNAmo FLASH SYBR Green qPCR kit (Finnzymes). The reaction was performed on a Rotor Gene 2000 thermocycler (Corbett Research). PCR conditions were as follows: 95°C for 2 min; followed by 40 cycles of 95°C for 15 s, 60°C for 10 s, and 72°C for 15 s; and then florescence acquisition at 79°C. PCR product specificity was verified by melting curve analysis and sequencing. For quantification, calibration curves were run simultaneously with experimental samples, and Ct (cycle number) calculations were performed by the Rotor-Gene 5.0 software (Corbett Research). The *Actin2* gene was used for normalization. The following primers were used for the PCR: for *Actin2* (L, 5′-CTGCTTGGTGCAAGTGCTGTGATT-3′; R, 5′-AGAAGTCTTGTTCCAGCCCTCGTT-3′), for 5PTase2 (L, 5′-CGGTGATCGAACACTTCAACTCCA-3′; R, 5′-TTCGATTCTGATCGTTACGCCGGA-3′), for 5PTase3 (L, 5′-ATCGCTGTAACCGGACGATGATCT-3′; R, 5′-TTCCGATGAGTACACCGCAAACGA-3′), for 5PTase7 (L, 5′-TCAGAAAACCGTGACTCCCCT-3′; R, 5′-GATGGAACGAAGGAAAGATATACT-3′), and for 5PTase11 (L, 5′-ACCCACTTCAAGCAAAGATCCGCT-3′; R, 5′-GCCATAAGATCAGGGTTCCAGCTT-3′).

## ROS Analysis

ROS was assayed with 10 μM $H_2$DCFDA, which is trapped in the cytoplasm following oxidation by hydrogen peroxide (Ubezio and Civoli, 1994). Epifluorescence and confocal microscopy were as described (Leshem et al., 2006). Quantification of ROS was performed by the ImagePro Plus analysis package (Media Cybernetics).

## PtdIns Treatments

PtdIns(3)P was purchased from Echelon Biosciences. The PtdIns were delivered into cells using carrier molecules according to the manufacturer's recommendations. The Shuttle carrier 2 was dissolved with PtdIns at a 1:1 ratio to form PtdIns:carrier complex, final 12.5 mM concentration, and incubated with the seedlings for 1 h before treatment with NaCl.

## Expression and Immunoprecipitation of At5PTases from S2 Cells

Expression and immunoprecipitation of At5PTases from *Drosophila* S2 cells were performed as described (Ercetin and Gillaspy, 2004). Briefly, S2 cells were transfected with 2 μg of pMTAt5PTase constructs using an Effectene transfection kit (Qiagen). Cells were harvested after 2 d of induction with 500 μM $CuSO_4$. Immunoprecipitation of the At5PTases and analysis of the resulting complexes were performed as described (Ercetin and Gillaspy, 2004).

## Activity Assays with Fluorescent and Radiolabeled Substrates

Activity assay conditions for fluorescent di-$C_6$-6(7Nitrobenz-2-oxa-1,3-diazol)-PtdIns-4,5-bisphosphate, di-$C_6$-6(7Nitrobenz-2-oxa-1,3-diazol)-PtdIns-3,4,5-trisphosphate, and $^3$H-labeled Ins(1,4,5)$P_3$ and Ins(1,3,4,5)$P_4$ were described before (Ercetin and Gillaspy, 2004). Recombinant At5PTases (15–80 ng) were incubated with 1.5 μg of fluorescent substrate in assay buffer containing 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 50 mM KCl. All activity assays were performed for 1 h at room temperature. Reaction products were separated by thin-layer chromatography and analyzed as described before (Ercetin and Gillaspy, 2004).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At5PTase1, At1g34120; At5PTase2, At4g18010; At5PTase3, At1g71710; and At5PTase7, At2g32010.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Unrooted phylogenetic N-J tree of the Arabidopsis 5PTases and conserved domain alignment.

**Supplemental Figure S2.** Expression of Arabidopsis *At5PTase* genes during abiotic stresses.

**Supplemental Figure S3.** Molecular characterization of the *At5ptase7* mutants.

**Supplemental Figure S4.** Arabidopsis *AtRboh* gene expression during salt stress.

**Supplemental Figure S5.** ROS-responsive elements in the promoters of *RD29A* and *RD22* genes.

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