

Nonspecific Phospholipase C NPC4 Promotes Responses to Abscisic Acid and Tolerance to Hyperosmotic Stress in *Arabidopsis*^{www}

Carlotta Peters,^{a,b} Maoyin Li,^{a,b} Rama Narasimhan,^{a,b} Mary Roth,^c Ruth Welti,^c and Xuemin Wang^{a,b,1}

^aDepartment of Biology, University of Missouri, St. Louis, Missouri 63121

^bDonald Danforth Plant Science Center, St. Louis, Missouri 63132

^cKansas Lipidomics Research Center, Division of Biology, Kansas State University, Manhattan, Kansas 66506

Diacylglycerol (DAG) is an important class of cellular lipid messengers, but its function in plants remains elusive. Here, we show that knockout of the *Arabidopsis thaliana* nonspecific phospholipase C (NPC4) results in a decrease in DAG levels and compromises plant response to abscisic acid (ABA) and hyperosmotic stresses. NPC4 hydrolyzes various phospholipids in a calcium-independent manner, producing DAG and a phosphorylated head group. NPC4 knockout (KO) plants display decreased ABA sensitivity in seed germination, root elongation, and stomatal movement and had decreased tolerance to high salinity and water deficiency. Overexpression of NPC4 renders plants more sensitive to ABA and more tolerant to hyperosmotic stress than wild-type plants. Addition of a short-chain DAG or a short-chain phosphatidic acid (PA) restores the ABA response of NPC4-KO to that of the wild type, but the addition of DAG together with a DAG kinase inhibitor does not result in a wild-type phenotype. These data suggest that NPC4-produced DAG is converted to PA and that NPC4 and its derived lipids positively modulate ABA response and promote plant tolerance to drought and salt stresses.

INTRODUCTION

Drought and high salinity are two crucial environmental stresses that limit plant growth, productivity, and geographic distribution. Studies in recent years have provided valuable insight into the molecular and cellular mechanisms by which plants respond to and tolerate salinity and drought stresses (Apse et al., 1999; Zhu, 2002; Chinnusamy et al., 2004; Chinnusamy and Zhu, 2009). Both drought and high salinity trigger the production of the phytohormone abscisic acid (ABA), which induces the expression of many genes involved in the stress responses (Finkelstein et al., 2002; Seki et al., 2002; Nambara and Marion-Poll, 2005). Manipulations of key ABA-responsive genes have led to changes in plant tolerance to drought and salt stresses (Kasuga et al., 1999; Umezawa et al., 2006a). Reception of ABA in the cell involves nuclear-localized ABA receptors (Ma et al., 2009; Park et al., 2009) and also membrane-bound G protein-coupled receptor (Pandey et al., 2009). The ABA receptor pyrabactin resistance 1 interacts with the protein phosphatase 2C-like ABA-INSENSITIVE1 (ABI1) (Ma et al., 2009). The function of ABI1 is also regulated by the lipid messenger phosphatidic acid (PA) (Zhang et al., 2004; Mishra et al., 2006).

Increasing evidence indicates that membrane lipids are rich sources for signaling messengers in plant response to hyperosmotic stresses (Testerink and Munnik, 2005; Mishra et al., 2006;

Hong et al., 2008). Specifically, PA is produced rapidly in response to hyperosmotic stress, and genetic and pharmacological manipulation of PA levels has resulted in alteration of plant response to ABA, drought, and salinity. Phospholipase D (PLD) hydrolysis of membrane lipids directly produces signaling PA. It has been shown recently that PLD α 3, ϵ , δ , and α 1 contribute to hyperosmotic stress-induced PA production and affect plant response to hyperosmotic stress in *Arabidopsis thaliana* (Hong et al., 2008, 2009; Munnik and Testerink, 2009). Activation of phospholipase C (PLC) is another major route for generating the PA signal. PLC produces diacylglycerol (DAG) that can be phosphorylated by DAG kinase (DGK) to generate PA (den Hartog et al., 2003; Zonia and Munnik, 2004). Plants have two distinctively different families of PLCs. PI-PLC uses primarily phosphoinositides (PIs), whereas nonspecific PLC (NPC) uses common membrane phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The signaling function of PI-PLC has been well established in animals. PLC hydrolysis of phosphatidylinositol 4,5-bisphosphate releases two potent second messengers, inositol 1,4,5-trisphosphate and DAG. DAG remains in the membrane and, in animals, activates certain members of the protein kinase C family and other proteins. However, in plants, there is no evidence for the existence of protein kinase C family members, and the target of DAG as a plant signaling molecule remains undefined. Yet, the plant PI-PLC family has been implicated in ABA signaling and salt and drought stress (Hirayama et al., 1995; Sanchez and Chua, 2001; Hunt et al., 2003).

Unlike PI-PLC, NPCs hydrolyze the common membrane lipids PC and PE in a calcium-independent manner (Nakamura et al., 2005). NPCs share sequence similarities with a bacterial PC-PLC

¹ Address correspondence to swang@danforthcenter.org.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Xuemin Wang (swang@danforthcenter.org).

^{www}The online version of this article contains Web-only data
www.plantcell.org/cgi/doi/10.1105/tpc.109.071720

that has been characterized in microbial systems (Wang, 2001). PC-PLC isolated from *Clostridium perfringens* is a potent toxin, with an α -toxin domain (Titball, 1993). This enzyme has hemolytic activity (Saint-Joanis et al., 1989), vascular permeabilization capability (Sugahara et al., 1977), and a platelet-aggregating property (Sugahara et al., 1976). Other nontoxic PC-PLCs are also found in bacteria such as *Bacillus cereus* and *Pseudomonas aeruginosa* (Titball, 1991, 1993). Based on sequence homology with bacterial PC-PLC, the *Arabidopsis* genome is predicted to contain six putative NPC genes designated NPC1-6. These genes encode ~60-kD proteins, consisting of 514– to 538–amino acid residues (Nakamura et al., 2005). NPCs lack the X-Y domains (catalytic domain) and the EF-hand motif (calcium binding domain) of PI-PLC and the calcium-dependent lipid binding C2 domain or PI binding PX and PH domains of PLD. NPC5 is involved in phospholipid degradation and digalactosyl diacylglycerol (DGDG) accumulation in *Arabidopsis* leaves, but not in roots (Gaude et al., 2008). NPC5 is a cytosolic protein, whereas NPC4 is associated with the plasma membrane (Nakamura et al., 2005; Gaude et al., 2008). Of all the NPC genes, NPC4 is the only one whose expression is greatly induced by phosphate deprivation (Misson et al., 2005), but genetic ablation of NPC4 resulted in a slight decrease of DGDG, whereas the content of major phospholipids did not change during phosphate deprivation (Nakamura et al., 2005). Thus, the metabolic and physiological functions of NPC4 remain elusive. Here, we show that NPC4 plays an important role in plant response to ABA, drought, and high salinity.

RESULTS

Decreased Plant Sensitivity to ABA in NPC4 Knockout *Arabidopsis*

A homozygous T-DNA insertion mutant of NPC4, *npc4-1* (SALK_046713), was isolated, and the elimination of NPC4 expression in *npc4-1* was verified by quantitative real-time PCR (Figure 1A). We first examined the effect of the *npc4-1* mutation on plant response to abiotic stresses and hormones by comparing seedling growth of *npc4-1* and the wild type. No overt differences in growth and development were observed when seedlings of the two genotypes were grown on media deficient in phosphate (1 μ M), nitrogen (0.6 mM), or potassium (0 μ M) or media containing 1 μ M indole-3-acetic acid (see Supplemental Figure 1 online). However, a drastic difference in growth occurred between *npc4-1* and wild-type seedlings grown on ABA-containing media. The seedlings of *npc4-1* had longer primary roots and more lateral roots than wild-type seedlings (Figures 1B and 1C; see Supplemental Figure 2 online). At 25 μ M ABA, flowering was inhibited in wild-type plants, but *npc4-1* plants were able to produce inflorescences (Figure 1B). The dry weights of *npc4-1* rosettes grown on 20 and 25 μ M ABA were more than threefold greater than the wild type and at 35 μ M ABA, twofold greater than the wild type (Figure 1D). The ABA-resistant root elongation and flowering phenotype of *npc4-1* was dosage dependent; at higher concentrations of ABA (35 and 50 μ M), root growth of *npc4-1* was comparable to the wild type. Genetic

complementation of *npc4-1* plants with the native NPC4 gene restored the wild-type phenotype to the mutant (Figures 1E and 1F). These results indicate that loss of NPC4 is responsible for reduced sensitivity toward ABA.

One of the best known effects of ABA is to induce seed dormancy and inhibit seed germination. In the absence of ABA, nearly 100% of seeds of the wild type and *npc4-1* germinated within 4 d (Figure 2A, left panel). In the presence of ABA, *npc4-1* seeds broke dormancy earlier than the wild type and were more resistant than the wild type to ABA inhibition of seed germination. In the presence of 1 μ M ABA for 4 d, 36% of *npc4-1* seeds germinated, whereas no wild-type seeds germinated (Figure 2A, middle panel). After 10 d at 1 μ M ABA, *npc4-1* seeds showed 33% more germination than wild-type seeds. At 5 μ M ABA, the germination of wild-type seeds was completely inhibited, but 10% *npc4-1* seeds still germinated (Figure 2A, right panel). Another well-documented function of ABA is to promote the stomatal closure. To test whether *npc4-1* was altered in ABA-mediated stomatal movement, stomatal peels of wild-type and *npc4-1* plants were incubated with 50 μ M ABA. ABA promoted stomatal closure and inhibited stomatal opening in the wild type but not in *npc4-1* leaf peels (Figure 2B).

We also identified T-DNA insertional knockout mutants for the other five NPCs and examined the effect of these mutations on plant response to ABA. At 25 μ M ABA, all other NPC-KOs, *npc1-1*, *npc2-1*, *npc3-1*, *npc5-1*, and *npc6-1*, displayed ABA-inhibited growth similar to wild-type seedlings and markedly different from *npc4-1* seedlings, which displayed ABA resistance (see Supplemental Figure 2 online). Lateral root number was higher in *npc4-1* than the wild type, whereas lateral root number was similar among the wild type, *npc1-1*, *npc2-1*, *npc3-1*, and *npc6-1* but lower in *npc5-1* in response to ABA (see Supplemental Figure 2 online). The results indicate that the decreased ABA sensitivity was specific to NPC4.

Different Expression Patterns of the NPC Family

The expression patterns of the NPC gene family were determined by quantitative real-time PCR using RNA from different tissues of 6-week-old *Arabidopsis* plants. The highest level of NPC4 expression was detected in mature leaves and then roots, and its expression in young leaves and reproductive tissues was low (Figure 3). By comparison, NPC5 was expressed mostly in inflorescences. The highest level of expression for NPC3 was in roots, whereas for NPC2 and NPC6, expression was highest in siliques. NPC1 was expressed in similar levels all the tissues examined (Figure 3). Analysis of available transcriptomic data (<http://bar.utoronto.ca>) indicates that the expression pattern of the NPCs varies in root cell types (see Supplemental Figure 3A online). The database data failed to distinguish NPC4 and NPC5 due to high sequence similarity. However, our analysis using gene-specific primers showed that NPC5 was mostly expressed in inflorescences, and its level of expression in leaves and roots was much lower than that of NPC4 (Figure 3). Thus, the combined NPC4/5 transcript level in leaves and roots may result mostly from the NPC4 expression. The level of NPC4 was higher in root stele and endodermis cells than cortex and lateral root caps. In guard cells, the transcript level of NPC4 was induced

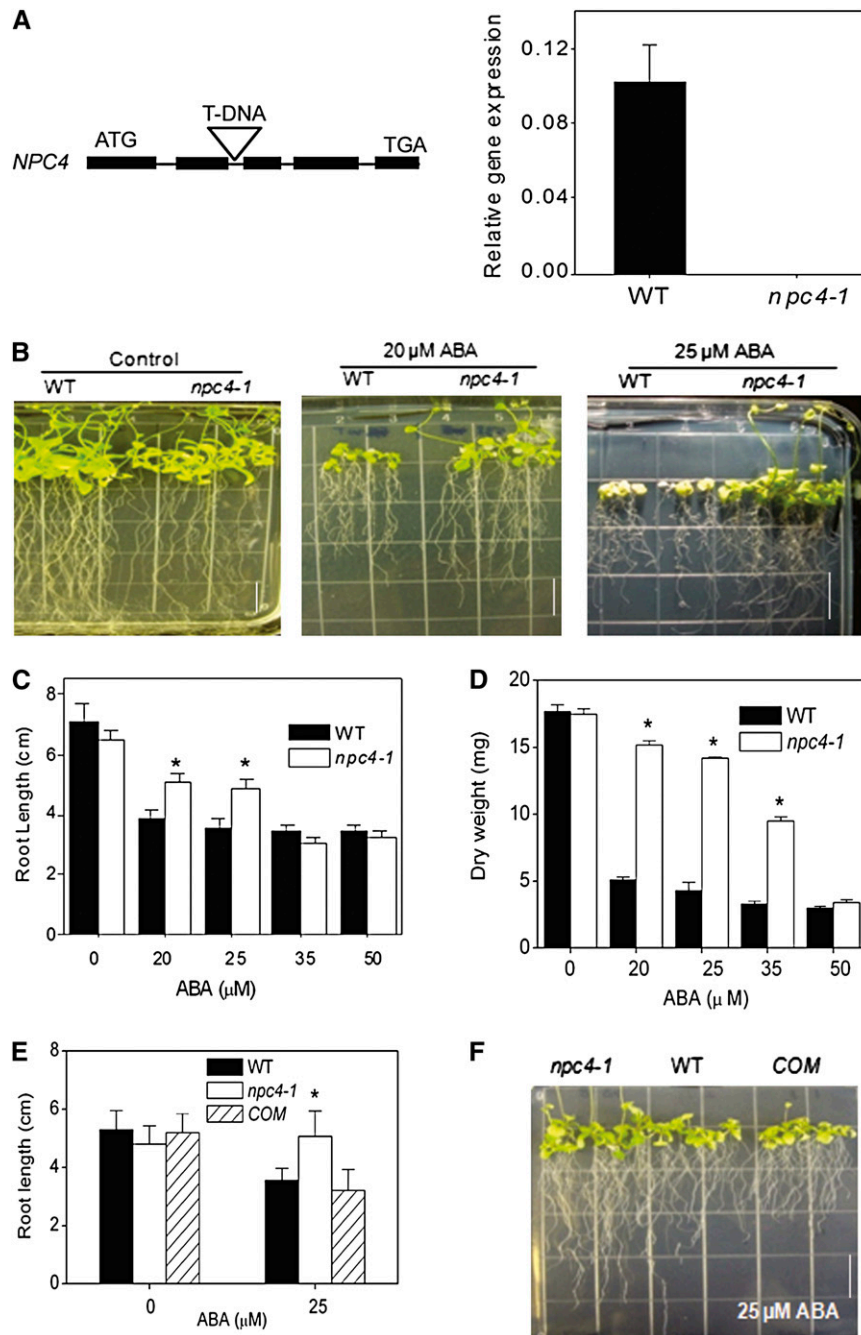


Figure 1. Decreased Sensitivity of *NPC4* Knockout Plants to ABA Inhibition of Plant Growth and Development.

(A) T-DNA insertional knockout of *NPC4*. Left: location of the T-DNA inserted in the *NPC4* gene. Solid bars represent exons, and thin lines represent introns. Right: level of *NPC4* expression in wild-type and *npc4-1* plants as measured by real-time PCR. Relative expression is based on the expression of *UBQ10*. Three independent experiments were performed; values are means \pm SD ($n = 3$) for one representative experiment.

(B) Seedlings on 0.5 \times Murashige and Skoog medium containing 0, 20, and 25 μ M ABA. Seedlings were germinated on media without ABA for 5 d and then transferred onto media with varied levels of ABA for 2 weeks. Bar = 1.3 cm.

(C) Root length on 0.5 \times Murashige and Skoog medium supplemented with 0 to 50 μ M ABA. Seedlings were germinated on media without ABA for 5 d and then transferred onto media with varied levels of ABA. Primary root length was measured 14 d after transfer. Values are means \pm SD ($n = 15$).

(D) Dry weight of seedlings grown in the presence of 0 to 50 μ M ABA. After growing on media with varied levels of ABA for 14 d, seedlings were harvested as measured. Values are means \pm SD ($n = 3$).

(E) Root length of the wild type, *npc4-1*, and *npc4-1* complemented with wild-type *NPC4* (COM). Seedlings were grown for 14 d on 0.5 \times Murashige and Skoog medium in the presence or absence of 25 μ M ABA. Values are means \pm SD ($n = 15$).

(F) Seedling phenotypes of the wild type, *npc4-1*, and *npc4-C* on 0.5 \times Murashige and Skoog medium containing 25 μ M ABA. Photograph was taken 14 d after transfer to ABA medium. Bar = 1.3 cm.

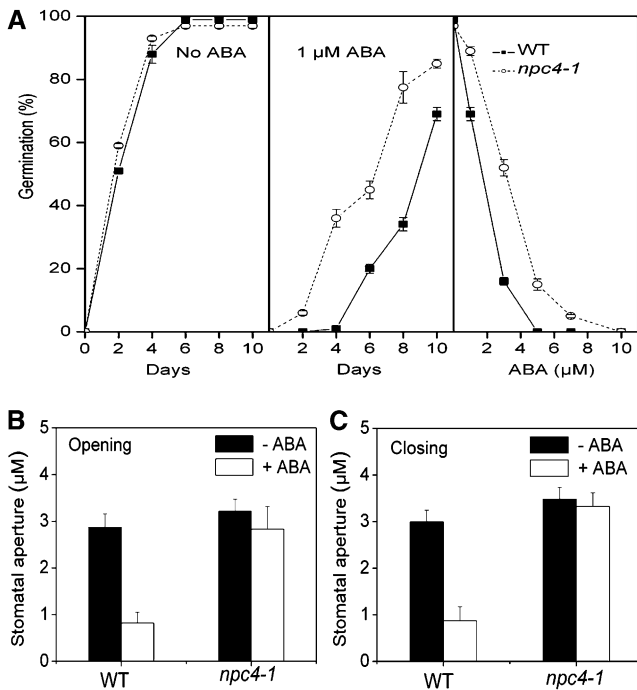


Figure 2. Decreased ABA Sensitivity of NPC4 Knockout in Seed Germination and Stomatal Movement.

(A) Seed germination rates in the absence of ABA (left), the presence of 1 μM ABA (middle), or different concentrations of ABA (right). Fully desiccated seeds were germinated on 0.5 \times Murashige and Skoog with or without ABA. Germination was scored at 10 d after transferring seeds from 4°C. One hundred seeds per genotype were measured in each experiment. Values are means \pm SD ($n = 3$).

(B) Opening of closed stomata as affected by ABA. Wild-type and mutant epidermal peels were incubated in the dark without ABA to close stomata. The peels were then placed under light for 3 h in the presence of 50 μM ABA. Stomata aperture was imaged using a light microscope. The images obtained were measured using Image Pro software. Values are means \pm SE ($n = 15$).

(C) Closure of open stomata in the presence of ABA. Epidermal peels were placed under light for 3 h to fully open stomata of both mutant and the wild type. The peels were treated with 50 μM ABA for 3 h. Values are means \pm SD ($n = 40$).

threefold by ABA (Yang et al., 2008; <http://bar.utoronto.ca>). The expression of other NPCs was detected in guard cells, but ABA treatments did not increase expression of NPC1, 2, or 6 (see Supplemental Figure 3B online). Some increase in NPC3 occurred, but its level of expression was lower than that of NPC4. These data indicate that NPC gene family members have overlapping but mostly distinguishable patterns of expression in *Arabidopsis* tissues and cells. ABA induction of NPC4 expression was detected in leaves; the increase was transient, with the highest level of expression at 12 h after ABA treatment (Figure 4).

ABA Content and the Expression of ABA-Responsive Genes

To investigate whether altered responses to ABA were due to changes in ABA levels, ABA content was measured in freshly

picked and desiccated seeds and leaf tissue of *npc4-1* and the wild type. The level of ABA in freshly picked *npc4-1* seeds was significantly higher (20%) than wild-type seeds (Figure 4A), and the difference became smaller in desiccated seeds (Figure 4A). However, the ABA content in rosette leaves was similar in *npc4-1* and the wild type (Figure 4A). These results indicate that the decreased sensitivity of *npc4-1* toward ABA is not attributable to lower ABA content than wild-type tissue, and NPC4 likely is involved in plant response to ABA.

We then tested whether *npc4-1* had altered expression of ABA-responsive genes (Figure 4). The basal level of expression of *ABI1*, *ENHANCED RESPONSE TO ABA1 (ERA1)*, *Response to ABA (RAB18)*, and *Open Stomata 1 (OST1)* were similar in wild-type and *npc4-1* leaves. The basal levels of *Response to Desiccation 29B (RD29B)* and *ABI2* were lower in *npc4-1* than the wild type, whereas *Root Curls in NPA1 (RCN1)* was higher in

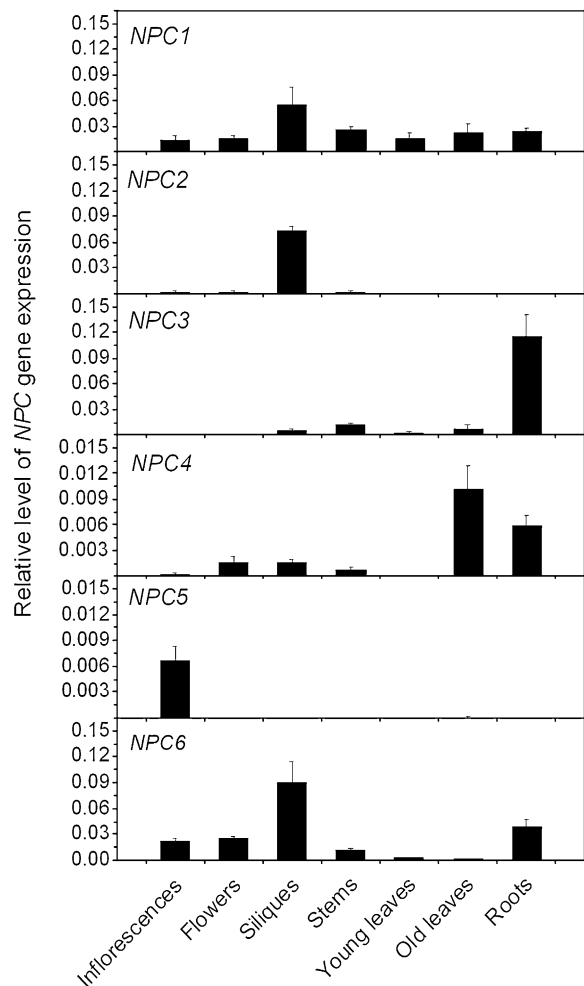


Figure 3. Expression Pattern of the NPC Gene Family Members in *Arabidopsis* Tissues.

Total RNA was isolated from 6-week-old soil-grown *Arabidopsis* plants. The level of gene expression was normalized to that of *UBQ10*. Values are means \pm SD; $n = 3$ biological replicates.

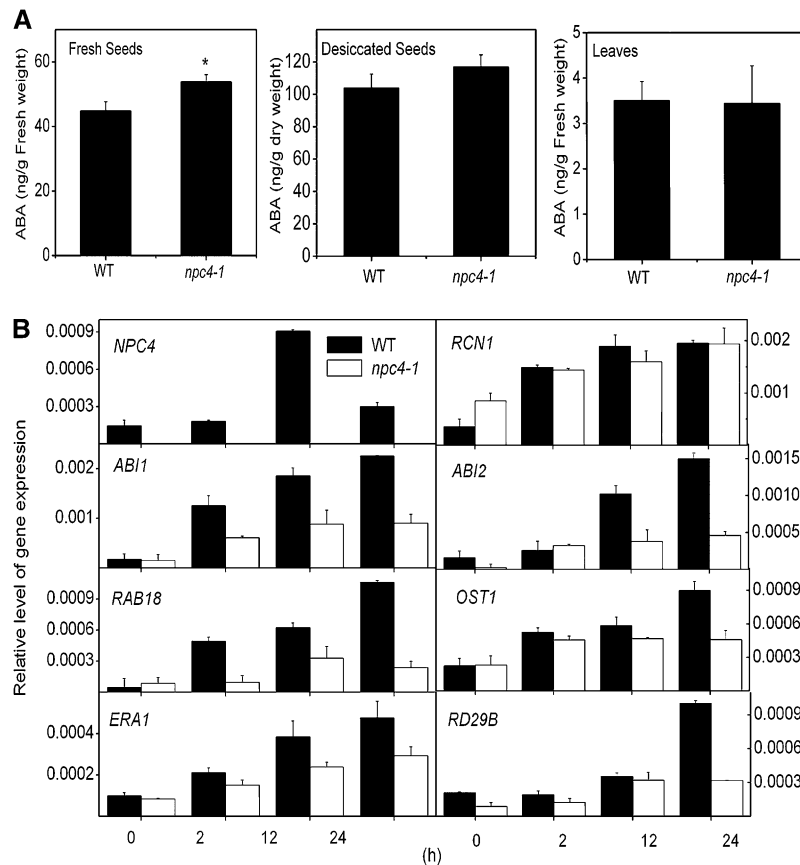


Figure 4. ABA Content and the Expression of *NPC4* and ABA-Responsive Genes in the Wild Type and *npc4-1*.

(A) ABA content of fresh seeds, desiccated seeds, and leaves of 4-week-old *Arabidopsis* plants. ABA was extracted and analyzed by the use of liquid chromatography-MS/MS. Values are means \pm SD ($n = 3$). *Significant at $P < 0.05$ compared with the wild type based on Student's t test.

(B) Levels of gene expression in response to ABA as measured by quantitative real-time PCR. Four-week-old *Arabidopsis* plants were treated with 50 μ M ABA, and samples were harvested at indicated time intervals for RNA extraction. Relative expression is based on the expression of *UBQ10*. Values are means \pm SD ($n = 3$).

npc4-1 (Figure 4B). When the plants were treated with ABA, the expression of the genes tested was induced in wild-type and *npc4-1* leaves. However, the level of induction of *ABI1*, *ABI2*, *RAB18*, *OST1*, *RD29B*, and *ERA1* was lower in *npc4-1* than in the wild type. The level of *RCN1* expression in response to ABA in the wild type and *npc4-1* was similar, but the magnitude of induction in *npc4-1* was lower than the wild type (Figure 4B). These results are consistent with the phenotypic changes indicating that *npc4-1* plants have decreased, but not abolished, sensitivity to ABA.

Decreased DAG Content in the *NPC4* Knockout

To determine the effect of the *NPC4* loss on DAG level, we analyzed the relative levels of DAG molecular species in the wild type and *npc4-1* using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Under normal growth conditions, the DAG level in 4-week-old wild-type leaves was $\sim 4\%$ of total glycerophospholipids and galactolipids, and the DAG content in *npc4-1* leaves was $\sim 80\%$ of the wild type (Figures 5A and 5B). Treatment of leaves with 50 μ M ABA resulted in a $>50\%$

decrease in DAG level in both the wild type and *npc4-1*, and the leaves of *npc4-1* still had a lower level of DAG than the wild type. The molecular species of DAG in *Arabidopsis* leaves that produced the largest mass spectral signals were 16:0/18:2, 16:0/18:3, and 18:2/18:3 combinations, followed by 18:2/18:2 and 18:3/18:3 DAG. The levels of all the major DAG species were decreased in *npc4-1* leaves (Figure 5B). Under normal and ABA-treated conditions, DGDG, monogalactosyldiacylglycerol (MGDG), PC, PE, PA, phosphatidylglycerol (PG), and phosphatidylinositol (PI) were similar in wild-type and *npc4-1* plants. However, phosphatidylserine (PS) was elevated in *npc4-1* compared with wild-type plants, suggesting that *NPC4* may use this lipid as a substrate (Figure 5C).

To test whether *NPC4* used PS and PA as substrates, we expressed *NPC4* in *Escherichia coli* and measured its activity toward different phospholipids (Figure 6A). *NPC4* displayed a high activity toward PC and PE (Figures 6B and 6C). In addition, *NPC4* used PG and PS as substrates, but these activities were lower than the hydrolytic activity with PC or PE (Figure 6B). Furthermore, using *NPC4* purified from *E. coli*, *NPC4* displayed

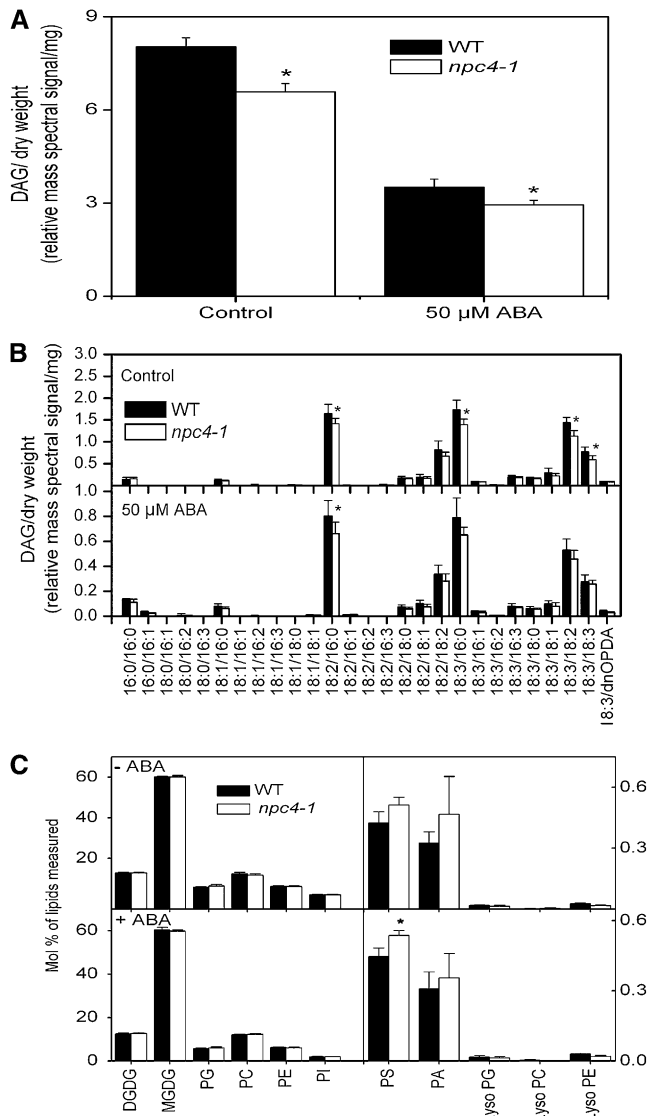


Figure 5. DAG, Phospholipid, and Galactolipid Content and Effect of ABA on the Wild Type and *npc4-1*.

(A) Total DAG content and its changes in response to ABA. Four-week-old soil-grown *Arabidopsis* plants were treated with 50 μ M ABA for 1 h. Total lipids were extracted from leaves and profiled using ESI-MS/MS. Asterisk denotes that the difference between the wild type and mutant is significant with $P < 0.05$ as determined by Student's *t* test. Values are means \pm SE ($n = 5$).

(B) DAG molecular species with and without ABA treatment. Molecular species are presented as a:b/c:d, where a and c represent the number of carbons in the two fatty acids of DAG, and c and d represent the number of double bonds in the two fatty acids, respectively. dnOPDA, dinoroxophytodienoic acid. Asterisk denotes that the difference between the wild type and mutant is significant with $P < 0.05$ as determined by Student's *t* test. Values are means \pm SE ($n = 5$).

(C) Total phospholipid and galactolipid content in leaves and its changes in response to ABA. Four-week-old soil-grown *Arabidopsis* plants were treated with ABA for 1 h. Asterisk denotes that the difference between the wild type and mutant is significant with $P < 0.05$ as determined by Student's *t* test. Values are means \pm SE ($n = 5$).

reproducible hydrolytic activity on 16:0/16:0 PA, although the activity on this PA was much lower than its activity on PC or PE (Figure 6C). However, no NPC4 activity was detected when 18:1/18:1 PA was used as substrate. Previously, NPC4 was reported to hydrolyze PC and PE; some activity toward phosphoinositol-4,5-bisphosphate was noted, but this activity was ~ 10 -fold less than that on PC (Nakamura et al., 2005).

Increased ABA Sensitivity in NPC4-Overexpressing Plants

To further investigate the function of NPC4, we generated transgenic *Arabidopsis* lines overexpressing STREP-tagged NPC4 (NPC4-OE) under the control of the cauliflower mosaic virus 35S promoter. Production of NPC4-STREP in plants was confirmed by immunoblotting using anti-STREP antibodies (Figure 7A). Several independent lines were tested for their stress responses, and two representative transgenic lines were analyzed further. NPC4-OE seeds were more sensitive to ABA inhibition of germination than wild-type and *npc4-1* seeds, and some of the seedlings did not develop green cotyledons but exhibited an etiolated phenotype after germination (Figure 7B). At 1 μ M ABA, unlike *npc4-1* seeds that displayed a higher germination rate than wild-type seeds, the germination of NPC4-OE seeds was 20% lower than the wild type (Figures 7B and 7C).

ABA maintains dormancy in seeds and prevents precocious germination. Genetic studies in *Arabidopsis* revealed that the first ABA peak occurs maternally right before the maturation phase of the offspring; the second ABA peak occurs in the embryo itself and is essential for the induction of dormancy (Karszen et al., 1983). To test precocious germination in these genotypes, freshly harvested wild-type, *npc4-1*, and OE seeds were germinated on filter paper. Fresh *npc4-1* seeds germinated at a much greater rate than wild-type seeds (80% versus 40%), whereas only $\sim 30\%$ of NPC4-OE seeds germinated (Figures 7D and 7E). These results suggest that NPC4 promotes ABA action and seed dormancy.

Opposite Responses to Hyperosmotic Stresses by NPC4 Knockout and Overexpression

ABA plays an important role in sensing and adaptation to abiotic stresses, such as drought and salinity (Yamaguchi-Shinozaki and Shinozaki, 1994). Since *npc4-1* plants exhibited reduced ABA sensitivity in both germination and vegetative growth, we investigated the effect of NPC4 alterations on plant tolerance to salinity and water deficits. Wild-type and *npc4-1* seeds were sown on 200 mM NaCl, and the percentage of plants in which emergence of the radicle was observed was scored as total germination; seedlings with green cotyledons were scored as viable seedlings. Seedlings of *npc4-1* were hypersensitive to NaCl; wild-type seeds produced more than twice as many viable seedlings as *npc4-1* (Figure 8A). By contrast, the number of viable OE (OE3 and OE8) seedlings was 60% higher than the wild type, and almost all germinated OE seedlings were viable. The total germination was lower in the wild type than *npc4-1* and OE seeds under high salinity.

To test the effect of NPC4 alterations on seedling growth in response to NaCl, 5-d-old seedlings grown on 0.5 \times Murashige

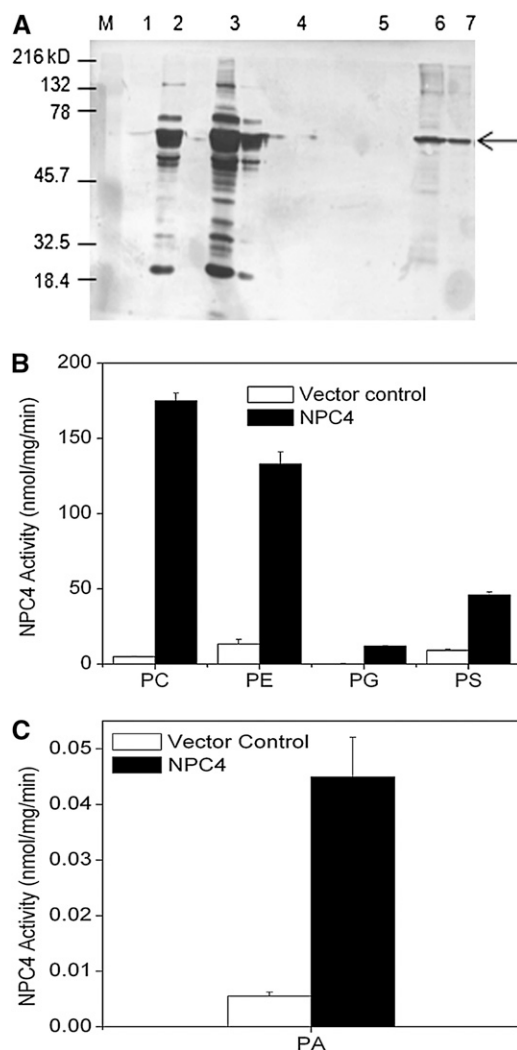


Figure 6. NPC4 Activity toward Different Phospholipids.

(A) Immunoblotting of NPC4 protein produced in *E. coli*. NPC4 from total *E. coli* lysate was purified and separated on 10% SDS-PAGE and transferred onto a filter and blotted with anti-His antibody. M, marker; 1, total lysate from empty vector; 2 and 3, total lysate harboring NPC4; 4, empty vector extract bound to His-agarose beads; 5, bound protein eluted from empty vector extract; 6, NPC4 extract bound to His-agarose beads; 7, NPC4 eluted from beads. Arrow indicates the 60-kD NPC4 protein.

(B) Lipid hydrolysis activity assayed in the presence of different phospholipids using crude *E. coli* lysate. DAG was quantified indirectly by measuring the release of the phosphorylated head group using a colorimetric phosphate assay. Values are means \pm SD ($n = 3$). Vector control refers to the lysate from *E. coli* harboring the empty vector. Lipid abbreviations are described in the legend to Figure 5.

(C) NPC4 activity on PA using purified NPC4. DAG was quantified using gas chromatography (GC) analysis of fatty acid methyl esters derived from the DAG. Vector control refers to the lysate from *E. coli* harboring the empty vector that was purified in the same manner as NPC4. Values are means \pm SD ($n = 3$).

and Skoog medium were transferred to NaCl-containing plates. Ten days after growth on 100 mM NaCl, *npc4-1* primary roots were 8% shorter than the wild type, whereas OE roots were 20% longer than wild-type roots. On plates containing 150 mM NaCl, the primary root length of *npc4-1* was 80% of the wild-type root length; OE roots were 55% longer than the wild type (Figures 8B and 8C). Biomass accumulation in *npc4-1*, NPC4-OE, and wild-type seedlings was similar in the absence of NaCl (Figure 8D). However, in the presence of 100 mM NaCl, the dry weight of *npc4-1* seedlings was 85% of wild-type seedlings, whereas NPC4-OE seedlings weighed 150% as much as wild-type seedlings (Figure 8D). At 150 mM NaCl, *npc4-1* seedlings accumulated \sim 70% of the biomass of wild-type seedlings, whereas OE accumulated twofold more biomass than the wild type (Figure 8D).

To determine whether the response was specific to salt stress, *npc4-1*, NPC4-OE, and wild-type seedlings were tested for their responses to water deficits. Murashige and Skoog plates were infused with polyethylene glycol (PEG) to a water potential of -0.25 or -0.45 MPa (Verslues and Bray, 2004). The length of *npc4-1* primary roots was \sim 90 and 80% of wild-type seedlings on -0.25 and -0.45 MPa, respectively (Figures 9A and 9B). The primary roots of NPC4-OE seedlings were \sim 20 and 15% longer than those of wild-type seedlings on -0.25 and -0.45 MPa, respectively. Compared with the wild type, *npc4-1* seedlings accumulated \sim 18 and 5% less biomass on -0.25 and -0.45 MPa, respectively (Figure 9C). By contrast, NPC4-OE seedlings accumulated \sim 35 and 30% more biomass than wild-type seedlings on -0.25 and -0.45 MPa, respectively (Figure 9C).

The effect of *npc4-1* and OE was investigated in plants grown in soil under drought conditions. Drought conditions were simulated by reducing and maintaining the soil water capacity to 35% and controls were maintained at 100%. Under well-watered control conditions, transpiration rate and stomatal conductance were lower in *npc4-1* than the wild type and higher in OE compared with the wild type. However, under drought conditions, transpiration rate and stomatal conductance were higher in *npc4-1* compared with the wild type and lower in OE compared with the wild type (Figure 10A). Lower levels of photosynthesis and water use efficiency were observed in *npc4-1* than wild-type plants under drought stress, whereas OE plants had higher water use efficiency than the wild type (Figure 10A). Under control growth conditions, no differences in phenotype were observed among the genotypes. Under drought conditions, a visible reduction in leaf turgor and size was observed in *npc4-1* plants compared with the wild type. By contrast, OE plants were more turgid than wild-type plants (Figure 10B). These results support the conclusion that NPC4 promotes tolerance to hyperosmotic stresses.

Restoring *npc4-1* to Wild Type–Like ABA Response by DAG and PA

To determine how NPC4 functions in plant response to stress, we tested whether the NPC product DAG restores the *npc4-1* phenotype to the wild type in the presence of ABA. Short-chain DAGs and short-chain phospholipids (8:0/8:0 PA, 8:0/8:0 PC, and 8:0/8:0 PG) were used in this experiment. The short chains

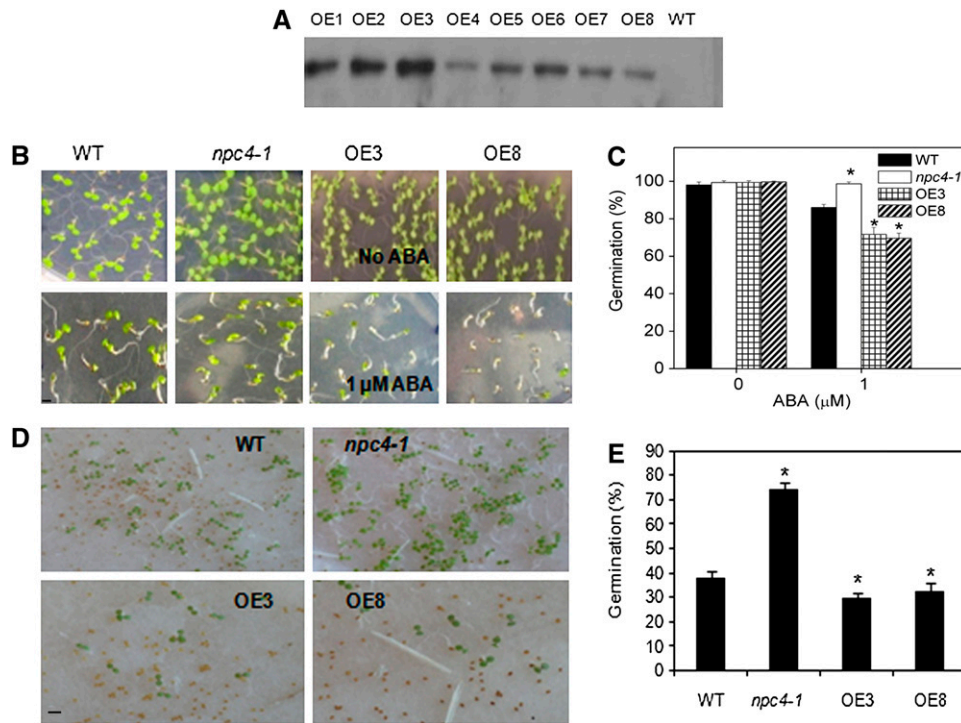


Figure 7. Increased Sensitivity of NPC4-Overexpressing Plants to ABA Inhibition of Seed Germination.

(A) Production of Strep-tagged NPC4 in *Arabidopsis* wild-type plants. Proteins were extracted from the leaves of NPC4-STREP transgenic plants and separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Ten micrograms of total protein was loaded onto each lane. The membrane was blotted with anti-STREP antibody conjugated with horseradish peroxidase. Lanes OE1 through OE8 represent different transgenic lines harboring the NPC4-STREP overexpression construct.

(B) Germination on 0.5× Murashige and Skoog medium in the presence of 1 μM ABA. Photographs were taken at 7 d after seeds were sown. Bar = 2 mm.

(C) Percentage germination of seeds of the wild type, *npc4-1*, OE3, and OE8 on 0.5× Murashige and Skoog medium with 0 or 1 μM ABA. One hundred seeds per genotype were measured. Values are means ± SD, $n = 3$, and experiments were repeated three times to ensure reproducibility.

(D) and **(E)** Precocious germination. Freshly picked seeds were germinated on Whatman filter paper **(D)**, and the germination percentage was quantified after 7 d **(E)**. One hundred and fifty seeds per genotype were measured. Values are means ± SD; $n = 3$. *Significant at $P < 0.05$ compared with the wild type based on Student's t test. Bar = 3 mm.

were chosen for addition to the growth media because these compounds are more water soluble than 18C- or 16C-acyl chain lipids. Short-chain analogs of DAG and phospholipids are also known to more readily undergo transbilayer movement (Horman and Pownall, 1988). The presence of 8:0/8:0 DAG restored *npc4-1* root growth so that in the presence of ABA, *npc4-1* roots were the same length as those of wild-type seedlings (Figure 11A). The addition of 8:0/8:0 PA also restored *npc4-1* root growth to the wild-type phenotype in the presence of ABA. Short-chain PC or PG had no such effect (Figure 11A). This result suggested that NPC4-produced DAG might be phosphorylated to PA by DGK. We then added the DAG kinase inhibitor 1 (DGKI1) to inhibit the conversion of DAG to PA. When DGKI1 was added together with DAG in the presence of ABA, DAG was no longer able to fully restore the *npc4-1* root growth to the wild-type phenotype. Instead, the root growth of *npc4-1* remained less sensitive to ABA inhibition than the wild type in the presence of 100 μM DGKI1 (Figure 11B). On the other hand, when PA and DGKI1 were added in the presence of ABA, PA was able to

increase the ABA sensitivity of *npc4-1* to the level of the wild type. DGKI1 by itself at this concentration displayed no significant effect on root growth. These results indicate that the DAG produced by NPC4 is converted to PA, and the NPC4/DGK pathway plays a role in ABA signaling events.

We then measured the level of DAG and PA, as well as other glycerolipids, in rosettes and roots of wild-type, *npc4-1*, and NPC4-OE seedlings. Under the control condition, DAG content in *npc4-1* rosette was lower than the wild type, whereas OE was higher. After treatment with ABA, a decrease in DAG content was observed in wild-type and *npc4-1* rosettes compared with the control, but an increase in DAG occurred in NPC4-OE rosettes (Figure 12A, left panel). The DAG content in *npc4-1* rosettes was ~85% that of the wild type, whereas DAG in NPC4-OE was 40% higher than the wild type after ABA treatment (Figure 12A, right panel). In roots without ABA treatment, the DAG content of NPC4-OE and the wild type was similar, but *npc4-1* contained ~20% less DAG than the wild type. ABA treatment resulted in an increase of DAG content in wild-type and *npc4-1* roots, but the

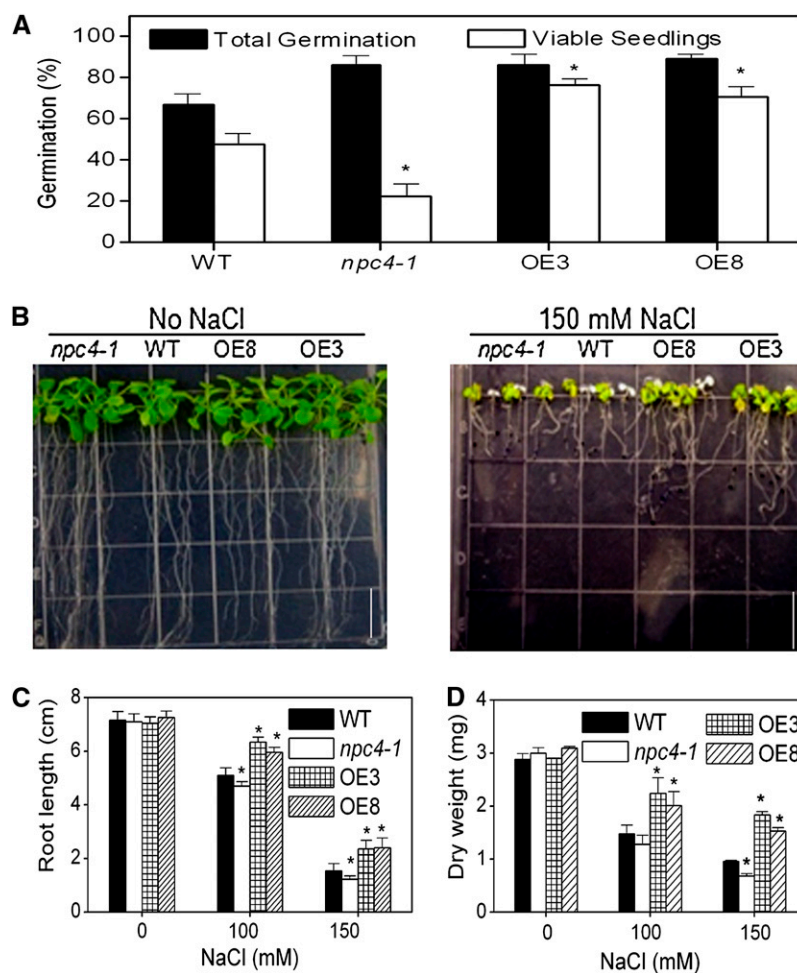


Figure 8. Opposite Effects of *npc4-1* and *NPC4*-OE on Tolerance to Salt Stress.

(A) Quantification of total germination and viable growth of wild-type, *npc4-1*, and *NPC4*-OE seedlings on 0.5× Murashige and Skoog medium plus 200 mM NaCl for 2 weeks. Total germination represents the percentage of radicle emergence, and viable seedlings refer to seedlings not bleached and remaining green. One hundred seeds per genotype were germinated. Values are means ± SD; $n = 3$. *Significant at $P < 0.05$ compared with the wild type based on Student's *t* test.

(B) Seedling phenotypes under salt stress. Five-day-old seedlings were transferred to 0.5× Murashige and Skoog medium with and without NaCl. Photographs were taken 10 d after transfer. Bar = 1.3 cm.

(C) Root length of wild-type, *npc4-1*, and OE seedling grown on 0.5× Murashige and Skoog medium plus 0, 100, or 150 mM NaCl for 10 d. Values are means ± SD ($n = 15$).

(D) Biomass production under salt stress. The rosettes from the wild type, KO, and OE were harvested on day 10 from plants subjected to salt stress as described in **(C)** and oven dried for 2 d at 60°C. Values are means ± SD ($n = 15$). Three independent experiments were performed with similar results, and the values of one experiment are presented.

DAG increase was subtle in *NPC4*-OE roots (Figure 12A, right panel). These results indicate that *NPC4* alterations directly impact DAG content both in roots and rosettes with or without ABA, but the effect differs between rosettes and roots.

Without ABA treatment, PA contents of the wild type, *npc4-1*, and *NPC4*-OE were similar in rosettes, but *npc4-1* roots had 20% more PA than wild-type and OE roots. After ABA treatment for 1 h, the PA content in the wild type remained unchanged, and PA in *npc4-1* decreased, whereas PA in *NPC4*-OE increased compared with corresponding roots without ABA treatment (Figure 12A, bottom right panel). In rosettes treated with ABA,

the PA content in the wild type and *npc4-1* increased, whereas the PA content in *NPC4*-OE decreased. In rosettes without ABA treatment, the contents of other glycerolipids were comparable among the wild type, *npc4-1*, and *NPC4*-OE, except that *npc4-1* rosettes had more PS than *NPC4*-OE and *npc4-1* and *NPC4*-OE had a higher level of PE than the wild type. ABA treatment for 1 h did not result in major changes, but there was a trend toward an increase in galactolipids MGDG and DGDG with a concomitant decrease in phospholipids PC, PG, and PS (Figure 12B, left panel). The trend of opposite changes in galactolipids versus phospholipids also occurred when lipids from ABA-treated

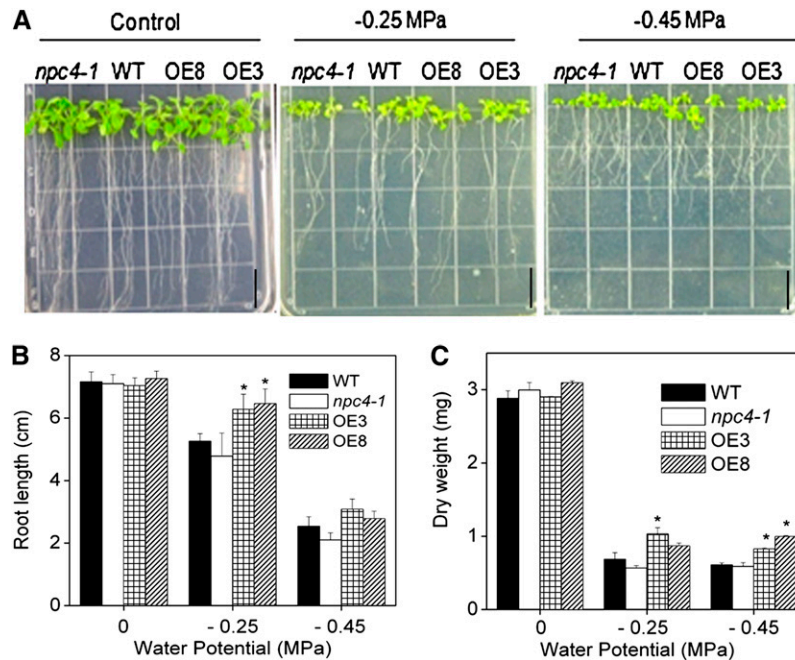


Figure 9. Opposite Effects of *npc4-1* and *NPC4*-OE on Tolerance to Water Deficiency.

(A) Seedling morphology of *npc4-1* and *NPC4*-OE plants germinated on PEG-infused plates. Four-day-old seedlings were transferred to 0.5× Murashige and Skoog medium and 1.5% agar plates infused with overlay solution containing 0 and 170 g/L⁻¹ PEG that corresponded to -0.25 and -0.45 MPa. Photographs were taken 10 d after seedlings were transferred. Control represents seedlings that were grown on 0.5× Murashige and Skoog 1% agar plates. Bar = 1.3 cm.

(B) Root length of wild-type, *npc4-1*, and OE seedlings grown on 0.5× Murashige and Skoog medium, -0.25 or -0.45 MPa PEG for 10 d. Values are means ± SD (*n* = 15). *Significant at *P* < 0.05 compared with the wild type based on Student's *t* test.

(C) Biomass production under drought stress. The rosettes from wild-type, *npc4-1*, and OE plants in **(B)** were harvested on day 10 from plants subjected to drought stress and oven dried for 2 d at 60°C. Dry weights were measured, and values are means ± SD (*n* = 15). Three independent experiments were performed with similar results, and the values represent one experiment.

roots were compared those without ABA, but PE was the phospholipid displaying a decrease (Figure 12B, left panel). In the absence of ABA treatment, the PC level in *npc4-1* roots was higher than the wild type and *NPC4*-OE. Under control and ABA-supplemented growth conditions, the levels of PE, PI, PG, and PS were similar in wild-type and *NPC4*-OE rosettes and roots. The levels of MGDG and DGDG tended to be higher in *npc4-1* roots than the wild type and *NPC4*-OE.

DISCUSSION

NPC4 Positively Modulates Plant Response to Hyperosmotic Stresses and ABA

Nonspecific PLC is a new family of phospholipases recently described in plants (Wang, 2001; Nakamura et al., 2005). The study of their function in plants has so far been limited to their role in phosphate deficiency (Nakamura et al., 2005; Gaude et al., 2008). Results from this work provide insight regarding the metabolic and physiological functions of *NPC4* and its derived DAG. Our results show that manipulations of *NPC4* alter plant response to high salinity and drought; *npc4-1* displayed hyper-

sensitivity to salt and water deficits, while OE plants were more tolerant to hyperosmotic stress and produced more biomass than did the wild type. In addition, the study indicates that the reduced tolerance of *npc4-1* plants to abiotic stress results from a positive role of *NPC4* in mediating ABA response. ABA is known to be involved in plant tolerance to abiotic stress, such as drought and salinity (Finkelstein et al., 2002). *NPC4*-KO plants showed reduced sensitivity to ABA in seed germination, seed dormancy, and root growth, whereas *NPC4*-OE was increased in ABA sensitivity. ABA levels in *npc4-1* were higher than in the wild type in seeds but similar in leaves, indicating that *NPC4* is most likely involved in ABA responses other than ABA accumulation. Additionally, stress-responsive and ABA-inducible genes, such as *RD29B*, *ABI1*, *ABI2*, *RAB18*, *ERA1*, and *OST1*, were down-regulated in *npc4-1*. The opposite effects of *NPC4* ablation and overexpression suggest that *NPC4* acts as a positive regulator of ABA and hyperosmotic stress responses in *Arabidopsis*.

NPC4 Is Involved in the Production of Basal and Stress-Induced Production of DAG

Ablation of *NPC4* decreases DAG in both leaves and roots, and the decrease occurred in plants grown two different conditions,

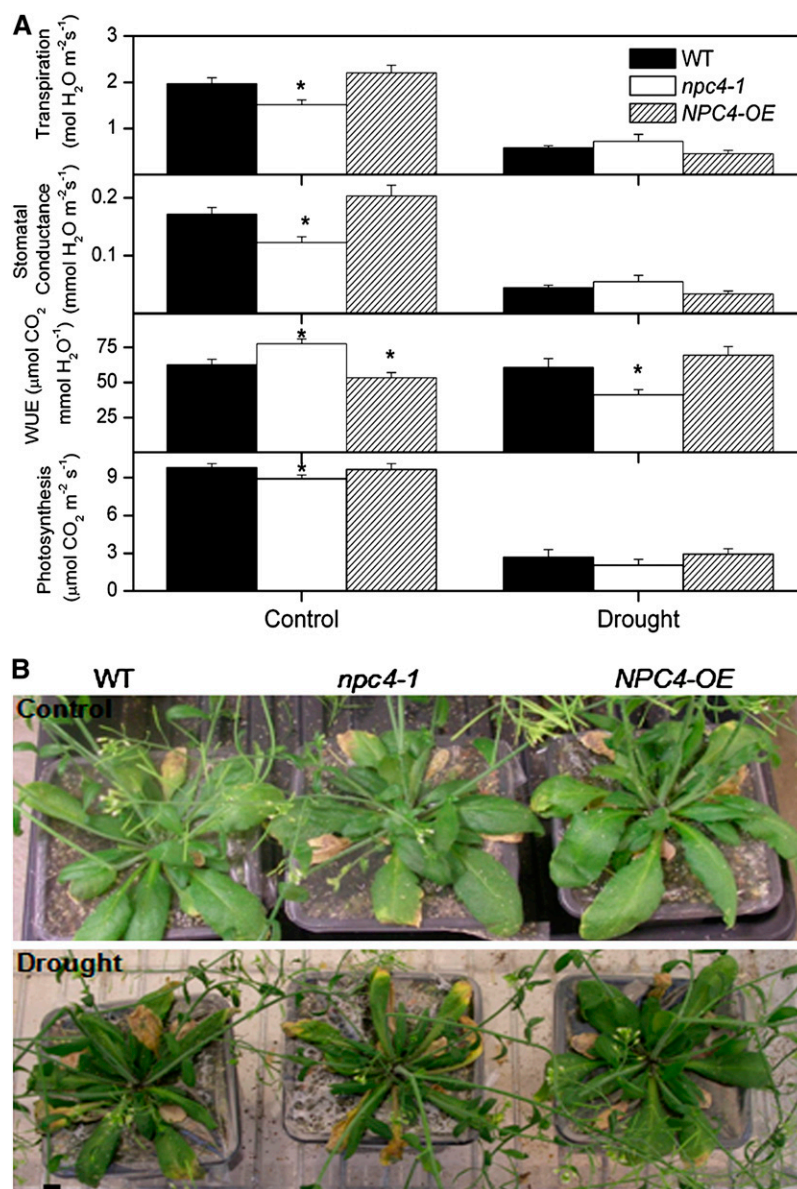


Figure 10. Effect of *npc4-1* and *NPC4-OE* on Tolerance to Water Deficiency in Soil-Grown Plants.

(A) Transpiration rate, stomatal conductance, water use efficiency (WUE) (A/g; i.e., photosynthetic rate/stomatal conductance), and photosynthetic rate of wild-type, *npc4-1*, and OE plants under control and water deficit conditions. Seven-day-old seedlings were transferred to pots containing water saturated soil. Control conditions were maintained at 100% field capacity, and water deficit conditions were maintained at 35% for a period of 10 d. Drought parameters were measured and recorded using a LI-6400 portable photosynthesis system (LI-COR) over a 10-d period. Six plants were measured per genotype. Experiments were conducted three independent times with similar results. Values are means \pm SE ($n = 24$) of one experiment. *Significant at $P < 0.05$ compared with the wild type based on Student's t test.

(B) Phenotypes of wild-type, *npc4-1*, and OE plants under control and water deficit conditions. Photographs were taken at the end of the experiments, and plants were 6 weeks old. Three independent experiments were performed with similar results, and the photos represent one experiment. Bar = 6 mm.

in leaves of soil-grown plants and in $0.5\times$ Murashige and Skoog plate-cultured rosettes under normal, unstressed growth conditions. The results suggest that NPC4 is involved in the production of a basal level of DAG in both tissues. Under stressed conditions, such as drought (see Supplemental Figure 4 online), and ABA treatments (Figure 5A), the steady level of DAG de-

creased in rosettes. The decrease may result from a decrease in the activity of de novo lipid biosynthesis, as much of the steady state DAG may be produced via de novo biosynthesis and used for the biosynthesis of PC, PE, and other glycerolipids. Separate pools may exist for DAG used for signaling and for neutral and polar lipid biosynthesis. In addition, the decrease of DAG could

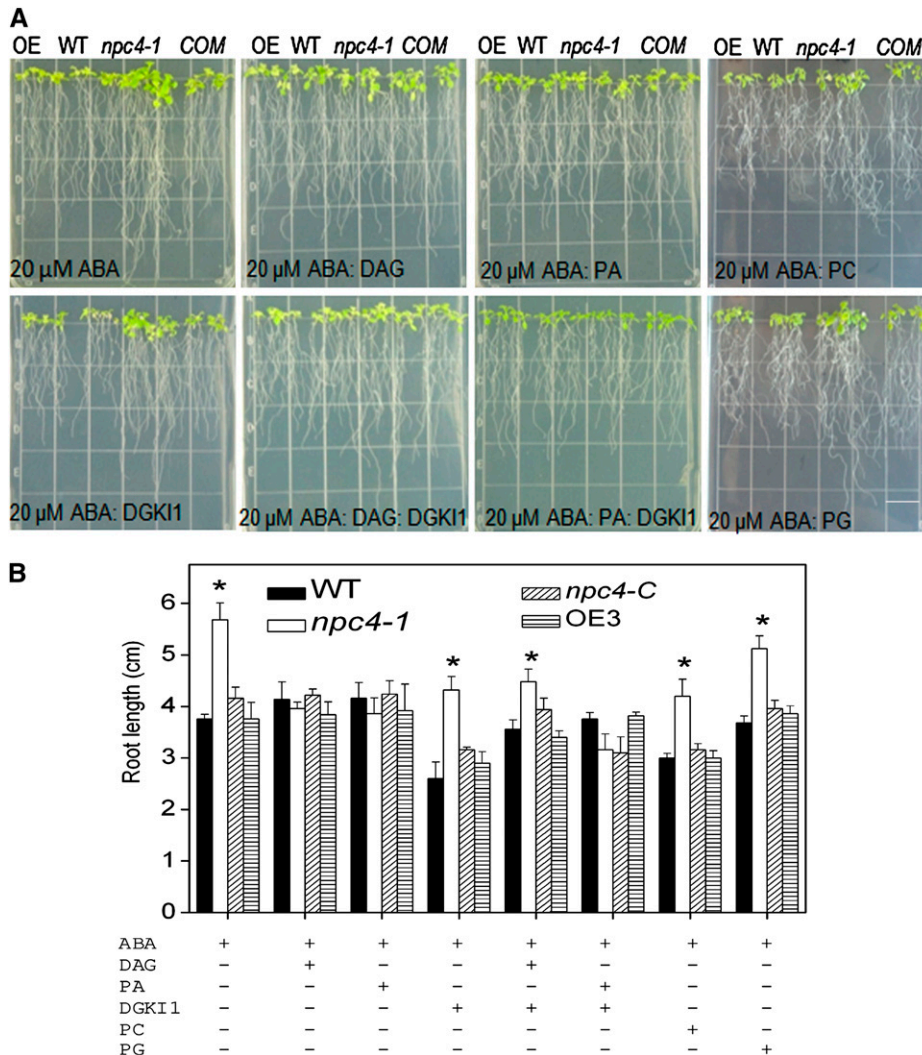


Figure 11. Addition of 8:0/8:0 DAG or 8:0/8:0 PA Restored *npc4-1* Seedlings' ABA Sensitivity to the Wild Type.

(A) Seedling morphology and root growth of *npc4-1*, OE, COM, and the wild type in the presence of 20 μ M ABA without or with 8:0/8:0 DAG, 8:0/8:0 PA, and/or DGKI1. Four-day-old seedlings were transferred to 0.5 \times Murashige and Skoog medium with or without 20 μ M 8:0/8:0 DAG, 8:0/8:0 PA, and DGKI1 and grown for 10 d. COM, the wild type complemented with *NPC4*. Bar = 1.3 cm.

(B) Root length of *NPC4*-KO, OE, COM, and the wild type in response to 20 μ M ABA in the presence of 8:0/8:0 DAG, 8:0/8:0 PA, 8:0/8:0 PG, 8:0/8:0 PC, and/or DGKI1. Values are means \pm SD ($n = 10$). *Significant at $P < 0.05$ compared with the wild type based on Student's t test. Experiments were performed at least three times with similar results, and the values presented represent one experiment. COM, the wild type complemented with *NPC4*. Lipid abbreviations are described in the legend to Figure 5. DAGKI1, DAG kinase inhibitor 1.

result from an increase in DGKs. On the other hand, DAG in roots increased in both the wild type and *npc4-1* in response to ABA, indicating that the production and regulation of DAG differs in roots and rosettes. Consistent with this notion, overexpression of *NPC4* increases the DAG level in leaves but not roots. This might be due to the higher level of expression of *NPC4* in leaves than roots and/or quick conversion of DAG to PA in *NPC4*-OE roots.

It is worth noting that very little is known about the steady state cellular level of DAG and its molecular species in plants. This analysis by ESI-MS/MS allows for measurement of DAG species

and comparative analysis of DAG in different plant samples. DAG levels are expressed as relative mass spectral signal where a value of 1 equals the signal for 1 nmol of a synthetic 15:0/15:0 DAG added as an internal standard. This mass spectral signal comparison is necessary because variation in ionization and fragmentation efficiency among different acyl glycerol species makes it impossible to use the MS responses of the plant and internal standard DAG species to determine DAG amount without knowing the mass spectral response factors for each DAG molecular species, and these response factors have not been determined for plant DAGs (Han and Gross, 2001).

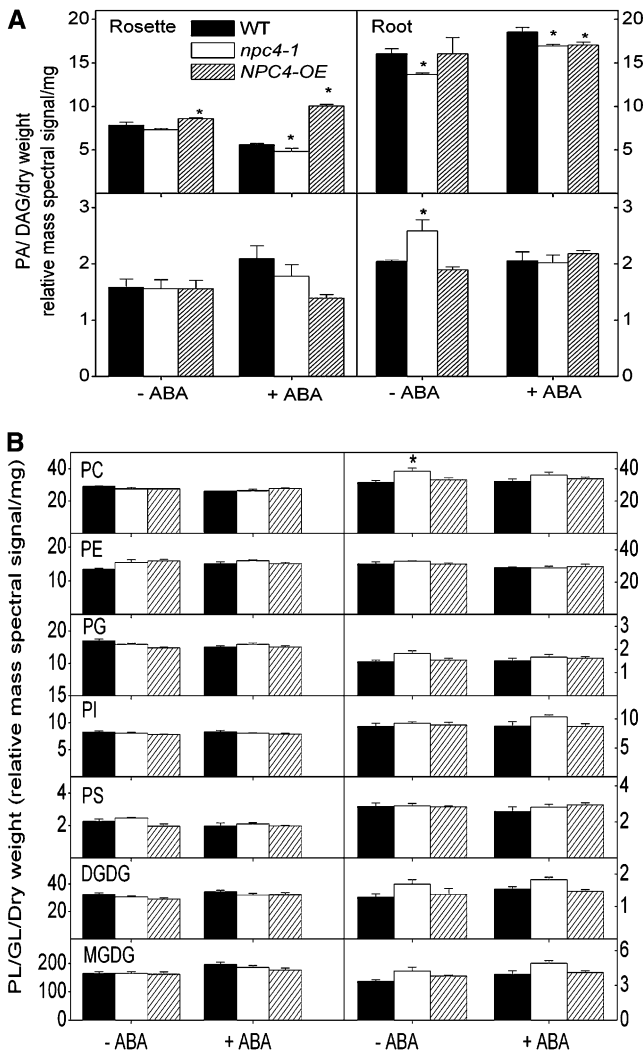


Figure 12. Glycerolipid Contents in Roots and Rosettes of Wild-Type, *npc4-1*, and OE Seedlings in Response to ABA.

(A) DAG and PA changes in the wild type, *npc4-1*, and OE in response to ABA. Five-day-old seedlings were transferred to 0.5× Murashige and Skoog vertical plates and grown for 10 d. Seedlings treated with ABA and infused with 50 μM ABA, and controls were infused with the carrier solution for 1 h. Total lipids were extracted and profiled using tandem ESI-MS/MS. Values are means ± SE ($n = 3$ to 5). *Significant at $P < 0.05$ compared with the wild type based on Student's t test.

(B) Phospholipid and galactolipid changes in response to ABA in the wild type, *npc4-1*, and OE. Plant growth conditions and treatment are same as described in **(A)**. Values are means ± SE ($n = 4$ or 5). *Significant at $P < 0.05$ compared with the wild type based on Student's t test. Lipid abbreviations are described in the legend to Figure 5.

NPC4 and DAG Promote Stomatal Opening under Well-Watered Conditions

The results suggest that the production of DAG is one route by which NPC4 mediates ABA and hyperosmotic stress response. DAG may function directly as a mediator and/or phosphorylated by DGK to PA. DAG in itself is a potent messenger in animal cells,

but its role in signaling in plants is unknown. A previous study using *Vicia faba* and synthetic DAG indicated that DAG promoted stomatal opening by activating an ion pump in the guard cell plasma membrane and inhibits stomatal closure by possibly inhibiting K^+ efflux (Lee and Assmann, 1991). This study shows that under well-watered control conditions, *npc4-1* plants had lower stomatal conductance and transpiration rates than the wild type, whereas *NPC4-OE* tended to have higher stomatal conductance than the wild type. These differences are associated with the opposite changes in DAG levels in the *NPC4-KO* and OE leaves. The stomata density of the *npc4-1* leaves was not altered, and detached leaves from well-watered *npc4-1* plants also lost less water than the wild type (see Supplemental Figure 5A online). Adding DAG to the closed stomata promoted stomatal opening more for *npc4-1* than the wild type (see Supplemental Figure 5B online). The results suggest that *npc4-1* leaves have a smaller stomatal aperture than those of the wild type and provide genetic evidence that NPC4-produced DAG is involved in promoting stomatal opening under well-watered conditions (Figure 13).

Under drought stress, however, *npc4-1* plants tended to lose more water and had lower water use efficiency than the wild type. The ratios of stomatal conductance in the well-watered plant to those under drought conditions were 3.3 for the wild type, 2.0 for *npc4-1*, and 4.5 for *NPC4-OE* (Figure 10A). Thus, in response to drought, the OE and wild-type plants displayed greater decreases than *npc4-1* in stomatal conductance to reduce transpirational water loss. This difference indicates that stomata in *NPC4*-ablated leaves are less responsive to water deficits than those in the wild type. Drought is known to increase ABA production, and the stomatal behavior in *npc4-1* epidermal peels suggests that NPC4 is involved in stomatal movements in response to ABA. These results support the conclusion that NPC4 renders plants more responsive to ABA and water availability in plants coping with hyperosmotic stress. Under water deficits and high levels of ABA, NPC4-derived DAG may be

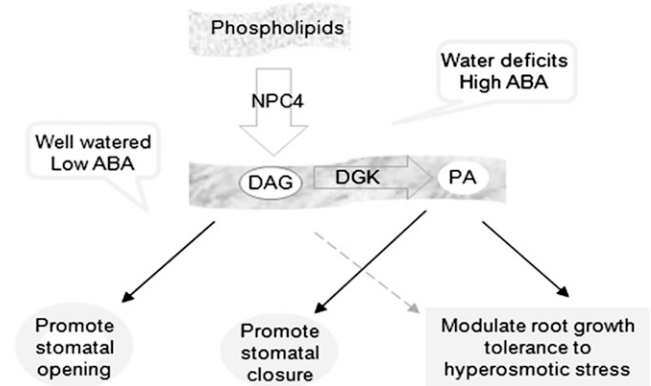


Figure 13. A Working Model for the Function of NPC4 and Derived DAG in Mediating Plant Response to ABA and Hyperosmotic Stress.

In well-watered plants, NPC4 contributes to the basal production of DAG that promotes stomatal opening. Water deficits increase the level of ABA, which stimulates NPC4 to produce DAG. The stress-induced DAG is phosphorylated by DGK to PA that promotes ABA-promoted stomatal closure and root response to ABA and hyperosmotic stress.

converted to PA that has been shown to promote stomatal closure (Zhang et al., 2004; Figure 13).

DAG Is Converted to PA That Mediates the NPC4 Effect on Hyperosmotic Response

The results from root growth indicate that NPC4-produced DAG is phosphorylated to PA that mediates the NPC4 effect on plant response to ABA and hyperosmotic stresses. Evidence for this proposition is that both DAG and PA restore the *npc4-1* response to ABA to the wild-type response, whereas addition of the DGK inhibitor that blocks the DAG to PA conversion prevents response restoration by DAG. In plants, DAG has been shown to be phosphorylated to PA under hyperosmotic stress conditions (Arisz et al., 2009; Darwish et al., 2009; Munnik and Testerink, 2009). PI-PLC has been thought to be the enzyme activated to produce DAG, but the enzymatic step for DAG production was not genetically established. NPC4 is associated with the plasma membrane (Nakamura et al., 2005), and these analyses show that its expression and activity are induced by ABA and hyperosmotic stresses (<http://bar.utoronto.ca>). The results suggest that NPC4 contributes to the production of DAG used for PA formation. The PA content in well-watered *npc4-1* roots was higher than in wild-type roots. NPC4 had PA-hydrolyzing activity, and the ablation of NPC4 might attenuate PA degradation in the knockout leaves. However, the reason for the increase requires further investigation because the PA-hydrolyzing activity of NPC4 was much lower than that of PC hydrolysis under these assay conditions. In addition, the steady state level of PA in plants can be influenced by various enzymes, including multiple forms of PLDs, DGKs, PA phosphatases, and other NPCs, as well as enzymes in de novo PA biosynthesis (Wang et al., 2006). The PA-hydrolyzing activity of NPC4 was not detected using NPC4 expressed in *E. coli* previously (Nakamura et al., 2005). Our study shows that using purified NPC4 is needed for the detection of NPC4 PA hydrolyzing activity since crude *E. coli* extracts harbor phosphatidylglycerolphosphate phosphatase activity that dephosphorylates PA (Icho and Raetz, 1983; Icho, 1988).

In summary, opposite physiological performances exhibited by NPC4-KO and OE plants indicate that NPC4 is a positive mediator in the plant response to ABA and hyperosmotic stress. Moreover, the results provide new insight into the roles of NPC4 and derived DAG in regulating stomatal movement and water loss in plants with varied water status. We propose that under well-watered conditions, the NPC4 and its derived DAG are involved in promoting stomatal opening (Figure 13). Under stress, however, DAG produced by NPC4 is converted to PA, and the NPC4/DGK pathway promotes root and plant growth in response to ABA and water deficit. These results provide an impetus for further investigation to determine the function of the new PLC family and the signaling function of DAG in plants.

METHODS

Plant Growth and Treatments

Seeds of NPC4-KO and the wild type of *Arabidopsis thaliana* (Columbia ecotype) were sown in potting soil (Fafard; superfine germinating mix

containing Canadian sphagnum peat moss, perlite, vermiculite, wetting agent, starter nutrients, and dolomitic limestone) and kept at 4°C for 2 d. Plants were grown in a growth chamber with cool white fluorescent light of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under 12-h-light/12-h-dark and 23°C/22°C cycles. Four- to six-week-old plants were used to prepare epidermal peels. Seed germination was performed on 0.5 \times Murashige and Skoog medium supplemented with 1.5% sucrose. Seeds were sterilized in 70% ethanol for 5 min followed by 20% bleach for 3 min and then rinsed three times with sterile, distilled water. Sterilized seeds were sown on medium containing 0 to 10 μM ABA and stratified at 4°C for 48 h. Germination was scored at 2-d intervals for 10 d. For precocious germination, fresh seeds of the wild type, NPC4-KO, and NPC4-OE were collected directly from yellow siliques on plants at the same time from the plants grown under the same conditions. Seeds were placed on moistened Whatman filter paper for germination, and germination percentage was calculated after 7 d of plating the seeds. For root elongation assays, 5-d-old seedlings were transferred to 0 to 50 μM ABA and grown under cool white fluorescent light of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under 16-h-light/8-h-dark and 23°C/22°C cycles for 2 weeks. For rescue experiments, 8:0/8:0 DAG, PC, PG, and PA (Avanti Polar Lipids) were prepared by drying the chloroform solvent under a stream of nitrogen gas and vortexing the dried lipids in water followed by sonication. DGK1 (R59022, 6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl] ethyl]-7-methyl-5H-thiazolo-[3,2-a]-pyrimidin-5-one) (Calbiochem) was dissolved in DMSO as described by Gomez-Merino et al. (2005). These solutions were added to the surface of 20 μM ABA agar plates (200 μL of 100 μM). For NaCl (0 to 150 mM) and PEG (−0.25 and −0.45 MPa) experiments, 5-d-old seedlings were transferred and grown under cool white fluorescent light of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under 12-h-light/12-h-dark and 23°C/22°C cycles for 10 d. PEG plates were prepared as described by Verslues and Bray (2004). Briefly, 1.5% agar plates containing 0.5 \times Murashige and Skoog medium were solidified and then were overlaid with a solution containing 6 mM MES with 0 or 170 g/L PEG (molecular weight, 8000; Sigma-Aldrich), which gave rise to an MPa of −0.25 and −0.45, respectively. The solution was allowed to sit for 24 h, and excess solution was removed from the plates. Control plates were 1% agar plates containing 0.5 \times Murashige and Skoog medium.

A gravimetric approach was adapted to create water limitation to study the effect of NPC4 alteration on stomatal regulation (Sheshshayee et al., 2005). *Arabidopsis* seeds were sown in potting soil and stratified for 2 d and then grown for 10 d under 12 h light/12 h dark. The seedlings were transplanted to pots containing soil saturated with a known weight of water (soil saturation was achieved by adding 5 times the weight of the soil in water). The pots were covered with thick polyethylene sheets to prevent evaporation. The first set of plants was maintained at 100% field water capacity (FC), and the second set was gradually brought down to 35% FC (drought stress). The pots were weighed daily during the experimental period, and the difference in weight in subsequent days was corrected by adding water to maintain the required FC. Stomatal conductance (gs) and other gas exchange-related parameters were measured on fully expanded leaf using a portable gas exchange system (LICOR6400-XT; LICOR Biosciences). Measurements were taken on the first 4 d following the onset of drought stress.

Knockout Mutant Isolation and Complementation

T-DNA insertion mutants in NPC1, NPC2, NPC3, NPC4, NPC5, and NPC6, designated *npc1-1-npc6-1*, were identified from the Salk T-DNA knockout collection. Seeds were obtained from the ABRC. The NPC-KO homozygous T-DNA insertion mutants were isolated by PCR screening using NPC gene-specific primers (see Supplemental Table 1 online) and a T-DNA left border primer: 5'-TGGTTCACGTAGTGGCCATC-3'. A single T-DNA insert was verified by cosegregation of the mutant allele with kanamycin resistance in a 3:1 ratio. The loss of transcription of NPC4 was

confirmed using *NPC4*-specific primers 5'-AGCATCAAATGCTGCTGCTCAACC-3' (forward) and 5'-TCCACCCACACACAAGAGAAGTGA-3' (reverse) by real-time PCR as described below. The real-time PCR cycling conditions comprised 90-s denaturation at 95°C and 50 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with the final cycle terminated at 72°C for an additional 10 min. For complementation of the *NPC4-KO* mutant, the native *NPC4* gene including its native promoter (1085 bp upstream of the start codon) was amplified from wild-type *Arabidopsis* plants and cloned into *Asc1* sites of PEC291 vector. The primers used for amplification were 5'-ATGGCGCGCCGACAGGTTTGTGATGCTCAATG-3' (forward) and 5'-ATGGCGCGCCGCGCGACTTTGTATTCC-TATTC-3' (reverse). The construct was introduced into the JM109 strain to test for positive clones. The resulting positive clone was transformed in *Agrobacterium tumefaciens* C58 and was used to transform *npc4-1* plants by floral dip (Clough and Bent, 1998). Hygromycin-resistant T3 transgenic plants were screened for homozygous lines. Homozygous lines did not segregate when placed on hygromycin.

Real-Time PCR

Total RNA was isolated using a rapid cetyl-trimethyl-ammonium bromide method (Stewart and Via, 1993), and RNA was precipitated using 2 M LiCl at 4°C. RNA integrity was checked on 1% (w/v) agarose gel. Eight micrograms of total RNA was digested with DNaseI according to the manufacturer's instructions (Ambion). Real-time PCR was performed as described by Li et al. (2006). Three independent experiments were performed; values are means \pm SD ($n = 3$) for one representative experiment. Briefly, RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad). The efficiency of cDNA synthesis was analyzed by real-time PCR amplification of *UBQ10* (At4g05320). The level of gene expression was normalized to that of *UBQ10*. PCR was performed with the MyiQ sequence detection system (Bio-Rad) using SYBR Green to monitor double-stranded DNA synthesis. Reaction mixes contained 7.5 μ L 2xSYBR Green Master Mix reagent kit (Bio-Rad), 1.0 ng cDNA, and 200 nM of each gene-specific primer in a final volume of 15 μ L. The primers for different genes were listed in Supplemental Table 1 online. The PCR cycling conditions were as follows: one cycle of 95°C for 90 s to facilitate denaturation and 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with the final cycle terminated at 72°C for an additional 10 min.

Measurement of Stomatal Aperture

Stomatal aperture was measured using a procedure described by Zhang et al. (2004). Briefly, epidermal peels from 6-week-old plants were floated in a solution of 5 mM MES-KOH, pH 6.15, 30 mM KCl, and 1 mM CaCl₂. After incubation for 3 h in the dark to ensure the closure of stomata, 50 μ M ABA was added and incubated under cool white light (150 μ mol m⁻² s⁻¹) at room temperature for 3 h to induce stomatal opening. To test the effect of ABA on stomatal closure, epidermal peelings were incubated in 5 mM MES-KOH, pH 6.15, 30 mM KCl, and 1 mM CaCl₂ for 3 h under cool white light (150 μ mol m⁻² s⁻¹) at room temperature to ensure the opening of stomata. After incubation, 50 μ M ABA was added and the peels incubated under cool white light (150 μ mol m⁻² s⁻¹) at room temperature for 3 h. Epidermal peels were observed under a microscope; stomatal aperture was recorded using a digital camera and analyzed with IMAGE-PRO software (Media Cybernetics).

ESI-MS/MS Analysis of DAG and PA

Lipid extraction was performed as described by Welti et al. (2002). Briefly, rosettes and roots were excised from *Arabidopsis* plants and immersed immediately in 3 mL of hot isopropanol containing 0.01% butylated hydroxytoluene at 75°C. Samples were kept at 75°C for 15 min, and 1.5 mL of chloroform and 0.6 mL of water were added. Samples were placed

on a shaker for 1 h, and the solvent was transferred to a new tube. The samples were reextracted using chloroform:methanol (2:1) at least four times for 30 min each time on a shaker. The lipid extracts were combined and washed with 1 M KCl followed by washing with 1 mL of water. The solvent was evaporated under a stream of nitrogen gas and the remaining tissue was oven dried at 100°C and weighed.

An automated ESI-MS/MS approach was used for DAG and phospholipid analysis. The samples were dissolved in 1 mL of chloroform. An aliquot of 5 to 300 μ L of extract in chloroform was used. Precise amounts of internal standards, obtained and quantified as previously described (Welti et al., 2002), were added. Phospholipid and galactolipid amounts were as described by Xiao et al. (2010), and 0.93 nmol 15:0/15:0 DAG was added. The sample and internal standard mixture were combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.4 mL.

Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole mass spectrometer (4000 Q-TRAP; Applied Biosystems). Samples were introduced using an autosampler (LC Mini PAL; CTC Analytics) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 μ L/min. Phospholipid and galactolipid scans and scan parameters were described by Xiao et al. (2010). DAG was detected by a series of neutral loss scans that detected DAG species as $[M + NH_4]^+$ ions. The scans targeted losses of various fatty acids as neutral ammoniated fragments: NL 259.2 (15:0, for the internal standard); NL 273.2 (16:0); NL 271.2 (16:1); NL 269.2 (16:2); NL 267.2 (16:3); NL 301.2 (18:0); NL 299.2 (18:1); NL 297.2 (18:2); NL 295.2 (18:3); NL 309.2 (18:4-O or oxophytodienoic acid); and NL 281.2 (16:4-O or dinor-oxophytodienoic acid). For DAG scans, the scan speed was 60 μ per second. The collision energy, with nitrogen in the collision cell, was +35 V, declustering potential was +100 V, entrance potential was +15 V, and exit potential was +12 V. Sixty of each DAG continuum scan were averaged in MCA mode.

For all analyses the collision gas pressure was set on low, and the mass analyzers were adjusted to a resolution of 0.7 μ m full width at half height. The source temperature (heated nebulizer) was 100°C, the interface heater was on, +5.5 kV was applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units). For all analyses, the background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software.

Phospholipids and galactolipids were determined by comparison of the peak for each lipid species to those of two internal standards of the same class (Welti et al., 2002; Xiao et al., 2010). Data were corrected for chemical or instrumental noise in the samples (Jao et al., 2009). The molar amount of each phospholipid species in the sample was calculated and normalized to the sample dry weights to produce data in the units nmol/mg.

For DAG analysis, data from the neutral loss scans were corrected for overlap of isotopic variants ($A + 2$ peaks) in higher mass lipids were applied. Both corrections for overlap within spectra, due to ($A + 2$ peaks) of $[M + NH_4]^+$ minus the neutral loss fragment, and overlap across spectra, due to the ($A + 2$ peaks) of the neutral loss fragments, were performed. The corrected signals corresponding to each DAG molecular species were combined, and DAG species were quantified in relation to the internal standard. Because there is variation in ionization efficiency among acyl glycerol species with different fatty acyl groups (Han and Gross, 2001), the ratio of the MS response of the plant DAG species to the MS response of the DAG internal standard does not provide a value that is directly proportional to the DAG content of each species. DAG amounts are thus expressed as relative mass spectral signal/mg dry weight, where a signal of 1.0 represents a signal equal to the signal of 1 nmol 15:0/15:0 DAG (the internal standard).

ABA Content

Seeds or leaf tissues were ground in liquid nitrogen and 0.5 mL of 1-propanol:H₂O:HCl (2:1:0.002 v/v) was added to the homogenate and vortexed well. One milliliter of dichloromethane and ABA internal standards were added. The samples were vortexed and centrifuged at 11,300g for 1 min. The lower phase was collected, and ABA was quantified by MS as described by Pan et al. (2008).

Enzyme Activity Assay of NPC4 Expressed in *Escherichia coli*

The NPC4 cDNA was amplified from 5'RACE pollen cDNA library using Phusion high fidelity Taq polymerase (New England Biolabs). The PCR product was cloned into pCR 2.1-TOPO (Invitrogen) vector to screen for a positive clone. A positive clone was selected, purified, and cloned into pET28a(+) expression vector with a His tag under the control of the T7 promoter and transformed into *E. coli* Rosetta (DE3). To induce the expression of NPC4, 0.1 mM isopropyl β-D-thiogalactopyranoside was added when the culture had reached an A₆₀₀ of ~0.4. The culture was then incubated for 36 h at 12°C. The cells were harvested by centrifugation (15,000g, 2 min) and resuspended in extraction buffer containing 50 mM Tris-HCl, pH 7.3, 50 mM NaCl, 5% glycerol, 1 mM DTT, and 0.5 mM PMSF. Cells were lysed by sonication and centrifuged (1500g, 10 min) to remove cell debris. The supernatant was assayed for PLC activity as described below.

Protein concentration in the supernatant was quantified using Bradford reagent (Bio-Rad) with BSA as a standard, according to the manufacturer's instructions. The PLC assay was performed using previously defined conditions (Nakamura et al., 2005). Briefly, the reaction mixture contained 100 μL of enzyme, 100 μL of substrate, and 300 μL of assay buffer (50 mM Tris-HCl, pH 7.3, 50 mM NaCl, and 5% glycerol). The substrate was prepared as follows: 100 μL of 10 μg/μL 18:1/18:1 PC (Avanti Polar Lipids), PE made by transphosphatidylolation of egg PC (Avanti Polar Lipids), soybean (*Glycine max*) PS (Avanti Polar Lipids), 16:0/16:0 PA (Matreya), and 18:1/18:1 PG (Avanti Polar Lipids) were dried under a stream of nitrogen gas and suspended in 1 mL of 250 mM Tris-HCl, pH 7.3, and 0.25% deoxycholate. Complete resuspension of the lipids was verified before addition to each reaction. The samples were incubated for 1 h at 37°C. The reaction was stopped by the addition of 1 mL of chloroform:methanol (1:2) or 0.75 mL ethyl acetate followed by vigorous vortexing and addition 0.75 mL of 0.45% NaCl. In reactions that were terminated with ethyl acetate, DAG was quantified by thin layer chromatography (TLC) followed by GC analysis. To measure phosphate released from the head group, EDTA was added to the aqueous phase removed from the reactions terminated using chloroform/methanol, to a final volume and concentration of 1 mL and 50 mM, respectively. Two units of calf alkaline phosphatase was added to each tube and incubated for 120 min at 37°C. Total phosphate released was quantified colorimetrically following a method described by Chen et al. (1956). For TLC, the solvent from the organic, upper phase of the ethyl acetate extract of the reaction was evaporated under a stream of nitrogen gas, and the residue was dissolved in 20 μL of chloroform/methanol (2:1, v/v) and separated on a TLC plate. The plate was developed in petroleum ether/ethyl ether/acetic acid (50:50:1, v/v/v). The DAG band was scraped from the TLC plate, 5 μL of 5.4 μM/mL 17:0 TAG was added to the samples as an internal standard, and the mixture was transmethylated in methanol containing 1% H₂SO₄ and 0.05% butylated hydroxytoluene at 90°C for 1 h. One milliliter of hexane and 1 mL of water were added, and the upper phase was removed for GC analysis. The fatty acid methyl esters were separated on a Shimadzu GC-17A gas chromatograph supplied with a hydrogen flame ionization detector and a capillary column DB-5MS (30 m; 0.25 mm i.d.) with helium carrier at 11 mL/min. The oven temperature was maintained at 170°C for 3 min and then increased linearly to 210°C (5°C min⁻¹). Fatty acid methyl esters were identified by comparison of their

retention times with known standards (37-component FAME mix; Supelco 47885-U).

Immunoblotting, Detection, and Purification of NPC4-His

Bacterial cells were harvested by centrifugation and resuspended in an extraction buffer (50 mM Tris-HCl, pH 7.3, 50 mM NaCl, 5% glycerol, 1 mM DTT, and 0.5 mM PMSF). Cells were lysed by sonication and centrifuged at 1500g for 5 min. The supernatant and total cell lysates were separated by 10% SDS-PAGE. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blotted with anti-His antibody (1:10,000) conjugated with alkaline phosphatase for 2 h. The protein bands were visualized by alkaline phosphatase reaction. For purification of NPC4-His, Ni-NTA agarose (Qiagen) was used according to the manufacturer's instructions. Briefly, 3 mL supernatant containing NPC4-His or empty vector control were added to 0.5 mL of Ni-NTA agarose and incubated for 1 h with gentle agitation at 4°C. Samples were washed four times, and Ni-NTA agarose was resuspended in 1 mL assay buffer (50 mM Tris-HCl, pH 7.3, 50 mM NaCl, and 5% glycerol). Samples were used for immunoblotting and PLC activity.

Immunoblotting and Detection of NPC4-STREP

Total proteins were extracted from 4-week-old *Arabidopsis* leaves using buffer A (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF). After centrifugation at 15,000g for 5 min, 10 μg of supernatant proteins were separated by 10% SDS-PAGE. The proteins (10 μg) were transferred to a polyvinylidene difluoride membrane, and the membrane was blotted with anti-STREP antibody (1:10,000) conjugated with horseradish peroxidase for 1 h. The membrane was washed four times for 10 min with PBS/T buffer (1× phosphate saline buffer and 0.05% Tween 20). The membrane was incubated with LumiGLO substrate for 1 min and then exposed to x-ray film.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: NPC1, Atg07230; NPC2, At2g26870; NPC3, At3g03520; NPC4, At3g03530; NPC5, At3g03540; NPC6, At3g48610; RD29B, At5g52300; RAB18, AT5g66400; UBG10, At4g05320; ABI1, At4g26080; ABI2, At5g57050; OST1, At4g33950; ERA1, At5g40280; and RCN1, At1g25490. Identification numbers for T-DNA mutants are as follows: npc1-1, SALK_027871; npc2-1, SALK_018011; npc3-1, SALK_065482; npc4-1, SALK_046713; npc5-1, SALK_045037; and npc6-1, SALK_077041.

Supplemental Data

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

Supplemental Figure 1. Root Length of *npc4-1* and Wild-Type Seedlings Grown in Media Deficient in Phosphorous, Potassium, or Nitrogen or Containing IAA.

Supplemental Figure 2. Phenotype of Six NPC Knockout Mutants and Wild-Type Seedlings in Response to ABA.

Supplemental Figure 3. Gene Expression of the NPCs in Root Cells and Guard Cells and Their Response to ABA.

Supplemental Figure 4. Drought Effect on DAG Content in the Wild Type and *npc4-1*.

Supplemental Figure 5. Water Loss from Detached Leaves and DAG and PG Effect on Stomatal Opening.

Supplemental Table 1. Primers for Real-Time PCR.**Supplemental References.****ACKNOWLEDGMENTS**

We thank Xiangqing Pan for hormone analysis, Sung Chul Bahn for help with gene cloning, Tara Wood for help on knockout mutant isolation, and Shivakumar Devaiah for help on water loss experiments, ABA-mediated stomatal movement, and knockout mutant isolation. The work was supported by grants from the USDA (2007-35318-18393) and the National Science Foundation (IOS-0818740) to X.W. Instrument acquisition and method development at the Kansas Lipidomics Research Center was supported by the National Science Foundation (EPS 0236913, MCB 0455318, and DBI 0521587), Kansas Technology Enterprise Corporation, K-IDeA Networks of Biomedical Research Excellence (INBRE) of the National Institutes of Health (P20RR16475), and Kansas State University.

Received September 29, 2009; revised June 18, 2010; accepted July 22, 2010; published August 10, 2010.

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