

A Nuclear Factor Regulates Abscisic Acid Responses in Arabidopsis^{1[W][OA]}

Min Jung Kim, Ryoung Shin², and Daniel P. Schachtman^{3*}

Donald Danforth Plant Science Center, St. Louis, Missouri 63132

Abscisic acid (ABA) is a plant hormone that regulates plant growth as well as stress responses. In this study, we identified and characterized a new Arabidopsis (*Arabidopsis thaliana*) protein, Nuclear Protein X1 (NPX1), which was up-regulated by stress and treatment with exogenous ABA. Stomatal closure, seed germination, and primary root growth are well-known ABA responses that were less sensitive to ABA in *NPX1*-overexpressing plants. *NPX1*-overexpressing plants were more drought sensitive, and the changes in response to drought were due to the altered guard cell sensitivity to ABA in transgenic plants and not to a lack of ABA production. The nuclear localization of NPX1 correlated with changes in the expression of genes involved in ABA biosynthesis and ABA signal transduction. To understand the function of NPX1, we searched for interacting proteins and found that an ABA-inducible NAC transcription factor, TIP, interacted with NPX1. Based on the whole plant phenotypes, we hypothesized that NPX1 acts as a transcriptional repressor, and this was demonstrated in yeast, where we showed that TIP was repressed by NPX1. Our results indicate that the previously unknown protein NPX1 acts as a negative regulator in plant response to changes in environmental conditions through the control of ABA-regulated gene expression. The characterization of this factor enhances our understanding of guard cell function and the mechanisms that plants use to modulate water loss from leaves under drought conditions.

In plant and animal genomes, there is a relatively high percentage of unknown genes that currently lack defined motifs or domains (Gollery et al., 2006). Most of the differences in the composition of genes between species have been attributed to proteins with obscure features (POFs). Arabidopsis (*Arabidopsis thaliana*) contains 5,069 POFs, and 2,045 have no obvious homologs in rice (*Oryza sativa*) or poplar (*Populus* species). These POFs have been suggested to be involved in species- or phylogenesis-specific functions in Arabidopsis. Therefore, the study of unknown genes or POFs is an important task that should provide information that will enhance our understanding of what makes plant species unique (Gollery et al., 2007). A couple of approaches have been suggested for defining the functions of proteins of unknown function. Genome-wide clustering and the analysis of gene function in clusters allowed for the association of 1,541 proteins of

unknown function with coexpressed genes for proteins of known function (Horan et al., 2008). Gene expression analysis was also used to identify a comprehensive set of abiotic stress-response genes (Horan et al., 2008). Coexpression and genome-wide transcript profiling data represent an important resource for guiding future functional characterization experiments of proteins or genes of unknown function. In this study, we were able to develop hypotheses regarding potential gene function based on available expression data in the AtGenExpress database (Arabidopsis eFP Browser; Winter et al., 2007).

Plants grow in environments where they encounter dynamic stresses such as cold, drought, and variations in nutrient concentrations. Molecular and cellular responses to abiotic stresses are becoming understood, which will allow for the engineering of how plants respond to such conditions (Zhu, 2002; Shinozaki and Yamaguchi-Shinozaki, 2007). The early events of plant adaptation to environmental stresses trigger specific and more general stress signal transduction networks, leading to specific changes in gene expression. The up-regulation of expression of several stress-regulated genes results in improved tolerance to drought, salt, and cold (Kasuga et al., 1999; Saijo et al., 2000).

The importance of potassium for plant growth and development is well documented. It plays a role in a wide range of functions in plants, including photosynthesis, enzyme activation, protein synthesis, osmotic adjustment (Marschner, 1995), and transpiration (Schroeder et al., 2001a). The protein of unknown function that we characterized in this work was identified as being regulated by potassium availability. Potassium is also known to be important under

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² Present address: RIKEN Plant Science Center, Yokohama, Kanagawa 203-0045, Japan.

³ Present address: Monsanto Company, St. Louis, MO 63017.

* Corresponding author; e-mail dschachtman@danforthcenter.org.

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drought conditions. Stomatal closure occurs following K^+ and anion efflux, resulting in the loss of water from the cell and leading to a reduction in cell turgor and pore closure (Schroeder et al., 2001b). Reducing water loss through stomatal closure is one strategy to minimize the adverse effects of drought (Riera et al., 2005); therefore, K^+ homeostasis may be considered as a key factor in drought adaptation.

The phytohormone abscisic acid (ABA) is also a key factor in regulating developmental and physiological processes in plants, including seed dormancy and germination and seedling growth, as well as in controlling responses to many abiotic stresses such as drought (Schroeder et al., 2001b; Assmann, 2003; Yamaguchi-Shinozaki and Shinozaki, 2006). Under drought conditions, the ABA concentrations in leaves increase and this increased ABA acts as a signal (Christmann et al., 2007; Schachtman and Goodger, 2008). The production of ABA in roots and its transport to the leaves also provides the plant with a mechanism for transmitting a signal to report on the water status of the soil. ABA is also required to maintain root growth in addition to its role in reducing transpiration by triggering stomatal closure under water deficit (Schachtman and Goodger, 2008). Additionally, ABA may act as a signal under reduced nutrient supply (Peuke et al., 1994, 2002). Although increased ABA in grains from K^+ -deficient wheat (*Triticum aestivum*) plants and increased ABA concentrations in K^+ -deficient wheat flag leaves (Haeder and Beringer, 1981) have been measured, the physiological consequences of this increase in ABA are unknown.

To identify the initial cellular responses to K^+ deprivation, we performed microarray experiments on Arabidopsis roots (Shin and Schachtman, 2004). From these experiments, the expression of the unknown gene, *Nuclear Protein X1* (*NPX1*), was found to be up-

regulated by potassium deprivation. In this study, we show that this protein of unknown function in Arabidopsis is a component in the ABA signaling pathway. We present evidence that *NPX1* acts on the ABA signal transduction pathway by repressing at least one transcription factor, which results in multiple, well-known ABA-associated phenotypes, including increased drought tolerance.

RESULTS

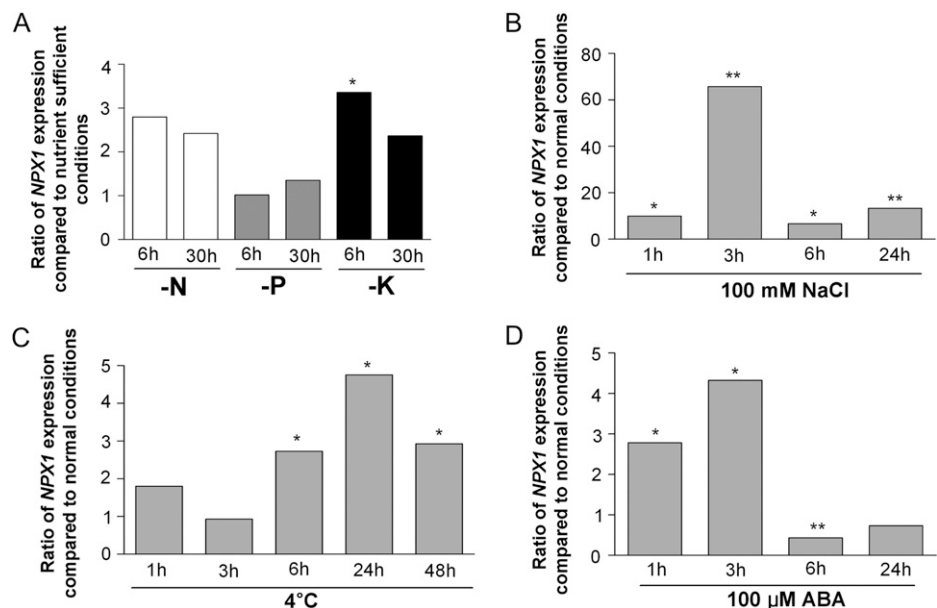
Expression of *NPX1* Was Enhanced under Stress Conditions

Previously, we found that the unknown Arabidopsis nuclear factor *NPX1* was up-regulated in roots under K^+ deprivation. To determine whether *NPX1* is regulated by other nutrient stresses, real-time PCR was performed on root RNA isolated from plants that had been deprived of potassium, nitrogen, or phosphorus. The abundance of the *NPX1* transcript increased significantly in roots starved of K^+ for 6 h (Fig. 1A). *NPX1* was widely expressed in different tissues (Supplemental Fig. S1) and was also found to be regulated by abiotic stresses, as determined by the AtGenExpress database (Arabidopsis eFP Browser; Winter et al., 2007). We verified that the expression of *NPX1* increased in response to salt stress (Fig. 1B), increased under cold stress (Fig. 1C), and responded to ABA treatment (Fig. 1D). The *NPX1* gene expression patterns suggested that *NPX1* may be involved in ABA-dependent abiotic stress signaling.

Overexpression of *NPX1* Causes ABA Insensitivity

To further elucidate the function of *NPX1*, a homozygous T-DNA inactivation line, *npX1-1* (SAIL_123),

Figure 1. The expression of *NPX1* was up-regulated by nutrient deprivation and abiotic stresses. Quantitative real-time PCR analysis of *NPX1* under nutrient-deprived and abiotic stress conditions and ABA treatment is shown. A, Arabidopsis ecotype Columbia roots grown under nutrient-sufficient conditions, K^+ deficiency ($-K$), N deficiency ($-N$), and P deficiency ($-P$) 6 or 30 h after incubation. B, Expression of *NPX1* in the whole seedlings after treatment with 100 mM NaCl for 1, 3, 6, and 24 h. C, Expression of *NPX1* in the whole seedlings after cold stress (4°C) for 1, 3, 6, 24, and 48 h. D, Expression of *NPX1* in the whole seedlings after 100 μM ABA treatment for 1, 3, 6, and 24 h. Data represent means of three biological replicates. Asterisks indicate significant differences ($* P < 0.05$, $** P < 0.01$, by Student's *t* test).



was isolated, and *NPX1*-overexpressing (*NPX1-ox*) lines under the control of the figwort mosaic virus promoter were created (for expression data on these lines, see Supplemental Fig S2). One *NPX1-ox* line with the highest expression level was chosen for further analysis. Multiple transgenic lines containing a 35S promoter-driven *NPX1* cDNA with a yellow fluorescent protein at the N terminus (*NYFP-NPX1-ox*) showed the same phenotypes described below (Supplemental Fig. S2). The growth of *npx1-1* was tested multiple times, and we found no significant differences in growth as compared with the wild type under control or K⁺-deprived conditions.

To elucidate the role of *NPX1* in ABA signaling, we tested whether the disruption or overexpression of *NPX1* affected ABA responses. Since ABA signaling is known to be an important component in seed germination, we determined the ABA sensitivity of germination (Fig. 2A). In the absence of exogenous ABA, *NPX1-ox* and *npx1-1* mutant seeds germinated as well as wild-type seeds (Fig. 2A); in the presence of 0.3 and 0.5 μM ABA, *NPX1-ox* seed germination was less sensitive to ABA, whereas *npx1-1* seed germination was more sensitive. *NYFP-NPX1-ox* seed germination was also less sensitive to ABA treatment (Supplemental Fig. S2B). After germination, ABA may still regulate seedling growth; therefore, we analyzed this aspect of ABA sensitivity by transferring 6-d-old seedlings on 0.25 \times Murashige and Skoog (MS) medium with sufficient potassium to plates containing 0, 3, 10, 20, and 50 μM ABA (Kuhn et al., 2006). The primary root of *NPX1-ox* and two *NYFP-NPX1-ox* lines was longer than the wild type under control conditions and also on medium containing 3 to 50 μM ABA (Fig. 2B; Supplemental Fig. S2C), whereas the primary root growth of *npx1-1* was more sensitive to ABA. The complemented *NPX1*-transformed *npx1-1* plants (*npx1-1-NPX1*) showed the same ABA sensitivity as the wild type in germination and root growth (Supplemental Fig. S2). When grown under K⁺-deprived conditions, the primary root length of *NPX1-ox* was significantly longer as compared with the wild type (Supplemental Fig. S3).

Since it is well known that both ABA and potassium are critical components in stomatal movement and response to drought, we analyzed stomatal responses to ABA in loss- and gain-of-function *NPX1* plants (Fig. 2C). Compared with the wild type, guard cells from *NPX1-ox* plants exhibited greater insensitivity to ABA-induced stomatal closure. Ten micromolar ABA (Fig. 2C) resulted in stomatal closure in wild-type plants but not in the *NPX1-ox* lines. In contrast, the disruption of *NPX1* increased stomatal sensitivity and stomatal closure responses at 1 μM . These data show that *NPX1* plays an important role in multiple processes that involve ABA signal transduction, including germination, root growth, and the regulation of stomatal aperture. Taken together, our data suggest an important role for *NPX1* as a negative regulator of ABA signaling.

Plants Overexpressing *NPX1* Show Hypersensitivity to Drought Stress

The control of water loss from the leaf surface triggered by ABA is a crucial survival mechanism for plants during drought periods. To investigate the role of *NPX1* in regulating drought tolerance and water loss, the soil of 3-week-old plants was covered with plastic and water was withheld for 2 weeks. *NPX1-ox* plants wilted more quickly than the other lines, whereas *npx1-1* remained turgid longer (Fig. 3A). Since survival depends on the rate at which water is depleted from pots, we also measured the stomatal conductance 3 and 7 d after watering was stopped and calculated the ratio of stomatal conductance to soil moisture. This provides a measure of the sensitivity of stomatal closure to soil moisture or stomatal response to soil water deficit. Three days after watering was stopped, the soil moisture levels in the pots were still relatively high (Fig. 3C), but the *npx1-1* plants had significantly lower rates of water loss (Fig. 3B). In contrast, the transpiration rate of *NPX1-ox* plants was not significantly different from the wild type (Fig. 3B). However, after 7 d, *NPX1-ox* plants showed a 1.4-fold increase in water loss compared with the wild type and *npx1-1* (Fig. 3, B and C). This result correlated with the different stomatal responses that we observed when adding ABA (Fig. 2C). From these results, we conclude that overexpression of *NPX1* reduces sensitivity to ABA, so those plants lose water faster and are more drought sensitive than *npx1-1* and the wild type.

NPX1 Is a Nuclear Protein

We identified three putative nuclear localization sequences (softberry ProtComp6.0; <http://linux1.softberry.com/berry.phtml?topic=protcompan&group=help&subgroup=proloc>) in the C-terminal region of *NPX1*, which suggested that *NPX1* could be localized to the nucleus. Therefore, we tested the cellular localization of *NPX1* fused to yellow fluorescent protein at the N terminus of *NPX1* (*NYFP-NPX1*) by transient expression in onion (*Allium cepa*) epidermal cells. Fluorescence was observed in the nucleus of cells where the *NYFP-NPX1* was expressed, whereas the *NYFP* empty vector control was mainly present in cytoplasm (Fig. 4A). To confirm the nuclear localization of *NPX1* protein, *NYFP-NPX1* was stably transformed into *Arabidopsis* and the fluorescence was measured in both leaves (Fig. 4B) and roots (Fig. 4C). Cells expressing the *NYFP-NPX1* also showed that the YFP signal appeared only in the nucleus.

NPX1 Regulates the Expression of ABA Signaling Genes

Since results suggested that *NPX1* may be involved in ABA signaling and was localized to the nucleus, we tested whether *NPX1* is involved in regulating the expression of genes important for ABA signaling in

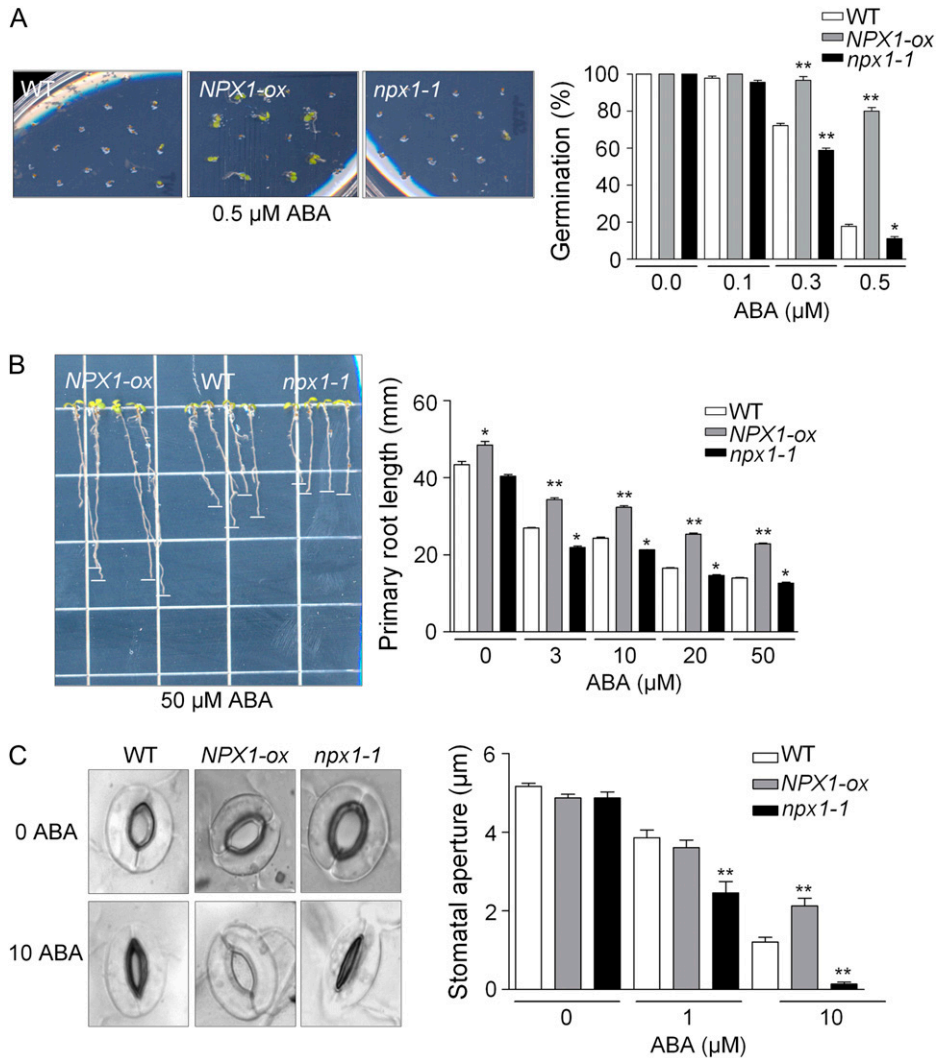


Figure 2. Constitutive expression of *NPX1* results in ABA insensitivity, while disruption of *NPX1* causes ABA hypersensitivity during seed germination, inhibition of primary root growth, and stomatal closure. **A**, Germination of wild-type (WT), *NPX1-ox*, and *npX1-1* seeds at 0.5 μM ABA after 5 d (left), and germination rates of seeds exposed to 0, 0.1, 0.3, and 0.5 μM ABA at 5 d (right). Data represent means ± SD of three independent experiments with 36 seeds per genotype and experiment. **B**, The photographs show plants of the wild type, *NPX1-ox*, and *npX1-1* treated with 0.25× MS medium supplemented with 50 μM ABA 10 d after transfer of 6-d-old seedlings from medium without ABA (left). At right is a comparison of root elongation of wild-type, *NPX1-ox*, and *npX1-1* seedlings; 6-d-old seedlings were transferred to plates supplemented with 0, 3, 10, 20, and 50 μM ABA, and root elongation was monitored after 6 d. Each data point represents the mean ± SD (*n* = 70). Asterisks indicate significant differences between the wild type and *NPX1-ox* or *npX1-1* plants (* *P* < 0.05, ** *P* < 0.01). **C**, Photographs show the stomatal apertures in ABA-induced stomata closure assays with 0 (top row) and 10 μM ABA (bottom row) after 2 h of incubation. At right are stomatal aperture measurements of wild-type, *NPX1-ox*, and *npX1-1* plants in response to 0, 1, and 10 μM ABA. Data represent means ± SD with 50 stomata per data point. Asterisks indicate significant differences between the wild type and *NPX1-ox* or *npX1-1* plants (***P* < 0.01).

the wild type, *NPX1-ox*, and *npX1-1* before and after treatment with 100 μM ABA. Our results clearly show that the expression of *ABA-Responsive Element Binding1 (AREB1)*, *RD29A*, and *ABI1* was down-regulated in *NPX1-ox* without ABA treatment (Fig. 5, A and B). In contrast, the expression of ABA biosynthesis genes *NCED3* and *ABA2* as well as *AtrbohF* was up-regulated in *NPX1-ox* without ABA treatment (Fig. 5C). In the

wild type, the expression of *MYB2*, *AtrbohD*, and *Open Stomata1 (OST1)* was up-regulated after treatment with ABA (Fig. 5). However, the induction of these genes was greatly attenuated or abolished in *NPX1-ox*. The *AtrbohF* gene was expressed at higher levels in the *NPX1-ox* lines even without the application of exogenous ABA, and the expression pattern was opposite that of *AtrbohD* (Fig. 5).

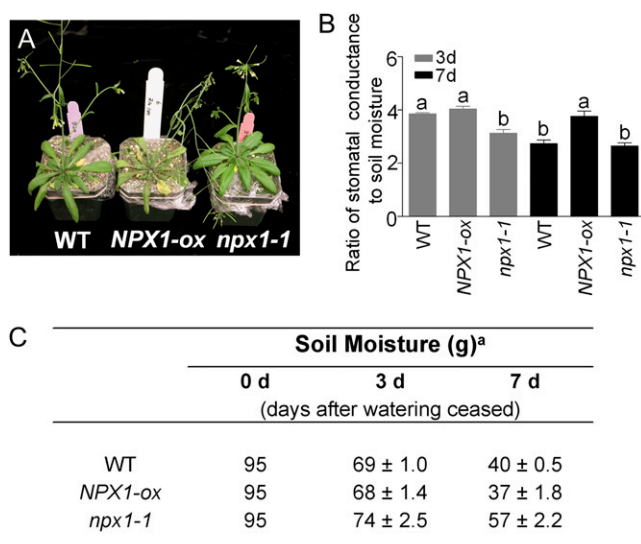


Figure 3. Plants overexpressing *NPX1* wilted more quickly under terminal drought stress. A, Photographs of plants after drought stress treatment that involves withholding water. Three-week-old plants of wild-type (WT), *NPX1-ox*, and *npx1-1* plants were grown for a period of 14 d without added water. B, Stomatal conductance and soil moisture after 3 and 7 d without added water. Different letters indicate significant differences between means at $P < 0.05$ (Tukey's honestly significant difference; $n = 20$ plants). C, Table shows the soil moisture during drought stress treatment ($n = 20$ for each group). ^aThe values are presented as means \pm SE.

NPX1 Contributes to the Regulation of ABA Biosynthesis

The fact that *NPX1* is coexpressed with the ABA biosynthesis gene *NCED3* (Ma and Bohnert, 2008) and that the expression of *NCED3* and *ABA2* is up-regulated in *NPX1-ox* without ABA treatment (Fig. 5C) suggest that *NPX1* may be involved in the regulation of biosynthesis of ABA. To investigate this possibility, we measured ABA in the wild type, *NPX1-ox*, and *npx1-1* using multiple plants in three sets of independent biological replicates. The ABA levels were significantly higher in *NPX1-ox* than in the other lines (Fig. 6A). We also measured ABA levels in leaves and roots after K^+ starvation. The level of ABA in leaves increased 6 and 30 h after deprivation, whereas the production of ABA in roots increased only 30 h after deprivation (Fig. 6B), which confirms previous results in *Ricinus communis* (Peuke et al., 2002) and helps to explain why this gene was up-regulated under potassium-deprived conditions.

NPX1 Interacts with TIP and Represses Transcription in Yeast

Because *NPX1* was localized to the nucleus and the expression of several genes involved in ABA biosynthesis and signaling was altered in *NPX1-ox* plants, we tested the transcriptional activation of *NPX1* even though it did not contain a classical DNA-binding domain (BD). For this experiment, *NPX1* was fused with GAL4 BD and transformed with GAL4 activation

domain (AD) containing empty vector. *NPX1* did not show transcriptional activation activity in yeast (data not shown). Therefore, we tested whether *NPX1* may regulate gene expression through interactions with other proteins such as transcription factors. To identify proteins that interact with *NPX1*, we used the yeast two-hybrid system with *NPX1* fused in-frame to the GAL4 BD (BD-*NPX1*). First, we checked for interactions between BD-*NPX1* and GAL4 AD fused to eight different transcription factors that were already known to be regulators of ABA signaling: AREB1, AREB2, AREB3, DREB2A, MYB2, MYC2, CBF4, and ATHB6 (Riera et al., 2005; Nilson and Assmann, 2007). However, none of these eight candidates interacted with *NPX1* (data not shown). Therefore, we screened an Arabidopsis root library fused to the GAL4 AD. Of the 1.5×10^6 transformants screened, 44 colonies initially grew on plates without Leu, Trp, and His. When these 44 clones were retransformed and subjected to colony-lift filter assay, 20 clones exhibited β -galactosidase activity. The cDNAs were recovered from these 20 colonies and sequenced (Supplemental Table S1). One of the cDNAs encoded a NAC transcriptional factor previously named *TIP* (Ren et al., 2000). The interaction between *NPX1* and *TIP* was confirmed using additional plasmid combinations and constructs to test for the following: autoactivation of the *HIS3* and *lacZ* reporter genes, possible direct interactions with the GAL4 BD or AD, and potential artifacts caused by high expression of the encoded GAL4 fusion protein. BD-*NPX1* alone did not activate the *HIS3* or *lacZ* reporter gene, whereas the cotransformation of BD-*NPX1* and AD-*TIP* activates *HIS3* or *lacZ* reporter gene (Fig. 7A). This interaction was confirmed by quantitative β -galactosidase assays using *o*-nitrophenyl- β -D-galactopyranoside as a substrate. The β -galactosidase activity of *NPX1*- and *TIP*-cotransformed cells was about 34% of that observed for the positive control (BD-Krev1 and AD-RalGDS-wt; data not shown).

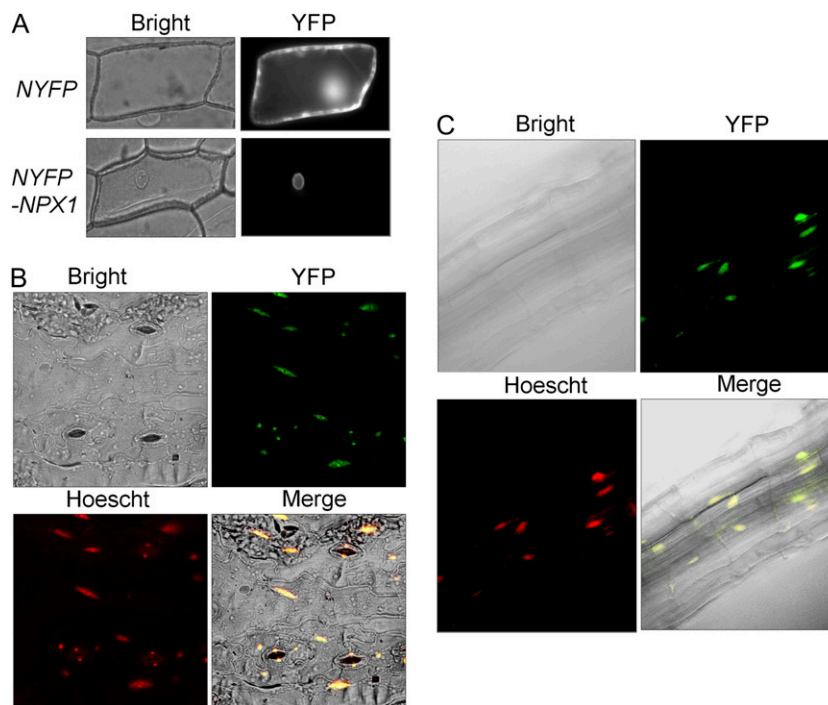
To reveal the underlying molecular mechanism of interaction between *NPX1* and *TIP*, we tested whether this direct interaction could lead to activation or repression of the reporter gene expression in yeast. Yeast containing an integrated GAL4-*lacZ* reporter was transformed with BD-*TIP* in the presence or absence of BD-*NPX1*. BD-*TIP* was previously reported to activate a *lacZ* reporter (Ren et al., 2000), and we confirmed this activation (Fig. 7B). The activity of BD-*TIP* was significantly reduced by 54% when BD-*NPX1* was present but not significantly reduced by BD-YFP, which was used as a control (Fig. 7B). Taken together, our results indicate that *NPX1* interacts with *TIP* and functions as a transcriptional repressor.

DISCUSSION

NPX1 Is a Novel Protein Involved in ABA Responses

The previously unknown gene *NPX1* was identified as a gene whose expression was up-regulated by potassium deficiency (Shin and Schachtman, 2004).

Figure 4. NYFP-NPX1 protein was localized to the nucleus of root and guard cells. A, Fluorescent proteins were transiently expressed in onion epidermal cells: NYFP (top) and NYFP-NPX1 (bottom). Signals from bright field (bright) and NYFP (YFP) are shown. B and C, NPX1 localization in stable transgenic Arabidopsis leaves (B) and roots (C). Signals from bright field (bright) and NYFP (YFP; green) and Hoescht signal (red) indicate nuclear localization, and the merge of the three signals (merge; yellow) is shown.



To gain insight into the function of the Arabidopsis *NPX1* gene, we searched databases for hormones and environmental conditions that regulated the transcription of this gene. *NPX1* was found to be up-regulated by dehydration, low temperature, and high salt and coexpressed with the ABA biosynthetic gene *NCED3* (Ma and Bohnert, 2008). The phytohormone ABA plays a central role in regulating plant responses to environmental stress, including water deficit, high salt, and low temperature (Leung and Giraudat, 1998). To confirm whether *NPX1* played a role in ABA responses, we studied how exogenously applied ABA affected germination, stomatal conductance, drought tolerance, and root growth, which are all known to be physiological responses that are influenced by ABA (Nilson and Assmann, 2007). In germination and root growth assays, we showed that the overexpression of *NPX1* decreased the sensitivity to exogenously applied ABA.

ABA plays a key role in reducing plant water loss and stimulating root growth under conditions of water deficit. When plants are drought stressed, ABA concentrations in leaves increase, which leads to stomatal closure ultimately controlling whole plant water loss (Nilson and Assmann, 2007). Guard cells, which make up each stomatal complex, shrink, which results in closure and swell, which results in opening. Stomatal apertures in response to ABA in isolated epidermal peels containing guard cells were compared between wild-type, overexpression, and knockout lines of *NPX1*. Those studies clearly showed that isolated guard cells from *NPX1-ox* plants were relatively insensitive to the application of low concentrations of ABA, which would normally close guard cells in wild-

type plants (Pei et al., 1997). To demonstrate that the ABA insensitivity of guard cells was also evident in transpiring leaves on whole plants, we carefully measured soil moisture content and water loss as well as plant survival in *NPX1-ox*, wild-type, and *np1-1* lines. Soil moisture content plays a key role in stomatal response, and it is well known that transpiration declines as a result of soil drying due to increased leaf ABA concentrations (Schachtman and Goodger, 2008). At the whole plant level, we clearly showed that guard cells in plants overexpressing *NPX1* did not close as rapidly upon soil drying, so *NPX1-ox* plants survived for a shorter time period as compared with other lines under water-limiting conditions, because they depleted the soil moisture more rapidly. This phenotype is reminiscent of other mutations such as *abi1-1* and *abi2-1* (Leung et al., 1997). Taken together, *NPX1-ox* plants were less sensitive to ABA at both the cellular and whole plant levels, indicating that *NPX1* acts a negative regulator of ABA response or biosynthesis. Measurement of ABA concentrations showed that insensitivity in the *NPX1-ox* lines does not occur due to reduced ABA biosynthesis, because *NPX1-ox* plants had higher levels of ABA than wild-type plants or the *np1-1* knockout. Other molecular events, including the action of transcription factors and phosphorylation, also serve as positive and negative regulators in ABA signaling (Nilson and Assmann, 2007).

NPX1 Is a Negative Transcriptional Regulator of ABA Responses

To understand how *NPX1* could modulate ABA responses, we scanned the encoded protein for known

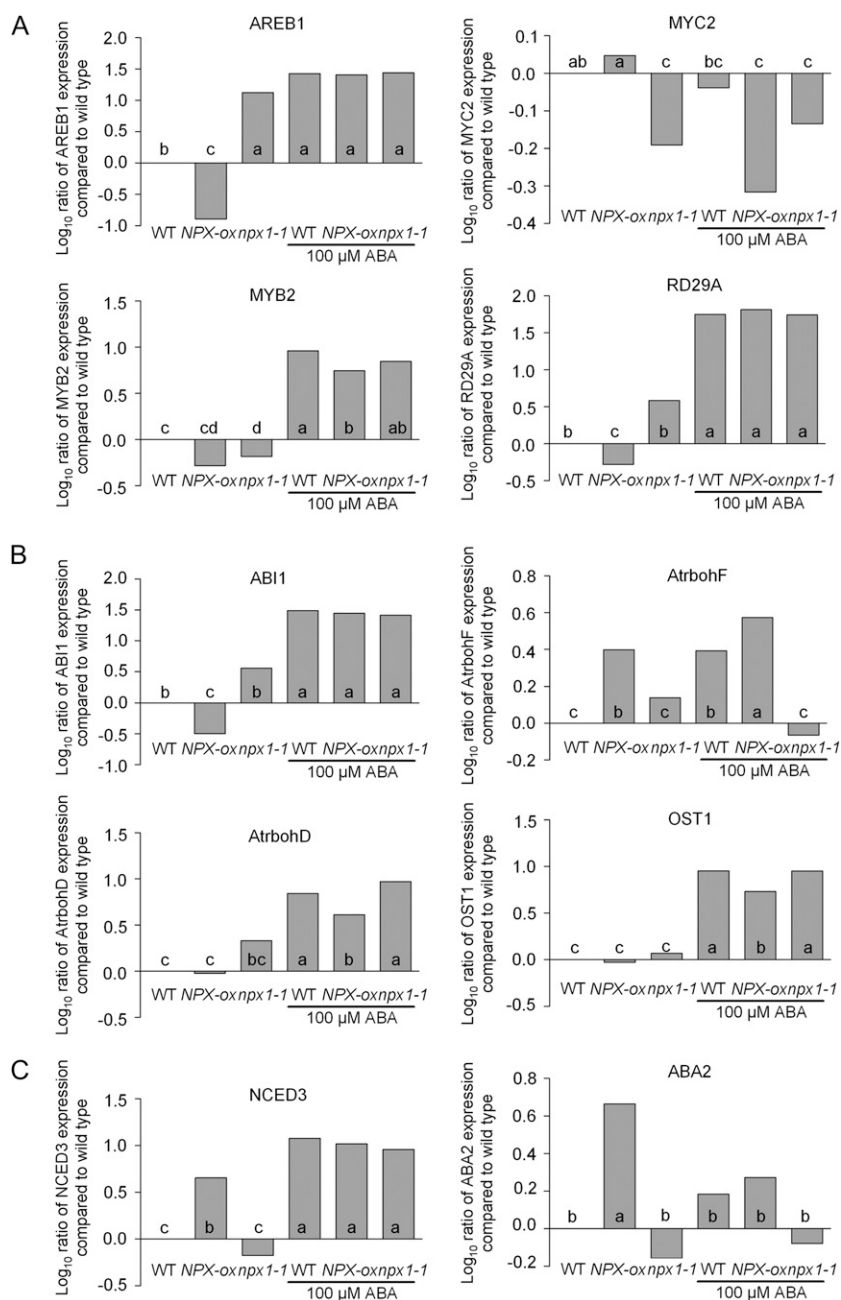


Figure 5. Constitutive expression of *NPX1* or disruption of *NPX1* alters the gene expression of ABA signaling genes. Seedlings growing on agar plates were untreated or treated with 100 μM ABA for 3 h. Gene expression before and after ABA treatment by real-time PCR analysis is shown. Real-time PCR for expression of genes involved in the ABA-dependent stress-responsive pathway (A), ABA-induced stomatal closing (B), or the ABA biosynthetic pathway (C). Different letters indicate significant differences between means at $P < 0.05$ (Student's t test). For full information on the genes represented in this figure, see Supplemental Table S2. WT, Wild type.

domains and found that *NPX1* had some limited similarity to the global transcription factor group E8 that contains a bromodomain. Although *NPX1* does not contain a functional DNA BD, we tested for transcriptional activity in yeast using β -galactosidase assays with GAL4 BD-fused *NPX1* and found that the protein does not show activity that might be associated with a transcription factor (data not shown). Localization studies showed that a GFP fusion of *NPX1* was localized to the nucleus and therefore may be involved in the regulation of transcription by binding to other proteins.

Previous studies aimed at revealing the molecular determinants of ABA signaling have highlighted reg-

ulators that act at the transcriptional and posttranscriptional levels (Riera et al., 2005; Nilson and Assmann, 2007). Since *NPX1* was localized to the nucleus and acts as a negative regulator of ABA responses, we determined whether there was a link between *NPX1* and transcription of many well-known genes involved in ABA signal transduction and biosynthesis. One SnRK2 kinase, OST1, plays a central role in ABA signaling events and the control of transpiration under abiotic stress (Mustilli et al., 2002; Yoshida et al., 2002). This kinase functions upstream of the NADPH oxidases *AtrbohD* and *AtrbohF* to activate reactive oxygen species production in guard cells and drive stomatal

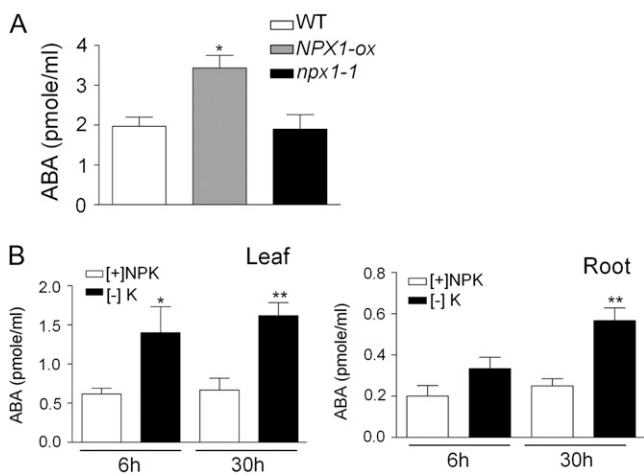


Figure 6. Constitutive expression of *NPX1* and potassium deprivation increased the biosynthesis of ABA. A, ABA concentrations in leaves of wild-type (WT), *NPX1-ox*, and *npx1-1* plants grown on agar plates. Data represent means of three biological replicates each containing multiple plants. B, Comparison of ABA concentrations in leaves or roots of plants grown under full nutrient conditions (+NPK) or K^+ deficiency (-K) for 6 or 30 h. Data represent means of six biological replicates. Asterisks indicate significant differences as compared with other means in each subfigure (* $P < 0.05$, ** $P < 0.01$, by Student's *t* test).

closure (Kwak et al., 2003). OST1 also phosphorylates proteins that contain AREB1, AREB2, and ABI5 (Furihata et al., 2006). The phosphorylation by OST1 activates transcription of these factors. In addition to changes in activity due to phosphorylation, expression studies revealed that transcript levels of *OST1*, *AREB1*, and *ABI1* are sensitive to ABA (Leonhardt et al., 2004; Fujita et al., 2005), but the upstream factors that activate OST1 are unknown. In this study, AREB1 and ABI1 expression was reduced in the *NPX1-ox* plants without added ABA, and OST1 expression was lower when ABA was added (Fig. 5). We also showed that the overexpression of *NPX1* inhibits the expression of ABA-induced *OST1* and *AREB1* (Fig. 5), which suggests that the decreased stomatal sensitivity to ABA in *NPX1-ox* might be caused in part by the repression of drought stress-regulated genes such as *OST1* and *AREB1*. Expression of *AtbohD* is also regulated by *NPX1*, but there appears to be some compensation in the form of the increased expression of *AtbohF*. We have seen this type of compensation in the expression of NADPH oxidase family members in roots of the *rhd2* mutant (Shin et al., 2005).

Other negative transcriptional regulators of ABA signaling have been identified, including ATHB6 and AtERF7 (Himmelbach et al., 2002; Song et al., 2005), which act to repress transcription through binding to DNA. Transgenic plants overexpressing *ATHB6* or *AtERF7* exhibited similar responses to *NPX1-ox*, such as increased water loss and reduced stomatal closure (Himmelbach et al., 2002; Song et al., 2005). However, these two transcriptional repressors act downstream of *NPX1* and do not appear to be direct targets

of *NPX1*. ATHB6 has been shown to target ABI1 (Himmelbach et al., 2002) and AtERF7 targets PKS3, which are Ser/Thr protein kinases in the pathway (Song et al., 2005). This is the first report, to our knowledge, of an upstream transcriptional regulator of the OST1-mediated signaling pathway.

ABA also induces the expression of stress-related genes (Shinozaki and Yamaguchi-Shinozaki, 2007) such as *RD29A*. More than half of drought-inducible genes such as *RD29A* are also induced by high salinity and/or ABA treatment, implicating significant cross talk between the drought, high salinity (Shinozaki and Yamaguchi-Shinozaki, 2007), and ABA response pathways. In our study, the ABA-inducible *NPX1* was also up-regulated by NaCl and cold stress (Fig. 1), and *NPX1-ox* plants were hypersensitive to drought stress (Fig. 3). The regulation of *RD29A* is enhanced in *npx1-1* plants without the addition of ABA, but upon addition

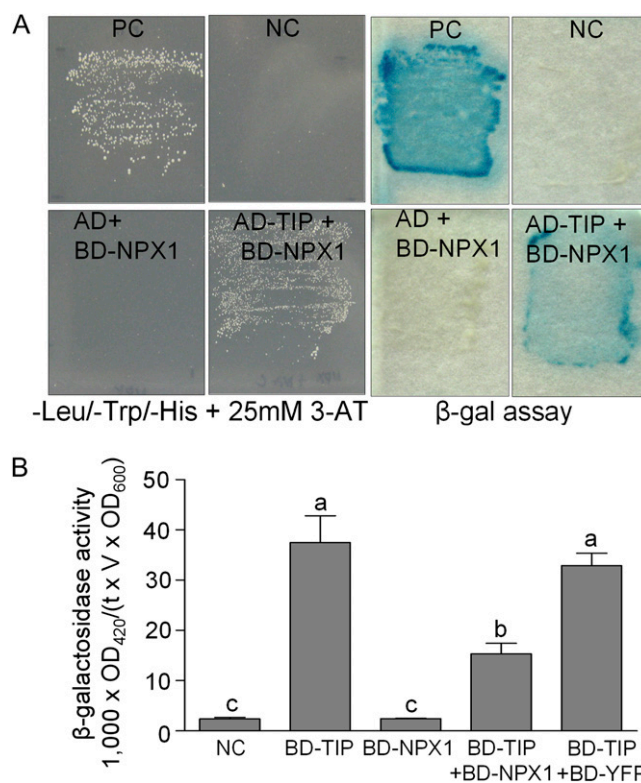


Figure 7. *NPX1* interacts with TIP and represses the transcription activity of TIP in yeast. A, Interaction analysis of *NPX1* using the yeast two-hybrid system. The yeast strain MaV203 was cotransformed as indicated. PC, Positive control (BD-Krev1 and AD-RalGDS-wt were used); NC, negative control (BD-Krev1 and AD-RalGDS-m2 were used). Activation of HIS3 and lacZ is indicated by growth on -His medium and by the blue color, respectively. White colonies indicate a lack of reporter activation. B, *NPX1* represses the activity of TIP. BD-NPX1 represses the activity of TIP in yeast, as indicated by the decreased β -galactosidase (β -gal) activity. BD-TIP but not NC (cotransformants of pGBKT7 and pDEST 32 vector) and BD-NPX1 activate lacZ expression. Data represent means \pm SD of three independent experiments. Different letters indicate significant differences between means at $P < 0.05$ (Student's *t* test). OD, Optical density.

of ABA *RD29A* expression increases in all lines tested. This suggests that either the application of exogenous ABA short circuits NPX1 effects or the effects of NPX1 are subtle and tightly regulated at endogenous levels of ABA.

The regulation of gene expression under drought stress is mediated by multiple transcriptional cascades (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). In each cascade, a set of transcription factors are induced by ABA, which in turn activates or represses downstream target genes important for drought resistance (Shinozaki and Yamaguchi-Shinozaki, 2007). From our results, we conclude that NPX1 negatively regulates ABA signaling in part by reducing the expression of ABA-inducible transcription factors such as AREB1 and MYB2 and stomata closure by regulating ABI1 and OST1 (Fig. 5). Thus, *NPX1-ox* plants are hypersensitive to drought, whereas *npx1-1* plants are more tolerant (Fig. 3). ABI1 is known as a negative regulator in stomatal closing and has been shown to be a negative regulator of OST1 (Yoshida et al., 2006). The changes in expression of *MYC2* with added ABA and the lower expression in the *npx1-1* line are also consistent with the conclusion that NPX1 modulates sensitivity to ABA through transcriptional changes (Abe et al., 2003). Therefore our data on the expression of genes involved in ABA signal transduction indicate that NPX1 defines one new component of these stress-responsive transcriptional cascades.

NPX1 Modulates ABA Signaling, Which Feeds Back to ABA Biosynthesis

NPX1-ox lines have higher ABA content, which is presumably due to the enhanced expression of ABA biosynthesis genes such as *ABA2* and *NCED3*, which are also strongly induced by drought (Iuchi et al., 2001; Figs. 5C and 6A). Even though levels of ABA are higher in *NPX1-ox*, these plants appear to be relatively less sensitive to ABA. Therefore, we suggest that NPX1 does not merely contribute to ABA signaling by increasing ABA content. Rather, we propose that the increased ABA concentrations are due to feedback that may be initially caused by insensitivity, which triggers increased ABA biosynthesis to overcome a perceived reduction in sensitivity. The idea of this type of feedback was suggested earlier (Xiong et al., 2001), and our study provides evidence that this occurs. The observation that *NPX1-ox* is less sensitive to but contains more ABA differs from other mutants such as *ahg2*, which accumulates 50% higher endogenous ABA but appears to be more hypersensitive to ABA (Nishimura et al., 2005). In contrast, the *sad1* mutant shows enhanced sensitivity to ABA but does not produce more ABA under stress conditions as compared with the wild type (Xiong et al., 2001). In the case of *sad1*, the authors suggested that ABA production was reduced in *sad1*, which resulted in enhanced sensitivity to ABA and abiotic stress. Multiple results from different groups suggest that ABA responses are complex and regu-

lated at different levels, including direct effects and feedback responses. Our results suggest that NPX1 modulates ABA signaling through effects on the signal transduction pathway, but the possibility still exists that it could act indirectly by inducing ABA biosynthesis. Thus, we conclude that NPX1 reduces ABA sensitivity through the repression of stress-inducible gene expression but activates the induction of ABA biosynthesis via positive feedback regulation.

A NAC Transcriptional Factor Is a Target of NPX1

To identify how NPX1 modulates ABA signaling, and since our hypothesis was that modulation occurred through protein-protein interactions rather than through direct binding to DNA, we employed yeast two-hybrid assays. We found that NPX1 interacted with multiple targets, including a NAC transcription factor. NACs are plant-specific transcription factors that participate in controlling plant development and are involved in pathogen attack and responses to various environmental stresses (Aida et al., 1997; Collinge and Boller, 2001; Hu et al., 2006). Several NAC transcriptional factors are induced by drought, high salinity, and ABA and regulate ABA- and stress-inducible gene expression (Hegedus et al., 2003; Fujita et al., 2004; Tran et al., 2004; Hu et al., 2006). Recently, a drought- and ABA-inducible NAC transcription factor (*RD26*) was identified, and *RD26-ox* plants were found to be hypersensitive to ABA whereas *RD26* dominant negative transgenic plants were insensitive (Fujita

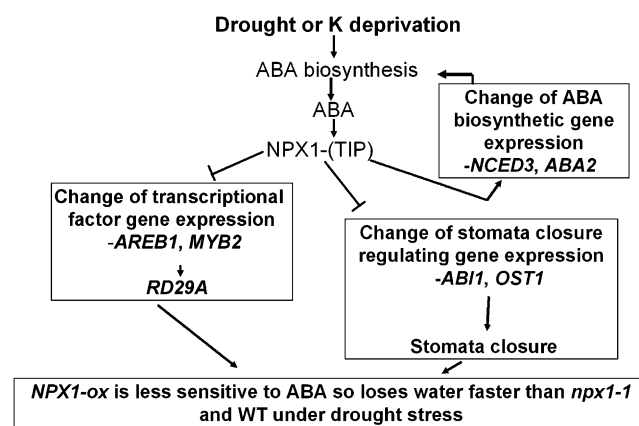


Figure 8. A model for NPX1 modulation of ABA signaling. NPX1 does not contain a classical DNA BD and does not exhibit transcriptional activity. NPX1 functions as a transcriptional regulator by interacting with factors such as TIP. This NPX1-TIP complex negatively regulates the expression of genes coding for ABA-dependent stress-responsive signaling and ABA-induced stomatal closing. The reduced expression of these genes may contribute to the reduced ABA sensitivity and faster water loss in the *NPX1-ox* plants. In addition, drought and low-potassium stresses induce ABA biosynthesis, and the increased ABA further stimulates the expression of some of the biosynthesis genes through positive feedback regulation. NPX1 may also regulate this feedback loop, thereby leading to increased ABA accumulation in *NPX1-ox* plants. WT, Wild type.

et al., 2004). In our study, one of the NAC transcription factors, previously named *TIP* (Ren et al., 2000), was isolated as an interacting protein of *NPX1*. Previously, *TIP* was shown to act as an important participant in the turnip crinkle virus resistance response through direct activation of the transcription of genes involved in basal resistance (Ren et al., 2000).

Plants have evolved a wide range of mechanisms to cope with biotic and abiotic stresses, and there is cross talk between these biotic and abiotic stress responses (Fujita et al., 2006). For example, the importance of ABA in pathogen resistance was highlighted by *OST1*'s involvement in part of the innate immunity defense against bacterial infection by regulating stomatal closure (Melotto et al., 2006), and ABA-responsive elements have also been identified in the promoters of defense-related genes (Adie et al., 2007). We found that *TIP* not only interacted with *NPX1* but was also induced under potassium-deprived conditions and by ABA and NaCl treatments (Supplemental Fig. S4). Furthermore, we identified regions in the promoters of *OST1* and *AREB1* that contained a CATGTG motif (data not shown) that have been shown to be NAC-binding motifs (Tran et al., 2004). Since *TIP* activates reporter gene expression in yeast cells (Ren et al., 2000), we used a yeast repressor assay to test whether *NPX1* may directly repress *TIP* activity. We showed that *TIP* activity was reduced when *NPX1* was expressed in the same yeast cells (Fig. 7B). *ABI1* expression was reduced in the *tip* plants without added ABA, and *OST1* expression was lower when ABA was added (Supplemental Fig. S5B). The regulation of these two genes in the *tip* knockout is as we would predict based on our results in *NPX1-ox* plants (Fig. 5).

Based on the yeast two-hybrid interaction, the fact that *NPX1* and *TIP* have overlapping expression patterns, and the repressor assay results, we suggest that *NPX1* acts as a repressor of *TIP* and perhaps other proteins and that this interaction modulates ABA responses. Although *NPX1* does not bind directly to DNA, it is up-regulated under abiotic stress conditions and may be targeted to relevant gene promoters via a complex with other proteins such as transcription factors.

***NPX1* Stimulates ABA Biosynthesis in Nutrient-Deficient Plants**

Our initial result showing that potassium deficiency induced the expression of *NPX1* led to the finding that ABA biosynthesis is enhanced when *Arabidopsis* is deprived of potassium (Jeschke et al., 1997; Peuke et al., 2002). At this time, it is still unclear what role ABA plays in plant response to nutrient deprivation. It is clear, however, that the *NPX1-ox* plants contained higher levels of ABA than other lines and that the overexpression of *NPX1* resulted in a moderate increase in tolerance to potassium deprivation (Supplemental Fig. S3). It is well known that root growth is

stimulated by ABA when applied at a low concentration, but it inhibits growth at higher concentrations (Pilet and Saugy, 1987). It is possible that ABA is needed for growth under normal conditions and that higher concentrations may be required for maintaining root growth under potassium-deprived conditions. This finding links ABA to the potassium deprivation response circuits, which include auxin (Shin et al., 2007), ethylene (Jung et al., 2009), and reactive oxygen species (Shin and Schachtman, 2004). Further work will be needed to fully understand the role of ABA plant response to low potassium.

In conclusion, the data presented here provide evidence that a novel nuclear factor modulates ABA signaling and plant sensitivity to abiotic stress, as summarized in Figure 8. The isolation and characterization of *NPX1* provides new insight into the control mechanisms for modulating the ABA responsiveness of plants and may provide new approaches for the genetic engineering of drought tolerance in plants.

MATERIALS AND METHODS

DNA Cloning

The *NPX1* (At5g63320) open reading frame was amplified using Pfu-polymerase (Stratagene) and the following primers: 5'-CCTGCAGAT-GCAAGTTGGAGTGTCTCGG-3' and 5'-TCCCGGGTCAATCAATTCTACTATATATC-3'. The YFP-*NPX1* and GAL4 DNA BD fusion proteins were constructed by amplifying the *NPX1* open reading frame using Pfu-polymerase (Stratagene) and the following primers: 5'-CACCATGGATGCAGTAG-TTCTCGTTCAG-3' and 5'-TCAATCAATTCTACTATATCTTCTCT-3'. The PCR product was introduced into pENTR D/Topo (Invitrogen) following the manufacturer's instructions. The insert was sequenced to ensure that no changes were introduced by PCR. The resulting entry clone was introduced into the destination plasmid pEARLYGATE N-YFP (Earley et al., 2006) to yield pEARLYGATE YFP-*NPX1*, which allows the expression of fusion proteins with YFP at the N-terminal position under control of the cauliflower mosaic virus 35S promoter.

Quantitative Real-Time PCR Analysis

To analyze the expression of *NPX1* by real-time PCR, the gene-specific primers (5'-AAAAGCAACCAGGAGTTGGTATAGACAAAA-3' and 5'-ATC-CATGGTGGTTGACTTTTCCCAA-3') were used. The gene-specific primers for quantifying the transcripts of ABA signaling genes were described previously (Chen et al., 2006; Shen et al., 2006). β -Tubulin gene (At5g62690) was amplified with the primers 5'-GCCAATCCGGTCTGGTAACA-3' and 5'-CATACCAGATCCAGTTCCTCTCCC-3' and was used to normalize the reactions. Real-time PCR was performed according to the instructions provided for the Bio-Rad iCycler iQ system (Bio-Rad Laboratories) with platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The fold change of transcripts was calculated based on an efficiency-calibrated model (Yuan et al., 2006) and compared with the transcript level under normal conditions. Statistical differences between samples were evaluated by Student's *t* test using delta Ct values (Yuan et al., 2006). In each experiment, the mean of three biological replicates is used to generate means and statistical significance. Two experiments on independently grown plant material were performed to confirm that the results are reproducible.

Plant Material and Growth Conditions

For constitutive expression of *NPX1* in planta, we used pCambia2300 with the figwort mosaic virus promoter (Sanger et al., 1990) and nopaline synthase terminator. *Arabidopsis* (*Arabidopsis thaliana*) plants were transformed using *Agrobacterium tumefaciens* by the floral dip method (Clough and Bent, 1998).

The insertion mutant in the *NPX1* locus (SAIL_123) or *TIP* (SALK_150356C) was obtained from the Arabidopsis Biological Resource Center. Homozygous plants were identified by PCR as described (<http://signal.salk.edu/tdnaprimers.2.html>). The absence of *NPX1* transcript or *TIP* was confirmed by reverse transcription-PCR. Prior to growth assays, both mutant alleles and the corresponding wild type (ecotype Columbia) were grown together under the same conditions to generate wild-type, overexpression, and knockout seeds for further experiments. For the nutrient-deficiency assays, plants were grown on plates containing a nutrient solution described previously (Shin et al., 2005) with the addition of 2% Suc and 0.6% SeaKem agarose (Cambrex). After stratification of the seeds at 4°C for 2 to 3 d, the plates were transferred to the growth chamber at 22°C with a 16-h daylength at 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For K^+ -deficiency growth assays, 10 μM KCl was used instead of 1.75 mM KCl. Four-day-old seedlings were transferred to nutrient-deficient medium, and then the length of primary roots and number of lateral roots were measured after 7 d.

Germination and Root Growth Assays

Germination and root growth assays with exogenous ABA were performed as described (Kuhn et al., 2006). Sterilized seeds were plated on minimal medium (0.25 \times Murashige and Skoog medium, no Suc) supplemented with increasing ABA concentrations. To score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined in three independent experiments (36 seeds per genotype and experiment).

Root growth assays to assess ABA sensitivity were carried out by transferring 6-d-old seedlings onto minimal medium supplemented with the indicated ABA concentrations on 0.8% agar (Phytigel; Sigma) plates. Root growth was measured 6 d after the transfer in three independent experiments with 70 individuals per genotype and experiment.

Measurements of the Stomatal Aperture

For stomatal closing experiments, fully expanded leaves from 3- to 4-week-old plants were excised, and epidermal pieces were peeled from the abaxial surface. The epidermal peels were floated for 2.5 h in stomatal opening solution (Pei et al., 1997) containing 50 mM KCl, 50 μM CaCl_2 , and 10 mM MES (pH 6.15). After incubation in ABA for 2 h, the stomatal aperture was measured. Control experiments were performed in parallel with no ABA added. Double-blind stomatal movement assays were performed such that the genotype and applied ABA concentrations were unknown. Three independent experiments were performed, and the same results were obtained.

Drought Tolerance Experiment

For the drought tolerance experiment, the soil in pots containing 3-week-old plants was covered with plastic film and exposed to a period of 14 d without added water. Stomatal conductance (g_s) was obtained using the AP3 porometer (Delta-T Devices) at 3 and 7 d after watering was stopped. Two independent experiments were performed, and similar results were obtained.

Visualization of YFP-NPX1 Localization

For transient expression of YFP-NPX1, biolistic bombardment of onion (*Allium cepa*) epidermal cells was performed as described previously (Marc et al., 1998) using 5 μg of plasmid DNA precipitated onto gold particles. Transformed Arabidopsis plants were generated expressing YFP-NPX1 as a fusion protein under the control of the cauliflower mosaic virus 35S promoter. Seedlings were grown on plates containing 0.25 \times MS + 0.8% agar and were imaged using a Nikon Eclipse E800 microscope. To stain the nucleus of Arabidopsis cells, 1 $\mu\text{g mL}^{-1}$ Hoescht dye (Sigma) was used for 30 min. Successive images were collected either with transmitted light or with appropriate YFP and Hoescht filters.

Yeast Two-Hybrid Screening

Full-length *NPX1* cDNA was amplified by PCR and subcloned into the vector pDEST32 (Invitrogen). An Arabidopsis root cDNA library was constructed using the activation domain expression vector pDEST22 (Invitrogen). Plasmids were cotransformed into *Saccharomyces cerevisiae* strain MaV203 according to the manufacturer's instructions (Invitrogen).

Yeast Repression Assay

Full-length *TIP* (At5g24590) cDNA was generated by PCR with the primers 5'-AGAATTCATGAAAGAAGACATGGAAGTACT-3' and 5'-ACTCGAGG-AATTGATCGGAACAAACATCAC-3' and subcloned into the *EcoRI/SalI* sites in pGBKT7 (Clontech). As a result, *TIP* was fused with GAL4 DNA BD. BD-NPX1 and BD-YFP were expressed from pDEST22 (Invitrogen). The plasmids were transformed into *S. cerevisiae* Y187, and β -galactosidase assays were performed as described (Sridhar et al., 2006). BD-*TIP*, BD-NPX1, or BD-YFP was selected by -Trp and -Leu. A liquid culture assay using *o*-nitrophenyl β -D-galactopyranoside as a substrate was performed to determine transcriptional activation. Data shown for these assays are averages of triplicates, and the experiment was repeated twice.

Measurement of ABA Concentration

The ABA concentrations in leaves or roots were measured with a Phytodetek ABA enzyme immunoassay test kit (Agdia). Mature leaves or roots of 3-week-old plants grown in medium were harvested, freeze dried, frozen in liquid nitrogen, and ground into powder. ABA was extracted by suspending 200 mg of lyophilized tissue in 1 mL of water and agitating overnight at 4°C. The suspension was centrifuged at 4,000 rpm for 20 min, and the supernatant was transferred to a clean tube and dried under vacuum. The dry residue was dissolved with 60 μL of water, and a 1:100 dilution with Tris-buffered saline (45 mM Tris-HCl, pH 7.8, 90 μM MgCl_2 , 0.135 M NaCl, and 3 mM sodium azide) was made. The ABA concentration was then determined with a Phytodetek ABA enzyme immunoassay test kit (Agdia). The color absorbances were detected with the Spectramax 250 microplate reader (Molecular Devices) at 405 nm. Two independent experiments were performed, and the same results were obtained.

Sequence data from this article can be found in the GenBank/EMBL data libraries as described in "Materials and Methods" and Supplemental Table S2.

Supplemental Data

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

Supplemental Figure S1. RT-PCR analysis of *NPX1* expression in Arabidopsis plants.

Supplemental Figure S2. Analysis of additional *NPX1* overexpression and knockout lines.

Supplemental Figure S3. Overexpression of *NPX1* enhances primary root growth.

Supplemental Figure S4. The expression of *TIP* was up-regulated by nutrient deprivation and abiotic stresses.

Supplemental Figure S5. Disruption of *TIP* alters the expression of ABA signaling genes.

Supplemental Table S1. Proteins that interact with *NPX1* as determined by yeast two-hybrid screening.

Supplemental Table S2. Information on genes used for real-time PCR analysis.

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