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Transgenic *Arabidopsis* Plants Expressing the Type 1 Inositol 5-Phosphatase Exhibit Increased Drought Tolerance and Altered Abscisic Acid Signaling [W]

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The phosphoinositide pathway and inositol-1,4,5-trisphosphate ($InsP_3$) are implicated in plant responses to stress. To determine the downstream consequences of altered $InsP_3$-mediated signaling, we generated transgenic *Arabidopsis thaliana* plants expressing the mammalian type I inositol polyphosphate 5-phosphatase (InsP 5-ptase), which specifically hydrolyzes soluble inositol phosphates and terminates the signal. Rapid transient Ca^{2+} responses to a cold or salt stimulus were reduced by ~30% in these transgenic plants. Drought stress studies revealed, surprisingly, that the InsP 5-ptase plants lost less water and exhibited increased drought tolerance. The onset of the drought stress was delayed in the transgenic plants, and abscisic acid (ABA) levels increased less than in the wild-type plants. Stomatal bioassays showed that transgenic guard cells were less responsive to the inhibition of opening by ABA but showed an increased sensitivity to ABA-induced closure. Transcript profiling revealed that the drought-inducible ABA-independent transcription factor DREB2A and a subset of DREB2A-regulated genes were basally upregulated in the InsP 5-ptase plants, suggesting that $InsP_3$ is a negative regulator of these DREB2A-regulated genes. These results indicate that the drought tolerance of the InsP 5-ptase plants is mediated in part via a DREB2A-dependent pathway and that constitutive dampening of the $InsP_3$ signal reveals unanticipated interconnections between signaling pathways.

INTRODUCTION

The membrane-associated phospholipids and the soluble inositol phosphates, collectively known as the phosphoinositides, are ubiquitous in eukaryotic cells and are involved in regulating a multitude of cellular functions (Martin, 1998; Cockcroft and De Matteis, 2001; Di Paolo and De Camilli, 2006). In the canonical phosphoinositide pathway, the membrane-associated phospholipid, phosphatidylinositol (PtdIns), is sequentially phosphorylated by specific lipid kinases to form phosphatidylinositol 4-phosphate (PtdInsP) and phosphatidylinositol 4,5 bisphosphate ($PtdInsP_2$). In response to a stimulus such as hyperosmotic stress, $PtdInsP_2$ is hydrolyzed by phospholipase C (PLC) to produce the soluble second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (Berridge, 1993). The phospholipids are ideally suited to mediate cellular signaling due to their high turnover rate and their spatial distribution, which is regulated in part by the concerted action of specific phosphoinositide kinases, phospholipases, and phosphatases (Mueller-Roeber and Pical, 2002; Boss et al., 2006). The phosphoinositide pathway integrates plasma membrane sensing with intracellular signaling and thereby provides a means of both sensing and propagating a signal.

Although the phosphoinositide pathway has been well studied in plants, there are fundamental differences between the plant and animal models (Stevenson et al., 2000; Meijer and Munnik, 2003; Wang, 2004). One critical difference is the relatively low steady state level of $PtdInsP_2$ in plants compared with animals. One of the reasons for this difference is that the plant PtdInsP kinases are not as active as their animal counterparts (Perera et al., 2005; Im et al., 2007).

Although rapid, transient increases in $InsP_3$ have been demonstrated in many different plant tissues in response to environmental stimuli (Stevenson et al., 2000; Meijer and Munnik, 2003; Krinke et al., 2007), the downstream consequences of the $InsP_3$ changes are not well understood. $InsP_3$ has been shown to trigger Ca^{2+} release from intracellular stores such as the vacuole (Sanders et al., 1999, 2002). $InsP_3$ is implicated in the propagation of Ca^{2+} oscillations in guard cells and root hair cells (Staxen et al., 1999; Engstrom et al., 2002), in cell-to-cell communication (Tucker and Boss, 1996), and in the regulation of diurnal Ca^{2+} fluctuations (Tang et al., 2007). However, genes encoding a canonical $InsP_3$ receptor are absent in the *Arabidopsis thaliana*

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genome (Hetherington and Brownlee, 2004; Krinke et al., 2007). In addition, only a few examples of a stimulus-generated increase in $InsP_3$ resulting in a change in cytosolic Ca^{2+} and a downstream response have been reported in higher plants (Franklin-Tong et al., 1996; DeWald et al., 2001).

In order to manipulate the plant phosphoinositide pathway and understand the downstream effectors of $InsP_3$-mediated signaling, we generated transgenic plants constitutively expressing the mammalian type I inositol polyphosphate 5-phosphatase (InsP 5-ptase), an enzyme that specifically hydrolyzes the soluble inositol phosphates $InsP_3$ and inositol tetrakisphosphate ($InsP_4$) and not the inositol phospholipids (Laxminarayan et al., 1993, 1994; Majerus et al., 1999). We chose this heterologous enzyme for several reasons. The mammalian type 1 inositol 5-phosphatase is well characterized and is more active than its plant counterparts. This enzyme is associated with the plasma membrane (De Smedt et al., 1997) and therefore should preferentially dampen $InsP_3$ signals generated at the plasma membrane. In previous work, we have shown that this enzyme is stably expressed in plants and produces an active, functional protein that is plasma membrane–localized (Perera et al., 2002, 2006). A further advantage of the heterologous genes is that they are not subject to gene-silencing effects. In *Arabidopsis*, the inositol 5-phosphatases are encoded by a multigene family of 15 genes (Berdy et al., 2001; Ercetin and Gillaspy, 2004), which increases gene redundancy.

Our hypothesis was that stimulus-induced increases in $InsP_3$ and $InsP_3$-mediated signaling cascades would be impaired in the transgenic plants and thereby the timing and/or magnitude of the response would be delayed or decreased. In previous work, we have shown that InsP 5-ptase transgenic *Arabidopsis* plants have normal growth and morphology under optimal growth conditions; however, basal $InsP_3$ levels are greatly reduced (to ~5% of wild-type levels) and the plants exhibit delayed and reduced gravitropic responses in roots, hypocotyls, and inflorescence stems (Perera et al., 2006). These data support the hypothesis that $InsP_3$ is a universal signal in plant gravitropism and that decreasing the signal attenuates the response.

In this work, we have used the InsP 5-ptase plants to dissect the function of $InsP_3$ in drought stress. Abscisic acid (ABA) is a prime mediator of plant responses to abiotic stresses such as drought, salt, and cold (Zhu, 2002). In the drought stress response, ABA acts at multiple cellular levels, including the regulation of stomatal aperture and the activation of gene expression. Many second messengers, including Ca^{2+}, $InsP_3$, cADP ribose, and others, are implicated in ABA-mediated signaling. In addition, there is crosstalk between both ABA-dependent and ABA-independent pathways involving other drought-responsive factors such as DREB2A and ERD1 (Shinozaki and Yamaguchi-Shinozaki, 2000, 2007; Yamaguchi-Shinozaki and Shinozaki, 2006).

Both $InsP_3$ and PLC (the enzyme responsible for the generation of $InsP_3$ from $PtdInsP_2$) have been shown to be important for ABA-mediated stomatal regulation. In early work in *Commelina communis* epidermal peels, the release of caged $InsP_3$ caused an elevation of cytosolic Ca^{2+} leading to stomatal closure (Gilroy et al., 1990). Also, treatment with the PLC inhibitor U73122 inhibited ABA-induced Ca^{2+} oscillations and stomatal closure (Staxen et al., 1999). More recently, Hunt et al., 2003 reported that transgenic tobacco (*Nicotiana tabacum*) plants with reduced PLC in their guard cells showed a wilty phenotype under water-limiting conditions and did not fully respond to ABA. Further characterization of the ABA-mediated stomatal regulation in these plants (Mills et al., 2004) revealed that the ability of ABA to inhibit stomatal opening was compromised (in the reduced PLC plants), although there was no difference in ABA promotion of stomatal closure. Stomatal conductance was also higher in plants with reduced PLC after a period of soil drying compared with controls.

Ectopic expression of endogenous plant InsP 5-ptase genes has also revealed differences in ABA responses compared with the wild type. Stomata of transgenic plants overexpressing *Arabidopsis* InsP 5-ptase, At5PTase1, were shown to be less open than the wild type and less responsive to ABA (Burnette et al., 2003), with delayed induction of ABA-responsive gene expression. Induction of ABA-responsive genes was also attenuated in transgenic plants expressing another *Arabidopsis* inositol phosphatase, At5PTase2 (Sanchez and Chua, 2001).

Based on our predictions and the work by others (described above), we expected that the InsP 5-ptase transgenic plants would be less drought-tolerant and unable to withstand prolonged drought stress. Surprisingly, the transgenic plants lose less water and are more drought-tolerant than the wild-type plants. We show here that the guard cells of transgenic plants show differential sensitivity to exogenous ABA compared with wild-type plants in bioassays to monitor stomatal opening and closure. Furthermore, with drought stress, ABA levels in the transgenic plants do not increase as much as in the wild type. A key to understanding the drought tolerance phenotype is the greater than twofold increase in basal transcript levels of the drought-inducible ABA-independent transcription factor DREB2A and a subset of DREB2A-regulated genes in the unstressed transgenic plants. These results suggest that $InsP_3$ acts as a negative regulator in the DREB2A drought signaling pathway and that in the absence of $InsP_3$, this and other compensatory pathways have been activated in the transgenic plants, leading to increased drought tolerance.

RESULTS

The InsP 5-ptase Plants Show Increased Tolerance to Drought and Lose Less Water Compared with Wild-Type Plants

Several independent stably transformed *Arabidopsis* lines expressing InsP 5-ptase have been generated and characterized as described previously (Perera et al., 2006). The transgenic plants have no morphological changes compared with the wild type and grow comparably under normal growth conditions. Furthermore, the InsP 5-ptase protein is produced and active, as evidenced by immunoblotting and the fact that the basal $InsP_3$ levels are reduced by >90% compared with wild-type plants (Perera et al., 2006).

In this work, we have studied the response of the transgenic plants to drought stress. When water is withheld, the transgenic

plants are able to withstand the drought conditions longer than the wild-type plants. After 12 d without water, the wild-type plants and plants from the vector control line are wilted and turning brown, while plants from three independent InsP 5-ptase lines remain green and turgid (Figure 1A). We next monitored water loss from detached leaves using fully expanded leaves of comparable size, weight, and development. Leaves were excised from the rosettes of well-hydrated wild-type and transgenic plants and kept at ambient light and temperature at 30% humidity (adaxial side up) for 1 to 2 h, and the fresh weight of the leaves was monitored periodically during this time. The transgenic plants show a 30% decrease in the rate of water loss from detached leaves compared with the wild-type plants (Figure 1B). We also monitored pot water loss over a period of 6 to 7 d to measure water loss during day and night. Over this time period, the transgenic plants lost less water during both the day and night (Figure 1C). The extent of root growth and root mass between wild-type and transgenic plants as observed at the end the experiment (see Supplemental Figure 1 online) was comparable, ruling out the possibility that reduced water loss from the transgenic plants was caused by greater water retention due to differential root growth.

We also measured stomatal conductance in well-watered plants over a period of 6 to 8 h of exposure to light. During the time course, maximal stomatal conductance was detected in all plant lines after ~4 h of light, which is consistent with the circadian rhythm of the plants. Stomatal conductance was comparable among the two independent transgenic plant lines

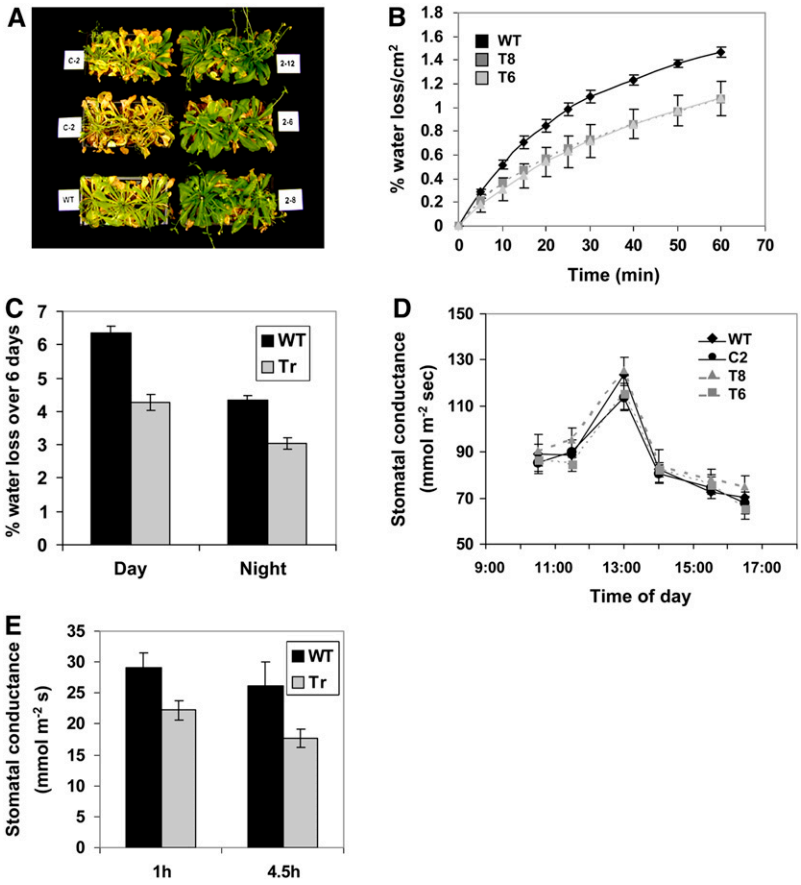

Figure 1. The InsP 5-ptase Transgenic Plants Lose Less Water and Are More Drought-Tolerant Compared with Wild-Type Plants.

(A) Eight-week-old plants were incubated at room temperature (30% humidity) and not watered for 12 d. C-2, vector control, hereafter denoted C2; 2-12, 2-6, and 2-8, three independent transgenic lines expressing InsP 5-ptase, hereafter denoted T12, T6, and T8, respectively.

(B) Leaf water loss in excised leaves was monitored at 5-min intervals over a period 1 to 2 h. Data are plotted as the decrease in fresh weight over time adjusted for potential differences in leaf surface area. Values are averages ± SE of four independent experiments.

(C) Water loss of intact plants in pots during the day and night was measured over a period of 6 d. The data plotted are averages ± SE from nine plants per line from either the wild type or two transgenic lines, T6 and T8, that were combined and denoted Tr.

(D) Stomatal conductance in well-watered plants measured over a period of 8 h. Data presented are averages ± SE of three measurements per plant and six plants per line for each time point. The experiment was repeated twice with similar results.

(E) Stomatal conductance in drought-stressed plants (without water for 8 d) measured at two time points after being exposed to light. Data presented are averages ± SE of three measurements per plant and 10 plants per line for each time point.

and the wild-type and vector control plants (Figure 1D). These results demonstrate that the transgenic plants are drought tolerant and that under well-watered conditions stomatal conductance was not affected, consistent with their normal growth phenotype. However, after 8 d without water, stomatal conductance was reduced in all plant lines and, importantly, was lower in the transgenic plants compared with the wild type (Figure 1E). This suggests that the transgenic plants are better able to adjust to the water stress compared with the wild-type plants.

The Transgenic Guard Cells Show Altered Responses to ABA

Because stomata regulate the major flow of water out of leaves during both day and night (Caird et al., 2007), we examined the response of guard cells from wild-type and transgenic plants to different stimuli. For these experiments, epidermal peels were prepared from the abaxial surface of intact healthy leaves of similar size and developmental stage. The presence of the InsP 5-ptase protein (~45 kD) in similar epidermal peel preparations was clearly visible by immunoblotting (see Supplemental Figure 2 online). $InsP_3$ levels were reduced by 95% in epidermal peel preparations (Table 1), as in all other tissues of the transgenic plants tested (Perera et al., 2006). We also monitored stomatal density in epidermal peels. There was no difference in the number of stomata/leaf area between wild-type and transgenic leaves (Table 1).

Stomatal aperture was monitored immediately after peeling or after incubation under dark or light conditions for 3 h (Figure 2A). There was no difference in stomatal aperture between wild-type and transgenic plants under these conditions. We also monitored the response of guard cells to high Ca^{2+} and external ABA. The transgenic guard cells were less responsive to closing in the presence of 1 mM Ca^{2+} compared with the wild type (Figure 2B). For the response to ABA, we measured both the promotion of stomatal closure, where epidermal peels were first incubated in the light and then treated with ABA (Figure 2C), and the inhibition of stomatal opening, where epidermal peels were prepared from plants incubated in the dark and then treated with light or ABA in the presence of light (Figure 2D). As seen in Figure 2C, at two different concentrations of ABA (10 and 100 μM), we observed that the transgenic guard cells were more responsive to ABA in the promotion of stomatal closure. However, ABA in the presence of light failed to inhibit stomatal opening in the transgenic guard cells, although opening of wild-type guard cells was strongly inhibited (Figure 2D). These results show that the transgenic guard cells have altered responses to ABA and Ca^{2+} compared with the wild type and suggest that the differential water loss in the transgenic leaves could in part be regulated at the level of guard cell dynamics (i.e., the higher sensitivity of the transgenic guard cells to ABA-induced closure).

Table 1. $InsP_3$ Levels and Stomatal Density in Epidermal Peels

Plant	$InsP_3$ Levels (pmol/g Fresh Weight)[a]	Stomatal Density (×20 Magnification)[b]
Wild type	233 ± 3.7	16.4 ± 0.8
Tr	14 ± 1.2	16.7 ± 0.5

[a] $InsP_3$ levels were measured in wild-type and transgenic (Tr = combined averages from both T6 and T8) epidermal peels. Data presented are averages ± SE of three experiments.

[b] Stomatal density was measured in wild-type and Tr epidermal peels at ×20 magnification. Data presented are averages ± SE of three experiments.

Whole Plant Responses to ABA

It has been shown that treatment with ABA can cause an increase in $InsP_3$ in guard cells (Lee et al., 1996) and seedlings (Sanchez and Chua, 2001; Burnette et al., 2003). Similarly, we found that 1- to 2-week-old wild-type seedlings sprayed with 100 μM ABA showed a biphasic increase in $InsP_3$ (Figure 3A, solid black line). $InsP_3$ levels increased rapidly with spraying and again after ~30 min. Plants were sprayed with water containing 0.1% ethanol as the solvent control (Figure 3A, dotted black line). $InsP_3$ levels were significantly reduced in the transgenic plants and showed no appreciable increase over the time course after spraying with ABA or solvent control (Figure 3A, solid gray and dotted lines).

We also monitored the expression of ABA-responsive genes after treatment with ABA and detected no impairment in the induction of select ABA-responsive genes in the transgenic lines compared with the wild type (Figure 3B). Expression of several ABA-responsive genes, including *RAB18*, *KIN2*, and *COR15a* (Kawaguchi et al., 2004; Shinozaki and Yamaguchi-Shinozaki, 2007), was induced equally well (if not better), with no delay in timing in the transgenic plants compared with the wild type. These results indicate that an ABA-mediated increase in $InsP_3$ is not essential for the induction of these ABA-responsive genes. We also monitored the seed germination response of transgenic lines compared with the wild type to increasing concentrations of ABA. Seed germination in the transgenic lines was less sensitive to 5 and 10 μM ABA compared with the wild type (Figure 3C); however, at 25 μM ABA, germination was dramatically inhibited in both transgenic and wild-type plants.

Changes in Inositol Phosphate Metabolism

In addition to $InsP_3$, it has been reported that inositol hexakisphosphate ($InsP_6$) is a signaling molecule in guard cells and that $InsP_6$ levels increase rapidly following ABA treatment (Lemtiri-Chlieh et al., 2000, 2003). Enzymes involved in the conversion of $InsP_3$ to $InsP_6$ have been functionally characterized in *Arabidopsis* (Stevenson-Paulik et al., 2002; Xia et al., 2003). Therefore, we set out to determine the impact of increased $InsP_3$ hydrolysis on the biosynthesis of inositol phosphate and, in particular, $InsP_6$. *Arabidopsis* seedlings were labeled with [^{3}H]inositol for 3 to 4 d, and the soluble inositol phosphates were extracted and analyzed by anion-exchange HPLC. The [^{3}H]inositol labeling results from whole seedlings (Figure 4A) or epidermal peels (Figure 4B) show altered levels of [^{3}H]inositol phosphates in the transgenic lines compared with the wild type. In the transgenic seedlings, consistent with increased $InsP_3$ hydrolysis, $InsP_2$ levels were slightly elevated and total $InsP_3$ isomer levels were decreased. Total $InsP_5$ isomer levels were also decreased. Under these labeling conditions, [^{3}H]$InsP_4$ species were undetectable over

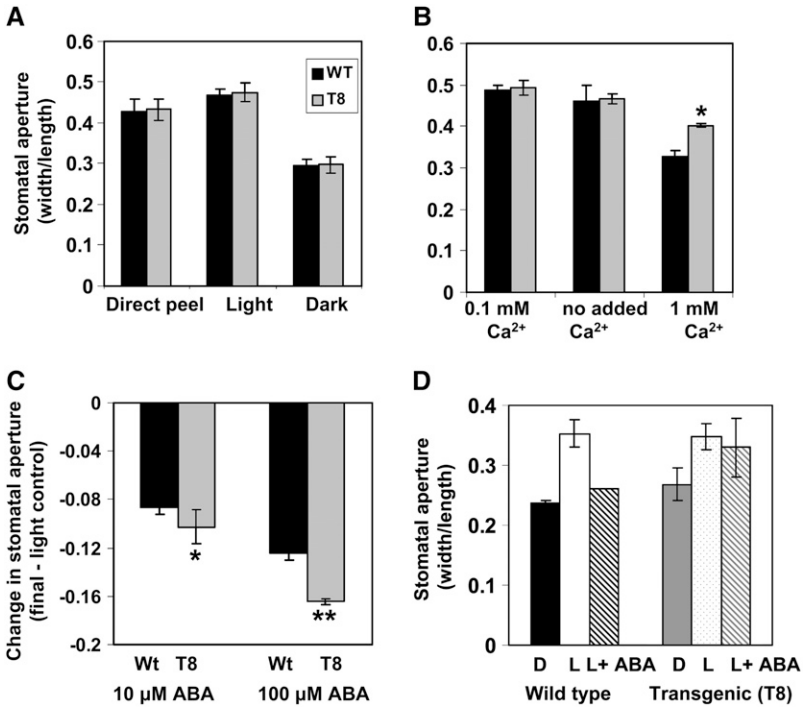

Figure 2. The InsP 5-ptase Plants Show Altered Responses to Ca^{2+} and ABA in Stomatal Bioassays.

(A) Stomatal aperture (presented as the ratio of width to length) was measured in epidermal peels prepared from the abaxial surface of wild-type and transgenic (T8) leaves. Peels were observed directly or after incubation for 3 h in the light or dark.

(B) Effect of high Ca^{2+}. Peels were incubated in buffer with or without Ca^{2+} or in the presence of 1 mM Ca^{2+}. The asterisk denotes a statistically significant difference compared with the wild type ($P < 0.005$).

(C) and **(D)** Effect of ABA. To determine differences in ABA-mediated stomatal closure **(C)**, peels were first incubated in the light for 3 h, and then ABA (10 or 100 μM) was added and the peels were incubated in the light for an additional 3 h. Data are shown as differences between the width:length ratios of the final aperture after ABA treatment and the initial light value (* $P < 0.1$, ** $P < 0.01$). To determine differences in inhibition of stomatal opening by ABA **(D)**, peels were taken from plants in the dark (D) and incubated in the light (L) for 3 h in buffer with or without 25 μM ABA. For all stomatal bioassays, ~15 images were captured per peel ($n = 45$ per experiment). Data presented are averages ± SE of three independent experiments.

background in both wild-type and transgenic plants and thus are not shown in the figure. By contrast, $InsP_6$ levels were readily detectable and were reduced in the transgenic seedlings (by 25%) and peels (by 50%) compared with the wild type. These results support a model for a direct route of $InsP_6$ biosynthesis from $InsP_3$ via the inositol polyphosphate kinases (Stevenson-Paulik et al., 2002; Raboy, 2003). The decrease in $InsP_6$ in the epidermal peels of the transgenic plants could contribute to the altered ABA and Ca^{2+} responses of the guard cells, since $InsP_6$ has been shown to affect stomatal regulation (Lemtiri-Chlieh et al., 2000, 2003).

The Onset of Water Stress Is Delayed in the Transgenic Plants

To further characterize the drought response, we monitored the relative water content (RWC) of wild-type and transgenic leaves during a period of dehydration (Griffiths and Bray, 1996). For these experiments, well-watered plants of similar size and maturity (~6 weeks old) were maintained in a growth chamber under short-day conditions at a relative humidity of 30%. RWC was measured on day 0 (well watered) and over a period of 8 d without water. A RWC of <60% is considered to be a severe water stress (Kawaguchi et al., 2003). As seen in Figure 5A, the transgenic lines maintain a RWC of >85% for longer than the wild type.

We also monitored ABA levels in the plants during the RWC time course. ABA levels were measured in leaf samples harvested at day 0 and at days 7 and 8 (Figure 5B). At day 0, ABA levels were equally low in all plant lines (see Supplemental Figure 3 online). As the drought stress progressed and the RWC decreased, the level of ABA increased in both wild-type and transgenic lines. However, ABA levels always remained lower in the transgenic plants compared with the wild type even at a RWC of ~60%. The higher RWC and lower ABA levels at both days 7 and 8 of a no-watering regime (Figure 5) indicate that the transgenic plants are experiencing less drought stress than the wild-type controls.

In some trials, the plants were rewatered on day 8. Upon rewatering, the transgenic plants recovered fully compared with the wild-type plants (see Supplemental Figure 4A online). The faster recovery is also evident by the expression patterns of

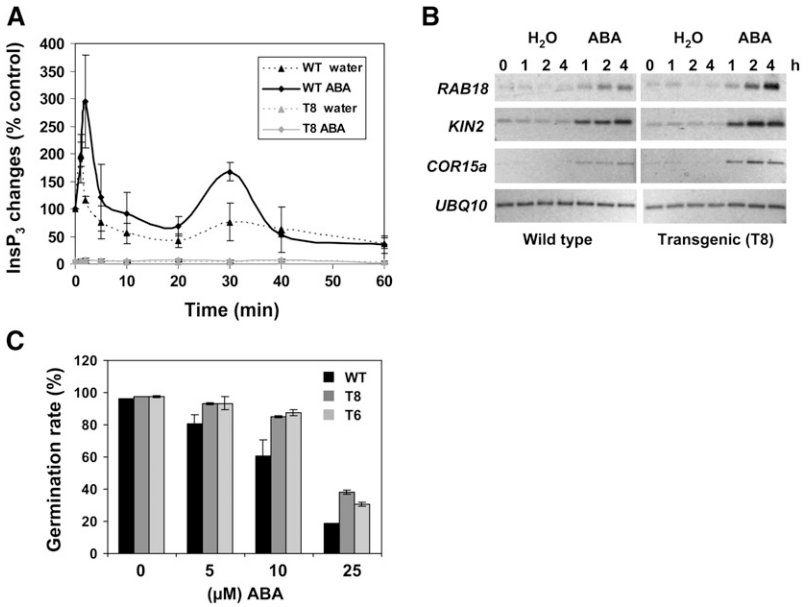

Figure 3. $InsP_3$ and Gene Expression Changes in Response to Exogenous ABA.

(A) Time course of $InsP_3$ changes in response to ABA. Two-week old *Arabidopsis* seedlings were sprayed with either water containing 0.1% ethanol as the solvent control (dashed line) or 100 μM ABA (solid line) and harvested at the indicated times for $InsP_3$ analysis. Data presented are averages ± SE of four independent experiments assayed in duplicate. The changes in $InsP_3$ are plotted as percentages of the wild-type basal level.

(B) Representative data of gene expression changes in response to ABA. RNA was isolated from 2-week-old seedlings sprayed either with water or ABA, and RT-PCR was performed using primers specific for *RAB18*, *KIN2*, and *COR15a*. The *Arabidopsis* polyubiquitin gene (*UBQ10*) was used as a control. Similar results were obtained in two independent experiments.

(C) Inhibition of seed germination by ABA. Seed germination assays were performed with increasing concentrations of ABA. Germination was scored after 3 d, and the data presented are averages ± SE of three independent experiments.

stress-inducible genes such as *RAB18* and *ERD1* (see Supplemental Figure 4B online). Both wild-type and transgenic plants showed high expression of these genes at day 8 (when the RWC was ≤60%). However, upon rewatering, the expression of these genes decreased in the transgenic plants while remaining elevated in the wild type.

Microarray Analysis of Transcript Changes with Drought Stress

In order to compare global expression profiles between wild-type and transgenic plants in response to water stress, we harvested leaves for transcript profiling at day 0 (well watered) and at day 7, when the transgenic plants were still at a RWC of >85% and the wild-type and vector control plants were at a RWC of ~50 to 60%. For each biological experiment, leaves were harvested and pooled from ~10 plants of each line/time point. Three biological replicates were performed with the wild type and two independent transgenic lines (T6 and T8). Two biological replicates of the vector control line (C2) were performed as an additional control.

Transcript profiling was performed using the *Arabidopsis* ATH1 arrays (Affymetrix). Array hybridization, data acquisition, and analysis were performed by Expression Analysis of Durham, NC, using the Affymetrix fluidics station and GCOS software. As indicated in Figure 6A, two-group comparisons were made in order to determine any differences in expression profiles between the basal (0) and drought-stressed (D) samples for each line and also between the basal expression profiles of the wild-type and transgenic lines. The two-group comparison incorporates a permutation analysis for differential expression and an estimate of the false discovery rate (Pawitan et al., 2005; Clarke and Zhu, 2006).

Comparison of Basal Expression Profiles

A comparison of the basal expression profiles of the two transgenic lines [T8(0) versus T6(0), comparison 7, Figure 6A] did not reveal any significant differences (false discovery rate > 0.9), which indicates that the transgenic lines are comparable and similar, thereby ruling out potential positional effects. We next looked at differences in basal expression profiles between transgenic and wild-type plants. Transcripts that were commonly upregulated or downregulated in both transgenic lines compared with the wild type (based on comparisons 4 and 5, Figure 6A) were further checked against basal levels of expression in the vector control line (C2) to ensure that the differences were specific for the transgene and not due to transformation.

The transgenic plants show no obvious morphological or developmental differences compared with wild-type plants

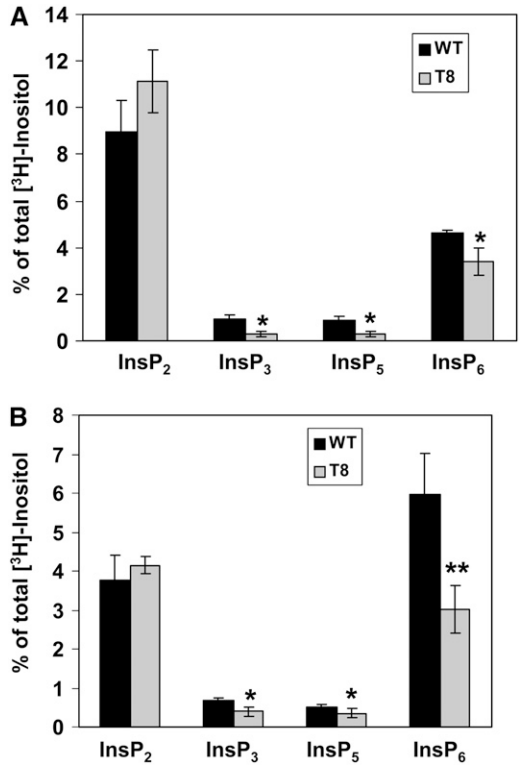

Figure 4. $InsP_6$ Is Reduced in InsP 5-ptase Seedlings and Epidermal Peels Compared with Wild-Type Plants.

(A) Inositol phosphate profiles from 1-week-old seedlings (wild type and T8) labeled for 4 d and analyzed by anion-exchange chromatography. Data presented are averages ± SE of five independent experiments (* $P < 0.1$).
(B) Inositol phosphate profiles from epidermal peel fragments labeled for 18 h. Data presented are averages ± SE of three independent experiments (* $P < 0.1$, ** $P < 0.01$).

under normal growth conditions. Consistent with this result, only a small fraction of transcripts showed altered basal expression in the transgenic lines compared with the wild type and the vector control (<0.4% of the transcriptome). There were more downregulated genes (62 transcripts; see Supplemental Table 1 online) compared with upregulated genes (20 transcripts; see Supplemental Table 2 online) in the transgenic lines. Of the downregulated genes, 18% fall into the defense-related category, including several pathogenesis-related genes (*PR-1*, *PR-2*, and *PR-5*). A second distinct group of downregulated genes (11%) represent proteins involved in Ca^{2+} binding and transport and protein folding, including CRT3 and BiP3 (Table 2). This downregulation may be an indication that the $InsP_3$-sensitive Ca^{2+} stores are underutilized in the transgenic plants due to the rapid metabolism of $InsP_3$. Conversely, in tobacco cells expressing the highly active human PtdInsP kinase Iα (which have basal $InsP_3$ levels ~ 50 fold higher than the wild type), we detected an upregulation of the endoplasmic reticulum Ca^{2+} binding proteins, presumably to maintain a high-capacity Ca^{2+} store (Im et al., 2007). We confirmed the transcript profile results for select genes from each category using quantitative RT-PCR (qRT-PCR), as shown in Figure 6B.

One of our more remarkable and unexpected findings is that the drought-responsive transcription factor *DREB2A* and a subset of *DREB2A*-regulated genes are basally upregulated in the transgenic plants. Of the 20 genes showing twofold higher basal expression in the InsP 5-ptase plants compared with wild-type and vector control plants (see Supplemental Table 2 online), the genes shown in Table 3 were found to be upregulated by greater than ninefold in *Arabidopsis* plants expressing a constitutively active form of DREB2A (Sakuma et al., 2006b). The basal upregulation in the InsP 5-ptase transgenic plants of the six genes shown in Table 3 was confirmed using qRT-PCR and additional independent biological replicates (Figure 6C).

Comparison of Expression Profiles in Response to Drought Stress

The two-group comparison analysis between the basal (0) and drought-stressed (D) samples (comparisons 1, 2, 3, and 6) were

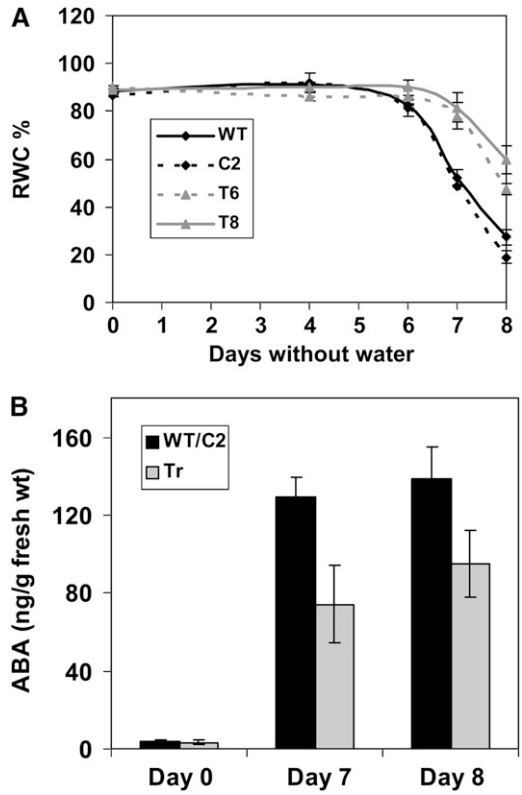

Figure 5. The InsP 5-ptase Plants Maintain Higher Relative Water Contents and Lower ABA Levels Compared with the Wild Type during Drought Stress.

(A) The RWC of leaves was measured on day 0 (well watered) and over a period of 8 d without water. Each time point represents the average value of 10 separate plants per line, and the data presented are averages ± SE from three independent experiments.
(B) ABA levels were measured in samples from day 0, 7, and 8 from three independent experiments. Data presented are averages ± SE from both the wild type and vector control (WT/C2) and from the two transgenic lines T6 and T8 (Tr).

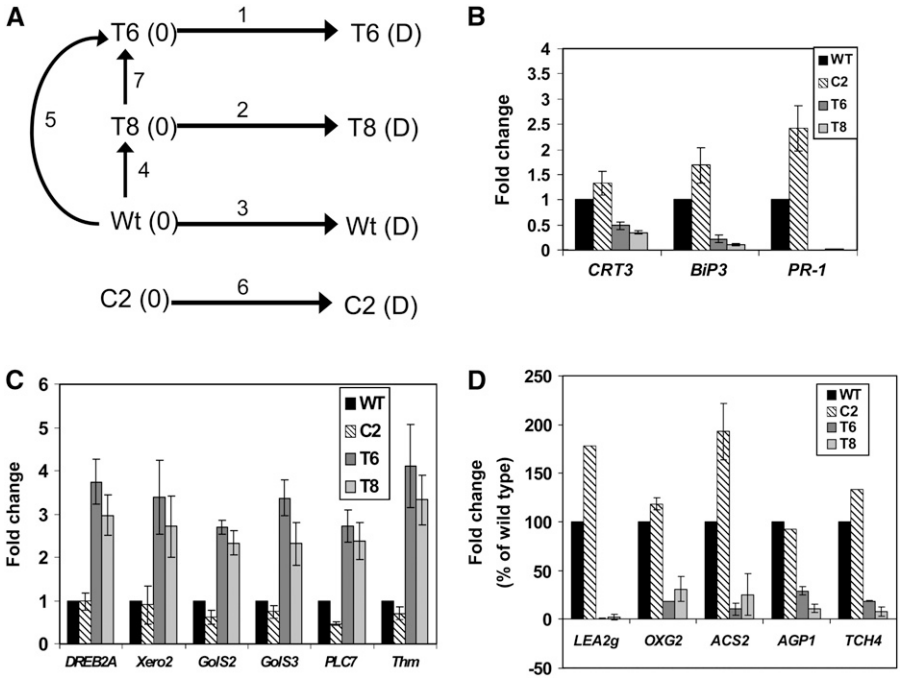

Figure 6. Schematic of the Microarray Experiment and Confirmation of Select Transcript Profiles Using qRT-PCR.

(A) Schematic showing the control (0) and drought-stressed (D) samples analyzed by microarray and the comparisons (numbered arrows) that were performed between samples.

(B) to **(D)** qRT-PCR of select transcripts to validate the array results.

(B) Select transcripts showing basal downregulation in transgenic lines compared with the wild type and vector control.

(C) Select transcripts showing basal upregulation in transgenic plants compared with the wild type and vector control.

(D) Select transcripts that are upregulated by drought stress in the wild type and vector control are significantly greater compared with transgenic plants.

The qRT-PCR results for **(B)** and **(C)** are presented as fold change compared with the wild type and are averages ± SE from four independent biological samples. For **(D)**, data are presented as percentage fold change of the wild type and are averages ± SE of three independent biological replicates (wild-type drought value was set to 100%).

used to compare gene expression profiles in response to drought stress. We generated four-way Venn diagrams using the upregulated or downregulated lists for each plant line and determined the common and unique patterns of expression between wild-type and transgenic lines in response to drought stress. The lists generated from the Venn comparison were further checked for overlap between the wild-type/C2 only and transgenic only groups (for genes that showed similar greater than twofold upregulation or downregulation, although with higher P values) to generate the lists of upregulated or downregulated genes unique to either the wild-type and vector control only or transgenic lines only (see Supplemental Tables 3 to 6 online). Table 4 summarizes the numbers of genes that fall into each of these categories. Interestingly, there was a greater number of upregulated or downregulated genes in the wild-type/C2 group than in the transgenic lines, which could be a reflection of the level of perceived stress (Table 4). For both upregulation and downregulation, there were ~150 to 200 genes that were either upregulated or downregulated with drought in all four plant lines tested (the complete lists of commonly upregulated and downregulated genes and pie charts depicting their functional categorization based on Gene Ontology annotation are shown in Supplemental Tables 7 and 8 and Supplemental Figures 5A and 5B online).

There were 244 transcripts that were exclusively upregulated (greater than twofold) with drought stress in both wild-type and vector control plants (see Supplemental Table 3 online). Interestingly, 30% of these transcripts are known to be regulated by ABA (Li et al., 2006b) and include a subset of late embyrogenic abundant (LEA) transcription factors and *PP2C* protein phosphatases. Four of five of this subset of LEAs belong to the same group recently classified as the LEA_4 group (Hundertmark and Hincha, 2008). The array results for some of the transcripts in Supplemental Table 3 online that are also regulated by ABA were confirmed by qRT-PCR (Figure 6D). By contrast, there were only four genes that were uniquely upregulated in the transgenic lines with drought (see Supplemental Table 4 online). The significance of this set of genes and what they share in common is not known at this point. In the downregulated with drought category, wild-type/C2 uniquely had 183 genes (listed in Supplemental Table 5 online). In the transgenic lines, there were 22 uniquely downregulated genes with drought (see Supplemental Table 6 online). These are predicted to encode unknown proteins (25%), proteins

Table 2. Subset of Genes Involved in Ca^{2+} Binding and Endoplasmic Reticulum Protein Folding and Transport That Are Basally Downregulated in the Transgenic Plants

Locus Identifier	Fold Change	P	Predicted Function and Gene Name
At2g18660	−9.15	0.01710	Expansin family protein (*EXPR3*)
At3g47480	−4.12	0.02440	Calcium binding EF hand family protein
At1g09080	−3.87	0.00640	Luminal binding protein 3 (*BiP-3*)
At3g60540	−3.57	0.00710	sec61 β family protein
At1g77510	−3.09	0.00350	Protein disulfide isomerase, putative
At4g16660	−2.57	0.01040	Heat shock protein 70, putative/HSP70, putative
At4g24920	−2.43	0.01090	Protein transport protein SEC61 γ subunit, putative
At1g08450	−2.40	0.00530	Calreticulin 3 (*CRT3*)
At3g51860	−2.35	0.00090	Cation exchanger, putative (*CAX3*)
At4g24190	−2.16	0.01730	Shepherd protein (*SHD*)/clavata formation protein, putative
At5g50460	−2.07	0.00900	Protein transport protein SEC61 γ subunit, putative

Subset of the basally downregulated genes in the two InsP 5-ptase transgenic lines (T6 and T8) compared with wild-type and C2 plants revealed by the microarray analysis. Data presented are from transgenic line T8. Transcripts were selected based on a greater than twofold decrease ($P < 0.1$).

involved in carbohydrate metabolism (17%), transport-related proteins (16%), signaling proteins (14%), transcription factors (11%), protein modification (10%), and calmodulin-related proteins (6%). The signaling-related proteins include a mitogen-activated protein kinase (At MPK11) that was reduced by approximately threefold in the transgenic plants compared with the wild type.

$InsP_3$-Mediated Ca^{2+} Signaling Is Attenuated in the Transgenic Plants

A well-characterized downstream consequence of increased $InsP_3$ is the release of Ca^{2+} from intracellular stores. Transient and rapid increases in intracellular Ca^{2+} have been demonstrated in response to many abiotic stresses, including cold, salt, and osmotic stress (Knight et al., 1996, 1997a; Knight and Knight, 2001). We predicted that if $InsP_3$ is involved in mediating a stress-induced Ca^{2+} signal, then the transgenic plants would have impaired Ca^{2+} signaling in response to the stress. In order to test this hypothesis, we generated transgenic plants expressing the Ca^{2+} binding photoprotein aequorin (Knight et al., 1997b). Two independent aequorin-expressing control lines and two independent aequorin-expressing InsP 5-ptase lines were selected and tested for Ca^{2+} response. Seedlings were incubated in the substrate coelenterazine and then subjected to a salt or a cold stimulus (by injecting 250 mM NaCl or ice-cold water), and luminescence was measured in a luminometer. Luminescence counts were collected every 0.2 s and converted to Ca^{2+} concentration as described (Knight et al., 1997b). The peak Ca^{2+} response was reduced in the InsP 5-ptase lines compared with the control in response to a cold or a salt stimulus (Figure 7). The Ca^{2+} response measured in these experiments is a reflection of the stress-induced increase in intracellular Ca^{2+} as result of entry from the outside as well as release from intracellular stores. Since $InsP_3$ triggers the release of Ca^{2+} from intracellular stores, these experiments reveal the $InsP_3$-mediated component of Ca^{2+} release from intracellular stores. Based on our results, the $InsP_3$-mediated component of the Ca^{2+} signal in these plants is ~150 to 200 nM (Table 5) and constitutes ~30% of the total Ca^{2+} signal under these conditions.

In separate experiments, aequorin-expressing wild-type and transgenic seedlings were treated with 100 μM ABA. Although the whole seedling Ca^{2+} response to ABA was less dramatic than with the salt or cold stimulus, there was a similar ~30% decrease in the peak Ca^{2+} in the transgenic seedlings compared with the control (Table 5).

DISCUSSION

In order to examine the downstream consequences of a constitutive reduction in $InsP_3$, we generated transgenic *Arabidopsis* plants expressing the mammalian type I inositol phosphatase.

Table 3. Subset of DREB2A-Regulated Genes That Are Basally Upregulated in the Transgenic Plants

Locus Identifier	Fold Change	P	Predicted Function and Gene Name
At5g05410	3.20	0.0001	DRE binding protein (*DREB2A*)
At3g55940	2.47	0.0012	Phosphoinositide-specific phospholipase C, putative (*PLC7*)
At4g36010	2.46	0.0238	Pathogenesis-related thaumatin family protein
At1g09350	2.36	0.0231	Galactinol synthase, putative (*GolS3*)
At1g56600	2.24	0.0857	Galactinol synthase, putative (*GolS2*)
At3g50970	2.05	0.0756	Dehydrin xero2 (*XERO2*)/low temperature–induced protein (*LTI30*)

Subset of the basally upregulated genes in the two InsP 5-ptase transgenic lines (T6 and T8) compared with wild-type and C2 plants revealed by the microarray analysis. Data presented are from transgenic line T8. Transcripts were selected based on a greater than twofold increase ($P < 0.1$).

Table 4. Transcripts That Are Upregulated or Downregulated with Drought in Wild-Type and Transgenic Plants

Regulation	Wild Type/C2 Only	Common	Tr Only
Upregulated with drought greater than twofold	244	219	4
Downregulated with drought greater than twofold	183	161	22

This enzyme is specific for $InsP_3$ hydrolysis and is involved in terminating the $InsP_3$ signal in animal cells (Mitchell et al., 1996; De Smedt et al., 1997). In the transgenic plants, basal levels of $InsP_3$ were ~5% of wild-type values (Perera et al., 2006) and $InsP_3$ remained below basal wild-type values even in response to stress.

$InsP_3$-Mediated Ca^{2+} Signaling Is Attenuated in the InsP 5-ptase Plants

According to the signaling paradigm, the first documented downstream effect of $InsP_3$ is to trigger the release of Ca^{2+} from intracellular stores. Using aequorin-expressing seedlings, we demonstrated that the $InsP_3$-mediated Ca^{2+} signal is reduced in the transgenic plants compared with wild-type plants in response to salt, cold, or exogenously added ABA and that the $InsP_3$-mediated Ca^{2+} contribution is ~30%. Aequorin-based technology is best suited for monitoring changes in Ca^{2+} with fast kinetics on the order of seconds to minutes; therefore, it was not possible to monitor gradual changes that may occur over a period of days with a physiological drought stress. Nevertheless, it is clear from our results that Ca^{2+} signaling and homeostasis are subtly altered in the transgenic plants. The transgenic guard cells were less responsive to high Ca^{2+}, and there was a downregulation of genes involved in Ca^{2+} binding and transport.

ABA Responses Are Altered in the Transgenic InsP 5-ptase Plants

It is well established that Ca^{2+} is a key intermediary in some but not all of the ABA-regulated processes. ABA is known to inhibit K^+-inward rectifying channels, a process that also involves an increase in Ca^{2+} and inositol phosphates (Ng et al., 2001; Hetherington and Brownlee, 2004). The pH-induced anion efflux in guard cells, however, is independent of Ca^{2+}, and activation of guard cell anion efflux channels by the application of ABA to the cytosol was not preceded by an elevation of Ca^{2+} and could not be mimicked by inositol phosphates or cADP-ribose (Levchenko et al., 2005).

The transgenic InsP 5-ptase guard cells showed an impaired response to the ABA inhibition of stomatal opening compared with wild-type plants. The decreased response of the transgenic guard cells to ABA and to high Ca^{2+} may also be explained by the fact that $InsP_6$ is reduced, since $InsP_6$ is important for guard cell regulation (Lemtiri-Chlieh et al., 2000, 2003). Plants with reduced PLC were similarly insensitive to ABA in the stomatal opening assay (Mills et al., 2004). Taken together, these results support the speculation that PLC (and/or $InsP_3$ and $InsP_6$) may only be required for the ABA-mediated inhibition of the opening response (Mills et al., 2004).

By contrast, the stomatal closure response to exogenous ABA was enhanced in the InsP 5-ptase plants. This could enhance sensitivity in the drought response, even though in planta the endogenous ABA levels did not increase as rapidly with drought stress. It is clear that guard cell regulation is a complex process

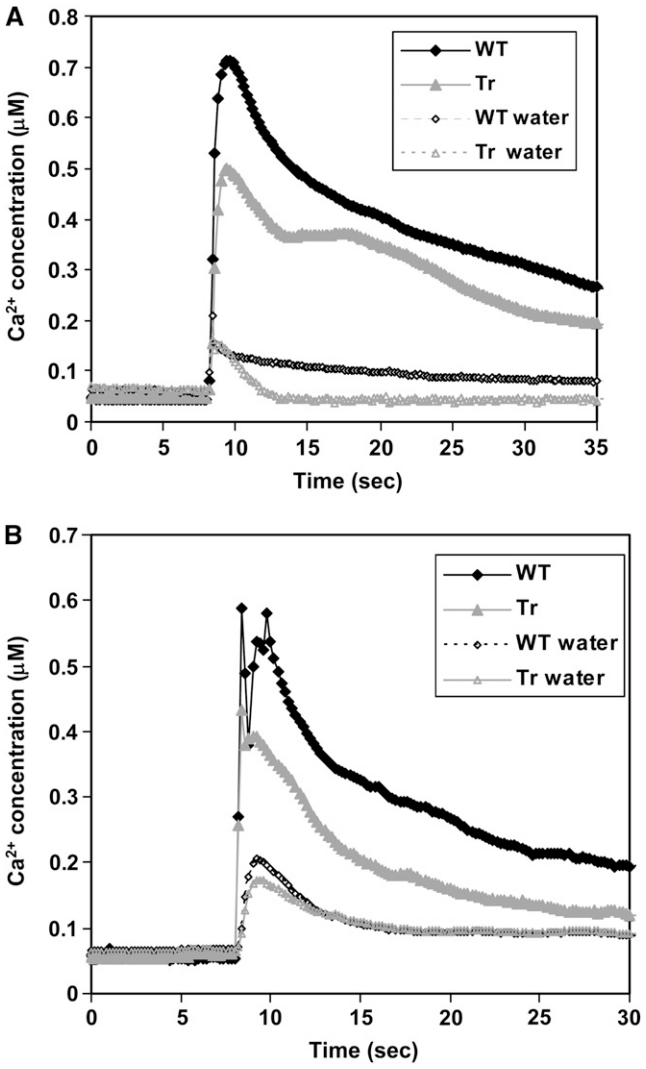

Figure 7. The InsP 5-ptase Plants Show an Attenuated Ca^{2+} Response to a Salt or a Cold Stimulus.

(A) Aequorin-expressing control (wild-type) and InsP 5-ptase (Tr) seedlings were treated with 0.25 M NaCl, and luminescence was measured in a luminometer.

(B) A similar experiment in which seedlings were treated with ice-cold water.

Representative data of salt- and cold-induced Ca^{2+} release are shown. Each trace is the average from three to four seedlings. The solid lines are traces from the stimulus, and the dotted lines are traces from adding water, which controls for mechanical or touch stimulation. The experiments were repeated four to five times with similar results.

Table 5. Peak Ca^{2+} Response to a Cold, Salt, or ABA Stimulus

Measurement	Cold[a]		Salt[b]		ABA[c]	
	Wild Type	Tr	Wild Type	Tr	Wild Type	Tr
Peak Ca^{2+} concentration (μM)	0.60 ± 0.1	0.35 ± 0.04	0.65 ± 0.04	0.48 ± 0.01	0.30 ± 0.02	0.22 ± 0.01
n	10	12	8	8	15	15

The average peak for the water control for the cold and salt experiments was 0.17 ± 0.02 μM, and the average peak for the solvent control for the ABA experiments (0.1% ethanol) was 0.11 ± 0.01 μM.

[a] Average ± SE of 10 to 12 experiments using two control (wild type) and two InsP 5-ptase (Tr) lines.

[b] Average ± SE of eight experiments using two control (wild type) and two InsP 5-ptase (Tr) lines.

[c] Average ± SE of 15 experiments using two control (wild type) and two InsP 5-ptase (Tr) lines.

involving several partially or completely independent pathways (Schroeder et al., 2001; Roelfsema and Hedrich, 2005; Israelsson et al., 2006; Li et al., 2006a) and that ABA-mediated opening and closure, although related, may be regulated via distinct mechanisms (Christmann et al., 2006; Li et al., 2006a).

With regard to ABA-mediated signaling, the induction of ABA-responsive genes with exogenous ABA treatment was comparable (if not stronger) in the InsP 5-ptase transgenic and wild-type plants. This is in contrast with plants overexpressing At5PTase1 and At5PTase2 (Sanchez and Chua, 2001; Burnette et al., 2003), which showed delayed or attenuated expression of select ABA-responsive genes.

We suspect that the unique features of the mammalian type I InsP 5-ptase enzyme used in our studies may help explain the differential responses observed with the InsP 5-ptase plants compared with the plant counterparts. Both At5PTase1 and At5PTase2 have been shown to hydrolyze the membrane phospholipids in addition to $InsP_3$ (Ercetin and Gillaspy, 2004), while the animal enzyme is specific for the soluble inositol phosphates such as $InsP_3$ (Majerus et al., 1999). Changes in stomatal aperture are regulated by volume changes of the guard cell, which could involve up to a twofold change in membrane surface area (Kubitscheck et al., 2000). This, in turn, would require significant reorganization of membrane lipids via vesicle secretion and endocytosis (Schroeder et al., 2001). Overexpressing At5PTases or decreasing production of PLC could have a negative impact on membrane biogenesis and, thereby, stomatal regulation. Guard cell responses are affected by PtdIns3P and PtdIns4P (Jung et al., 2002), and $PtdInsP_2$ was recently shown to be important for regulating stomatal opening (Lee et al., 2007). The reduced stomatal opening observed in the At5TPase1-overexpressing plants could in part be due to reduced levels of $PtdInsP_2$, although *Arabidopsis 5ptase1* null mutants do not show any increases in $PtdInsP_2$ levels (Gunesekera et al., 2007). The effect of increased $PtdInsP_2$ was evident in the *sac9* mutant (which is defective in a $PtdInsP_2$-hydrolyzing 5-phosphatase and overaccumulates $PtdInsP_2$), which shows a constitutive stress phenotype with closed stomata (Williams et al., 2005). $PtdInsP_2$ homeostasis is clearly important for normal growth and development in plants, and altering this balance can lead to severe consequences.

When comparing other methods for altering endogenous $InsP_3$, it is important to note that the inhibition of PLC will have far-reaching effects on phosphoinositide turnover in addition to reducing $InsP_3$, making it difficult to compare our results with those from studies in which $InsP_3$ is lowered by blocking PLC. Reduced PLC hydrolysis could lead to a buildup of $PtdInsP_2$ (unless the lipid phosphatases are activated) and would also have an impact on phosphatidic acid, either via diacylglycerol or by activating specific phospholipase D isoforms (Qin and Wang, 2002). Phosphatidic acid plays an important role in promoting ABA-mediated guard cell closure by the interaction with both ABI1, a negative regulator of ABA (Zhang et al., 2004, 2005), and Gpa1 (Mishra et al., 2006). Phosphatidic acid levels have been shown to increase in response to hyperosmotic stress (Munnik et al., 2000) as well as several other stresses (Wang, 2004; Testerink and Munnik, 2005), and blocking this increase may adversely affect the plant's ability to respond to stress.

Basal and Drought-Induced Transcriptional Changes in the InsP 5-ptase Transgenic Plants May Contribute to the Enhanced Drought Tolerance

The microarray results have revealed interesting and important differences in transcript abundance between the wild-type and transgenic plants both in basal levels and also in the response to drought stress. The most striking result in the basal comparison is the upregulation of *DREB2A* and the subset of *DREB2A*-regulated genes in the transgenic plants. DREB2A belongs to a family of AP2 domain–containing transcription factors. The DREB1/CBF group is involved in cold stress (Thomashow, 1999), while DREB2A and DREB2B have been implicated in responses to dehydration, salt, and, more recently, heat stress (Sakuma et al., 2006a, 2006b; Schramm et al., 2007). In early experiments (Liu et al., 1998), *DREB2A* expression was found to be highly induced by dehydration and salt treatment, but not by ABA, leading to the conclusion that DREB2A functions mainly in an ABA-independent drought stress–responsive pathway (Shinozaki and Yamaguchi-Shinozaki, 2000, 2007; Yamaguchi-Shinozaki and Shinozaki, 2006). DREB2A has been shown to bind to DRE elements in stress-responsive gene promoters; binding is regulated by a negative regulatory domain in the DREB2A protein (Sakuma et al., 2006b) and may also be influenced by phosphorylation (Agarwal et al., 2007). Although constitutive overexpression of specific DREB family transcription factors conferred improved stress tolerance, it caused severely retarded growth under normal conditions (Liu et al., 1998; Kasuga et al., 1999). However, if only a subset of these genes were upregulated, it might be possible to induce tolerance without impairing growth.

We propose that the constitutive dampening of the $InsP_3$ signal and the increased turnover of pathway intermediates could lead to the selective derepression of stress-induced transcripts and to increased stress tolerance without inhibiting growth under nonstressed conditions.

In the InsP 5-ptase plants, there were five basally upregulated genes (excluding *DREB2A*), and all of these contain a DRE binding motif in their promoter sequences (Sakuma et al., 2006b). Three of the genes (*XERO2*, *GolS2*, and *GolS3*) are implicated in osmoprotection and drought tolerance. Xero2 is a dehydrin, and galactinol synthase catalyzes the first committed step in the synthesis of the raffinose family of oligosaccharides (RFOs). RFOs are known to protect seeds during desiccation (Obendorf, 1997). In *Arabidopsis*, the At *GolS1*, *-2*, and *-3* genes are upregulated by drought and temperature stress (Taji et al., 2002; Nishizawa et al., 2006), and plants overexpressing At *GolS2* showed reduced transpiration, increased drought tolerance, and an increase in galactinol and raffinose sugars in leaves (Taji et al., 2002). Another factor that is critical for the production of RFOs is the availability of *myo*-inositol. Mutant soybean (*Glycine max*) having reduced *myo*-inositol had drastically reduced galactinol and RFOs (Hitz et al., 2002; Karner et al., 2004). It is possible that the increased hydrolysis of $InsP_3$ in the transgenic InsP 5-ptase plants could lead to an increased flux through this pathway, resulting in increased levels of RFOs. Preliminary work suggests that basal levels of raffinose and inositol are similar in InsP 5-ptase plants compared with wild-type plants (see Supplemental Table 9 online).

It is curious that a putative PLC gene (At *PLC7*) is upregulated, although this gene is not predicted to encode a functional protein (Hunt et al., 2004); therefore, the significance of this result is unclear at present. In addition to the six listed genes, two other DREB2A-regulated genes, *COR47* (At1g20440) and *LEA14* (At1g01470), were found to be expressed by >1.5 fold in the transgenic lines over the wild type and vector control ($P < 0.05$). Basal upregulation of select stress-responsive genes was found to be one of the critical differences between *Arabidopsis* and its halotolerant relative, *Thellungiella halophila* (Taji et al., 2004; Gong et al., 2005). It is likely that the basal upregulation of select DREB2A-regulated genes in the transgenic InsP 5-ptase plants contributes to their increased ability to withstand drought stress.

Comparison of the drought-stressed samples has also revealed some important insights. There were ~150 to 200 genes that were commonly upregulated or downregulated in both transgenic and wild-type plants. Many of these are consistent with other published studies and show similar patterns of expression with abiotic stress. The list of commonly upregulated transcripts from this study shares 75% overlap with a recently reported list of drought-induced genes in wild-type plants (Catala et al., 2007). These genes probably represent pathways and processes that are not altered in the InsP 5-ptase plants. In a comparison of three separate microarray studies on osmotic and drought stress, Bray (2004) found that there were only 27 commonly upregulated genes. Since the experimental methods and developmental stages of plants used varied between the experiments (Seki et al., 2001; Kreps et al., 2002; Kawaguchi et al., 2004), it is not surprising that this number is low. Interestingly, in this work, we found 15 of the common 27 genes upregulated in both the transgenic and wild-type/C2 plants, and 5 of these 15 transcripts showed a >10 fold increase in all plant lines with drought stress.

Further support for ABA-independent pathways contributing to drought tolerance in the transgenic plants comes from the observation that, of the genes that were uniquely upregulated in wild-type/C2, ~30% are known to be regulated by ABA (Li et al., 2006b) and many of these did not show any increase in the transgenic plants with drought stress. It is possible that the expression of these genes requires a certain threshold of ABA that has not been reached in the transgenic plants, or, alternatively, that induction of these specific genes is impaired in the transgenic plants. There were four genes that were uniquely upregulated by drought stress in the transgenic plants, and it will be interesting to determine if these genes have any common features and if their patterns of expression are similar in the transgenic plants when the drought stress is prolonged.

In related work, the constitutive expression of the InsP 5-ptase in tomato (*Solanum lycopersicum*) plants resulted in plants with increased drought tolerance (M. Khodakovskaya, C. Sword, I.Y. Perera, W.F. Boss, C.S. Brown, and H. Winter-Sederoff, unpublished data). In contrast with the *Arabidopsis* plants, the transgenic tomato plants have increased biomass and increased levels of soluble sugars, suggesting that basal metabolism is differentially sensitive to the decrease in $InsP_3$ in tomato versus *Arabidopsis*. The increase in soluble sugars seen in the InsP 5-ptase tomato plants may play a protective role during drought stress. Stress tolerance is often attributed to the ability to maintain higher levels of several metabolites, including hexose sugars, in both the presence and absence of stress (Bartels and Sunkar, 2005; Valliyodan and Nguyen, 2006). Because we did not detect comparable differences in soluble sugars in the InsP 5-ptase *Arabidopsis* plants under normal conditions (see Supplemental Table 9 online), we anticipate that the mechanisms of tolerance in the two plants are different. Further comparisons will be necessary to identify the $InsP_3$-mediated response in these two systems.

In summary, although constitutively decreasing $InsP_3$ was predicted to render plants more sensitive to drought stress, our findings indicate that decreasing $InsP_3$-mediated signaling leads to increased drought tolerance. The increased drought tolerance in the transgenic plants cannot be explained by the altered responses to ABA. Apart from the increased response to exogenously added ABA in the stomatal closure assay, the transgenic plants show reduced ABA levels with drought stress and lack of induction of several ABA-regulated genes compared with the wild type. However, the transgenic plants show a compensatory upregulation of an ABA-independent pathway involving DREB2A and a select subset of DREB2A-regulated genes. These results suggest that the coordinated regulation of these genes in the InsP 5-ptase plants contributes to conferring drought tolerance without adversely affecting plant growth. The selective regulation of this subset of the DREB2A pathway could be promising as a mechanism for increasing drought tolerance in crop plants.

METHODS

Plant Material and Growth Conditions

Wild-type (ecotype Columbia) and InsP 5-ptase transgenic *Arabidopsis thaliana* plants (Perera et al., 2006) were grown in the North Carolina State

University Phytotron in a growth chamber under short-day conditions (8 h of light/16 h of dark) at 21°C with a light intensity of ~150 μmol·m^{-2}·s^{-1}. Six-week-old, fully-grown plants (prior to bolting) were used for most experiments. For all soil-grown experiments, a large batch of soil mix (Promix PGX; Hummert International) was moistened well with water and the pots were filled with an equal amount of soil prior to planting the seeds. For experiments using seedlings, seeds were surface-sterilized as described previously (Perera et al., 2006) and stratified for 48 h at 4°C prior to plating on Murashige and Skoog medium (Caisson Labs) containing 1% sucrose and 0.8% agar type M (Sigma-Aldrich). Plates were incubated vertically in a growth chamber under short-day conditions as described above.

Measurements of Water Loss and Drought Stress

Leaf water loss was measured in fully expanded leaves of comparable size, weight, and development that were excised from the rosettes of well-hydrated wild-type and transgenic plants. The excised leaves (adaxial side up) were kept at 25°C room temperature in the light (30% humidity) for 1 to 2 h. Fresh weight of the leaves was monitored at 5-min intervals. After the water loss measurements, the surface area of the leaves was measured using a LI 3100 area meter (Li-Cor). For whole plant water loss measurements, plants were well watered at the start of the experiment and the surface of the pot was covered with plastic wrap to prevent any evaporation from the soil. Pots were weighed twice daily (in the morning just prior to when the lights came on in the chamber and immediately after the lights were turned off in the evening) for 6 d. Stomatal conductance was measured using an AP4 cycling porometer (Dynamax). At least three to four measurements were made per individual plant, and four to five plants per line were monitored. RWC was measured as described (Griffiths and Bray, 1996) using 6-week-old plants over a period of 8 d without water in a chamber with relative humidity of 30%. (RWC = [FW − DW]/[TW − DW], where FW = fresh weight of leaf, DW = dry weight of leaf, and TW = rehydrated weight of leaf after incubating in water overnight.) The average value for 10 separate plants per line is indicated at each time point.

Stomatal Bioassays

Epidermal peels were prepared from the abaxial surface of wild-type and transgenic *Arabidopsis* leaves in buffer (5 mM MES/KOH, pH 6.5, 50 mM KCl, and 0.1 mM $CaCl_2$) as described by Roelfsema and Prins (1995). Guard cells were observed using a 100×/1.25 oil objective on a Leica DMLS microscope. Aperture widths and lengths were measured, and stomatal apertures were determined as the ratio of width to length. For each treatment, ~45 measurements (from three separate peels) were collected, and each experiment was repeated at least three times. Peels were observed directly (after preparing peels from plants kept in the light) or incubated in the light for 3 h. For dark measurements, plants were kept in darkness for 3 h prior to making the epidermal peels. For Ca^{2+} treatment, peels were incubated for 3 h in the light in the buffer described above (0.1 mM Ca^{2+}) or in this buffer with no added Ca^{2+} or with 1 mM Ca^{2+}. To determine differences in ABA-mediated stomatal closure, peels were first incubated in the light for 3 h, and then 10 or 100 μM ABA (*cis*, *trans*-ABA from A.G. Scientific) was added and the peels were incubated in the light for 3 h. Data are shown as the difference between the width:length ratios of the final aperture after ABA treatment and the initial light value. The differences in inhibition of stomatal opening by ABA were determined using peels taken from plants that had been in the dark for 3 h and that were then incubated in the light for 3 h in buffer with or without 25 μM ABA.

Seed Germination Assays

Surface-sterilized, stratified seeds were plated on Murashige and Skoog plates containing a filter paper soaked in water or different concentrations of ABA as described (Weigel and Glazebrook, 2002). Germination was scored as the emergence of green cotyledons at 3 to 4 d after plating.

ABA Measurements and Sugar Analysis

ABA levels and soluble sugars were measured in leaf samples from the RWC time course. Leaves were frozen and ground in liquid N_2 and extracted in a 60:40 methanol:water solution. Samples were analyzed at the Metabolomics and Proteomics Laboratory at North Carolina State University. ABA was measured using a Thermo LTQ ion-trap mass spectrometer operating in electrospray ionization mode. Briefly, a 5-μL aliquot was injected onto a 150- × 2-mm Pursuit XRs column (Varian) equilibrated in 80:20 A:B, where A = 0.1% ammonium formate in water, pH 3.5, and B = acetonitrile, with a flow rate of 250 μL/min. A linear gradient to 65% B was utilized to elute ABA. Quantification was against an external standard (reserpine, 2 μM; Sigma-Aldrich), which was spiked into samples. Data were acquired in tandem mass spectrometry mode, ABA *m/z* 263 > total ion chromatogram; reserpine *m/z* 609.3 > total ion chromatogram; ABA negative ion mode, reserpine positive ion mode. Soluble sugars and inositol were analyzed by gas chromatography–mass spectrometry. Leaf tissue was ground in cold solvent, mixed with acetonitrile, and dried under vacuum. The sugars were converted to trimethylsilyl derivatives, and gas chromatography–mass spectrometry was performed using a ThermoTrace GC Ultra gas chromatograph coupled to a Thermo DSQ II mass spectrometer. The mass spectrometer was operated with an electron-impact source in positive mode monitoring *m/z* 191, 204, 217, 361, and 437. Quantitation was conducted by comparing peak areas obtained for trimethylsilyl derivatives of fructose, glucose, and sucrose in the samples with a series of reference standards analyzed concurrently, and data were processed using Thermo's Xcalibur software. Data presented are averages from three independent biological replicates.

Labeling Studies with [^{3}H]Inositol

One-week-old *Arabidopsis* seedlings (~10 seedlings per well) were transferred to a multiwell plate containing 800 μL of 0.5× Murashige and Skoog medium containing 45 μCi of [^{3}H]*myo*-inositol. Plates were incubated in a growth chamber under long-day conditions with gentle rotation to ensure aeration for 4 to 5 d. After incubation, the seedlings were quickly blotted on tissue and ground in liquid N_2. The frozen ground powder was incubated in 0.75 N HCl containing 0.2% phytate (as carrier) on ice for 20 min. After incubation, the samples were centrifuged and the supernatants were analyzed for inositol phosphates by anion-exchange chromatography as described by Stevenson-Paulik et al. (2005). Inositol phosphate species were identified based on the coelution of standard [^{3}H]Ins(1,4)P_2, [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(1,3,4)P_3, [^{3}H]Ins(1,3,4,5)P_4, [^{3}H]Ins(1,4,5,6)P_4, [^{3}H]Ins(1,3,4,6)P_4, [^{3}H]Ins(1,3,4,5,6)P_5, [^{3}H]Ins(1,2,4,5,6)P_5, [^{3}H]Ins(1,2,3,4,6)P_5, and [^{3}H]Ins(1,2,3,4,5,6)P_6. Standards were either purchased from Perkin-Elmer Life Sciences or generated as described (Stevenson-Paulik et al., 2002, 2005). Because many of the inositol phosphate isomers are in low abundance in ^{3}H-labeled *Arabidopsis* seedlings and cannot be easily resolved using the strong anion exchange column, peaks for [^{3}H]InsP_3 and [^{3}H]InsP_5 are reported here without specific isomer identification. It should be noted that [^{3}H]InsP_4 was not detectable above background in these samples. Epidermal peel fragments were incubated in guard cell incubation buffer (described above, containing 0.15 M mannitol) and 25 μCi of [^{3}H]*myo*-inositol for 18 h and processed as described above.

InsP_3 Assays

Epidermal peels were prepared as described above and incubated in buffer in the light. After ~2 h, peels were immediately frozen and ground

in liquid N_2 and assayed for Ins-1,4,5-trisphosphate content using the receptor binding kit (GE Healthcare) as described previously (Perera et al., 2006). For $InsP_3$ measurements after ABA treatment, seedlings were sprayed with either 100 μM ABA or water containing 0.1% ethanol as a solvent control. Samples were harvested at the indicated times, frozen immediately in liquid N_2, and assayed for $InsP_3$ content. The cross-reactivity of the bovine brain extract for other $InsP_3$ stereoisomers or $InsP_4$ or $InsP_5$ isomers is <1.0% and <0.03% for $InsP_6$, according to the manufacturer's specifications (http://www.gehealthcare.com/lifesciences). With plant extracts, >90% of the $InsP_3$ content measured with this assay can be removed by treatment with the type I InsP 5-ptase, indicating that the data reported reflect total cellular Ins-1,4,5-P_3 (Perera et al., 2001).

Aequorin Measurements

Wild-type *Arabidopsis* and InsP 5-ptase plants (lines T6 and T8) were stably transformed with *Agrobacterium tumefaciens* containing the pGIF2-35SAq plasmid (a kind gift from Marc and Heather Knight). Transgenic plants expressing aequorin were selected by RT-PCR and maintained until the T3 or T4 generation. At least two to three aequorin-expressing control and InsP 5-ptase lines were selected, and extracts were tested for aequorin luminescence by in vitro reconstitution as described (Knight et al., 1997b). For stimulation experiments, aequorin-expressing control and InsP 5-ptase seedlings were first incubated overnight in the luminophore (2.5 μM coelenterazine; Nanolight Technology) in the dark with gentle shaking in order to reconstitute functional aequorin. Individual seedlings were transferred to a cuvette, and luminescence was monitored in a Sirius single-tube luminometer (Berthold Detection Systems). The salt stimulus (final concentration of 0.25 M NaCl) or a water control was injected at ~10 s, and the total reconstitutable Ca^{2+} was discharged at ~70 s by a second injection of 2 M $CaCl_2$ in 20% ethanol. Luminescence was monitored at 0.2-s intervals and converted to Ca^{2+} concentration as described (Knight et al., 1996, 1997b). Each trace is the average from three seedlings. For the cold stimulus, ice-cold water was injected at ~10 s followed by the Ca^{2+} discharge at 70 s. For ABA treatments, ABA (final concentration of 100 μM in 0.1% ethanol) was injected at ~10 s followed by the Ca^{2+} discharge at 70 s as described above. The data presented in Table 5 are compiled from two independent aequorin-expressing control lines (wild type) and two independent aequorin-expressing InsP 5-ptase lines (one each of T6 and T8; denoted Tr).

Microarray Analysis

For the microarray analysis, leaf samples (pooled from 10 plants per line) were collected on day 0 and day 7 of the RWC time course and frozen immediately in liquid N_2. Three biological replicates were performed for the wild type and two independent transgenic lines (T6 and T8) as well as two biological replicates for the vector control (C2). RNA was isolated using the Plant RNeasy kit (Qiagen), and biotinylated target cRNA was synthesized using the two-cycle target-labeling protocol (Affymetrix). *Arabidopsis* arrays (ATH1 from Affymetrix) were hybridized, and data acquisition and analysis were performed by Expression Analysis using the Affymetrix fluidics station and GCOS software.

Statistical Analysis of Microarray Data

For the two-group analyses, an estimate of signal for each transcript was calculated based on the Microarray Suite 5.0 algorithm (Affymetrix), and raw fold change for each transcript was calculated by taking the simple ratio of the geometric means of the signal values for each respective group (i.e., 0 versus D). For the wild type, T8, and T6, which were represented by three arrays per group, differential expression was determined using a robust implementation of permutation testing as described at the Expression Analysis website (http://www.expressionanalysis.com/pdf/PADE_technote.pdf).

In brief, a modified t-statistic (Di) was calculated for each transcript when comparing groups, and a difference (Δ) was computed between Di and the average or expected t-statistic ordered values from a reference distribution (D[i]), calculated by computing all possible random permutations of our samples. A list of differentially expressed transcripts was then created by selecting for transcripts with an estimated absolute raw fold change of ≥2. The drought-responsive differential expression lists were further selected based on a false discovery rate of ≤5% (Pawitan et al., 2005; Clarke and Zhu, 2006). For the vector control C2 (which had two biological replicates), we used a cutoff of $P < 0.05$ (comparison 6). For the basal comparisons between the transgenic lines versus the wild type and vector control, where the differential expression lists were limited in size, we used a cutoff of fold change ≥ 2 and $P < 0.1$ (Tables 2 and 3; see Supplemental Tables 1 and 2 online). Results for several of the genes showing differential basal expression were confirmed using qRT-PCR as described below. To determine the common patterns of expression between the wild type, the vector control, and the two transgenic lines with drought stress, we generated four-way Venn diagrams using the four-way Venn diagram generator at http://www.pangloss.com/seidel/Protocols/venn4.cgi.

RNA Isolation, RT-PCR, and qRT-PCR Analysis

RNA was isolated from harvested leaves using the plant RNeasy Mini kit (Qiagen) with the on-column RNase-free DNase I treatment. The first-strand cDNA was synthesized from total RNA using either the StrataScript QPCR cDNA synthesis kit (Stratagene) or the High-Capacity cDNA Archive kit (Applied Biosystems) and random primers. For RT-PCR, cDNAs were amplified using HotStar Taq DNA Polymerase (Qiagen) and gene-specific primers (see Supplemental Table 10 online). For qRT-PCR, the cDNAs were amplified using specific primers and the Full Velocity SYBR-Green QPCR Master Mix (Stratagene) on the Mx3000P thermocycler (Stratagene). Gene-specific primers for select genes were designed with the help of AtRTPrimer, a database for generating specific RT-PCR primer pairs (Han and Kim, 2006), and are shown in Supplemental Table 10 online. PCR was optimized, and reactions were performed in triplicate. The transcript level was standardized based on cDNA amplification of reference genes such as *ACTIN2/8* and *UBIQUITIN10*. Relative gene expression data was generated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using either the wild type as the reference (for basal expression comparisons) or the 0 time point of each plant line as the reference (for drought-induced expression).

Accession Numbers

Sequence data from the genes studied in this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *RAB18*, At5g66400; *KIN2*, At5g15970; *COR15a*, At2g42540; *ERD1*, At5g51070; *ACTIN2*, At3g18780; *UBIQUITIN10*, At4g05320; *CRT3*, At1g08450; *BiP3*, At1g09080; *PR-1*, At2g14610; *DREB2A*, At5g05410; *Xero2*, At3g50970; *GolS2*, At1g56600; *GolS3*, At1g09350; *PLC7*, At3g55940; *Thm*, At4g36010; *LEA2g*, At2g42560; *OXG2*, At5g43450; *ACS2*, At1g01480; *AGP1*, At5g64310; *TCH4*, At5g57560.

Supplemental Data

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

Supplemental Figure 1. Comparison of Root Growth in Drought-Stressed Plants.

Supplemental Figure 2. Detection of InsP 5-ptase Protein in Epidermal Peels.

Supplemental Figure 3. Basal ABA Levels in Wild-Type and Transgenic Plants.

Supplemental Figure 4. Recovery of Transgenic Plants after Rewatering.

Supplemental Figure 5. GO Annotation for Transcripts Commonly Upregulated and Downregulated (Greater Than Twofold) with Drought.

Supplemental Table 1. Transcripts Showing Reduced Basal Expression in InsP 5-ptase Plants Compared with the Wild Type and C2 (62 Transcripts).

Supplemental Table 2. Transcripts Showing Increased Basal Expression in InsP 5-ptase Plants Compared with the Wild Type and C2 (20 Transcripts).

Supplemental Table 3. Transcripts Upregulated (Greater Than Twofold) with Drought in the Wild Type and C2 Only (244 Transcripts).

Supplemental Table 4. Transcripts Upregulated with Drought (Greater Than Twofold) in InsP 5-ptase Plants Only (Four Transcripts).

Supplemental Table 5. Transcripts Downregulated (Greater Than Twofold) with Drought in the Wild Type and C2 Only (183 Transcripts).

Supplemental Table 6. Transcripts Downregulated with Drought (Greater Than Twofold) in InsP 5-ptase Plants Only (22 Transcripts).

Supplemental Table 7. Transcripts Commonly Upregulated (Greater Than Twofold) with Drought (219 Transcripts).

Supplemental Table 8. Transcripts Commonly Downregulated (Greater Than Twofold) with Drought (161 Transcripts).

Supplemental Table 9. Comparison of Basal Levels of Soluble Sugars and Inositol in Wild-Type and Transgenic (T8) Plants.

Supplemental Table 10. Primers Used for RT-PCR and qRT-PCR.

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Transgenic *Arabidopsis* Plants Expressing the Type 1 Inositol 5-Phosphatase Exhibit Increased Drought Tolerance and Altered Abscisic Acid Signaling

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