

Arabidopsis PLC1 Is Required for Secondary Responses to Abscisic Acid Signals

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The role of inositol 1,4,5-trisphosphate (Ins[1,4,5]P₃) in transducing the abscisic acid (ABA) signal during seed germination and in the stress responses of mature plants is poorly understood. We have considered the contributions of the phospholipase C1 (encoded by *AtPLC1*) and an Ins(1,4,5)P₃ 5-phosphatase (encoded by *AtIP5P1I*) to ABA signaling by using a modified version of the glucocorticoid-inducible system to regulate transgene expression. In the presence of the dexamethasone (Dex) inducer, transgenic lines expressing the *AtPLC1* antisense and *AtIP5P1I* sense transgenes showed no inhibition of germination and growth by ABA, whereas in the absence of the inducer they were sensitive. In the presence of Dex, these lines accumulated lower Ins(1,4,5)P₃ levels upon ABA treatment compared with that of the control transgenic lines. RNA gel blot analysis revealed a decrease in the induction of the ABA-responsive genes *RD29a*, *KIN2*, and *RD22* but not *COR47* in the Dex-induced transgenic plants. In transgenic lines expressing the inducible *AtPLC1* sense transgene, an increase in *AtPLC1* expression was not sufficient to activate the expression of ABA-responsive genes in vegetative tissues. In vitro experiments demonstrated the induced PLC1 expression when extracts were assayed in the presence of calcium, but no increase in Ins(1,4,5)P₃ levels in vivo was detected, suggesting that the PLC1 enzyme was latent. Our results indicate that although an increase in PLC1 activity and increased Ins(1,4,5)P₃ levels are necessary for maximal gene induction by ABA, overexpression of *AtPLC1* itself is not sufficient to trigger the expression of ABA-responsive genes. We propose that *AtPLC1* plays a role in secondary ABA responses.

INTRODUCTION

The phytohormone abscisic acid (ABA) regulates seed maturation and maintains seed dormancy. In adult plants, ABA acts as a negative regulator of growth by controlling the adaptive responses of many physiological and developmental processes to environmental stresses such as cold, drought, and high salinity (Giraudat, 1995; reviewed in Merlot and Giraudat, 1997). At the physiological level, this stress hormone is responsible for decreasing the turgor of the guard cells that surround the stomatal pore, resulting in a reduction of pore size and an increase in water retention (MacRobbie, 1998). These adaptive developmental responses are supported in large part by changes in the transcription of a set of genes triggered by ABA (Shinozaki and Yamaguchi-Shinozaki, 2000). Transduction of the ABA signal for gene expression (Wu et al., 1997) and for stomatal closure (Gilroy et al., 1990; McAinsh et al., 1990; Schroeder and Hagiwara, 1990) has been shown to require an increase in the cytosolic Ca²⁺ concentration.

Ca²⁺ is a major component in many signaling pathways in

animal and plant systems, and it is thought that information is encoded in the period and amplitudes of Ca²⁺ waves (reviewed in Berridge, 1993; Clapham, 1995). In animal cells, there are two major mechanisms for the release of Ca²⁺ from internal stores. The first is the inositol 1,4,5-trisphosphate (Ins[1,4,5]P₃)-dependent mobilization of Ca²⁺ by activation of the Ins(1,4,5)P₃ receptor, and the second is the cyclic ADP ribose (cADPR)-mediated release of stored Ca²⁺ via the ryanodine receptor (Lee and Aarhus, 1991; Galione, 1993; Lee et al., 1995). Ca²⁺ also can activate the ryanodine receptor to release Ca²⁺ from internal stores by a process called Ca²⁺-induced Ca²⁺ release (Clapham, 1995). Several studies in animal cells suggest that cADPR, Ins(1,4,5)P₃, and Ca²⁺ may participate in the regulation of Ca²⁺ oscillations by determining the onset, persistence, and intensity of a signal (Clapham, 1995; Cancela et al., 1998, 2000; Trewavas, 1999). In the case of plant cells, ABA treatment has been shown to cause a transient increase in the levels of cADPR (Wu et al., 1997) and Ins(1,4,5)P₃ (Lee et al., 1996). However, the relative importance of the two compounds in mediating calcium release and ABA responses has not been addressed.

We have shown previously that cADPR can activate the expression of two ABA-responsive fusion genes, *RD29a-GUS* and *KIN2-GUS*, in single cells of tomato and that this activation

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is mediated by calcium (Wu et al., 1997). Moreover, in Arabidopsis plants, cADPR levels increased after ABA treatment and before changes in transcription of ABA-responsive genes. Using the same single-cell assay, we showed that $\text{Ins}(1,4,5)\text{P}_3$ also can activate the expression of *RD29a-GUS* and *KIN2-GUS* (Wu et al., 1997) and that this activation is sensitive to heparin, a specific inhibitor of $\text{Ins}(1,4,5)\text{P}_3$ -gated channels. In contrast, ABA induction of these two promoters is insensitive to heparin (Wu et al., 1997), suggesting that $\text{Ins}(1,4,5)\text{P}_3$ is not absolutely required for the induction of these two ABA-responsive genes. This observation is consistent with the observation that transcript levels of the Arabidopsis (*AtPLC1*) gene are low or undetectable in untreated plants and are induced upon ABA treatment (Hirayama et al., 1995). Together, these results suggest that cADPR mediates the primary ABA response and that *AtPLC1* and $\text{Ins}(1,4,5)\text{P}_3$ may be involved as a secondary signal to potentiate the primary ABA response.

To expand our knowledge of the role of $\text{Ins}(1,4,5)\text{P}_3$ in the transduction of the ABA signal during germination and in the stress response of mature plants, we have used a modified version of the glucocorticoid-inducible system for regulated gene expression in plants (Aoyama and Chua, 1997). We have investigated the contributions of phospholipase C1 (encoded by *AtPLC1*) as well as an $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase (encoded by *AtIP5PII*) to ABA signaling. Transgenic plants expressing these inducible genes, in either the sense or the antisense orientation, were generated and analyzed.

RESULTS

We have used a modified version of the two-component glucocorticoid-inducible system (Aoyama and Chua, 1997) for the present work. Figure 1A shows the T-DNA region of

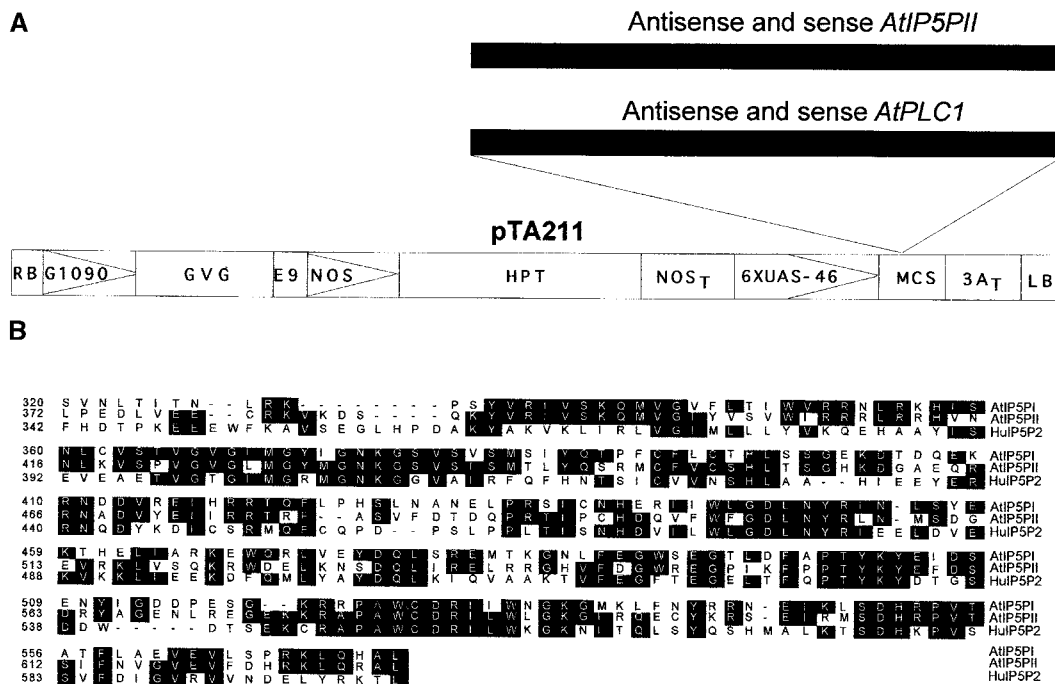


Figure 1. Structures of the Transgenic Constructs and Alignment of the $\text{Ins}(1,4,5)\text{P}_3$ 5-Phosphatase Amino Acid Sequences. **(A)** Structure of the pTA211 plasmid with the left and right T-DNA borders. *AtPLC1* and *AtIP5PII* cDNAs were cloned in both the sense and antisense orientations into the multiple cloning site of the binary plasmid pTA211, which is a modification of the original pTA7001 vector (Aoyama and Chua, 1997) (see Methods). RB, right T-DNA border; G1090, G10-90 promoter (Ishige et al., 1999); GVG, glucocorticoid-inducible chimeric transcription factor; E9, pea *rbcs-E9* polyadenylation sequence; NOS, nopaline synthetase promoter; HPT, hygromycin phosphotransferase coding sequence; NOS_T, nopaline synthetase polyadenylation sequence; 6XUAS-46, GVG-regulated promoter; MCS, multiple cloning site; 3A_T, *rbcs-3A* polyadenylation sequence; LB, left T-DNA border. **(B)** Alignment of the conserved amino acid sequences of the human $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase type II (HuIP5P2; Jefferson and Majerus, 1995) and Arabidopsis AtIP5PI and AtIP5PII. Identical amino acids are shaded in black. Alignment was performed using Clustal methods with the DNASTAR (Madison, WI) program.

pTA211, in which two series of constructs were made containing the *AtPLC1* and *AtIP5PII* coding sequences in either the sense or the antisense orientation. pTA211 encodes a glucocorticoid-regulated transcription factor, GVG (for Gal4-VP16-glucocorticoid binding domain; Aoyama and Chua, 1997). The GVG fusion protein is composed of a Gal4 DNA binding domain, a transactivating domain from the herpes simplex virus VP16 protein, and the hormone binding domain of the rat glucocorticoid receptor (Aoyama and Chua, 1997). GVG is expressed from the constitutive G10-90 promoter (Ishige et al., 1999), which has higher expression levels than does the cauliflower mosaic virus 35S promoter used in the previous pTA7001 version. The GVG fusion protein mediates dexamethasone (Dex)-inducible transcription of promoters containing Gal4 upstream activation sequence (or UAS) (Aoyama and Chua, 1997; McNellis et al., 1998).

Arabidopsis cDNAs encoding *AtPLC1* (GenBank accession number D38544) and Ins(1,4,5)P₃ 5-phosphatase (*AtIP5PII* cDNA; GenBank accession number AF289634) were obtained from Dr. K. Shinozaki (Tsukuba Life Science Center of Plant Molecular Biology, Ibaraki, Japan) and Dr. W. Gruissem (Swiss Federal Institute of Technology, Zurich, Switzerland), respectively. Arabidopsis *AtPLC1* has been previously characterized by Hirayama et al. (1995). Analysis of the putative coding sequence of *AtIP5PII* indicates an extensive homology (57%) with the human Ins(1,4,5)P₃ 5-phosphatase type II (Jefferson and Majerus, 1995) in the catalytic domain (Figure 1B). We expressed Arabidopsis *AtIP5PII* in *Escherichia coli*, purified it as a His-tagged protein, and assayed the purified protein for 5-phosphatase activity. The *AtIP5PII* protein had Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ 5-phosphatase activities 100-fold greater than those of a control protein (glutathione S-transferase; Table 1). No 5-phosphatase activity was found by using phosphatidylinositol 4, 5 bisphosphate as a substrate.

Six to eight independent transgenic lines were produced for each construct. All lines were selected for hygromycin resistance, and several T3 homozygous lines were selected for further studies. As controls, a number of transgenic lines were generated containing the pTA211 vector alone. In this work, we focused on the analysis of transgenic plants containing the *AtPLC1* sense and antisense transgenes and the *AtIP5PII* sense transgene.

Recently, the pTA7001 Dex-inducible transcription system has been found to cause growth defects in certain Arabidopsis transgenic lines (Kang et al., 1999; T. Aoyama and N.-H. Chua, unpublished data) and to induce defense-related genes such as *PDF1.2*. Most of the transgenic plants (ecotype Columbia or C24) carrying the G10-90 GVG transgene from the new pTA211 vector did not display any growth defects or unusual phenotypes upon Dex induction. In the experiments of Kang et al. (1999), plants were grown in liquid medium with constant agitation, whereas in our experiments, plants were grown on plates and induced for 1 to 24 hr by using a hydroponics system. We found that GVG transcript levels did not vary significantly between transgenic lines and did not correlate with morphological defects

Table 1. Inositol 5-Phosphatase Activity of the *AtIP5PII* Gene Product^a

Protein	PIP ₂ ^b	Ins(1,4,5)P ₃	Ins(1,3,4,5)P ₄
Control (GST) ^c	0.0	0.01	0.01
His- <i>AtIP5PII</i>	0.0	2.50	1.20

^a *AtIP5PII* was expressed in *E. coli* as a His-tagged protein. Values shown are nanomoles hydrolyzed per 50 ng of protein.

^b PIP₂, phosphatidylinositol 4, 5 bisphosphate.

^c GST, glutathione S-transferase.

in some transgenic plants. Induction of *PDF1.2* was observed rarely in the transgenic lines (1 of 15) and did not correlate with Dex induction. However, we found that improper handling of plants induced *PDF1.2* transcript expression (data not shown).

When treated with Dex, transgenic lines carrying the pTA211 vector alone did not display any induction or repression in the presence of ABA of >20 genes (*ABI1*, *ABI2*, *COR47*, *COR15a*, *COR15b*, *CDP kinase*, *CDP synthase*, *4-5IP kinase*, *IP5PI*, *IP5PII*, *KIN1*, *KIN2*, *PLC1*, *PLC2*, *RAB18*, *RD22*, *RD29a*, *RAC1*, *PDF1.2*, *PR1*, and genes encoding ζ-crystallin) (data not shown).

Dex Induction of the *AtPLC1* Antisense and *AtIP5PII* Sense Transcripts in Transgenic Plants

Transgenic lines containing the *AtPLC1* antisense transgene, the *AtIP5PII* sense transgene, and the pTA211 vector alone were analyzed by RNA gel blotting after treatment with either Dex or ABA or both. The vector control line and the uninduced transgenic lines had low levels of *AtPLC1* transcript in the absence of ABA (Figure 2A, lane ABA-0). After a 4-hr exposure to ABA, however, these lines showed a fivefold induction of the endogenous *AtPLC1* transcript (Figure 2A, lane ABA-4). Although a 20-hr exposure to Dex did not affect the basal expression of *AtPLC1*, Dex-treated transgenic *AtPLC1* antisense lines did not show an increase in *AtPLC1* transcript levels after a subsequent 4-hr incubation with ABA (Figure 2A, lanes DEX-24 and DEX-24/ABA-4). Under our experimental conditions, the *AtPLC1* probe hybridized specifically with the *AtPLC1* but not the *AtPLC2* transcript.

Two transgenic lines expressing *AtIP5PII* in the sense orientation displayed the normal *AtIP5PII* expression profile, as did a vector control line in the absence of Dex (Figure 2B). In all of these plants, the *AtIP5PII* transcript level was not affected by ABA. However, a 24-hr incubation with Dex resulted in a significant (sixfold) increase in *AtIP5PII* transgene expression levels, which were not affected significantly by ABA treatment (Figure 2B, lanes DEX-24 and DEX-24/ABA-4). Under our experimental conditions, the *AtIP5PII* probe

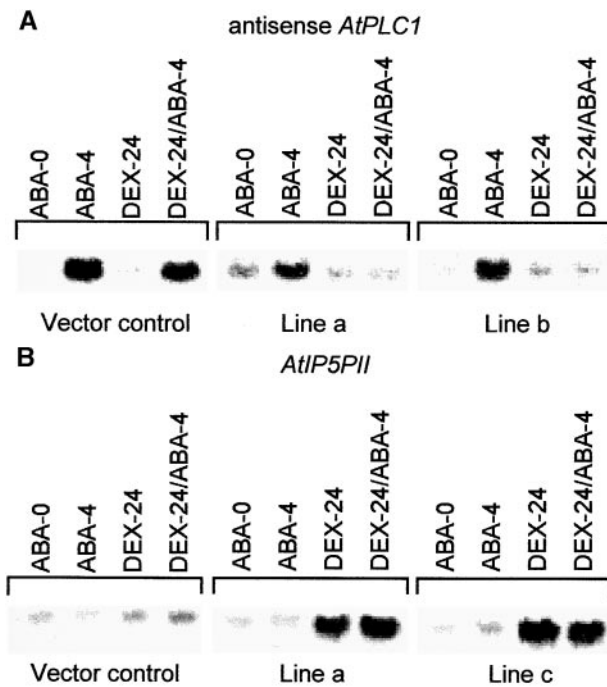


Figure 2. RNA Gel Blot Analysis of Transgenic Lines Expressing *AtIP5P1I* Sense and *AtPLC1* Antisense Transgenes.

For each construct, two independent lines were selected, grown, and treated as described in Methods.

(A) Induction of the *AtPLC1* transcript in two *AtPLC1* antisense lines and a vector control transgenic line. ABA-0, plants collected at 0 hr in the ABA medium; ABA-4, plants incubated with ABA (50 μ M) for 4 hr; DEX-24, plants incubated with Dex (30 μ M) for 24 hr; DEX-24/ABA-4, plants incubated with Dex (30 μ M) for 24 hr followed by an additional 4 hr with ABA (50 μ M). For details, see Methods.

(B) Induction of the *AtIP5P1I* transcript in two *AtIP5P1I* sense transgenic lines and a control transgenic line containing the pTA211 vector. Lanes are the same as in **(A)**.

hybridized specifically with the *AtIP5P1I* but not the *AtIP5PI* transcript. These results indicate that antisense *AtPLC1* transgenic plants showed greatly diminished *AtPLC1* expression in response to ABA. Moreover, expression of the transgenic *AtIP5P1I* transcript can be induced by Dex.

Effect of ABA on Transgenic Seed Germination

In addition to mediating stress responses in adult plants, ABA also inhibits seed germination. Screening for mutants insensitive to ABA in a germination assay has yielded several mutants altered in components of the ABA signal transduction pathway (Giraudat, 1995). To determine whether the transgenic plants display altered ABA signaling, we tested their ABA sensitivity during germination. Seed from transgenic lines containing an *AtIP5P1I* sense construct and an *AtPLC1* antisense construct were germinated on 2 μ M ABA in the presence and absence of Dex (Table 2). Seed from transgenic lines carrying the *AtPLC1* antisense transgene displayed 93% germination in the presence of Dex, whereas only 6% of the *AtPLC1* antisense plants germinated in the absence of Dex (Table 2). In comparison, 2 to 3% germination was observed in vector control lines and in transgenic lines carrying an inducible *AtPLC1* sense transgene with or without Dex (Table 2). As expected, the ABA-insensitive mutant *abi1* was unaffected by the presence of Dex and germinated at a frequency of 95% (Table 2). Similarly, 95% of seed of transgenic lines containing the *AtIP5P1I* sense transgene germinated in the presence of the Dex; in the absence of inducer, however, germination was inhibited by ABA (Table 2). No effects on germination were observed in several antisense *AtIP5P1I* lines (Table 2). There was no significant difference in the germination frequencies of the different transgenic lines in the presence or absence of Dex without ABA (data not shown). At higher concentrations of ABA (2 to 10 μ M), the *AtIP5P1I* sense and *AtPLC1* antisense lines in the presence of Dex were also insensitive to ABA with a 40 to 90% germination frequency (see Figures 3A to 3C).

We next examined the effects of ABA on growth in the *AtIP5P1I* sense, *AtPLC1* antisense, and vector control transgenic lines after 16 days of growth under normal conditions in the presence and absence of Dex. A marked difference was observed in the growth of *AtIP5P1I* sense and *AtPLC1* antisense lines. In the presence of Dex, the plants grew normally, but almost no growth was detected in the absence of the chemical inducer (Figure 3D). These results suggest that ABA inhibition of germination and seedling growth can be overcome in lines expressing *AtPLC1* antisense or *AtIP5P1I* sense transgenes.

Table 2. Germination Frequency of Transgenic Lines in the Presence of ABA^a

	Vector Control	<i>AtPLC1</i>		<i>AtIP5P1I</i>		<i>abi1-1</i>
		Antisense	Sense	Antisense	Sense	
+Dex	1.9 (0.6)	93.3 (5.2)	2.4 (1.0)	1.8 (0.7)	94.9 (4.1)	95.9 (8.1)
-Dex	2.5 (0.9)	6.5 (1.5)	1.5 (0.5)	2.9 (1.0)	2.1 (0.8)	93.2 (6.5)

^a Germination was measured in the presence of 2 μ M ABA ($n = 200$ to 300 seeds). Values shown are % germination, expressed as average value (standard deviation).

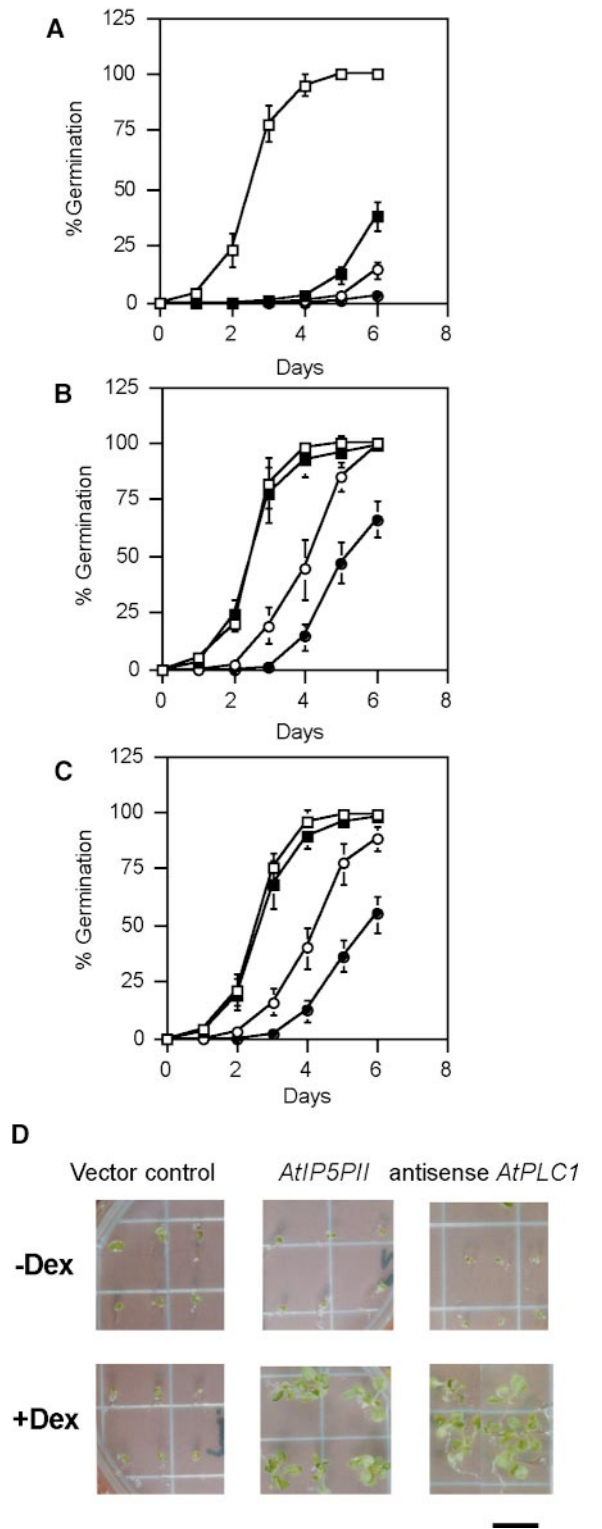


Figure 3. Effects of ABA on the Germination and Growth of Transgenic Plants.

(A) to (C) Seeds from transgenic lines carrying the pTA211 vector

Analysis of ABA-Responsive Gene Expression in Transgenic Plants Carrying *AtPLC1* Antisense and *AtIP5P1I* Sense Transgenes

The ABA insensitivity of transgenic plants containing either the *AtPLC1* antisense or the *AtIP5P1I* sense transgene prompted us to analyze the expression of ABA-responsive genes in these plants. Induction of *RD29a*, *KIN2*, and *RD22* (Giraudat, 1995) by ABA was normal in vector control transgenic lines and *AtPLC1* antisense and *AtIP5P1I* sense transgenic lines not treated with Dex (Figure 4). However, in the presence of Dex, although the accumulation of these transcripts was still responsive to ABA, it was threefold to sevenfold lower in the *AtPLC1* antisense and *AtIP5P1I* sense transgenic lines than in the vector control lines (Figure 4). In contrast, *COR47* expression was unaffected by Dex in either the *AtPLC1* antisense and *AtIP5P1I* sense transgenic lines or the vector control transgenic lines. These results show that in the presence of Dex, induction of the ABA-responsive genes *RD29a*, *KIN2*, and *RD22* was diminished in transgenic lines containing either the *AtPLC1* antisense transgene or the *AtIP5P1I* sense transgene. Analysis of the *AtIP5P1I* antisense transgenic lines revealed no effects on the expression of ABA-responsive genes (data not shown). Together, our results suggest that a decrease in *AtPLC1* levels or an increase in *AtIP5P1I* levels interferes with normal ABA signaling, leading to attenuated gene expression.

Analysis of *Ins(1,4,5)P3* Levels in Transgenic Plants in Response to ABA

To determine whether expression of the *AtPLC1* antisense gene and the *AtIP5P1I* sense genes led to the expected changes in *Ins(1,4,5)P3* levels, we measured the latter in transgenic lines treated with ABA in the presence and absence of Dex. Treatment of the vector control transgenic line with 30 μM ABA resulted in a 3.5-fold increase in *Ins(1,4,5)P3* levels after 1 hr (Figure 5A). Although a small increase in the levels of *Ins(1,4,5)P3* was detected in transgenic plants expressing the *AtPLC1* antisense and *AtIP5P1I*

(A), from transgenic lines carrying the *AtPLC1* antisense gene (B), and from transgenic lines carrying the *AtIP5P1I* sense gene (C) were incubated on plates containing 0 μM (open squares), 2.5 μM (closed squares), 5 μM (open circles), and 10 μM (closed circles) ABA under normal light conditions (see Methods) for 1 to 6 days in the presence of Dex (30 μM). Germination frequency was measured by scoring for radicle emergence. Average values ±SD are shown for three independent lines of each construct. Approximately 100 to 150 seeds of each line were plated in duplicates.

(D) Transgenic plants were grown in ABA (2 μM) for 18 days in the absence (-) or presence (+) of Dex (30 μM). Representative plants of three lines for each transgenic construct tested and a vector control transgenic line are shown. Bar = 3.2 mm.

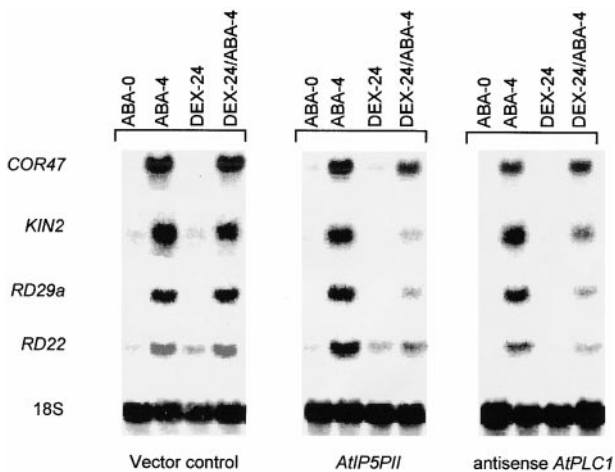


Figure 4. Induction of ABA-Responsive Genes in Transgenic Plants Expressing the *AtPLC1* Antisense and *AtIP5P11* Sense Transgenes.

Two independent lines for each construct were grown on plates for 2 weeks, and the seedlings were transferred to a hydroponic medium (Aoyama and Chua, 1997). After 2 days, the medium was replaced with fresh medium containing either Dex (30 μM) or ABA (50 μM) or both. Total RNA was isolated at various times (0, 4, and 24 hr), and RNA gel blots were hybridized with *RD29a*, *KIN2*, *RD22*, and *COR47* probes and 18S rDNA. The vector control line contained the pTA211 plasmid alone. For details, see Methods. ABA-0, plants collected at 0 hr in the ABA medium; ABA-4, plants incubated with ABA (50 μM) for 4 hr; DEX-24, plants incubated with Dex (30 μM) for 24 hr; DEX-24/ABA-4, plants incubated with Dex (30 μM) for 24 hr followed by an additional 4 hr with ABA (50 μM).

sense transgenes, the levels were significantly lower than the levels in vector control plants. The reduced accumulation of $\text{Ins}(1,4,5)\text{P}_3$ in ABA-treated transgenic lines expressing the *AtPLC1* antisense transgene likely was due to a decrease in PLC1 activity in vivo.

We analyzed the $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase activity in total extracts prepared from *AtIP5P11* sense transgenic lines after Dex induction. A fourfold to fivefold increase in phosphatase activity was found after 20 hr of induction with Dex (Figure 5B), suggesting that an active *AtIP5P11* was produced. The increase of the $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase would result in the hydrolysis of $\text{Ins}(1,4,5)\text{P}_3$ that accumulated in response to ABA, thus leading to lower steady state levels.

Analysis of ABA-Responsive Gene Expression in Transgenic Plants Carrying an Inducible *AtPLC1* Sense Transgene

To further examine the contribution of PLC1 to ABA signaling, we analyzed the transgenic lines containing the *AtPLC1* sense transgene and the pTA211 vector alone by RNA gel

blotting after treatment with Dex. *AtPLC1* transgenic transcript was detected after 3 hr of Dex induction and reached a maximal level at 6 hr, whereas no transcript was detected in the vector control line (Figure 6A). Biochemical experiments showed that 3 to 6 hr after the induction, there was a threefold to fourfold increase in phospholipase C activity when the extracts were assayed in the presence of calcium in vitro (Figure 6C). However, almost no increase in $\text{Ins}(1,4,5)\text{P}_3$ levels in vivo was detected in these transgenic plants (Figure 6D). As expected, a vector control transgenic line showed no increase in PLC1 activities or $\text{Ins}(1,4,5)\text{P}_3$ levels after Dex treatment. Notwithstanding the increase in Dex-induced *PLC1* expression, the transgenic lines did not express any of the ABA-responsive genes (*KIN2*, *RD29a*, or *RD22*) even after 20 hr of induction (Figure 6A). Moreover, the induction of *KIN2* and *RD29a* by ABA was normal in the presence or absence of Dex in the *AtPLC1* sense transgenic lines (Figure 6B).

A vector control line showed similar levels of ABA-induced expression of *KIN2* and *RD29a*. In addition, the transgenic lines were neither insensitive (Table 2) nor hypersensitive (data not shown) to ABA during germination. Together, these results suggest that in the absence of ABA, an increase in *AtPLC1* expression is not sufficient to cause an increase in $\text{Ins}(1,4,5)\text{P}_3$ levels and to induce the expression of ABA-responsive genes in vegetative tissues. It is likely that the expressed *AtPLC1* enzyme is latent because it requires a calcium signal for activation in vivo. Furthermore, the increase in *AtPLC1* apparently did not interfere with the induction of *KIN2* and *RD29a* by ABA.

DISCUSSION

The Dex-inducible system (Aoyama and Chua, 1997) can be used for the regulated expression of transgenes in plants and is a useful tool to probe the function of proteins in plant signaling pathways. We have used a modified version of this system to generate a series of Arabidopsis transgenic lines carrying cDNAs encoding either the phospholipase C1 (*AtPLC1*) transgene or the $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase (*AtIP5P11*) transgene in the sense and antisense orientations. The germination, biochemical, and molecular phenotypes of the transgenic lines reported here are gene specific and completely dependent on whether the cDNA is expressed in the sense or the antisense orientation.

Decreased Expression of *AtPLC1* and Increased Expression of *AtIP5P11* Reduce $\text{Ins}(1,4,5)\text{P}_3$ Levels in ABA-Treated Plants

RNA gel blot analysis showed that ABA induction of the endogenous *AtPLC1* in the transgenic lines carrying the *AtPLC1* antisense transgene was decreased markedly only

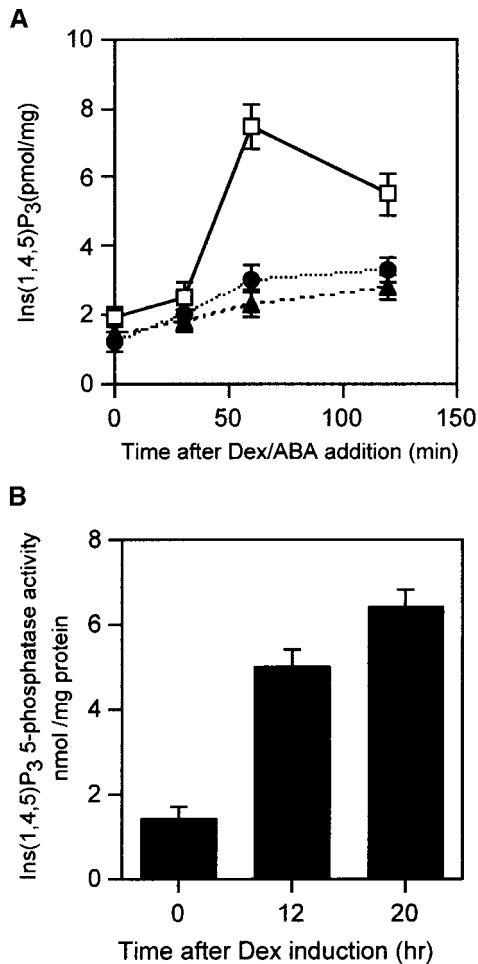


Figure 5. Analysis of Ins(1,4,5)P₃ Levels and Ins(1,4,5)P₃ 5-Phosphatase Activity in Transgenic Lines.

(A) Changes in Ins(1,4,5)P₃ levels in transgenic lines expressing the *AtPLC1* antisense and *AtIP5P1I* sense transgenes in response to ABA. Fourteen-day-old transgenic seedlings were transferred to a hydroponic system (Aoyama and Chua, 1997) for 2 days before the addition of fresh medium containing Dex (30 μ M) or ABA (50 μ M) or both. Plants were collected at 0, 30, 60, and 120 min after ABA addition, and the amount of Ins(1,4,5)P₃ was determined as described in Methods. Vector control transgenic plants contained the pTA211 plasmid alone. Open squares, vector control line with ABA (50 μ M); closed circles, *AtPLC1* antisense line with Dex (30 μ M) and ABA (50 μ M); closed triangles, *AtIP5P1I* sense line with Dex (30 μ M) and ABA (50 μ M). Mean values \pm SD are provided for three independent induction experiments. **(B)** Ins(1,4,5)P₃ 5-phosphatase activity in transgenic lines expressing the Dex-responsive *AtIP5P1I* sense transgene. Fourteen-day-old transgenic seedlings were transferred to a hydroponic system (Aoyama and Chua, 1997) for 2 days before the addition of fresh medium containing Dex (30 μ M). Plants were collected from different lines at 0, 14, or 20 hr after Dex addition, and Ins(1,4,5)P₃ was assayed as described in Methods. Vector control lines displayed only background levels upon the addition of Dex (0-hr time point; data not shown). Each bar represents the average value of three independent induction experiments with standard deviations.

after Dex-induced expression. The Dex induction of the *AtPLC1* antisense transgene did not affect *AtPLC2* expression levels, indicating specificity (data not shown). Upon Dex treatment, the Ins(1,4,5)P₃ levels in *AtPLC1* antisense transgenic lines showed only a small increase in response to ABA (Figure 4A). These results demonstrate a decrease in ABA-induced PLC1 enzymatic activity as well as in Ins(1,4,5)P₃ levels in these transgenic lines.

In the *AtIP5P1I* sense transgenic lines, the Dex-induced increase in *AtIP5P1I* transcript levels was accompanied by a corresponding increase in specific Ins(1,4,5)P₃ 5-phosphatase activity. When treated with ABA, these plants showed only a small increase in Ins(1,4,5)P₃ levels compared with that of vector control transgenic lines (Figure 5A). These results suggest that the increase in the Ins(1,4,5)P₃ 5-phosphatase activity in *AtIP5P1I* sense transgenic lines most likely is responsible for the reduced Ins(1,4,5)P₃ levels.

AtPLC1 Expression Is Necessary but Not Sufficient for the Maximal Induction of ABA-Responsive Genes

Analysis of ABA-responsive gene expression in Dex-induced adult transgenic lines carrying the *AtIP5P1I* sense and *AtPLC1* antisense genes revealed lower levels of *KIN2*, *RD29a*, and *RD22* induction by ABA, whereas *COR47* was induced normally. In addition, we found that the *abi1-1* mutant was impaired in the ABA induction of these three genes but not *COR47* (J.-P. Sanchez and N.-H. Chua, unpublished results), suggesting that the action of the two transgenes is specific to the ABI1-mediated ABA signaling pathway. Our results indicate that increases in the PLC1 and Ins(1,4,5)P₃ levels are required for maximal induction of a subset of genes by ABA.

Transgenic lines containing the *AtPLC1* sense transgene displayed induction of the *AtPLC1* transgenic transcript only upon the addition of Dex, but no induction in the expression of *KIN2*, *RD29a*, and *RD22* was observed in the Dex-treated lines. Therefore, in the absence of ABA, the expression of *AtPLC1* itself is not sufficient to trigger expression of the three ABA-responsive genes. At different times after Dex induction, extracts prepared from the transgenic plants showed an increase in PLC1 activity when assayed in vitro in the presence of calcium, demonstrating the expected induction of the enzyme. However, there was no difference in the Ins(1,4,5)P₃ levels between *AtPLC1* sense and vector control transgenic lines. These results can be explained if the expressed PLC1 is latent and requires an increase in cytosolic Ca²⁺ for activation.

We also examined the induction of the *AtPLC1* transgene in the presence of Dex and ABA. We found that in the *AtPLC1* sense transgenic lines, the induction of *KIN2* and *RD29a* by ABA was similar to that in a vector control line and was not affected by Dex. These results suggest that *AtPLC1* expression apparently does not interfere with the ABA induction of *KIN2* and *RD29a*.

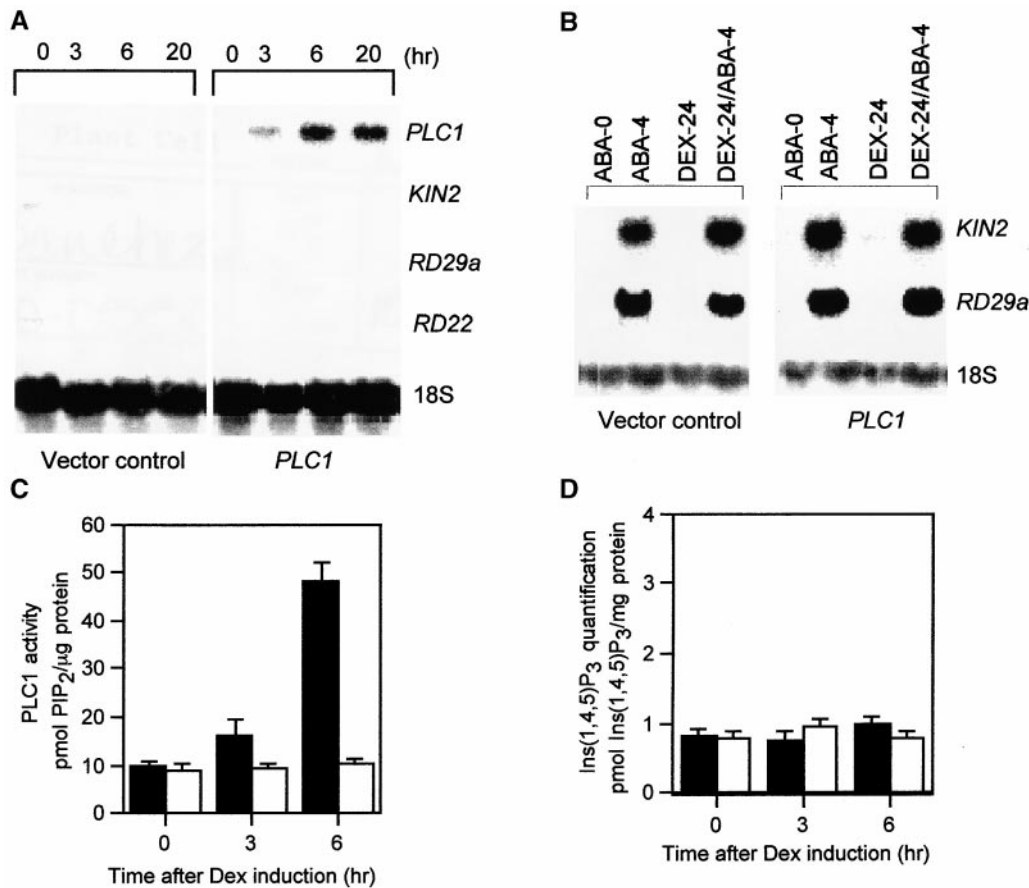


Figure 6. Molecular and Biochemical Analysis of Transgenic Lines Expressing the Dex-Inducible *AtPLC1* Sense Transgene.

(A) Dex induction of the *AtPLC1* transgenic transcript in *AtPLC1* sense lines and in a vector control transgenic line. Plants were collected at 3, 6, and 20 hr after treatment with Dex (30 μ M), and the expression of *PLC1*, *KIN2*, *RD29a*, and *RD22* probes and 18S rDNA was examined by RNA gel blot hybridization.

(B) Transgenic lines containing the *AtPLC1* sense transgene were grown on plates for 2 weeks, and the seedlings were transferred to a hydroponic medium (Aoyama and Chua, 1997). After 2 days, the medium was replaced with fresh medium containing either Dex (30 μ M) or ABA (50 μ M) or both. Total RNA was isolated at various times (0, 4, and 24 hr), and the gel blots were hybridized with *RD29a* and *KIN2* probes and 18S rDNA. Vector control transgenic lines contained the pTA211 plasmid alone. For details, see Methods. ABA-0, plants collected at 0 hr in the ABA medium; ABA-4, plants incubated with ABA (50 μ M) for 4 hr; DEX-24, plants incubated with Dex (30 μ M) for 24 hr; DEX-24/ABA-4, plants incubated with Dex (30 μ M) for 24 hr followed by an additional 4 hr with ABA (50 μ M).

(C) and **(D)** Seedlings of a transgenic line containing the inducible *AtPLC1* sense transgene were grown and treated with Dex as described in **(B)**, except that plants were collected at 0, 3, and 6 hr after treatment. Total protein extracts were assayed for PLC1 enzyme activity **(C)**, and the amount of Ins(1,4,5)P₃ was determined **(D)**. Each bar represents the average value of three independent induction experiments with standard deviations. For details, see Methods. Solid bars, transgenic line with the inducible *AtPLC1* sense gene; open bars, vector control transgenic line. PIP₂, phosphatidylinositol 4, 5 bisphosphate.

We propose that the Ca²⁺ signal required for activation of the expressed latent PLC1 is induced by cADPR, which shows a rapid and transitory increase upon ABA treatment and likely mediates the primary ABA response (Wu et al., 1997). This notion is consistent with the observation that the expression of *AtPLC1* itself is induced by ABA (Hirayama et al., 1995). The subsequent activation of *AtPLC1* and in-

crease in Ins(1,4,5)P₃ levels are needed as secondary signals to amplify the primary response and to maximize the induced gene expression. In previous single-cell experiments, the induction of *RD29a-GUS* and *KIN2-GUS* by cADPR was observed, and this was likely to be the primary ABA response. In pancreatic acinar cells treated with the hormone cholecystokinin, mobilization of calcium from the

endoplasmic reticulum and amplification of the calcium signal are mediated by cADPR and nicotinic acid adenine dinucleotide phosphate, and $\text{Ins}(1,4,5)\text{P}_3$ participates only as a terminal amplifier of the calcium wave signal (Cancela et al., 2000).

AtPLC1-Dependent Events Are Required for Sensitivity of Germination and Seedling Growth to ABA

To date, limited information is available concerning the molecular events that take place during Arabidopsis seed germination other than the fact that ABA and gibberellic acid (GA) have opposing effects. The germination of Arabidopsis seed is sensitive to ABA, suggesting that an ABA signaling pathway operates in seed to block this process. This observation has been used to identify ABA signaling components through the isolation and characterization of mutants insensitive to ABA. In principle, the inducible expression of antisense and sense transcripts encoding a positive regulatory component of the ABA signaling pathway should phenocopy loss-of-function and gain-of-function mutations, respectively, in the pathway. A well-known regulator of the ABA signaling pathway is *ABI1*, a protein phosphatase 2C. A dominant mutation (*abi1-1*) in this phosphatase can render plants insensitive to ABA (Giraudat, 1995). We have observed that transgenic plants expressing *abi1-1* can become insensitive to ABA, whereas transgenic plants expressing *ABI1* have the same sensitivity as wild-type plants (Y. Wu, J.-P. Sanchez, and N.-H. Chua, unpublished results).

Transgenic seeds were germinated in 2 μM ABA in the presence or absence of Dex to examine the effect of regulated transgene expression on ABA signaling pathways that operate during germination. In the case of the *AtPLC1* transgenic plants, only lines expressing the antisense transgene were able to germinate in the presence of Dex (93%), whereas in the absence of Dex almost no germination was observed. In the absence of Dex, 3% germination was observed in the *AtIP5PII* sense transgenic lines, compared with the 95% germination frequency observed in the presence of the inducer. No ABA insensitivity was seen in lines expressing *AtIP5PII* in the antisense orientation.

Figure 3D shows that growth of *AtIP5PII* sense and *AtPLC1* antisense transgenic plants in the presence of ABA (without Dex) was arrested for >18 days, whereas in the presence of Dex they were able to grow. Furthermore, after 2 to 3 weeks of incubation in the presence of Dex, *AtIP5PII* sense and *AtPLC1* antisense transgenic lines showed no growth inhibition when challenged subsequently with ABA. Mild inhibition was observed if the plants were first germinated (4 to 5 days) in the absence of ABA and subsequently transferred to medium containing 2 μM ABA (previously induced for 24 hr in Dex), suggesting that the major effect occurs during germination (data not shown). These results also suggest that the effects exerted by ABA during germination are mediated by PLC1 and increased $\text{Ins}(1,4,5)\text{P}_3$ levels. Similar experiments were performed with transgenic plants

harboring the antisense *AtPLC2* transgene, which encodes a phospholipase C2 (Hirayama et al., 1997). Seed of these transgenic lines, however, displayed no ABA insensitivity during germination, suggesting that the effect seen with *AtPLC1* is specific to the PLC1 gene product (data not shown).

Analysis of *AtPLC1* sense transgenic seed germinated in 2 μM ABA in the presence or absence of Dex to examine the effect of regulated transgene expression on the ABA signaling pathway revealed neither a hypersensitivity nor an insensitivity to ABA (hypersensitivity, data not shown; see Table 2 for insensitivity).

During Arabidopsis seed germination, it is possible that the ABA signal is mediated by a Ca^{2+} -dependent pathway acting in parallel with the GA signal. Studies of barley aleurone cells suggest that GA stimulates Ca^{2+} production in an $\text{Ins}(1,4,5)\text{P}_3$ -independent manner and that ABA inhibits this response (reviewed in Lovegrove and Hooley, 2000). Furthermore, ABA has been postulated to mediate the repression of an α -amylase through a Ca^{2+} -dependent pathway (Chen, 1997; Lovegrove and Hooley, 2000). How these ABA signaling events in barley aleurone cells relate to the Arabidopsis system remains to be determined.

METHODS

Reagents

AtPLC1 (GenBank accession number D38544) and *AtPLC2* (GenBank accession number D50804) cDNAs were kindly provided by Dr. K. Shinozaki. *AtIP5PI* (GenBank accession number AF289633) and *AtIP5PII* (GenBank accession number AF289634) cDNA were provided by Dr. W. Gruissem. *KIN2* and *RD29a* cDNAs were obtained as expressed sequence tag (EST) clones (Arabidopsis Information Management System [AIMS] accession numbers 103216T7 and ATT53026; Arabidopsis Biological Resource Center [ABRC], Ohio State University, Columbus). *COR47* and *RD22* were amplified by polymerase chain reaction (PCR) from a cDNA library provided by Dr. Qi Xie (Institute of Molecular Agrobiolology, Singapore), with primers flanking the coding sequence. The 18S rDNA probe was obtained from Dr. Taku Takahashi (Department of Biology, Hokkaido University, Sapporo, Japan). The authenticity of the probes was verified by sequencing.

Cloning of *AtIP5PI* and *AtIP5PII* cDNAs

An EST database search with sequences from the human inositol 1,4,5-trisphosphate ($\text{Ins}[1,4,5]\text{P}_3$) 5-phosphatase revealed two different groups of *Arabidopsis thaliana* ESTs that contain the highly conserved domain for $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase. The EST used for the isolation of the *AtIP5PII* full-length clone was AIMS accession number ATT0437 obtained from ABRC. A cDNA fragment of 1.2 kb was excised with EcoRI and XhoI and used as a probe for cDNA library screening. A similar procedure was used to isolate a full-length cDNA of *AtIP5PI* by use of the EST clone AIMS accession number ATT04703. The EST was used as a probe to screen an Arabidopsis cDNA library CD4-15 (ABRC) to obtain full-length cDNAs for *AtIP5PI* and *AtIP5PII*.

Transformation Constructs

A modified version of the binary transformation plasmid pTA7001 containing the complete two-component Gal4-VP16-glucocorticoid binding domain (GVG) (Aoyama and Chua, 1997) was used. In contrast to pTA7001, which was derived from pBI101, the new plasmid, pTA211 (P. Spielhofer and N.-H. Chua, unpublished results), is derived from a pPZP vector (Hajdukiewicz et al., 1994); in addition, the cauliflower mosaic virus 35S promoter for transcription of the GVG fusion gene was replaced by the G10-90 promoter (Ishige et al., 1999). The pTA211 plasmid was digested with XhoI and SpeI (New England BioLabs, Beverly, MA). The coding region of the *AtIP5P1I* gene was engineered by PCR to have a XhoI restriction site at the 5' end and a SpeI site at the 3' end. The sequences of the forward and reverse primers were 5'-GGCTCGAGATGGTATTCGCGGCCGCGT-TTGACTCTCC-3' and 5'-GCAC TAGTTCAAAAAGAAGGTT CAGGA-TGAACAGCAGAA-3', respectively. For the *AtIP5P1I* antisense construct, the same gene-specific sequences were used as primers, except that the restriction sites were switched. The *AtPLC1* antisense construct was engineered by PCR to have a XhoI site at the 3' end and a SpeI site at the 5' end by using PCR amplification. The sequences of the forward and reverse primers were 5'-GGCTCG-AGCTAACGAGGCTCCAAGACAAACCGCATGAGC-3' and 5'-GGA-CTAGTATGATATGTTGTGTAAGAACTTCAAGGTGA-3', respectively. For the *AtPLC1* sense construct, the same primers were used, except that the restriction sites were switched. The PCR-amplified DNA fragment was digested with XhoI and SpeI, gel purified using the Qiaquick Gel extraction protocol (Qiagen, Valencia, CA), and ligated into the pTA211 plasmid by use of T4 DNA ligase (New England BioLabs). A clone for each construct was verified by restriction analysis. All constructs were introduced into *Agrobacterium tumefaciens* strain ABI (Aoyama and Chua, 1997).

Plant Transformation

Arabidopsis plants (ecotypes C24 and Landsberg) were transformed with *Agrobacterium* by use of the vacuum infiltration procedure (Bent et al., 1994). The C24 line contains a transgene consisting of the ABA-responsive *KIN2* promoter fused to the coding sequence of firefly luciferase (Foster and Chua, 1999). Seed collected from the vacuum-infiltrated plants were surface sterilized by treating them with a solution of 1.5% sodium hypochlorite and 0.01% Tween 20 (Sigma) for 10 min and washed three times with sterile water. The sterilized seed were then resuspended in 0.1% agarose and sown in Petri dishes containing medium A (full-strength Murashige and Skoog [1962] salts, pH 5.7, and 1% sucrose solidified with 0.8% Bactoagar [Gibco BRL, Grand Island, NY]) and 20 μ g/mL hygromycin B (Sigma). The plated seed were vernalized for 4 days and then transferred to a growth chamber maintained at 22°C under long-day conditions (16 hr of light/8 hr of dark). Transgenic T1 seedlings were selected on a plate containing hygromycin (20 mg/mL), and after 2 to 3 weeks of growth, the presence of the transgene was confirmed by PCR analysis.

Dexamethasone Treatments

ABA (Sigma) was dissolved in 100% methanol to make a 100 mM stock solution, and dexamethasone (Dex; Sigma) was dissolved in DMSO to make a 100 mM stock solution. Both solutions were stored at -20°C. To monitor transgene expression, we surface sterilized

transgenic seed and sowed them in Petri dishes as described above. After vernalization at 4°C for 4 days, the plates were incubated for 2 weeks in a growth chamber maintained at 22°C under long-day conditions (16 hr of light/8 hr of dark). Seedlings were removed from the plates and grown for 2 days in a hydroponic system containing liquid medium A (full-strength Murashige and Skoog salts, pH 5.7, and 1% sucrose; Gibco BRL). Fresh medium containing either Dex (30 μ M) or ABA (50 μ M) or both was added, and plants were removed at the designated times and then washed and frozen in liquid nitrogen. For the vector control transgenic lines, identical conditions were used, and the experiments were performed in parallel.

RNA Analysis

Total RNA was isolated from seedlings and adult plants using the Qiagen RNA purification kit. RNA gel blot analysis was performed according to Ausubel et al. (1994). Each lane contained 10 μ g of total RNA. *KIN2*, *RD29a*, *COR47*, *RD22*, and 18S rDNA fragments were obtained by PCR amplification with Pfu polymerase (*Pyrococcus furiosus*; Stratagene, La Jolla, CA) as described above. For the *AtPLC1* and *AtIP5P1I* genes, we used the coding region or the 3' noncoding sequence as a probe. Fragments were purified using the Qiaquick Gel extraction protocol (Qiagen). All DNA fragments were labeled with ³²P-dCTP and ³²P-dATP by random priming (Amersham, Arlington Heights, IL). Hybridization signals were quantified using the PhosphorImager STORM system (Molecular Dynamics, Sunnyvale, CA), and the data were analyzed with the Image Quant program (version 1.1).

Quantification of Ins(1,4,5)P₃

Twenty-day-old plants treated with ABA (50 μ M) and Dex (30 μ M) as described above were collected and frozen rapidly in liquid nitrogen. Frozen tissue (including stem, roots, and leaves) were ground to a fine powder with a porcelain mortar and pestle precooled in liquid nitrogen. The frozen powder was resuspended in 0.2 volume of ice-cold 20% perchloric acid and incubated on ice for 20 min. Extracts were sonicated for 10 sec to disrupt any aggregates, and samples were purified as described by the manufacturer of the Ins(1,4,5)P₃ assay system kit (Amersham) except that we used a Partisil 5 SAX column (Whatman) (Shears, 1997). Ins(1,4,5)P₃, Ins(1,4)P₂, and Ins(1,3,4,5)P₄ standards were prepared according to the manufacturer's specifications (Sigma).

Inositol Polyphosphate 5-Phosphatase Purification and Assay

A full-length cDNA of *AtIP5P1I* was cloned in the vector pET28a (Novagen, Madison, WI) and transformed into *Escherichia coli* BL21-Codon Plus (DE3) (Stratagene, La Jolla, CA). The pET28a vector without an insert served as a control. *E. coli* cells were grown at 37°C overnight. A 0.5-mL overnight culture was used to inoculate 100 mL of 1 \times Luria-Bertani medium, and the culture was induced at 37°C for 2 hr with a final concentration of 0.1 mM isopropyl- β -D-thiogalactopyranoside (Sigma). The culture was grown for another 2 hr at 24°C. Cells were harvested by centrifugation, and the pellets were resuspended in ice-cold bacterial lysis buffer (50 mM phosphate buffer, pH 7.4, 100 mM NaCl, and 100 μ g/mL lysozyme). Bacterial cells were lysed by sonication, and the cell lysate was centrifuged at 15,000g for 30 min at 4°C. The supernatant was collected, and the

recombinant protein was purified as recommended by the manufacturer (Histrap kit; Amersham Pharmacia Biotech) except that a chromatography system (Perfusion Chromatography System; Biocad-Sprint, Cambridge, MA) was used with the column. The protein was incubated at 37°C for 30 min with 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM MgCl₂, and ³H-labeled Ins(1,4,5)P₃ or Ins(1,3,4,5)P₄ (10 μM ³H, 0.01 μCi) in 50 μL. The reaction was initiated by the addition of the substrate and terminated by the addition of 1 mL of distilled water followed by immediate heating at 100°C for 3 min (Connolly et al., 1985). After cooling, the samples were applied to a Sep-Pak Accell Plus QMA cartridge (Waters; Millipore, Milford, MA) and eluted (Maslanski and Busa, 1990). One-milliliter fractions were collected, and the radioactivity was determined by scintillation counting. Samples were then analyzed by HPLC using a Partisil 5 SAX column (250 × 4.6 mm) according to the procedure described by Shears (1997).

Phospholipase C Assay

Twenty-day-old plants were treated with Dex (30 μM) as described above, collected, and frozen rapidly in liquid nitrogen. Extracts were prepared as described (Deutscher, 1990), and phosphatidylinositol 4, 5 bisphosphate hydrolysis was assayed according to Hirayama et al. (1995).

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