

Trivalent Ions Activate Abscisic Acid-Inducible Promoters through an *ABI1*-Dependent Pathway in Rice Protoplasts¹

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The plant hormone abscisic acid (ABA) mediates many vital processes in plant growth and development, including seed dormancy, cell division, water use efficiency, and adaptation to drought, salinity, chilling, pathogen attack, and UV light. Our understanding of ABA signal transduction is fragmentary and would benefit from specific and facile probes of the process. Protoplasts from rice (*Oryza sativa* L. cv IR54) embryonic suspension cultures cotransformed with effector plasmids encoding the maize (*Zea mays*) *VIVIPAROUS1* cDNA and/or the Arabidopsis dominant negative mutant (*abi1-1*) *ABA-insensitive* cDNA demonstrated genetic interactions of *VIVIPAROUS1* and *abi1-1* in transactivation of the ABA-inducible *HVA1* promoter from barley (*Hordeum vulgare*), suggesting the mechanisms of these effectors are conserved among monocots and dicots. Trivalent ions have been shown to act as an effector of gene expression in plants and animals, although the mechanism of action is unknown. We show in two complementary transient ABA-inducible gene expression assays (β -glucuronidase and luciferase enzymatic activities and quantitative flow cytometry of green fluorescent protein) that trivalent ions specifically interact with an *ABI1*-dependent ABA-signaling pathway leading to gene expression. Trivalent ions mimic ABA effects on gene expression and may be a useful tool to study ABA signaling.

Abscisic acid (ABA) acts via multiple pathways, for example by inducing rapid closure of stomatal pores by ion efflux from guard cells and by slower changes in gene expression. Despite the complex multitude of data (physiological, molecular, genetic, biochemical, and pharmacological) that implicate ABA in stress responses, the adaptive responses of plants to ABA, stresses, and the pathways that trigger them are largely unknown (Grill and Himmelbach, 1998; Hetherington et al., 1998; Leung and Giraudat, 1998).

Rapid progress in understanding ABA signaling has been made with reverse genetic approaches to reconstruct minimal cascades leading to gene expression (for review, see Shen and Ho, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Busk and Pagès, 1998). Sheen (1996) showed in maize (*Zea mays*) protoplasts that exogenous calcium and/or overexpressed protein kinases from Arabidopsis could activate the ABA- and stress-inducible barley (*Hordeum vulgare*) *HVA1* promoter. In barley aleurone, an ABA-inducible protein kinase and phospholipase D activity have been implicated in ABA-regulated gene repression (Ritchie and Gilroy, 1998; Gómez-Cadenas et al., 1999). A cADP-Rib-dependent ABA-signaling pathway involving intracellular calcium and protein phosphorylation has

been shown to exist by micro-injection experiments in tomato hypocotyl cells (Wu et al., 1997). cADP-Rib has recently also been shown to mediate rapid ABA-induced stomatal closure in *Commelina communis* guard cell protoplasts (Leckie et al., 1998), suggesting that rapid ABA responses at the plasma membrane and slower nuclear responses may share some common intermediates such as calcium transients.

Genetic studies in the model organisms maize and Arabidopsis have resulted in the identification of mutants altered in ABA- and environmental stress signaling (McCarty, 1995; Bonetta and McCourt, 1998; Grill and Himmelbach, 1998; Foster and Chua, 1999; Lee et al., 1999). Several ABA-signaling genes have been cloned and shown to encode protein phosphatases, transcription factors, and a subunit of farnesyl transferase (Cutler et al., 1996; Finkelstein et al., 1998; Luerksen et al., 1998). The *ABA INSENSITIVE1* (*ABI1*) and *ABI2* ABA response genes encode homologous protein Ser/Thr phosphatases that act as negative regulators of ABA sensitivity (Leung et al., 1997; Rodriguez et al., 1998; Sheen, 1998; Gosti et al., 1999). The *VIVIPAROUS1* (*VP1*) gene of maize is orthologous to *ABI3* of Arabidopsis and encodes a DNA-binding protein (Carson et al., 1997; Suzuki et al., 1997). Although the exact molecular mechanisms of these signaling effectors are not known, VP1 potentiates ABA-inducible gene expression by remodeling of chromatin architecture and forms a DNA binding complex with 14-3-3 and basic Leu zipper proteins (Schultz et al., 1998; Li et al., 1999).

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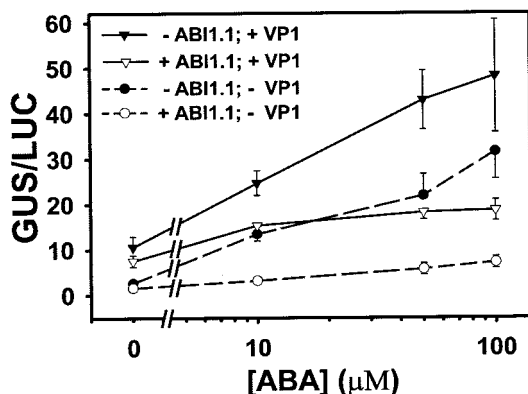


Figure 1. Single and combined effects of VP1 and *abi1-1* overexpression on *HVA1* promoter- β -glucuronidase (GUS) expression in transiently transformed rice protoplasts. The non-ABA-inducible ubiquitin promoter-luciferase cDNA reporter construct (Christensen and Quail, 1996) was cotransformed as an internal control for transcription activity. Treatments were performed in triplicate; variance bars are \pm SE.

Lanthanide salts inhibit ion channels in plants and animals and have been used extensively as plasma membrane calcium channel antagonists (Huang et al., 1994; Bush, 1995; Van der Meulen et al., 1996; Gelli and Blumwald, 1997; Tähtiharju et al., 1997; Clayton et al., 1999). There is recent evidence for non-specific inhibition of ion channels by lanthanum (Lewis and Spalding, 1998). We have previously observed lanthanide and trivalent ion effects on gene expression (Rock and Quatrano, 1996). Here we have used ABA-inducible and noninducible enzymatic reporter gene expression, flow cytometry of protoplasts expressing ABA-inducible green fluorescent protein (GFP), and the dominant-negative *abi1-1* gene to provide evidence for specific activation by trivalent ions of ABA-inducible gene expression.

RESULTS AND DISCUSSION

The ABA response genes *ABI3* (orthologous to maize *VP1*) and *ABI1* have been shown to genetically

interact in planta (Finkelstein and Somerville 1990; Parcy et al., 1997), although their mechanisms of action are not known. Because we use a heterologous, artificial system of transiently expressed promoters and effector cDNAs in protoplasts to assay ABA signaling, we sought to validate the fidelity of the system by testing whether the Arabidopsis *abi1-1* cDNA could function as a bona fide effector of ABA responses in rice (*Oryza sativa* L. cv IR54) protoplasts. Figure 1 shows the results of transient gene co-expression assays testing the interactions of ABA and overexpressed maize *VP1* cDNA and/or the dominant negative Arabidopsis *abi1-1* cDNA on transactivation of the barley *HVA1* promoter. There is an antagonistic effect of overexpressed *abi1-1* on both ABA-dependent and *VP1*-dependent *HVA1* activation. This result is consistent with *abi1-1* dominant negative effects observed in transgenic tomato, tobacco, and maize (Grabov et al., 1997; Parcy and Giraudat, 1997; Carrera and Prat, 1998; Sheen, 1998) and a model whereby *abi1-1* negatively affects ABA-signaling amplitude and *VP1* indirectly potentiates it (Gosti et al., 1999; Li et al., 1999).

We showed previously that lanthanum ions had a specific agonist effect on ABA-inducible endogenous *Em* expression in rice suspension cells (Rock and Quatrano, 1996). We further investigated the lanthanum specificity toward ABA-inducible gene expression in transient co-expression assays using reference promoter-reporter constructs as internal controls for transformation and transcription. The maize ubiquitin promoter is not inducible by ABA (Shen and Ho, 1997). We tested the effects of ABA and lanthanum chloride on activation of the wheat (*Triticum aestivum*) *Em*, barley *HVA1* and *HVA22*, rice actin, and cauliflower mosaic virus 35S promoters. Figure 2A shows that the *Em* promoter was specifically activated (46-fold) by a saturating concentration of ABA (100 μ M; Desikan et al., 1999), almost 6-fold by lanthanum chloride (10 mM), and exogenous ABA and lanthanum together acted in a synergistic manner relative to expression of the reference ubiquitin-

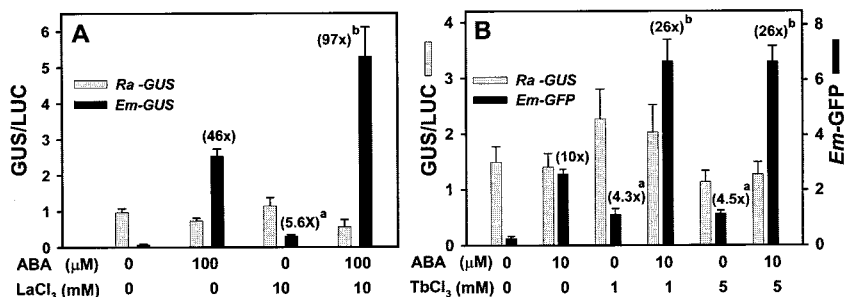


Figure 2. Specific synergistic effect of lanthanide ions on the ABA-inducible *Em* promoter, but not the non-ABA-inducible rice actin (*Ra*) promoter. Numbers in parentheses show fold induction over untreated controls. A, Effects of lanthanum with or without ABA cotreatment on the actin and *Em* promoters, measured by GUS/LUC reporter enzyme assays. B, Effects of terbium on the actin and *Em* promoters, cotransformed and measured in the same samples by reporter enzyme activities and GFP flow cytometry, respectively. Results are the average of three to nine replicates (\pm SE). a, Significantly higher than control treatment (paired *t* test, $P < 0.02$). b, Significantly higher than ABA ($P < 0.05$).

Table 1. Specificity of the lanthanide effect on the ABA-inducible *Em* and *HVA1* promoters in transiently transformed protoplasts

Induction was calculated as the average ratio of relative reporter gene activities (promoter-GUS/*Ubi-LUC*) between treatment and control (set to unity) from three- or four-paired replicates in two independent experiments.

Treatments		Promoter Fold-Induction (\pm SE)		
ABA	Salt	35S	<i>Em</i>	<i>HVA1</i>
μ M	mM			
0	0	1.0	1.0	1.0
100	0	0.8 (0.1)	16.6 (2.0)	13.7 (1.0)
0	1 LaCl ₃	1.1 (0.0)	2.2 (0.6)	3.2 ^a (0.5)
0	2 TbCl ₃	1.1 (0.1)	4.6 ^a (0.2)	n/a ^c
0	5 LaCl ₃	1.4 (0.3)	2.7 (0.8)	3.8 ^a (0.4)
100	1 LaCl ₃	1.1 (0.1)	32.6 ^b (2.9)	29.6 ^b (5.6)
100	2 TbCl ₃	1.2 (0.1)	31.9 ^b (1.5)	n/a ^c
100	5 LaCl ₃	1.1 (0.4)	38.8 ^b (9.9)	24.1 (5.5)

^a Significantly higher than control, $P < 0.03$ (one-sided paired *t* test). ^b Significantly higher than ABA treatment, $P < 0.06$ (one-sided paired *t* test). ^c n/a, Not analyzed.

firefly luciferase (LUC) reporter (Fig. 2A). Similar results were obtained when sub-saturating concentrations of ABA (10 μ M) and the lanthanide terbium were used to activate the *Em* promoter (Fig. 2B); however, the rice actin promoter was not significantly affected by ABA or lanthanide treatments (Fig. 2). Likewise, the lanthanide agonist and synergistic effects were significant and specific for the ABA-inducible *HVA1* promoter (Table 1) and the ABA-inducible *HVA22* promoter (D. Hagenbeek and C.D. Rock, unpublished data) but not the control cauliflower mosaic virus 35S promoter (Table 1).

Because protoplasts are a heterogeneous population with potentially different characteristics that might complicate analysis of signaling pathways, we have developed a novel, quantitative, ABA-inducible reporter gene assay based on GFP and flow cytometry (Desikan et al., 1999). This method allows quantitation of thousands of gene expression events (and other correlative cell biology parameters) in potentially complex populations on a per cell basis. We further tested the specificity and synergy of trivalent ion action on ABA signaling by observing the effects of overexpressed *abi1-1* cDNA on ABA- and lanthanide-inducible *Em*-GFP reporter gene expression. Figure 3 shows representative scatter plots (cell size versus GFP fluorescence) of 10,000 protoplasts ex-

pressing *Em*-GFP after 20-h treatments of 10 μ M ABA, 5 mM lanthanum chloride, or both. The transformation efficiency was observed to be approximately 4% by this method. On the basis of scoring a fixed number of cells, it is apparent that the cells expressing, *Em*-GFP generally respond to treatments uniformly as a population, both in terms of cell numbers and fluorescence intensities (Fig. 3). For reference, *Em*-GFP-transformed control samples that were not treated with ABA or lanthanides resulted in 18 protoplasts above the arbitrary background threshold per 10,000 (D. Hagenbeek and C.D. Rock, unpublished data), whereas the number of *Em*-GFP expressing protoplasts in Figure 3C is 340. The quantitative measurement of a large number of GFP-expressing cells by flow cytometry yields results comparable with those obtained with enzymatic reporter assays (Fig. 2) with the advantage of being able to observe population dynamics. Expression of *Em*-GFP was positively correlated with exogenous ABA concentration (Desikan et al., 1999; D. Hagenbeek and C.D. Rock, unpublished data). From these results we conclude that heterogeneity of ABA and/or lanthanum sensitivity/responses by sub-populations of rice protoplasts does not play a major role in ABA- and lanthanide-signaling pathways, in contrast to reports

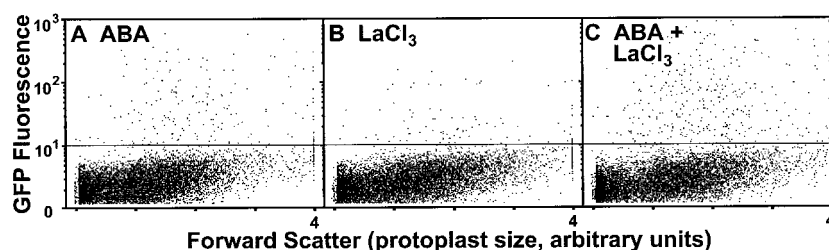


Figure 3. Scatter plot (cell size versus GFP fluorescence) of *Em*-GFP-transformed protoplasts treated for 20 h with 10 μ M ABA (A), 5 mM LaCl₃ (B), or both ABA plus LaCl₃ (C). Ten-thousand protoplasts were measured by flow cytometry, and those with fluorescence above an arbitrary background (horizontal line) were gated for quantitation of fluorescence intensity.

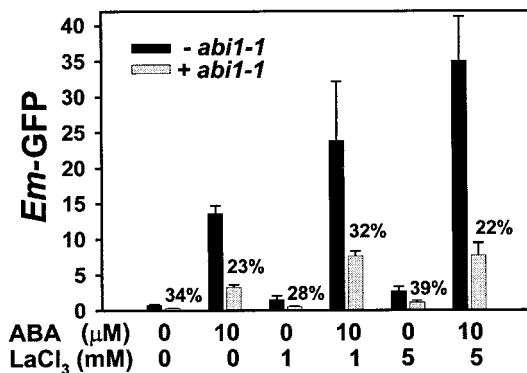


Figure 4. Overexpression of *abi1-1* inhibits lanthanum-induced *Em* expression to a similar extent as ABA-induced *Em* expression. Rice protoplasts were transformed with *Em-GFP* in the presence of overexpressed *abi1-1* (pG2) or a null *ABI1* control (pG1; Sheen, 1998). Numbers above the *abi1-1*-treated samples (white bars) indicate the percentage *Em* expression relative to control. Transformations were performed in triplicate and flow cytometry measurements in duplicate; variance bars are \pm SE.

of heterogeneity of gibberellin sensitivity in aleurone protoplasts (Ritchie et al., 1999).

A quantitative, flow-cytometric analysis of the interaction of *abi1-1* gene, ABA, and lanthanum ions on *Em-GFP* expression is shown in Figure 4. ABA (10 μ M) gave a 21-fold activation of the *Em* promoter, whereas lanthanum gave a dose-dependent 2- to 4-fold activation that was synergistic with the ABA induction. Overexpression of the dominant negative *abi1-1* cDNA resulted in a similar (60%–75%) inhibition of *Em-GFP* expression in untreated controls, ABA, lanthanum, and ABA plus lanthanum treatments (Fig. 4). This result supports the hypothesis that lanthanum acts on an ABA signal transduction pathway upstream from *ABI1* and *VPI1*. Furthermore, quantitative flow cytometry of *Em-GFP* was sensitive enough to reveal that *Arabidopsis abi1-1* significantly

inhibits ($P < 0.03$) *Em* promoter activity in the absence of exogenous ABA (Fig. 4), which was not obvious in reporter enzyme assays (Fig. 1). This observation underscores the conservation of ABA-signaling mechanisms among various species including rice.

The viability of protoplasts has been shown to be negatively correlated with ABA concentration (Desikan et al., 1999); therefore, the effect of lanthanides on protoplast viability was tested. Results of several experiments (Table II) demonstrated that day-to-day variation in protoplast viability was substantial and not strictly correlated with ABA or lanthanide treatments. The significance of this environmentally induced cell death is not known, but the observation clearly points out that the inductive effects of ABA and lanthanum on individual cells (Fig. 3) are stronger than measured by reporter enzyme assays, which are often normalized to total protein concentration. Our previous results showing lack of specificity of lanthanide effects in protoplasts may have been confounded by the effects of cell death on protein concentrations (Rock and Quatrano, 1996). Flow cytometry permits quantitative and qualitative analysis of the effects of ABA and other signaling factors on heterogeneous cell populations as well as simultaneous correlation of multiple cell biological parameters in single cells and populations.

We reported previously that lanthanide and trivalent aluminum ions had inductive effects on transient gene expression in protoplasts, whereas monovalent salts such as potassium, sodium, and lithium had no such effect (Rock and Quatrano, 1996). We further analyzed the lower limits of the lanthanide and the trivalent aluminum ion effects on *Em* promoter activity and extended the analysis of possible salt effects to the divalent ions magnesium and manganese. Representative results of experiments performed with different concentrations of salts are shown in

Table II. ABA and lanthanide salt effects on viability of transformed protoplasts after an 18- to 20-h treatment

Samples were assayed by flow cytometry of protoplasts treated with 0.01% (w/v) fluorescein diacetate for 5 min.

Treatment		Viability Percentage (\pm SE)		
ABA ^a	Salt ^b	Experiment I	Experiment II	Experiment III
μ M	mM			
0	0	36.2 (6.7)	15.0 (0.6)	45.4 (0.6)
+	0	31.7 (4.0)	15.5 (0.7)	43.8 (0.6)
0	1	14.9 (1.2)	15.5 (0.6)	49.6 (1.7)
+	1	9.4 (4.6)	17.7 (0.7)	48.5 (0.7)
0	5	12.8 (11.0)	17.4 (0.3)	48.5 (1.4)
+	5	3.4 (1.8)	16.5 (0.4)	47.5 (1.3)

^a Ten micromolar ABA in experiments I and II; 100 μ M ABA in experiment III. ^b Experiment I, LaCl₃ (two independent transformations); experiment II, TbCl₃ (five–nine independent transformations); experiment III, average of parallel LaCl₃ and TbCl₃ treatments (three–nine independent transformations).

Table III. Trivalent, but not divalent, ions stimulate *Em* promoter activity in transiently transformed protoplasts

Induction was calculated as the average ratio of relative reporter gene activities (*Em*-GUS/*Ubi*-LUC) between treatment and control (set to unity) from three paired replicates for the LiCl, MgCl₂, MgSO₄, and MnCl₂ treatments. For the AlCl₃, LaCl₃, and TbCl₃ treatments, induction was calculated as the average ratio of relative weighted *Em*-GFP expression between treatment and control from three paired replicates.

Treatment		<i>Em</i> Promoter Fold-Induction (±SE)	<i>P</i> Value ^a
Concentration	Salt		
<i>mM</i>			
5.0	LiCl	0.9 (0.1)	0.29
5.0	MgCl ₂	1.0 (0.1)	0.44
5.0	MgSO ₄	1.0 (0.1)	0.48
10.0	MnCl ₂	1.0 (0.3)	0.44
0.13	AlCl ₃	3.7 (0.6)	0.03
0.13	LaCl ₃	2.4 (0.4)	0.05
0.13	TbCl ₃	1.6 (0.2)	0.09

^a One-sided paired *t* test.

Table III. Lithium, manganese, and magnesium salts had no effect on *Em* promoter activity at more than 10-fold higher concentrations than those where lanthanides and the trivalent ion aluminum had significant effects (Table III). These results indicate that the trivalent ion effect is not a general stress or salt response.

We have demonstrated, using various reporter genes and salts that the synergistic effect of trivalent ions on ABA-inducible promoters in transiently transformed rice protoplasts is specific and dependent upon the activity of *ABI1*. In previous transient gene expression experiments with trivalent ions (Rock and Quatrano, 1996), no internal reference reporter was used to normalize gene transcription against environmental factors such as transformation efficiency or cell viability, and specificity for ABA signaling was not established. Here we have used the dominant negative ABA regulatory gene *abi1-1* and two independent reporter assays (a ubiquitin promoter-LUC internal reference reporter gene construct [Christensen and Quail, 1996] and flow cytometry of large populations of GFP-expressing protoplasts) to demonstrate specificity and homogeneity of the trivalent ion effect on ABA-inducible promoters.

The mechanism of trivalent ion activity on gene expression is not known. Aluminum ions have also been reported to activate *c-fos* gene expression in fibroblasts (Hughes and Pennington, 1993). It is unlikely that lanthanides increase the sensitivity of cells to ABA, since the synergistic effect of lanthanum is observed at saturating concentrations of ABA (Fig. 2A; Desikan et al., 1999). Because lanthanum binds with high affinity to the plasma membrane and intracellular vesicles (van Steveninck et al., 1976), we speculate that it may exert effects on ABA-inducible gene expression via ion channels (Lewis and Spald-

ing, 1998) or other membrane-associated proteins. Lanthanum is routinely used in plants as a calcium channel blocker and alters gene expression (Tähtiharju et al., 1997; Clayton et al., 1999). Calcium is required for ABA-inducible gene expression (Sheen, 1996; Van der Meulen et al., 1996), and lanthanum can substitute for the calcium requirement for *Em* gene expression (Rock and Quatrano, 1996). However, the role of extracellular calcium in ABA signaling is not known, and lanthanides are not specific for calcium channels (Lewis and Spalding, 1998). The calcium channel blockers bepridil, nifedipine, and verapamil had no effect on *Em* promoter activity, although bepridil did block calcium uptake in rice protoplasts (C.D. Rock, unpublished data). Relatively high concentrations of lanthanides were required for maximum agonist activity in these experiments, suggesting a possible intracellular site of lanthanide action. In this context it is interesting to note that lanthanum has been shown to inhibit calcium-dependent protein kinases (Polya et al., 1987), which have been implicated in ABA signaling (Sheen, 1996). It is plausible that an integrin-like molecule in plants (Zhang et al., 1996; Faik et al., 1998; Nagpal and Quatrano, 1999) could be activated by trivalent ion binding (D'Souza et al., 1994; Obsil et al., 1999). Lanthanide ions act as agonists of integrin expression in animal cells (Ahmad et al., 1999). Recent characterization of a glycoprotein in rice plasma membranes that may undergo conformational changes (both are properties of integrins) and is involved in ABA signaling (Desikan et al., 1999) is consistent with a hypothetical role for integrins in ABA responses. Multidisciplinary and integrative approaches such as flow cytometry, patch-clamping of rice protoplasts, and an in vitro biosensor assay for ABA interaction with plasma membrane vesicles (Desikan et al., 1999) may provide insights into the cell biology of ABA signaling and the mechanism of action of trivalent ions.

MATERIALS AND METHODS

Plant Materials

Rice (*Oryza sativa* L. cv IR54) suspension cultures from the International Rice Research Institute, Los Baños, Philippines), initiated from germinating embryos, were propagated and digested for making protoplasts as previously described (Marcotte et al., 1988; Desikan et al., 1999). Protoplasts were transiently transformed with polyethylene glycol as described by Maas et al. (1995) with modifications (Desikan et al., 1999). It was typical that 70 µg of reporter plasmid and 20 µg of effector plasmids were mixed with 2.5 × 10⁶ rice protoplasts per transformation. Transformations were split into four- or six-paired samples and treated for 20 h in a final volume of 0.8 mL of Krens solution.

Plasmid Constructions

The plasmid pCR559 contains the wheat (*Triticum aestivum*) *Em* promoter driving a modified *Aequoria victoria* GFP (Chiu et al., 1996; Desikan et al., 1999). Plasmids pBM207 and pBM314 contain the wheat *Em* and cauliflower mosaic virus 35S promoters, respectively, driving *uidA* expression (GUS; Marcotte et al., 1988; Hill et al., 1996). Plasmids pQS264 and pLSP contain the barley (*Hordeum vulgare*) *HVA1* and *HVA22* promoters driving GUS expression, respectively (Shen and Ho, 1997). Plasmid pAct1D contains the rice actin promoter driving GUS expression (Zhang et al., 1991). Plasmid pAHC18 contains the maize (*Zea mays*) ubiquitin promoter driving LUC (Christensen and Quail, 1996) and was included in transformations as an internal reference for non-ABA-inducible transient transcription. Plasmid pG2 encodes the chimeric 35S*C4PPDK* (cauliflower mosaic virus 35S-maize C₄ pyruvate orthophosphate dikinase) promoter driving the coding region of the *Arabidopsis abi1-1* dominant negative G180D mutant allele (Sheen, 1996, 1998). Plasmid pG1 is a control construct that is identical to pG2 except it is wild type at aa180 (Gly), and the phosphatase active site has been mutated (G174D) to produce a null mutant (Sheen, 1998).

Functional Assays

Flow cytometry of live protoplasts expressing GFP was performed on a dual beam instrument (FACS Vantage, Becton-Dickinson, San Jose, CA) equipped with a 200- μ m nozzle and Lysis II acquisition and analysis software. The excitation wavelength was 488 nm, and emission detection was with a fluorescein isothiocyanate 530/30-nm filter set. In order to take advantage of the large numbers of individual measurements inherent in flow cytometry, a quantitative method, "weighted fluorescence intensity," was developed that proved more sensitive than previous reporter gene measurements (Desikan et al., 1999). For each sample, 10,000 protoplasts were gated, and the weighted-GFP fluorescence per 10,000 cells was calculated as the product of the average fluorescence intensity of the population expressing above background threshold and the number of individual cells. Cell viability was determined by flow cytometry of an aliquot of live protoplasts treated 5 min with 0.01% (w/v) fluorescein diacetate (Molecular Probes, Eugene, OR). Enzyme-based reporter assays were as described previously (Desikan et al., 1999).

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