

## Functional Interactions of Lanthanum and Phospholipase D with the Abscisic Acid Signaling Effectors VP1 and ABI1-1 in Rice Protoplasts\*

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Srinivas S. L. Gampala, Dik Hagenbeek, and Christopher D. Rock‡

From the Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

*cis,trans*-Abscisic acid (ABA) plays an important role in plant growth and development, regulation of seed maturation, germination, and adaptation to environmental stresses. Knowledge of ABA mechanisms of action and the interactions of components required for ABA signal transduction is far from complete. Using transient gene expression in rice protoplasts, we observed additive and inhibitory effects between maize VP1 (Viviparous-1, a transcriptional activator) and a dominant-negative mutant protein phosphatase, ABI1-1 (ABA-insensitive-1-1), from *Arabidopsis*. Lanthanide ions were shown to be specific agonists of ABA-inducible gene expression and to interact synergistically with ABA and overexpressed VP1. Both VP1 and lanthanum activities could be antagonized by coexpression of ABI1-1, which demonstrates the specific ABA dependence of these effectors on ABA-regulated gene expression. We obtained pharmacological evidence that phospholipase D (PLD) functions in ABA-inducible gene expression in rice. Antagonism of ABA, VP1, and lanthanum synergy by 1-butanol, a specific inhibitor of PLD, was similar to the inhibition by coexpression of ABI1-1. These results demonstrate that ABA, VP1, lanthanum, PLD, and ABI1 are all involved in ABA-regulated gene expression and are consistent with an integrated model whereby La<sup>3+</sup> acts upstream of PLD.

*cis,trans*-Abscisic acid (ABA)<sup>1</sup> modulates seed development, dormancy, cell division, stomatal movements, and cellular responses to environmental stresses such as drought, cold, salt, pathogen attack, and UV light (1–3). Despite its importance in plant growth and development, the mechanisms of ABA action are largely unknown, and there may be more than one ABA signal transduction pathway leading to both fast (guard cell ion fluxes) and slow (gene regulation) cellular responses (4–6). Transient gene expression studies with *LEA* (late embryogenesis abundant) and drought-inducible gene promoters have defined the *cis*-acting elements necessary and sufficient to con-

fer ABA-inducible transcription (7–10). Separate ABA-responsive elements and coupling elements function cooperatively and redundantly as an ABA response complex (9, 11, 12).

Recent insight into the mechanisms of ABA-inducible transcription have come from cloning of TRAB1 (transcription factor responsible for ABA regulation), a basic leucine zipper transcription factor that binds ABA response complex promoter elements and VP1 (Viviparous-1), a transcriptional activator required for ABA-regulated gene expression during seed maturation (13–15). Other proteins interact with ABA response complexes, VP1 orthologs, and basic leucine zipper factors, but there is no direct evidence that these factors function in ABA signaling (16–27). Regulation by ABA of TRAB1 and VP1 transactivation of ABA response complexes is not at the level of DNA binding, based on transient gene expression and *in vivo* footprinting assays (13, 28). Consistent with this model is the finding that the bean ortholog of VP1, PvALF, functions to remodel chromatin independent of exogenous ABA (29). VP1 and orthologs have distinct functions that are not ABA-dependent, and the precise mechanism(s) of VP1 action in ABA signaling are not known (14, 15, 27, 30–35).

Genetic analysis (36, 37) of seed maturation and germination processes in *Arabidopsis* has resulted in map-based cloning of the *ABI1–ABI5* (*ABA-insensitive*) genes. *ABI3* is the genetic equivalent of the maize *Vp1* gene and is conserved both functionally and at the nucleotide level in monocots and dicots (3, 4, 38). The *ABI4* gene shows homology to the *APETELA2* family of transcriptional regulators (39). The *ABI5* gene encodes a member of the basic leucine zipper family of transcription factors (40) and is highly homologous to rice TRAB1, sunflower DPBF1, and *Arabidopsis* ABF1–ABF4 and AREB1–AREB3 (10, 13, 24, 25).

The semidominant *abi1* and *abi2* mutations are the most pleiotropic ABA-insensitive mutants in terms of physiological and tissue-specific ABA processes (4, 41). The *ABI1* and *ABI2* genes encode homologous type 2C protein Ser/Thr phosphatases with partially redundant but distinct tissue-specific functions in the regulation of ABA-, cold-, or drought-inducible genes and ion channels (42, 43). Remarkably, the sole mutant alleles, *abi1-1* and *abi2-1*, are both missense mutations of a conserved Gly-to-Asp mutation (G180D in *abi1-1* and G168D in *abi2-1*) that results in a dominant phenotype *in vivo* and reduced phosphatase activity *in vitro* (44–46). Plants homozygous for intragenic null suppressor alleles of *abi1-1* exhibit higher seed dormancy and enhanced ABA sensitivity to germination inhibition and stomatal movements, suggesting that *ABI1* and probably *ABI2* act as negative regulators of ABA signaling (47). Overexpression of *abi1-1* antagonizes both up- and down-regulation of ABA-responsive promoters (48). *ABI1* acts downstream of the ABA agonist lanthanum (48). The mo-

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‡ To whom correspondence should be addressed. Tel.: 852-2358-8634; Fax: 852-2358-1559; E-mail: borock@ust.hk.

<sup>1</sup> The abbreviations used are: ABA, *cis,trans*-abscisic acid; PLC, phospholipase C; PLD, phospholipase D; GFP, green fluorescent protein; GUS,  $\beta$ -glucuronidase.

lecular mechanisms and targets of *ABI1* and lanthanum action are not known.

Phospholipase C (PLC) and phospholipase D (PLD) are phosphodiesterases that hydrolyze phospholipids, producing inositol 1,4,5-trisphosphate and diacylglycerol or phosphatidic acid and the head group, respectively. Phospholipases have been proposed to play a major role in mediating a wide range of cellular processes in plants such as membrane trafficking, cell proliferation, cytoskeletal organization, defense responses, differentiation, reproduction, and hormone action (49, 50). PLC and PLD have been implicated in ABA regulation of stomatal movements (51, 52). Richie and Gilroy (53) have shown that application of phosphatidic acid to barley aleuron inhibits gibberellin-inducible  $\alpha$ -amylase production and triggers synthesis of an ABA-inducible amylase inhibitor. They have also recently shown *in vitro* that ABA stimulates PLD activity in plasma membrane extracts (54). PLC and PLD genes are up-regulated by ABA (55, 56). 1-Butanol, a specific inhibitor of PLD (49, 57–59), inhibits ABA-induced accumulation of the RAB (response to ABA) protein (53). However, the specificity of PLD action in secondary messenger formation and ABA-regulated gene expression is far from understood.

We are interested in the cell biology of ABA signal transduction, from events at the cell surface such as ligand binding (60) through secondary messengers such as calcium, inositol 1,4,5-trisphosphate, and cyclic ADP-ribose (61) to changes in gene expression. The activities of overexpressed ABA signaling effectors are easily and rapidly assayed in rice protoplasts. This capacity allows facile analysis of pharmacological agents for interaction with ABA signaling components as well as characterization of the molecular mechanisms of such interactions. In this study, we have obtained pharmacological evidence that PLD functions in ABA-inducible reporter gene expression in rice and acts downstream of the ABA effector lanthanum. VP1 transactivation of ABA-inducible promoters is inhibited by 1-butanol, and the extent of 1-butanol inhibition is comparable to that effected by coexpression of the dominant-negative *ABI1-1* protein. These results are consistent with genetic studies showing an ABA requirement for VP1 action and suggest that PLD and *ABI1-1* act upstream of VP1 on a single ABA signaling pathway.

#### EXPERIMENTAL PROCEDURES

**Plant Materials**—Embryonic rice suspension cultures (*Oryza sativa* L. cv. IR-54) were kindly provided by Dr. W. M. Marcotte, Jr. (Clemson University, Clemson, SC) and propagated in MS basal medium (62). Three days after subculturing, protoplasts were prepared and transformed with various mixtures of DNA constructs using polyethylene glycol precipitation as previously described (48, 60). Aliquots of transformed protoplast samples were treated with or without pharmacological agents and ABA for 18 h in the dark in a final volume of 0.8 ml of Krens solution (7).

**Plasmid Constructs**—Plasmid pBM207 contains the wheat (*Triticum aestivum*) *Em* (early methionine-labeled) promoter driving expression of  $\beta$ -glucuronidase (GUS; encoded by *uidA* from *Escherichia coli*) (7). Plasmids pQS264, pLSP, and pDHN7 contain the barley (*Hordeum vulgare*) *Hva1*, *Hva22*, and dehydrin (*Dhn7*) promoters driving GUS expression, respectively (8, 9). Plasmids pBM314 and pAHC27 contain the cauliflower mosaic virus 35 S promoter (referred to below as 35S) and the maize (*Zea mays*) *Ubi* (ubiquitin) promoter driving GUS expression, respectively (7, 63). Plasmid pCR559 contains the *Em* promoter driving a modified S65T green fluorescent protein cDNA from *Aequoria victoria* (60, 64). A construct (pDH559) containing the maize *Ubi* promoter driving S65T GFP was created by digesting pCR559 with *XhoI* and filling in the linearized product with reverse transcriptase before digesting with *SphI* to release the *Em* promoter. The resulting 3.7-kilobase pair fragment (pCR559 minus the *Em* promoter) was then gel-purified and ligated with the 2-kilobase pair *SphI/SmaI* fragment of pAHC27, encoding the maize *Ubi* promoter (63). Plasmid pCR349.13S contains the 35 S promoter driving VP1 sense cDNA (18). Plasmid pG2 encodes the cauliflower mosaic virus 35 S promoter-maize C4 pyruvate-

orthophosphate dikinase (*Ppdk35S*) promoter chimera driving the coding region of the *Arabidopsis thaliana abi1-1* dominant-negative G180D mutant allele (46). Plasmid pDirect2.6 contains the *Ubi* promoter in a reversed orientation and was used as control construct to balance the total amount of input plasmid DNA between various treatments. Plasmid pAHC18 contains the *Ubi* promoter driving firefly (*Photinus pyralis*) luciferase (63) and was included in transformations to provide an internal reference for non-ABA-inducible transient transcription in reporter enzyme assays.

**Chemicals**—1-Butanol was obtained from Acros Organics (Geel, Belgium). 2-Butanol was from Nacal Tesque, Inc. (Kyoto, Japan). Synthetic abscisic acid (( $\pm$ )-*cis,trans*-abscisic acid) and lanthanum chloride were obtained from Sigma (St. Louis, Missouri). ABA was dissolved and stored in absolute ethanol at  $-20^{\circ}\text{C}$  as a 0.1 M stock solution. Prior to use, required dilutions of ABA were made in Krens solution, and control samples received the same volumes of solvents as in ABA treatments.

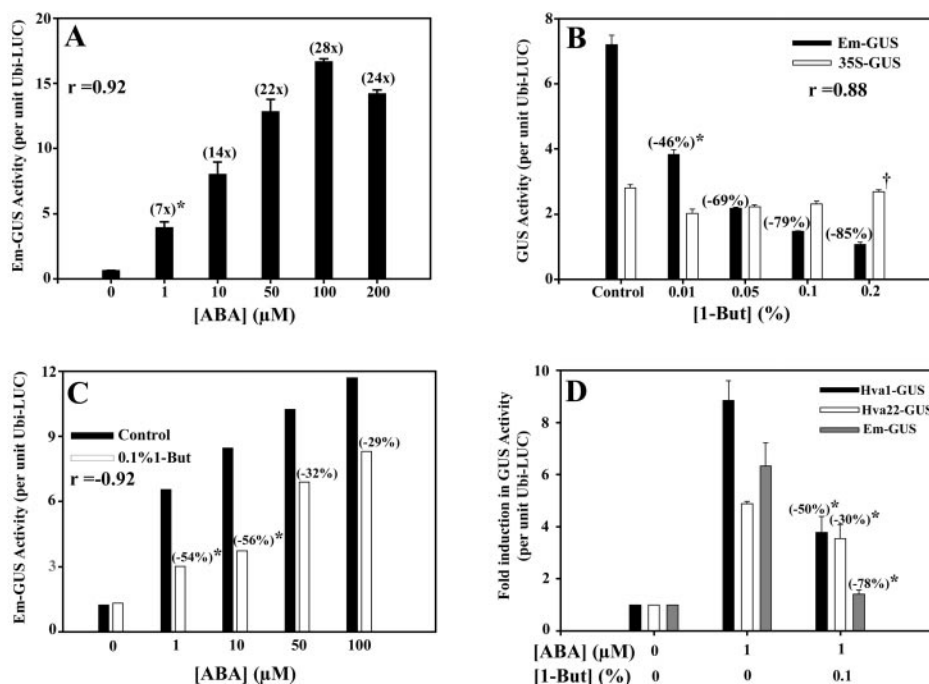
**Functional Assays**—Flow cytometry of live protoplasts expressing GFP was performed on a Becton-Dickinson FACS Vantage dual-beam instrument equipped with a 200- $\mu\text{m}$  nozzle, Lysis II acquisition and analysis software, and an Enterprise argon-ion laser (1.3 watts) tuned to 488 nm. The sheath fluid used was Krens (7). GFP emission detection was carried out with a fluorescein isothiocyanate 530/30-nm band-pass filter. 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 and the number of individual cells (48) expressing above a background threshold. After 16 h of incubation, cell viability was determined by flow cytometry of an aliquot of protoplasts treated for 5 min with 0.01% (w/v) fluorescein diacetate (Molecular Probes, Inc., Eugene, OR).

For reporter enzyme assays, protoplasts were lysed in 250  $\mu\text{l}$  of lysis buffer and spun at maximum speed for 3 min in a microcentrifuge, and the supernatant was retained. Luciferase substrate (Promega, Madison, WI) was prepared according to the manufacturer's instructions. 10  $\mu\text{l}$  of sample extract was mixed with 50  $\mu\text{l}$  of substrate, and the luciferase activity was measured on a Zylux FB15 luminometer (Fisher Scientific, Pittsburgh, PA). GUS activities were determined by fluorometry with 4-methylumbelliferyl glucuronide as substrate according to Desikan *et al.* (60). The relative reporter gene activity was represented as the ratio of GUS to luciferase activities, expressed in units of 4-methylumbelliferyl fluorescence/40  $\mu\text{l}$  of extract/h and photons/10  $\mu\text{l}$  of extract/min, respectively.

#### RESULTS

To characterize the maximum and specific effects of pharmacological agents on ABA-inducible expression, it was considered important to perform interaction experiments with concentrations of exogenous ABA that are physiologically relevant. Therefore, an ABA dose response of *Em*-GUS expression was performed. The results are shown in Fig. 1A. There was a log-linear relationship of ABA concentration to *Em* promoter expression up to 100  $\mu\text{M}$ , with a correlation coefficient of  $r = 0.92$ . There was a significant increase ( $p < 0.0005$ ) in *Em*-GUS transcription in response to 1  $\mu\text{M}$  ABA treatment. The maximum induction of *Em*-GUS (28-fold) was observed with 100  $\mu\text{M}$  ABA treatment. The slight decrease in relative *Em*-GUS induction observed with a saturating ABA concentration (200  $\mu\text{M}$ ) may have been caused by a negative effect of high ABA concentrations on protoplast viability (60).

PLD is competitively inhibited by 1-butanol due to its ability to transfer the phosphatidyl moiety of the substrate to 1-butanol instead of water, producing phosphatidylbutanol that can be used as a quantitative assay of PLD activity (57). The *trans*-phosphatidyl reaction is unique to PLD, making 1-butanol a suitable pharmacological agent to study PLD-regulated processes (57–59). To test the role of PLD in ABA signaling in rice embryonic tissue, we measured the effect of various concentrations of 1-butanol on ABA-inducible gene expression in protoplasts at a subsaturating concentration of ABA (1  $\mu\text{M}$ ). The results are shown in Fig. 1B. 1-Butanol antagonized ABA-inducible *Em*-GUS expression in a dose-dependent manner. Significant inhibition of ABA-induced *Em*-GUS expression was seen with 0.01% (v/v) 1-butanol ( $p < 0.01$ ). The maximum inhibition of *Em*-GUS expression (85%) was



**FIG. 1. 1-Butanol-specific inhibition of ABA-inducible gene expression in transiently transformed rice protoplasts.** *A*, ABA induces *Em-GUS* expression in a dose-dependent manner. Numbers in parentheses indicate the -fold induction of ABA-inducible *Em-GUS* expression relative to the zero ABA treatment. The correlation coefficient ( $r$ ) for linear regression of the -fold induction between 0 and 100  $\mu\text{M}$  ABA is shown. The asterisk indicates significantly different from no ABA treatment ( $p < 0.0005$ , two-sided Student's  $t$  test, equal variance assumed). *B*, 1-butanol specifically antagonizes ABA-inducible *Em-GUS* expression in a dose-dependent manner, while having no effect on 35 S promoter activity. The asterisk indicates significantly different from control ( $p < 0.01$ , two-sided Student's  $t$  test, equal variance assumed). The single dagger indicates no significant difference from control ( $p \geq 0.34$ , two-sided Student's  $t$  test, equal variance assumed). The correlation coefficient ( $r$ ) is for linear regression of percent inhibition of *Em-GUS* expression and 1-butanol concentration. *C*, increasing concentrations of ABA decrease the inhibition of *Em-GUS* expression by 1-butanol. Numbers in parentheses are the percent relative inhibition by 0.1% 1-butanol at the given ABA concentration. The asterisks indicate significantly different from control ( $p < 0.04$ , two-sided Student's  $t$  test, equal variance assumed). There is a negative correlation ( $r = -0.92$ ) between ABA concentration and percent inhibition of *Em-GUS* expression. Data are the average of two replicate experiments. *D*, 1-butanol antagonizes expression of the ABA-inducible *Hva1* and *Hva22* promoters. -Fold induction was calculated relative to untreated, paired controls (set to unity) from four samples. The asterisks indicate significantly different from ABA-treated, no 1-butanol control ( $p < 0.04$ , two-sided paired Student's  $t$  test, equal variance assumed). Error bars in *A*, *B*, and *D* are the means  $\pm$  S.E. of three to six replicates/sample. *LUC*, luciferase.

observed with 0.2% 1-butanol treatment. There was a good linear correlation ( $r = 0.88$ ) between inhibition of *Em-GUS* expression and 1-butanol concentration. The 1-butanol effect was specific for ABA-inducible *Em* promoter activity because the non-ABA-inducible promoter constructs *Ubi-GUS* (data not shown) and *35S-GUS* (Fig. 1*B*;  $p > 0.34$ ) were not significantly affected.

Since pharmacological agents used at high concentrations have negative effects on cell viability, it was important to rule out the trivial possibility that 1-butanol affected transcription by a general decrease in cell activity. The results of an experiment to correlate protoplast viability with 1-butanol doses are shown in Table I. Treatment of protoplasts with 0.5% 1-butanol decreased cell viability 26% in untreated control cells, whereas 0.01% 1-butanol, a concentration that antagonized *Em-GUS* expression significantly (Fig. 1*B*), decreased cell viability only 6% ( $p > 0.2$ ). These results indicate that 1-butanol inhibition of ABA-inducible gene expression is not due to its effect on cell viability.

To provide insight into the interaction of ABA and PLD signaling, the effect of 1-butanol on the dose dependence of ABA-inducible gene expression was tested. The results are shown in Fig. 1*C*. In response to increasing concentrations of ABA, the inhibition of *Em-GUS* caused by 0.1% 1-butanol steadily decreased from above 50% at subsaturating ABA concentrations to 29% at a saturating concentration of ABA (100  $\mu\text{M}$ ) (Fig. 1*C*). The linear correlation coefficient for 1-butanol inhibition of *Em-GUS* expression and ABA concentration was  $r = -0.92$ .

TABLE I

## 1-Butanol does not significantly affect protoplast viability

Rice protoplasts were treated for 18 h with various concentrations of 1-butanol and then stained with 0.01% fluorescein diacetate for 5 min and measured in triplicates by flow cytometry. The percentage of viable protoplasts was calculated from the number of cells (out of 10,000 gated) above the fluorescence threshold set for dead, EtOH-fixed cells.

1-Butanol treatment	Viability	Decrease (from control)
	%	%
Control (0%)	69	
0.01%	65 <sup>a</sup>	6
0.05%	58	16
0.1%	59	14
0.2%	54	22
0.5%	51	26

<sup>a</sup> No significant difference between control and treatment ( $p \geq 0.2$ , two-sided Student's  $t$ -test, equal variance assumed).

We extended our experiments to include the ABA-inducible promoters *Hva1* and *Hva22* (9). Fig. 1*D* shows the results of 1-butanol (0.1%) inhibition experiments on promoters induced by ABA. 1-Butanol significantly antagonized expression of the ABA-inducible *Em-GUS*, *Hva1-GUS*, and *Hva22-GUS* reporter constructs ( $p < 0.04$ ), albeit to different extents (Fig. 1*D*).

We sought further evidence for the specificity of 1-butanol effects on PLD activity by testing a non-active isomer of butanol, 2-butanol (52, 53, 57, 59), for antagonistic activity against ABA-inducible gene expression. The results are shown in Table II. Increasing concentrations of 1-butanol ( $p < 0.0002$ ), but not



TABLE II

The biologically inactive PLD inhibitor 2-butanol does not affect ABA-inducible *Em-GUS* expression

Protoplasts were transformed and treated with 10  $\mu\text{M}$  ABA, and aliquots were treated with either water (control) or two concentrations of 1- or 2-butanol. Results are the means  $\pm$  S.E. of four transformation experiments.

Alcohol treatment	Inhibition	
	1-Butanol	2-Butanol
	%	
Control (0%)		
0.1%	65 $\pm$ 1 <sup>a</sup>	0 $\pm$ 3 <sup>b</sup>
0.2%	75 $\pm$ 1 <sup>a</sup>	5 $\pm$ 2 <sup>b</sup>

<sup>a</sup> Significantly different from control ( $p < 0.0002$ , two-sided Student's *t*-test, equal variance assumed).

<sup>b</sup> No significant difference from control ( $p \geq 0.25$ ).

2-butanol ( $p > 0.25$ ), significantly inhibited ABA-inducible *Em-GUS* expression (Table II).

To place the site of action of PLD in an ABA signaling cascade, we performed interaction studies between 1-butanol and two agonists of ABA-inducible gene expression, the transcription factor VP1 and the trivalent ion lanthanum (18, 48, 65, 66). The results are shown in Table III. Overexpression of VP1 by cotransformation of the 35S-VP1 cDNA construct transactivated *Em-GUS* expression by 20-fold and acted in synergy with a subsaturating concentration of ABA to give 40-fold transactivation. 1-Butanol treatment of cotransformed protoplasts resulted in significant antagonism of 35S-VP1 transactivation and ABA plus 35S-VP1 synergy to a similar extent as 1-butanol inhibition of ABA induction alone (Table III). Lanthanum ion treatment (1 mM) activated *Em-GUS* expression by 4-fold, and a 30-fold synergistic induction was observed in response to 10  $\mu\text{M}$  ABA plus lanthanum treatment. 1-Butanol also significantly inhibited *Em-GUS* expression induced by lanthanum or lanthanum plus ABA in synergy (Table III).

Previous studies have placed the action of ABI1 downstream of lanthanum (48). To further integrate the action of PLD into a single cell model of ABA signaling, interaction and specificity studies were performed by treating the protoplasts with 35S-VP1, lanthanum, and *Ppd35S-ABI1-1* to compare the inhibitory effects of ABI1-1 with those of 1-butanol. In the experiments shown in Fig. 2, the specificity and activity of effectors for ABA-inducible gene expression were quantified by flow cytometry and traditional enzyme assays (48, 60). The non-ABA-inducible *Ubi-GFP* and the ABA-inducible *Dhn-GUS* reporter constructs were cotransformed with or without cotransformation of 35S-VP1 and/or *Ppd35S-ABI1-1*. Aliquots of various cotransformations were then treated with 100  $\mu\text{M}$  ABA and 1 mM lanthanum alone or in combination, and GFP and GUS expression was quantified by flow cytometry or reporter gene assays. In Fig. 2 (C and D), the cotransformed reporters were the ABA-inducible constructs *Em-GFP* and *Hva22-GUS*. The *Ubi* promoter was not activated by ABA, lanthanum, or cotransformed 35S-VP1 and was not inhibited by cotransformed *Ppd35S-ABI1-1* ( $p > 0.2$ , two-sided Student's *t* test, equal variance assumed) (Fig. 2A). In the same samples, *Dhn-GUS* was significantly induced 5.6-fold by 100  $\mu\text{M}$  ABA ( $p < 0.002$ ), 2.6-fold by lanthanum ( $p < 0.07$ ), and 12.6-fold by the synergistic effect of lanthanum plus ABA treatment ( $p < 0.006$ , this induction is greater than ABA or lanthanum alone, one-sided Student's *t* test, equal variance assumed). In parallel experiments with the cotransformed ABA-inducible reporters *Em-GFP* and *Hva22-GUS* (Fig. 2, C and D), significant induction of the *Em* and *Hva22* promoters by 100  $\mu\text{M}$  ABA (58.9- and 16-fold, respectively;  $p < 0.007$ ) and 1 mM lanthanum (5- and 3.4-fold, respectively;  $p < 0.03$ ) was observed. There was also

TABLE III

Effect of 1-butanol on ABA, VP1, and lanthanum transactivation of *Em-GUS* expression

Activation and inhibition of *Em-GUS* expression were calculated as the -fold induction relative to the control (no effector added) in paired samples. Values are the means  $\pm$  S.E. of three replicate transformations.

Effector	Activity	Inhibition by 1-butanol
	(-fold induction over control)	%
Control (none)	1	
10 $\mu\text{M}$ ABA	15 $\pm$ 1	50 $\pm$ 4 <sup>a</sup>
35S-VP1	20 $\pm$ 1	48 $\pm$ 4 <sup>a</sup>
10 $\mu\text{M}$ ABA + 35S-VP1	40 $\pm$ 2	43 $\pm$ 2 <sup>a</sup>
1 mM La <sup>3+</sup>	4 $\pm$ 0.3	61 $\pm$ 3 <sup>a</sup>
10 $\mu\text{M}$ ABA + La <sup>3+</sup>	30 $\pm$ 0.5	52 $\pm$ 3 <sup>a</sup>

<sup>a</sup> Significantly different from control ( $p < 0.06$ , two-sided Student's *t*-test, equal variance assumed).

significant synergism upon induction of the *Hva22* promoter by treatment with ABA plus lanthanum (28.3-fold;  $p < 0.005$ , one-sided Student's *t* test, equal variance assumed). Overexpression of the cotransformed 35S-VP1 construct significantly ( $p < 0.02$ ) transactivated the *Dhn*, *Em*, and *Hva22* promoters by 2.2-, 23.8-, and 3.4-fold, respectively, in the absence of exogenous ABA or lanthanum (Fig. 2, B-D). The inhibition of ABA-inducible promoter expression by the overexpressed *Ppd35S-ABI1-1* cDNA effector construct was similar when compared separately or together for control untreated, 100  $\mu\text{M}$  ABA-, lanthanum-, or ABA/lanthanum-treated samples (Fig. 2, B-D). Taken together, the average inhibition of the *Em*, *Dhn*, and *Hva22* promoters by *abi1-1* overexpression was 54% in control, 64% in 100  $\mu\text{M}$  ABA-, 69% in 1 mM lanthanum-, and 69% in ABA/lanthanum-treated protoplasts. The average *abi1-1* inhibition of the promoters transactivated by overexpressed VP1 in the absence or presence of ABA and lanthanum, alone or together, was 28% (control), 44% (ABA), 35% (lanthanum), and 45% (ABA plus lanthanum).

In the presence of overexpressed 35S-VP1 and a saturating ABA concentration (100  $\mu\text{M}$ ), an increase in ABA-inducible reporter gene expression over 100  $\mu\text{M}$  ABA alone was observed (Fig. 2, B-D), as previously reported for the *Em* promoter (18, 48, 66). Furthermore, there was a significant ( $p < 0.07$ ) interaction between lanthanum and VP1 in ABA-inducible gene expression that could be seen in the presence or absence of 100  $\mu\text{M}$  ABA (Fig. 2, B-D). The observed magnitudes of the interactions between ABA and 35S-VP1 and between 35S-VP1 plus lanthanum plus ABA were additive rather than synergistic (Fig. 2, B-D).

## DISCUSSION

The rice protoplast transient reporter assay system has a large dynamic range of ABA sensitivity (Fig. 1A). This attribute, together with the ease of handling large numbers of samples, makes it an ideal system to screen and characterize effector molecules that interact with ABA signaling pathways. Previously, Northern blots of rice suspension culture RNAs showed interactions between salt stress (67) or lanthanum treatment (65) and ABA-inducible *Em* gene expression. We have demonstrated here that 1-butanol, a specific inhibitor of PLD (49, 52, 53, 57-59), but not the biologically inactive analog 2-butanol, specifically antagonized ABA-inducible gene expression in rice protoplasts (Fig. 1 and Table II). Taken together with the similar results of Richie and Gilroy (53) and Jacob *et al.* (52) in barley aleurone and guard cell protoplasts, respectively, these data suggest that PLD is a conserved element in ABA signaling cascades in plants. 1-Butanol inhibition of ABA-inducible gene expression was strongly dose-dependent (Fig. 1B), and increasing concentrations of ABA could partially over-

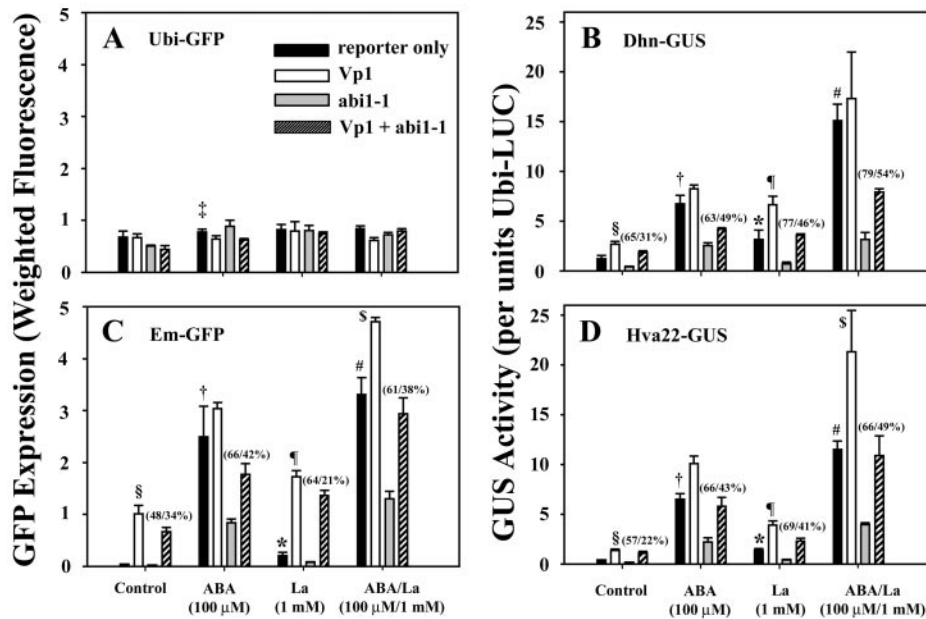


FIG. 2. Specificity of interaction of ABA, lanthanum ions, and overexpressed VP1 and ABI1-1 upon expression of the ABA-inducible *Dhn*, *Em*, and *Hva22* promoters in rice protoplasts. *A*, expression of the non-ABA-inducible *Ubi*-GFP reporter construct is not significantly affected by ABA, lanthanum, 35S-VP1, or *Ppdk35S*-ABI1-1 treatment. The double dagger indicates that control and ABA treatments are not different ( $p > 0.2$ , two-sided Student's *t* test, equal variance assumed). *B*, the same samples as *A* were cotransformed with *Dhn*-GUS and *Ubi*-luciferase reporter constructs, and GUS activity was normalized to the *Ubi*-luciferase internal control. *C*, transactivation of the *Em*-GFP reporter construct by treatment with 35S-VP1, ABA, and lanthanum and interactions between the effectors and antagonism by *Ppdk35S*-ABI1-1. *D*, the same samples in *C* were cotransformed with *Hva22*-GUS and *Ubi*-luciferase reporter constructs, and GUS activity was normalized to the *Ubi*-luciferase internal control. For *B*–*D*, the numbers in parentheses indicate percentage inhibition by overexpressed ABI1-1 in the absence/presence of cotransformed 35S-VP1, respectively. Values are the means  $\pm$  S.E. of three replicate experiments. §, significant VP1 transactivation compared with control ( $p < 0.02$ , one-sided Student's *t* test, equal variance assumed); †, significant effect by ABA compared with control ( $p < 0.007$ , one-sided Student's *t* test, equal variance assumed); ‡, significant effect by lanthanum (*La*) compared with control ( $p < 0.07$ , one-sided Student's *t* test, equal variance assumed); \*, significant effect of 35S-VP1 plus lanthanum compared with VP1 alone ( $p < 0.03$ , one-sided Student's *t* test, equal variance assumed); ††, significant additive effect of ABA plus lanthanum compared with ABA alone ( $p < 0.006$ , one-sided Student's *t* test, equal variance assumed); §, significant effect of 35S-VP1 plus lanthanum plus ABA compared with 35S-VP1 plus ABA ( $p < 0.07$ , one-sided Student's *t* test, equal variance assumed).

come the inhibition by 0.1% 1-butanol (Fig. 1C). These results are consistent with the competitive inhibition of PLD by 1-butanol and suggest that PLD is a major element in ABA signaling leading to gene expression. Because the inhibition by 1-butanol was not complete, other ABA signaling mechanisms may be operating in parallel to PLD. Fan *et al.* (68) have shown that antisense suppression of PLD $\alpha$  expression retards ABA-inducible leaf senescence. Expression of PLD mRNA is induced by ABA and stresses (55), consistent with the hypothesis that PLD activity is rate-limiting for ABA signal amplitude. We are currently testing if overexpression of PLD isoforms can increase ABA perception in protoplasts. Staxén *et al.* (51) have provided biochemical and pharmacological evidence for the role of PLC in ABA-mediated stomatal closure. It remains to be determined if PLC is involved in ABA-regulated gene expression and whether there is cross-talk between PLC and PLD pathways. PLC produces diacylglycerol and inositol 1,4,5-trisphosphate, which trigger some cellular responses to ABA such as calcium transients and stomatal closure (51). Wu *et al.* (61) have shown that inositol 1,4,5-trisphosphate is able to induce ABA-inducible gene expression in tomato; therefore, a PLC pathway may also operate in ABA-inducible gene expression. Diacylglycerol can be interconverted to phosphatidic acid by diacylglycerol kinase (50). Interestingly, major isoforms of PLD require micromolar quantities of calcium for their optimal activity (50, 69), suggesting that PLC action may precede PLD-dependent ABA signaling.

We have demonstrated that 1-butanol antagonized the VP1 transactivation of *Em*-GUS expression to the same extent as that of ABA induction. Since both VP1 and ABA are required for *Em* expression *in planta* (66), we interpret 1-butanol inhi-

bitation of VP1 transactivation as primarily an effect on ABA-dependent processes required for VP1 activity. However, other interpretations are plausible. For example, VP1/ABI3 also has ABA-independent genetic interactions with developmental factors and some ABA-regulated promoters (30, 70, 71), and PLD may be involved downstream of these VP1-related pathways.

Lanthanide ions have been extensively used as plasma membrane calcium channel blockers (72). Hagenbeek *et al.* (48) have demonstrated that trivalent ions such as lanthanum and terbium specifically activate ABA-inducible promoters through an ABI1-dependent pathway in rice protoplasts. We have shown that 1-butanol inhibits lanthanum-activated and lanthanum/ABA synergistic *Em*-GUS expression, suggesting that PLD plays a significant downstream role in a lanthanum-mediated ABA signaling pathway. The mechanism of action of lanthanum on ABA-inducible gene expression is not known. Lanthanum interacts with membranes and is hypothesized to promote calcium release (73, 74), processes that are associated with PLC and PLD activities. The effects of ABA plus 35S-VP1 on ABA-inducible gene expression are inhibited by overexpression of ABI1-1 cDNA (Fig. 2, *B*–*D*), but to a lesser extent (39% average for *Em*, *Hva22*, and *Dhn* promoters) compared with 1-butanol inhibition (Table III). These results suggest that PLD and ABI1 may affect the same or similar ABA signaling pathway(s). Lanthanum and ABA act in synergy on ABA-inducible gene expression with or without coexpressed VP1, suggesting that ABA, lanthanum, and VP1 act on the same pathway. Overexpression of ABI1-1 inhibits ABA and lanthanum activation, either alone or in synergy, of the *Dhn*, *Em*, and *Hva22* promoters to a similar extent (68% on average). However, inhibition by ABI1-1 of the VP1 interactions with lantha-

num and ABA is, on average, lower (42%) (Fig. 2, B–D) than inhibition of ABA and lanthanum effects. This result is consistent with the existence of an ABA-independent mechanism of VP1 transactivation (30, 71). The molecular mechanisms of VP1 and ABI1 activity are not known. ABI1 may negatively regulate ABA signaling by interacting with a transcription complex that includes VP1.

Transient gene expression studies permit integration of diverse (e.g. interspecies) *trans*-acting effectors into a single system, thereby facilitating characterization and testing of molecular mechanisms. Our results provide insight into the action of ABA, lanthanum, PLD, ABI1, and VP1 in regulating ABA-inducible promoter activity. The data are consistent with, but do not derive, a model of ABA action: receptor, La<sup>3+</sup> → ABI1, PLD → VP1 → gene expression. However, it is also possible that these effectors act in parallel rather than sequentially.

With the exception of an unconfirmed report in 1984 (75), no ABA receptors have been described. Using surface plasmon resonance in conjunction with flow cytometry, Desikan *et al.* (60) provided indirect evidence for a putative ABA-receptor complex that interacts with a cell-surface glycoprotein. A monoclonal antibody (JIM19) generated against pea guard cell protoplasts specifically binds to plasma membrane glycoproteins and antagonizes ABA-inducible gene expression in rice and barley (60, 76). It is noteworthy in this context that PLC is involved in shedding of arabinogalactan proteins by plasma membrane vesicles (77). The precise role of arabinogalactan proteins in ABA signaling is yet to be established (78). We are currently pursuing multiparameter correlated flow cytometric analysis<sup>2</sup> of cell-surface glycoprotein markers and *Em*-GFP expression in response to ABA and effectors to critically analyze the relationship of cell-surface glycoproteins and ABA perception and signaling from the receptor through lanthanum, PLD, ABI1, and VP1, leading to gene expression.

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