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ABI5 Interacts with Abscisic Acid Signaling Effectors in Rice Protoplasts*

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Abscisic acid (ABA) regulates seed maturation, germination, and adaptation of vegetative tissues to environmental stresses. The mechanisms of ABA action and the specificity conferred by signaling components in overlapping pathways are not completely understood. The *ABI5* gene (ABA insensitive 5) of *Arabidopsis* encodes a basic leucine zipper factor required for ABA response in the seed and vegetative tissues. Using transient gene expression in rice protoplasts, we provide evidence for the functional interactions of ABI5 with ABA signaling effectors VP1 (viviparous 1) and ABI1 (ABA insensitive 1). Co-transformation experiments with *ABI5* cDNA constructs resulted in specific transactivation of the ABA-inducible wheat *Em*, *Arabidopsis AtEm6*, bean *β-Phaseolin*, and barley *HVA1* and *HVA22* promoters. Furthermore, ABI5 interacted synergistically with ABA and co-expressed VP1, indicating that ABI5 is involved in ABA-regulated transcription mediated by VP1. ABI5-mediated transactivation was inhibited by overexpression of *abi1-1*, the dominant-negative allele of the protein phosphatase ABI1, and by 1-butanol, a competitive inhibitor of phospholipase D involved in ABA signaling. Lanthanum, a trivalent ion that acts as an agonist of ABA signaling, potentiated ABI5 transactivation. These results demonstrate that ABI5 is a key target of a conserved ABA signaling pathway in plants.

Abscisic acid (ABA)[1] is one of the major plant hormones and functions in regulation of seed maturation, germination, and adaptation of vegetative tissues to environmental stresses (1, 2). ABA acts to effect changes on multiple physiological processes such as inducing the rapid closure of stomatal pores to limit transpiration and by triggering slower changes in gene expression (see Refs. 3–5 for reviews). Although these disparate processes share genetic elements (some ABA mutants affect both processes) and signaling intermediates such as phospholipases, cADP-ribose, inositol 1,4,5-trisphosphate, and calcium ions (6–9), these secondary messengers are not specific to ABA pathways. Our knowledge of separate yet overlapping ABA and stress signal transduction pathways is fragmentary.

Genetic analyses (10, 11) of germination processes in *Arabidopsis* have resulted in map-based cloning of the ABA-insensitive genes, *ABI1–5* (12–19). The *ABI1* and *ABI2* genes encode homologous type 2C protein Ser/Thr phosphatases (PP2Cs) with partially redundant but distinct tissue-specific negative regulator functions in the regulation of ABA-, cold-, or drought-inducible genes and ion channels (20–24). The original 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*. The substrates for ABA-regulatory protein phosphatases 2C are not known (15, 16, 25).

The *ABI3*, *ABI4*, and *ABI5* genes encode proteins belonging to three distinct classes of transcription factors: the basic B3 domain, APETALA2 domain, and the basic leucine zipper (bZIP) domain families, respectively. Physiological, genetic, and transgenic analyses of *abi3*, *abi4*, and *abi5* mutants show cross-regulation of expression, suggesting that these genes function in a combinatorial network rather than a regulatory hierarchy controlling seed development and ABA responses (26).

Despite numerous biochemical studies showing binding of bZIP factors to ABA-responsive promoter elements (27–31), until recently there was no functional evidence for the role of bZIP factors in ABA signaling. Cloning of *ABI5* and its homologs, the Dc3-Promoter binding factors, ABA response element-binding factors (ABFs), ABA-responsive element-binding proteins (AREBs), and TRAB1 (transcription factor responsible for ABA regulation 1), has demonstrated a correlation between these bZIPs and ABA signaling. Members of this family of bZIPs can bind ABA-responsive elements, heterodimerize, and have limited transactivating activities (18, 32–36). ABI5 transcript and protein accumulation, phosphorylation state, stability, and activity are highly regulated by ABA during germination and early seedling growth (18, 37). Similarly, expression of some of the ABA-responsive element-binding protein genes is induced by ABA, and their ability to transactivate an ABA-responsive promoter is inhibited by the *abi1-1* mutation (35).

The *VP1* (viviparous 1) gene of maize (38) is orthologous to *ABI3* of *Arabidopsis* (12) and encodes a transcription factor required for ABA-regulated seed development. Structure-function studies with VP1 in transient gene expression assays have demonstrated that the N-terminal acidic domain functions as both a transcriptional activator and repressor (39). The conserved B2 domain is required for transactivation of the ABA-inducible *Em* promoter and for enhancing the *in vitro* binding of various bZIP proteins to their cognate targets (40). The B3 domain binds specifically to promoter sequences required for

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[1] The abbreviations used are: ABA, *cis*,*trans*-abscisic acid; PLD, phospholipase D; GUS, β-glucuronidase encoded by *uidA*; bZIP, basic leucine zipper transcription factor; *Em*, early methionine-labeled; *Ubi*, *Zea mays* ubiquitin promoter; *35S*, cauliflower mosaic virus 35S promoter; *β-Phas*, *β-Phaseolin*.

transactivation but not to ABA-responsive *cis*-elements (41). The exact molecular mechanisms of VP1/ABI3 action are not known, but it interacts genetically with ABI4 and ABI5, possibly forming a regulatory complex mediating seed-specific and/or ABA-inducible gene expression (26).

Recently, TRAB1 was shown to bind both ABA-responsive promoter elements and VP1, thereby providing a mechanism for bZIP and VP1 transactivation of ABA signaling (33). Similarly, two-hybrid assays in yeast have shown that ABI5 forms homodimers and binds to ABI3; the B1 domain of ABI3 was essential for these interactions (42). Regulation by ABA of TRAB1 and VP1 transactivation was not at the level of DNA binding (33), suggesting the existence of additional regulatory mechanisms. PvALF, a bean ortholog of VP1 that transactivates the *β-Phas* promoter, has been proposed to function by remodeling chromatin independent of exogenous ABA (43).

We are interested in elucidating the molecular mechanisms of ABA signaling. In this study, we utilized transient gene expression in protoplasts from embryonic rice callus cultures to functionally analyze the interactions of genetically defined ABA regulatory genes (*ABI5*, *ABI1-1*, and *VP1*) and pharmacological effectors (La^{3+}, 1-butanol, and an inhibitor of phospholipase D) (44, 45) in ABA-inducible gene expression. We have obtained evidence that ABI5 specifically interacts with all tested ABA signaling effectors and promoters from both monocots and dicots, demonstrating the conservation of ABA signaling in plants and the utility of rice protoplasts for molecular and cell biological dissection of ABA regulatory mechanisms.

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 Murashige and Skoog medium (46). Three days after subculturing, protoplasts were isolated and transformed with various mixtures of DNA constructs using polyethylene glycol precipitation as previously described (47, 48). Aliquots of transformed protoplast samples were treated with or without ABA and pharmacological agents for 16 h in the dark in a final volume of 0.8 ml of Krens solution.

Chemicals—1-Butanol was obtained from Acros Organics (Geel, Belgium). Synthetic ABA and lanthanum chloride were obtained from Sigma. Fluorescein diacetate was obtained from Molecular Probes Inc. (Eugene, OR) and was stored as 1% stock solution in acetone at −20 °C. ABA was dissolved and stored in absolute ethanol at −20 °C as a 0.1 M stock solution. Prior to use, required dilutions of ABA, lanthanum chloride, and 1-butanol were made in Krens solution, and control samples received the same volumes of solvents as in ABA and pharmacological treatments.

Plasmid Constructs—Plasmid pBM207 contains the wheat (*Triticum aestivum*) early methionine-labeled (*Em*) promoter driving the expression of β-glucuronidase (GUS; encoded by *uidA* from *Escherichia coli*) (40). The *AtEm6*::GUS fusion is a translational fusion including nine codons of the *AtEm6* gene, created by ligating a 1.2-kb *Xba*I-*Pvu*II fragment of the *AtEm6* gene cloned into the *Xba*I and *Sma*I sites of pBI101.3. Plasmid pTZ207 containing the *Vicia faba β-Phaseolin* promoter (49) was created by digesting pTZ/Phas with *Acc*I to release the 1.5-kb *β-Phaseolin* cDNA. The vector was end-filled with Klenow fragment, dephosphorylated with calf intestinal phosphatase, and ligated to the 2-kb *Nco*I/*Eco*RI end-filled fragment of pBM207 encoding GUS. Plasmids pQS264 and pLSP contain the barley (*Hordeum vulgare*) *Hva1* and *Hva22* promoters driving GUS expression, respectively (50). Plasmid pBM314 (51) contains cauliflower mosaic virus 35S (*35S*) promoter driving GUS expression. A construct (pDH359) containing the maize *Ubi* promoter (52) driving 1.4-kb *ABI5* cDNA was created by digesting pDH349 (*Ubi*::VP1-Myc) with *Eco*RI and filling in the linearized product with Klenow fragment before digesting with *Bam*HI to release the VP1-Myc fragment. The resulting 4.8-kb vector was then ligated with the 1.4-kb *Bam*HI/*Hin*dIII end-filled fragment of pBKA5 (18) encoding the *Arabidopsis thaliana* (L.) Heynh *ABI5* cDNA. Plasmid pCR349.13S contains the *35S* promoter driving the *VP1* sense *cDNA* (40). Plasmid pG2 encodes the *35S*-maize C4 pyruvate-orthophosphate dikinase (*Ppdk-35S*) promoter chimera driving the coding region of *Arabidopsis abi1-1* dominant-negative G180D mutant allele (25). Plasmids pG1 and pDirect2.6 were used as controls to demonstrate the protein-specific nature of the ABI5 and ABI1-1 effects. Plasmid pG1 is identical to pG2 except that it is wild type at amino acid 180 (Gly) and that the phosphatase active site has been mutated (G174D) to express a null mutant (25). Plasmid pDirect2.6 contains the *Ubi* promoter in a reverse orientation and was used as a control construct to balance the total amount of input plasmid DNA between various treatments and as a potential target for binding of endogenous transcription factors. Plasmid pAHC18 contains the *Ubi* promoter driving firefly (*Photinus pyralis*) luciferase (52) and was included in transformations to provide an internal reference for non-ABA-inducible transient transcription in reporter enzyme assays. Typically 60 μg of DNA for reporter constructs and 40 μg of DNA for effector constructs were used for transformations. Cell viability was measured by staining the protoplasts with 0.01% fluorescein diacetate, and batches of protoplasts with viability higher than 90% were used for transformations.

TABLE I

ABI5 transactivates the ABA-inducible barley promoters Hva1, Hva22, and bean β-Phas promoter in rice

Fold induction (ABA treatment and/or ABI5 transactivation) were calculated relative to control (zero ABA added, equal to unity) in paired samples co-transformed with either *Ubi*::ABI5 cDNA effector construct or *Ubi*::expression vector alone and treated as described under "Experimental Procedures." For *Em*, *Hva1*, and *Hva22* experiments, the concentration of ABA used was 10 μM; for *β-Phas* experiments, it was 100 μM. The values are the averages ± S.E. of four replicate transformations.

Construct	Fold induction in GUS expression	
	No ABA	+ ABA
Em::GUS	1	12 ± 0.6[a]
Em::GUS + *Ubi*::ABI5	2.3 ± 0.12[a]	28 ± 2.6[c]
Hva1::GUS	1	5.3 ± 0.9[a]
Hva1::GUS + *Ubi*::ABI5	3.6 ± 0.8[a]	16.9 ± 3.0[c]
Hva22::GUS	1	3.4 ± 0.5[a]
Hva22::GUS + *Ubi*::ABI5	3.5 ± 0.5[a]	8.1 ± 0.9[c]
β-Phas::GUS	1	3.6 ± 0.5[a]
β-Phas::GUS + *Ubi*::ABI5	4.7 ± 0.4[a]	52 ± 7.2[c]
35S::GUS	1	1.0 ± 0.2[b]
35S::GUS + *Ubi*::ABI5	0.8 ± 0.1[b]	0.9 ± 0.1[b]

[a] Significantly different than control, $p < 0.003$ (two-sided Student's *t* test, equal variance assumed).

[b] Not significantly induced by ABA nor *trans*-activated by ABI5, $p > 0.50$ (one-sided Student's *t* test, equal variance assumed).

[c] Significantly higher than either ABA or *Ubi*::ABI5 reference, $p < 0.03$ (one-sided Student's *t* test, equal variance assumed).

RESULTS

Previous results have demonstrated a specific log linear dose response to exogenous ABA of various promoters in synergy with transgene effectors in transiently transformed rice protoplasts (44, 45). To test the role of ABI5 in ABA signaling in rice protoplasts and further examine the conservation of ABA signaling machinery among species, we measured the effect of overexpressed ABI5 cDNA, driven by the *Ubi* promoter, on various ABA-inducible promoters. Table I shows the results of numerous promoter activation experiments that tested the specificity and extent of functional interactions of ABA and co-expressed ABI5. In these experiments a construct containing the *Ubi* promoter alone was transformed in the negative control samples to account for possible DNA effects or titration of endogenous transcription factors. Therefore, the effects observed by *Ubi*::ABI5 co-transformation are due to ABI5 overexpression. There was a significant 12-fold induction of wheat *Em*::GUS expression observed with 10 μM ABA treatment. Co-transformation of *Ubi*::ABI5 cDNA specifically and significantly transactivated *Em*::GUS expression more than 2-fold over control, in the presence or absence of ABA (Table I). Co-expression of ABI5 also specifically and significantly transactivated the ABA-inducible *Hva1* and *Hva22* promoters of barley, the *β-Phas* promoter of bean, but not the non-ABA-inducible *35S* promoter of cauliflower mosaic virus (Table I). The ABI5 transactivation functioned in synergy with exoge-

TABLE II
Lanthanum synergizes with and a PLD inhibitor (1-But) antagonizes ABA induction of Phas::GUS expression

Fold induction was calculated for ABA induction, lanthanum activation, and ABA/lanthanum synergy by comparing with the control (no ABA/lanthanum added) in paired samples treated as described under "Experimental Procedures." Inhibition of *Phas*::GUS expression by 1-butanol is expressed as the percentage of inhibition (in parentheses) relative to control samples (zero 1-butanol added). The values are the averages ± S.E. of three replicate transformations. NA, not analyzed.

Treatments			Promoter fold induction	
ABA	La^{3+}	1-Butanol	*35S*	*β-Phas*
μM	mM	%		
0	0	0	1	1
100	0	0	0.9 ± 0.04[a]	23 ± 0.001[c]
100	0	0.1	NA	9.7 ± 0.08 (−58%)
100	0	0.2	NA	7.1 ± 0.11 (−69%)
0	1	0	1.1 ± 0.3[a]	1.5 ± 0.006[c]
100	1	0	1.1 ± 0.13[a]	45 ± 0.08[b]

[a] Not significantly affected by lanthanum or ABA, $p > 0.4$ (two-sided Student's t test, equal variance assumed).
[b] Significantly higher than ABA or La^{3+} treatments alone, $p < 0.03$ (one-sided Student's t test, equal variance assumed).
[c] Significantly different than control, $p < 0.001$ (two-sided Student's t test, equal variance assumed).

nous ABA, based on the observed factorial rather than additive responses of promoters to ABA plus ABI5 treatments compared with either treatment alone (Table I). This result demonstrates that ABI5 transactivation acted via an ABA-specific pathway.

Rice protoplasts are a facile model system for cell biological analyses of signaling mechanisms (4, 53). We extended our analyses of the ABA agonist lanthanum and ABA antagonist 1-butanol, a competitive and specific inhibitor of PLD and ABA-regulated gene expression (44, 54, 55), to the *β-Phas* promoter of bean (49). Zheng *et al.* (56) have shown that β-Phaseolin accumulates up to 4% of the total endosperm protein in transgenic rice. The *β-Phas* promoter exhibited a relatively weak response to a saturating dose (100 μM) of ABA in rice protoplasts (Tables I and II), ranging from 4- to 20-fold induction because of experimental variation. 1-Butanol treatment specifically inhibited ABA-inducible *β-Phas* promoter activity in a dose-dependent manner (Table II). The biologically inactive isomer 2-butanol had no effect on ABA-inducible promoter expression (44). Lanthanum ions had a small but significant agonist effect on the *β-Phas* promoter and acted in synergy with ABA (Table II), as previously observed for other ABA-inducible promoters (44, 45, 57).

To determine whether ABI5 is regulated by the lanthanum effect, we tested the interaction of lanthanum with ABI5 transactivation of *Em*::GUS expression, and the results are shown in Table III. Lanthanum ion treatment (1 mM) significantly activated *Em*::GUS expression by 1.7-fold, and a synergistic induction was observed in response to 10 μM ABA plus lanthanum treatment (35-fold *versus* 17-fold induction in response to ABA alone). Co-transformed ABI5 potentiated both ABA and lanthanum induction of *Em*::GUS alone and in combination, because co-transformation of ABI5 resulted in a factorial increase in *Em*::GUS expression of 1.5-fold, 2-fold, and 1.3-fold over the 10 μM ABA treatment, the 1 mM lanthanum treatment, or both treatments, respectively, similar to the 1.7-fold transactivation over control (no ABA; Table III).

Phospholipase C and PLD have been implicated in ABA signaling (8, 9, 44, 54, 55). To test the dependence of ABI5 transactivation of *Em*::GUS on PLD activity, protoplasts were co-transformed with *ABI5* cDNA and were treated with or without a competitive inhibitor of PLD, 1-butanol (54). 1-Butanol significantly antagonized ABA induction and ABI5 transactivation of *Em*::GUS in a dose-dependent manner (Table IV), and the inhibitions by 1-butanol of ABA induction *versus* ABI5 transactivation were not significantly different from each other (Table IV).

Previous studies have shown that ABA induction and VP1 transactivation of ABA-inducible promoters are antagonized by overexpression of the dominant-negative allele of *ABI1* (25, 44, 45), with greater than 90% inhibition possible with increasing concentrations of effector *abi1-1* construct (53). We co-expressed ABI5 and *abi1-1* (or $ABI1^{null}$ as a negative control) in rice protoplasts and observed that *abi1-1* significantly inhibited dose-dependent ABA induction (by 68%) and ABI5 transactivation/ABA synergy of *Em*::GUS expression by 68 and 62%, respectively (Fig. 1*A*). Overexpression of *abi1-1* also inhibited ABA-inducible *Phas*::GUS expression and ABA synergy with ABI5 (Fig. 1*B*).

Protein interaction assays in yeast have identified the domains required for the physical interaction of ABI5 with ABI3 (42); however, the functional significance of the interactions is

TABLE III
ABI5 potentiates lanthanum-activated and lanthanum/ABA synergistic activation of Em::GUS expression

Fold induction and activation were calculated for ABA induction, lanthanum activation, and ABA/lanthanum synergy by comparing to the control (zero ABA/lanthanum added, equal to unity) in paired samples as described under "Experimental Procedures." The fold induction over non-ABI5-co-transformed samples is shown in parentheses.

Treatments		Promoter fold induction	
ABA	La^{3+}	*Em*::GUS	*Em*::GUS + *Ubi*::ABI5
μM	mM		
0	0	1	1.7 ± 0.06[a]
10	0	17 ± 1.4[a]	26 ± 0.50 (1.5×)[c]
0	1	1.7 ± 0.06[a]	3.4 ± 0.14 (2.0×)[c]
10	1	35 ± 1.4[b]	49 ± 2.40 (1.3×)[c]

[a] Significantly different than control, $p < 0.008$ (two-sided Student's t test, equal variance assumed).
[b] Significantly higher than ABA or La^{3+} treatments alone, $p < 0.0005$ (one-sided Student's t test, equal variance assumed).
[c] Significantly higher than without *Ubi*::ABI5, $p < 0.002$ (one-sided Student's t test, equal variance assumed).

TABLE IV
1-Butanol antagonizes ABA induction and ABI5 trans-activation of Em::GUS expression

Inhibition of reporter gene expression was calculated as the percentage of inhibition of GUS/luciferase activity relative to control samples (zero 1-butanol added) treated with or without ABA and/or co-transformed with *Ubi*::ABI5 or *Ubi*::vector alone. The values are the averages ± S.E. of three replicate transformations.

Treatments		Relative inhibition of reporter activity	
ABA	1-Butanol	*Em*::GUS	*Em*::GUS + *Ubi*::ABI5
μM	%		
10	0.1	45 ± 3[a]	57 ± 6[a,b]
10	0.2	53 ± 6[a]	60 ± 1[a,b]

[a] Significantly different than control, $p < 0.002$ (two-sided Student's t test, equal variance assumed).
[b] Not significantly different from *Em*::GUS transformation alone, $p > 0.25$ (two-sided Student's t test, equal variance assumed).

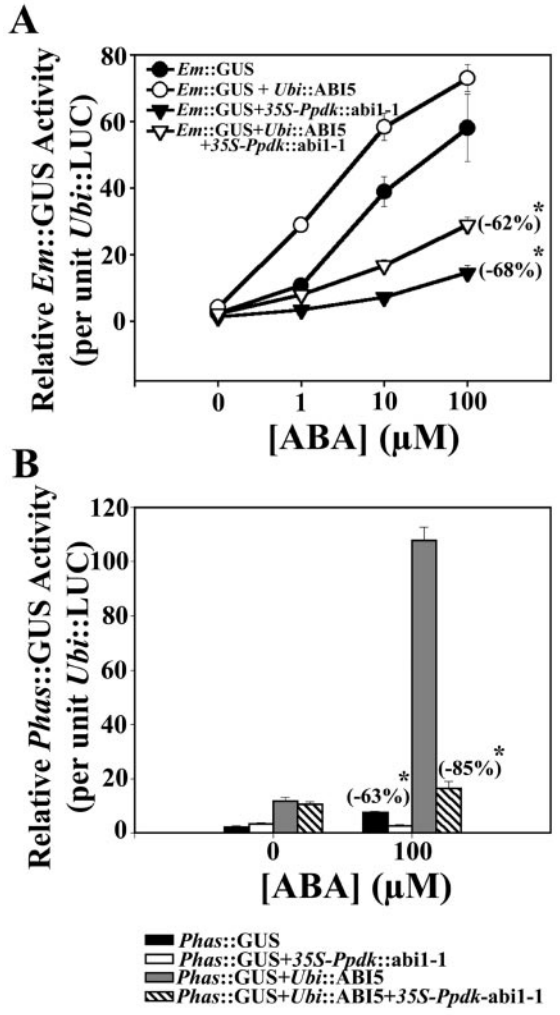

FIG. 1. **Overexpression of dominant-negative *abi1-1* antagonizes ABI5 transactivation of *Em*::GUS (*A*) and *Phas*::GUS (*B*) expression and ABA/ABI5 synergy.** Control samples were co-transformed with a *35S*-Ppdk::ABI1^{null} expression construct (25). The *numbers* in *parentheses* indicate the relative percentages of inhibition compared with control. An *asterisk* indicates a value significantly different from control, $p < 0.0004$ (paired Student's *t* test, equal variance assumed). The *error bars* are ± S.E., three or four replicates/sample. LUC, luciferase.

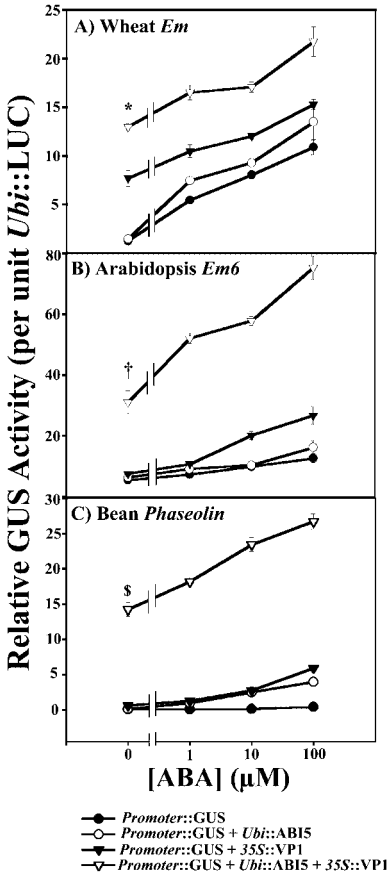

FIG. 2. **Overexpressed ABI5 interacts synergistically with ABA and VP1 to transactivate wheat *Em*, *Arabidopsis AtEm6*, and bean *β-Phas* promoters.** Protoplasts were transformed with either *Em*::GUS, *AtEm6*::GUS, or *β-Phas*::GUS constructs alone and in combination with *Ubi*::ABI5 and/or *35S*::VP1 or *Ubi*:: vector alone. The *symbols* (*,†, and $) indicate significantly different from the activation by any of the effectors alone, $p < 0.0012$, 0.01, and 0.02, respectively (paired Student's *t* test, equal variance assumed). The *error bars* are ± S.E., three or four replicates/sample. *LUC*, luciferase.

unknown. We tested for functional interactions of ABI5 with the maize ortholog of ABI3, VP1, on heterologous ABA-inducible promoters. Fig. 2 shows the results from ABI5 and VP1 cDNA effector construct co-transformation experiments on transactivation and ABA synergy of the wheat *Em* (Fig. 2*A*), *Arabidopsis AtEm6* (Fig. 2*B*), and bean *β-Phas* (Fig. 2*C*) promoters. Overexpression of ABI5 and VP1 alone transactivated all three promoters, and both effectors synergized with ABA (Fig. 2). Interestingly, VP1 and ABI5 had different modes of synergy with ABA on *Em*::GUS expression than with *AtEm6* or *β-Phas*. Overexpression of VP1 had a relatively stronger transactivating effect with low (especially zero) dose treatments of ABA (Fig. 2*A*). Conversely, the synergy between VP1 and ABA was more apparent for *AtEm6* and *β-Phas* at high ABA concentrations (Fig. 2, *B* and *C*). When both VP1 and ABI5 were co-expressed with the *AtEm6*::GUS or *β-Phas*::GUS reporters, strong synergies between both the effectors and ABA were observed (Fig. 2, *B* and *C*). Most strikingly, strong and significant synergistic interactions of ABA, ABI5, and VP1 were observed with all promoters over the range of ABA concentrations tested (Fig. 2).

DISCUSSION

We have demonstrated synergistic interactions of ABA with ABI5 and VP1, alone and in combination, in transient gene expression of both monocot and dicot ABA-inducible promoters in rice protoplasts. The data presented here consistently point toward the conservation of ABA signaling pathways between plant species. All tested ABA-inducible promoters from monocots (*Hva1*, *Hva22*, and *Em*) and dicots (*AtEm6* and *β-Phas*) were regulated by ABA in rice protoplasts, including the barley *Dehydrin* promoter studied previously (45). The ABA pathway-specific pharmacological agents La^{3+} and 1-butanol acted predictably on the ABA-regulated promoters, as did the maize *VP1* and *Arabidopsis abi1-1* gene products that have previously been shown to interact with each other and the above pharmacological agents (44, 45). The strong transactivation by VP1 of the *Em* promoter in the absence of ABA (Fig. 2*A*) is likely explained by the observation that the *Em* promoter elements sufficient for activation by ABA and VP1 are partially separable (58).

It was shown previously that a GAL4AD-ABI5 fusion activates an *AtEm6*-LacZ reporter in yeast by 2–3-fold in absence of ABA (42). Presumably, this reflects an ABA-independent DNA binding event targeted to the ABI5-binding site, with transactivation accomplished by the GAL4 activation domain. Our results showing synergy of ABI5 with ABA suggest that ABA is required for ABI5 transactivation of ABA-inducible

promoters. More significantly, the *Arabidopsis* ABI5 gene product interacted with all the tested ABA effectors, firmly supporting the conclusion that the ABA signaling mechanisms operating in rice embryonic protoplasts are conserved with those in other plants and tissues and that ABI5 activation may be the consequence, directly or indirectly, of the effectors. A similar conclusion was drawn for ABA activation of TRAB1 by Hobo *et al.* (33) based on observed ABA-dependent transactivation but ABA-independent DNA binding by TRAB1. The ABI5-related ABA-responsive element-binding proteins 1 and 2 did not transactivate the *RD29* promoter in the absence of (AREB1,2) ABA in *Arabidopsis* leaf protoplasts (35), whereas another *Arabidopsis* ABI5 family member, ABA-response element-binding factor 3, transactivated the *Em* promoter in rice protoplasts in the (ABF3) absence of exogenous ABA (36), similar to ABI5 shown here.

The molecular mechanisms of the effectors studied here are not known, but there is evidence that La^{3+} and PLD act at the plasma membrane, suggesting that they function near to a postulated membrane-bound ABA receptor that may interact with G-protein subunits coupled to calcium and ion channels (59–62). Some early ABA signal transduction components exist in animals, suggesting that ABA signaling mechanisms may be even more broadly conserved than previously thought (63). We are currently testing whether La^{3+} can modulate ABA activation of PLD in plasma membrane fractions (60).

Because the *abi1-1* allele acts as a dominant-negative protein phosphatase possibly acting on targets other than those of wild type PP2Cs (23, 25), it is difficult to interpret its antagonistic action on ABI5 activity (or any other ABA activity). For example, if *abi1-1* "poisons" or traps some necessary ABA sensitivity components, then theoretically ABI1 could function either upstream or downstream of ABI5 activation without a discernible end result of lower ABA-inducible gene expression. Allen *et al.* (21) observed reduced ABA-inducible $[Ca^{2+}]_{cyt}$ concentrations and S anion channel currents in the *abi1-1* and *abi2-1* mutants that were restored by external Ca^{2+}, suggesting that ABI1 and ABI2 act upstream of $[Ca^{2+}]_{cyt}$ to regulate anion channels. However, Grabov *et al.* (64) showed that *abi1-1* dominant-negative protein had no detectable effect on the ABA-activation of the S-anion channel in transgenic tobacco while decreasing ABA sensitivity of K^+ channels, suggesting that ABI1 function may be more flexible. Consistent with this hypothesis, Shen *et al.* (65) have shown that *abi1-1* antagonizes only the ABA-inducible pathway of gene expression but not the ABA suppression pathway of gibberellin-inducible gene expression. Taken together, we interpret these results to support the hypothesis that ABI1 could act at or near ABI5 during transcriptional activation of ABA-inducible genes in rice. ABI5 and homologs are phosphorylated *in planta* (35) and are plausible targets for ABI1 activity *in vitro* (37), because the conserved regions contain consensus residues for protein kinases (18, 35). ABI1 did not physically interact with ABI5 in yeast two-hybrid assays (42), but this result could be due to the absence of a phosphorylated ABI5 substrate in yeast.

The activities of overexpressed ABI5 and VP1 on seed-specific reporter gene expression demonstrated here suggest that spatial, temporal, and quantitative expression of transcription factors may constitute a combinatorial mechanism conferring specificity and amplitude of ABA-inducible gene expression in plants (66). Consistent with this model is the observation that the promoters studied here are also expressed to a lesser degree in vegetative tissues in response to ABA and/or stress[2] or when VP1 orthologs are ectopically expressed (26, 43). The physiological significance of a 2–4-fold increase in ABA-inducible gene expression by ABI5 in rice protoplasts is corroborated by overexpression studies with *35S*::ABI5 transgenic *Arabidopsis*. Lopez-Molina *et al.* (37) have shown that there is a limited developmental time window immediately after germination when ABA-inducible ABI5 accumulation correlates with ABA-mediated growth quiescence. Three days after germination, ABI5 expression was no longer ABA-inducible, but in *35S*::ABI5 transgenic plants expressing ABI5 to varying degrees there was a good correlation between ABA sensitivity to root and embryo growth inhibition and ABI5 protein levels, and *35S*::ABI5 plants retained water more efficiently than wild type (37). Because the rice callus cultures used in our studies are derived from embryonic tissue, it is possible that endogenous ABA regulatory factors (such as OsVP1, OsABI5, and OsABI4) interact with overexpressed ABI5 and contribute to the observed transactivations. Two-week-old transgenic *35S*::ABI5 *Arabidopsis* plants do not exhibit significantly elevated *AtEm1* or *AtEm6* expression, perhaps because of the absence of embryonic factors in vegetative tissue that interact with ABI5.[3] However, bZIP protein binding to ABA-responsive elements is independent of VP1/PvAlf and dependent on ABA *in vivo* (33, 43, 67).

Although the exact mechanisms of VP1 action are not known, it is postulated based on several protein-protein interaction studies in yeast that VP1 could potentiate ABA-inducible gene expression by forming DNA-binding complexes with 14-3-3, histone, bZIP, zinc finger, RNA polymerase II subunit RPB5 or other proteins (43, 68–71). Mutations in *VP1* and *ABI3* loci have a range of pleiotropic effects on a number of developmental markers for seed maturation and germination that have different degrees of ABA-responsiveness, and ABI3 genetically interacts with developmental mutants that are not ABA-insensitive (39, 72, 73). These results suggest that *VP1* and *ABI3* do not have entirely conserved functions and may serve to integrate ABA signaling into a network regulating development.

Because of the ease of manipulation and high throughput of transient gene expression assays, rice protoplasts are a good model system to address the molecular mechanisms of ABA responses. ABI5 and VP1 are the prototypical bZIP and ABA transcription factors, based on mutant phenotypes (18, 19, 38). The B1 domain of ABI3 binds to the N-terminal charged domains of ABI5 (42). There are eight closely related bZIP members in the *ABI5* family in *Arabidopsis*; for many of these there exists circumstantial or functional evidence for their involvement in ABA signaling (34–36). Likewise, there are 14 members of the *VP1/ABI3* B3 domain family in *Arabidopsis* (74), including two known regulators of embryonic development: *FUS3* and *LEC2*. Therefore, it is likely that genetic redundancy may mask subtle, tissue-specific ABA mutant phenotypes *in planta*. Structure/function analysis, domain swapping, and co-transformation experiments with ABI5, VP1, and family members in rice protoplasts will facilitate unraveling the complexity of ABA-inducible transcription. For example, generating an allelic series of ABI5 mutant cDNAs could address whether ABA, La^{3+}, PLD, VP1, and ABI1 modulation of ABI5 activity is mediated through the same activation domains. Likewise, there are over 30 PP2C homologs in *Arabidopsis* that have conserved amino acid residues found in ABI1 and ABI2 critical for ABA signaling (4, 25), and it is feasible to test the efficacy of these family members as effectors of ABA-inducible gene expression in transiently transformed protoplasts. The outcomes of these studies should provide ample resources and strategies

[2] S. Grillo and R. S. Quatrano, personal communication.

[3] R. Finkelstein, manuscript in preparation.

for practical applications to genetic engineering of crops with value-added seed qualities and improved productivity under environmental stress conditions.

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