Characterization and functional analysis of ABSCISIC ACID INSENSITIVE3-like genes from Physcomitrella patens

Heather H. Marella†, Yoichi Sakata†,‡ and Ralph S. Quatrano*
Department of Biology, Washington University, 1 Brookings Drive, St Louis, MO 63130, USA

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*For correspondence (fax +1 314 935 8137; e-mail rsq@wustl.edu).
‡Present address: Department of Bioscience, Tokyo University of Agriculture, Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan.
†These authors contributed equally to this work.

Summary
Although the moss Physcomitrella patens is known to respond to abscisic acid (ABA) by activating gene expression, the transcriptional components involved have not been characterized. Initially, we used the ABA-responsive Em promoter from wheat linked to β-glucuronidase (GUS) to determine whether ABI3/VP1, transcriptional regulators in the ABA-signaling pathway in angiosperms, were similarly active in the ABA response of P. patens. We show by particle bombardment that ABI3 and VP1 affect Em–GUS expression in P. patens in a manner similar to angiosperms. We also show the involvement of ABI1 in the pathway, utilizing the abi1-1 mutant allele. We isolated three ABI3-like genes from P. patens. Using an Em-like ABA-responsive promoter from P. patens (PpLea1), we demonstrate that PpABI3A, only in the presence of ABA, strongly enhances PpLea1–GUS expression in P. patens. PpABI3A also enhances ABA-induced Em–GUS expression in P. patens. In barley aleurone, PpABI3A transactivates Em–GUS but to a lesser extent than VP1 and ABI3. PpABI3A:GFP is localized to the nucleus of both protonemal cells and barley aleurone, indicating that the nuclear localization signals are conserved. We show that at least a part of the inability of PpABI3A to fully complement the phenotypes of the Arabidopsis abi3-6 mutant is due to a weak interaction between PpABI3A and the bZIP transcription factor ABI5, as assayed functionally in barley aleurone and physically in the yeast-two-hybrid assay. Our data clearly demonstrate that P. patens will be useful for comparative structural and functional studies of components in the ABA-response pathway such as ABI3.

Keywords: ABI3, ABI5, abscisic acid, Physcomitrella, transcriptional regulation, VP1.

Introduction
The phytohormone abscisic acid (ABA) not only regulates processes occurring during seed development (e.g. desiccation tolerance), but also controls processes associated with responses to water stress during vegetative development of seed plants (e.g. stomatal opening and closing). Because ABA is found in most land plants (Finkelstein and Rock, 2002), and has demonstrated physiological (Goode et al., 1993; Minami et al., 2003, 2005; Werner et al., 1991) and molecular responses in non-seed plants such as mosses (Knight et al., 1995), we have decided to take a comparative approach to determine the evolution of this response pathway as well as the role of specific regulatory proteins and protein domains that may be conserved.

Genetic approaches in Arabidopsis have been primarily responsible for identifying several components involved in the ABA-response pathway in seed plants. Arabidopsis ABA-insensitive (abi) mutants were isolated by their ability to germinate in the presence of ABA (Brocard-Gifford et al., 2004; Finkelstein, 1994; Koornneef et al., 1984) and have been extensively characterized. The abi genes that have been cloned revealed a diverse set of proteins, including ABI1 and ABI2 which encode type 2C protein phosphatases (PP2Cs) and are ABA-signaling intermediates that act as negative regulators (Gosti et al., 1999; Leung et al., 1994, 1997). The ABI3 (Giraudat et al., 1992), ABI4 (Finkelstein et al., 1998) and ABI5 (Finkelstein and Lynch, 2000) genes each encode a different type of transcriptional regulator, while ABI8 represents a novel plant-specific protein (Brocard-Gifford et al., 2004). We have used a comparative approach to characterize the ABA response in...
Physcomitrella patens, focusing initially on the plant-specific transcriptional regulator ABI3 from Arabidopsis, and its ortholog from maize VP1 (Giraudat et al., 1992; McCarty et al., 1991) to determine whether a similar regulator is found in P. patens.

ABI3/VP1-like genes have been found in various seed plants (Bobb et al., 1995; Chandler and Bartels, 1997; Footitt et al., 2003; Hattori et al., 1994; Lazarova et al., 2002; Rohde et al., 2002; Shiota et al., 1998), and regulate a set of proteins expressed during the later stages of seed development. One such gene, the Em gene (Marcotte et al., 1989), requires both ABA and ABI3/VP1 for expression (Bies-Etcheve et al., 1999; McCarty et al., 1991; Vasil et al., 1995). All of the ABI3/VP1-like genes cloned from seed plants revealed highly conserved protein domains, designated A1, B1, B2 and B3, starting from the N-terminal (Suzuki et al., 1997). In fact, maize VP1 can complement the major phenotypes of the Arabidopsis abi3-6 mutant (Suzuki et al., 2001). The B3 domain has been shown to bind DNA in vitro (Suzuki et al., 1997), whereas the B1 domain is involved in the physical interaction with the bZIP transcription factor, ABI5 (Nakamura et al., 2001). The B2 domain has been shown to be responsible for the ABA-dependent activation of ABA-regulated genes, such as Em, through the ABA-responsive element (ABRE) (Bies-Etcheve et al., 1999; Ezcurra et al., 2000; Hill et al., 1996), and facilitates the interaction with bZIP transcription factors (Hill et al., 1996) such as ABI5. However, the mechanism by which this domain functions in seed plants remains to be elucidated. Until recently, ABI3/VP1 was thought to function exclusively during seed development, specifically as a component of the ABA-signaling pathway involved in the maturation and germination of seeds (Giraudat et al., 1992; McCarty et al., 1991; Nambara et al., 1995, 2000; Parcy et al., 1997). However, recent reports have indicated that ABI3 might have broader functions outside of the seed, such as plastid development, control of flowering time, and outgrowth of axillary meristems (reviewed in Rohde et al., 2000). These analyses also revealed a novel cross-talk between ABA and auxin in seed germination and lateral root formation in Arabidopsis (Brady et al., 2003; Suzuki et al., 2001). These data clearly indicate that ABI3/VP1 is not only involved in the ABA regulation of seed development and germination, but also has broader functions in vegetative growth.

The ABA-response pathway in the moss P. patens has been demonstrated previously using the ABA-responsive promoter of the wheat Em gene. The wheat Em promoter can be activated by exogenous ABA in a transient assay using protoneval tissue as well as in stable expression lines of P. patens (Knight et al., 1995). Also, the in vitro footprint of proteins of P. patens on the Em promoter was identical to that of seed plants, indicating that the moss transcriptional machinery recognizes the same promoter region as the seed plant factors (Knight et al., 1995). This suggests that higher plants and P. patens share common ABA regulatory components, and as such will be suitable for a comparative approach to elucidate the mechanism(s) involved and the evolution of the transcriptional response to ABA. Furthermore, a homolog of the wheat Em gene from P. patens, PpLEA1, has recently been described (Kamisugi and Cuming, 2005). Similar to the wheat Em gene, the expression of PpLEA1 is highly inducible by ABA and is mediated through an ACGT motif in the promoter, a common feature of ABA-inducible genes in seed plants (Kamisugi and Cuming, 2005). This provides another comparative tool for the study of the ABA-response pathway in P. patens.

We have started to dissect the ABA-regulated transcriptional mechanism in P. patens by characterizing the structure and function of ABI3-like genes. We show by particle bombardment that the seed plant proteins, ABI1, ABI3 and VP1 can affect Em–β-glucuronidase (GUS) expression in P. patens. We successfully cloned three P. patens cDNAs encoding ABI3/VP1-like genes and tested their activity in both P. patens and barley aleurone cells using ABA-responsive group 1 LEA promoters from P. patens and wheat (Em). PpABI3A enhances ABA-induced PpLea1–GUS and Em–GUS expression in protonemal tissue and in barley aleurone cells, similar to the response elicited by ABI3/VP1. However, PpABI3A cannot significantly enhance GUS expression in the absence of ABA unlike ABI3/VP1. We also demonstrate that PpABI3A is able to function in certain cellular and molecular functions both in protonemal tissue and in aleurone cells. We show that at least a part of the inability of PpABI3A to fully complement the molecular response in the Arabidopsis abi3-6 mutant is due to a weak interaction between PpABI3A and the bZIP transcription factor ABI5, as assayed functionally in barley aleurone and physically in the yeast-two-hybrid assay. Our data clearly demonstrate that P. patens will be useful for comparative structural and functional studies of components in the ABA-response pathway such as ABI3.

Results

ABA transcriptional regulation and signaling in P. patens is similar to that of higher plants

In order to determine whether the seed plant proteins ABI1, VP1 and ABI3 can act in the ABA-induced gene expression pathway, we tested their function in P. patens protonemal tissue. Co-bombardment of the reporter Em–GUS with either Ubi-VP1 or Ubi-ABI3 demonstrated that both VP1 and ABI3 were able to transactivate Em–GUS expression, 4- and 10-fold respectively (Figure 1a). This response to VP1 and ABI3, in the absence of ABA, is similar to the effect each has in the seed plant ABA-response pathway. In Arabidopsis and barley, ABI3 and VP1 characteristically act downstream from ABI1 in the ABA-signaling pathway (Brady et al., 2003; Casaretto and Ho,
Predictably, over-expression of the dominant negative allele of ABI1, 35S–abi1-1 (Armstrong et al., 1995), with Em–GUS completely repressed induction of Em–GUS expression by ABA (Figure 1b). Furthermore, Ubi–VP1 could overcome the repression effect of abi1-1, also placing VP1 downstream from ABI1 in the ABA-signaling pathway (Figure 1b). These results suggest the involvement of molecules in *P. patens* with the same or similar functions as the regulatory ABI3/VP1 proteins from angiosperms.

**Identification of ABI3/VP1-like proteins from *P. patens***

By searching public EST and genomic databases, we cloned three PpABI3 genes. The *PpABI3A* gene encodes a 658 amino acid product, while the *PpABI3B* and *PpABI3C* gene encode 515 and 539 amino acid products, respectively. The domain structure of PpABI3A, PpABI3B, PpABI3C, AtABI3 and VP1 is relatively conserved (Figure 2a). However, PpABI3B is missing the acidic activation and serine-rich domains, while PpABI3C contains a serine-rich region, but lacks the activation domain (Figure 2a). The position of three of the introns of the B3 domain is conserved across all species, but *PpABI3C* has all five of the intron positions found in the higher plant ABI3/VP1 (Figure 2a).

The amino acid sequence alignment of PpABI3A, PpABI3B, PpABI3C, AtABI3 and VP1 reveals that the B3 domain, underlined in blue, is the most highly conserved, with 50% identity in all five sequences (Figure 2b). The B1 domain, underlined in red, and the B2 domain, underlined in green, have 23% and 40% identity in the five proteins, respectively (Figure 2b). The *P. patens* PpABI3 sequences are more closely related to each other than the higher plant sequences, and share 34% identity over their entirety. PpABI3A, PpABI3B and PpABI3C share higher identity in the conserved basic domains; B1 is 68% identical, while B2 and B3 are 60% and 64% identical, respectively. These data strongly suggest that higher plant VP1/ABI3 and *P. patens* ABI3A, ABI3B and ABI3C originated from a common ancestor gene.

**Does PpABI3A act as a transcriptional enhancer of ABA-responsive promoters?**

In order to address the question of whether the PpABI3 proteins from *P. patens* were active, we utilized the ABA-responsive promoter of the *P. patens* Late Embryogenesis Abundant (PpLEA1) gene linked to GUS (Kamisugi and Cuming, 2005). Only PpABI3A strongly enhanced PpLea1–GUS expression in the presence of ABA (Figure 3a) to a level comparable to that of VP1 (Figure 3b). All three PpABI3 proteins had little or no activity in activating GUS expression in the absence of ABA. Interestingly, when comparing the level of enhancement between VP1 and PpABI3 with and without ABA, PpABI3A exhibits a 10-fold increase in GUS activity versus a twofold increase with VP1 (Figure 3b). VP1, however, clearly shows an enhancement of the ABA response in the absence of ABA (Figure 3b). These results clearly show that the *PpABI3A* gene can act as a strong transcriptional enhancer of an ABA response from a *P. patens* promoter of an ABA-responsive gene.

Using the Em promoter (group 1 LEA from wheat), we observed a similar pattern with PpABI3A, with an enhancement effect only when ABA is present in *P. patens* (Figure 4a). However, neither VP1 nor ABI3 showed an enhancement of GUS activity in combination with ABA (Figure 4a). Comparing these results with those observed...
with the PpLea1 promoter, we see that the enhancement effect is much greater (10-fold) with the endogenous PpLea1 promoter (Figure 3b) than with the wheat Em promoter (threefold) (Figure 4a).

To determine whether PpABI3A could function in higher plants, we analyzed the ability of PpABI3A, VP1 and ABI3 to transactivate Em–GUS in barley aleurone cells (Figure 4b). Co-bombardment of Ubi–PpABI3A and Em–GUS resulted in a threefold activation of expression. Em–GUS is more strongly activated by VP1 and ABI3 (4- and 13-fold, respectively) in barley aleurone cells.

What higher plant functions are conserved in PpABI3A?

For analysis of the subcellular localization of PpABI3A, a GFP fusion construct (PpABI3A:GFP) was introduced into cells via particle bombardment. In both barley aleurone cells and *P. patens* protonemal cells bombarded with PpABI3A:GFP, the GFP signal localizes at the nucleus (Figure 5b,e). The AtABI3:GFP control also localizes to the nucleus of both barley aleurone cells and *P. patens* protonemal cells (Figure 5a,d). The GFP control shows a diffuse localization, in both the cytoplasm and the nucleus (Figure 5c,f). These results demonstrate that the localization of PpABI3A is consistent with its function as a transcriptional activator, and that the nuclear localization signals of PpABI3A and AtABI3 are recognized in both *P. patens* and barley cells.

To determine whether PpABI3A is capable of functioning in higher plants, the Arabidopsis abi3-6 mutant, a severe mutant allele of abi3 (Nambara et al., 1994), was stably transformed with PpABI3A under the control of the Arabidopsis ABI3 promoter. As a control, abi3-6 plants were also transformed with a PAtABI3–AtABI3 construct.

The seeds of abi3-6 plants are green due to a failure to degrade chlorophyll and appear shriveled up because they are desiccation intolerant, as previously reported (Figure 6b) (Nambara et al., 1994). In complementation lines expressing PAtABI3–PpABI3A, the seeds are brown (Figure 6c), just like Columbia wild-type and the PAtABI3–AtABI3 control (Figure 6a,d, respectively). Interestingly, in the PAtABI3–PpABI3A complementation lines, even though the seeds are brown, they are not desiccation-tolerant (data not shown).

The germination of abi3-6 seeds is insensitive to ABA; however, ABA inhibits germination at levels higher than 3 μM in Columbia wild-type. At 100 μM ABA, 100% of the abi3-6 mutant seeds germinate, while a 40% reduction in germination is seen in the PAtABI3–PpABI3A complementation line (Figure 7a). Germination of the PAtABI3–AtABI3 control is sensitive to ABA like Columbia wild-type (Figure 7a). Therefore, expression of PpABI3A can partially complement the ABA insensitivity of germination of the abi3-6 mutant.

Figure 2. Comparison of the PpABI3 proteins with Arabidopsis ABI3 and maize VP1.

(a) Schematic representation of PpABI3A, PpABI3B, PpABI3C, ABI3 and VP1 proteins. Conserved basic regions (B1–B3) and the serine-rich regions (S-rich) are indicated by boxes. Asterisks indicate the position of introns.

(b) Comparison of amino acid sequences of PpABI3A, PpABI3B, PpABI3C, ABI3 and VP1. The alignment was made by the T-COFFEE program. Identical residues between PpABI3A, PpABI3B, PpABI3C, and ABI3 or VP1 are shaded black and similar residues are shaded gray. The B1, B2 and B3 domains are indicated respectively by the red, green, or blue underlining.
What molecular responses are not complemented by PpABI3A?

As the P_AtABI3–PpABI3A/abi3-6 lines show partial complementation of the abi3-6 phenotypes, we tested for the expression of several seed genes controlled by ABI3 to determine whether PpABI3A could properly regulate their expression (see Table 1). Semi-quantitative reverse transcription-PCR was performed on RNA extracted from siliques of Columbia wild-type, P_AtABI3–AtABI3/abi3-6, P_AtABI3–PpABI3A/abi3-6, and abi3-6 harvested 11 days after pollination. In abi3-6 mutant seeds, the expression of these genes is reduced (Figure 7b). In the P_AABB–PpABI3A/abi3-6 complementation lines, the expression levels of CruciferinC, Napin, Oleosin2 and Rab18 are complemented to near wild-type levels (Figure 7b). However, the expression of AtEm1 and AtEm6 is not complemented in the P_AABB–PpABI3A/abi3-6 siliques (Figure 7b). The gene expression in the P_AABB–AtABI3/abi3-6 line behaves similarly to Columbia wild-type (Figure 7b). These data suggest that PpABI3A is able to partially complement gene expression in the abi3-6 mutant.

Figure 3. PpABI3A enhances ABA-regulated gene expression in P. patens.
(a) The PpLea1–GUS reporter construct and each of the PpABI3 effector constructs were introduced into P. patens protonemal tissue by particle bombardment. The protonemal tissue was allowed to recover for 24 h untreated and then subsequently incubated with (black bars) or without (white bars) 10 µM ABA for 24 h. Protein extracts were made from the treated tissue for GUS and LUC assays. Bars indicate the relative GUS activities ± SE (n = 4).
(b) The PpLea1–GUS reporter construct and each of the effector constructs were introduced into P. patens protonemal tissue by particle bombardment. The protonemal tissue was allowed to recover for 24 h untreated and then subsequently incubated with (black bars) or without (white bars) 10 µM ABA for 24 h. Protein extracts were made from the treated tissue for GUS and LUC assays. Bars indicate the relative GUS activities ± SE (n = 4).

Figure 4. PpABI3A functions as a transcriptional activator in P. patens and barley.
(a) PpABI3A functions as a transcriptional activator in P. patens. The Em–GUS reporter construct was introduced into P. patens protonemal tissue with each of the effector constructs by particle bombardment. P. patens protonemal tissue was incubated with (black bars) or without (white bars) 10 µM ABA for 48 h, and protein extracts were made for GUS and LUC assays. Bars indicate the relative GUS activities ± SE (n = 4).
(b) PpABI3A functions as a transcriptional activator in barley aleurone. The Em–GUS reporter construct was introduced into barley aleurone cells with each of the effector constructs by particle bombardment. Barley half seeds were incubated for 24 h and protein extracts were made for GUS and LUC assays. Bars indicate the relative GUS activities ± SE (n = 4).

Because PpABI3A appeared to have a defect in the complementation of ABI5-dependent gene expression (i.e. AtEm1 and AtEm6, see Table 1) in the abi3-6 background, we tested for a functional interaction between PpABI3A and barley ABI5 (HvABI5) in barley aleurone cells. It is known that ABI5 alone is not sufficient to completely activate ABA-responsive promoters, but co-expression of ABI3/VP1 with ABI5 compensates for the effect of ABA (Casaretto and Ho, 2003; Hobo et al., 1999). In the case of the Em promoter in barley aleurone cells, HvABI5 alone did not show strong activation (1.5-fold) (Figure 8a). When HvABI5 was over-expressed with VP1 or ABI3 in the absence of ABA, Em promoter activity was almost the same level as that of ABA induction (Figure 8a). In the presence of ABA, HvABI5 co-expressed with either VP1 or ABI3 enhanced the Em–GUS transactivation. PpABI3A in combination with HvABI5 also showed synergistic enhancement (11.5-fold) in the absence of ABA; however, the activation level was far lower than that of ABA induction of the Em promoter (Figure 8a). Also, co-expression of PpABI3A and HvABI5 in the presence of ABA lacked the enhancement of Em–GUS transactivation (Figure 8a). These data show that PpABI3A is capable of a basal interaction with HvABI5 in the transactivation of Em–GUS; however, it is not functionally equivalent to the interaction between ABI3/VP1 and HvABI5 in barley aleurone cells.

We performed yeast two-hybrid assays to further test the physical interaction between PpABI3A and HvABI5 in a more quantitative manner. In the nutrient-requiring growth assay, the colony size of the yeast expressing AD-PpABI3A with BD-HvABI5 is smaller than for the control yeast expressing AD-AtABI3 with BD-HvABI5 (Figure 8b). In the β-galactosidase assay, the yeast expressing AD-PpABI3A with BD-HvABI5 yielded 25% of the activity of the AD-AtABI3 control (Figure 8c). These data reveal that PpABI3A is able to interact with HvABI5, although the interaction is weak.

Discussion

ABA-signaling pathway in P. patens

The wheat Em promoter was previously shown to respond to ABA in P. patens protonemal tissue, similar to the re-
response in seed plants (Knight et al., 1995). It was shown that the ABA response was dependent on the ABRE (two ACGT boxes separated by 46 bp) (Marcotte et al., 1989). The activation of genes in seed plants by ABA required not only the ABRE and its bZIP transcription factor but also the transcriptional activator VP1. VP1 can transactivate the Em promoter in a transient assay in the absence of ABA and further transcriptional regulation was overcome by the co-expression of VP1, showing that abi1-1 acts upstream of the transcriptional activator VP1. The involvement of abi1-1 in the ABA-signaling pathway in P. patens and its relationship with VP1 appears identical to its role in seed plants (Gampala et al., 2002). At least five ESTs show significant similarity to ABI1 and ABI2 in the P. patens database (data not shown), allowing for future functional comparisons of this class of phosphatases in ABA signaling. Hence, results using the ABA-signaling intermediate ABI1, and the transcription factors ABI3/VP1, demonstrate that at least these components of the angiosperm ABA-signaling pathway function in a similar manner in a bryophyte.

Characterization of the PpABI3 proteins

As VP1 and ABI3 were active in P. patens, we proceeded to isolate three ABI3-like genes from P. patens: PpABI3A, PpABI3B and PpABI3C. Three copies of ABI3 are present in the P. patens genome as compared to one in angiosperms, and although smaller than their seed plant counterparts, the overall structure of the PpABI3 proteins follows the same pattern with the three basic domains: B1, B2, and B3. Vari-
We demonstrate that, like ABI3/VP1 in angiosperms (Gampa et al., 2002; Vasil et al., 1995), PpABI3A functions in the ABA-response pathway as a transcriptional enhancer of ABA-responsive promoters. When PpABI3A is over-expressed in the presence of exogenous ABA, enhanced expression from both P. patens (PpLea1) and wheat (Em) promoters is observed in P. patens protonemal tissue. This enhancement is similar to what has been reported in angiosperms, when VP1 is over-expressed in ABA-treated cells. PpABI3A also functions weakly (compared to ABI3 and VP1) as a transcriptional activator of the Em promoter in barley aleurone cells. In fact, the length of the activation domain of these three proteins is proportional to their enhancement of the ABA response. We also showed that PpABI3A and AtABI3 localize to the nucleus of both barley aleurone and P. patens protonemal cells. The fact that PpABI3A localizes to the nucleus and enhances expression in both P. patens and barley aleurone cells indicates that the core transcriptional regulatory properties of PpABI3A are at least partially conserved.

We also show that PpABI3A, but not PpABI3B and PpABI3C, enhances expression from a P. patens ABA-responsive promoter, PpLea1 (Kamisugi and Cuming, 2005). PpABI3A contains all the conserved domains found in the seed plant orthologs, including the activation domain, while PpABI3B and PpABI3C lack the N-terminal activation domain. The B1 and B2 domains of ABI3 and VP1 are responsible for some unique functions of these proteins (Bies-Etheve et al., 1999; Ezcurra et al., 2000; Hill et al., 1996; Nakamura et al., 2001). For example, the Arabidopsis abi3-7 mutant has a point mutation that converts an alanine into threonine in the B2 domain and prevents the expression of AtEm1 and AtEm6 (Bies-Etheve et al., 1999). This alanine residue is perfectly conserved not only in all known ABI3/VP1s from seed plants, but also in the PpABI3 proteins. Similarly, the abi3-8 mutation alters a conserved leucine in B1, which is possibly involved with ABI5 interaction (Nambara et al., 2002). However, this leucine is not conserved in P. patens. Alignments such as these will target those amino acids/domains for further analysis to determine their conserved function during the evolution of land plants.

**Functional analysis of PpABI3A**

In the B1 and B2 domains of ABI3 and VP1 are responsible for some unique functions of these proteins (Bies-Etheve et al., 1999; Ezcurra et al., 2000; Hill et al., 1996; Nakamura et al., 2001). For example, the Arabidopsis abi3-7 mutant has a point mutation that converts an alanine into threonine in the B2 domain and prevents the expression of AtEm1 and AtEm6 (Bies-Etheve et al., 1999). This alanine residue is perfectly conserved not only in all known ABI3/VP1s from seed plants, but also in the PpABI3 proteins. Similarly, the abi3-8 mutation alters a conserved leucine in B1, which is possibly involved with ABI5 interaction (Nambara et al., 2002). However, this leucine is not conserved in P. patens. Alignments such as these will target those amino acids/domains for further analysis to determine their conserved function during the evolution of land plants.

**ABA-responsive pathway as a transcriptional enhancer of ABA-responsive promoters.** When PpABI3A is over-expressed in the presence of exogenous ABA, enhanced expression from both P. patens (PpLea1) and wheat (Em) promoters is observed in P. patens protonemal tissue. This enhancement is similar to what has been reported in angiosperms, when VP1 is over-expressed in ABA-treated cells. PpABI3A also functions weakly (compared to ABI3 and VP1) as a transcriptional activator of the Em promoter in barley aleurone cells. In fact, the length of the activation domain of these three proteins is proportional to their enhancement of the ABA response. We also showed that PpABI3A and AtABI3 localize to the nucleus of both barley aleurone and P. patens protonemal cells. The fact that PpABI3A localizes to the nucleus and enhances expression in both P. patens and barley aleurone cells indicates that the core transcriptional regulatory properties of PpABI3A are at least partially conserved.

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This will allow further elucidation of this interaction by the use of domain swaps of the PpABI3 proteins with the angiosperm orthologs, along with the construction of specific promoter sequences.

With respect to the specific ABRE in each of these promoters, PpLea1 contains a single ACGT motif while the ABRE from angiosperms have either two ACGT motifs or one ACGT motif and a coupling element (Kamisugi and Cuming, 2005; Shen et al., 2004). While we have demonstrated that the wheat Em promoter functions in an ABA-responsive manner in both barley and P. patens, the same cannot be said of the PpLea1 promoter, which fails to express in barley aleurone cells (Kamisugi and Cuming, 2005). These studies appear to indicate that the ABA-responsive promoter in P. patens (PpLea1) represents the ancestral state, while ABA-responsive promoters from angiosperms (wheat Em) have additional factors/sequences involved in the response. (Kamisugi and Cuming, 2005). A further comparative analysis of the cis-elements in the wheat Em and PpLea1 promoters that are required in aleurone and protoneal cells for the level of ABA responsiveness will aid in our understanding of the evolution of the response elements and the factors that bind to them.

Complementation of abi3-6

Given the transcriptional activation and nuclear localization properties of PpABI3A in barley, we further showed that PpABI3A could partially complement the Arabidopsis abi3-6 mutant. Similar results have been reported for ABI3/VP1 orthologs from seed plants; abi3-6 lines expressing maize VP1 were unable to properly express CruciferinC (Suzuki et al., 2001), while the yellow cedar ABI3 failed to fully complement the ABA sensitivity of germination (Zeng and Kermode, 2004). However, in both of these complementation experiments, ABI3/VP1 expression was driven by the enhanced 35S promoter, which is known to have weak promoter activity during seed development (Benfey et al., 1990; Sunilkumar et al., 2002; Terada and Shimamoto, 1990). Hence, the failure of these higher plant proteins to complement fully may be due to an improper spatial, temporal or level of expression of ABI3 or VP1. In our hands, complementation of the abi3-6 mutant using 35S–PpABI3A or 35S–AtABI3 showed no detectable complementation of the abi3-6 phenotypes (data not shown). However, when the AtABI3 promoter was used to drive expression of PpABI3A or AtABI3, AtABI3 fully complemented the mutant while PpABI3A only partially complemented it.

The seed color phenotype of the abi3-6 was fully complemented (from green to brown) in lines expressing PpABI3A. However, full ABA sensitivity was not observed in PpABI3A/abi3-6 seeds, where only 60% of the seeds germinate on 100 μM ABA. Seeds from abi3-6 germinated at 100 μM ABA, whereas seeds from wild-type and AtABI3-complemented lines did not germinate above 10 μM ABA. This partial complementation of ABA sensitivity by PpABI3A may be due to a failure of PpABI3A to interact with the Arabidopsis ABA-response factors, such as ABI5, because certain conserved amino acids in the B1 domain are lacking. The PpABI3 proteins lack the conserved leucine of the Arabidopsis abi3-8 mutation, which alters the B1 domain and possibly the interaction with ABI5 (Nambara et al., 2002).

Transcript levels of several ABI3-regulated, seed-specific genes suggest that this may indeed be the case. Expression of AtEm1 and AtEm6, both of which require ABI5 for expression (Finkelstein and Lynch, 2000), are not complemented in the PpABI3A lines. However, other seed-specific genes that are not regulated by ABI5 are fully complemented by PpABI3A, such as Napin and CruciferinC, which require another member of the bZIP family (OPAQUE2-like) (Lara et al., 2003), and Oleosin2 and Rab18 (see Table 1).

PpABI3A interaction with ABI5

In this study, we showed that PpABI3A and HvABI5 functionally interact in aleurone cells, but that this was not functionally equivalent to the interaction of ABI3/VP1 with HvABI5 in the same cells. This conclusion is also supported by our demonstration of a weak physical interaction between PpABI3A and HvABI5 in the yeast two-hybrid assays; AtABI3 and HvABI5 have a strong interaction even though one protein is from a dicot and the other from a monocot. The B1 domain of AtABI3 is necessary for the interaction with AtABI5 in yeast two-hybrid assays (Nakamura et al., 2001), highlighting this domain for functional comparisons of ABI5 interactions. Several sequences for bZIP transcription factors are in the P. patens genome, at least two of which fall into the ABI5 subfamily. This will enable a functional comparison of the interaction between ABI3 and ABI5 in P. patens. In this same regard, ABI3/VP1 are able to physically interact not only with ABI5 and OPAQUE2-related bZIP transcription factors, but also other proteins such as 14-3-3 (Schultz et al., 1998), the transcription factor CONSTANS, a protein involved in cell-cycle regulation, and an E3 ligase with a RING motif (Jones et al., 2000; Kurup et al., 2000; Zhang et al., 2005). It will be interesting to determine how the composition of the ABI3/VP1 transcriptional complex varies and how this relates to the different functions of the members of the ABA-signaling networks in P. patens and higher plants.

Experimental procedures

Plant material

P. patens subspecies patens (Grandsen) was used as the wild-type strain. The strain was maintained as previously described (Bezanilla

Barley seeds (Hordeum vulgare cv. Himalaya) from 1998 harvests at Washington State University in Pullman (WA, USA) were used in all experiments. Arabidopsis thaliana ecotype Columbia was used as the wild-type. The abi3-6 mutant was a gift from Dr Peter McCourt (University of Toronto). Plants were grown in a mixture of 60% Redi-Earth and 40% vermiculite at 22°C under a 16 h light/8 h dark regime in a growth chamber. Seeds were surface-sterilized for 5 min in 95% ethanol, dried, plated on MS medium containing 0.8% agar and subjected to 4 days of cold treatment in the dark before being moved to a growth chamber at 22°C under a 16 h light/8 h dark regime.

Cloning of PpABI3 cDNAs

The B3 domain of PpABI3 from Arabidopsis was used as a query sequence for the BLAST program (Altschul et al., 1990) against Physcobsbase (http://moss.nibb.ac.jp/) (Nishiyama et al., 2003) to retrieve cDNAs encoding B3 domain proteins. As a result, three independent cDNA clones were identified as candidates. From the sequence information of the contigs, we set up primers to amplify the full-length cDNAs. The primers to amplify the cDNA clone pphn12b05, which we named PpABI3A, are 5'-AT-GTGAAGAGTGACCTGAGC-3' and 5'-CTCACCCCTGTTAGACAGAAA-3' for the 5' and 3' ends, respectively. PCR of P. patens against the full-length cDNA library (Nishiyama et al., 2003), a gift from Dr M. Hasebe (National Institute for Basic Biology, Japan) was performed with KlenTaq LA (Clontech, Mountain View, CA, USA) and the supplied buffer with 25 µM of each dNTP and 3.5 µM MgCl2, using 35 cycles of 96°C for 1 min, 56°C for 1 min, and 68°C for 2 min. The DNA fragment was subcloned into pGEM-T Easy (Promega, Madison, WI, USA) and subjected to DNA sequencing using the M13 forward and reverse primers and Big Dye Terminator mix (Applied Biosystems, Foster City, CA, USA). The amino acid sequence was analyzed using Genetics software (Hitachi Software Engineering, Tokyo, Japan).

PpABI3B was cloned from a partial EST sequence (Frank et al., 2005) using 5' and 3' RACE. RNA was extracted from P. patens gametophore tissue using the RNeasy Kit (Qiagen, Valencia, CA, USA) for use with the 5' and 3' RACE kit (Invitrogen). The coding sequence of PpABI3B was amplified by RT-PCR and cloned into the pGEM-T Easy (Promega) or pCR 2.1-TOPO vector (Invitrogen). Accuracy of the amplified fragments was checked by sequencing, then each fragment was placed downstream of the maize Ubi1 promoter (Christensen and Quail, 1996), followed by the terminator of the nopaline synthase (nos) gene. The GFP fusions were generated by PCR to add GFP to the C-terminal end as a translational fusion in the respective Ubi–PpABI3/PpABI3A–nos vector. Em–GUS (pBM113KP) (Marcotte et al., 1989), Ubi–LUC (pAHCl19) (Bruce et al., 1989), Ubi–HvAB15 (Casaretto and Ho, 2003), Ubi–GFP (Zentella et al., 2002), PpLea1–GUS (Kamisugi and Cuming, 2005) and 35S–abi1-1 (Armstrong et al., 1995) were as described previously.

Transient assays

In principle, DNA delivery to barley aleurone cells was performed as described previously (Shen et al., 1993). Two microgram of each reporter construct (Em–GUS and Ubi–LUC) and effector constructs were used to prepare tungsten particles for three shots. Bombarded seeds were treated with or without 20 µM ABA for 24 h at room temperature. Transient transformation of P. patens by particle bombardment was carried out as described previously (Bezania et al., 2003). We used 0.8 µg of each reporter construct (Em–GUS/PpABI3A–GUS and Ubi–LUC) and effector construct to prepare gold particles for four shots. One-week-old protocormal tissue was used for bombardment, and then incubated on PpNH4 agar medium with or without 10 µM ABA for 48 h under the conditions described in the Plant Material section.

Four barley seeds or moss protocormal tissue were ground in extraction buffer (Lanahan et al., 1992) and cell debris was spun down at 20 000 g. GUS and luciferase activities were measured as described previously (Lanahan et al., 1992). GUS activity was normalized by the luciferase activity and represented as relative GUS activity (GUS/LUC) ±SE. All the experiments consisted of four replicates.

Microscopy

Confocal microscopy was performed using barley aleurone tissue or protocormal tissue of P. patens placed in water on a glass slide and covered with a cover slip. A Leica Confocal System TCS SP2 (Mannheim, Germany) was used for imaging GFP signal with an argon laser.

Light microscopy of Arabidopsis seeds and siliques was performed using an Olympus SZX12 system (Melville, NY, USA).
Arabidopsis complementation

The vector pCHF3 (Jarvis et al., 1998), a gift from Michael Neff (Washington University), which contains the CaMV 35S promoter and pea RBCS terminator, was used to express PpABI3A or ABI3 in Arabidopsis. The abi3-6 mutant plants were transformed by Agrobacterium tumefaciens strain GV3101 using the floral dip technique (Clough and Bent, 1998). Transformsants were obtained by selection on MS plates containing kanamycin (50 mg l\(^{-1}\)). 3SS–ABI3 and 3SS–PpABI3A plants in the T\(_3\) generation, which were segregating 3:1 for kanamycin resistance, failed to show any complementation of the abi3-6 phenotypes. Therefore, we removed the CaMV 35S promoter from pCHF3 and replaced it by EcoRI and BamHI digestion with the first 1752 bp of the Arabidopsis ABI3 promoter, which we amplified by PCR from the genomic sequence of the At3g24650 locus. The primers used to amplify the ABI3 promoter were 5'-GAATTCCC-GTGTACGTCTTATGTTGGC-3' and 5'-GAATTCAGAAGCAGAAGCTTT-3'. The P\(_{\text{GABI}}\)-PpABI3A and P\(_{\text{GABI}}\)-AtABI3 constructs were then transformed into the abi3-6 mutant as described above. We obtained four independent P\(_{\text{GABI}}\)-PpABI3A complementation lines and seven of the P\(_{\text{GABI}}\)-AtABI3 lines, which had a single T-DNA insertion as determined by kanamycin segregation. Seed color was scored after the siliques had turned yellow, approximately 18 days after flowering.

ABA germination assay

Seeds were harvested at 15 days after flowering from Columbia wild-type, abi3-6, P\(_{\text{GABI}}\)-PpABI3A/abi3-6 and P\(_{\text{GABI}}\)-AtABI3/abi3-6 complementation lines and surface-sterilized. Seeds were plated on MS medium containing 0, 0.1, 1, 10 or 100 \(\mu\)M ABA, and cold-treated for 4 days at 4°C in the dark. The plates were then moved to 22°C with a 16 h light/8 h dark regime. The germination rate was scored after 4 days using at least 40 seeds for each assay condition. The assay was performed in triplicate.

RT-PCR analysis

RNA was extracted from 11-day-old siliques using an RNAqueous kit with the Plant RNA isolation aid (Ambion, Austin, TX, USA). Total RNA (500 ng) was first treated with DNaseI (amplification grade; Invitrogen) for 15 min at room temperature. The enzyme was inactivated by heating at 65°C for 10 min in the presence of 2.5 mM EDTA. The RNA was used for making cDNA by the ThermoScript RT-PCR system (Invitrogen). The RNA was first pre-incubated with oligo(dT)\(_{20}\) and dNTPs at 65°C for 5 min, then placed on ice. The cDNA Synthesis Mix was added to the RNA and incubated at 55°C for 1 h. The reaction was terminated by heating at 85°C for 5 min, and then RNaseH was added and incubated at 37°C for 20 min to remove the RNA template. PCR was carried out using 2 \(\mu\)l of the cDNA with 0.4 \(\mu\)l of Taq polymerase (Invitrogen) in a 50 \(\mu\)l reaction. The primer sequences are 5'-AGGTCAGTATGAGCAGCTT-3' and 5'-ACGCGGAAGCTTTTCTTGC-3'; AtEm1 (5'-AGAGCATCAGAAGCTTTTCTTCTG-3'; CruciferinC (5'-AGGTCAGTATGAGCAGCTT-3'; Napin1 (5'-AGGTCAGTATGAGCAGCTT-3'; Oleosin2 (5'-AGGTCAGTATGAGCAGCTT-3'; Ubiquitin10 (5'-AAAGATCCAGAAGACT-3').

References


Yeast two-hybrid assay

For constructs used in yeast two-hybrid experiments, the entire coding sequences of HvABI5, ABI3 and PpABI3A were amplified by PCR. The HvABI5 fragment was cloned into the multi-cloning site of pGBTK (Clontech) in-frame with the GAL4 binding domain sequence (BD-HvABI5), and fragments of ABI3 and PpABI3A were cloned into the multi-cloning site of pGAD424 (Clontech) in-frame with the GAL4 activation domain sequence (AD-ABI3 and AD-PpABI3A).

Media were prepared as described (Sambrook and Russell, 2001). Yeast two-hybrid assays were carried out as described previously (James et al., 1998). In this system, three different reporter genes, GAL2-ADE2, GAL1-HIS3 and GAL7-LacZ, are available. A host strain PJ69-4A-4A was used for BD constructs, and PJ69-4A-AD-4A was used for AD constructs. Mating was carried out by co-inoculation of a strain harboring the BD construct and a strain harboring the AD construct. The diploid strains were selected on SD medium without leucine and tryptophan (SD-LW), and used for analyses of the interaction between the transcription factors. For the plate assay, SD-LW medium without histidine and adenine (SD-LWHA) with 35 \(\mu\)g 3-aminoazolone (3-AT) was used for culture. For the quantification assay, GAL4-DB-driven β-galactosidase gene expression was analyzed using ONPG (O-nitrophenylgalactoside) as the substrate according to the protocol described at http://www.fhcrc.org/labs/gottschling/yeast/Bga.html.

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