Characterization and Functional Analysis of *ABSCISIC ACID INSENSITIVE3*-like Genes from *Physcomitrella patens*

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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 if 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.
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). Since ABA is found in most land plants (Finkelstein and Rock, 2002), and has demonstrated physiological (Goode et al., 1993; Minami et al., 2003; Minami et al., 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; Leung et al., 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 will take a comparative approach to characterize the ABA response in Physcomitrella patens and will initially focus on the plant specific transcriptional regulator ABI3 from Arabidopsis,
and its ortholog from maize VP1 (Giraudat et al., 1992; McCarty et al., 1991) and
determine if 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-Etheve 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, like Em, through the ABA-responsive
element (ABRE) (Bies-Etheve 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; Nambara et al., 2000; Parcy et al., 1997). However, recent reports
have pointed out that ABI3 might have broader functions outside of the seed, such as
plastid development, flowering time, and outgrowth of axillary meristems (reviewed in Rohde et al., 2000). These analyses also revealed a novel crosstalk 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* was 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 protonemal 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-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 \textit{P. patens} is similar to that of higher plants

In order to determine if the seed plant proteins ABI1, VP1 and ABI3 can act in the ABA induced gene expression pathway, we tested their function in \textit{P. patens} protonemal tissue. Co-bombardment of the reporter \textit{Em-GUS} with either \textit{Ubi-VP1} or \textit{Ubi-ABI3} demonstrated that both VP1 and ABI3 were able to transactivate \textit{Em-GUS} expression, four- and ten-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, 2003). Predictably, over-expression of the dominant negative allele of \textit{ABI1, 35S-abi1-1} (Armstrong et al., 1995), with \textit{Em-GUS} completely repressed induction of \textit{Em-GUS} expression by ABA (Figure 1B). Furthermore, \textit{Ubi-VP1} could overcome the repression effect of \textit{abi1-1} placing VP1 also downstream from ABI1 in the ABA signaling pathway (Figure 1B). These results suggest the involvement of molecules in \textit{P. patens} with the same or similar functions as the regulatory ABI3/VP1 proteins from angiosperms.
Figure 1

Seed plant ABA response factors function in *P. patens*.

**A)** Maize VP1 and Arabidopsis ABI3 transactivate *Em-GUS* expression in *P. patens*. *Em-GUS* and *Ubi-LUC* were co-bombarded into *P. patens* protonemal tissue with or without the effector constructs, *Ubi-VP1* or *Ubi-ABI3*, using 0.2µg of each construct. Bars indicate the relative GUS activities ±SE after 48h incubation (n=4).

**B)** *Em-GUS* expression in *P. patens* is repressed by *abi1-1* and restored by VP1. *Em-GUS* and *Ubi-LUC* were co-bombarded into *P. patens* protonemal tissue with or without the effector constructs, *35S-abi1-1* and *Ubi-VP1*, using 0.2µg of each construct. Bars indicate the relative GUS activities ±SE after 48h incubation with (black bars) or without (white bars) 10µM ABA (n=4).
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* gene 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 the 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.
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 underlines.

**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 *Lea1-GUS* expression in the presence of ABA (Figure 3A) 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 PpABI3A with and without ABA, PpABI3 exhibits a ten-fold increase in GUS activity versus a two-fold 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.
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 24h untreated and then subsequently incubated with (black bars) or without (white bars) 10µM ABA for 24h. 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 24h untreated and then subsequently incubated with (black bars) or without (white bars) 10µM ABA for 24h. Protein extracts were made from the
treated tissue for GUS and LUC assays. Bars indicate the relative GUS activities ±SE (n=4).

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 (ten-fold) with the endogenous *PpLea1* promoter (Figure 3B) than with the wheat *Em* promoter (three-fold) (Figure 4A).

To determine if *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 three-fold activation of expression. *Em-GUS* is more strongly activated by VP1 and ABI3 (four- and thirteen-fold, respectively) in barley aleurone cells.
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) 10µM ABA for 48h 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 24h and protein extracts were made for GUS and LUC assays. Bars indicate the relative GUS activities ±SE (n=4).

**What higher plant functions are conserved in PpABI3A?**

For analysis of the sub-cellular of 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 (Figures 5B and 5E). The AtABI3:GFP control also localizes to the nucleus of both barley aleurone cells and *P. patens* protonemal cells (Figures 5A and 5D). The GFP control shows a diffuse localization, in both the cytoplasm and the nucleus (Figures 5C and 5F). 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.
Figure 5

A PpABI3A:GFP fusion protein localizes to the nucleus in barely aleurone and *P. patens* cells.

Constructs for the over-expression of AtABI3:GFP (A and D), PpABI3A:GFP (B and E), and GFP (C and F) protein were delivered into barley aleurone cells (A-C) and *P. patens* protonemal tissue (D-F) by particle bombardment. Images of transformed cells were taken by confocal microscopy. The GFP signal and auto-fluorescence of chloroplasts are shown by green and red color, respectively.

To determine if PpABI3A is capable of functioning in higher plants, the Arabidopsis *abi3*-6 mutant, a severe 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 *P ABI3*-AtABI3 construct. The seeds of *abi3*-6 plants are green due to a failure to degrade chlorophyll and appear shriveled up because of the inability to undergo desiccation, as previously reported (Figure 6B) (Nambara et al., 1994). In complementation lines expressing *P ABI3*-PpABI3A the seeds
are brown (Figure 6C), just like Columbia wild type and the \( P_{\text{AtABI3-AtABI3}} \) control (Figure 6A and 6D respectively). Interestingly, in the \( P_{\text{AtABI3-PpABI3A}} \) complementation lines, even though the seeds are brown, they are not desiccation tolerant (data not shown).

Figure 6

\( \text{PpABI3A} \) complements the green seed phenotype of \( abi3-6 \).

Seeds from mature siliques were harvested from (A) Columbia wild type, (B) \( abi3-6 \), and the transgenic lines of (C) \( P_{\text{AtABI3-PpABI3A}}/abi3-6 \) and (D) \( P_{\text{AtABI3-AtABI3}}/abi3-6 \).

The germination of \( abi3-6 \) seeds is insensitive to ABA, however, ABA inhibits germination at levels higher than 3µM in Columbia wild type. At 100µM ABA, 100% of the \( abi3-6 \) mutant seeds germinate, while a 40% reduction in germination is seen in the \( P_{\text{AtABI3-PpABI3A}} \) complementation line (Figure 7A). The germination of the \( P_{\text{AtABI3}}\)
*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 7

PpABI3A partially complements the *abi3-6* mutant.

A) PpABI3A partially complements the ABA insensitivity of *abi3-6* seed germination. Seeds of Columbia wild type (red circle), *abi3-6* (green square), *P*<sub>AtABI3</sub>*AtABI3A*/abi3-6 (orange cross), and *P*<sub>AtABI3</sub>*AtABI3*/abi3-6 (blue triangle) were plated on MS media containing 0, 0.1, 1, 10, or 100 μM ABA and cold treated for 4 days at 4°C in the dark. The plates were then moved to 22°C with a 16-hour light and 8-hour dark light regime. The germination rate was measured after 4 days using at least 40 seeds per assay. Bars indicate ±SE of three independent replicates.

B) PpABI3A partially complements the expression of AtABI3 regulated genes in *abi3-6* seeds.
RNA was extracted from siliques 11 days after pollination of Columbia wild type, \( P_{AtABI3}\text{-}AtABI3/abi3-6 \), \( P_{AtABI3}\text{-}PpABI3A/abi3-6 \), and \( abi3-6 \). RT-PCR was performed using 0.5 \( \mu \)g of total RNA and the expression of various seed genes controlled by ABI3 was analyzed.

**What molecular responses are not complemented by PpABI3A?**

Since the \( P_{AtABI3}\text{-}PpABI3A/abi3-6 \) lines show partial complementation of the \( abi3-6 \) phenotypes, we tested for the expression of several seed genes controlled by ABI3 to determine if PpABI3A could properly regulate their expression (see Table 1). Semi-quantitative RT-PCR was performed on RNA extracted from siliques of Columbia wild type, \( P_{AtABI3}\text{-}AtABI3/abi3-6 \), \( P_{AtABI3}\text{-}PpABI3A/abi3-6 \), and \( abi3-6 \) harvested 11 days after pollination. In \( abi3-6 \) mutant seeds, the expression of these genes is reduced (Figure 7B). In the \( P_{AtABI3}\text{-}PpABI3A/abi3-6 \) complementation lines, the expression level of \textit{CruciferinC}, \textit{Napin}, \textit{Oleosin2}, and \textit{Rab18} is complemented to near wild type levels (Figure 7B). However, the expression of \textit{AtEm1} and \textit{AtEm6} is not complemented in the \( P_{AtABI3}\text{-}PpABI3A/abi3-6 \) siliques (Figure 7B). The gene expression in the \( P_{AtABI3}\text{-}AtABI3/abi3-6 \) 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.

Because PpABI3A appeared to have a defect in the complementation of ABI5 dependent gene expression (i.e. \textit{AtEm1} and \textit{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 also 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 with 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 on 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 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.
Figure 8

PpABI3A interacts weakly with HvABI5.

A) PpABI3A interacts weakly with HvABI5 in barley aleurone cells.

The Em-GUS reporter construct was introduced into barley aleurone cells in various combinations with each of the effector constructs. Barley half seeds were incubated with (black bars) or without (white bars) 20µM ABA for 24h. Bars indicate the relative GUS activities ±SE (n=4).

B) PpABI3A interacts weakly with HvABI5 in yeast-two-hybrid plate assay for analysis of reporter genes, GAL2-ADE2, GAL1-HIS3. The diploids harboring indicated BD and AD constructs were grown for one week on SD-LW medium lacking adenine and histidine containing 35mM 3-AT (see Materials and Methods). Bar=50mm.
C) β-galactosidase activity of the diploids indicated in (B). Black bars represent the BD-HvABI5 interaction and white bars represent the pGBD control. The individual data is the average ± SD (n=3). pGBD (pGBTK) and pGAD (pGAD424) are vector controls. [The differences between pGAD/ABI5 and PpABI3/ABI5, pGAD/BD-ABI5 and ABI3/ABI5, and PpABI3/HvABI5 and ABI3/HvABI5 were significant (P<0.05, P<0.01 and P<0.01, respectively)]
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 response in seed plants (Knight et al., 1995). They showed 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 enhance expression in the presence of ABA (Vasil et al., 1995). Mutants of VP1, and its ortholog from Arabidopsis ABI3, failed to accumulate the *Em* transcript during seed development (Bies-Etheve et al., 1999; McCarty et al., 1991; Parcy et al., 1994). In this study, we confirmed the results of Knight et al. (1995), and further showed that both VP1 and ABI3 can transactivate this promoter in *P. patens* without exogenous ABA but unlike the response in angiosperms, they failed to enhance expression with ABA.

The activation of ABA-regulated genes in seed plants also requires the signaling intermediate *ABI1*, which encodes a PP2C phosphatase and acts as a negative regulator of ABA signaling in Arabidopsis. The dominant negative *abi1-1* allele was shown to inhibit the ABA signaling not only in Arabidopsis but other seed plants such as barley, rice, and tomato (Carrera and Prat, 1998; Casaretto and Ho, 2003; Gampala et al., 2002; Grabov et al., 1997; Hagenbeek et al., 2000). We showed that *abi1-1* blocks the ABA induction of *Em-GUS* expression in *P. patens*. In addition, this negative 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**

Since 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. Variation in the structure occurs primarily at the N-terminus, where the PpABI3 proteins have either a shorter acidic activation domain (PpABI3A) or lack this domain (PpABI3B and PpABI3C). The serine-rich region found in the seed plant orthologs is also found in PpABI3A and PpABI3C. The intron-exon junctions between the angiosperm orthologs and PpABI3C are perfectly conserved. In contrast, PpABI3A and PpABI3B only share three of the five intron positions in the B3 domain, suggesting that these paralogs were lost in the seed
plant lineage. These observations strongly suggest that the VP1/ABI3 gene family arose prior to the divergence between mosses and seed plants.

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 in determining their conserved function during the evolution of land plants.

**Functional analysis of PpABI3A**

We demonstrate that like VP1/ABI3 in angiosperms (Gampala 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-GUS* (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. All three ABI3 proteins from *P. patens* either lack the ability or show weak transactivation in the absence of ABA. Interestingly, these results in protonemal tissues are specific to *PpLea1-GUS* promoter, since all three PpABI3 proteins are able to transactivate the *Em-GUS* promoter (data not shown). Hence, the structure/function relationship of the ABI3 proteins from *P. patens* reflects not only their domain structure but also their interaction with specific ABA-responsive promoters. This will allow further understanding 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 demonstrate 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 which are required in aleurone and protonemal 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 fully complement may be due to 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 drives expression of PpABI3A or AtABI3, AtABI3 fully complemented the mutant while PpABI3A only partially complemented.

The seed color phenotype of the \textit{abi3-6} was fully complemented (from green to brown) in lines expressing PpABI3A. \textbf{However}, full ABA sensitivity was not observed in \( P_{\text{AtABI3}} \)-\( PpABI3A/abi3-6 \) seeds, where \textbf{only} 60\% of the seeds germinate on 100\( \mu \text{M} \) ABA. Seeds from \textit{abi3-6} germinated at 100\( \mu \text{M} \) ABA, whereas seeds from wild type and AtABI3 complemented lines did not germinate above 10\( \mu \text{M} \) 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, since certain conserved amino acids in the B1 domain are lacking. \textbf{The PpABI3 proteins lack the conserved leucine of the \textit{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 \textit{AtEm1} and \textit{AtEm6}, both of which require ABI5 for expression (Finkelstein and Lynch, 2000), are not complemented in the \( P_{\text{AtABI3}} \)-\( PpABI3A \) lines. However, other seed-specific genes that are not regulated by ABI5 are fully complemented by PpABI3A, such as \textit{Napin} and \textit{CruciferinC}, which require another member of the bZIP family (OPAQUE2-like) (Lara et al., 2003), and \textit{Oleosin2} and \textit{Rab18} (see Table 1).
**PpABI3A interaction with ABI5**

In this study, we showed that PpABI3A and HvABI5 functionally interact in aleurone cells but it 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) and now targets 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 the 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 (Gransden) was used as the wild type strain. The strain was maintained as previously described. Briefly, protonemal tissue were ground with a PowerGen 125 homogenizer (Fisher Science, Hampton, NH) and inoculated onto cellophane overlaid on 0.7 % agar plates of PpNH₄ medium (1.8 mM KH₂PO₄, pH7.0, 3.4 mM Ca(NO₃)₂, 1 mM MgSO₄, 45mM FeSO₄, 0.22 mM CuSO₄, 0.19 µM ZnSO₄, 9.9 µM H₃BO₃, 2 µM MnCl₂, 0.23 µM CoCl₂, 0.17 µM KI, 0.1 µM Na₂MoO₄, 2.7 µM ammonium tartrate). The tissue was cultured at 25°C under the cycle of 16 hours light (90 µmol m⁻² s⁻¹) and 8 hours dark.

That tissue was cultured at 25°C under the cycle of 16 hours light (90 µmol m⁻² s⁻¹) and 8 hours dark.

Barley seeds (*Hordeum vulgare cv Himalaya*) from 1998 harvests at Washington State University in Pullman were used in all experiments.

*Arabidopsis thaliana* ecotype Columbia was used as the wild-type. The abi3-6 mutant was a gift of Dr. Peter McCourt. Plants were grown in a mixture of 60% Redi-Earth and 40% vermiculite at 22°C under a 16-hour light and 8-hour dark light regime in a growth chamber. Seeds were surface sterilized for 5 minutes in 95% ethanol, dried, and plated on MS media 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-hour light and 8-hour dark light regime.

Cloning of *PpABI3* cDNAs

The B3 domain of ABI3 from Arabidopsis was used as a query sequence for the BLAST program (tblastn) (Altschul et al., 1990) against Physcobase (http://moss.nibb.ac.jp/)
(Nishiyama et al., 2003) to retrieve cDNAs encoding the B3 domain protein. 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 pphp12b05, which we named \textit{PpABI3A}, are 5’-ATG TGA GAG TGA GCC TGC AG-3’ and 5’-TCC ACC CCT GTA ACA GAA AA-3’ for 5’ and 3’ end, respectively. PCR of \textit{P. patens} against the full-length cDNA library, a gift from Dr. M. Hasebe (Nishiyama et al., 2003) was performed with KlenTaq LA (Clontech, Mountain View, CA) and the supplied buffer with 25 µM each dNTP and 3.5mM MgCl$_2$ by 35 cycles of 96°C for 1 minute, 56°C for 1 minute, and 68°C for 2 minutes. The DNA fragment was subcloned into pGEM-T Easy (Promega, Madison, WI) and subjected to the DNA sequencing using the M13 forward and reverse primers and Big Dye Terminator mix (Applied Biosystems, Foster City, CA). The amino acid sequence was analyzed using DNASIS software (Hitachi Software Engineering, Tokyo, Japan).

\textit{PpABI3B} was cloned from a partial EST sequence (Frank et al., 2005) using 5’ and 3’ RACE. RNA was extracted from \textit{P. patens} gametophore tissue using the RNeasy Kit (Qiagen, Valencia, CA) for use with the 5’ and 3’ RACE kit (Invitrogen, Carlsbad, CA) to amplify the remaining sequence. Three sequential rounds of 5’RACE were required to reach the 5’ end of the PpABI3B mRNA. The full-length coding sequence of PpABI3B was amplified by RT-PCR and cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA). The DNA sequence was checked using M13 forward and reverse primers with the Big Dye Terminator mix (Applied Biosystems, Foster City, CA).
PpABI3C was obtained by a BLAST search (tblastx) (Altschul et al., 1997) of the P. patens JGI raw sequences in Physcobase (Nishiyama et al., 2003) using the Arabidopsis ABI3 coding sequence from the start codon through the B2 domain (bases 1-1401) as the query. This search identified P. patens sequences with B1 and B2 domains that did not match to either PpABI3A or PpABI3B. We assembled a contig from the available genomic sequences (ASYA169080.bl, ASYA 384419.gl, AXOS216104.xl, and AXOS216373.yl) and used this to design primers for 5’ and 3’ RACE based on predicted amino acid conservation with the other PpABI3 proteins. One round of 3’RACE and two rounds of 5’RACE were required to obtain the full cDNA. RNA for the RACE reactions was extracted from protonema treated with 10µM ABA for two hours using the RNeasy Kit (Qiagen, Valencia, CA) for use with the 5’ and 3’ RACE kit (Invitrogen, Carlsbad, CA). The coding sequence of PpABI3C was amplified by RT-PCR and cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA). The DNA sequence was checked using M13 forward and reverse primers with the Big Dye Terminator mix (Applied Biosystems, Foster City, CA).

Amino acid alignments were created by the T-coffee program (Notredame et al., 2000).

**DNA constructs**

The effector constructs of Ubi-PpABI3A, Ubi-PpABI3B, Ubi-PpABI3C, Ubi-VP1 and Ubi-ABI3 were made as follows. The coding sequence of PpABI3A, PpABI3B, PpABI3C, VP1 and ABI3 were amplified by PCR from the cDNAs, and subcloned into pGEM-Teasy (Promega, Madison, WI) or pCR 2.1-TOPO vector (Invitrogen, Carlsbad,
CA). 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 nopalinsynthetase gene. The GFP fusions were
generated by PCR to add GFP to the C-terminal end as a translational fusion in the
respective Ubi-ABI3/PpABI3A-nos vector. Em-GUS [pBM113KP, (Marcotte et al.,
1989)], Ubi-LUC [pAHC18, (Bruce et al., 1989)], Ubi-HvABI5 (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 described previously.

**Transient assays**

In principle, DNA delivery to barley aleurone cells was performed as described
previously (Shen et al., 1993). Two µg of each reporter constructs (Em-GUS and Ubi-
LUC) and effector constructs were used to prepare tungsten particles for three shots.
Bombarded seeds were treated with or without 20µM ABA for 24 h at room temperature.

Transient transformation of P. patens by particle bombardment was carried out as
described (Bezanilla et al., 2003). We used 0.8 µg of each reporter construct (Em-
GUS/PpLea1-GUS and Ubi-LUC) and effector construct to prepare gold particles for four
shots. One-week old protonemal tissue was used for bombardment, and then incubated
on PpNH₄ agar media with or without 10µM ABA for 48 h under the conditions
described above.

Four barley seeds or moss protonemal tissue were ground in extraction buffer
(Lanahan et al., 1992) and cell debris was spun down. GUS and luciferase activities were
measured as described (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 protonemal 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).

**Arabidopsis Complementation**

The vector *pCHF3* (Jarvis et al., 1998), a gift from Michael Neff, 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). Transformants were obtained by selection on MS plates containing kanamycin (50 mg/L). 35S-*ABI3* and 35S-*PpABI3A* plants in the T3 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’ GAATTCCGTGTACGTTTAGGTGGCATG-3
and 5’-GGATCCAGAAGACTCTCTTTTTTCTTC-3’. The $P_{AtABI3}$-$PpABI3A$ and the $P_{AtABI3}$-$AtABI3$ constructs were then transformed into the abi3-6 mutant as described above. We obtained four independent $P_{AtABI3}$-$PpABI3A$ complementation lines and seven of the $P_{AtABI3}$-$AtABI3$ 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 wildtype, abi3-6, $P_{AtABI3}$-$PpABI3A/abi3$-6 and $P_{AtABI3}$-$AtABI3/abi3$-6 complementation lines and surface sterilized. Seeds were plated on MS media containing 0, 0.1, 1, 10, or 100 µM ABA and cold treated for 4 days at 4°C in the dark. The plates were then moved to 22°C with a 16-hour light and 8-hour dark light 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 RNAqueous kit with the Plant RNA isolation aid (Ambion, Austin, TX). 500 ng of total RNA was first treated with DNaseI (Amplification grade, Invitrogen, Carlsbad, CA) for 15 minutes at room temperature. The enzyme was inactivated by heating at 65°C for 10 minutes in the presence of 2.5 mM EDTA. The RNA was used for making cDNA by the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA). The RNA was first pre-incubated with oligo(dT)$_{20}$ and dNTPs at 65°C for 5 minutes, then placed on ice. The cDNA Synthesis Mix was added
to the RNA and incubated at 55°C for 1 hour. The reaction was terminated by heating at 85°C for 5 minutes and then RNaseH was added and incubated at 37°C for 20 minutes to remove the RNA template. PCR was carried out using 2 µL of the cDNA with 0.4 µL of Taq polymerase (Invitrogen, Carlsbad, CA) in a 50 µL reaction. The primer sequences are AtEm1 (5’-CGTCAAAGCAACTGAGCA-3’ and 5’-TCCATCGTACTGAGTCTCC-3’); AtEm6 (5’-TGGCGTCTCAACAAGAGAAG-3’ and 5’-TCTCCGGTGCTAAGACCA-3’); Napin (5’-GCAATCACAACACCTAAGAGCT-3’ and 5’-CTGGATTGGAATGGTCCGTG-3’); CruciferinC (5’-AGCTCAGCAATCTCTCGTT-3’ and 5’-TTTCCGCGCCAATGGAACAC-3’); Rab18 (5’-ATCCAGCACGAGCGATGACGAGT-3’ and 5’-ACCGGAAGCTTTTCTTGATC-3’); Oleosin2 (5’-AGGTCAGTATGAAGGTGACGGTG-3 and 5’-TGCTCACAGCAGTTCTCCTT-3’); Ubiquitin10 (5’-AAGATCCAGGACAAGGAGGTATTCC-3’ and 5’-CATAACAGAGACGATTTAGAAACC-3’). The cycling parameters for AtEm1 and AtEm6 were 35 cycles of 94°C for 30 seconds, 52°C for 45 seconds and 72°C for 1 minute. The cycling parameters for Ubq10, Oleosin2, and Rab18 were 21 cycles of 94°C for 30 seconds, 58°C for 45 seconds and 72°C for 1 minute. The cycling parameters for Napin and CruciferinC were 28 cycles of 94°C for 30 seconds, 56°C for 45 seconds and 72°C for 1 minute. Ten µL of each PCR reaction was run out on a 1.5% agarose gel.
Yeast Two Hybrid

Plasmid construction

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

Yeast two hybrid assays

Media was prepared as described (Sambrook and Russell, 2001). Yeast two hybrid assays were carried out as described before (James et al., 1996). In this system three different reporter genes, \textit{GAL2-ADE2}, \textit{GAL1-HIS3} and \textit{GAL7-LacZ} are available. A host strain PJ69-4A-a was used for BD constructs, whereas PJ69-4A-AD-a was used for AD constructs. Mating was carried out by co-incubation of a strain harboring the BD construct and a strain harboring the AD construct. The diploid strains were selected on SD media 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 35mM 3-aminotriazole (3-AT) was used for culture. For the quantification assay, GAL4-BD driven $\beta$-galactosidase gene expression was analyzed using ONPG (O-nitrophenylgalactoside) as the substrate according to the protocol described at

\url{http://www.fhcrc.org/labs/gottschling/yeast/Bga37.html}.
ACKNOWLEDGEMENTS

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Table 1. AtABI3 regulated genes grouped by co-regulator for RT-PCR analysis.

<table>
<thead>
<tr>
<th>Co-Regulator</th>
<th>Gene</th>
<th>Reference</th>
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<tbody>
<tr>
<td>ABI5</td>
<td><em>AtEm1</em></td>
<td>Finkelstein and Lynch, 2000</td>
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<tr>
<td></td>
<td>(At3g51810)</td>
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<tr>
<td></td>
<td><em>AtEm6</em></td>
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<tr>
<td></td>
<td>(At2g40170)</td>
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<tr>
<td>OPAQUE2-like bZIPs</td>
<td><em>CruciferinC</em></td>
<td>Lara et al., 2003</td>
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<tr>
<td></td>
<td>(At4g28520)</td>
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<tr>
<td></td>
<td><em>Napin</em></td>
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<td>(At4g27160)</td>
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<td>Unknown*</td>
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<td>Finkelstein and Lynch, 2000</td>
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<td>(At5g40420)</td>
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<tr>
<td></td>
<td><em>Rab18</em></td>
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* Not ABI5