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Functional analyses of the ABI1-related protein phosphatase type 2C reveal evolutionarily conserved regulation of abscisic acid signaling between Arabidopsis and the moss *Physcomitrella patens*

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Abstract We employed a comparative genomic approach to understand protein phosphatase 2C (PP2C)-mediated abscisic acid (ABA) signaling in the moss *Physcomitrella patens*. Ectopic expression of Arabidopsis (*Arabidopsis thaliana*) *abi1-1*, a dominant mutant allele of *ABI1* encoding a PP2C involved in the negative regulation of ABA signaling, caused ABA insensitivity of *P. patens* both in gene expression of late embryogenesis abundant (LEA) genes and in ABA-induced protonemal growth inhibition. The transgenic *abi1-1* plants showed decreased ABA-induced freezing tolerance, and decreased tolerance to osmotic stress. Analyses of the *P. patens* genome revealed that only two (*PpABI1A* and *PpABI1B*) PP2C genes were related to *ABI1*. In the *ppabi1a* null mutants, ABA-induced expression of *LEA* genes was elevated, and protonemal growth was inhibited with lower ABA concentration compared to the wild type. Moreover, ABA-induced freezing tolerance of the *ppabi1a* mutants was markedly enhanced. We provide the genetic evidence that PP2C-mediated ABA signaling is evolutionarily conserved between Arabidopsis and *P. patens*.

Keywords ABI1 · Abscisic acid · Gene targeting · Negative regulation · *Physcomitrella patens* · PP2C

Abbreviations

ABA	Abscisic acid
ABI	ABA-insensitive
ABRE	ABA-response element
DRE	Drought-responsive element
GUS	β-Glucuronidase
JA	Jasmonic acid
LEA	Late embryogenesis abundant
MPK	Mitogen-activated protein kinase
PP2C	Protein phosphatase 2C

Introduction

Abscisic acid (ABA) regulates the maturation, dormancy and germination of seeds (Finkelstein et al. 2002), and postembryonic development, such as lateral root development (Brady et al. 2003; De Smet et al. 2003). ABA also plays an important role in plant adaptation to stresses, such as drought, high salinity and low temperature, and regulates the expression of genes involved in the response to abiotic stresses (Finkelstein et al. 2002).

A genetic approach using the model plant Arabidopsis (*Arabidopsis thaliana*) contributed to the identification of important factors involved in the ABA signal transduction pathway. ABA-insensitive (*abi*) mutants were identified by screening for seedlings that could germinate in the presence of ABA (Koornneef et al. 1984). Subsequent cloning

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identified *ABI1* and *ABI2*, which encode homologous type 2C protein phosphatases (PP2Cs) (Leung et al. 1994, 1997; Meyer et al. 1994), and *ABI3*, *ABI4* and *ABI5*, which encode transcription factors (Giraudat et al. 1992; Finkelstein et al. 1998; Finkelstein and Lynch 2000). Many other factors involved in ABA signaling were also identified through non-ABA based mutant screenings and reverse genetics (Finkelstein et al. 2002). In spite of these studies, our knowledge of how these gene products function in ABA signaling is still fragmentary.

Among the *ABI* genes, *ABI1* and *ABI2* are unique in that they encode PP2Cs, which are ubiquitously found in all eukaryotes and involved in phosphorylation-mediated signaling; in addition, these genes function through seed maturation and germination to vegetative growth. The *abi1-1* and *abi2-1* mutations, dominant alleles of *ABI1* and *ABI2*, respectively, have the same single amino acid substitution (Gly to Asp) in the highly conserved phosphatase catalytic domain, and are capable of blocking ABA response (Leung et al. 1994, 1997; Meyer et al. 1994). The mutants show a broad range of ABA-related phenotypes, including reduced seed dormancy, ABA-resistant seed germination and seedling growth, abnormal stomatal regulation, and defects in various responses to drought (Koornneef et al. 1984; Finkelstein and Somerville 1990). Isolation of intragenic suppressors of *abi1-1* and *abi2-1* provided crucial evidence that these proteins act as negative regulators of the ABA signaling pathway (Gosti et al. 1999).

Database analysis of the Arabidopsis genome identified 76 PP2C genes, and phylogenetic analysis separated these genes into 10 groups (A–J) (Schweighofer et al. 2004). Interestingly, Group A contains most of the genes associated with ABA signaling, including *ABI1* and *ABI2*. *HAB1* was originally identified based on its sequence homology to *ABI1* and *ABI2*, and the loss-of-function approach provided the concrete genetic evidence of the function of a PP2C as a negative regulator of ABA signaling (Saez et al. 2004). T-DNA or Ds insertion mutants of 8 Group A PP2C genes were analyzed for ABA sensitivity, and insertion mutations in *ABI1*, *ABI2*, *HAB1*, *HAB2* and *AtPP2CA* were found to increase ABA sensitivity during germination (Yoshida et al. 2006b). These and other genetic studies (Sheen 1998; Tähtiharju and Palva 2001; Kuhn et al. 2006; Nishimura et al. 2007) have demonstrated the significant role of Group A PP2Cs in the regulation of ABA-signaling in Arabidopsis, and also revealed the redundant and distinct functions of Group A PP2Cs in the negative regulation of ABA signaling.

In contrast, the role of PP2Cs in ABA signaling in other plant species has not been well characterized. Arabidopsis abi1-1 protein has been shown as a strong negative regulator of ABA signaling in other seed plant species, such as *Nicotiana benthamiana* (Armstrong et al. 1995), tomato (Carrera and Prat 1998), rice protoplasts (Hagenbeek et al. 2000) and barley aleurone layer (Casaretto and Ho 2003) by gain-of-function analyses. These results suggested the likely existence of a PP2C-mediated ABA signaling pathway in these seed plants. However, no loss-of-function analysis has been carried out in these plants due to the technical difficulties. Thus, the significance of PP2Cs in ABA-signaling regulation in plants other than Arabidopsis has yet to be elucidated.

The nuclear genes of the moss *Physcomitrella patens* can be efficiently targeted by homologous recombination, therefore this system is useful for loss-of-function analysis of gene function (Schaefer and Zrÿd 2001). *P. patens* accumulates ABA and responds to ABA in a similar manner as angiosperms (Knight et al. 1995). In fact, our previous report demonstrated that the regulatory factor of seed maturation, ABI3, is conserved in the non-seed plant *P. patens* and activates ABA responsive promoters of late embryogenesis abundant (LEA) genes in barley as well as *P. patens* (Marella et al. 2006). As the evolutionary view considers bryophytes to be the basal land plants, comparative studies between seed plants and bryophytes could provide insights into the molecular evolution of ABA function and signaling in land plants. This evolutionary position, coupled with the sequenced genome (Rensing et al. 2008) and advanced tools for studying gene function, such as RNA interference, inducible promoters and gene targeting (Frank et al. 2005a; Quatrano et al. 2007), supports *P. patens* as a model system for such comparative and functional genomics on ABA studies.

To undertake comparative analysis of PP2Cs in ABA signaling, we generated transgenic *P. patens* expressing Arabidopsis *abi1-1* to evaluate the ABA signaling of *P. patens*.

Materials and methods

Plant materials

P. patens subspecies patens (Gransden) was the wild-type strain. Protonemal tissue was grown on BCD or BCDAT medium at 25°C under continuous light (50–80 μmol m^{-2} s^{-1}) (Nishiyama et al. 2000). The gametophores were induced on sterile peat pellets (Jiffy-7; Sakata seed, Japan) in a plant culture box by culturing at 25°C under continuous light for 1–1.5 months. For induction of sporophytes, gametophore cultures were transferred to 15°C under 8 h light/16 h dark conditions. PEG-mediated protoplast transformation was performed as previously described (Schaefer and Zrÿd 1997).

Phylogenetic analysis

We searched the annotated *P. patens* genome database ver. 1.0 (http://genome.jgi-psf.org/Phypa1_1/) with "Ser/Thr protein phosphatase" as a keyword and retrieved 75 putative protein phosphatase genes. We analyzed each annotation, and selected 42 putative PP2C genes. We then performed a tblastn search against the database using putative amino acid sequences encoded by the 42 genes as the queries and identified 9 additional putative PP2C genes. The catalytic domains of PP2Cs from 73 (*A. thaliana*) and 50 (*P. patens*) PP2C genes identified with PlantsP Motif/Domain Scan (http://plantsp.genomics.purdue.edu/html/) were used for phylogenetic analysis. The multiple sequence alignment was performed by MAFFT ver. 6 (Katoh et al. 2005) using the L-INS-i strategy, and gaps were excluded manually. For the maximum-likelihood (ML) analyses, the ProtML and NJdist programs in MOLPHY ver. 2.3b3 package (Adachi and Hasegawa 1996) were used. The ML distances were calculated using ProtML under the JTT model (Jones et al. 1992), and a Neighbor-Joining (NJ) tree was obtained with NJdist. A local rearrangement search was performed with the NJ tree as the starting tree. The local bootstrap probability of each branch was calculated with re-sampling of estimated log likelihood (RELL) method (Kishino et al. 1990; Hasegawa and Kishino 1994). Bootstrap test was performed with 100 replications.

Cloning of *PpABI1A*, *PpABI1B* and *pphn39k21*

The full-length cDNA clone (*pphn8m22*) corresponding to *PpABI1A* gene was obtained from RIKEN Bio Resource Center (BRC), and used as the representative sequence of *PpABI1A*. Since the full-length cDNA clone for *PpABI1B* was not in the PHYSCObase, we used information from the predicted transcript model of *PpABI1B* in the annotated *P. patens* genome database. We designed primers corresponding to the 5′- and 3′-end of the predicted transcript model, respectively, and obtained the corresponding cDNA fragment by RT-PCR, and subcloned the fragment into the Zero Blunt TOPO vector (Invitrogen, USA). The full-length cDNA clone *pphn39k21*, corresponding to Phypa1_1 160175 and encoding a PP2C from a different clade, was obtained from RIKEN BRC. The complete sequences of the clones and the cDNA fragment were determined.

DNA constructs

The stable transformation construct *Act:abi1-1* and the effector constructs *Act::ABI1*, *Act::PpABI1A* and *Act::pphn39k21* were generated as follows. The coding sequences of *abi1-1*, *ABI1*, *PpABI1A* and *pphn39k21* were amplified by PCR from the cDNAs, and subcloned into the Zero Blunt TOPO vector. The amplified fragments were confirmed by sequencing, and each fragment was placed downstream of the rice Actin 1 promoter (McElroy et al. 1990) with the terminator of the CaMV 35S gene at the 3′ end of the fragment. The cassettes were then transferred to a vector containing the hygromycin resistance cassette (Schaefer and Zrÿd 1997). The Em-GUS and Ubi-*LUC* constructs were described previously (Marella et al. 2006). To create the *PpABI1A* targeting construct, the upstream (1,383 bp) and downstream regions (953 bp) of the ORF were amplified by PCR. PCR products were cloned into the pGEM-T Easy vector (Promega, USA), and the fragments were then excised and cloned into the both ends of the resistant cassette *Lox-CaMV35S::NptII::NOS-Lox* with *Sfi*I (5′ fragment) and *Asc*I (3′ fragment).

Measurement of plant growth

One-week-old protonemal tissues were harvested and the fresh weight was measured after aseptic removal of excess water. A tenth g of fresh tissue was homogenized in 2 ml of 0.1% (w/v) agar solution, and spotted on BCD agar medium (10 spots/plate, 5 µl each spot). After 2 weeks of culture, colony areas were digitally measured using LIA for Win32 image analysis software (freely available from http://www.agr.nagoya-u.ac.jp/%7Eshinkan/LIA32/index.html). To confirm the accuracy of the image analysis, we evaluated the colony growth of wild type protonemata on various concentration of ABA, and the growth of colonies was evaluated by the image analysis as well as chlorophyll contents, which is conventionally used for growth quantification of moss plants (Supplemental Fig. S1). Chlorophyll was extracted from each colony using dimethylformamide. Chlorophyll contents (Chl a + Chl b = $17.67A_{646.8}$ + $7.12A_{663.8}$) were measured with spectrophotometer, and calculated according to Porra et al. (1989).

Measurement of freezing tolerance

One-week-old protonemal tissues were treated with various concentrations of ABA for 24 h. Then freezing tolerance was determined by the measurement of electrolyte leakage from cells after equilibrium freezing to −5°C, as previously described (Minami et al. 2003).

Transient assay

Particle bombardment was carried out as previously described (Marella et al. 2006). We used 0.8 µg each of the reporter constructs (Em-GUS and Ubi-*LUC*) and the effector construct to prepare DNA-coated gold particles for four shots. One-week-old protonemal tissue was used, and

incubated on BCDAT agar medium with or without 10 μM ABA for 48 h.

RNA analysis

Total RNAs were extracted from protonemal tissues using the RNeasy plant mini Kit (QIAGEN, USA). About 10 μg of total RNA was separated by a formaldehyde-denaturing agarose gel and transferred onto a nylon membrane. The *PpABI1A* probe was amplified by PCR from full-length cDNA clone. Probes for *19C6* (Minami et al. 2005) and *PpLea1* (Kamisugi and Cuming 2005) were amplified by PCR from cDNA prepared from protonemal tissue. PCR fragments were purified using the Gel Extraction kit (QIAGEN, USA). Hybridization with ^{32}P-labeled DNA probes was carried out as described previously (Yamaguchi-Shinozaki and Shinozaki 1994). BAStaion 2500 (Fuji Film, Japan) was used for visualization of the blot.

RT-PCR

For cDNA synthesis, 1 μg of total RNA was first treated with DNaseI (Sigma-Aldrich, USA) for 15 min at room temperature, and the enzyme was inactivated by heating at 70°C for 10 min. Reverse transcription was performed with the ThermoScript RT-PCR system (Invitrogen, USA) according to the manufacturer's instructions. Synthesized cDNAs were purified using the Gel Extraction kit. Semi-quantitative RT-PCR analysis for *PpABI1A* and *PpABI1B* expression was performed using 1 μl of the cDNA, the primer set D and ExTaq polymerase with the supplied buffer and dNTP (Takara-Bio, Japan). The PCR conditions were as follows: 30 (*PpABI1A* and *PpABI1B*) or 27 (*PpActin5*, AY382284) cycles of 95°C for 30 s, 45°C (*PpABI1A* and *PpABI1B*) or 55°C (*PpActin5*) for 30 s and 72°C for 15 s. A 5 μl aliquot of each PCR reaction was separated on an agarose gel. As a negative control for the absence of genomic DNA contamination in the cDNA, we performed an PCR with a primer set that is designed to amplify the 4th intron of *PpABI1A*, and confirmed the absence of a DNA fragment that is corresponding to genomic DNA. *abi1-1* expression in *Act::abi1-1* plants was analyzed by quantitative PCR with LightCycler Systems for Real-Time PCR (Roche Applied Science, Japan) using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Applied Science, Japan) according to the manufacturer's instructions. The PCR conditions were as follows: 40 cycles of 95°C for 10 s, 55°C for 10 s and 72°C for 10 s. The quantities of cDNA were calculated using the Second Derivative Maximum Method on LightCycler Data Analysis software (Roche Applied Science, Japan). The plastocyanin-like gene (GenBank Accession AW509984) was used to normalize *abi1-1* expression.

Analysis of *ppabi1a* mutants

Genomic DNA was isolated using the Nucleon PhytoPure system (Amersham biosciences, USA). To check homologous recombination at the 5′ and 3′ flanking regions of the *PpABI1A* genomic locus, PCR was performed using primer set A for the 5′ recombination, and primer set B for the 3′ recombination. The absence of the *PpABI1A* genomic region was confirmed by PCR with primer set C. The PCR condition was 30 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 1 min. The expressions of *PpABI1A* and *PpABI1B* were analyzed by non-quantitative RT-PCR using the primer set D.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AB369256 (PpABI1A), AB369255 (PpABI1B), and AB369257 (pphn39k21).

Results

Ectopic expression of Arabidopsis *abi1-1* reduces ABA sensitivity of *P. patens*

Previously we demonstrated that transient expression of Arabidopsis *abi1-1* represses ABA-dependent transcriptional activation of the wheat Em promoter in *P. patens* protonemata (Marella et al. 2006). To confirm the effect of *abi1-1* on ABA signaling of *P. patens* at the whole plant level, we established stable transformants expressing HA-tagged *abi1-1* under the control of the constitutive rice actin promoter (Perroud and Quatrano 2006). Three independent transformants (*Act::abi1-1*) A, D and P, which showed different levels of *abi1-1* expression as judged by quantitative RT-PCR (Fig. 1a), were used for subsequent analyses.

We first analyzed the expression of endogenous ABA-inducible genes in *Act::abi1-1* plants (Fig. 1b). *PpLea1* and *19C6* encode LEA-like proteins and are induced by both ABA and abiotic stresses, such as hyper-osmolarity and cold temperatures (Minami et al. 2003; Kamisugi and Cuming 2005). Expression levels of these genes were low in the absence of exogenous ABA, and no significant differences in expression levels between the wild type and *Act::abi1-1* plants were detected. Notably, treatment of *Act::abi1-1* plants with ABA (1 μM) showed reduced gene induction compared to wild-type plants. Down-regulation was most prominent in *Act::abi1-1* line P, the highest expressor of *abi1-1*, suggesting a correlation between the level of *abi1-1* expression and the degree of down-regulation.

To further confirm the reduced ABA sensitivity in *Act::abi1-1* plants, we measured the growth of protonemata

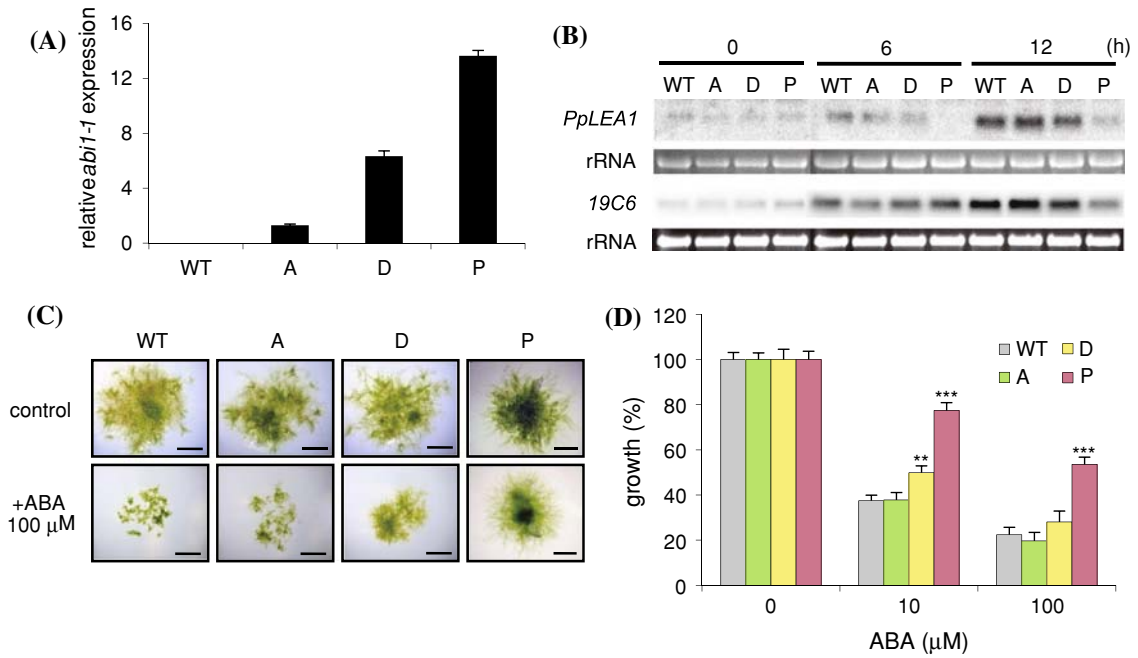


Fig. 1 *abi1-1* functions as a negative regulator of ABA signaling in *P. patens*. **a** One-week old protonemal tissue from *Act::abi1-1* plants line A, D and P were subjected to QRT-PCR analysis for detection of the *abi1-1* transgene. Values are average ± SE of triplicate samples from three independent experiments. **b** Northern blot analysis of ABA-inducible genes in *Act::abi1-1* plants treated with 1 μM ABA. Ethidium bromide-stained bands of rRNA (*bottom panels*) confirmed equal loading. Representative images (**c**) and quantitative analysis (**d**) of protonemal growth of *Act::abi1-1* plants with and without ABA. Wild type (WT) and *Act::abi1-1* plants were grown on ABA media for 2 weeks, and the area of the protonemal colonies was measured by image analysis. The values in (**d**) are means ± SE ($n = 20$). Asterisks indicate significant changes between *Act::abi1-1* plants and wild type (** $P < 0.01$, *** $P < 0.001$). Scale bars: 2 mm

on ABA media. Protonemal growth of transformants on media containing various concentrations of ABA was evaluated by measuring the colony area (Fig. 1c, d). In the presence of ABA, protonemal growth of the wild type was inhibited by 63% (at 10 μM) and 78% (at 100 μM) compared to the untreated control. Although the lowest expressing line A behaved similarly to the wild type in response to ABA, line D showed slightly but significantly better growth than wild type at 10 μM ABA. The highest expressing line P showed clear ABA-resistance compared to wild type with both 10 and 100 μM ABA. These results indicated that *Act::abi1-1* plants were less sensitive to ABA than wild type, and that the insensitivity correlated with the level of *abi1-1* expression. This demonstrates that *abi1-1* functions as a negative regulator of ABA signaling in *P. patens*.

Arabidopsis *abi1-1* reduces tolerance of *P. patens* to abiotic stresses

Although *P. patens* protonemata are susceptible to freezing stress, exogenous ABA markedly increases the freezing tolerance (Minami et al. 2003). Thus, we evaluated the tolerance of *Act::abi1-1* plants to freezing (Fig. 2a). As reported, ABA treatment of the wild type protonemata enhanced the tolerance to freezing in a dose-dependent manner, and most protenemal cells survived when treated with 10 μM ABA for 24 h. In contrast, all *Act::abi1-1* plants treated with 0.1 and 1 μM ABA showed significant reduction of freezing tolerance in proportion to the level of *abi1-1* expression. The highest expressing line P exhibited significantly lower freezing tolerance, even at 10 μM ABA, compared to the wild type.

We next investigated the physiological role of ABA signaling regulated by *abi1-1* in *P. patens*. Since mosses accumulate ABA under dry conditions, and exogenously applied ABA enhances drought resistance in *Funaria hygrometrica* (Cove et al. 2006), endogenous ABA signaling is likely involved in the drought tolerance of *P. patens*. Therefore, we analyzed their tolerance to osmotic stress (Fig. 2b). *Act::abi1-1* protonemata were cultured on media containing various concentrations of mannitol, which causes changes in media osmolality. As the mannitol concentration increased, the growth of the wild type protonemata was inhibited. Growth inhibition caused by mannitol-induced osmotic stress was more pronounced in all *Act::abi1-1* plant lines. Together, these results demonstrate that ectopic expression of Arabidopsis *abi1-1* reduces

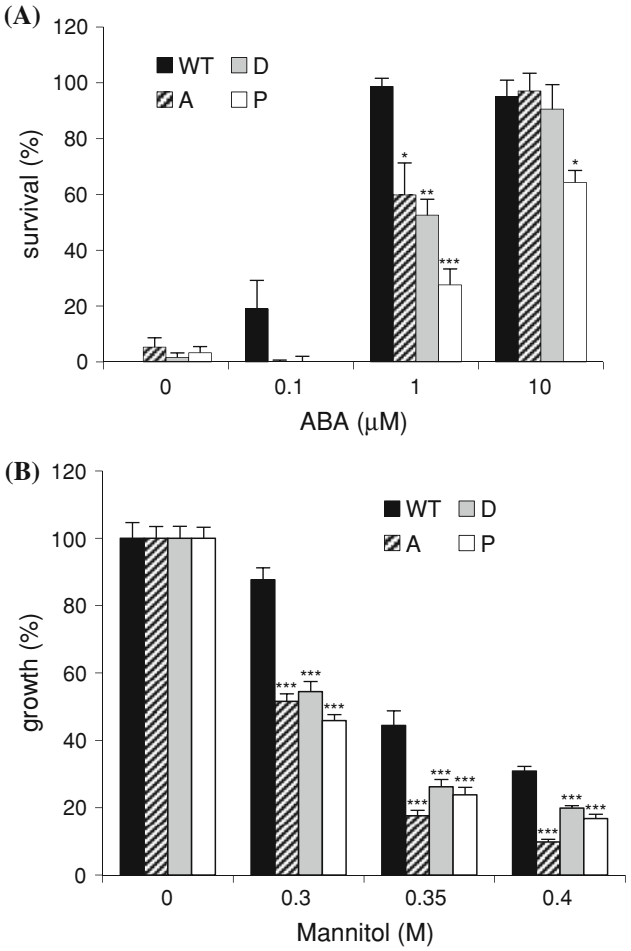


Fig. 2 *abi1-1* plants exhibit reduced ABA-induced tolerance to freezing and reduced tolerance to osmotic stress. **a** Wild type (WT) and *Act::abi1-1* plants protonemata were treated with various concentrations of ABA for 24 h, and then subjected to freezing stress at −5°C. Cell survival was determined by measurement of electrolyte leakage after freeze-thawing. Values are means ± SE of three different experiments. **b** Wild type (WT) and *Act::abi1-1* plants protonemata were grown on BCD medium containing the indicated concentration of mannitol. The area of protonemal colonies was measured by image analysis. The values shown are means ± SE ($n = 20$). Asterisks indicate significant changes between *Act::abi1-1* plants and wild type (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

tolerance of *P. patens* protonemata to freezing stress and osmotic stress.

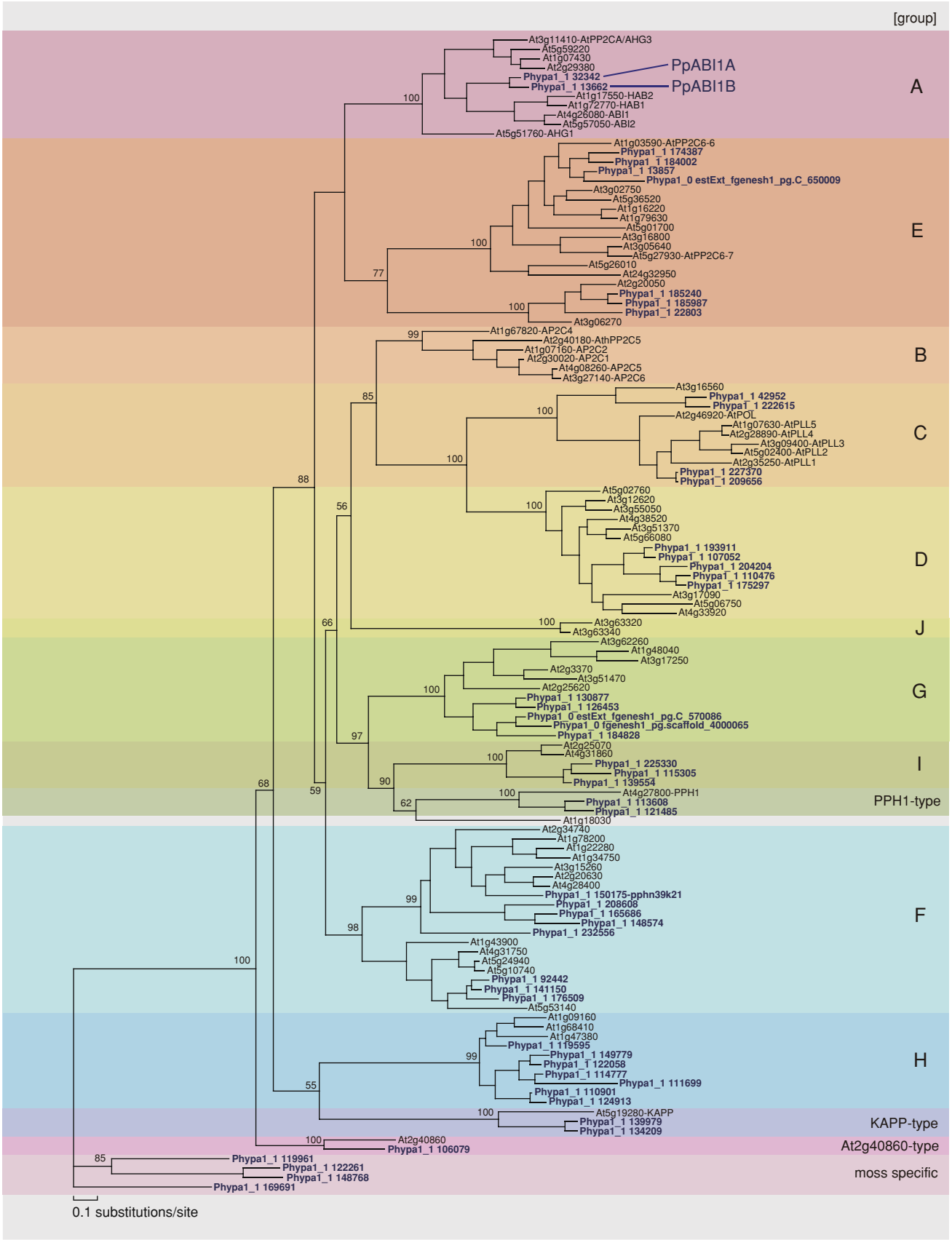
Two *ABI1*-related genes exist in the genome of *P. patens*

Studies of ectopically expressed Arabidopsis *abi1-1* strongly supported the presence of a PP2C-mediated ABA signaling pathway in *P. patens*. We therefore searched for genes encoding PP2Cs in the *P. patens* EST database (Nishiyama et al. 2003) as well as in the genome database (http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html) and identified 51 genes containing a highly conserved

Fig. 3 The *P. patens* genome contains two ABI1-related genes. The maximum-likelihood tree of plant PP2C genes, including 73 *A. thaliana* PP2C genes (*black*) and 50 *P. patens* PP2C genes (*blue*). The local bootstrap probabilities are shown on the branches. The horizontal branch lengths are proportional to the estimated evolutionary distance. Each cluster is categorized according to the phylogenetic analysis of Arabidopsis PP2C genes (Schweighofer et al. 2004)

catalytic domain of PP2C. Among them, two closely related genes showed high similarity to Arabidopsis Group A PP2Cs. We generated a phylogenetic tree based on sequence alignment of the catalytic domains encoded by the *P. patens* PP2C genes with those of Arabidopsis PP2Cs (Fig. 3). Each cluster was categorized according to the phylogenetic analysis of Arabidopsis PP2Cs (Schweighofer et al. 2004). Almost all groups contained both Arabidopsis PP2Cs and *P. patens* PP2Cs. However, Group B consisted of only Arabidopsis PP2Cs, suggesting that Group B PP2Cs evolved after the separation of the bryophytes and the vascular plants from a common ancestor, and that these genes are likely involved in physiological events specific to the vascular plants. We also found that some PP2Cs were moss-specific. Only two genes, *PpABI1A* and *PpABI1B*, positioned in the same cluster with Arabidopsis Group A PP2Cs with a high bootstrap value. These analyses suggest that the Group A PP2C genes involved in the regulation of Arabidopsis ABA signaling are also conserved in the *P. patens* genome, although the number of genes is lower than that of Arabidopsis. The exon–intron structures of *PpABI1A* and *PpABI1B* were similar to that of Arabidopsis *ABI1* (Supplemental Fig. S2), and the coding sequences of *PpABI1A* and *PpABI1B* were highly similar to one another (76% amino acid identity and 83% nucleotide identity). In contrast, the sequence similarity between Arabidopsis *ABI1* and *PpABI1A* or *PpABI1B* was restricted only to the catalytic domains. Two Gly residues in the catalytic domain of ABI1 that correlated with regulation of ABA signaling were also conserved in PpABI1A and PpABI1B (Finkelstein and Rock 2002, Supplemental Fig. S3).

Semi-quantitative RT-PCR analysis, using a primer set that is able to distinguish the PCR products of *PpABI1A* from that of *PpABI1B* by the size, revealed that *PpABI1A* and *PpABI1B* were expressed ubiquitously during the moss life cycle although *PpABI1A* exhibited higher expression than *PpABI1B* (Fig. 4a, b). Arabidopsis Group A PP2C genes are transcriptionally induced in response to exogenous ABA. Similarly, *PpABI1A* and *PpABI1B* quickly responded to exogenously applied ABA, and sustained high levels of expression even after 12 h of ABA treatment, eventually decreasing (Fig. 4c). *PpABI1A* and *PpABI1B* were also responsive to osmotic stress. Interestingly, *PpABI1A* showed enhanced transcriptional induction in response to cold treatment. These data suggest that while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Phypa1_1 150175-pphn39k21
Phypa1_1 208608
Phypa1_1 165686
Phypa1_1 148574
Phypa1_1 232556
At1g43900
At4g31750
At5g24940
At5g10740
Phypa1_1 92442
Phypa1_1 141150
Phypa1_1 176509
At5g53140
At1g09160
At1g68410
At1g47380
Phypa1_1 119595
Phypa1_1 149779
Phypa1_1 122058
Phypa1_1 114777
Phypa1_1 111699
Phypa1_1 110901
Phypa1_1 124913
At5g19280-KAPP
Phypa1_1 139979
Phypa1_1 134209
At2g40860
Phypa1_1 106079
Phypa1_1 119961
Phypa1_1 122261
Phypa1_1 148768
Phypa1_1 169691
0.1 substitutions/site

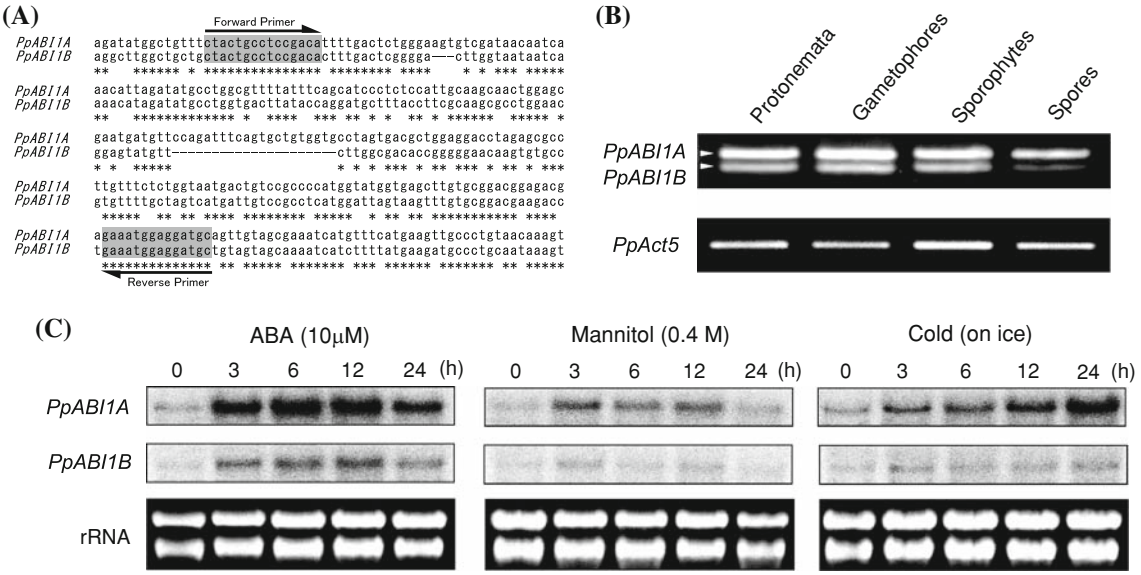

Fig. 4 *PpABI1A* and *PpABI1B* are ubiquitously expressed through the life cycle, and are induced by ABA and abiotic stresses. **a** Schematic of the corresponding regions of the primers in *PpABI1A* and *PpABI1B* for RT-PCR analysis. **b** Semi-quantitative RT-PCR of *PpABI1A* and *PpABI1B* was performed using total RNA isolated at different stages of development. Expression of *PpAct5* was used as an internal control. **c** Northern blot analysis of *PpABI1A* and *PpABI1B* expression. Total RNA was isolated from protonemata treated with 10 μM ABA, 0.4 M mannitol, or cold stress (on ice) at indicated times after treatment. Ethidium bromide-stained bands of rRNA confirm equal loading

PpABI1A and *PpABI1B* are ubiquitously expressed and responsive to various stresses, *PpABI1A* is predominantly expressed and strongly induced by low temperature stress. Therefore, we focused on PpABI1A in subsequent experiments.

Transient expression of *PpABI1A* in *P. patens* protonemata blocks the ABA-induced expression of Em-GUS

We previously developed a useful transient assay to assess the ABA sensitivity of *P. patens*, in which the wheat Em gene promoter fused to the *β*-glucuronidase (GUS) gene was introduced into protonemal cells and the Em promoter activity was assessed by measuring GUS activity (Marella et al. 2006). We used this system to analyze the function of PpABI1A in ABA signaling (Fig. 5). The entire *PpABI1A* coding sequence was inserted downstream of the rice actin 1 promoter, and this construct was introduced into protonemal cells along with the Em-GUS construct via particle bombardment. Arabidopsis *ABI1* and a PP2C gene from *P. patens* (*pphn39k21*) that is distinct from group A PP2Cs (Fig. 3) were used as a positive and negative control, respectively. PpABI1A completely suppressed the ABA-dependent activation of the Em promoter, and the degree of repression was comparable to that of Arabidopsis ABI1. In contrast, pphn39k21 hardly affected the Em promoter activity irrespective of ABA treatment. These data suggest that PpABI1A specifically functions as a negative regulator of ABA signaling in *P. patens*.

Targeted disruption of *PpABI1A* causes ABA hypersensitive phenotypes

To confirm the function of PpABI1A in ABA signaling, we performed a loss-of-function analysis and disrupted *PpABI1A* by gene targeting via homologous recombination (Fig. 6a). Two independent disruptants (lines 3 and 7) were selected after PCR analysis confirmed the complete deletion of the *PpABI1A* coding sequence (Fig. 6b). RT-PCR analysis confirmed that both transgenic lines lost the expression of *PpABI1A* while retaining *PpABI1B* expression (Fig. 6c). We next evaluated ABA sensitivities of the disruptants by analyzing temporal expression patterns of ABA-inducible genes, *PpLea1* and *19C6*, in protonemata (Fig. 7a). ABA treatment enhanced induction of *PpLea1* and *19C6* in the *ppabi1a* plant (line 7) compared to wild type plants, suggesting a negative regulatory role of PpABI1A in ABA-regulated gene expression in *P. patens*.

Growth of the *ppabi1a* plant protonemata on media with or without 0.01 μM ABA was similar to that of wild type. Statistically significant and reproducible growth inhibition of the *ppabi1a* plants in response to 0.1 μM ABA was detected, although the growth of wild type plant was unaffected at this concentration (Fig. 7b). Protonemal wild type cells can differentiate into spherical brood cells in

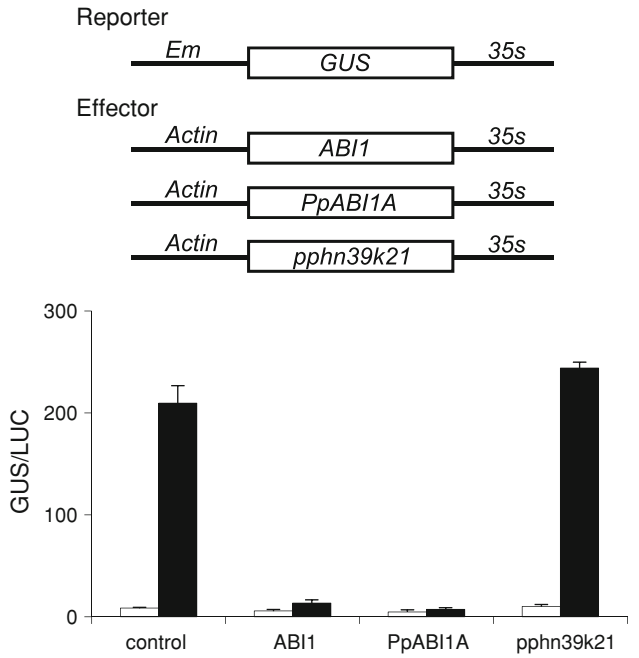

Fig. 5 Transient expression of PpABI1A blocked ABA-induced transcription of Em-GUS in *P. patens* protonemata. The Em-GUS reporter construct and each of the effector constructs were introduced into *P. patens* protonemal tissue by particle bombardment. After bombardment, the protonemal tissue was incubated with (*black bars*) or without (*white bars*) 10 μM ABA for 48 h. Protein lysates were extracted from the treated tissues for GUS and LUC assays. Bars indicate the relative GUS activities ± SE ($n = 4$)

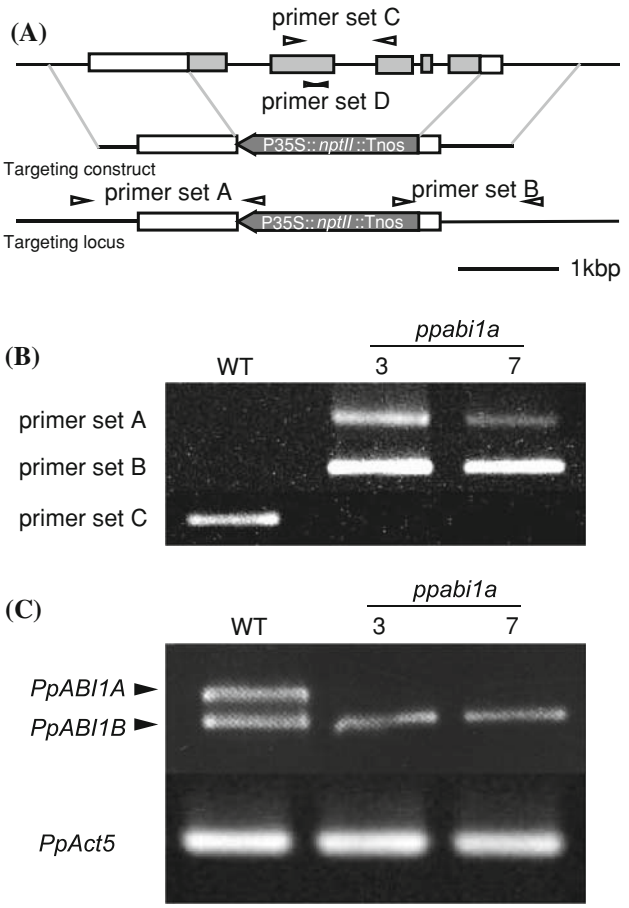

Fig. 6 Generation of *PpABI1A* disruptant by gene targeting. **a** Schematic of the *PpABI1A* disruption by homologous recombination. The locations of the primer sets used in (**b**) and (**c**) are shown by *arrowheads*. The 5′ and 3′ untranslated regions and the coding region of *PpABI1A* are shown by *open boxes* and *gray boxes*, respectively. The *dark gray box* represents the *nptII* cassette. **b** Disruption of *PpABI1A* is confirmed by PCR analysis with the primer set A and the primer set B, respectively. Deletion of *PpABI1A* sequence is confirmed with the primer set C. **c** Non-quantitative RT-PCR analysis of the expression of *PpABI1A* and *PpABI1B* in the wild type, the *ppabi1a* mutant line 3 and line 7 using the primer set D. Expression of *PpAct5* was used as an internal control

response to stresses or after long periods of culture (Goode et al. 1993), which are also induced by relatively high concentrations of ABA (>10 μM). Therefore we examined brood cell formation of the *ppabi1a* plants in response to exogenous ABA. To obtain better images of protonemal filaments under a microscope, we examined protonemata cultured between two cellophane sheets. Under these culture conditions, the effect of ABA was weakened due to the presence of a cellophane sheet on the medium. We observed brood cell formation on many side branches of the *ppabi1a* plants cultured on media containing 1 μM ABA (Fig. 7c), although no brood cells in the wild type plants were detected. We also measured the freezing tolerance (Fig. 7d) and found that even in the absence of ABA treatment, the *ppabi1a* plants showed slightly higher survival rates than the wild type plants. In the presence of ABA, the survival of the *ppabi1a* plants markedly increased even with treatment as low as 0.01 μM ABA, whereas wild type plants were unaffected at this concentration. Upon treatment with 0.1 μM ABA, 88.7% (line 3) and 95.3% (line 7) of the *ppabi1a* plant protonemata survived, while in contrast only 23.3% of wild type survived. These data demonstrate that disruption of *PpABI1A* leads to ABA-hypersensitive phenotypes, providing concrete evidence for the role of PpABI1A as a negative regulator of ABA signaling in the moss. These data also suggest that the acquisition of freezing tolerance of *P. patens* involves PpABI1A-mediated ABA signaling.

Discussion

Evolutionarily conserved function of PP2Cs in ABA signaling

The *abi1-1* dominant mutation allele of *ABI1* functions as a strong negative regulator of ABA signaling in angiosperms. Here we showed that the negative regulatory

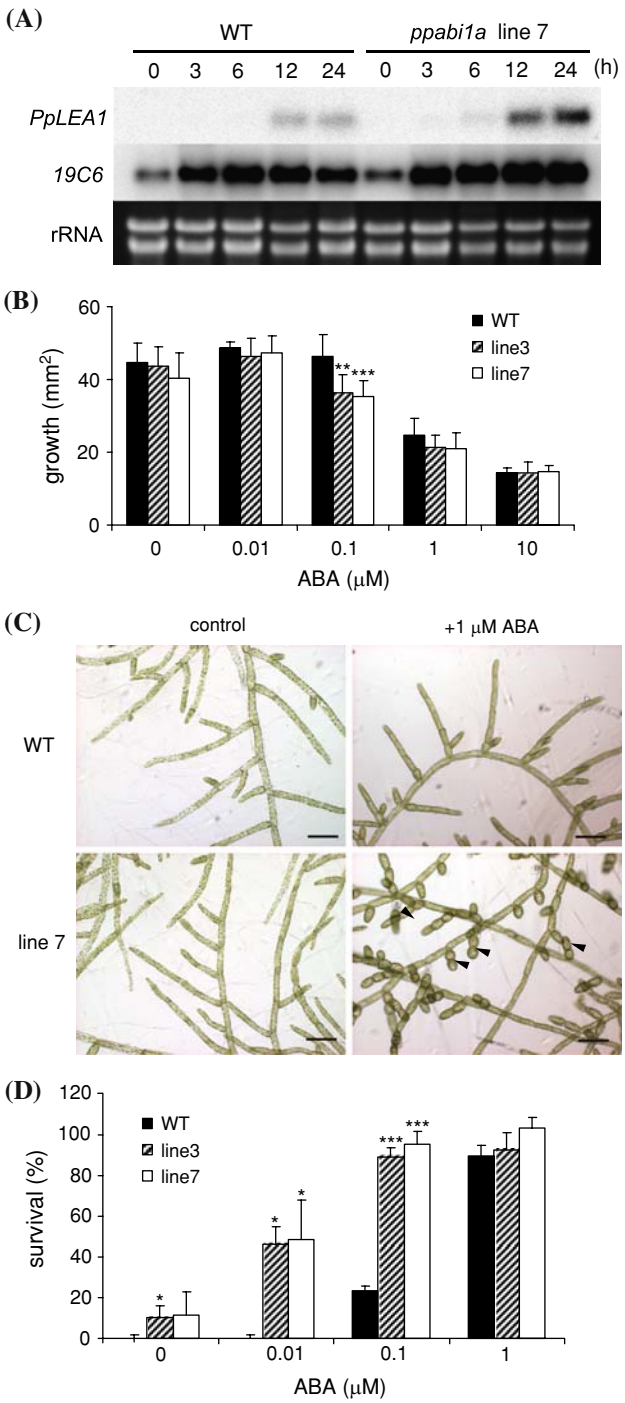

Fig. 7 Disruption of *PpABI1A* resulted in an ABA-hypersensitive phenotype. **a** RNA analysis of ABA-inducible genes in the *ppabi1a* mutant line 7. Protonemata were treated with 1 μM ABA for 0, 3, 6, 12, and 24 h, and isolated total RNA (10 μg) was subjected to RNA blot analysis for the indicated genes. **b** Protonemal growth of the *ppabi1a* mutants on ABA medium. Wild type, *ppabi1a* mutant line 3, and mutant line 7 were grown on ABA medium with the indicated concentration of ABA for 2 weeks. Areas of protonemal colonies were measured by image analysis. The values are mean ± SE ($n = 10$). **c** Phenotypes of protonemal cells of the wild type and the *ppabi1a* mutant line 7. Protonemata were cultured on BCDAT agar plates for 2 weeks with or without 1 μM ABA treatment. The brood cells are indicated by arrowheads. Scale bars: 100 μm. **d** Freezing tolerance of wild type and the *ppabi1a* mutants treated with various concentrations of ABA for 24 h. The values are mean ± SE ($n = 3$). Asterisks indicate significant changes between *ppabi1a* mutant lines and wild type (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

function of *abi1-1* is intact even in the evolutionarily distant plant species *P. patens*, suggesting that PP2C-mediated ABA signaling is evolutionarily conserved between angiosperms and bryophytes.

The mechanism by which the *abi1-1* dominant mutation exerts its negative regulation of ABA signaling is still unclear. The *abi1-1* mutation reduced the phosphatase activity of ABI1 in vitro (Bertauche et al. 1996), however, recent studies suggested that the *abi1-1* mutation is not a dominant negative mutation but rather a hypermorphic mutation (Saez et al. 2006; Yoshida et al. 2006b; Moes et al. 2008). In this scenario, the abi1-1 protein might irreversibly bind to its substrate(s), a potential master positive regulator(s) of ABA signaling, thus interrupting the positive signaling cascade. If this scenario is accurate, this master positive regulator(s) will likely also be conserved in *P. patens*. Several ABI1-related PP2C interacting proteins have been identified thus far. The PKS3 protein kinase interacts with ABI2 and, to a lesser extent, with ABI1, and negatively regulates ABA signaling in Arabidopsis (Guo et al. 2002). Another PKS protein kinase, SOS2, interacts with ABI2 but not ABI1 (Ohta et al. 2003). More recently, the SNF1-related protein kinase SnRK2E (or OST1) was found to specifically interact with ABI1, but not with ABI2 or abi1-1 (Yoshida et al. 2006a). Molecules other than protein kinases also interact with Group A PP2Cs (Cherel et al. 2002; Himmelbach et al. 2002; Zhang et al. 2004). None of these interactions, however, provide a mechanism for the dominant effect of abi1-1 in ABA signaling. The moss protonemata consist of only chloronema and caulonema cells, and the large-scale culture is possible in fermenters (Cove et al. 1997). Thus, the protonemata of *Act::abi1-1* plants may be a good source for the biochemical identification of the putative master positive regulator in ABA signaling.

Complete genome sequencing of *P. patens* revealed at least 51 genes encoding putative PP2Cs, enabling us to construct a comparative phylogenetic tree of *P. patens* PP2Cs with Arabidopsis PP2Cs. We found that Group B consisted of only Arabidopsis PP2Cs, suggesting that this class of PP2Cs are likely involved in physiological events specific to the vascular plants. Although few reports have been made on Group B PP2Cs, it is noteworthy that AP2C1 has been shown recently to play a role in regulating stress responsive mitogen-activated protein kinases (MPK4 and MPK6) and jasmonic acid (JA) levels to modulate innate immunity in Arabidopsis (Schweighofer et al. 2007). The

presence or function of JA in *P. patens* is yet to be investigated (Decker et al. 2006).

Only two genes (*PpABI1A* and *PpABI1B*) were closely related to Arabidopsis Group A PP2Cs. Although the two genes encoded proteins highly homologous to one another and were expressed through the moss life cycle, the expression levels and responses to abiotic stresses varied between the genes. Notably, the *PpABI1A* single disruption was sufficient to alter ABA sensitivity to freezing tolerance as well as gene expression in *P. patens* protonemata, providing concrete evidence for a conserved PP2C-mediated ABA signaling pathway in the moss. Although the disruption phenotype of *PpABI1B* is yet to be examined, these data may indicate that PpABI1A and PpABI1B are functionally distinct from each other, possibly due to different expression profiles. A similar idea was also proposed in regards to the function of Arabidopsis group A PP2Cs (Yoshida et al. 2006b). *ABI1*, *ABI2*, *HAB1*, *HAB2* and *AtPP2CA/AHG3* encode PP2Cs closely related to one another within the catalytic domain, and loss-of-function mutations of each gene resulted in ABA-hypersensitivity in seed germination with varying intensities. Among the mutants, the *AtPP2CA/AHG3* mutant, which was expressed the highest in seeds compared to others, showed the strongest ABA-hypersensitivity. The authors concluded that the level of gene expression may be the primary factor for their distinct functions. Other factors, however, may also determine functional specificity. Arabidopsis PP2Cs are highly similar within their catalytic domains, but divergent in the N-terminal regions, suggesting that other post-transcriptional mechanisms, such as substrate specificity or subcellular localization, could also play a role in the functional specificities of PP2Cs. However, the functions of PpABI1A and PpABI1B are distinct especially in tolerance to low temperature stress, despite the high homology throughout the coding sequences of these two genes. Together this strongly supports the idea that different expression levels of the PP2Cs are a critical contributor to the regulation of their distinct functions.

Our study suggests that the Group A PP2C clade evolved before the separation of the bryophytes and vascular plants from a common ancestor, and that angiosperms increased the number of PP2C genes with differential expression profiles during evolution to enable tissue- and organ-specific tuning of ABA signaling.

PP2C-mediated regulation of ABA-inducible genes in *P. patens*

PpLea1 encodes a Group I LEA protein, which is expressed in a seed-specific manner in angiosperms and is activated by exogenous ABA (Kamisugi and Cuming 2005). We previously demonstrated that *PpLea1* is also activated by, thus one of the target genes of, PpABI3A (Marella et al. 2006). Ectopic expression of *abi1-1* suppressed the ABA-induced expression of *PpLea1* in proportion to the *abi1-1* expression level. Accordingly, disruption of *PpABI1A* activated the ABA-responsive expression of *PpLea1*. These results suggest that *PpLea1* is involved downstream of ABA signaling mediated by PpABI1A and PpABI3A. The ABA signaling pathway in protonemata closely resembled that of ABA signaling in the seeds of Arabidopsis, suggesting that ABA signaling in the vegetative tissue of the ancestor land plants has been functionally diverted specifically to seeds during the evolution of land plants.

To date very little is known about the *cis*-elements in ABA- or stress-inducible promoters from *P. patens*, except *PpLea1*. The *PpLea1* promoter contains one ABA-response element (ABRE)-like motif, and mutation of this element abolished the ability for ABA induction in *P. patens*, however, the ABRE-like motif was not active in barley aleurone protoplasts (Kamisugi and Cuming 2005). Saez et al. (2006) reported the differential up-regulation of ABA-inducible genes in loss-of-function Arabidopsis PP2C mutants associated with the presence of the ABRE, drought-responsive element (DRE), or a combination of the two. These observations suggested that PP2Cs are involved in at least two independent pathways for the regulation of ABA-inducible genes in Arabidopsis, possibly through different trans-acting factors. Cuming et al. (2007) reported transcriptomic analysis of *P. patens* treated with ABA, or subjected to osmotic, salt and drought stress. Many ABA- and drought responsive genes are homologues of angiosperm genes expressed during drought stress and seed development, including *LEA* genes. They also showed that ABREs and DREs are significantly over-represented in the 5′-proximal sequences of the ABA- and stress-induced gene set. In the present study, we demonstrated that ABA induction of a Group I LEA *PpLea1* and a Group II LEA *19C6* is under the control of PpABI1A. We found that *cis*-element databases for angiosperms also predicted ABREs and DREs in the 5′-upstream region of *19C6* (data not shown). Although true *cis*-elements for the ABA induction of *19C6* have yet to be elucidated experimentally, these facts suggest that *cis*-elements required for PP2C-mediated regulation of ABA-inducible genes in *P. patens* is comparable to Arabidopsis.

Physiological role of PP2C-mediated ABA signaling in *P. patens*

Bryophytes are considered as a modern representation of the first land plants, which were subjected to stresses novel to these plants, such as drought and freezing. Many land plants including bryophytes have been shown to

accumulate ABA, and bryophytes also respond to exogenous ABA, suggesting that ABA evolved to protect the land plants from such stresses. However, the role of endogenous ABA or the signaling pathways in response to environmental stresses has yet to be elucidated. *P. patens* displays a high degree of tolerance against osmotic stress (Frank et al. 2005b), and ABA levels in protonemata increase upon 0.5 M mannitol treatment (Minami et al. 2005). Our results demonstrated that ectopic expression of *abi1-1* significantly reduced the ABA sensitivity and tolerance against osmotic stress of protonemata, strongly indicating that endogenous ABA signaling is involved in the water stress response of bryophytes. *PpABI1A* and *PpABI1B* levels were ubiquitously detectable even under normal conditions; however, these genes were quickly activated by exogenous ABA and mannitol treatment, indicating that PP2C-mediated ABA signaling is involved in the osmotic tolerance of *P. patens* protonemata. Thus far, no significant difference in the osmotic tolerance between the protonemata of wild type and the *ppabi1a* mutants was detectable. Disruption of both the PP2Cs will confirm the role of PP2C-mediated ABA signaling in the osmotic stress response.

ABA levels in seed plant vegetative tissues increase in response to low temperatures (Xiong and Zhu 2003). Antisense inhibition of *AtPP2CA* also enhanced cold acclimation in Arabidopsis (Tähtiharju and Palva 2001). Thus ABA signaling is considered as one of the important factors involved in the freezing tolerance of Arabidopsis. Exogenous ABA also dramatically increases the freezing tolerance of *P. patens* protonemata in a dose-dependent manner (Minami et al. 2003). Interestingly, treatment with low temperature increased the freezing tolerance of *P. patens*, but did not change ABA accumulation, suggesting that cold acclimation in *P. patens* does not involve ABA biosynthesis (Minami et al. 2005). Our study demonstrated that ectopic expression of *abi1-1* or disruption of *PpABI1A* dramatically affected the ABA-induced freezing tolerance, clearly indicating the indispensable role of PP2C-mediated ABA signaling for the acquisition of freezing tolerance. The expression of *LEA* genes that are thought to play a role in freezing tolerance was upregulated in the moss upon cold treatment (Minami et al. 2005), suggesting a role for these genes in enhancing the freezing tolerance of the moss. We showed that PpABI1A negatively regulates these *LEA* genes, and that *PpABI1A* is upregulated by cold treatment. This suggests that ABA signaling is indeed activated during the cold acclimation without an apparent change of ABA accumulation, and that negative regulation of ABA signaling through PpABI1A is also activated to fine tune the process. Another molecule(s) generated during low temperature treatment might activate ABA signaling to enhance the freezing tolerance in *P. patens*.

Conclusion

We provided the solid molecular evidence that PP2C-regulated ABA signaling is not specific to the model plant Arabidopsis but is also present in the basal land plant *P. patens*, suggesting that the function of ABI1-related PP2Cs in the negative regulation of ABA signaling is evolutionarily conserved in land plants. Compared to the number and partially redundant function of Group A PP2Cs in Arabidopsis, *P. patens*, with only two Group A PP2Cs, is a promising model to understand the function of Group A PP2Cs in ABA signaling.

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