AP2-type transcription factors determine stem cell identity in the moss Physcomitrella patens

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SUMMARY
Stem cells are formed at particular times and positions during the development of multicellular organisms. Whereas flowering plants form stem cells only in the sporophyte generation, non-seed plants form stem cells in both the sporophyte and gametophyte generations. Although the molecular mechanisms underlying stem cell formation in the sporophyte generation have been extensively studied, only a few transcription factors involved in the regulation of gametophyte stem cell formation have been reported. The moss Physcomitrella patens forms a hypha-like body (protonema) and a shoot-like body (gametophore) from a protonema apical cell and a gametophore apical cell, respectively. These apical cells have stem cell characteristics and are formed as side branches of differentiated protonema cells. Here, we show that four AP2-type transcription factors orthologous to Arabidopsis thaliana AINTEGUMENTA, PLETHORA and BABY BOOM (APB) are indispensable for the formation of gametophore apical cells from protonema cells. Quadruple disruption of all APB genes blocked gametophore formation, even in the presence of cytokinin, which enhances gametophore apical cell formation in the wild type. All APB genes were expressed in emerging gametophore apical cells, but not in protonema apical cells. Heat-shock induction of an APB4 transgene driven by a heat-shock promoter increased the number of gametophores. Expression of all APB genes was induced by auxin but not by cytokinin. Thus, the APB genes function synergistically with cytokinin signaling to determine the identity of the two types of stem cells.

KEY WORDS: Stem cell, Apical cell, Meristem, Physcomitrella patens, PLT, Gametophore

INTRODUCTION
Stem cells are characterized by their ability to self-renew and give rise to differentiated cells (Lajtha, 1979). Land plants have a haplodiplontic life cycle, in which gametophyte and sporophyte generations alternate (Prigge and Bezanilla, 2010). Stem cells are formed only during sporophyte development in flowering plants, whereas mosses, a basal group of land plants, form stem cells in both the sporophyte and gametophyte generations (Sakakibara et al., 2008; Kofuji et al., 2009). Because the closest relatives of land plants, the charophytes, have a haplontic life cycle and retain stem cells only in the gametophyte generation, it is hypothesized that the molecular mechanisms underlying stem cell regulation in sporophytes were co-opted from pre-existing mechanisms in gametophytes (Kenrick and Crane, 1997; Nishiyama et al., 2003; Friedman et al., 2004). However, previous studies in the moss Physcomitrella patens showed that class 1 KNOX genes, which regulate the initiation and maintenance of stem cells in flowering plant shoot meristems, did not function in the haploid stem cells of the moss (Sakakibara et al., 2008). In contrast to the advancements made in our understanding of the molecular mechanisms underlying stem cell regulation in the sporophyte generation of flowering plants, only a few genes responsible for gametophyte stem cell formation have been reported (Jang et al., 2011). It is important to identify the genes that regulate gametophyte stem cell formation in order to elucidate the general principles of stem cell formation in plants.

P. patens is a useful model organism for investigating the regulation of stem cells in the gametophyte generation. Two types of bodies are formed in this moss: filamentous bodies, called protonemata, and gametophores, which consist of stems and leaves. The two main types of protonemata (chloronemata and caulonemata) can be distinguished by their chloroplast morphology, cell length, tip growth rate and cross wall orientation (Cove et al., 2006; Prigge and Bezanilla, 2010). A primary chloronema apical cell is initiated during spore germination and exhibits tip growth. The primary chloronema apical cell is a stem cell, which is maintained by self-renewal and produces chloronema cells by continuous cell divisions. Primary chloronema apical cells develop into caulonema apical cells, which produce caulonema cells. Caulonema cells form side branch initial cells of which ~87% become secondary chloronema apical cells, 5% secondary caulonema apical cells, 5% gametophore apical cells and 3% undivided cells under regular culture conditions (Fig. 1) (Cove and Knight, 1993). Gametophore apical cells divide to form gametophores, which develop stems and leaves, and later archegonia and antheridia, which facilitate sexual reproduction. Apical cell formation is regulated by two phytohormones, cytokinin and auxin. The exogenous application of cytokinin enhances the formation of gametophore apical cells (Ashton et al., 1979; Cove et al., 2006). In addition, the exogenous application of auxin to cytokinin-resistant mutants restored gametophore formation. This suggests that these mutants had some defects in auxin biosynthesis and that auxin is necessary for cytokinin signaling during gametophore apical cell formation (Ashton et al., 1979). Furthermore, it was shown that auxin and cytokinin signaling act in the same pathway (Prigge et al., 2010).
The coordination of auxin and cytokinin signaling in the regulation of stem cell formation in flowering plants is mediated by several transcription factors, including WUSCHEL, AUXIN RESPONSE FACTORS (ARFs) and ARABIDOPSIS RESPONSE REGULATORS (ARRs) (Zhao et al., 2010). However, no transcription factors have been shown to regulate secondary protonema apical cell and gametophore apical cell formation via phytohormone signaling.

The AP2-type transcription factors, which are characterized by the AP2/ERF DNA-binding domain, form a plant-specific protein family (Riechmann and Meyerowitz, 1998). The AINTEGUMENTA (ANT) subfamily consists of eight genes: ANT, AINTEGUMENTA-LIKE (AIL) 1, AIL5 [PLETHORA (PLT) 5], PLT1, PLT2, AIL6 (PLT3), AIL7 (PLT7) and BABY BOOM (BBM), which are involved in the development of flowering plants (Elliott et al., 1996; Boutillier et al., 2002; Aida et al., 2004; Nole-Wilson et al., 2005; Galinha et al., 2007; Prasad et al., 2011). In A. thaliana, PLT genes are required for stem cell niche formation in root apical meristems, and loss of function of these genes causes a defect in stem cell maintenance (Aida et al., 2004). A gradient of PLT proteins controls stem cell programming, mitotic activity and cell differentiation (Galinha et al., 2007). ANT, another gene of this subfamily in A. thaliana, regulates cell proliferation and organ growth during auxin signaling (Elliott et al., 1996; Mizukami and Fischer, 2000; Hu et al., 2003). BBM is thought to be involved in embryogenesis, because its overexpression induces somatic embryogenesis in A. thaliana (Boutillier et al., 2002). However, the functions of genes in this subfamily have not been characterized in non-flowering plants.

Here, we show that four P. patens genes orthologous to ANT, PLT and BBM are indispensable for the formation of gametophore apical cells. We also reveal that these genes are transcriptionally regulated by auxin, and are required for the cytokinin-dependent induction of gametophore apical cells. We conclude that the AP2-type genes function as a molecular switch to promote the development of different types of stem cells in the P. patens gametophyte generation.

MATERIALS AND METHODS

Plant material and culture conditions

P. patens Bruch and Schimp. subsp. patens collected in Gransden Woods (Ashton and Cove, 1977) was used as the wild-type line and was propagated on BCDAT medium (Nishiyama et al., 2000) at 25 °C under continuous light. For heat-shock induction, protonemata were cultured at 25 °C and kept at 38 °C for 1 hour of every 12 hours. To analyze the effect of auxin and cytokinin on the activity of APB genes, protonemata were cultured on BCDAT medium for four or five days under continuous light and then transferred to BCDATG medium and cultivated for seven days under unilateral red light. After cultivation, the moss plants were transferred to a twofold dilution of liquid BCD medium (Nishiyama et al., 1995) under unilateral red light. After cultivation, the moss plants were transferred to BCDATG medium and cultivated for seven days.

Phylogenetic analysis

We used a data set of AP2-type transcription factor homologs obtained using the amino acid sequence of APB1 protein as a query for a BLASTP search (Altschul et al., 1997) against the non-redundant protein data set from the National Center for Biotechnology Information. Deduced amino acid sequences were aligned with clustalW (Thompson et al., 1994) in a MEGA5 program (Tamura et al., 2011) and then revised manually. MOLPHY version 2.36 program package (Adachi and Hasegawa, 1996) was used for construction of phylogenetic trees. After exclusion of short or redundant sequences, 14 representative land plants (P. patens, Selaginella moellendorffii, Ceratopteris thalictroides, Gnetum parvifolium, Cycas revoluta, Ginkgo biloba, Pinus thunbergii, Picea abies, Vitis vinifera, Glycine max, Populus trichocarpa, Arabidopsis thaliana, Zea mays and Oryza sativa) were selected and 92 amino acid sequences were used to calculate maximum-likelihood (ML) distances for 107 genes using the JTT model (Jones et al., 1992) with a ProtML program and to construct a neighbor-joining (NJ) tree (Saitou and Nei, 1987) with a NJdist program. The ML tree was searched by local rearrangement using this NJ tree as a start tree under the JTT model with a ProtML program. Local bootstrap probability was estimated using the resampling-of-estimated-log-likelihood (RELL) method (Kishino et al., 1990; Hasegawa and Kishino, 1994).

Construction of plasmids for gene targeting

A schematic of the disruption construct and the primer sequences are given in supplementary material Fig. S1 and Table S1, respectively. To delete APB1, a genomic DNA fragment of APB1 was amplified using the PpAPB1-HI and RpAPB1+5-E primers. The amplified fragment was digested with HindIII and EcoRI, and cloned into the HindIII-EcoRI site of the p35S-Zeo plasmid (EF451822), which contains the bleomycin expression cassette (Hwataishi et al., 2008). This plasmid was named pAPB1-dis-5. A genomic DNA fragment was amplified with the PpAPB119+X and RpAPB15+S primers. The amplified fragment was digested with XbaI and SacII and inserted into the XbaI-SacII site of pAPB1-dis-5, to generate the pAPB1-KO construct. The pAPB4-KO construct was made in the same way using the PpAP4T4F1n, RpPpAnt12+E, PpPpAnt4F2XbaI and PpANT42SacII primers and the p5Ss-lox-P-Bsd plasmid (AB37973), which contains the CaMV 3SS promoter, blasticidin S deaminase gene and CaMV polyadenylation signal. For gene targeting, DNA fragments amplified with the PpPpAnt11+H and RpPant15+S primers using pAPB1-KO as template and with those with the PpAP4T4F1n and PpANT42SacII primers using pAPB4-KO as template were used.

For construction of pAPB2-KO and pAPB3-KO plasmids, APB2 and APB3 genomic DNA amplified using the PpPant2-3-1-all-nest and PpAnt2-5-1-nest-all primers, and the PpAnt3-3-1 nest and PpAnt3-5-1 primers, were cloned into the p Gemini T vector (Promega, Madison, WI, USA), thereby generating pPpAnt2tg and pPpAnt3tg plasmids, respectively. A DNA fragment conveying the neomycin phosphotransferase II expression cassette was excised from the pGFpmutNPTII plasmid (Hiwatashi et al., 2008) with EcoRI and Sphi, blunt-ended, and inserted into a blunt-ended Aor5HI site of the pAPB2tg plasmid. The recombinant plasmid was designated as pAPB2-KO. The fragment was amplified with PpPant2-3-1-all-nest and PpPant2-5-1-nest-all primers using pAPB2-KO as template and was used for gene targeting. To make the pAPB3-KO plasmid, a DNA fragment was amplified using the hygromycin phosphotransferase expression cassette (aphIv; pTN86 [AB267705]) was excised from pHTS14 (Tanahashi et al., 2005) with XbaI and HindIII, blunt-ended, and cloned into a blunt-ended Bsi1107 site of the pPpAnt3tg plasmid. The fragment was amplified with the PpAnt3-5-1-nest and PpAnt3-3-1 primers using pAPB3-KO as template and was used for gene targeting.

Schematics of a reporter knock-in construct are shown in supplementary material Fig. S2. To generate APB1-Citrine and APB4-Citrine lines, an APB1 genomic DNA fragment just prior to the stop codon was PCR-amplified with the PpAPB1&4-ctr-5F1 and PpAPB1-ox-R5 primers, and cloned into the EcoRv site of pCtRN-NPTII 2, thereby creating an in-frame fusion of the APB1 or APB4 coding sequence with the Citrine yellow fluorescent protein gene (Heikal et al., 2000) to produce pPpAPB1&4Citrine-5. A genomic fragment downstream of the APB1 stop codon was amplified with the PpAPB1-citrine-3F2 and PpAPB1-citrine-3R2 primers, and cloned into the Smal site of pPpAPB1&4Citrine-5 to generate pAPB12Citrine. A genomic fragment downstream of the APB4 stop codon was amplified with the PpAPB4-ctr-3F2 and PpAPB4-citrine-3R2 primers, and cloned into the Smal site of pPpAPB1&4Citrine-5 to generate pAPB42Citrine. pAPB12Citrine and pAPB42Citrine were digested with HindIII and XbaI for gene targeting. To fuse the udA gene, which encodes β-glucuronidase (GUS) (Jefferson, 1987), with APB2 and APB3, the genomic DNA fragments of APB2 and APB3 were respectively amplified with the PpAnt2+H and PpAnt2-M primers, and the PpAnt3-5-2 and PpAnt3-HindIII primers, using pPpAnt2tg and pPpAnt3tg plasmids as templates, and digested with HindIII. These digested fragments were
cloned into the EcoRv-HindIII site of the pGUS-NPTII-2 plasmid (Sakakibara et al., 2003) to create an in-frame fusion of each APB2 and APB3 coding sequence with uidA and to produce pAPB2-GUS-S5 and pAPB3-GUS-5. A genomic DNA fragment downstream of the APB2 stop codon was amplified by TAIL-PCR (Li and Whittier, 1995), cloned into pGEM-T (Promega) and named pPpANT2-tail. The DNA fragment was amplified with the T7 and PpA2ta3 primers using pPpANT2-tail as template, and cloned into the blunt BamHI site of pAPB2-GUS-5 to generate pAPB2-GUS. pPpANT3g was digested with PsI and the DNA fragment was blunt-ended and inserted into the blunt-blunted BamHI site of pAPB3-GUS-5 to make pAPB3-GUS. The generated constructs were digested with suitable restriction enzymes for gene targeting.

To construct HSP-APB4 lines and HSP-Cerulean-APB4 lines (supplementary material Fig. S3), cDNA was synthesized using total RNA extracted from protonemata of the apb1apb2apb3-56 line as template using Superscript III (Invitrogen, Carlsbad, CA, USA). APB4 cDNA was amplified using the cDNA as template and the PpAP4-4spf1 and PpAP4-3-1-all primers. The amplified fragment was cloned into the pcRII-Blunt II vector (Invitrogen) and named APB4-CDNA plasmid. A PCR fragment amplified using the APB4-CDNA plasmid as template with APB1-ox-F1 and APB1-ox-R5 primers was cloned into the pENTR/D-TOPO vector (Invitrogen). This plasmid was named pAPB4-entry. The pHSP-APB4 plasmid was constructed using the LR clonase reaction between the pAPB4-entry and pHSP140 vectors to replace the insert DNA fragment with the HSP promoter region (Scaffold_7: 2739557-2749001) was constructed using the LR clonase reaction between the pAPB4-entry and pT1OG vectors to insert the putative neutral site PIG1 (Okano et al., 2009). The pHSP-Cerulean-APB4 plasmid was constructed using the LR clonase reaction between pAPB4-entry and pPIG1HG (AB472844) vectors to insert the emission vector to the scaffold_310: 80872-81503 (p. patens targeting site 1 (PTA1)) of the version 1.1 genome (http://genome.jgi-psf.org/Phypa11/Phypa11.home.html). The plasmid was linearized with Pmel and used for gene targeting.

To construct EF1-α-APB4 lines (supplementary material Fig. S4), the pEF1-1-APB4 plasmid containing a pEF1-α promoter region (Scaffold_7: 2739557-2749001) was constructed using the LR clonase reaction between the pEF1-α-entry and pT1OG vectors to replace the insert DNA fragment with the scaffold_310: 80872-81503 (p. patens targeting site 1 (PTA1)) of the version 1.1 genome (http://genome.jgi-psf.org/Phypa11/Phypa11.home.html). The plasmid was linearized with Pmel and used for gene targeting.

Transformation
Polyethylene glycol-mediated transformation was performed as described previously (Nishiyama et al., 2000). Two double disruption lines of APB2 and APB3 (apb2apb3-60 and -97) were generated by inserting IAPB2 into the background of the APB3 disruption (apb3-30). Two triple disruption lines of APB1, APB2 and APB3 (apb1apb2apb3-3 and -86) were generated by the deletion of APB1 in the background of the double disruption line of APB2 and APB3 (apb2apb3-111). Two quadruple disruption lines (apb1apb2apb3apb4-57 and apb1apb2apb3apb4-73) were generated by the deletion of APB4 into the background of the quadruple disruption lines, apb1apb2apb3apb4-86 and -3, respectively. Correct gene targeting was confirmed by Southern hybridization.

Southern hybridization
Southern hybridizations were conducted as described by Hiwatashi et al. (Hiwatashi et al., 2001) using a BcaBEST DNA Labeling Kit (Takara Bio, Otsu, Japan). DNA fragments for the APB3ko and APB4ko probes were amplified using the PpAP4F1Sall and RPpANT12-E, and PpAP2-3 and APB3-3ox-R4 primers, respectively. Probes for the APB1-Citrine, APB2-GUS and APB3-GUS, and APB4-Citrine lines were amplified using the PpAP1-1cr-3F2 and PpAP1-1cr-3R2, PpAP2-3-5 and PpAP3-3HindIII, and PpAP4-3cr-3F2 and PpAP4-3cr-3R2 primers, respectively. A PIG1 probe for the HSP-APB4 and HSP-Cerulean-APB4 lines was amplified using the SSP-PIG1striction and Xb-PIG1striction primers. A PTA1 probe for EF1-α-APB4 lines was amplified using the PTA1-3F1 and PTA1-3R1 primers.

Quantitative RT-PCR
A mixture of chloronemata and caulonemata was homogenized by vortexing for 1 minute using a six-well tube and ceramic balls (KURABO, Osaka, Japan), and vegetatively propagated on a BCDAT medium agar plate for four days. Then, the tissues were treated with or without phytohormones. The collected protoneema were ground in liquid nitrogen and total RNA was extracted using the RNeasy Plant Mini Kit (QUIAGEN, Hilden, Germany). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (QUIAGEN), and quantitative RT-PCR was performed using the QuantiTect SYBR Green PCR Kit (QUIAGEN). The following primer pairs were used: for APB1, PpAPB1GSP-F1 and PpAPB1GSP-R1; for APB2, PpAPB2GSP-F1 and PpAPB2GSP-R1; for APB3, PpAPB3GSP-F1 and PpAPB3GSP-R1; for APB4, PpAPB4GSP-F3 and PpAPB4GSP-R3; and for PpTUA1, PpTUA1F and PpTUA1R.

Southern hybridization
GUS staining was basically conducted according to the methods of Nishiyama et al. (2000). Each line was cultured on BCDAT or BCDATG medium. The tissues were not fixed before GUS staining, were infiltrated for 20 or 30 minutes in a substrate solution [50 mM NaH2PO4 (pH 7.0), 0.5 mM 5-bromo-4-chloro-3-indolyl β-d-glucuronide (X-Gluc), Wako Pure Chemical Industries, Osaka, Japan], 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6, and 0.05% (v/v) Triton X-100] and were then stained at 37°C for 6-24 hours. After the incubation, the tissues were fixed in 5% (v/v) formalin for 10 minutes and then soaked in 5% (v/v) acetic acid for 10 minutes. The tissues were then dehydrated through an ethanol series. Images of the stained tissues were digitized with a charge-coupled device (CCD) camera (FUJIX HC300Z, Fuji Photo Film, Japan or CoolSNAP, Roper Scientific Photometrics, Germany).

Microscopy
To observe fluorescence proteins, moss was cultured on glass-bottom dishes with BCDAT medium for 7 to 10 days. Digital images were obtained using a confocal microscope (A1, Nikon, Japan) with a 20×/0.75 NA objective lens. The fluorescence excitation was performed with a 514-nm Argon laser and the emission spectra were collected using a 540/30 band pass filter.

To perform time-lapse observation, moss was cultured on glass-bottom dishes with BCD medium for 5 to 8 days. After cultivation, the moss plants were treated with 1/2 BCD liquid medium containing 1 µM BAP and time-lapse observation was started. Digital images were obtained using a microscope (IX81, Olympus, Japan).

RESULTS
Gametophores were not formed in the APB quadruple disruptants
The APB subfamily of AP2/ERF transcription factors includes ANT, PLT1, PLT2, BBM, AIL1, AIL5, AIL6 and AIL7. These genes regulate various developmental processes, including organ size determination, formation of the stem cell niche, control of phyllotaxis, and embryogenesis in A. thaliana (Elliott et al., 1996; Mizukami and Fischer, 2000; Boutilier et al., 2002; Hu et al., 2003; Aida et al., 2004; Nole-Wilson et al., 2005; Galinha et al., 2007; Prasad et al., 2011). We focused on their orthologs in P. patens. In addition to three reported orthologs named PpANT1, PpANT2 and PpANT3 (Shigyo et al., 2006), we found a fourth ortholog in the genome sequence. Based on a phylogenetic analysis, the four genes belonged to same group with ANT, PLT1, PLT2, BBM, AIL1, AIL5, AIL6 and AIL7 (supplementary material Fig. S5) (Kim et al., 2006; Shigyo et al., 2006; Floyd and Bowman, 2007). We therefore renamed the four P. patens genes APB1 (AB675589), APB2 (AB675590), APB3 (AB675591) and APB4 (AB675592), based on the initials of ANT, PLT and BBM. For loss-of-function analysis of these genes, each APB gene was inserted or replaced with a different antibiotics-resistant gene cassette by homologous recombination (supplementary material Fig. S1) to form single gene disruption lines (apb1-7, apb1-119, apb2-55, apb2-61, apb3-30, apb3-32, apb4-103 and apb4-104). The protoneema and gametophores of the apb1, apb2 and apb3 single disruption lines were not distinguishable from those of the wild type, but the apb4 single disruption lines produced...
fewer gametophores (Fig. 2). We then constructed double (apb2apb3-60 and -90), triple (apb1apb2apb3-3 and -56) and quadruple disruption lines (apb1apb2apb3apb4 [apb-quadruple]-57 and -73). The apb2apb3 double disruption lines were indistinguishable from the wild type. By contrast, the number of gametophores per protonema culture decreased in the apb1apb2apb3 triple disruption lines, although the decrease was not greater than in the apb4 single disruption lines. Finally, quadruple disruption of APB genes completely blocked gametophore formation. These results suggest that the four APB genes function redundantly in gametophore formation and that APB4 has the major role in this process; however, it is necessary to analyze other double and triple disruption mutants to determine the precise function of each gene.

**Protonema apical cells replace gametophore apical cells in apb-quadruple mutant lines**

As the apb-quadruple lines do not form gametophore apical cells, we next investigated whether the gametophore apical cells were replaced by secondary chloronema apical cells, secondary caulonema apical cells or undivided cells. During the early stages of development, we could not distinguish between secondary chloronema apical cells and secondary caulonema apical cells and treated them together as secondary protonema apical cells. It is also difficult to quantify the number of secondary protonema apical cells and gametophore apical cells in regular culture conditions, because side branch formation is not synchronous. Therefore, we pre-cultured protonemata in unilateral red light for one week and then moved them to polarized white light (Okano et al., 2009). Under unilateral red light, caulonema cells produced almost no side branch initial cells (Fig. 3A). When caulonema filaments of both the wild-type and the apb-quadruple lines were transferred to polarized white light and cultured for a further two days without any exogenous phytohormone, most caulonema cells synchronously underwent tip growth and formed secondary protonema apical cells. However, no gametophore apical cells formed in either the wild-type or the apb-quadruple lines (Fig. 3B,C). When wild-type protonemata were cultured in the presence of benzylaminopurine (BAP), 5.8±2.3 (s.d., n=42) and 2.0±1.7 (s.d., n=42) of the ten caulonema cells adjacent to the caulonema apical cell gave rise to gametophore apical cells and secondary protonema apical cells, respectively (Fig. 3D; supplementary material Table S2). By contrast, no gametophore apical cells were formed and 5.4±1.5 (s.d., n=42) and 4.4±1.6 (s.d., n=43) secondary protonema apical cells were formed on the corresponding cells in the apb-quadruple-57 and -73 lines, respectively, upon BAP treatment (Fig. 3E; supplementary material Table S2). Although the total number of side branches was slightly lower in the apb-quadruple lines [8.9±1.5 (s.d.), n=42 in wild type; 7.7±1.4 (s.d.), n=42 in apb-quadruple-57; 7.4±1.9 (s.d.), n=43 in apb-quadruple-73], these results indicate that gametophore apical cells were, at least partially, replaced by secondary protonema apical cells in the apb-quadruple lines. Whereas the number of gametophore apical cells decreased in the quadruple mutants, that of secondary protonema apical cells increased. This finding suggests that a mechanism that forms secondary protonema apical cells is activated when gametophore apical cells fail to form. The exogenous application of auxin could exacerbate defects in gametophore formation in some cytokinin-resistant mutants (Ashton et al., 1979). However, the exogenous application of both cytokinin and auxin did not induce gametophore apical cells in the apb-quadruple-57 and -73 lines (Fig. 3F,G) with few exceptions (supplementary material Table S3).

Although side branch initial cells differentiate into protonema apical cells in the apb-quadruple lines, such side branch initial cells might have gametophore apical cell identity transiently. Therefore, time-lapse observation of secondary apical cell formation process was performed (supplementary material Fig. S6, Movies 1-3, Table S3). It seems that, at least based on their morphology, side branch initial cells do not acquire gametophore apical cell identity and instead directly differentiate into protonema apical cells.

**APB genes were continuously expressed during gametophore apical cell formation**

To examine the functions of the APB genes, we analyzed the expression patterns of the APB proteins during the formation of secondary protonema apical cells and gametophore apical cells. The Citrine yellow fluorescent protein gene (Heikal et al., 2000) or the uidA gene, which encodes β-glucuronidase (GUS) (Jefferson, 1987), was inserted just before the stop codon of each APB gene by means of homologous recombination. We selected single insert lines, determined by Southern hybridization, for further analysis (supplementary material Fig. S2). All four fusion proteins localized to caulonema cells before the formation of apical cells (Fig. 4A,F,K,P,U,Z). A side branch initial cell is initiated as a protrusion at the surface of caulonema cells and divides from the parent caulonema cell. Citrine and GUS signals were detected in both the side branch initial and parental caulonema cells in the APB1-Citrine, APB3-GUS and APB4-Citrine lines (Fig. 4B,Q,V,AA), whereas signals were detected only in the side branch initial cells in the APB2-GUS lines (Fig. 4G,L). At this stage, the morphology of a side branch initial cell did not reveal whether the cell was fated to become a gametophore or a secondary protonema apical cell. Subsequently, ~5% of side branch initial cells swelled up and divided obliquely to form a gametophore apical cell, whereas the remaining 95% of side branch initial cells continued to undergo tip growth without swelling and became secondary protonema cells.
The APB-Citrine and APB-GUS signals were detected in swollen cells (Fig. 4C,H,M,R,BB; supplementary material Table S4). We detected APB-reporter signals of all APB genes in the gametophore apical cell and in the gametophore cells derived from the gametophore apical cell (Fig. 4D,I,N,S,X,CC). By contrast, APB-reporter signals were not usually detected in apical cells undergoing tip growth to become secondary protonema apical cells (Fig. 4E,J,O,T,Y,DD), and signals were occasionally observed in secondary protonema apical cells in each reporter line. To quantitatively examine the expression patterns, we analyzed APB1-Citrine and APB4-Citrine lines further, because the apb4 single deletion mutant showed the strongest phenotype and APB1 is a sister gene to APB4 (supplementary material Table S4). APB-Citrine signals were detected in all mother protonema cells and >85% side branch initial cells. More than 70% side branch initial cells with swelling showed Citrine signals, whereas less than 15% side branch initial cells with secondary protonema characteristics showed signals.

**Induction of APB4 increased the number of gametophores**

To examine further the roles of APB in the regulation of gametophore apical cell formation, we generated heat-shock-inducible transgenic lines of APB4, which has the most important function of the four APB genes (supplementary material Fig. S3). In addition to lines containing APB4 fused to the soybean Gmhspl7.3B heat-shock promoter (Saidi et al., 2005) (HSP:APB4-12 and HSP:APB4-120 lines), transgenic lines containing APB4 fused to a cyan fluorescent protein gene, Cerulean (Rizzo et al., 2004), and HSP (HSP:Cerulean-APB4-264 and HSP:Cerulean-APB4-284 lines) were generated to monitor induction of the gene (supplementary material Fig. S3). Transgenic lines containing Cerulean fused with HSP were also generated as a control (HSP:Cerulean-2 line). The amount of APB4 mRNA increased in HSP:APB4 and HSP:Cerulean-APB4 lines upon heat-shock induction, but did not increase in the HSP:Cerulean line (Fig. 5A). APB4 transcripts were more strongly induced in HSP:Cerulean-
ABP4 lines than in HSP:APB4 lines, for unknown reasons. The Cerulean signal of HSP:Cerulean-APB4 lines was detected in all protonema cells exposed to heat shock (supplementary material Fig. S3). In the absence of induction, the APB4 inducible lines were indistinguishable from the wild type and formed a similar number of gametophores (Fig. 5B). By contrast, the number of gametophores increased in HSP:APB4 and HSP:Cerulean-APB4 lines upon induction, but not in HSP:Cerulean lines (Fig. 5B). Although the number of gametophores increased, other side branch initial cells of HSP:APB4 and HSP:Cerulean-APB4 lines changed to secondary protonema apical cells.

To investigate the proportion of gametophore apical cells to secondary chloronema apical cells with APB4 induction, we cultivated HSP:Cerulean-APB4 lines under red light conditions. However, side branch formation was arrested with heat shock under red light conditions for unknown reasons. We therefore generated constitutive overexpression lines of APB4 (supplementary material Fig. S4). When protonemata were treated with 0.1 μM BAP, 2.7±2.0 (s.d., n=36) gametophore apical cells in the ten caulonema cells adjacent to the caulonema apical cell were formed in wild type, whereas the number increased in EF1-α:APB4-82 and EF1-α:APB4-259 lines [3.8±1.6 (s.d., n=44) and 4.9±1.6 (s.d., n=57), respectively] (supplementary material Table S5).

The overexpression of APB4 gene also affects caulonema differentiation. The formation of caulonemata was observed within two weeks in wild-type and HSP:APB4 lines without heat shock (supplementary material Fig. S7A,E). However, caulonema formation of HSP:APB4 lines was arrested with heat shock (supplementary material Fig. S7B,D,F-H). The formation of caulonemata is positively regulated by auxin (Cove et al., 2006). The defect in caulonema formation in the overexpression line was exacerbated by the addition of NAA to the medium (supplementary material Fig. S7I,J).

**Auxin induces the expression of APB genes, but cytokinin functions in parallel with auxin and APBs**

We have demonstrated that APBs are indispensable for the formation of gametophore apical cells and that overexpression of APB4 enhances the formation of gametophore apical cells. The formation of gametophore apical cells is known to be regulated by the phytohormones auxin and cytokinin (Ashton et al., 1979; Cove et al., 2006). We thus analyzed the regulation of APB transcripts by these phytohormones using quantitative real-time RT-PCR (Fig. 6). The accumulation of each APB transcript significantly increased after auxin application, whereas cytokinin application did not have a significant effect on the accumulation. The amount of APB1, APB3 and APB4 transcript was approximately fivefold higher 12 hours after the addition of auxin than in the absence of auxin (Fig. 6A,C,D). APB2 transcript levels were strongly induced (by up to ~15-fold) by auxin (Fig. 6B). Small synergistic effects of cytokinin and auxin were observed. APB2 expression is enhanced by the combined addition of auxin and cytokinin whereas cytokinin slightly suppresses the auxin-induction of APB3 (Fig. 6). These results indicate that all four APB genes are positively regulated by auxin, but not by cytokinin. We also found that expression patterns were not changed by the phytohormones in both APB1-Citrine and APB4-Citrine lines (supplementary material Fig. S8).

**DISCUSSION**

**APB genes are master regulators of gametophore apical cell formation in P. patens**

This study shows that disruption of all of four P. patens AP2-type transcription factor APBs caused defects in the formation of gametophore apical cells (Fig. 2) and promoted the formation of secondary protonema apical cells as compensation (Fig. 3). An APB-reporter protein fusion was detected in gametophore apical cells, but was largely absent from secondary protonema apical cells (Fig. 4). APB expression in side branch initial cells continues during gametophore apical cell formation, but disappears when secondary protonema cells are formed. This suggests that continuous expression of APBs is required for gametophore apical cell formation. Future experiments to manipulate APB expression levels during each apical cell formation will be useful to examine this hypothesis and to reveal the timing of fate decision (Fig. 4; supplementary material Table S4). Overexpression of APB4 increased the proportion of gametophore apical cells to secondary protonema apical cells (Fig. 5; supplementary material Table S5), although secondary protonema apical cells were still formed. Together, these results suggest that APBs are indispensable but not sufficient molecular switches in the formation of gametophore...
apical cells. APB-quadruple lines produced a few gametophore apical cells with the addition of exogenous cytokinin and auxin (supplementary material Table S2), suggesting that other redundant genes or pathways are involved.

**Interaction of APBs with auxin and cytokinin**

Gametophores are induced by exogenously applied cytokinin (Cove et al., 2006). Previously isolated *P. patens* mutants, BAR, PC22, and P24, exhibited a reduced number of gametophores in comparison with the wild type, although the genes responsible have not been identified. This defect was augmented by the exogenous addition of cytokinin or the induction of a cytokinin biosynthesis gene (Ashton et al., 1979; Abel et al., 1989; Reutter et al., 1998).

In addition, the number of gametophores decreased in the BAR 77 mutant line, and the exogenous addition of auxin exacerbated the defect, indicating that auxin is also involved in the formation of gametophore apical cells (Ashton et al., 1979). This study showed that auxin positively regulates the expression of APB genes (Fig. 6) and that APBs are necessary for the cytokinin signaling-mediated formation of gametophore apical cells (Fig. 3). The regulation of APBs is reminiscent of the auxin-mediated regulation of *ANT* via ARGOS (Hu et al., 2003) and PLTs (Aida et al., 2004; Galinha et al., 2007). As the auxin perception pathway using TIR1 and Aux/IAA in *A. thaliana* is conserved in *P. patens* (Prigge et al., 2010), the regulatory gene networks of the ANT subfamily might also be conserved between these two distantly related plants. However, we could not find *ARGOS* orthologs in the *P. patens* genome (Banks et al., 2011), and the network is therefore expected to be partly different.

As proteins in the AP2 family function as transcription factors and APBs are necessary for cytokinin signaling, APB proteins might regulate the expression of cytokinin signaling genes, such as...
response regulators. Alternatively, given the role of the AP2 domain in protein-protein interactions (Chandler et al., 2007; Chandler et al., 2009; Lee et al., 2010), APB proteins might interact with proteins that function in cytokinin signaling. Indeed, it was recently demonstrated that AP2-type transcription factors form heterodimers that control embryogenesis or the stress response (Chandler et al., 2007; Chandler et al., 2009; Lee et al., 2010). Similar interactions between APB and other factors might regulate stem cell formation in *P. patens*. Future isolation of the targets and factors that interact with APB will provide insight into the role of APB in cytokinin signaling.

**APBs are candidate targets of local cues for fate determination**

As gametophore apical cells and secondary protonema apical cells are sometimes observed next to each other on the same caulonema cell, it is speculated that intracellular cues exist in a parent caulonema cell that determine the fate of each gametophore apical cell and secondary protonema apical cell (Harrison et al., 2009). These authors discussed the involvement of a local cytokinin gradient and auxin transport to a restricted region in establishing the local cue that determines the fate of side branch initial cells. An APB-reporter fusion protein was broadly expressed in caulonema

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**Fig. 5. Induction of APB4 transcripts increased the number of gametophores.** (A) Quantitative real-time RT-PCR analysis of APB4 transcripts in transgenic lines subjected or not to heat-shock induction. Protonemata were collected after cultivation at 38°C for 1 hour. Error bars represent the mean ± s.e.m. of three independent quantitative real-time RT-PCR experiments. The TUA1 alpha-tubulin gene (AB096718) was used as an internal control. (B) The number of gametophores per protonema culture grown in white light for two weeks with or without heat-shock at 38°C for 1 hour of every 12 hours. Bars represent the mean ± s.e.m. of data derived from five independent cultures. Asterisk indicates a significant difference relative to non-heat-shock conditions (*P*<0.01, *t*-test).

**Fig. 6. Auxin induces APB, whereas cytokinin does not.** (A-D) Relative transcript levels of APB1 (A), APB2 (B), APB3 (C) and APB4 (D), as determined by quantitative real-time RT-PCR, in wild-type moss cultured in mock solution and in the absence of exogenously applied phytohormones (circle), with 1 μM NAA (triangle), 1 μM BAP (diamond) or 1 μM of both NAA and BAP (square). Bars represent the mean ± s.e.m. of three independent quantitative real-time RT-PCR experiments. The TUA1 alpha-tubulin gene was used as an internal control. The value at 0 hour was taken as 1.0.
cells and in side branch initial cells, which become either gametophore apical cells or secondary protonema apical cells (Fig. 4). The fusion protein disappeared in secondary protonema apical cells upon the initiation of tip growth, but was continuously detected in gametophyte apical cells, which swell but do not undergo further tip growth. Incorporating the auxin-mediated regulation of APB (Fig. 6), a local loss of auxin in presumptive protonema apical cells might serve as a cue during cell fate determination. To elucidate this unique developmental mechanism that relies on local cues, it is necessary to determine the local distribution of active auxin, the localization of auxin transporters, the expression of genes involved in auxin metabolism, and the effect of cytokinin signaling.

The function of APBs in other developmental processes

Although this study focused on the formation of gametophore and secondary protonema apical cells, in addition to the increase in gametophores, we observed several other phenotypes in the APB4 overexpression lines, such as partial defects in caulonema formation (supplementary material Fig. S7). Caulonema formation is regulated by auxin and the defect in these lines was augmented by the addition of auxin (supplementary material Fig. S71). This finding implies that endogenous auxin levels in the overexpression lines are lower than those in the wild type and raises the possibility that APB4 negatively regulates auxin biosynthesis. As the accumulation of APB4 transcripts was upregulated by auxin (Fig. 6), there might be a negative feedback loop between auxin biosynthesis and APB genes.

Acknowledgements

We thank Kazuyoshi Someya, Misako Gotoh and other members of the Division of Evolutionary Biology, NIBB for technical assistance; and Younousse Saiidi and Pierre Goloubinoff for providing us with a heat-shock promoter. We also thank the Functional Genomics Facility, Model Plant Research Facility, and Data Integration and Analysis Facility of NIBB; and Tomoaki Nishiyama, Keiko Inoue, H., Sakakibara, K., Hiwatashi, Y., Kurata, T., Kishino, H., Miyata, T. and Hasegawa, M.

Funding

This work was supported by Japan Science and Technology Agency (JST), Ministry of Education, Culture, Sports, Science and Technology (MEXT) and Japan Society for the Promotion (JSPS) [13970036, 21370107, 22128002 and 23012002 to M.H.]. This work was also supported by JSPS fellowships [No.22-1868 to T.A.].

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org lookup/suppl doi:10.1242/dev.076091-/-/DC1

References


Regulation of stem cell identity


