Involvement of auxin and a homeodomain-leucine zipper I gene in rhizoid development of the moss *Physcomitrella patens*

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Summary

Differentiation of epidermal cells is important for plants because they are in direct contact with the environment. Rhizoids are multicellular filaments that develop from the epidermis in a wide range of plants, including pteridophytes, bryophytes, and green algae; they have similar functions to root hairs in vascular plants in that they support the plant body and are involved in water and nutrient absorption. In this study, we examined mechanisms underlying rhizoid development in the moss, *Physcomitrella patens*, which is the only land plant in which high-frequency gene targeting is possible. We found that rhizoid development can be split into two processes: determination and differentiation. Two types of rhizoids with distinct developmental patterns (basal and mid-stem rhizoids) were recognized. The development of basal rhizoids from epidermal cells was induced by exogenous auxin, while that of mid-stem rhizoids required an unknown factor in addition to exogenous auxin. Once an epidermal cell had acquired a rhizoid initial cell fate, expression of the homeodomain-leucine zipper I gene *Pphb7* was induced. Analysis of *Pphb7* disruptant lines showed that *Pphb7* affects the induction of pigmentation and the increase in the number and size of chloroplasts, but not the position or number of rhizoids. This is the first report on the involvement of a homeodomain-leucine zipper I gene in epidermal cell differentiation.

Key words: Homeobox, Leucine zipper, Epidermal cell, Rhizoid, Auxin, Chloroplast, *Physcomitrella patens*, Cell differentiation

Introduction

Multicellular organisms are composed of diverse cell types and the definition of the molecular mechanism of cell-type determination is a fundamental problem in developmental biology (Alberts et al., 2002). In land plants, the epidermis is the outermost cell layer, which functions as a barrier to retain water and to defend against pathogens and predators. The compact arrangement of small cells with thick cell walls and the presence of a tough cuticle of the epidermis provide the mechanical strength to withstand gravity and the extracellular environment (Bold et al., 1987; Esau, 1977; Glover, 2000). Parts of the epidermal cells of land plants are specialized. Stomatal guard cells are specialized for gas exchange, trichomes and scales are for protection against environmental changes and predators, and root hairs in vascular plants and rhizoids in pteridophytes and non-vascular plants including green algae are for the absorption of water and nutrients. Rhizoid morphology varies from unicellular to multicellular filamentous structures. In addition to absorption, the rhizoids attach the plant body to the substratum, which is especially important in the gametophytes of pteridophytes and non-vascular plants, which do not have roots (Bell, 1992; Bold et al., 1987; Raghavan, 1989).

Molecular genetic studies of individual specialized cell types in angiosperms have provided information on the mechanism behind the patterns of cell differentiation (Brownlee, 2000; Hülskamp et al., 1994; Marks, 1997). Positioning of presumptive cells is likely regulated by cell-cell communication via molecular signals, such as the phytohormone ethylene, which functions in root hair formation (Tanimoto et al., 1995), and the serine protease subtilisin, which participates in stomatal guard cell differentiation (Berger and Altmann, 2000). The factors involved in trichome differentiation, as well as additional determinants of root hair and stomatal guard cell differentiation, are unknown. Communication usually occurs between clonally unrelated cells in root hairs and trichomes, while the determination of stomatal guard cells occurs via cell-cell interactions among clonally related cells (Brownlee, 2000; Glover, 2000). Such cell-cell communication triggers signal transduction cascades and induces transcription of downstream genes involved in later events of cell differentiation. Various classes of transcription factors are involved in these cascades (Lee and Schiefelbein, 1999; Oppenheimer et al., 1991; Payne et al., 2000; Wada et al., 1997). *GLABRA2* (*GL2*), a member of the homeodomain-leucine zipper (HD-Zip) gene family, functions in the promotion and repression of specialization in trichome and root hair cells, respectively (Di Cristina et al., 1996; Rerie et al., 1994), in co-operation with other transcription factors

(Lee and Schiefelbein, 1999; Oppenheimer et al., 1991; Payne et al., 2000; Wada et al., 1997).

The HD-Zip genes, characterized by a homeodomain and an adjacent leucine zipper motif, form four subfamilies (Sessa et al., 1994) and are found only in green plants [the HD-Zip I-IV subfamilies (Sakakibara et al., 2001)]. In addition to *GL2* of the HD-Zip IV subfamily, some members of the HD-Zip gene family are involved in cell differentiation. Overexpression of the HD-Zip I gene *Athb-1* produces pleiotropic effects on leaf cell differentiation (Aoyama et al., 1995), and most members of the HD-Zip III subfamily play roles in the cellular differentiation of stems (Baima et al., 2001; Zhong and Ye, 1999) and leaves (McConnell et al., 2001).

Rhizoids are widely observed in green plants, but their development has not been studied at molecular level, mainly because useful model systems were lacking. The moss *Physcomitrella patens* is a plant suitable to study rhizoid development, since techniques for transformation and gene targeting have been established (Cove et al., 1997; Schaefer, 2001; Schaefer and Zrÿd, 1997). The rhizoids of *Physcomitrella patens* are multicellular filamentous structures that develop from the epidermal cells of the stem of a leafy shoot (gametophore). The rhizoids function in the attachment of leafy shoots to the substratum and in the uptake of nutrients (Bates and Bakken, 1998; Duckett et al., 1998). Auxin is reported to increase the number of rhizoids (Ashton et al., 1979), although the details of their spatial patterns are unknown.

In this study, we report that rhizoid development can be divided into two processes, determination and differentiation. The cell-cell communication via auxin and unknown factor(s) regulates the former process, while a member of the HD-Zip I subfamily, *Pphb7* induced by auxin is involved only in the latter process.

Materials and methods

Culture conditions

Physcomitrella patens Bruch & Schimp subsp. *patens* Tan (Ashton and Cove, 1977) was cultured with 16 hours light and 8 hours dark. To observe juvenile gametophores, protonemata were cultured for 1 week in 6 cm Petri dishes with 5 ml of liquid 0.5×BCD medium (Nishiyama et al., 2000) that contained 0.1 mM $CaCl_2$ and 2% (w/v) sucrose. To examine short-term effects of exogenous auxin, gametophores grown on G medium (Sakakibara et al., 2001) for 6 weeks were cultured for 1 week in water that contained 1-naphthalene acetic acid (NAA) (N1641, Sigma, MO). To examine long-term effects, protonemata were cultured for 6 weeks on G medium that contained NAA. To test the effect of phytohormones on mRNA accumulation, gametophores were cultured for 4 weeks on G medium covered with cellophane. The cellophane sheets with gametophores were transferred to Petri dishes with 10 ml of NAA, 6-benzylamino purine (BA) (B-3274, Sigma), gibberellin A_3 (GA) (Wako, Japan), or abcisic acid (ABA) (A1049, Sigma) solution. To examine the effect of dehydration and osmotic stress, the cellophane sheets with gametophores were transferred to empty Petri dishes and Petri dishes with 6% mannitol, respectively.

Microscopy

The histochemical detection of β-glucuronidase (GUS) activity followed the method of Nishiyama et al. (Nishiyama et al., 2000). Leaves were observed after clearing with chloral hydrate (Tsuge et al., 1996).

Images of rhizoid cells were digitized with a CCD camera (CoolSNAP, Roper Scientific Photometrics, Germany) to quantify rhizoid pigmentation. Light transmittance was calculated as the ratio of light intensity in each, red, green and blue channel on the rhizoid to that outside the rhizoid. The light intensity was determined as the mean in an area of 2500 pixels. In order to quantify chloroplast size, an outline of the chloroplast was traced on the image and the area was measured.

For the sectioning of resin-embedded tissues, gametophores were fixed in 2.5% formaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0), dehydrated in a graded ethanol series, and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). Sections (5 μm thick) were made with a microtome and stained with 0.05% (w/v) Toluidine Blue in 0.1 M sodium phosphate buffer (pH 7.0).

For scanning electron microscopy (SEM), gametophores were fixed with modified Karnovsky's fixative (3% glutaraldehyde, 1.5% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4) (Karnovsky, 1965) for 2 hours, and soaked in 1% (w/v) tannic acid overnight. The samples were then fixed in 2% osmium tetroxide for 2 hours and in 1% uranyl acetate overnight before dehydration through a graded ethanol series. The fixed preparations were soaked once in an isoamyl acetate and ethanol mixture (1:1) for 30 minutes and twice in isoamyl acetate for 30 minutes, and were critical-point dried using carbon dioxide. The dried tissues were mounted on stubs and coated with gold with an ion spatter. The preparations were observed using a scanning electron microscope (S-800; Hitachi Ltd., Tokyo, Japan).

For transmission electron microscopy, rhizoids were fixed in the Karnovsky's fixative for 2 hours and 2% osmium tetroxide for 2 hours, stained with 1% uranyl acetate en bloc before dehydration, and embedded with Epon. A JEM 100CX (JEOL Ltd, Akishima, Japan) was operated at 100 kV.

Gene targeting

Most of the *Pphb7* genomic DNA (2851 bp) amplified using Pphb7-52 (5′-TAGCGGCCGCGGAAAGGGGAGGGAAGGGTGTAA-3′) and Pphb7-32 (5′-TAGCGGCCGCTCAGGGACGCACAACAGCGAC-AA-3′) primers were cloned into pGEM3z (Promega, WI), thereby generating pgPphb7.

A *Sal*I site was added to the end of the coding sequence of pgPphb7 with the SalI-Pphb7S primer (5′-ACGCGTCGACTCCGAGCTC-GATGGTTAAG-3′). The 693 bp *Sal*I fragment from the internal *Sal*I site to the new *Sal*I site was cloned into the *Sal*I site of the pGUS-NPTII-2 plasmid, which contained the coding sequence of *uidA*, nopaline synthase polyadenylation signal (nos-ter) and NPTII cassette (nptII; Nishiyama et al., 2000), thereby creating an in-frame fusion of the *Pphb7* and *uidA* genes (Fig. 4B). A 1.2 kb *Xba*I fragment of pgPphb7 that contained the 3′ region was inserted into this plasmid and the recombinant plasmid was designated as pPphb7-GUS. This plasmid was linearized with *Apa*I and *Not*I for gene targeting (Fig. 4B).

The plasmid pKS1, which contained the coding sequence of sGFP from pTH-2 (Chiu et al., 1996), nos-ter, and nptII, was used for construction of GFP-Pphb7 (Fig. 4C). The 891 bp *Bam*HI (blunt-ended)-*Not*I fragment from pgPphb7, which contained the 3′-untranslated region, was inserted into the *Sph*I (blunt ended)-*Not*I site (3′ to nptII). The 1842 bp coding region and the 740 bp 5′-flanking region were amplified and inserted into the *Eco*RV (3′ to sGFP) and *Kpn*I-*Apa*I (5′ to sGFP) sites. The plasmid was linearized with *Kpn*I and *Not*I for gene targeting.

A 4.4 kb fragment that encompassed *uidA*, nos-ter, and nptII was cloned into the *Pma*CI site of pgPphb7. The plasmid was linearized with *Not*I for gene targeting (Fig. 4D). PEG-mediated transformation followed the method of Hiwatashi et al. (Hiwatashi et al., 2001).

RNA and DNA gel blot analyses

$Poly(A)^+$ RNA and DNA extraction, blotting and hybridization

followed the method of Hiwatashi et al. (Hiwatashi et al., 2001). A *Bam*HI-digested fragment of the plasmid p3Pphb7 (Sakakibara et al., 2001) was used as the cPphb7 probe for northern analysis (Fig. 4A). A fragment amplified with the Pphb7-52 and Pphb7-32 primers using pgPphb7 plasmid as template was used as the gPphb7 probe (Fig. 4A). A *GAPDH* (Leech et al., 1993) cDNA fragment was amplified with the PpgapC5′ (5′-GAGATAGGAGCATCTGTACCGCTTGTGC-3′) and PpgapC3′ (5′-CATGGTGGGATCGGCTAAGATCAAGGTC-3′) primers, using pPpGapC (Hiwatashi et al., 2001) as template. Radioactivity of hybridization signals was quantified with FUJIX BAS2000 Bio Analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Reverse transcription-polymerase chain reaction (RT-PCR)

Rhizoids were collected from gametophores cultured on the G medium for 6 weeks under continuous light. Total RNA was extracted using RNeasy Plant mini kit (QIAGEN, CA). cDNA was synthesized with a mixture of dT_{20} primer and chloroplast gene specific primers (5′-CAGATGGCTCGATTCGAGCAA-3′, 5′-CAGCAGCTAATTCAGGACTCCA-3′, and 5′-TCTAGAGGGAAGTTGTGAGCGT-3′). PCR was performed using *PfuTurbo* Hotstart DNA polymerase. The products were analyzed by electrophoresis and directly sequenced. The primer pairs were as follows: for *PpPOR1*, 5′-CATTCGGGCTCAGGGTGTTG-3′ and 5′-CCCATCAGCATATTAGCCAAGAGGA-3′; for *PpPOR2*, 5′-GCCAGCGTACAATCATCAGCA-3′ and 5′-TGAGGACAGATCACAGTGCATCA-3′; for *PprbcS1*, 5′-TTGGCTGCATTGCCCTTGCGAT-3′ and 5′-ATCAAAGCTACTGCTACCCGACC-3′; for *chlB*, 5′-AGTAATTCCTGAAGGAGGCTCTGT-3′ and 5′-CGAGTTATTGAAGCTGCGTGAGT-3′; for *rbcL* (AB066207), 5′-TACCCATTAGATTTATTTGAAGAAGGTTC-3′ and 5′-CGTTCCCCTTCAAGTTTACCTACTACAGT-3′; and for *psbA*, 5′-CTTGCTACATGGGTCGTGAGTG-3′ and 5′-TGCTGATACCTAATGCAGTGAACC-3′.

Results

Two developmentally different types of rhizoid

Two distinct developmental stages are observed in the haploid generation of *P. patens*: the protonema, a filamentous network of the chloronemata and caulonemata; and the gametophore, a leafy shoot (Reski, 1998). The gametophore is derived from a side-branch initial cell, which arises from a sub-apical cell of the caulonema. The gametophore leaves are divided into juvenile and adult leaves according to the absence and presence of a midrib, respectively (Fig. 1A,B). Adult leaves appear after several juvenile leaves have formed. A gametophore with only juvenile leaves is designated as a juvenile gametophore, and a gametophore with mature adult leaves is termed an adult gametophore.

Rhizoids are brown-pigmented filaments that develop from epidermal cells of a gametophore stem. The first rhizoid forms at the base of a gametophore, below the first juvenile leaf (Fig. 1C). Subsequent rhizoids developed from the epidermal cells of the gametophore stem, below the subsequent juvenile leaves (Fig. 1D). We describe a rhizoid with a juvenile leaf as a ‘basal rhizoid’ and a rhizoid formed below the adult leaf as a ‘mid-stem rhizoid’. The two types of rhizoids were distinguishable by position but not morphologically. While the basal rhizoids were formed concomitant with the growth of juvenile leaves, the mid-stem rhizoids developed with a stereotypic spatial pattern once the growth of the corresponding adult leaf ceased. When a gametophore grew with approximately 8 adult leaves that were longer than 300 μm, the first mid-stem rhizoid started to develop on the gametophore stem, below the first adult leaf and above the uppermost juvenile leaf (Fig. 1E,F). SEM

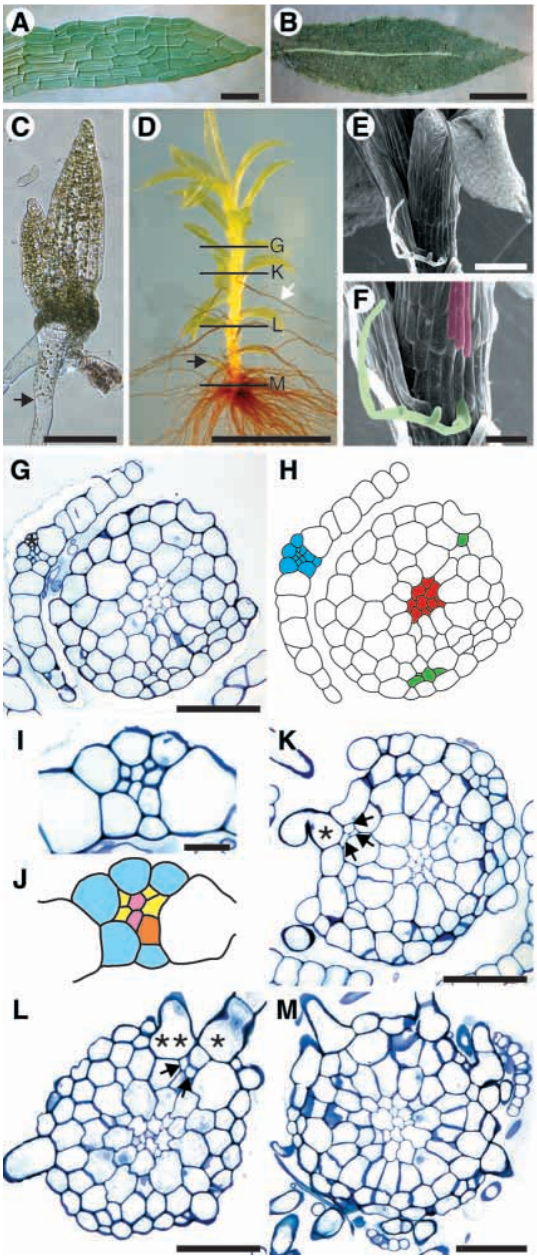


Fig. 1. Rhizoid development in the wild type. (A) A juvenile and (B) an adult leaf. (C) A juvenile gametophore with the first rhizoid (arrow). (D) An adult gametophore with 17 leaves. Lines G, K, L and M indicate transverse sections in G, K, L and M. The white and black arrows indicate the uppermost mid-stem rhizoid and the uppermost juvenile leaf, respectively. (E) A mid-stem rhizoid formed below an adult leaf. (F) The midrib cells (pink) and a mid-stem rhizoid (green). (G-M) Transverse sections of a gametophore with 18 leaves. The asterisk in G indicates the abaxial epidermal cell of a midrib in the same longitudinal cell file as the uppermost mid-stem rhizoid. (H) A line drawing of G showing hydroids in the center of the stem (red), leaf traces (green) and a midrib (blue). (I) A higher magnification of the midrib. (J) A line drawing of I showing epidermal cells (light blue), stereids (yellow), hydroids (pink) and deuters (orange). The asterisk in K indicates the uppermost mid-stem rhizoid. The single and double asterisks in L indicate the first and the second mid-stem rhizoid, respectively. The arrows in K and L indicate leaf trace cells. Scale bars: 100 μm for A,F,G,K-M; 250 μm for B,E; 50 μm for C; 1 mm for D; 20 μm for I.

showed that mid-stem rhizoids developed from stem epidermal cells of the same longitudinal cell files as abaxial epidermal cells of a midrib (Fig. 1F). The positions of the rhizoid cells in relation to leaf traces were examined by serial sectioning (Fig. 1G-M). The midrib contained abaxial and adaxial epidermal cells, hydroids, stereids and conducting parenchymal elements (deuters) (Wiencke and Schulz, 1983) (Fig. 1G-J). Adult leaves merged with stems, and parts of the hydroids and stereids in the midrib were connected to leaf traces in the stem. The leaf traces (Fig. 1I,J) were not connected to hydroid cells in the center of the stem. The mid-stem rhizoid developed from a stem epidermal cell adjacent to a leaf trace (Fig. 1K). Serial sectioning also confirmed that the rhizoid-forming cell was located in the same cell file as the abaxial epidermal cell of a midrib (Fig. 1G,K). Following mid-stem rhizoid development, the next rhizoid developed from an adjacent epidermal cell (Fig. 1L).

Effect of exogenous auxin on rhizoid development

Ashton et al. (Ashton et al., 1979) described increase in the number of rhizoids by exogenous auxin. In this study, we observed spatial patterns of increased rhizoids. When adult gametophores with 12-16 leaves were cultured in 0-, 0.1-, 1.0-, or 10 μM NAA for 1 week, the numbers of mid-stem rhizoids per gametophore increased relative to NAA concentration (Table 1; Fig. 2A). With exogenous auxin, the uppermost rhizoids formed in the more apical part of gametophores, and the number of leaves above the uppermost mid-stem rhizoid decreased (Table 1). The additional rhizoids developed from stem epidermal cells in the cell files of the midrib cells, as was the case with non-treated gametophores (Fig. 2B). Adventitious gametophores were formed from gametophore stems, and several rhizoids were observed on the adventitious gametophores (Fig. 2A).

Long-term effects of NAA were also examined. Gametophores with leaves formed on the 0.1 μM NAA medium (Fig. 2C) as on NAA-free medium. However, leafless gametophores with numerous rhizoids formed on medium with either 1 or 10 μM NAA (Fig. 2D,E). The leafless gametophores grown on 10 μM NAA were taller than those grown on 1 μM NAA medium (Fig. 2D,E). The total number of rhizoids per gametophore was increased by exogenous auxin in a dose-dependent manner (Table 2). The numbers of both mid-stem and basal rhizoids increased in gametophores grown on 0.1 μM NAA compared to gametophores on NAA-free medium (Table 2), although the rhizoids of leafless gametophores could not be classified into mid-stem and basal rhizoids. The additional, NAA-induced mid-stem rhizoids developed from epidermal cells that were located close to a leaf trace (Fig. 2F,G), as was the case without exogenous auxin (Fig. 1K). The leafless gametophores lacked leaf traces and central strands, and most epidermal cells developed into rhizoids (Fig. 2H,I).

The first step in rhizoid development was the protrusion of an epidermal cell from the gametophore stem. The protrusion elongated, and divided into a rhizoid apical cell and a rhizoid subapical cell, which were separated by an oblique septum (Fig. 3A). Rhizoids continued to elongate with the division of apical cells, so that a filamentous rhizoid with several cells was formed. Brown pigmentation was first seen in the third cell from the apical cell, which became more extensive (Fig. 3B-D).

Pphb7 is expressed in rhizoid cells

We isolated partial cDNA clones of ten HD-Zip genes of *P. patens* (Sakakibara et al., 2001), and found in a preliminary analysis that *Pphb7* of the HD-Zip I subfamily was expressed in rhizoids. The *Pphb7* cDNA and corresponding genomic DNA were isolated and sequenced (Fig. 4A: GenBank Accession Number AB084623). Northern analysis of *Pphb7* using a *Pphb7*-specific probe showed the expression of *Pphb7* in gametophores with rhizoids (Fig. 4E).

In order to characterize the detailed expression pattern of *Pphb7*, the coding sequence of β-glucuronidase (GUS) (Jefferson et al., 1987) was inserted in-frame just before the stop codon of *Pphb7* by homologous recombination (Fig. 4B), enabling histochemical detection of GUS activity of the Pphb7-GUS fusion protein. Stable transformants were obtained, two of which had the fusion targeted to the *Pphb7* locus, as confirmed by Southern analysis (Fig. 4F). The blot showed that one line (Pphb7-GUS-1) contained a single copy of the fusion inserted in the *Pphb7* locus (Fig. 4B,F), which was confirmed by sequencing the region. The second transformant contained multiple copies of the fusion construct (Fig. 4F). Since the GUS activity patterns of the two Pphb7-GUS lines were not distinguishable, the results for Pphb7-GUS-1 are shown (Fig. 5).

No signal was detected in juvenile gametophores before the development of the first rhizoid (Fig. 5A). GUS activity was

Table 1. The number of rhizoids per gametophore in wild type and the *Pphb7* disruptants following treatment with NAA for one week

		Medium			
		0 μM NAA	0.1 μM NAA	1 μM NAA	10 μM NAA
Wild type	Mid-stem rhizoids	14.0±8.2	26.4±9.2*	41.0±15.6*	52.0±28.0*
	Basal rhizoids	40.4±6.2	38.7±13.3	40.5±18.3	36.8±21.0
	Leaves†	8.2±2.5	5.3±1.3*	2.0±3.3*	1.9±0.7*
Pphb7dis-2	Mid-stem rhizoids	16.1±9.1	28.4±10.8*	41.2±9.0*	52.4±15.7*
	Basal rhizoids	40.0±10.6	37.4±7.1	39.5±12.7	35.1±12.9
	Leaves†	8.3±2.3	5.7±1.4*	3.3±1.7*	1.5±0.5*
Pphb7dis-3	Mid-stem rhizoids	17.9±9.8	26.6±10.3*	43.1±19.3*	53.3±25.5*
	Basal rhizoids	38.1±5.2	35.1±6.3	37.4±18.3	36.8±12.9
	Leaves†	7.5±2.4	5.8±1.48*	3.0±3.1*	1.6±1.2*

Gametophores were grown on the G medium for 6 weeks, and then cultured for a week in water that contained 0, 0.1, 1.0 or 10 μM NAA. The means and standard deviations for the numbers of rhizoids are indicated. Ten gametophores were examined in each line and condition.

*The values are significantly different by the Student's *t*-test ($P<0.05$) from those obtained following treatment without NAA (0 μM).

†Number of adult leaves above the uppermost mid-stem rhizoid that measured more than 300 μm in length.

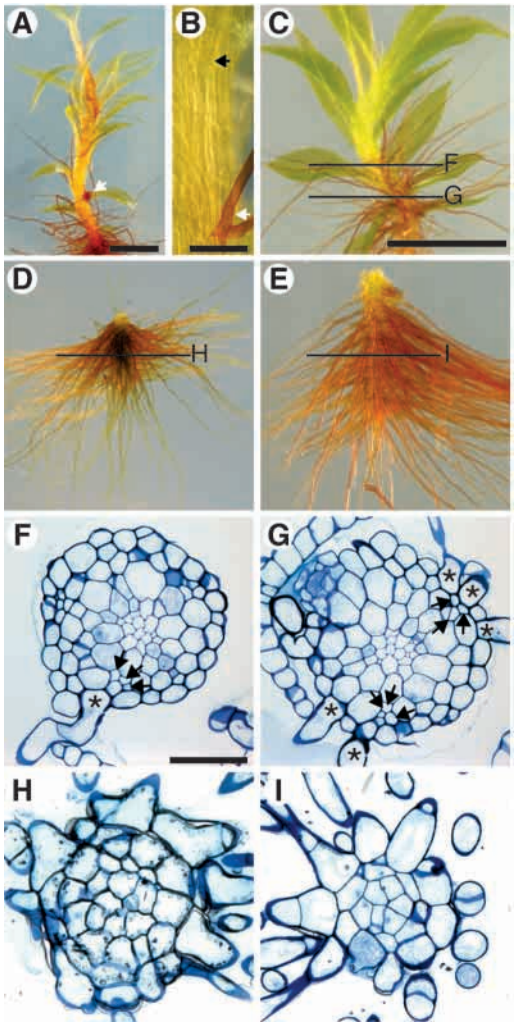


Fig. 2. The effects of exogenous auxin in wild type. (A) A gametophore cultured in 1 μM NAA for a week with adventitious gametophore (white arrow). (B) The uppermost mid-stem rhizoid of the gametophore (white arrow) in A. The black arrow indicates a midrib. (C-E) Gametophores grown with 0.1 (C), 1.0 (D), and 10 (E) μM NAA for 6 weeks. The positions of the transverse sections in F-I is indicated by the lines. The asterisks and arrows in F and G indicate rhizoids and cells of the leaf traces, respectively. Scale bars: in A, 1 mm for A; in C, 1 mm for C-E; in B, 100 μm for B; in F, 100 μm for F-I.

initially detected in the epidermal cells at the base of juvenile gametophores (Fig. 5B). GUS activity was detected in the base of adult gametophores, which corresponds to the rhizoid initial cells (Fig. 5C). In the case of mid-stem rhizoids, one cell below the leaf midrib, considered as a rhizoid initial cell, showed a strong GUS signal (Fig. 5D). Cells with protrusions also showed GUS signals (Fig. 5E). GUS signals were detected in several rhizoid cells close to the rhizoid apical cell (Fig. 5F), while the signals were weaker in brown-pigmented rhizoid cells (Fig. 5C). Although chloronemata and caulonemata are filamentous tissues like the rhizoids, no signal was detected in these tissues (Fig. 5G,H).

The subcellular localization of the protein was not readily detected in the histochemical GUS staining of Pphb7-GUS lines, because subcellular membranes were broken with the detergent. To determine the subcellular localization, the GFP-Pphb7 fragment, in which the coding sequence of sGFP (Chiu et al., 1996) was inserted in-frame just before the start codon of *Pphb7*, was introduced into *P. patens* (Fig. 4C). Southern analysis showed that one line (GFP-Pphb7-1) contained a single copy of the insertion in the *Pphb7* locus, while other tested lines contained multiple copies of the fusion construct (Fig. 4C,G). The intracellular localization of GFP-Pphb7-2 and -3 was not distinguishable from that of GFP-Pphb7-1.

Green fluorescence was detected in the nucleus and cytoplasm of apical cells (Fig. 6A), but the nuclear signal was stronger. The nuclear GFP signal was equally strong in the second cell (Fig. 6B), but weaker in subsequent cells (Fig. 6C,D). GFP fluorescence was detected in both the nucleus and cytoplasm of a rhizoid in which the intact GFP was expressed under the control of the GH3 promoter (Li et al., 1999) (Sakaguchi, Fujita, and Hasebe unpublished) (Fig. 6E-G). The wild-type rhizoid emitted very weak fluorescence (Fig. 6H-J).

Pphb7 mRNA expression is regulated by auxin

Rhizoid development was induced by exogenous auxin (Tables 1,2). We examined whether the expression of *Pphb7* mRNA was induced by auxin using northern analyses. Exogenous auxin increased the level of *Pphb7* mRNA expression in a dose-dependent manner within 1 hour (Fig. 7A). To examine the effect of exogenous auxin on the spatial expression patterns of Pphb7 protein, gametophores of the Pphb7-GUS-1 line were

Table 2. The number of rhizoids per gametophore in wild type and the *Pphb7* disruptants following growth for 6 weeks in media that contained various concentrations of NAA

		Medium			
		0 μM NAA	0.1 μM NAA	1 μM NAA	10 μM NAA
Wild type	Mid-stem rhizoids	5.0±2.0	16.2±3.4*	n.d.	n.d.
	Basal rhizoids	37.1±8.5	62.5±13.0*	n.d.	n.d.
	Total number of rhizoids	42.1±8.6	78.7±13.0*	89.3±19.4*	113.3±19.3*
Pphb7dis-2	Mid-stem rhizoids	5.3±2.0	16.9±2.3*	n.d.	n.d.
	Basal rhizoids	36.3±7.2	62.6±13.0*	n.d.	n.d.
	Total number of rhizoids	41.6±8.1	79.5±13.2*	88.1±24.0*	110.9±21.3*
Pphb7dis-3	Mid-stem rhizoids	5.6±1.9	16.5±3.1*	n.d.	n.d.
	Basal rhizoids	34.7±11.9	66.8±10.9*	n.d.	n.d.
	Total number of rhizoids	40.3±12.6	83.3±12.1*	89.1±24.8*	108.1±20.7*

Protonemata were cultured for 6 weeks on G medium that was supplemented with 0, 0.1, 1.0 or 10 μM NAA. The means and standard deviations for rhizoid numbers are indicated. n.d., no leafy gametophores were differentiated. Ten gametophores were examined in each line and condition.

*The values are significantly different ($P<0.001$) from those obtained following treatment without (0 μM) NAA.

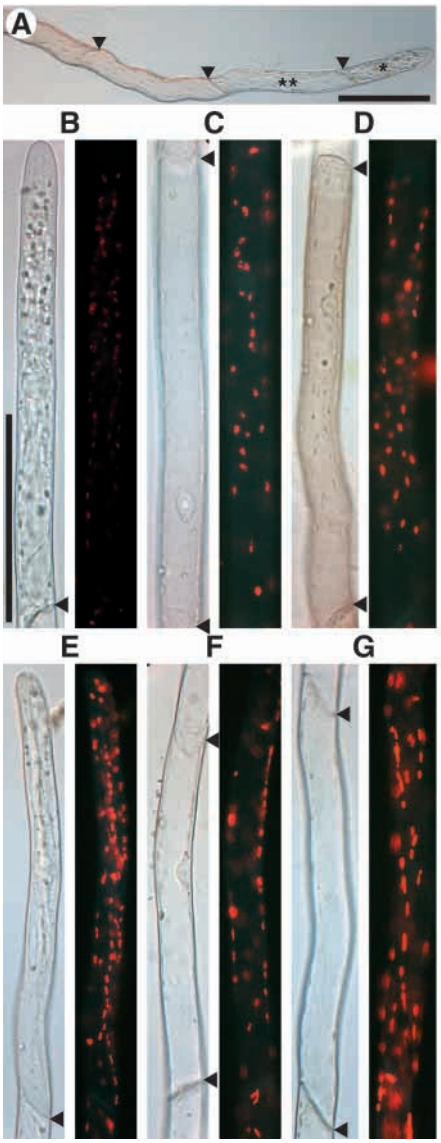


Fig. 3. Rhizoid cells of wild type and *Pphb7* disruptant. (A) A wild-type rhizoid composed of 4 cells, viewed with Nomarski optics. The single asterisk indicates a rhizoid apical cell and the double asterisk indicates a rhizoid subapical cell. (B-G) A rhizoid composed of 8 cells in wild type (B-D) and Pphb7dis-3 (E-G). The apical cell (B,E), third cell (C,F), and fifth cell (D,G) viewed with Nomarski optics (right), and imaged under UV excitation (left). The arrowheads indicate septa between the cells. Scale bar: in A, 100 μm for A; in B, 50 μm for B-G.

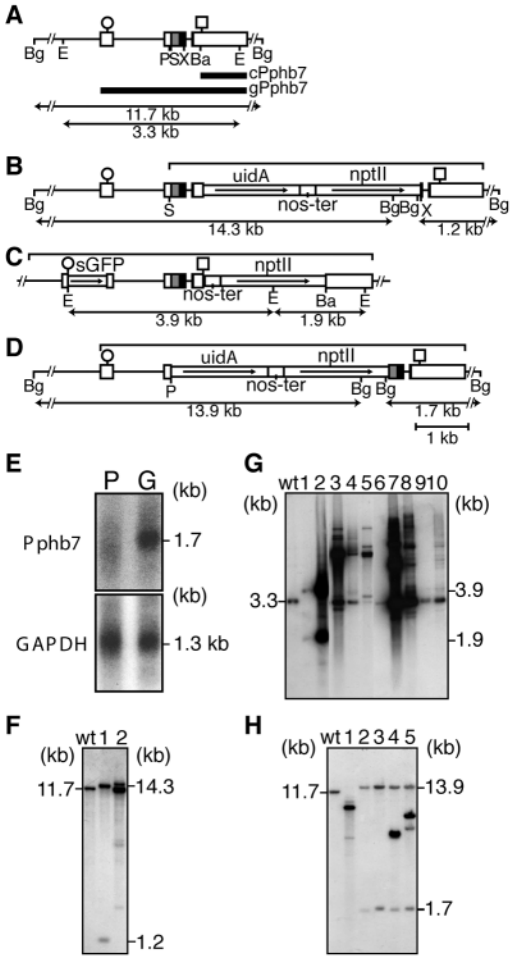


Fig. 4. Genomic structures of *Pphb7* in wild type and transformants and northern analysis of *Pphb7*. Genomic structure of *Pphb7* in wild type (A), Pphb7-GUS (B), GFP-Pphb7 (C) and *Pphb7* disruptant (D). The boxes and the lines between the boxes indicate the exons and introns. The circle and square indicate putative start and stop codons. The gray and black boxes indicate a homeodomain and a leucine-zipper motif. The *uidA*, sGFP, nos-ter, and nptII designate the *uidA*-coding region, GFP-coding region, nopaline synthase polyadenylation signal, and NPTII expression cassette, respectively. Each bracket above the genome structure indicates the region contained in the plasmid used for gene targeting. P, *Pma*CI;, S, *Sal*I; X, *Xba*I; Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*T14I. (E) Northern analysis of *Pphb7*. Poly(A)$^+$ RNA (1.0 μg) from protonemata (P) and gametophores with rhizoids (G) of wild type was hybridized with cPphb7 probe in (A). As an internal control, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a probe. (F-H) Southern analyses to confirm gene targeting. Genomic DNA of wild type (wt) and transformants was digested with *Bgl*II (F,H) or *Eco*T14I (G), and the gPphb7 probe was used. (F) Pphb7-GUS-1 and -2 lines (lanes 1,2); (G) GFP-Pphb7-1 to -10 lines (lanes 1-10); (H) Pphb7dis-1 to -5 lines (lanes 1-5).

soaked in NAA solution. The GUS signals on gametophore stems were stronger closer to the apex (Fig. 5I), where exogenous auxin induced ectopic rhizoids (Fig. 2A). The GUS signals in these rhizoids were stronger than in those without NAA treatment (Fig. 5C,I).

Exogenous phytohormones induce several HD-Zip I genes (Lee et al., 1998; Söderman et al., 1996; Söderman et al., 1999). We examined the effect of the phytohormones BA, GA, ABA and the ethylene precursor ACC (Fig. 7B). Treatment with water for 24 hours resulted in an increased level of *Pphb7* expression. The *Pphb7* expression levels increased more with BA treatment than with water. The increase of *Pphb7* expression by GA was less than that with water, indicating that GA represses *Pphb7* expression. ABA treatment decreased *Pphb7* expression. When treated with ACC, no signals of *Pphb7* or *GAPDH* were detected (data not shown), possibly because of immediate senescence after incubation in ACC. To examine the spatial change in *Pphb7* expression with water and

the phytohormones, gametophores of the Pphb7-GUS-1 line were soaked in water, or 1- or 10-μM solutions of BA, GA and ABA. The spatial expression patterns after water, GA and ABA treatments were not distinguishable from the pattern with no treatment, although the GUS signals became weaker with GA and ABA than with water (data not shown). Gametophores soaked in 10-μM BA formed adventitious buds within 24 hours, and the basal parts, where rhizoids develop, showed GUS activity. GUS activity was unchanged except for the additional signals in the buds.

As dehydration and osmotic stress induce expression of several HD-Zip I genes (Lee et al., 1998; Söderman et al., 1996; Söderman et al., 1999), gametophores with rhizoids were left without water or were soaked in 6% mannitol (Fig. 7C). Dehydration induced *Pphb7* expression in 1 and 6 hours, while 6% mannitol treatment did not induce *Pphb7* expression.

Disruption of *Pphb7* does not affect the number and position of rhizoids with or without exogenous auxin

A DNA fragment that contained the NPTII cassette upstream from the homeobox of *Pphb7* was introduced into *P. patens* to obtain *Pphb7* disruptants (Fig. 4D). Five of 20 randomly selected stable lines in 107 independent stable transformants were found by PCR analysis to have a disrupted *Pphb7* locus (data not shown). Southern analysis showed that two lines (Pphb7dis-2 and -3) contained a single insertion in the *Pphb7* locus, while the other lines contained multiple copies (Fig. 4H).

To examine whether *Pphb7* is involved in the determination of rhizoids, the number and position of rhizoids in the wild type and in *Pphb7* disruptants were compared (Fig. 8A). The number of mid-stem and basal rhizoids per gametophore with 11-14 leaves did not differ (Table 2). The number of leaves above the uppermost rhizoid was similar in the wild type and disruptants (Table 1), indicating that the position of the uppermost mid-stem rhizoid of *Pphb7* disruptants was indistinguishable from wild type. The relationship between the leaf trace and mid-stem rhizoid cell of the *Pphb7* disruptant was identical to that of the wild type (Fig. 8B-D).

To compare the effects of exogenous auxin on the wild type and on the *Pphb7* disruptants, both Pphb7dis-2 and -3 lines were cultured with various concentrations of NAA. The number of rhizoids in the *Pphb7* disruptant increased to the same extent as in the wild type during both short (Table 1) and long (Table 2) culture periods.

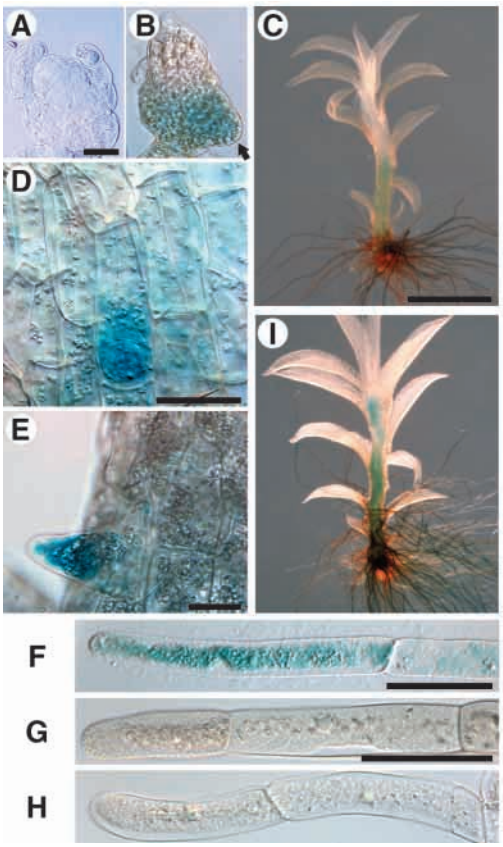


Fig. 5. Histochemical detection of GUS activity in Pphb7-GUS-1 line. A juvenile gametophore without (A) and with (B) a basal rhizoid protrusion (arrow). (C) A gametophore cultured for 6 weeks on G medium. (D) Epidermal cells of an adult gametophore stem. (E) A mid-stem rhizoid initial cell with protrusion. (F) The apical and subapical cells of a rhizoid with 8 cells. (G,H) Apical parts of a chloronema (G) and a caulonema (H). (I) Gametophores cultured in 10 μM NAA solution for 6 hours. Scale bars: in A, 50 μm for A,B; in C, 1 mm for C,I; in D, 50 μm for D; in E, 25 μm for E; in F, 100 μm for F; in G, 50 μm for G,H.

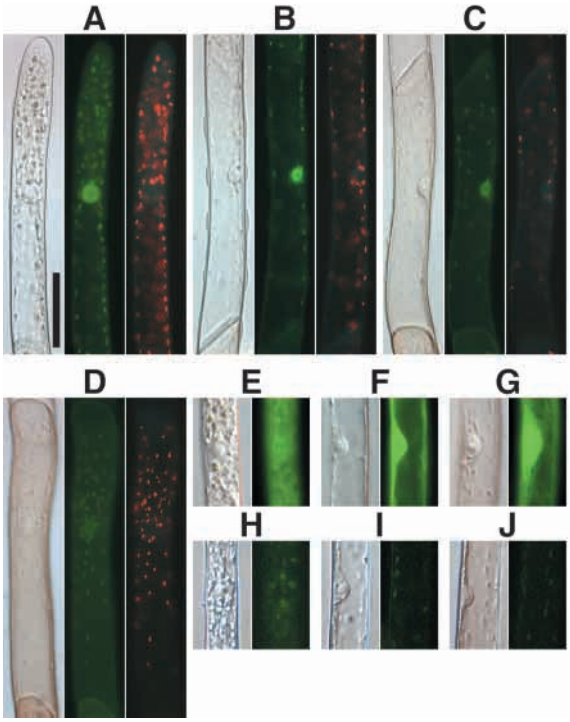


Fig. 6. Subcellular localization of the GFP-Pphb7 fusion protein. (A-D) A rhizoid from GFP-Pphb7-1. The apical (A), second (B), third (C) and fourth (D) cells were examined using Nomarski optics (left), by GFP fluorescence (middle), and by chlorophyll fluorescence (right). (E-H) A rhizoid that expressed an intact GFP under the control of the GH3 promoter (E-G) and a wild-type rhizoid (H-J). The apical (E,H), third (F,I), and fourth (G,J) cells were examined using Nomarski optics (left) and GFP fluorescence (right). Scale bar: 50 μm for all panels.

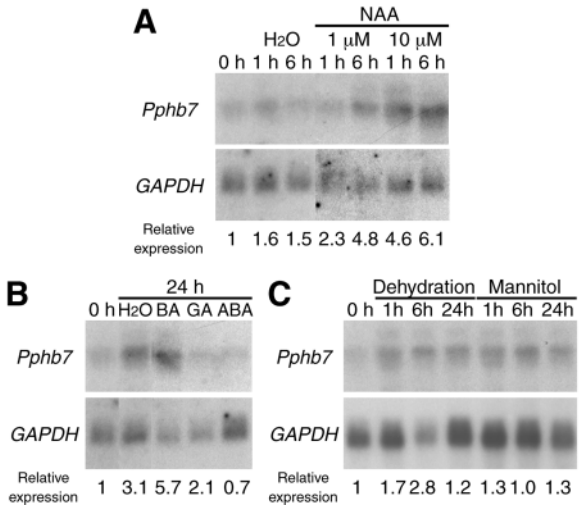


Fig. 7. Effects of phytohormones, dehydration, and osmotic stress on *Pphb7* expression. *Pphb7* expression in gametophores treated with (A) exogenous NAA, (B) other phytohormones (BA, GA and ABA) and (C) dehydration and osmotic stress (6% mannitol). *GAPDH* was used as an internal control. Relative expression levels are indicated below each panel.

Pphb7 disruption alters rhizoid differentiation

The size and shape of rhizoids were similar in wild type and disruptants (data not shown). The wild-type rhizoids contained brown pigment, while those of both *Pphb7* disruptants were greener (Fig. 3, Fig. 8A). Light transmittance in the red, green and blue channels was estimated for rhizoid cells using CCD images. The mean light transmittance of Pphb7dis-2 (data not shown) and Pphb7dis-3 (Fig. 9A) at the fifth cell was significantly higher than that of wild type ($P<0.001$ by Student's *t*-test), indicating that rhizoids of *Pphb7* disruptants were less pigmented than those of the wild type.

Since the rhizoids of *Pphb7* disruptants were greener in appearance than those of wild type (Fig. 8A), the number and size of chloroplasts in the rhizoid cells were compared. The numbers of chloroplasts per cell in the second to fourth cells from the rhizoid apical cell were determined. The number of chloroplasts in the apical cell could not be determined reliably. The number of chloroplasts in wild type was constantly around 30 through the second to fourth cells, while the numbers in *Pphb7* disruptants increased, and the fourth cell of *Pphb7* disruptants contained twice as many chloroplasts as in the wild type (Fig. 9B).

The project areas of the chloroplasts were measured to quantify chloroplast size. The mean chloroplast size was significantly larger in *Pphb7* disruptant rhizoids than in those of wild type ($P<0.01$; Fig. 9C). To compare the structure of chloroplasts between the wild-type and *Pphb7* disruptants, nucleoids were observed by staining with SYBR Green I (Fig. 8H,I). The number and size of nucleoids of wild type and of *Pphb7* disruptants were similar. The internal structure of chloroplasts was further observed with electron microscopy. Well-developed thylakoid membranes with grana were observed in both wild type and *Pphb7* disruptants (Fig. 8J,K).

To see the effects of auxin in rhizoid differentiation, the light transmittance and the number and size of chloroplasts in wild-type and disruptant rhizoids cultured in 10-μM NAA were

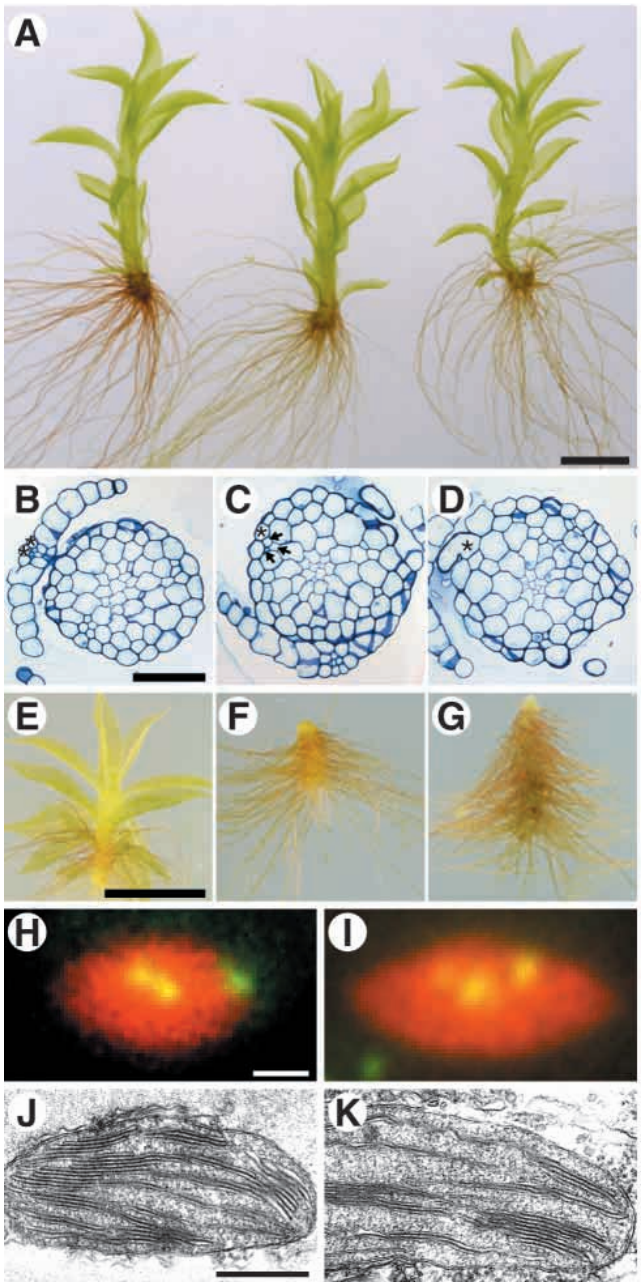
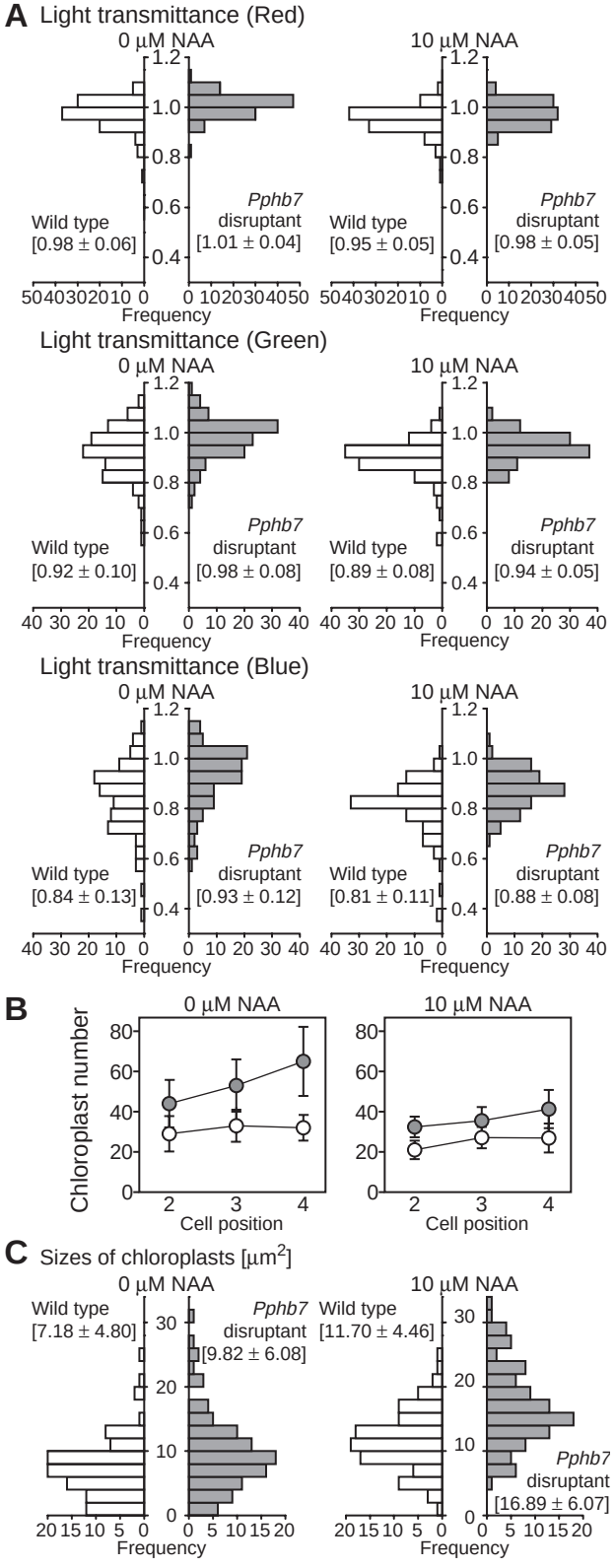


Fig. 8. Rhizoids of the wild-type and *Pphb7* disruptant lines. (A) Gametophores with 12 leaves in the wild type (left), Pphb7dis-2 (middle), and Pphb7dis-3 (right). (B-D) A Pphb7dis-3 gametophore with 18 leaves was serially sectioned at the position of the leaf above the uppermost mid-stem rhizoid (B), where the leaf merges to a stem (C), and where the uppermost mid-stem rhizoid (asterisk) emerges (D). The abaxial leaf epidermal cells (asterisk in B) and the stem epidermal cells (asterisk in C) in the same longitudinal cell file as the uppermost mid-stem rhizoid. Arrows in C indicate leaf traces. Gametophores of the Pphb7dis-3 grown for 6 weeks in the presence of 0.1 (E), 1.0 (F) and 10 (G) μM NAA. (H,I) Merged images of chlorophyll autofluorescence by UV excitation and SYBR Green I staining of chloroplasts in the fifth cell from the rhizoid apical cell of wild type (H) and Pphb7dis-3 (I). The yellow spots are chloroplast nucleoids. (J,K) Transmission electron micrographs of rhizoid chloroplasts from wild type (J) and Pphb7dis-3 (K). Scale bars: in A, 1 mm for A; in E, 1 mm for E-G; in B, 100 μm for B-D; in H, 2.5 μm for H,I; in J, 0.5 μm for J,K.

compared (Fig. 9). The light transmittance and the number of chloroplasts were decreased by the NAA treatment in both wild type and *Pphb7* disruptants (Fig. 9A,B). The size of chloroplasts increased in both wild type and disruptants (Fig. 9C). These results indicate that auxin is involved in both determination and differentiation of rhizoids.



To further characterize the effects of *Pphb7* and auxin on chloroplasts, mRNA expression of the following genes involved in the photosynthesis was compared between wild-type and *Pphb7*-disruptant rhizoids by RT-PCR: a ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*) and a small subunit gene (*PprbcS1*) as Calvin cycle enzymes (reviewed by Hartman and Harpel, 1994), NADPH-protochlorophyllide oxidoreductase genes (*PpPOR1* and *2*) and light-independent protochlorophyllide reductase gene (*chlB*) as the key enzymes in chlorophyll biosynthesis (reviewed by Armstrong, 1998) and the *psbA* gene encoding D1 protein as a component of photosystem II (reviewed by Zhang and Aro, 2002). Levels of *rbcL* and *PprbcS1* expression were higher in disruptants than in the wild type, while levels of *PpPOR1*, *PpPOR2*, *chlB* and *psbA* did not differ from those of the wild type (Fig. 10). The expression levels of each gene in auxin-treated rhizoids were not different from those without auxin (Fig. 10).

Discussion

Developmental and hormonal control of rhizoid determination

This study has enabled us to propose a model of rhizoid development in *P. patens* gametophores (Fig. 11A). Our model suggests that rhizoid development can be separated into two processes, which we call determination and differentiation. The determination of presumptive cells and the subsequent signal transduction cascades of cell differentiation have been recognized as important processes in epidermal cell differentiation. Hülskamp et al. (Hülskamp et al., 1994) suggested that trichome differentiation could be separated into these two processes, because several mutants that regulate only one of the two processes were identified. These processes have not been clearly identified in other model systems of epidermal cell differentiation, such as root hairs and stomatal guard cells.

Rhizoids develop from epidermal cells of a gametophore stem below a leaf. Basal and mid-stem rhizoids are distinguished according to their developmental patterns. The former rhizoid develops below a juvenile leaf, while the latter develops below an adult leaf. A mid-stem rhizoid always originates from an epidermal cell that lies close to a leaf trace (Fig. 1). Basal rhizoids develop at epidermal cells below juvenile leaves, which do not have a midrib (Fig. 1), and no stereotypic spatial pattern of rhizoid development was observed. Our model distinguishes between the two types of rhizoid development mentioned above, which probably have different determination processes (Fig. 11A).

Fig. 9. Comparison of the rhizoid characters of wild type and *Pphb7* disruptant with or without NAA. (A) Frequency distributions of the light transmittance of rhizoids in the wild-type (white bars) and Pphb7dis-3 (gray bars) gametophores. The red, blue, and green channels from RGB images are shown. The means and standard deviations are indicated in brackets (*n*=100). (B) The number of chloroplasts per cell at each cell position from the rhizoid apical cell in the wild type (white circles) and Pphb7dis-3 (gray circles) (*n*=10). Bars indicate the standard deviations. (C) Frequency distribution of chloroplast size in rhizoid cells from the wild type (white bars) and Pphb7dis-3 (gray bars). The means and standard deviations are indicated in brackets (*n*=100).

The number of mid-stem rhizoids increased with exogenous NAA (Tables 1, 2), indicating that auxin promotes mid-stem rhizoid development from stem epidermal cells below adult leaves. The number of basal rhizoids increased when juvenile gametophores were cultured with 0.1-μM NAA (Table 2), but did not increase when adult gametophores were cultured in NAA (Table 1). This indicates that auxin promotes basal rhizoid development from epidermal cells below juvenile leaves, before the cells are fully matured. While auxin plays inductive roles in both mid-stem and basal rhizoids, the patterns of additional rhizoid development differ. The increased mid-stem rhizoids always developed from stem epidermal cells that lay adjacent to a leaf trace (Fig. 1K), whereas additional basal rhizoids originated from epidermal cells in a random manner (Fig. 1M). These results suggest that auxin is sufficient for the induction of basal rhizoids, but that an unknown factor related to leaf traces is required for mid-stem rhizoid induction (Fig. 11A).

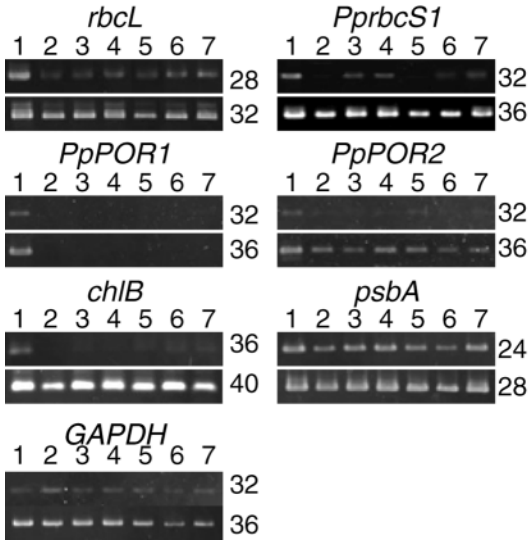


Fig. 10. Expression of the genes involved in photosynthesis. RT-PCR analyses of transcripts of photosynthesis genes in the wild type and *Pphb7* disruptants. The number of PCR cycles is indicated to the right of each panel. Gametophores of the wild type (lane 1), rhizoids of the wild type treated without (lane 2) or with (lane 5) NAA, and rhizoids of Pphb7dis-2 and -3 treated without (lanes 3 and 4) or with (lanes 6 and 7) NAA.

The midrib differentiates in the adult leaf, and probably functions as a conducting tissue (Ligrone et al., 2000). The aforementioned unknown factor may be produced in the leaf and pass through the midrib and the leaf trace as a signal, which, in conjunction with auxin, directs the epidermal cells around the leaf trace to form rhizoids. In the absence of NAA, the uppermost mid-stem rhizoid is usually associated with the eighth leaf from the gametophore apex, which implies that it takes a set amount of time for the unknown factor to accumulate in sufficient amounts to induce rhizoid development. Although ectopic induction of root hairs by ethylene or its precursor ACC has been reported (Tanimoto et al., 1995), ACC did not increase the number of rhizoids (data not shown), suggesting that different signals are involved in rhizoid induction.

Gametophores grown without NAA formed several mid-stem rhizoids around the first mid-stem rhizoid during later stages of development (Fig. 1L). Rose and Bopp (Rose and Bopp, 1983) reported basipetal auxin transport in rhizoids. The auxin transport from the rhizoid to the stem, together with the unknown factor from the leaf trace, may enhance the induction of mid-stem rhizoids. During *Arabidopsis* trichome differentiation, previously differentiated trichomes inhibit surrounding cells to differentiate into trichomes (Marks, 1997). However, mid-stem rhizoids promote subsequent rhizoid development.

Two explanations are possible for the differences in patterning between mid-stem and basal rhizoids. First, the different patterns may reflect differences in the distribution of the unknown factor (Fig. 11B). If the unknown factor is abundant in the basal part of a gametophore and is restricted around the leaf trace in the middle, every epidermal cell in the basal part can form a rhizoid in response to auxin, whereas only the epidermal cells around the leaf trace can form rhizoids. The structural differences between juvenile and adult leaves probably account for differences in the distribution of the unknown factor. In juvenile leaves, the unknown factor emanates from leaves, passes through the entire juvenile leaf, and broadly diffuses in epidermal cells, below the juvenile leaves without a midrib. Second, the differences in patterning may reflect

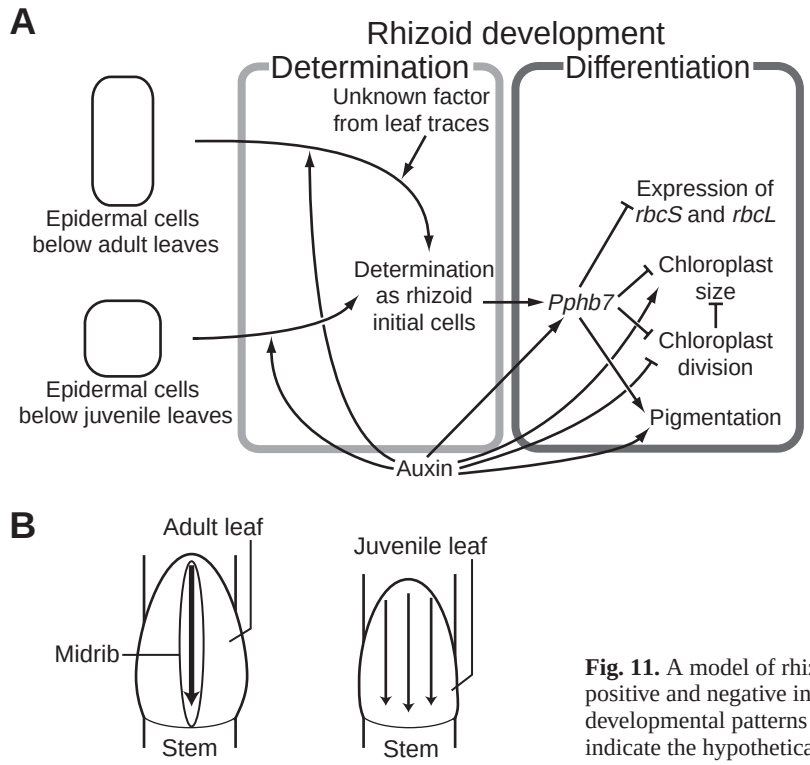


Fig. 11. A model of rhizoid development. (A) Arrows and barred lines indicate the positive and negative interactions, respectively. (B) An explanation for the different developmental patterns observed between mid-stem and basal rhizoids. The arrows indicate the hypothetical flow of an unknown factor.

different mechanisms for the determination of basal and mid-stem rhizoids. When basal rhizoids develop, the epidermal cells are formed while the gametophore is juvenile, but when mid-stem rhizoids develop, the epidermal cells are formed after the gametophore reaches adulthood. Different mechanisms could be involved during different developmental stages. Therefore, the unknown factor that is required for mid-stem rhizoid development may not be essential for basal rhizoid development.

Auxin and *Pphb7* function in rhizoid cell differentiation

The specific expression of *Pphb7* was observed in the rhizoid initial and rhizoid cells (Fig. 5), indicating that *Pphb7* is involved in rhizoid development. Since the *Pphb7* disruptants formed rhizoids in positions indistinguishable from those in wild-type plants (Fig. 8), it appears that *Pphb7* is not involved in the positional determination of rhizoids, but is involved in their differentiation (Fig. 11A).

Once an epidermal cell had acquired a rhizoid initial cell fate, *Pphb7* regulates various features of the rhizoids, including pigmentation and the size and number of chloroplasts in the rhizoid cell. Chloroplasts in wild-type rhizoids are smaller than those in protonemata and gametophores (data not shown), consistent with the notion that rhizoids are not predominant photosynthetic organs (Duckett et al., 1998). However, the structure of rhizoid plastids resembles that of chloroplasts in photosynthetic organs (Kasten et al., 1997). Rhizoid plastids possess chlorophylls, as observed by UV excitation (Fig. 3), well-developed thylakoid membranes with grana stacks at maturity (Fig. 8J,K) and randomly distributed plastid nucleoids (Fig. 8H,I). Rhizoid chloroplasts of *Pphb7* disruptants had all the three characteristics observed in wild-type rhizoid chloroplasts, although the size and number of chloroplasts in disruptants were increased (Fig. 3E-F, Fig. 9B,C). This indicates that *Pphb7* does not affect plastid differentiation, but regulates chloroplast size and number in rhizoid subapical cells. This is concordant with the results in *Arabidopsis*, in which different genes regulate these two processes. The double mutant of *AtGlk1* and *AtGlk2* exhibits reduction in granal thylakoids, but not in the number of plastids (Fitter et al., 2002). The *accumulation and replication of chloroplasts* (*arc*) mutants are affected in chloroplast division, but the chloroplasts develop normally in these loss-of-function mutants (Pyke, 1999).

The size and number of chloroplasts are negatively correlated in many *Arabidopsis* mutants, suggesting that the genes are involved in chloroplast division (Marrison et al., 1999; Pyke, 1997; Pyke, 1999). The phenotype of *Pphb7* disruptants clearly differs from these mutants. Both the size and number of plastids were increased in the rhizoids of disruptants; therefore, *Pphb7* is considered as a new class of regulator that down-regulates total chloroplast mass per cell. When the gametophytes were treated with NAA, the expression of *Pphb7* mRNA increased within 1 hour and further increased up to 6 hours (Fig. 7A). The kinetics of induction was comparable to auxin-responsive genes, such as *PpIAA1* (Imaizumi et al., 2002), suggesting that *Pphb7* is regulated by auxin fairly directly. Histochemical staining of GUS activity in the Pphb7-GUS line confirmed that the expression was up-regulated in rhizoid cells (Fig. 5). As auxin induces *Pphb7*, which is involved in rhizoid differentiation, auxin is likely a positive regulator of both rhizoid differentiation and determination (Fig. 11A). Furthermore, auxin is likely involved in rhizoid differentiation in another way, which can be the major auxin response pathway promoting rhizoid differentiation, because exogenous auxin enhanced all the three rhizoid characters, pigmentation, chloroplast number and chloroplast size even in the *Pphb7* disruptant (Fig. 9). These responses were slightly stronger in *Pphb7* disruptant, which may indicate that *Pphb7* could function as negative regulator in such a second, independent pathway.

BA also induced *Pphb7* expression as determined by the northern analysis (Fig. 7B). Microscopic observation showed that new adventitious buds differentiated after BA treatment, and new rhizoids formed at the bases of the newly formed adventitious buds. The GUS signals of the Pphb7-GUS lines were visible at the bases of the adventitious buds, but those in rhizoid cells were not different from the controls. Therefore, *Pphb7* induction by BA is likely caused by increase of rhizoid initials accompanying adventitious bud formation. While exogenous cytokinin induces chloroplast division in protonemata (Abel et al., 1989), the numbers of plastids in wild-type and *Pphb7*-disrupted rhizoids treated with 10-μM BA for 48 hours were similar to that in non-treated rhizoids (data not shown). This implies that the regulation of plastid division in rhizoids differs from that in protonemata.

Other phytohormones (ABA and GA), dehydration and osmotic stress did not induce *Pphb7* more than water, which induced *Pphb7* three fold. This is concordant with the observation that rhizoids are induced by low nutrient condition in some mosses (Duckett, 1994). Although submersion is known to induce stress response mediated by ethylene, we could not detect the *Pphb7* induction with the ethylene precursor ACC. ABA and GA likely reduce *Pphb7* expression, and further studies on regulation of *Pphb7* by phytohormones are necessary.

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