Diversification of gene function: homologs of the floral regulator FLO/LFY control the first zygotic cell division in the moss Physcomitrella patens

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Accepted 19 January 2005

Development 132, 1727-1736
Published by The Company of Biologists 2005
doi:10.1242/dev.01709

Summary

After fertilization, the zygote undergoes dynamic changes in chromosomal and cytoplasmic organization, and begins the cell cycles that eventually lead to formation of the multicellular embryo. Specific transcription factors that initiate this cascade of events in land plants have not been identified. We have identified two FLO/LFY genes, PpLFY1 and PpLFY2, that regulate the first cell division after formation of the zygote in the moss Physcomitrella patens. The deduced amino acid sequences of the two PpLFY genes are 94.8% identical to each other and show similar expression patterns. While fertilization occurred in the PpLFY disruptants, the development of double disruptant zygotes was arrested at the single-cell stage. When the double disruptants, as the female parent, were crossed with the wild type, as the male parent, normal sporophytes were formed, supporting the notion that the PpLFY genes function after fertilization to regulate the first mitotic cell division in zygotes. The rare sporophytes that formed on the PpLFY double disruptants showed mostly normal organogenesis, but had abnormalities in the pattern of cell division, supporting a role of PpLFY genes in regulating cell division. The FLO/LFY genes in angiosperms are conserved master regulators of floral identity without any obvious effects on cell division. By contrast, our study suggests that FLO/LFY genes have functions throughout sporophyte development in the basal land plant lineages.

Key words: LEAFY, FLORICAULA, Fertilization, Zygote, Physcomitrella patens

Introduction

Multicellular organisms begin their diploid development from a single-cell stage: the zygote. The proper initiation of mitotic cell cycles in the fertilized egg is a key to the progression of development in both land plants and metazoans. In land plants, however, the regulatory mechanisms of this critical step remain mostly unknown. The first cell division of the zygote in land plants is usually asymmetric, forming a cytoplasm-rich apical cell and a vacuole-rich basal cell, although the plane of cell division and the orientation of the apical-basal axis to the principal axis of the egg-bearing organ vary among plant lineages (Wardlaw, 1955). Among land plants, only one gene, GNM, which functions in vesicle trafficking, has been characterized to be involved in apical-basal polarity and in the normal division of the zygote (Mayer et al., 1993; Geldner et al., 2003). However, transcription factors affecting the first cell division of zygotes have not been identified in land plants.

The moss Physcomitrella patens has been recognized as a useful model for the study of plant embryogenesis and development (Cove et al., 1997; Sakakibara et al., 2003) for several reasons. First, the exceptionally high efficiency of homologous recombination in P. patens makes it the only land plant in which gene targeting is feasible (Schaefer and Zryd, 1997). Second, the eggs, zygotes and young sporophytes of P. patens are located solely in the cavity of its egg-bearing organ, the archegonium, and are thus easily accessible for observation, while those in seed plants are relatively difficult to observe. Third, P. patens has a multicellular autotrophic body in its haploid generation, permitting maintenance of mutants with severe defects in their diploid generation.

While searching for genes involved in sporophyte development in P. patens, we found that the homologs of the transcription factor FLORICAULA/LEAFY (FLO/LFY) are required for the first cell division in the zygote. FLO/LFY genes have been reported only in land plants; a single copy or a few copies exist in each genome (Himi et al., 2001). In Arabidopsis thaliana, LFY plays a central role in a gene network that controls flower development (Ng and Yanofsky, 2000). LFY integrates environmental and endogenous signals that control the timing of the transition from the vegetative to the reproductive phase (Blázquez and Weigel, 2000) and interacts with other transcription factors to activate the transcription of floral homeotic genes that control the identity of floral organs (Busch et al., 1999; Lamb et al., 2002; Lohmann et al., 2001; Parcy et al., 1998; Wagner et al., 1999). FLO/LFY functions are largely conserved in flowering plants, even in distantly related species (Ahearn et al., 2001; Bomblies et al., 2003;
Coen et al., 1990; Hofer et al., 1997; Molinero-Rosales et al., 1999; Schultz and Haughn, 1991; Souer et al., 1998; Weigel et al., 1992). The functions of FLO/LFY genes are also probably conserved in gymnosperms, including *Pinus* (Mouradov et al., 1998) and *Gnetum* (Shindo et al., 2001). Although FLO/LFY homologs have been cloned from several ferns and bryophytes (Frohlich and Parker, 2000; Himi et al., 2001), their functions in these plants have not been characterized.

We report the characterization of two FLO/LFY genes in *P. patens*, and show that the FLO/LFY genes play a key role in the progression of the first cell division of the zygote. In addition, they are important for later sporophyte development.

**Materials and methods**

**Culture conditions**

*Physcomitrella patens* Bruch & Schimp subsp. *patens* (Ashton and Cove, 1977) was cultured as described previously (Nishiyama et al., 2000). To observe the gametangia and sporophytes, protonemata that had been vegetatively propagated on 9 cm Petri dishes with BCDAT medium (Nishiyama et al., 2000) were inoculated onto sterile 42-mm diameter peat pellets (Jiffy-7; Jiffy Products International AS, Kristiansand, Norway) using tweezers. The cylindrical pellets had been prepared by immersion in distilled water to permit them to expand, and then autoclaved in a plastic box. Distilled water was then poured to just below the upper surface of the expanded cylindrical pellets. The mosses inoculated on the peat pellets were cultured at 25°C and were collected after an additional month of culture for 15°C and were collected after an additional month of culture.

**Southern analyses**

Materials and methods for induction of gametangia development. Under these conditions for induction of gametangia development. Under these conditions, first antheridia and then archegonia differentiated at the shoot apices of the gametophores. Because of the wet conditions, fertilization occurred without any additional treatment. Sporophytes were visible under stereomicroscope 4 weeks after the transfer to 15°C and were collected after an additional month of culture for experiments using spores. To estimate the germination rate, the spores were spread on solidified BCDAT medium, and the germinated protonemata were counted after 1 week.

**Reverse transcription-polymerase chain reaction**

Total RNA was extracted from protonemata, gametophores, or sporophytes using RNasey plant mini-Kit (Qiagen, Hilden, Germany). The protonemata were grown on solidified BCDAT medium for 6 days. The gametophores were grown on the solidified BCD medium (Nishiyama et al., 2000) for 80 days. Various developmental stages of sporophytes, excluding archegonium tissue, were collected from mosses grown on peat pellets for a month after the transfer to 15°C. The cdnas were synthesized from 1 mg of total RNA from each tissue using TranscriptII reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT)16 primer according to the manufacturer’s instructions. PCR was performed with TaKaRa Ex Taq Hot Start Version (Takara Bio Inc., Otsu, Japan), using the following gene-specific primers: for *PpLFY1*, pL1-Nsense1 (5′-CGGTGCCGGGA-AGTGTTGAAAATGTGGAAGCA-3′) and L1e2spe (5′-CTTCCCTC-CAAGAATTTCCACATGCGTCG-3′), and for *PpLFY2*, *PpLFY2-5′* (5′-GCTCGGCTGCTGATCCAGCCTCCTGCTC-3′) and L2e2-5-2 (5′-AATGCTGCTGACCTTCCAGAAACCTCC-3′). The PCR products were sequenced directly to confirm the amplification of the expected fragments.

**Construction of targeting plasmids and generation of transformants in *P. patens***

Based on the cdNA sequences of *PpLFY1* and *PpLFY2* (Himi et al., 2001), the 5′ and 3′ genomic regions of these cdNAs were amplified using TAIL-PCR (Liu and Whittier, 1995) and sequenced. These cdNA and genomic DNA sequences were then used to construct targeting plasmids to make *PpLFY1-GUS*, *PpLFY2-GUS*, *PpFLY1-dis*, *PpLFY2-dis* and *PpLFY1-PpLFY2-dis* lines in addition to *PpLFY* ectopic expression lines in *A. thaliana*.

Genomic sequences of *PpLFY1* and *PpLFY2* were used to design and synthesize primer sets for the amplification of the majority of both *PpLFY1* (2871 bp) and *PpLFY2* (2852 bp) genomic DNA: L1-1int1 (5′-CTTGGCTTATGACCTTCCTCATCGGTGC-3′) and L1-3U1 (5′-TTGTTCAAGATCTCTGCGGCGACGTG-3′), and *PpLFY2-5′* (5′-GCTCGGCTGATCCAGCCTCCTGCTC-3′) and *PpLFY2-3′* (5′-TCAAAAAATGTTGAAAAGGCTCATTG-3′). Each amplified DNA fragment was cloned into the *pGEM-T* Easy vector (Promega, Madison, WI, USA), thereby generating *pgPpLFY1* and *pgPpLFY2*.

A partial 1367 bp fragment of *PpLFY1* genomic DNA ranging from the second exon to just before the stop codon was also cloned into the *pGEM-T* vector (Promega). A *Sall*-Snai fragment was excised from this plasmid and inserted into a *SalI*-Clai site of the *pGSU-NPTII-1* plasmid that contained the coding sequence of *uidA* (Jefferson et al., 1987), the nopaline synthase polyadenylation signal (nos-ter), and an NPTII cassette (Nishiyama et al., 2000), thereby creating an in-frame fusion of the *PpLFY1* and *uidA* genes. A 943 bp DNA fragment containing the 3′ region of *PpLFY1* gene, produced by TAIL-PCR using the gene-specific primer *PpLFY-LA1* (5′-CGGATCTGGTTATGCTCCAGAAACCTCC-3′), was inserted into the *Smal* site of this plasmid. The resulting plasmid was linearized with *BamHI* and *SalI* for generation of the *PpLFY1-GUS* lines. Of 78 independent stable transformants, 18 were identified by PCR to have the fusion construct in the *PpLFY1* locus. Southern analyses of 12 of the 18 candidate lines showed that three lines (*PpLFY1-GUS-1, 2 and 3*) contained a single insertion in the *PpLFY1* locus (see Fig. SIA,C in the supplementary material), while the other nine lines tested contained multiple copies (data not shown).

A 1460 bp fragment containing a partial sequence of the 5′ region of the *PpLFY2* genomic DNA was amplified using the *PpLFY2-5′* and *L2a-Hi3* (5′-CAAGGCTCGGCTTCTCTGCGGCCGGCC-3′) primers. A 1398 bp fragment of the 3′ region of the *PpLFY2* genomic DNA was amplified using the *L2s-Ba1* (5′-CGGATCCGGGTTGAAGGCTCATTAG-3′) and *PpLFY2-3′* primers. These fragments were inserted into *pgUS-NPTII-1*, creating an in-frame fusion of the *PpLFY2* and *uidA* genes. The resulting plasmid was linearized with *BamHI* and *SalI* and used to generate the *PpLFY2-GUS* lines. Of 80 independent stable transformants, 44 were identified by PCR to have the fusion construct in the *PpLFY2* locus. Southern analyses of 15 of the 44 candidate lines showed that 11 lines (*PpLFY2-GUS-1 to -11*) contained a single insertion in the *PpLFY2* locus (see Fig. SIB,D in the supplementary material; data not shown), while the other lines tested contained multiple copies (data not shown).

To disrupt the *PpLFY1* gene, the 5′ *SalI*-HindIII fragment and the 3′ *XbaI* fragment of *pgPpLFY1* were inserted into the *SalI*-HindIII and *XbaI* sites of *pHTS14*, respectively. *pHTS14* contains an HPT cassette composed of the CaMV 35S promoter, the hygromycin B phosphotransferase (*hpt*) gene, and the nos-ter sequence between multiple cloning sites. The resulting plasmid was used for transformation after linearization with *NotI* and then used to generate the *PpLFY1-dis* and *PpLFY1-PpLFY2-dis* lines. To generate *PpLFY1-dis* lines, 17 of 38 lines randomly selected from 81 independent stable transformants were identified by PCR as having a disrupted *PpLFY1* locus (data not shown). Southern analyses of 10 of
the 17 candidate lines showed that 8 lines (PpLFY1-dis-1 to 8) contained a single insertion in the PpLFY1 locus (see Fig. S2A,C in the supplementary material), while the other lines contained multiple copies (data not shown).

To disrupt the PpLFY2 gene, the fragment of PpLFY2 between the HindIII sites located in the second intron and the third exon of pgPpLFY2 was replaced with an NPTII cassette. The resulting plasmid was used for transformation after linearization with EcoRI and NotI. To generate the PpLFY2-dis line, 5 of 56 lines randomly selected from 79 independent stable transformants were identified by PCR as having a disrupted PpLFY2 locus (data not shown). Southern analyses of the 5 candidate lines showed that a single line (PpLFY2-dis-1) contained a single insertion in the PpLFY2 locus (see Fig. S2B,D in the supplementary material), while the other lines contained multiple copies (data not shown).

To generate double disruptants, an HPT cassette was inserted into the third exon of PpLFY1 in the PpLFY2-dis-1 line. Of 47 independent stable transformants, 20 were identified by PCR as having a disrupted PpLFY1 locus (data not shown). Southern analyses of 18 of the 20 candidate lines showed that 6 lines (PpLFY1-PpLFY2-dis-1 to -6) contained a single insertion in the PpLFY1 locus, in addition to the disrupted PpLFY2 locus (see Fig. S2D,E in the supplementary material), while the other lines contained multiple copies (data not shown).

Ectopic expression plasmids of PpLFY1 or PpLFY2 in A. thaliana

The PpLFY1 cDNA fragment was amplified by PCR using PpLFY-sense2 (5′-CCCCGGGTTTTGAAATGGGAGCGACGCGCGG-3′) and PpLFY1-antisense1 (5′-ATGTCACCGGGTTTATCCACCGTCGTC-3′) primers. The PpLFY2 cDNA fragment was amplified with PpLFY-sense2 and PpLFY2-3′. Each DNA fragment was cloned into the pBI121Hm-G vector, which was generated by excising the hpt gene and the nptII gene. These vectors were transferred into Agrobacterium tumefaciens strain C58C1RIf and used for transformation of A. thaliana (Columbia).

Transformation of P. patens

Polyethylene glycol-mediated transformation was performed as described previously (Nishiyama et al., 2000). Stable transformants were further screened with PCR using appropriate primer sets to confirm the insertion of the construct in each locus. PCR-positive candidates were further analyzed by Southern analyses.

Confocal laser scanning microscopy

Shoot apices with gametangia and several young leaves were collected 3-4 weeks after the induction of gametangia and fixed in a solution of 4% (v/v) glutaraldehyde and 1 µg/ml DAPI in 12.5 mM cacodylate (pH 6.9) overnight at 4°C (Christensen et al., 1997). The fixed materials were then dehydrated in a graded ethanol series and cleared with a 2:1 mixture of benzyl benzoate and benzyl alcohol for observation on a Leica TCS SP2 confocal system equipped with a UV laser (Leica, Wetzlar, Germany). Fluorescence between 400 and 700 nm was detected under excitation with 351 nm and 364 nm excitation beams.

Sectioning and histochemical assay for GUS activity

For observation of the egg cells, the plant tissues were fixed in 5% glutaraldehyde in phosphate buffer (pH 6.9) for 5 hours and in 2% osmium tetroxide for 2 hours at room temperature, and then dehydrated with acetone and embedded in Quetol 651 epoxy resin (Nisshin EM, Tokyo, Japan). Semi-thin, 0.5 µm sections were examined after staining with 0.5% (w/v) Toluidine Blue O solution.

For observation of the sporophytes, the plant tissues were fixed in 4% (w/v) paraformaldehyde in cacodylate (pH 7.2) overnight at 4°C. After dehydration in an ethanol series, the samples were embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). The 5 µm sections were examined after Toluidine Blue O staining.

The histochemical assay for GUS activity was performed as previously described (Nishiyama et al., 2000). The incubation time was adjusted from 2.5 to 12 hours, depending on the tissues examined.

Genetic crosses

The wild-type and double disruptants were cultured on 30-mm diameter peat pellets in separate plastic boxes. After cultivation at 25°C for 1 month, followed by 3 weeks at 15°C, both antheridia and archegonia matured. A peat pellet with wild-type plants and one with double disruptants were then transferred into the same plastic box and the two peat pellets were submerged in distilled water. After 30 seconds, the water above the mosses was decanted and the culture was continued at 15°C for 5 weeks before harvesting sporophytes.

The obtained spores were spread on BCDAT medium overlaid with a sheet of cellophane. After determining the germination rate of the spores, the cellophane was cut into quarters, and transferred to BCDAT medium containing either no antibiotics or hygromycin (30 mg/l), G418 (20 mg/l), or both hygromycin (30 mg/l) and G418 (20 mg/l).

Ectopic expression of PpLFY1 or PpLFY2 in A. thaliana

Transformations of A. thaliana (Columbia) using 35S::PpLFY1 and 35S::PpLFY2 (see above) were performed by the floral dip method (Clough and Bent, 1998). The T3 seedlings of transformed A. thaliana were grown at 22°C under conditions of 16 hours light/8 hours dark or 10 hours light/14 hours dark. The 35S::LFY7-7 and 35S::LFY16-7 lines are subsequent generations of 35S::LFY7 and 35S::LFY16, respectively (Shindo et al., 2001).

Results

Two FLO/LFY homologs in Physcomitrella patens

We have previously isolated cDNA clones of two FLO/LFY homologs from P. patens, designated PpLFY1 and PpLFY2 (Himi et al., 2001). The nucleotide sequences of their coding regions have 89.5% identity. The putative PpLFY1 and PpLFY2 proteins are 94.8% identical to each other and have 49.5% and 48.5% identity with A. thaliana LFY, respectively. The genomic DNA corresponding to each cDNA was isolated and sequenced (Fig. 1A,B; GenBank accession numbers AB192348 and AB192349, respectively). There are three introns in the coding region of each gene, and the exon-intron borders are conserved in both genes. The positions of the second and third introns are conserved with those of most other known FLO/LFY genes (Frohlich and Meyerowitz, 1997), while the first intron is specific to the PpLFY genes. Southern blot analyses under high and low stringency conditions showed that other FLO/LFY homologs are unlikely to exist in the P. patens genome (Fig. 1C).

PpLFY1 and PpLFY2 show similar expression patterns

Analysis by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) revealed higher expression levels of PpLFY1 and PpLFY2 in gametophores and sporophytes than in protonemata (Fig. 2). To investigate spatiotemporal expression patterns in more detail, the uidA gene, which codes for β-glucuronidase (GUS), was inserted just before the stop codon of PpLFY1 (Fig. 1D), or in-frame in the second exon of PpLFY2 (Fig. 1E). Multiple single insertion
lines were identified for each construct (see Fig. S1 in the supplementary material).

The expression patterns of PpLFY1-GUS and PpLFY2-GUS were similar among all of the isolated lines (Fig. 3). GUS signal was first detected in the young gametophores before leaf differentiation (Fig. 3A,M) and detected continuously in the main and lateral shoot apices of the leafy gametophores (Fig. 3B,N). GUS signal was also detected in the developing archegonium, in which the egg cell differentiates (Fig. 3C-F,O-R), but not in the sperm-forming organ, the antheridium (Fig. 3G,S). The GUS signal was detected in the whole part of the developing sporophyte after fertilization (Fig. 3H,I,T-W).

Approximately 90% of the gametophores of the PpLFY2-GUS lines formed sporophytes, while only about 1% of the gametophores of the PpLFY1-GUS lines formed sporophytes, approximately 80% of which had abnormal morphology. After the differentiation of the sporangium, the seta and the foot (Fig. 3X), GUS activity was predominantly detected in the sporangium and the foot (Fig. 3J-L,Y-AA). GUS activity weakened and became undetectable as the sporangium matured (Fig. 3AB).

Because of technical problems and a lack of PpLFY antibodies. We note that GUS fusion renders PpLFY1 proteins inactive because PpLFY1-GUS lines showed similar phenotypes to PpLFY1-dis lines as mentioned below. Although care has to be taken to interpret the results, we believe that the observed GUS pattern is authentic because the expression pattern of PpLFY2-GUS, which had very weak disruptant phenotypes, was not distinguishable from that of PpLFY1-GUS in addition to the concordant results between the GUS lines (Fig. 3) and RT-PCR (Fig. 2).

**Double disruption of PpLFY genes causes defects in sporophyte formation**

To investigate the functions of PpLFY1 and PpLFY2, disruptants for each gene and for both genes were generated (Fig. 1F,G; see Fig. S2 in the supplementary material). For PpLFY1, 8 lines (PpLFY1-dis-1 to 8) contained a single insertion of an HPT cassette, which confers resistance to hygromycin, in the PpLFY1-GUS lines (D), PpLFY2-GUS lines (E), PpLFY1-dis and PpLFY1-PpLFY2-dis lines (F), PpLFY2-dis and PpLFY1-PpLFY2-dis lines (G). uidA, uidA coding region; nos-ter, nopaline synthase polyadenylation signal; NPTII, NPTII expression cassette; HPT, HPT expression cassette. The sense directions of uidA, nptII, and hpt are indicated with arrows. The scale bar applies to A,B,D-G.
sporophyte development and PpLFY1-dis-1, 3 and 8 showed a severe decrease. The two types of phenotypes caused by the same gene disruption are unusual and further studies are necessary. The sporophytes in the double disruptants had abnormal morphology, which is described below.

**Zygotes of the double disruptants do not progress to the first cell division**

To identify the developmental stage that was arrested in the PpLFY disruptants, the development of egg cells, zygotes and young sporophytes was examined using confocal laser scanning microscopy (CLSM). The morphology of the egg cell during all stages before the entry of sperm was indistinguishable between the wild type (Fig. 5A,B), the single disruptants (data not shown), and the double disruptants (Fig. 5H). The egg cell had weaker fluorescence in the nucleus than in the cytoplasm (Fig. 5B,H). Although the boundary between the nucleus and cytoplasm was often indistinct in CLSM, a distinct nucleus was observed in semi-thin sections with light microscopy (Fig. 5A). Sperm were observed in the archegonium cavities of both the wild type (Fig. 5C) and the disruptants (Fig. 5I and data not shown). After an archegonium opened (Fig. 3D,P), its neck canal, a narrow path that permits sperm entry, turned brown (Fig. 3E,Q). When sperm were observed in the archegonium cavity of the wild type and the disruptants, the neck canal was always brown. In contrast, we did not observe any sperm in the cavities of open archegonia with the neck canal uncolored (Fig. 3D,P).

In both the wild type and the disruptants, after the neck canal had turned brown, one of two types of cell was observed in the cavity (Table 2 and data not shown). One cell type had stronger fluorescence in the nucleus than in cytoplasm (Fig. 5B,H), similar to the zygote (Fig. 5E) and the cells of young embryos with more than two cells (Fig. 5F); the other had weaker fluorescence in the nucleus than in cytoplasm (Fig. 5G,K), similar to the egg cells observed in uncolored open archegonia (Fig. 5B,H). The former and latter cell types probably corresponded to fertilized and unfertilized egg cells, respectively, as we discuss below.

Before the first cell division, the zygote expanded to fill the archegonium cavity in the wild type (Fig. 5E). Young wild-type sporophytes developed with continuous cell divisions (Fig. 5F). The zygote and subsequent sporophyte development proceeded normally in approximately 80% of wild-type archegonia with a brown neck canal (Table 2).
Development resistant to both hygromycin and G418, indicating that these were close to zero. The rare protonemata that germinated were abnormal morphology and/or the germination rates of the spores parent. Among 140 sporophytes obtained, 135 sporophytes had the female parents, were crossed with the wild type, as the male normal, fertilizable egg cells, the PpLFY1-PpLFY2-dis lines, as

To examine whether the PpLFY1-PpLFY2-dis lines can form disruptants

Crosses between the wild type and the double disruptants

Phenotypes of PpLFY2-dis-1, PpLFY2-GUS-3, and PpLFY2-GUS-5 lines were not distinguishable from those of the wild type in preliminary analyses (data not shown), which is concordant with the slight difference of sporophyte formation rate shown in Table 1. In contrast, a much smaller number of expanded zygotes and embryos were observed in the PpLFY1-dis lines than in the wild type and the PpLFY2-dis disruptants (Table 2 and data not shown). Neither expanded zygotes nor young sporophytes with more than two cells were found in PpLFY1-PpLFY2-dis lines (Table 2). These results indicate that the expansion and the subsequent first cell division of the zygote were arrested in the PpLFY1 single disruptants and the double disruptants.

**Crosses between the wild type and the double disruptants**

To examine whether the PpLFY1-PpLFY2-dis lines can form normal, fertilizable egg cells, the PpLFY1-PpLFY2-dis lines, as the female parents, were crossed with the wild type, as the male parent. Among 140 sporophytes obtained, 135 sporophytes had abnormal morphology and/or the germination rates of the spores were close to zero. The rare protonemata that germinated were resistant to both hygromycin and G418, indicating that these sporophytes were not formed by a cross between the double disruptant and the wild type. Five of the 140 sporophytes obtained had morphology that was indistinguishable from that of the wild type, and the germination rates of these spores were largely similar to that of the wild type (Table 3). Approximately half of the protonemata that germinated from these spores were resistant to either hygromycin or G418, and approximately a quarter was resistant to both drugs. These frequencies were consistent with the expected values if these five sporophytes resulted from a cross between double disruptant eggs and wild-type sperm.

**Defects in sporophyte development in the disruptant lines**

After fertilization, a spindle-like sporophyte developed in an archegonium cavity with concurrent enlargement of a venter in the wild type (Fig. 6A,B). Then, mature sporophytes were formed in which spores are produced via meiosis (Fig. 6C,D). Approximately 0.7 and 0.5% of the PpLFY1-dis-1, 3 and 8 single disruptant and the PpLFY1-PpLFY2-dis double disruptant gametophores, respectively, formed sporophytes that had abnormal morphology (Table 1), as observed in the 135 sporophytes obtained by the crossing experiments. In the PpLFY1-dis-2, 6 and 7 single disruptants, approximately 0.8% of gametophores formed sporophytes that had abnormal morphology, while other sporophytes were normal with regular fertile spores. Such abnormal sporophytes were not observed in the wild type or the PpLFY2-dis-1, PpLFY2-GUS-3 and PpLFY2-GUS-5 lines. The abnormal sporophytes emerged from the side of the archegonium, rupturing the archegonium wall (Fig. 6E). Several sporophytes also formed multiple sporangia (Fig. 6F). As we discuss later, these abnormal morphologies were observed in sporophytes produced by parthenogenesis (Lal, 1984).

The development of these abnormal sporophytes was arrested at various developmental stages (Fig. 6G-K). Some sporophytes ceased their development at an early stage (Fig. 6H). Other sporophytes differentiated a sporangium, a seta and a foot with aberrant morphologies (Fig. 6G-I-K). In these sporophytes, oriented cell divisions, cell number and cell differentiation were all defective to different degrees and they were usually more fragile than those of the wild type (Fig. 6G). When spores were formed (Fig. 6J,K), the number of spores was variable and less than that produced by wild-type sporophytes [approx. 5000 (Reski, 1998)], and the spore germination rates were significantly lower than that of the wild type. When all of the spores in the sporangia of the double disruptant lines (PpLFY1-

**Table 1. The percentage of sporophyte formation in the wild type and the PpLFY disruptants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Sporophyte formation (n)</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>93.9 (441)</td>
</tr>
<tr>
<td>PpLFY1-disrupted mutants</td>
<td></td>
</tr>
<tr>
<td>PpLFY1-dis-1</td>
<td>1.3 (318)</td>
</tr>
<tr>
<td>PpLFY1-dis-2</td>
<td>26.0 (648)</td>
</tr>
<tr>
<td>PpLFY1-dis-3</td>
<td>0 (307)</td>
</tr>
<tr>
<td>PpLFY1-dis-6</td>
<td>23.0 (417)</td>
</tr>
<tr>
<td>PpLFY1-dis-7</td>
<td>24.4 (328)</td>
</tr>
<tr>
<td>PpLFY1-dis-8</td>
<td>0.9 (438)</td>
</tr>
<tr>
<td>PpLFY2-disrupted mutants</td>
<td></td>
</tr>
<tr>
<td>PpLFY2-dis-1</td>
<td>84.0 (194)</td>
</tr>
<tr>
<td>PpLFY2-GUS-3</td>
<td>88.5 (340)</td>
</tr>
<tr>
<td>PpLFY2-GUS-5</td>
<td>89.6 (365)</td>
</tr>
<tr>
<td>PpLFY1-PpLFY2-disrupted mutants</td>
<td></td>
</tr>
<tr>
<td>PpLFY1-PpLFY2-dis-1</td>
<td>0.4 (936)</td>
</tr>
<tr>
<td>PpLFY1-PpLFY2-dis-2</td>
<td>0.8 (1777)</td>
</tr>
<tr>
<td>PpLFY1-PpLFY2-dis-3</td>
<td>0.3 (1429)</td>
</tr>
<tr>
<td>PpLFY1-PpLFY2-dis-5</td>
<td>0.6 (724)</td>
</tr>
<tr>
<td>PpLFY1-PpLFY2-dis-6</td>
<td>0.5 (1768)</td>
</tr>
</tbody>
</table>

*The number of gametophores with archegonia after two month cultivation under gametangia-inductive conditions.

**Fig. 4.** The phenotypes of the single and double disruptants of the PpLFY1 and PpLFY2 genes. (A) A sporophyte (arrow) is formed on the shoot apex of a gametophore. (B-E) Gametophores of wild-type P. patens (B), PpLFY1-dis-1 (C), PpLFY2-dis-1 (D), and PpLFY1-PpLFY2-dis-2 (E) lines, photographed from above. The sporangia are ocher to dark brown spheres, two of which are indicated in each panel by arrowheads. Scale bars: 1 mm.
PpLFY2-dis-1, 2, 3, 5 and 6) and the PpLFY1 single disruptant line (PpLFY1-dis-1) were spread, 5.1±9.7 and 4.0±2.9 spores germinated per sporangium (n=25 and 5), respectively. Spore germination rates of the sporangia with normal morphology in the wild type, the PpLFY1 single disruptant lines (PpLFY1-dis-2 and PpLFY1-dis-7), and the PpLFY2 single disruptant lines (PpLFY2-dis-1 and PpLFY2-GUS-5) were 95.0±2.6, 93.5±2.5, 91.4±1.2, 94.5±1.3 and 92.4±1.2%, respectively (n=5).

Constitutive expression of PpLFY1 and PpLFY2 in A. thaliana
In contrast to the moss, there is not a zygotic phenotype in flo/lfy mutants of angiosperm (Bomblies et al., 2003; Coen et al., 1990; Hofer et al., 1997; Molinero-Rosales et al., 1999; Shultz and Haughn, 1991; Souer et al., 1998; Weigel et al., 1992). To learn whether the function of FLO/LFY genes in flowering plants has diverged from that of PpLFY genes, we generated A. thaliana plants that constitutively expressed PpLFY1 or PpLFY2. Constitutive expression of A. thaliana LFY mRNA under the control of the CaMV 35S promoter has been shown to induce early flowering and the formation of terminal flowers (Weigel and Nilsson, 1995; Blázquez et al., 1997; Blázquez and Weigel, 2000). Expression of PpLFY mRNAs in A. thaliana plants transformed with 35S::PpLFY1 and 35S::PpLFY2 constructs was verified by northern analyses (data not shown). We could not examine whether transcribed mRNAs were translated because of the lack of PpLFY antibodies. Further studies are necessary to confirm PpLFYs are translated in A. thaliana, although unusual codons in A. thaliana were not found in PpLFYs (data not shown). Under either long- or short-day conditions, the 35S::PpLFY plants flowered at the same time as the wild type and there was no abnormal phenotype (see Table S1 in the supplementary material). Control experiments, in which the A. thaliana LFY genes were constitutively expressed, showed early flowering and terminal flowers.

Discussion
PpLFY1 and PpLFY2 regulate zygote development in P. patens
The PpLFY1-PpLFY2 double disruptants and the PpLFY1-dis single disruptants showed substantial decreases in the number of gametophores that formed a sporophyte (Table 1),

**Table 2. The developmental stage of the cell or tissue in archegonia after the neck canal changed brown**

<table>
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<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Unexpanded zygote</td>
<td>9</td>
<td>57</td>
<td>54</td>
<td>47</td>
<td>46</td>
<td>43</td>
<td>41</td>
</tr>
<tr>
<td>Expanded zygote</td>
<td>7</td>
<td>0</td>
<td>3</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>More than two cell embryo</td>
<td>71</td>
<td>0</td>
<td>6</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unfertilized egg cell or dying zygote*</td>
<td>14</td>
<td>43</td>
<td>37</td>
<td>34</td>
<td>34</td>
<td>54</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>35</td>
<td>35</td>
<td>32</td>
<td>28</td>
<td>28</td>
<td>37</td>
</tr>
</tbody>
</table>

*It was not always possible to distinguish between an unfertilized egg cell and a dying zygote without expansion by confocal laser scanning microscopy.

Analyses using semi-thin sections showed both types of cells are included in this category.

1The number of archegonia examined between the third and fourth weeks after the induction of gametangia. The most-developed archegonium with a brown neck canal in all archegonia of a shoot apex was selected.
while all gametophytic tissues, including protonemata, gametophores and gametangia were normal. The PpLFY1-dis lines showed much more severe phenotypes than the PpLFY2-dis and PpLFY2-GUS lines, indicating that PpLFY1 plays a major role in the wild type. Because neither the PpLFY1-GUS nor the PpLFY2-GUS fusion proteins were detected in the antheridia and sperm (Fig. 3G,S), the PpLFY genes are unlikely to play a role in sperm differentiation. Also, sperm were observed in the archegonia of the double disruptant lines (Fig. 5I), indicating that normal sperm were produced in these lines.

A change in fluorescence appears to indicate fertilization (Fig. 5), similar to what has been reported in some flowering plants (Hoshino et al., 2004; Webb and Gunning, 1994; Zhou, 1987). By this criterion, fertilization was seemingly normal in the PpLFY disruptants. After fertilization, however, the putative zygote of the double disruptants remained in the single-cell stage, indicating that the PpLFY proteins are important for the first cell division of the zygote, along with the normal development of the zygote, including the cellular expansion and the accompanying extensive vacuolation. Since LFY protein from A. thaliana has been shown to be a DNA-binding transcription factor (Parcy et al., 1998), the PpLFY gene products may regulate the expression of genes responsible for the zygote development and the zygotic cell division.

**Possible involvement of PpLFY genes in sporophyte development**

The few sporophytes formed by the PpLFY1-PpLFY2-dis double disruptants and the severe PpLFY-1 single disruptants had abnormal morphology. These sporophytes were possibly generated by parthenogenesis, which is common in the three species of *Physcomitrium* under laboratory culture conditions (Lal, 1984). The mode of emergence of sporophytes from the archegonium venter and the multiple sporangia occasionally observed in the disruptant lines are reminiscent of the parthenogenetic sporophytes of *Physcomitrium cyathicarpum* (Fig. 6E,F). Abnormal sporophytes were not observed in the wild-type plants, most likely because the sporophytes resulting from fertilization develop much earlier than those resulting from parthenogenesis. Normally, a single sporophyte matures on the apex of a gametophore, even though several archegonia are fertilized, suggesting that the early growth of a sporophyte inhibits the development of the embryos on the same gametophore apex that were fertilized later. In the PpLFY disruptant lines, normal sporophytes resulting from fertilization do not grow, and thus sporophytes arising from parthenogenesis can survive and grow.

In addition to having the morphological characteristics commonly observed in sporophytes that arise from parthenogenesis, the PpLFY disruptant sporophytes had other abnormalities. The sporangia of the disruptants were much softer than those of the wild type and often withered during development (Fig. 6G). The number of differentiated spores present in each sporangium varied in the disruptants. Germinations occurred in only a few spores of several tens to several thousands of spores per sporangium. This is in contrast to the case in parthenogenetic sporophytes, which regularly

<table>
<thead>
<tr>
<th>Sporophyte obtained</th>
<th>Maternal strain</th>
<th>% Germination (n*)</th>
<th>% Drug resistant (n*)</th>
<th>Hygromycin</th>
<th>G418</th>
<th>Hygromycin + G418</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PpLFY1-PpLFY2-dis-1</td>
<td>58.3 (571)</td>
<td>50.8 (378)</td>
<td>49.7 (473)</td>
<td>27.9 (322)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PpLFY1-PpLFY2-dis-2</td>
<td>95.1 (1634)</td>
<td>50.3 (597)</td>
<td>46.5 (553)</td>
<td>27.5 (404)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PpLFY1-PpLFY2-dis-3</td>
<td>85.2 (1722)</td>
<td>49.0 (351)</td>
<td>44.7 (358)</td>
<td>29.1 (477)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>PpLFY1-PpLFY2-dis-4</td>
<td>56.0 (343)</td>
<td>52.1 (192)</td>
<td>51.8 (328)</td>
<td>27.9 (348)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>PpLFY1-PpLFY2-dis-5</td>
<td>47.6 (479)</td>
<td>52.6 (228)</td>
<td>46.9 (292)</td>
<td>23.8 (324)</td>
<td></td>
</tr>
</tbody>
</table>

*The number of spores examined.
form viable spores (Lal, 1984). These additional abnormal phenotypes may be related to the loss of \textit{PpLFY} gene function and may indicate that the \textit{PpLFY} genes are involved in sporophyte development in addition to zygote development. The expression of both \textit{PpLFY} mRNAs and proteins in the developing sporophytes (Figs 2, 3) is consistent with this hypothesis.

The sporophytes of the \textit{PpLFY} double disruptants exhibited abnormalities in the patterns of cell division, but apparently not in organogenesis. Normal sporophytes consist of three organs: the sporangium, the seta and the foot (Fig. 3X); all of these organs were formed even in the severely defective sporophytes (Fig. 6G, I-K). An aberrant pattern of cell division is the probable cause of the abnormalities in cell number, cell shape and cell arrangement in the sporophytes of disruptants (Fig. 6H-K). These abnormalities are reminiscent of those observed in \textit{fass} mutants of \textit{A. thaliana} (Torres-Ruiz and Jürgens, 1994), which showed defects in the pattern of cell division but not in organogenesis during embryogenesis. This may suggest that the \textit{PpLFY} proteins regulate the transcription of genes involved in cell division in the sporophytes. Whether the decreased spore germination rates in the double disruptants are caused by a defect in mitosis of sporegonous cells or by a defect in meiosis of the spore mother cells, or both, will be the subject of future research. Our results also suggest that cell division is regulated differently in sporophytes and gametophytes in \textit{P. patens}, because the protonemata and gametophores of the \textit{PpLFY} disruptants had no morphological differences from the wild type.

\textbf{Evolution of \textit{FLO/LFY} genes and body plan in land plants}

The developmental processes from gametogenesis to the first cell division of the zygote commonly occur in both unicellular and multicellular organisms with sexual reproduction, and also probably occurred in the unicellular common ancestor of land plants and metazoa. This suggests that at least some of the molecular mechanisms involved in the developmental processes should be shared among all organisms. However, \textit{FLO/LFY} genes have been found only in land plants and the involvement of the \textit{PpLFY} genes in the initiation of the first cell division in the zygote suggests that plants have evolved molecular mechanisms that differ from those of animals, even for very early stages of development. This is concordant with the fact that plant cells have a unique mode of cytokinesis involving cell plate formation.

The \textit{FLO/LFY} genes of flowering plants, such as the \textit{A. thaliana LFY} gene, function in the transition from the vegetative to the reproductive phase (Blázquez et al., 1997; Weigel and Nilsson, 1995), where one of its principal targets is \textit{APETAL1} (\textit{API}) (Liljegren et al., 1999; Parcy et al., 1998; Wagner et al., 1999). In addition, \textit{LFY} activates floral homeotic genes and their cofactors (Busch et al., 2001; Lamb et al., 2002; Parcy et al., 1998; Schmid et al., 2003; Weigel and Meyerowitz, 1993; William et al., 2004). These functions are generally preserved in other angiosperms, although \textit{FLO/LFY} has acquired additional roles in some plants such as pea (Ahearn et al., 2001; Bommilies et al., 2003; Coen et al., 1990; Hofer et al., 1997; Molinero-Rosales et al., 1999; Schultz and Haughn, 1991; Sosere et al., 1998; Weigel et al., 1992). Gymnosperm \textit{LFY} homologs from \textit{Gnetum} (\textit{GpLFY}) and \textit{Pinus} (\textit{NEEDLY}) are predominantly expressed in reproductive meristems (Mouradov et al., 1998; Shindo et al., 2001), and the overexpression of \textit{GpLFY} or \textit{NEEDLY} complements \textit{LFY} function in the \textit{Ify} null mutant and enhances early flowering, similar to that observed with overexpression of \textit{LFY}. Therefore, these gymnosperm homologs probably have similar functions to those of the \textit{A. thaliana LFY} gene. These findings suggest that the common ancestor of gymnosperms and angiosperms had a \textit{FLO/LFY} gene with similar functions to those of the \textit{A. thaliana LFY} gene. The fern \textit{LFY} homologs \textit{CrLFY} are also predominantly expressed in the reproductive meristems, and not in the vegetative ones, but their expression patterns are more restricted than those of the five fern MADS-box genes (Hasebe et al., 1998; Himi et al., 2001). This may imply that the \textit{CrLFY} genes have a function in the transition from vegetative to reproductive growth, but that they are not indispensable for the induction of the fern MADS-box genes analyzed.

In contrast to the partial or full functional conservation of the \textit{FLO/LFY} genes among vascular plants, the \textit{FLO/LFY} homologs in the moss \textit{P. patens} function differently from those of vascular plants. Although the \textit{PpLFY} genes function in the sporophytic generation, as is the case in vascular plants, \textit{LFY} and its homologs that have been reported in vascular plants do not function in embryogenesis. Ectopic overexpression of \textit{PpLFY} genes did not cause early flowering in \textit{A. thaliana} (see Table S1 in the supplementary material), and \textit{PpLFY} fused with a \textit{A. thaliana LFY} promoter did not complement the \textit{Ify} mutant (Maizel et al., 2005). These observations suggest that the function of the \textit{FLO/LFY} genes in the transition from the vegetative to the reproductive phase and in the induction of the floral homeotic MADS-box genes evolved in the vascular plant lineage after the divergence of mosses.

The basic body plans and the functions of signaling molecules in metazoa are well conserved among a wide variety of lineages from \textit{Hydra} to vertebrates (Carroll et al., 2001; Hobmayer et al., 2000). Green plants emerged on land approximately 480 million years ago, and the moss lineage diverged from the vascular plant lineage almost immediately after that time, which is more recent than the Cambrian explosion, when most of the major lineages of extant metazoa diverged. The \textit{FLO/LFY} homologs are key developmental regulators in both vascular plants and mosses; the differences in the functions of these genes in the two lineages suggest that the body plans of land plants diverged unexpectedly.

We would like to thank Rumiko Kofuji and Ryosuke Sano for helping to clone \textit{PpLFY1}; R. Kofuji for help with Southern analyses; Tomoaki Nishiyama, Alexis Maizel, Detlef Weigel, Tomomichi Fujita and Naoki Aono for discussion and comments on manuscript; A. Maizel and D. Weigel for unpublished data; Yoshikatsu Sato and Takashi Murata for microscopy; Keiko Sakakibara for discussion; Kenzo Nakamura for pBI121Hm; Hirokazu Tsukaya for pHITS14; Chieko Nanba for Arabidopsis culture; Masae Umeda, Yoshimi Bitoh, Kana Yano and Yukiko Dodo for technical assistance; and Akio Toh-e for BAS 1000. Sequencing analyses and CLSM analyses were performed in part at the NIBB Center for Analytical Instruments. This study was carried out under the NIBB Cooperative Research Program (4-125) and was supported by grants from MEXT and JSPS.

\textbf{Supplementary material}

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/7/1727/DC1
automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. *Genomics* 25, 674-681.


