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Regulation of stem cell maintenance by the Polycomb protein FIE has been conserved during land plant evolution

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The Polycomb group (PcG) complex is involved in the epigenetic control of gene expression profiles. In flowering plants, PcG proteins regulate vegetative and reproductive programs. Epigenetically inherited states established in the gametophyte generation are maintained after fertilization in the sporophyte generation, having a profound influence on seed development. The gametophyte size and phase dominance were dramatically reduced during angiosperm evolution, and have specialized in flowering plants to support the reproductive process. The moss *Physcomitrella patens* is an ideal organism in which to study epigenetic processes during the gametophyte stage, as it possesses a dominant photosynthetic gametophytic haploid phase and efficient homologous recombination, allowing targeted gene replacement. We show that *P. patens* PcG protein FIE (PpFIE) accumulates in haploid meristematic cells and in cells that undergo fate transition during dedifferentiation programs in the gametophyte. In the absence of *PpFIE*, meristems overproliferate and are unable to develop leafy gametophytes or reach the reproductive phase. This aberrant phenotype might result from failure of the PcG complex to repress proliferation and differentiation of three-faced apical stem cells, which are designated to become lateral shoots. The *PpFIE* phenotype can be partially rescued by *FIE* of *Arabidopsis thaliana*, a flowering plant that diverged >450 million years ago from bryophytes. *PpFIE* can partially complement the *A. thaliana fie* mutant, illustrating functional conservation of the protein during evolution in regulating the differentiation of meristematic cells in gametophyte development, both in bryophytes and angiosperms. This mechanism was harnessed at the onset of the evolution of alternating generations, facilitating the establishment of sporophytic developmental programs.

KEY WORDS: Apical cell, *Arabidopsis thaliana*, BiFC, CLF, PcG complex, *Physcomitrella patens*, Protein-protein interaction

INTRODUCTION

All land plants are characterized by an alteration of two generations: the haploid gametophyte and the diploid sporophyte. In flowering plants, the sporophyte comprises complex organs including leafy shoots and flowers. Here, this phase dominates over the diminutive and ephemeral gametophytic phase. The gametophytes of flowering plants, namely the pollen and female embryo sacs in which fertilization occurs, are epiphytic to the diploid plant body. In contrast to flowering plants, in bryophytes, the earliest diverging group in land plant evolution, the gametophytic generation is photosynthetically active and dominates the epiphytic sporophyte (reviewed by Reski, 1998a). Consequently, bryophytes propagate through haploid spores, whereas flowering plants propagate via diploid seeds. The last common ancestor of bryophytes and flowering plants was estimated to live around 500 million years ago (Zimmer et al., 2007), an evolutionary distance similar to that between human and fish.

In recent years it has become evident that mechanisms for gene silencing play a role in regulating developmental programs. In general, silencing involves both nucleic acid-based mechanisms, such as small RNA molecules (Bartel, 2004; Jones-Rhoades et al., 2006; Zhang et al., 2007) or DNA methylation (Ginder et al., 2008; Henderson and Jacobsen, 2007; Reik et al., 2001; Saurin et al., 2001), as well as histone-based modifications (Jenuwein and Allis, 2001), such as methylation of lysine 27 on histone 3 (H3K27me3) (Lachner et al., 2003). Methylation of H3K27 is mediated by the Polycomb recruiting complex 2 (PRC2) (Czermin et al., 2002; Ketel et al., 2005; Muller et al., 2002; Nekrasov et al., 2005), also designated the Polycomb group protein (PcG) complex. The PcG complex was first identified in *Drosophila melanogaster* (Jurgens, 1985; Lewis, 1978) and subsequently in *Caenorhabditis elegans* (Holdeman et al., 1998), *Homo sapiens* (Chen et al., 1996; Denisenko and Bomsztyk, 1997), as well as in flowering plants (Goodrich et al., 1997; Grossniklaus et al., 1998; Ohad et al., 1999; Luo et al., 1999).

In *A. thaliana* genetic and biochemical analyses predict several PcG-like PRC2 complexes (Goodrich et al., 1997; Grossniklaus et al., 1998; Ohad et al., 1999; Luo et al., 1999; Chanvivattana et al., 2004), some of which have been isolated and identified (De Lucia et al., 2008; Wood et al., 2006). All PcG complexes in *A. thaliana* comprise the WD40 motif-containing proteins FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPRESSOR OF IRA 1 (MSI1) (Kohler et al., 2003b; Ohad et al., 1999). In addition, each PcG complex is predicted to contain one of the three SET domain proteins CURLY LEAF (CLF), SWINGER (SWN) or MEDEA (MEA) (Chanvivattana et al., 2004; Luo et al., 1999; Katz et al., 2004; Yadegari et al., 2000). The SET domain protein acts as the catalytic subunit and methylates H3K27me3 (Czermin et al., 2002; Ketel et al., 2005; Muller et al., 2002; Nekrasov et al., 2005). Members of the SET domain PcG proteins may interact with one of the zinc-finger PcG proteins, including EMBRYONIC FLOWER 2 (EMF2), VERNALIZATION 2 (VRN2) or FERTILIZATION INDEPENDENT SEED 2 (FIS2), via the VEFS domain (Chanvivattana et al., 2004).

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The modular nature of flowering plant PcG complex composition leads to the formation of individual PcG complexes, which facilitate the control of different developmental programs along the plant life cycle. Whereas PcG complexes containing either CLF or SWN act during the sporophytic stage, for example in flowers and fruits (Chanvivattana et al., 2004; Katz et al., 2004), PcG complexes containing either MEA or SWN act at the gametophytic stage, as evident from their mutant phenotype characterized by abnormal endosperm in the absence of fertilization and embryo abortion after fertilization.

In view of the central role of the FIE PcG complex in regulating the transition of the female gametophyte to the sporophyte in flowering plants (Goodrich, 1998; Guitton et al., 2004; Kohler et al., 2003a; Ohad et al., 1996; Ohad et al., 1999), it is intriguing to analyze the evolution of this function in basal land plants. For this purpose, we chose the model bryophyte *Physcomitrella patens*, with its dominant gametophytic phase. Furthermore, different types of stem cells can be analyzed in this plant, as the juvenile gametophyte (the protonema) is a filamentous tissue growing solely by apical cell division, whereas the transition to the adult gametophyte (the gametophore) is characterized by a cell-fate transition to a three-faced apical cell (the bud). This transition can specifically be triggered by the plant hormone cytokinin (reviewed by Decker et al., 2006). Unique to land plants, reverse genetics approaches via gene targeting are highly efficient in *P. patens* (Reski, 1998b). In addition, the genome of *P. patens* has been entirely sequenced (Rensing et al., 2008), facilitating evo-devo studies with emphasis on the evolution of specific transcription factors (Maizel et al., 2005; Sakakibara et al., 2008).

Here we show that *P. patens* FIE protein (PpFIE) accumulates only in gametophyte apical cells and cells that undergo fate transition. Moreover, using targeted gene deletion and replacement, in the absence of PpFIE moss gametophore meristems overproliferate, but fail to develop and reach the reproductive phase, illustrating the key role of FIE in regulating proper differentiation and proliferation of *P. patens* gametophytic stem cells. This aberrant phenotype can be partially rescued by the *FIE* gene of *A. thaliana*, indicating functional conservation over more than 450 million years. Accordingly, PpFIE can partially complement the *A. thaliana fie* mutant. Thus the essential FIE PcG function in regulating developmental programs along the plant life cycle was established early in evolution, around the water-to-land transition of plant life.

MATERIALS AND METHODS

Plant material, culture conditions and treatments

The 'Gransden 2004' strain of *P. patens* (Ashton and Cove, 1977; Rensing et al., 2008) was propagated on BCD and BCDAT media (Ashton and Cove, 1977) at 25°C under a 16-hour light and 8-hour dark cycle (Frank et al., 2005). All transgenic lines described in this study are deposited in the International Moss Stock Center with the accessions IMSC 40319-40324 and 40265-40267 (*PpFIE-GUS*, *ΔPpFIE*- and *AtFIE-co* mutants).

Construction of the phylogenetic tree

Initially, sequences for which BLAST hits were at least 30% identical over a length of 80 amino acids were selected in order to avoid inclusion of false-positive hits from the twilight zone of protein alignment (Rost et al., 1999). Only FIE homologs from organisms (plants and animals) for which the whole genome sequence had been determined were taken into account. An amino acid sequence alignment was generated using MAFFT G-INS-i version 5.860 (Katoh et al., 2005) and manually curated using Jalview version 2.4 (Clamp et al., 2004). Based on the conserved core of this alignment (essentially comprising several WD40 domains), a hidden Markov model was generated (HMMER 2.3.2; http://hmmer.janelia.org/), a gathering cutoff of 400 was defined based on searches against several plant and animal genomes, and this was subsequently used to detect and retrieve FIE homologs from a larger set of completely sequenced genomes. Based on this set of sequences, a second alignment was constructed. The most appropriate evolutionary model was selected using ProtTest version 1.3 (Abascal et al., 2005) and turned out to be WAG (Whelan and Goldman, 2001) with gamma-distributed rate categories. Bayesian inference (BI) was carried out with the predetermined model using MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) with eight gamma-distributed rates categories (four chains, two runs) until convergence (average s.d.<0.01). Trees were visualized using FigTree version 1.2.2 (http://tree.bio.ed.ac.uk/software/figtree/) and manually rooted. Support values at the nodes (Fig. 1) are BI posterior probabilities. In addition, a neighbor-joining (NJ) tree was calculated using QuickTree version 1.1 (Howe et al., 2002) with bootstrap resampling 1000 times. Neither the NJ nor the ProtTest maximum likelihood tree was found to be significantly different from the BI tree.

Accession numbers are as follows: PpFIE, Phypa_61985 (*Physcomitrella Patens*); AtFIE, AAD23584 (*Arabidopsis thaliana*); ARALY_898508 (*Arabidopsis lyrata*); EED, AAB38319 (*Mus musculus*); EED, AAH47672 (*Homo sapiens*); CAG31770 (*Gallus gallus*); AAV36839 (*Drosophila melanogaster*); Esc2, AAA86427 (*D. melanogaster*); EED, BAD22546 (*Oryzias latipes*); AAH93351, LOC550463 (*Danio rerio*); POPTR_688045 (*Populus trichocarpa*); FIE1, Os08g04270.1 (*Oryza sativa*); FIE2, Os08g04290.1 (*O. sativa*); XENTR_293769 (*Xenopus laevis*); FUGRU_713547 (*Takifugu rubripes*); OSTLU_37673 (*Ostreococcus lucimarinus*); OSTTA_22117 (*Ostreococcus tauri*); PHATR_9860 (*Phaeodactylum tricornutum*); THAPS_118885 (*Thalassiosira pseudonana*); CHLRE_193732 (*Chlamydomonas reinhardtii*); CYAME_CMK173C (*Cyanidioschyzon merolae*); Selmo1_2_143777 (*Selaginella moellendorffii*); VITVI_A7P0Y9 (*Vitis vinifera*); ZEAMA_148924 P.01 (*Zea mays1*); ZEAMA_118205 P.01 (*Z. mays2*) (Danilevskaya et al., 2003); ZEAMA_006312 P.01 (*Z. mays3*); RICCO_28166 (*Ricinus communis*); FIE1, GLYMA_10g02690.1 (*Glycine max*); FIE2, GLYMA_02g17110.1 (*G. max*); FIE3, GLYMA_13g36310.1 (*G. max*); FIE4, GLYMA_12g34240.1 (*G. max*); CARPA_7.67 (*Carica papaya*); FIE1, SORBI_4986219 (*Sorghum bicolor*); FIE2, SORBI_4838275 (*S. bicolor*); NEMVE_102199 (*Nematostella vectensis*); VOLCA_58949 (*Volvox carteri*); MICP1_49065 (*Micromonas pusilla*); CHLSP_19370 (*Chlorella sp. NC64A*).

Gene isolation

The *PpFIE* and *PpCLF* complete coding regions were amplified from cDNA by PCR using primers based on the 'Joint Genome Institute' (JGI) *P. patens* version 1.1 database (http://www.jgi.doe.gov) and were subsequently cloned and sequenced. *PpFIE* turned out to be identical to Phypa_61985 (as available on www.cosmoss.org, version 1.2). The cDNA of *PpCLF* has been submitted to GenBank (accession: bankit1178009 FJ917288, www.ncbi.nlm.nih.gov), as no appropriate gene model is present at the genomic locus (scaffold_100:687012-693616) in genome versions 1.1 and 1.2. *PpFIE* is encoded by 1089 bp organized in a single exon, whereas *PpCLF* is encoded by 3000 bp spliced from 19 exons.

Construction of transformation vectors

PpFIE coding sequence (1089 bp) was amplified using the following primer set: PpFIE-Fw-1, 5′-AAGCTTCTCGAGATGGGAGATCTTGTCCCGGACAAG-3′; and PpFIE-Rev-1, 5′-AAGCTTTCAGCTAGCAGACACAGCATCCCAGCGCCAAAT-3′. In addition, the 5′ (805 bp) and 3′ (598 bp) of the untranslated region (UTR) of *PpFIE* were amplified using the following primers: PpFIE5′-UTR-Fw, 5′-GAAGCTTGACTAGAGCAAAAAAATTGTGATAGTGTGT-3′; PpFIE5′-UTR-Rev, 5′-GAAGCTTCACGGGATCCGTGCCGA-3′; PpFIE3′-UTR-Fw, 5′-GCATGCTGATCGTGGATATCTGGAGCCA-3′; and PpFIE3′-UTR-Rv, 5′-GCATGCCGCTAGGGTCATAGCCATATAAACA-3′. All amplified fragments were subcloned into the pTZ 57 vector (Fermentas, Lithuania) and sequenced to ensure their integrity. The PCR-amplified *PpFIE* genomic sequence was used for constructing the disrupted vector by the insertion of the selection cassette at the *Bal*I site. The same genomic fragment was cloned in-frame to the *uidA* reporter gene at the *Nhe*I site to obtain a protein fusion between PpFIE and GUS, and then cloned into the pMBL5 vector,

followed by a nopaline synthase polyadenylation signal (NOS-ter), a *nptII* cassette, as described in (Nishiyama et al., 2000), and the PpFIE3′-UTR (see Fig. S1 in the supplementary material). *AtFIE* full-length cDNA was amplified using the primers AtFIE-F 5′-CCCGGGATGTCGAAGATAACCTTAGGG-3′ and AtFIE-R 5′-CAAGGTCGACGGGAGTAGCAACAT-3′. The *AtFIE* cDNA was cloned into pMBL5 vector flanked by PpFIE5′-UTR at the 5′ end and by the NOS terminator, the *nptII* cassette and PpFIE3′-UTR, respectively, at the 3′ end. Prior to transformation the vectors were linearized.

Protoplast isolation and PEG-mediated transformation of *P. patens*

PEG transformation was performed as described in PHYSCObase (http://moss.nibb.ac.jp). Six days after regeneration, transformants were selected on BCDAT medium containing 20 mg/l of G418.

RT-PCR

Total RNA extraction from protonemata or leaves followed by RT-PCR were performed as described (Katz et al., 2004), using the following gene-specific primers: for *PpFIE*, PpFIE-Fw-1 and PpFIE-Rev-1; for *AtFIE*, AtFIE-F and AtFIE-350-R 5′-GATGCTCGTTTCTTCGATGT-3′.

Real-time PCR analysis of gene expression

Real-time quantitative PCR analysis was performed by ΔΔCt method of relative quantification with a StepOne Thermal Cycler (Applied Biosystems, Foster City, CA, USA), using SYBR Green to monitor dsDNA synthesis. The following primers were used to detect PpFIE and the housekeeping genes: PpFIE-left-261-278, 5′-AGATGGCAACCCCTTGCT-3′; PpFIE-Right-302-320, 5′-CAATCAATGATGCGGAGGA-3′; 60s-Left, 5′-GGGAACACTATCTTTTTCCTGGT-3′; 60s-Right, 5′-TGAAATCATGCGATTAGTCCTC-3′; TATA-Left, 5′-GATCTAGCTATAAGCCTGATCTACCG-3′; TATA-Right, 5′-CAGGAGCAGGGAGAGATTTG-3′. The amount of cDNA for each gene was quantified using a log-linear regression curve of the threshold cycle and the amount of standard template prepared from a cDNA clone.

Identification of *A. thaliana fie* mutant allele via RFLP analysis

A. thaliana fie alleles carry a single point mutation resulting in a polymorphic site recognized by the *Dra*I restriction enzyme. PCR analysis was carried out on genomic DNA as a template using the primers AtFIE-2277-F 5′-GCTTGTGGTTCGTTTGTATG-3′ and AtFIE-3143-R 5′-CCTATATGGCAACAGAAAAT-3′ followed by restriction with the *Dra*I enzyme, which enabled us to distinguish between the wild-type and mutant alleles. The resulting wild-type fragment was 866 bp, and the mutant gave rise to two products of 492 bp and 374 bp.

GUS assay

The histochemical assay for GUS activity was performed as described by Nishiyama et al. (Nishiyama et al., 2000). The incubation time was adjusted from 2 to 24 hours, depending on the tissues examined.

Electron microscopy

Cryoscanning electron microscopy samples were frozen in liquid nitrogen on a copper sample holder, sputtered with 20 nm gold particles and visualized using a JEOL 6300 cryoscanning electron microscope. Environmental scanning electron microscopy was performed using the Quanta 200 FEG with a field-emission gun and a gaseous secondary electron detector for surface imaging in wet mode.

Bimolecular fluorescence complementation analysis

Protein-protein interactions in plants were examined by bimolecular fluorescence complementation (BiFC) assay. *PpFIE*, *PpCLF*, *AtFIE*, *AtMEA*, *AtCLF* and *AtSWN* full-length cDNAs were cloned into pSY 735 and pSY 736 vectors at the *Spe*I site, which contain the N-terminal (YN) and the C-terminal (YC) fragments of the YFP protein, respectively (Bracha-Drori et al., 2004). Equal concentrations of *Agrobacterium tumerfaciens* strain GV3101/pMp90 containing plasmids of interest (see Table 1) were transiently coexpressed in *N. benthamiana* leaves via the leaf injection procedure (Bracha-Drori et al., 2004). Image annotation was performed with Zeiss AxioVision, Zeiss CLSM-5 and Adobe Photoshop 7.0 (Mountain View, CA, USA). The expression of each construct was verified by its ability to interact with AtFIE (see Fig. S4G-I in the supplementary material). Negative controls with vectors bearing only YN or YC alone were carried out in every experiment to verify the specificity of the interactions.

RESULTS

The FIE sequence is highly conserved among the eukaryotic crown kingdoms

Putative homologs of *A. thaliana* FIE were collected using hidden Markov model (HMM) searches from organisms for which the whole genome sequence had been determined. The phylogeny of the FIE protein superfamily is presented in Fig. 1. Potential FIE homologs are also present in the genomes of organisms, including *C. elegans* and *Saccharomyces cerevisiae*; however, their low conservation grade does not allow for unambiguous assignment to the FIE superfamily. It is evident from the phylogenetic tree that FIE is essentially a single-copy ortholog that was already present in the last common ancestor of all eukaryotes and might subsequently have been lost in some (unicellular) lineages. The FIE phylogeny approximately reflects the taxonomic relationships of the species involved. Paralog retention occurred occasionally and relatively late during evolution (after the insect-vertebrate split and the monocot-eudicot split, as can be seen from the *D. melanogaster*, *O. sativa*, *Z. mays*, *S. bicolor* and *G. max* paralogs, Fig. 1). The high conservation of the FIE proteins (e.g. 66% identity, 81% similarity over the whole protein length between *A. thaliana* and *P. patens* and 41% identity,

Table 1. Vectors used in this study

Vector	Description	Source
pMBL5	SK backbone with *nptII* cassette	GenBank accession number DQ228130
pTZ 57	TA cloning vector	Fermentas, Lithuania
pTZ ΔPpFIE	pTZ *PpFIE* genomic *nptII* (*Bal*l)	This study
pMBL5 PpFIE GUS	pMBL *PpFIE* GUS NOS *nptII* 3′ UTR	This study
pMBL5 AtFIE	pMBL cDNA *AtFIE* NOS *nptII* 3′ UTR	This study
pCmbia 4.2 P:PpFIE	pCambia 4.2 *AtFIE* promoter:*PpFIE*	This study
pSY 736	YN only (for negative control)	(Bracha-Drori et al., 2004)
pSY 735	YC only (for negative control)	(Bracha-Drori et al., 2004)
pSY 736-AtFIE	YN-MSI1	(Bracha-Drori et al., 2004)
pSY 736-AtMEA	YN-AtMEA	(Bracha-Drori et al., 2004)
pSY 736-AtCLF	YN-AtCLF	This study
pSY 736-AtSWN	YN-AtSWN	This study
pSY 736-PpFIE	YN-PpFIE	This study
pSY 735- PpFIE	YC-PpFIE	This study
pSY 736-PpCLF	YN-PpCLF	This study
pSY 735-PpCLF	Yn-PpCLF	This study

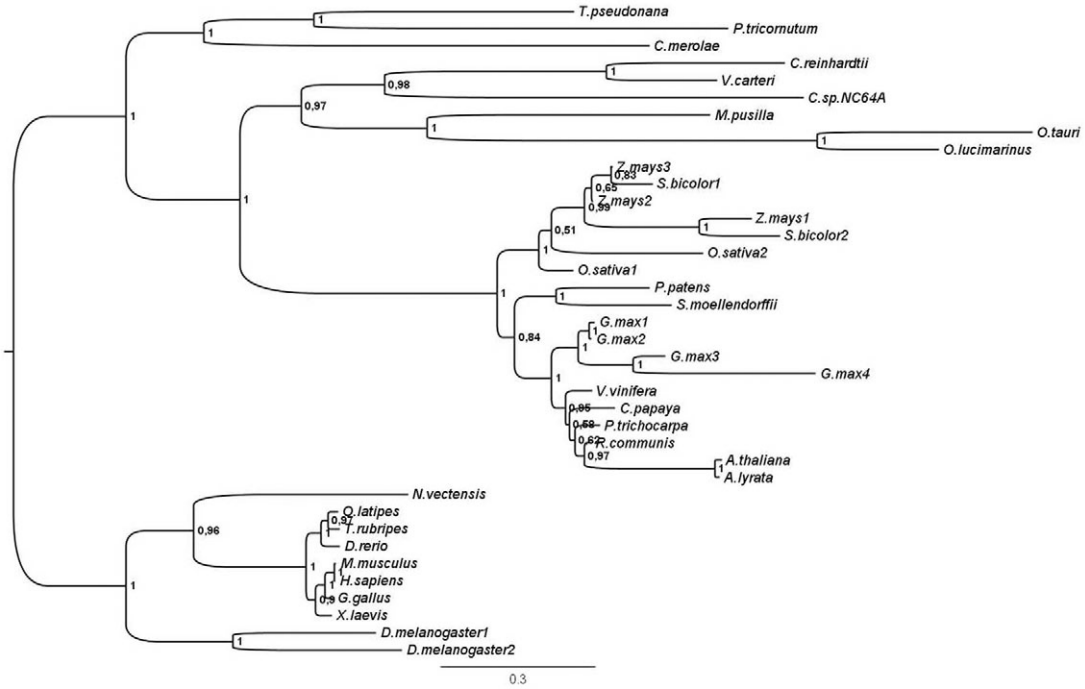

Fig. 1. Phylogenetic tree (Bayesian inference) of the FIE protein superfamily. Numbers at the nodes represent posterior probabilities. The tree was rooted at the branch separating multicellular animals from plants and algae. Only clear true-positive homologs from organisms for which the whole genome sequence had been determined were included. In the case of paralogy the proteins are labeled using index numbers.

60% similarity between *A. thaliana* and *H. sapiens*) across the millennia underlines its conserved structure and indicates its crucial function for higher eukaryotes (Fig. 1).

PpFIE-GUS accumulates in all meristematic cells and gametophytic cells undergoing fate transition

To determine PpFIE temporal and spatial accumulation during *P. patens* development, we generated five transgenic plants in which the *uidA* (GUS) reporter gene was inserted via homologous recombination, replacing the *PpFIE* stop codon. The resulting plants express a PpFIE-GUS fusion protein under control of its native promoter within the endogenous genomic environment.

The PpFIE-GUS staining pattern was identical among all five moss transgenic lines generated. None of these lines exhibited any obvious abnormalities as compared with wild-type plant morphology, nor were any changes detected in the course and timing of their life cycle. Therefore, we conclude that PpFIE function for developmental control in PpFIE-GUS plants was not impaired.

During the life cycle of wild-type *P. patens*, the fertilized zygote develops into a sporophyte consisting of a reduced seta and the spore capsule. Several days after spore dispersal, the wild-type haploid spores germinate forming the juvenile gametophyte, a branched filamentous tissue growing by apical cell division. This protonema tissue comprises two subsequently occurring cell types, the chloronemata and the caulonemata. The division of the apical cell produces protonemal filaments, whereas subapical cells may divide to produce either side branch initials or three-faced apical cells, the buds. These buds subsequently develop into the adult gametophyte, the leafy shoots (gametophores) that bear the sex organs (Cove et al., 2006; Cove and Knight, 1993; Decker et al., 2006; Reski, 1998a; Schaefer and Zryd, 2001).

No FIE was detected by GUS staining in the spores after dispersal, but the protein appeared during imbibition before spore germination (Fig. 2A,B). Upon germination (Fig. 2B,C) and throughout the protonema phase, a weak GUS staining was detected in the apical cells of caulonemata and chloronemata (Fig. 2D,E).

A strong GUS staining was visible at the time of transition from the juvenile to the adult gametophyte, marked by the transition to three-faced apical cells, the buds (Fig. 2F). Subsequently, strong GUS staining was consistently detected in the apical and lateral shoot apices of the leafy gametophores (Fig. 2G-J). In the course of the GUS staining process, the apical cell of the gametophore stained first, followed by the adjacent cells (Fig. 2I,H). During gametophore development, PpFIE-GUS staining decayed gradually from the apical cell towards differentiated leaves (Fig. 2H). Throughout lateral shoot formation, the PpFIE protein could be monitored as early as in a single cell designated to form a lateral shoot (Fig. 2J, arrowheads).

Upon transition from the gametophyte to the reproductive phase, GUS signals were associated with the male and female reproductive organs antheridia and archegonia, respectively. Before fertilization, GUS activity was detected in the developing archegonia (Fig. 2K,L), where staining was particularly strong in the unfertilized egg cell (Fig. 2L, see arrow), whereas the signal gradually decayed after fertilization (Fig. 2M-O). Likewise, GUS signals were also evident in the young antheridia carrying the spermatozoids (Fig. 2P), whereas no signal was detected upon sperm maturation (Fig. 2Q). After fertilization no GUS signal was detectable, neither in the developing embryo, nor in the mature sporophyte until the stage of spore formation (Fig. 2R). Here, GUS staining was only found during spore formation in the tetrads after meiosis (Fig. 2S) and was undetectable in mature spores (Fig. 2T).

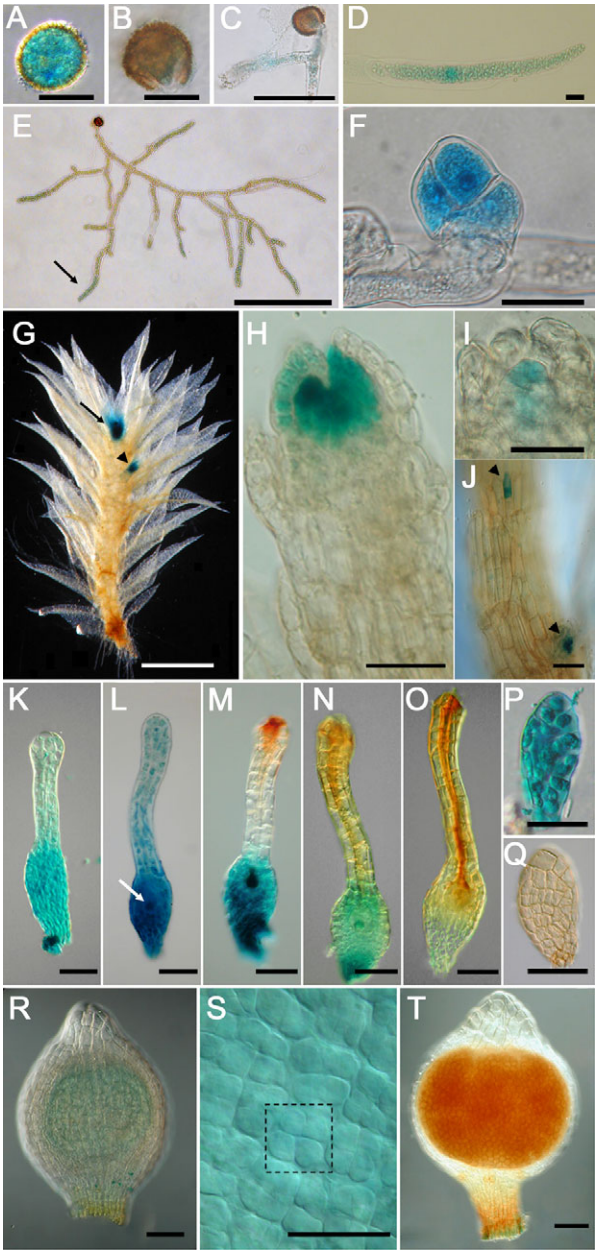

Fig. 2. PpFIE-GUS protein expression pattern determined by histochemical GUS assays. Analysis of *PpFIE-GUS* lines. (**A-C**,**E**) Germinating spores, arrow indicates the apical protonema cell. (**D**) The protenema tip. (**F**) A juvenile bud. (**G-J**) Leafy gametophore (G) and a subtending meristematic cell (J) exposed after removal of surrounding leaves. The apices of the main (arrow) and lateral shoots (arrowhead) are indicated in G and J. Staining of the apical cell; 5 hours in H and 2 hours in I. (**K-O**) A developing archegonia. GUS activity was detected in the egg cell as well as in the archegonia tissue; arrow in L marks the egg cell. (**P**,**Q**) Antheridia. (**R**,**T**) A sporophyte. (**S**) Spore formation; box shows tetrad after meiosis. Scale bars: 25 μm in A-C; 50 μm in I-Q,S; 100 μm in R,T,E; 1 mm in G.

PpFIE-GUS protein accumulation patterns reveal that the protein is present in all meristematic cells and gametophytic cells undergoing fate transition. The complex pattern of PpFIE-GUS localization suggests that it is involved in various developmental processes restricted to the haploid tissue during the *P. patens* life cycle.

ΔPpFIE mutants overproliferate three-faced apical cells

To study PpFIE function during *P. patens* development, we generated four independent disruptant mutant lines, designated *ΔPpFIE*, by gene targeting via homologous recombination (see Fig. S1A in the supplementary material). Proper integration of the disrupting construct into the *PpFIE* locus was verified by amplifying and sequencing the junction sites between the insert and the *PpFIE* locus (see Fig. S1A in the supplementary material). Single event of integration was determined by Southern blot analysis (see Fig. S6 in the supplementary material) and complete loss of *PpFIE* transcripts due to the disruption was confirmed by RT-PCR analysis (see Fig. S2A in the supplementary material). Subsequently, the phenotype of the *ΔPpFIE* mutants was monitored along with their development. *ΔPpFIE* protonemata and bud initials appeared indistinguishable from those of wild type (Fig. 3A,F). However, during the transition from juvenile to adult gametophytes, marked by the transition from apical cells to three-faced apical bud initials, *ΔPpFIE* mutants displayed dramatic morphological alterations (Fig. 3B,G). Whereas wild-type protonema gave rise to buds that further developed into leafy gametophores (Fig. 3B-E), *ΔPpFIE* developed a mass of cone-shaped buds (Fig. 3H, insertion) that grow further, thus harboring multiple apices (Fig. 3G-J). These mutant buds developed into cone-like leafless gametophores (Fig. 3J), whereas cones remote from the main apex initiated the differentiation of leaf primordia (Fig. 3J, insertion). Each main bud continued to repeatedly produce additional successive orders of primordia, until numerous apices accumulated on the main bud surface (Fig. 3I,J).

This aberrant phenotype indicates that *ΔPpFIE* mutant plants fail to restrict bud proliferation, resulting in ectopic initiation and differentiation of multiple bud apices. Furthermore, these buds failed to mature or to form normal gametophores and thus are prevented from reaching the reproductive phase. However, as some of the mutated structures resembled the morphology of a sporophyte (Fig. 3H, insertion), we tested whether such structures acquire sporophytic identity. To this end we have tested the expression of the genes *MKN2* and *MKN5*, which were shown to be expressed specifically in sporophytic tissue (Sakakibara et al., 2008). Our results show that *MKN2* and *MKN5* are expressed in *ΔPpFIE* protonemata bearing abnormal buds but not in wild-type protonemata bearing gametophores (Fig. 3N).

PpFIE is associated with the maintenance of pluripotency and cell reprogramming

P. patens cells have remarkable regenerative plasticity following tissue damage (Cove and Knight, 1993), indicating that cells retain their capability to exit their determined state after differentiation. During *P. patens* regeneration, all differentiated cells undergo division, giving rise to protonemata. As our results indicate that PpFIE is involved in maintaining the undifferentiated state of apical cells, we examined whether PpFIE protein accumulation correlates with the regeneration process. To induce this process, leaves from *PpFIE-GUS* transgenic lines were detached from mature gametophores and placed on BCD media for different periods of time up to 88 hours and subsequently stained for GUS (Fig. 4). Following detachment, we monitored the regeneration process at the surface of the distal leaf region, rather than the proximal marginal region of the incision site where wounding might affect *FIE* expression. Forty-eight hours after induction, single scattered cells expressing PpFIE-GUS were observed on the leaflets (Fig. 4C,I). At 72 hours some of the GUS-expressing cells were observed to

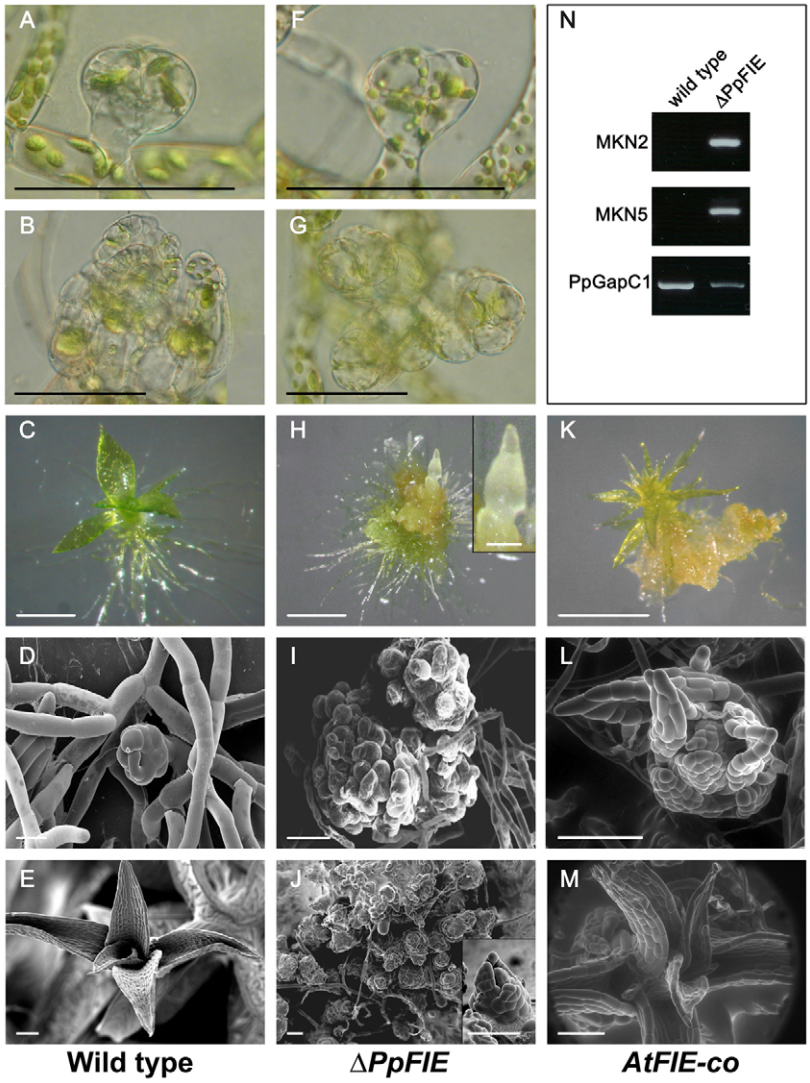

Fig. 3. Morphological analysis of wild-type, *ΔPpFIE* and *P. patens* plants in which *PpFIE* is replaced by the *AtFIE* gene. Wild-type (**A-E**) and *ΔPpFIE* (**F-J**) plants, and *P. patens* plants containing *AtFIE* instead of *PpFIE* (**K-M**). (A,F) Light microscopy of juvenile bud consisting of few cells. (B) Bud with leaf primordia. (G) *ΔPpFIE* bud bearing multiple apices. Two-week-old colonies of wild type (C), *ΔPpFIE* (H) and *AtFIE-co* (K) grown on BCD media. Insertion in H is magnified view. Color images were taken by light stereomicroscopy. CryoSEM images of wild-type bud (D), *ΔPpFIE* overproliferating buds (I,J) and a mature bud of *AtFIE-co* (L). Wild-type (E) and *AtFIE-co* (M) gametophore. (J) Mature *ΔPpFIE* overproliferating buds. (**N**) RT-PCR analysis of *MKN2* and *MKN5* gene expression in wild type and *ΔPpFIE*, as compared with *PpGAPC1* levels to monitor input amounts. Scale bars: 100 μm in A,B,E,F,G-J,L,M; 1 mm in C,H,K; 50 μm in D; 200 μm in insertion in H.

undergo cell division (Fig. 4K, arrows). After 88 hours protonema filaments emerged from cells in which GUS staining was visible (Fig. 4L, arrowhead).

In order to examine whether *PpFIE* transcription is upregulated during the regeneration process, wild-type leaves were detached and incubated as described above. RNA was purified from approximately 1000 leaves collected at time '0' and 72 hours after detachment. Quantitative RT-PCR analysis showed that the *FIE* transcript is upregulated at least twofold 72 hours after leaf detachment, as compared with time '0' (Fig. 4M).

PpFIE upregulation and the spatial and temporal pattern of accumulation of the protein during the regeneration process indicate that the epigenetic machinery is involved during cell reprogramming and acquisition of pluripotency.

A. thaliana FIE is able to complement *P. patens* FIE

To determine whether FIE protein function is conserved between bryophytes and angiosperms, we performed a cross-species complementation assay. To this end, transgenic *P. patens* lines were generated, in which the endogenous *PpFIE* was replaced via homologous recombination with an *AtFIE* cDNA driven by the native moss *PpFIE* promoter (designated *AtFIE-co*). Four independent *P. patens AtFIE-co* lines were isolated expressing *AtFIE* (see Fig. S1B and Fig. S2B in the supplementary material). All four *AtFIE-co* lines partially complemented the lack of the endogenous *PpFIE* (Fig. 3K-M, Fig. 5B). Similar to *ΔPpFIE* mutant lines, *AtFIE-co* lines exhibited abnormal buds with multiple primordial leaves at the early phase of growth (Fig. 3K). However, in contrast to *ΔPpFIE* plants, *AtFIE-co* lines were able to develop mature gametophores (Fig. 3L,M), although *AtFIE-co* lines exhibited distinct abnormal phenotypes. The distance between leaves along the gametophore was shorter, resulting in a denser appearance as compared with wild-type gametophores (compare Fig. 5A and 5B). At the apex of mature gametophores, clusters of shoot apices developed, bearing juvenile leaves (Fig. 5C, arrows). This phenomenon was also observed in lateral shoot apices (data not shown). Under reproduction-inducing conditions (Hohe et al., 2002), *AtFIE-co* lines failed to produce sex organs. The PpFIE-GUS accumulation pattern overlapped with the sites in which the above mentioned abnormalities were observed. The failure of AtFIE to support the proper development of reproductive organs could be due to the inability of AtFIE to recognize additional subunits present in the PcG complex at this particular *P. patens* developmental stage. These results demonstrate that *FIE* has been functionally conserved through evolution, thus allowing partial rescue of the *ΔPpFIE* mutant using *AtFIE*.

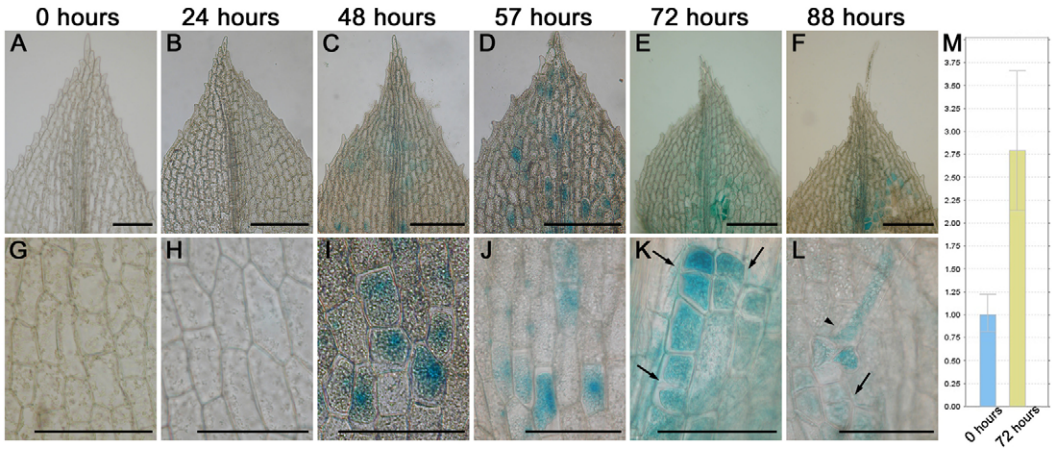

Fig. 4. PpFIE expression and protein accumulation in detached, somatic leaves. GUS staining of detached leaves from the gametophore, incubated in BCD medium for time intervals of 24, 48, 57, 72 and 88 hours. (**A-F**) PpFIE-GUS staining of gametophore leaves. (**G-L**) Magnification of the respective images. Dividing cells are marked with an arrow, differentiated protonema grown on the leaf in L is marked with an arrowhead. (**M**) Quantitative RT-PCR analysis of *PpFIE* expression at time '0' and 72 hours after leaf detachment. Scale bars: 0.1 mm.

PpFIE partially complements the gametophytic lesion of the *A. thaliana fie* mutant

The ability of *AtFIE* to rescue *P. patens* plants lacking PpFIE indicates that *AtFIE* can functionally recognize components of the *P. patens* PcG complex. Thus we performed the reciprocal experiment and analyzed whether PpFIE can functionally complement the absence of AtFIE in the *A. thaliana* PcG complex.

To this end, we tested whether PpFIE can rescue the aborted embryo of *A. thaliana* plants in which the female gametophyte contributes a *fie* mutant allele (Ohad et al., 1996; Ohad et al., 1999; Chaudhury et al., 1997). To test this possibility we established six independent transgenic lines expressing *PpFIE* under the *A. thaliana FIE* native promoter (*ProAtFIE:PpFIE*) as described by Kinoshita et al. (Kinoshita et al., 2001). To establish the complementation assay, we first crossed *ProAtFIE:PpFIE A. thaliana* lines as female recipients with pollen from heterozygous *FIE/fie* plants. F1 progenies from the six parents were selected for kanamycin resistance as an indication for the presence of the *ProAtFIE:PpFIE* transgene. These lines were than screened for the presence of the *fie* allele as determined by seed abortion (Ohad et al., 1996). In addition, the same heterozygous F1 plants (*FIE/fie*) hemizygous for the transgene (*PpFIE/~*; plant genotype designated as *FIE/fie,PpFIE/~*) were tested by DNA restriction analysis for the presence of the *fie* mutant allele, monitoring for the unique *Dra*I polymorphic site (see Fig. S3C in the supplementary material). The expression of the *PpFIE* transgene in these lines was confirmed by RT-PCR analysis (see Fig. S3B in the supplementary material). F2 progenies were collected and germinated, out of which *FIE/fie,PpFIE/~* plants were selected as described above. Complementation was assessed by scoring F3 progeny seed abortion ratio in siliques from individual F2 plants, derived from self pollination.

In the case that *PpFIE* would fully complement the *fie* mutant allele, one would expect that F2 plants carrying both alleles would display 25% seed abortion in the F3 generation (Ohad et al., 1999), in contrast to 50% seed abortion if no complementation occurs. Out of six independent lines, two displayed abortion of approximately 45% (Table 2, rows 1 and 2), which is significantly lower than 50% (χ^2 test, $P<0.001$), in which the morphology of aborted embryos was not different from *FIE/fie* aborted embryos. These results indicate that the bryophyte FIE protein partially complements for the absence of a functional *FIE* allele in the flowering plant female gametophyte, thus supporting early seed development and allowing embryo rescue.

An additional genetic approach was employed to determine whether *PpFIE* is able to complement the *A. thaliana fie* allele. The *A. thaliana fie* mutant allele causes embryo lethality when transmitted through the female parent, with 100% penetration (Ohad et al., 1996). Thus, the *fie* mutant allele is not transmitted by the female parent but only through the male. A cross between a *FIE/fie* female with a wild-type male will result in 100% wild-type F1 plants (Table 2, row 6). However, if *PpFIE* can compensate for *A. thaliana fie* lack of function in the female

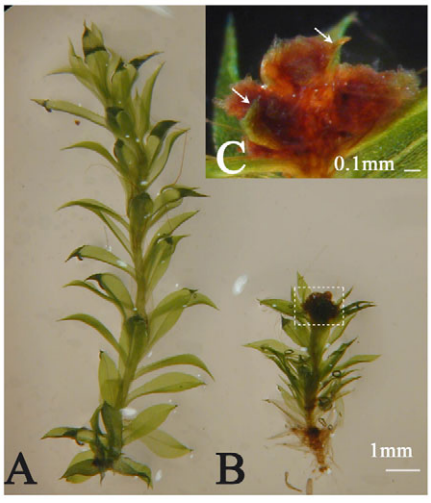

Fig. 5. Morphological analysis of mature gametophores from wild type and plants in which *PpFIE* is replaced by the *AtFIE* gene. (**A**) Wild-type adult gametophore. (**B**) Adult *AtFIE-co* gametophore exhibiting clusters of shoot apices bearing juvenile leaves at the shoot apex. (**C**) Magnification of an *AtFIE-co* apex (box in B). Arrows mark the leaflets that formed on the proliferated apex. The images were taken by light stereo-microscopy. Scale bars: 1 mm in A,B; 0.1 mm in C.

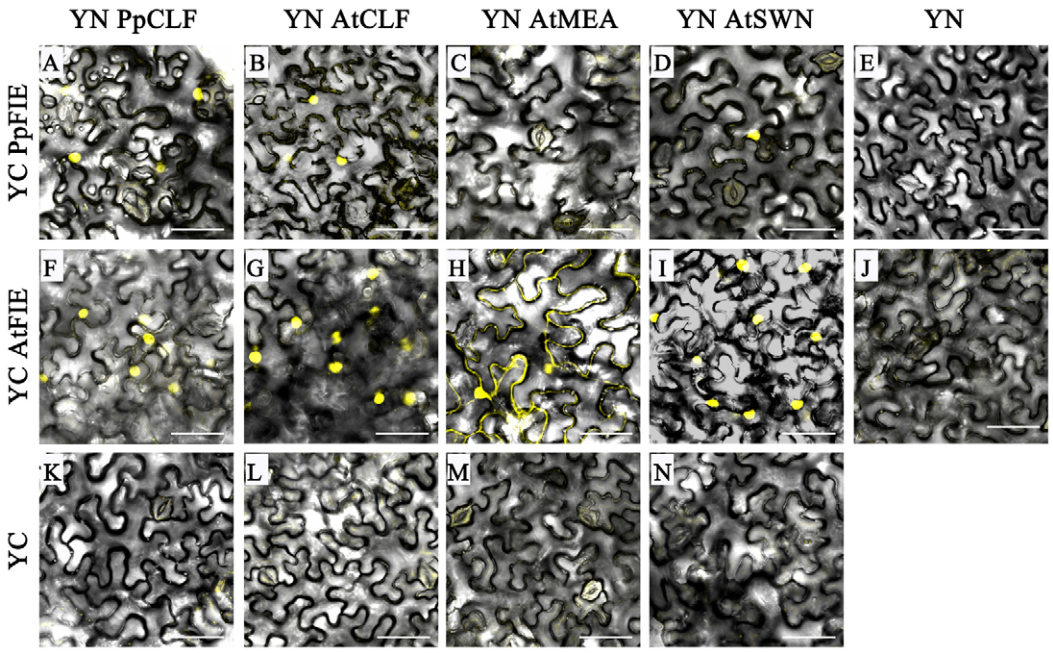

Fig. 6. PpFIE and AtFIE interact with PcG SET domain proteins in planta. BiFC analysis of in planta interactions between YC-PpFIE and the following: YN-PpCLF (**A**), YN-AtCLF (**B**), YN-AtMEA (**C**), YN-AtSWN (**D**) and YN-only as a negative control (**E**). As a positive control YC-AtFIE was tested with YN-PpCLF (**F**), YN-AtCLF (**G**), YN-AtMEA (**H**), YN-AtSWN (**I**) and YN-only as a negative control (**J**). Negative controls examining YC-only with YN-PpCLF (**K**), YN-AtCLF (**L**), YN-AtMEA (**M**) and YN-AtSWN (**N**). Localization was determined in the leaf epidermis of *Nicotiana benthamiana*. YFP fluorescence from single confocal sections is overlaid with Nomarsky differential interference contrast (DIC) images. Scale bars: 50 μm.

gametophyte, then a heterozygous *FIE/fie* female carrying the *PpFIE* transgene is expected to transmit the *A. thaliana fie* mutant allele to the next generation.

F1 progeny resulting from a cross between a *FIE/fie,PpFIE/~*female and a wild-type male (see Fig. S4A,B, column P and Fig. S4C,D in the supplementary material) carrying a GFP marker were screened by RFLP analysis for the presence of the *fie* mutant allele (see Fig. S4A,B, column F1 in the supplementary material). In addition, progenies obtained from self-fertilization of the above F1 plants were tested morphologically for seed abortion (see Fig. S4E in the supplementary material). Out of 67 F1 progenies, four carried the *fie* allele, whereas in the control experiment in which a *FIE/fie* female was crossed with the same wild-type male donor, all 192 resulting plants were homozygous for the *FIE* wild-type allele as expected. The presence of the GFP marker provided by the male donor assured that the outcrossing occurred properly.

Although *PpFIE* was able to facilitate the transmission of the female *A. thaliana fie* allele, none of the F3 progenies in the complementation experiment was homozygous for *A. thaliana fie*. Since the paternal *AtFIE* allele is apparently not expressed until late embryogenesis (Yadegari et al., 2000), we suggest that under the experimental conditions we have used PpFIE complementation is delimited to the early phase, during which PpFIE is provided maternally.

Based on our findings that PpFIE can partially complement the *A. thaliana fie* lesion at particular developmental stages, and that AtFIE can replace PpFIE at the bud stage, we conclude that there has been partial functional conservation of FIE during land plant evolution.

PpFIE and PpCLF PcG proteins interact in planta

In *A. thaliana*, FIE and SET domain proteins were shown to interact directly (Katz et al., 2004), as in the case for the homologous PcG proteins in *D. melanogaster*, *Mus musculus* and *H. sapiens* (reviewed by Berger and Gaudin, 2003; Hsieh et al., 2003; Simon and Tamkun, 2002). To test whether PpFIE and PpCLF are able to interact, as expected from their proposed function, we used the BiFC assay (Bracha-Drori et al., 2004). To this end we cloned the full-length cDNAs of *PpFIE* and *PpCLF*, each fused to either the N-terminal (YN) or C-terminal (YC) fragments of the YFP encoding

Table 2. Abortion rates of wild type, *A. thaliana fie* mutants and *A. thaliana fie* mutants complemented with *PpFIE*

Genotype	Abortion ratio	Abortion average ratio	χ^2	*P* value/H_0
PpFIE/~,FIE/fie line 1	469:586, 1:1	44.45	13.00	0.003 (50%)*
PpFIE/~,FIE/fie line 17	611:728, 1:1	45.59	10.4	0.001 (50%)*
FIE/fie	879:804, 1:1	52.22	3.34	0.068 (50%)
PpFIE/~,FIE/FIE	3:1162, 0:1	0.002	–	–
FIE/FIE – wild type	3:1328, 0:1	0.002	–	–
F1 of *FIE/FIE* female × *FIE/fie* male	2:880, 0:1	0.002	–	–

χ^2 test was used to determine whether the H_0 hypothesis of 50% seed abortion fit with the different genetic categories. Lines marked with * exhibit a significantly lower statistical rate of seed abortion than the expected 50%.

sequence. Members of each protein pair were transiently coexpressed via *Agrobacterium tumefaciens*-mediated transformation in leaf epidermal cells of *Nicotiana benthamiana*. YFP fluorescence was observed in cells expressing both YC-PpFIE and YN-PpCLF (Fig. 6A). No fluorescence was observed when expressing YC-PpFIE with YN only (Fig. 6E) or YN-PpCLF with YC only (Fig. 6K), both serving as negative controls. Interaction between both proteins was localized in the nucleus, which is in agreement with the known functions of the PcG in regulating chromatin structure (Dingwall et al., 1995; Francis et al., 2004; Paro and Hogness, 1991). The above result supports the hypothesis that PpFIE and PpCLF interact in vivo to form the core of a PcG complex.

The complementation tests described above suggest that both *P. patens* and *A. thaliana* FIE proteins interact with the respective PcG complex subunits in the other species. We next tested whether FIE proteins from either *P. patens* or *A. thaliana* were able to interact with their counterpart SET domain proteins. Using the BiFC assay we show that PpFIE can interact with AtCLF and AtSWN (Fig. 6B and 6D), but fails to interact with AtMEA (Fig. 6C). All interactions occurred in the nucleus. Whereas the interaction between PpFIE and PpCLF gave rise to a reconstitution of the YFP in almost all cells, the interaction with AtSWN appeared only sporadically. As negative controls, neither the SET domain proteins nor PpFIE or AtFIE interacted with the half-complementing counterpart YFP protein alone (Fig. 6K-N,E,J, respectively). In addition, we observed an interaction between AtFIE and PpCLF (Fig. 6F). These cross-species protein interactions are in agreement with the genetic complementation assays described above. *P. patens* FIE could interact only with *A. thaliana* CLF or SWN, which are SET domain proteins that have evolved earlier during plant evolution compared with AtMEA, the most recently derived gene among the *A. thaliana* SET domain family (Spillane et al., 2007).

DISCUSSION

PcG function has been conserved during plant evolution

The Polycomb group (PcG) complex controls gene expression profiles epigenetically. In this study we identified *P. patens* single-copy orthologs to the *A. thaliana* PcG complex core (*FIE*) and SET domain catalytic (*CLF*) subunits. Two lines of evidence indicate that *PpFIE* is a true functional ortholog of the PcG core subunit. First, even though the reciprocal complementation assays between *P. patens* and *A. thaliana* were limited to specific developmental stages, they demonstrate that the *FIE* genes have maintained their function during evolution. Second, our BiFC experiments show that PpFIE interacts with two *A. thaliana* SET domain PcG proteins, AtCLF and AtSWN.

Further studies will reveal the extent of functional conservation of PpFIE as a member of a transcriptional repressor complex (PRC2) alongside the *A. thaliana* life cycle. To this end, the ability of PpFIE to regulate AtFIE target genes, evident by either ChIP assay or the analysis of marker genes in vivo, could be applied.

Interestingly, PpFIE did not interact with AtMEA, which is the most recently diverged member in the SET domain protein family present in flowering plants (Chanvivattana et al., 2004; Spillane et al., 2007). Although all three *A. thaliana* SET domain proteins are expressed in the ovule (Chanvivattana et al., 2004; Goodrich et al., 1997; Grossniklaus et al., 1998; Wang et al., 2006; Xiao et al., 2003), MEA has a more prominent function in regulating central cell development (Grossniklaus et al., 1998; Kiyosue et al., 1999; Wang et al., 2006). The lack of interaction between PpFIE and AtMEA in the BiFC assay agrees with the partial complementation in *A. thaliana* by PpFIE, which could result from the inability of PpFIE and AtMEA to form a complex in the central cell. Consistent with this hypothesis, it was recently shown that the apomictic *Hieracium* (*H. piloselloides*) and non-apomictic *Hieracium* (*H. pilosella*) FIE proteins differentially bind members of the PcG complex, thus limiting their later function. The inability to bind particular partners was attributed to specific modifications at the protein level, which may lead to structural changes between the two proteins (Rodrigues et al., 2008).

In mammals, the PcG complex exerts its function by methylating H3K27 via the SET domain of the enhancer of zeste subunit (Czermin et al., 2002; Muller et al., 2002). Our analysis shows that PpFIE and the SET domain protein PpCLF interact in vivo (Fig. 6), which supports the possibility that in *P. patens* these proteins form a complex to perform a similar biochemical function as in mammals. This hypothesis is supported by the conservation of the catalytic PpCLF SET domain protein in *P. patens*, as this is also present in *A. thaliana*, *H. sapiens* and *D. melanogaster* (see Fig. S5 in the supplementary material).

FIE function in *P. patens*

During wild-type bud formation the apical cell divides, giving rise to a subset of three-faced apical daughter cells. After several consecutive cell divisions some of the peripheral cells along the surface of the young bud give rise to either leaf primordia (Schumaker and Dietrich, 1997) or meristematic cells that subsequently develop into lateral shoots. PpFIE-GUS protein levels are correlated with organ differentiation, as they gradually decline from the bud apex towards the region where leaf initials emerge, until the fusion protein can no longer be observed. Bud initiation in *ΔPpFIE* mutants is indistinguishable from wild type (Fig. 3A,F), thus PpFIE is not essential for the initiation of the three-faced apical cell. However, soon after the *ΔPpFIE* three-faced apical cell gives rise to several daughter cells, secondary buds are initiated, forming multiple apices in a repeatable pattern (Fig. 3G). These secondary buds initiate leaf primordia only after they grow further apart from the center of the main bud cluster (Fig. 3J).

Taking the PpFIE protein expression pattern and *ΔPpFIE* mutant phenotype together, we hypothesize that in wild-type *P. patens* FIE functions to maintain an undifferentiated state of meristematic cells within the apex. These cells are designated to become lateral shoots, probably by the epigenetic repression of gene expression. This repression can be relieved by as yet unknown signals, thus allowing the initiation of lateral shoot formation, as the apex is pushed upwards by its daughter cells. Thus the *ΔPpFIE* mutant phenotype might result from failure of the PcG complex to repress such meristematic cells from pursuing their default program to differentiate into lateral buds in due time.

Whereas in the wild type each bud gives rise to individual gametophores bearing leaves, *ΔPpFIE* mutants develop multiple bud apices that fail to form leafy gametophores. However, mutant buds continue to proliferate, until marginal buds generate undeveloped leaf primordia, thus being relieved from the repressed state they were in. These results indicate that the potential to develop leaves exists in these mutant apices (Fig. 3J, see insertion).

This phenomenon might not necessarily derive directly from the absence of PpFIE. Rather, it might result from the presence of multiple apices, leading to either overproduction of a signal inhibiting leaf differentiation, or it might be due to the dilution of a required signal consumed by the overproliferating buds situated within close vicinity.

The proposed role of PpFIE, as revealed from the *ΔPpFIE* phenotype and *PpFIE* expression pattern, is to maintain the pluripotency of the three-faced apical daughter cell. This fits well with the known function of PcG proteins during stem cell differentiation (for reviews, see Kanno et al., 2008; Pietersen and van Lohuizen, 2008). Mammalian EED and ESC PcG proteins, homologs of FIE and CLF, respectively, take part in the maintenance of stem cells by preventing their differentiation (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006). Mutant stem cells lacking EED cannot maintain their pluripotency and are prone to differentiate (Boyer et al., 2006; O'Carroll et al., 2001). Similarly, in the *A. thaliana fie* mutant the central cell, serving as a stem cell, proliferates and differentiates precociously into juvenile endosperm without fertilization (Chaudhury et al., 1997; Ingouff et al., 2005; Ohad et al., 1996; Ohad et al., 1999), demonstrating that the PcG complex represses the central cell from differentiating to endosperm prior to fertilization.

Thus, the role of FIE in bryophyte development, as revealed in this study, agrees with the basic function of the PcG complex to regulate self-renewal and inhibit differentiation. This developmental role is conserved in both the plant and the animal kingdom.

PpFIE function during reproductive development

ΔPpFIE mutants were unable to develop gametophores, thus remaining infertile. However, PpFIE-GUS was detected during sex organ and gamete formation, yet was excluded from the zygote once fertilization took place, implying that it has a possible function during sexual development. Furthermore, when replacing the native PpFIE with AtFIE, the resulting transgenic plants were able to develop gametophores. However, the apical cells of these plants failed to produce sex organs, and developed multiple apices on top of the gametophore apex instead. It is possible that at this developmental stage, AtFIE, in contrast to PpFIE, fails to recognize particular *P. patens* PcG subunits, which are crucial for proper development. Taken together, the above results indicate that PpFIE takes part in regulating the transition from the vegetative to the reproductive phase.

In support of the above we found that *ΔPpFIE* mutants form cone-like structures resembling young sporophytes. These mutants also express the sporophytic marker genes MKN2 and MKN5 (Sakakibara et al., 2008). Thus, these results indicate that PpFIE controls the transition of particular developmental stages along the *P. patens* life cycle, including the transition from the gametophytic to the sporophytic stage. This function of the PcG complex has been retained for more than 450 million years of plant evolution.

PpFIE function during redifferentiation

PpFIE is present mainly in apical meristematic cells but is absent from cells that have already differentiated, such as in mature leaves. Our data show that *PpFIE* expression and protein accumulation precede the regeneration processes in which leaf somatic cells are about to regenerate, giving rise to protonema (Fig. 4), as well as in wounded tissue (data not shown), and further support the proposed function in establishing self-renewal and pluripotency. We predict that leaf regeneration first requires de-differentiation of leaf cells, allowing them to pass through a meristematic state before they can acquire a new identity. Upregulation of PpFIE might allow the cell to enter into a reprogrammable state facilitated by chromatin remodeling, as expected from PcG function.

So far the analysis of plant cell de-differentiation and regeneration has been performed mainly with protoplasts (for a review, see Grafi, 2004). Our results now show that the PpFIE protein might serve as a novel molecular marker, highlighting cells that are about to divide and differentiate, thus serving as a tool to monitor these processes during the development of the entire plant and leaf regeneration.

Evolution of the PcG role in development

PpFIE is mainly expressed in gametophytic tissues. The transition of land plants from haploid dominancy to diploid dominancy may have evolved either by utilizing established developmental pathways acting within the gametophyte (the 'homologous' theory) or via novel genes and networks that arose specifically to support this process (the 'antithetic' theory) (Bennici, 2005). Support for the homologous model was suggested in the case of *PpRSL1* and *PpRSL2*, which control gametophytic rhizoid formation in *P. patens*, whereas their orthologs *AtRSL1* and *AtRHD6* control sporophytic root hair development in *A. thaliana* (Menand et al., 2007). Our results show that the PRC2 epigenetic machinery, in which FIE is included, was maintained through the evolution of land plants, repressing the differentiation of meristematic cells in the gametophyte. This is supported by the observation that PpFIE-GUS was detected only in haploid tissues.

During the evolution of land plants the PcG machinery was recruited to regulate the proper development of sporophytic programs, such as transition from the vegetative to the reproductive phase, flowering time and flower organ formation (for reviews, see Guitton and Berger, 2005; Hsieh et al., 2003; Kohler and Makarevich, 2006). This was accompanied by the diversification of the SET domain in *P. patens* to a gene family in *A. thaliana*, allowing the formation of diverse PcG complexes, as seen in the case of MEA, which is specialized in regulating endosperm development (Kawabe et al., 2007; Miyake et al., 2009; Spillane et al., 2007).

Thus, the findings described here highlight an example whereby, instead of harnessing pre-existing transcription factors, higher-order epigenetic machinery was recruited to regulate the expression of particular gene sets to control evolving developmental programs.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/14/2433/DC1

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