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Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in *Arabidopsis*

Mitsutomo Abe[1,*], Hiroshi Katsumata[1], Yoshibumi Komeda[1,2] and Taku Takahashi[1,†]

[1]Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan
[2]Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo, Tokyo 113-0033, Japan
*Present address: Department of Botany, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan
[†]Author for correspondence (e-mail: perfect@sci.hokudai.ac.jp)

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SUMMARY

In higher plants, the outermost cell layer (L1) of the shoot apex gives rise to the epidermis of shoot organs. Our previous study demonstrated that an 8-bp motif named the L1 box functions as a cis-regulatory element for L1-specific gene expression in the shoot system of *Arabidopsis*. We show here that *PROTODERMAL FACTOR2* (*PDF2*), a member of the HD-GL2 class of homeobox genes, is expressed exclusively in the L1 of shoot meristems and that recombinant PDF2 protein specifically binds to the L1 box in vitro. Although knockout mutants of *PDF2* and *ATML1*, another L1-specific HD-GL2 class gene sharing the highest homology with *PDF2*, display normal shoot development, the double mutant results in severe defects in shoot epidermal cell differentiation. This suggests that *PDF2* and *ATML1* are functionally interchangeable and play a critical role in maintaining the identity of L1 cells, possibly by interacting with their L1 box and those of downstream target-gene promoters.

Key words: *Arabidopsis*, Epidermis, Homeodomain, L1, *MERISTEM LAYER1*, *PROTODERMAL FACTOR2*, Shoot apical meristem

INTRODUCTION

The shoot apical meristem of angiosperms consists of clonally distinct cell layers. The outermost layer (L1) gives rise to the epidermis of the primary shoot by anticlinal cell division (for a review, see Howell, 1998; Lyndon, 1998). Although several genes that are expressed exclusively in L1 have been identified, the molecular mechanisms by which the L1 is established and maintained remain obscure. The *Arabidopsis thaliana MERISTEM LAYER1* (*ATML1*) gene encodes a transcription factor of the homeodomain-GLABRA2 (HD-GL2) class and is expressed specifically in the protoderm of developing embryos and the L1 of the shoot apex (Lu et al., 1996). Similar expression patterns have also been reported for its homologous genes in *Phalaenopsis* (Nadeau et al., 1996), maize (Ingram et al., 1999; Ingram et al., 2000) and rice (Ito et al., 2002; Yang et al., 2002). HD-GL2-class transcription factors are characterized by an amino-terminal homeodomain followed by a leucine-zipper motif (Ruberti et al., 1991) and a region similar to the mammalian StAR-related lipid-transfer (START) domain (Ponting and Aravind, 1999). The class includes *Arabidopsis* GL2, which promotes trichome differentiation in the shoot epidermis (Rerie et al., 1994) and suppresses root-hair formation in the root epidermis (Di Christina et al., 1996). Another member of the class, *Arabidopsis ANTHOCYANINLESS2* (*ANL2*), is involved in anthocyanin distribution in subepidermal cells (Kubo et al., 1999). Thus, HD-GL2-class genes have been implicated in regulating cell-layer-specific gene expression.

An important issue regarding transcription factors is identification of their target genes, but information on plant homeodomain target genes is limited. The *Arabidopsis WUSCHEL* (*WUS*) gene encodes a homeodomain protein belonging to a distinct class and functions in specifying stem cell identity in shoot and floral meristems (Mayer et al., 1998). Lohmann et al. have identified WUS-binding sites in the second intron of the floral homeotic gene *AGAMOUS* (*AG*) and revealed that WUS acts together with another transcription factor, LEAFY (LFY), as a direct activator of *AG* (Lohmann et al., 2001). *Arabidopsis* ATHB-2, which belongs to a subgroup of the HD-ZIP proteins and plays a role in the shade avoidance response in photomorphogenesis (Steindler et al., 1999), has been shown to bind its own promoter and create a negative autoregulatory loop (Ohgishi et al., 2001). Given the functional significance of individual members of the homeodomain proteins in plant growth and development, their target DNA sequences and downstream genes must be investigated further.

Arabidopsis PROTODERMAL FACTOR 1 (*PDF1*) encodes a proline-rich cell-wall protein that is expressed exclusively in the L1 of shoot meristems. By using progressive deletions of a promoter fragment of the *PDF1* gene, we previously showed that a cis-regulatory element named the L1 box is required for the L1-specific gene expression (Abe et al., 2001). The L1 box is well-conserved within the promoter regions of all L1-specific

genes analyzed so far. Furthermore, recombinant ATML1 specifically binds to the L1 box in vitro (Abe et al., 2001). Here we report on the characterization of the *PROTODERMAL FACTOR2* (*PDF2*) gene, which shares the highest homology with *ATML1* in the *Arabidopsis* genome. *PDF2* also shows L1-specific expression. *atml1 pdf2* double mutation results in severe defects in shoot epidermal cell differentiation, which are not observed in plants carrying mutations of only one of the genes. Our results suggest that *PDF2* and *ATML1* play a critical role in maintaining the L1 cells, possibly by regulating the expression of essential L1-specific proteins.

MATERIALS AND METHODS

Plant material

The wild-type control used in all experiments was the Columbia (Col-0) ecotype. Plants were grown in MS agar plates with 3% sucrose or on rock-wool bricks supplemented with vermiculite in growth chambers at 22°C under continuous light.

The T-DNA insertion alleles of *PDF2* and *ATML1* were isolated by screening a total of 60,480 T-DNA-tagged lines generated at the University of Wisconsin Knockout Arabidopsis Facility (Krysan et al., 1999). A primer specific for the T-DNA left border (LB, 5′-CATTT TATAA TAACG CTGCG GACAT CTAC-3′) was used in tandem with *PDF2*-specific primers (PDF2-F, 5′-ATATT GATCA GTGCC TTGAA GGAAA CCAA-3′ and PDF2-R, 5′-CTTGT TACTT GCTCC ACAAG AATCC CATT-3′) or *ATML1*-specific primers (ML1-F, 5′-TGGGA TATAC AGGCA GAAGA AAATC GAGA-3′ and ML1-R, 5′-ACCTT CTGCA AAAAC ACAAA CCAAA ACAT-3′). These T-DNA-tagged mutants had been created in the Ws ecotype, and were backcrossed at least three times into the wild-type Col-0 before analysis. Primers used for the mutant screen were also employed for PCR-based genotyping.

Cloning of *PDF2*

The partial *PDF2* cDNA fragment that was originally isolated by cDNA subtraction (Abe et al., 1999) was used as a probe to screen an *Arabidopsis* cDNA library derived from wild-type inflorescences (kindly provided by Drs J. Mulligan and R. Davis, Stanford University). Plaque hybridization and sequencing were performed as described previously (Abe et al., 1999). An almost full-length cDNA fragment was isolated and cloned as an *Eco*RI fragment into pBluescript II (Stratagene) to generate pPDF2-02. The 5′ end of the *PDF2* transcript was determined by using a 5′-RACE kit (Takara, Kyoto, Japan). One μg of total RNA from inflorescences was used as a template for first-strand cDNA synthesis. The full-length *PDF2* cDNA sequence has been deposited with GenBank under the accession number AB056455.

Expression analyses

Preparation of total RNA and RNA gel blot analyses were performed as described previously (Abe et al., 1999). For the *PDF2* probe, gel-purified *Eco*RI fragment of pPDF2-02 was labeled by the random-priming method. The *ATML1* probe was prepared from an *Eco*RI-*Pst*I fragment of the *ATML1* cDNA clone (Abe et al., 2001). Probes for *PDF1* and *EF1α* have been described (Abe et al., 1999). For reverse transcription (RT)-PCR, one μg of total RNA from aerial parts of 10-day-old seedlings was used as a template for first-strand cDNA synthesis with an oligo(dT) primer. Nucleotide sequences of PCR primers were PDF2-F and PDF2-R for *PDF2*, ML1-F and ML1-R for *ATML1*, PDF1-F (5′-TCCCT CTGGC TCACA TGGAA-3′) and PDF1-R (5′-GTCTC TAACT TGAGG GGTTG-3′) for *PDF1*, ACR-F (5′-TGAAG AACAC AATGC TCGAG-3′) and ACR-R (5′-TATCT CTTCC TCAAG ACTCC-3′) for *ACR4* (Tanaka et al., 2002), and STM-F (5′-ACAGC ACTTC TTGTC CAATG GCTT-3′) and STM-R (5′-GAAGA CCATA GCTTC CTTGA AAGG-3′) for *SHOOTMERISTEMLESS* (*STM*) (Long et al., 1996).

In situ hybridization was performed as described previously (Abe et al., 1999). To prepare a *PDF2* gene-specific riboprobe, the *PDF2* 5′-untranslated region (UTR) was amplified by PCR using pPDF2-02 as a template with *PDF2* 5′-specific primers (PDF2-5′F, 5′-CTGAG TGATC ATAGT CAATC ATCC-3′ and PDF2-5′R, 5′-AGTAG TGACT TCGGT ACCTG ACTT-3′), and was cloned as a *Bcl*I-*Kpn*I fragment into pBluescript II. Sense and antisense probes were generated by using T7 and T3 RNA polymerases with digoxigenin-11-UTP (Boehringer), respectively.

Gel shift assays

The *PDF2* protein-coding sequence was amplified by PCR using pPDF2-02 as a template with specific primers (PDF2P-F, 5′-GATCA TAGTG AATTC TCCAT AACAA-3′ and PDF2P-R, 5′-GAAAC CATAA CCAAG CTTAA TCCT-3′). The sequence was cloned as an *Eco*RI-*Hin*dIII fragment into pMAL-p2X (New England Biolab) to make a maltose-binding protein (MBP)-PDF2 fusion construct. The fusion protein was produced in *E. coli* TB1 and was purified according to the manufacturer's protocol. Gel retardation assays were performed as described previously (Abe et al., 2001).

Microscopy

For scanning electron microscopy (SEM), seedlings grown in MS plates were fixed in FAA (50% ethanol, 5% formaldehyde and 5% acetic acid), dehydrated in an ethanol series and critical point-dried using liquid CO_2. After coating with gold, samples were viewed using a Hitachi scanning electron microscope. For light microscopy, tissue samples were fixed in FAA, dehydrated in an ethanol series and embedded in Technovit 7100 resin (Kulzer GmbH, Wehrheim, Germany). Sections (10 μm) were stained for 2 minutes in an aqueous 0.1% Toluidine Blue solution.

Plant transformation

For the *35S::PDF2* sense and antisense constructs, gel-purified *Eco*RI fragment of pPDF2-02 was blunt-ended with T4 DNA polymerase and inserted into a *Sma*I site of pUC-NOS, a pUC18 derivative containing an *Sac*I-*Eco*RI fragment of the nopaline synthase gene (*NOS*) terminator from pBI101 (Jefferson et al., 1987). The resulting clones carrying the *PDF2* cDNA in sense or antisense orientation were used as *Xba*I-*Eco*RI fragments to replace the β-glucuronidase gene downstream of the cauliflower mosaic virus (CaMV) 35S promoter of the pBI121 Ti-vector (Clontech). The constructs were introduced into *Agrobacterium* strain C58C1 by electroporation and transformed into wild-type plants by the floral dip method (Clough and Bent, 1998).

RESULTS

Structure of *PDF2*

The *Arabidopsis* genome contains a large number of gene duplications (Vision et al., 2000). *ATML1* is located in the duplicated block 90b on chromosome 4, and there is a homologous gene in block 90a on the same chromosome. A partial cDNA clone of the homolog has been identified as a meristem-specific gene in our previous study (Abe et al., 1999). We named this gene *PROTODERMAL FACTOR2* (*PDF2*; GenBank accession number AB056455), and attempted to evaluate its functional relationship to *ATML1*. The longest *PDF2* cDNA isolated from an *Arabidopsis* inflorescence-derived cDNA library was 2742 bp. Comparison of the cDNA

with the corresponding genomic sequences and determination of a transcription start site of *PDF2* by the 5′-RACE method revealed a transcription unit of 12 exons including a 5′ untranslated exon located 1.8 kb upstream of the predicted translation start site (Fig. 1A). The open reading frame of *PDF2* encodes a putative protein of 743 amino acids (Fig. 1B). The predicted amino acid sequence shares 82.6% identity with ATML1 and shows high sequence similarity to other plant homeodomain proteins of the HD-GL2 class.

The 5′ promoter region of *PDF2* contains an L1 box which has been identified as a cis-regulatory element responsible for L1-specific gene expression (Abe et al., 2001), as shown in Fig. 1A. The L1 box of *PDF2* is preceded by a TTAATGG heptamer, a potential target sequence of WUS (Lohmann et al., 2001). The 100-bp region between the L1 box and the predicted transcription start site does not contain a putative TATA box. A similar arrangement of such elements is also found in the *ATML1* promoter region (Fig. 1A).

Expression pattern of *PDF2*

We examined the expression pattern of *PDF2* by RNA gel blot analysis. Total RNA samples prepared from each tissue were probed with a *PDF2*-specific probe. *PDF2* expression was detected mainly in flower bud clusters including shoot apices (Fig. 2A). *PDF2* mRNA was also present in leaves, stems, siliques and 10-day-old seedlings. We detected only a faint signal of *PDF2* expression in root tissue.

The spatial expression pattern of the *PDF2* gene was further examined by RNA in situ hybridization. *PDF2* mRNA was readily detected in the L1 layer of vegetative shoot meristems and the protoderm of leaf primordia (Fig. 2B). L1-layer-specific expression was also found in floral and apical inflorescence meristems (Fig. 2C). In developing flowers, *PDF2* mRNA was present in protodermal cells of primordia of all floral organs (Fig. 2D), but later the signal became restricted to the protodermis of developing ovules (Fig. 2E). *PDF2* expression was evenly distributed in the quadrant-stage embryo (Fig. 2F), but was confined to the outermost cell layer in the early globular-stage embryo (Fig. 2G). These expression patterns are indistinguishable from those of *ATML1* (Lu et al., 1996).

PDF2 binds to the L1 box

Our previous study demonstrated that the recombinant ATML1 protein can bind to the L1 box within the *PDF1* promoter in vitro (Abe et al., 2001). To determine whether PDF2 also interacts with the L1 box, we performed gel retardation assays using the recombinant PDF2 protein. PDF2 was produced as a fusion protein with a maltose-binding protein (MBP) in *E. coli* cells, purified and then tested for its binding ability to the L1 box probe. Complex formation was observed with the recombinant PDF2 but not with MBP alone (Fig. 3). Specific interaction of PDF2 with the authentic L1 box was confirmed with effective competition with the unlabeled probe and no complex formation with the mutated L1 box (Fig. 3).

Isolation of *pdf2* and *atml1* knockout mutants

To define the role of PDF2 in L1 layer differentiation, we screened a collection of 60,480 T-DNA tag lines (Krysan et al., 1999) for knockout mutants of the *PDF2* gene by using a PCR-based screening strategy, and we identified one allele

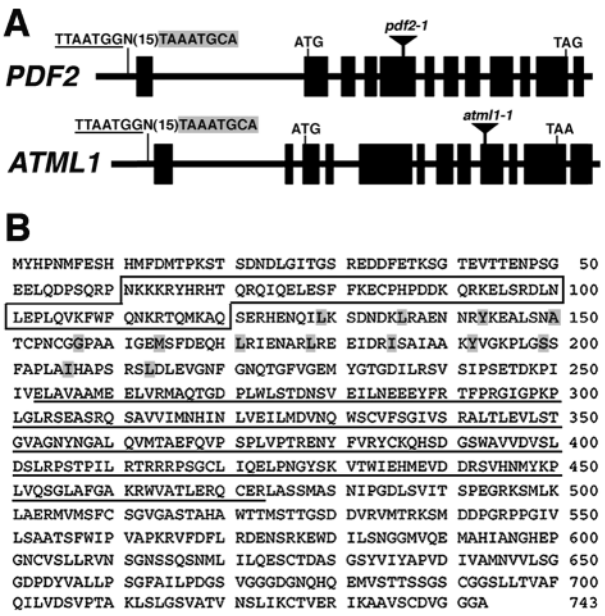

Fig. 1. Structure and deduced amino acid sequence of *PDF2*. (A) Genomic structure of *PDF2* and *ATML1*. Black boxes represent exons. Start ATG and termination codons are indicated. T-DNA insertion sites in *pdf2-1* and *atml1-1* are shown. The upstream regions of both genes contain L1 box sequences (gray boxes) associated with putative WUS target sites (underlined). (B) The predicted amino acid sequence of the PDF2 protein. The boxed area indicates a homeodomain. Amino acids forming a ZIP motif are shaded. A START domain is underlined.

Table 1. F_1 segregation of double mutants

Parental genotype	F_1 genotype						
Female×Male	*ppAA**	*ppAa*	*ppaa*	*Ppaa*	*PPaa*	*PpAA*	*PpAa*
ppAa×ppAa	26 (0)	48 (0)	17 (17)	–	–	–	–
Ppaa×Ppaa	–	–	14 (14)	39 (0)	22 (0)	–	–
ppAa×PPAA	–	–	–	–	–	9 (0)	7 (0)
PPAA×ppAa	–	–	–	–	–	8 (0)	10 (0)

**p*, the *pdf2-1* allele; *P*, the wild-type *PDF2* allele; *a*, the *atml1-1* allele; *A*, the wild-type *ATML1* allele.

Figures in parentheses indicate the number of plants showing abnormal seedling growth.

designated *pdf2-1*. The *pdf2-1* allele has a T-DNA insertion in the fifth exon of the *PDF2* gene (Fig. 1A) but exhibits no abnormal phenotype with respect to growth and morphology. We further screened for *ATML1* knockout mutants and isolated one allele designated *atml1-1*. The *atml1-1* allele has a T-DNA insertion in the ninth exon of the *ATML1* gene (Fig. 1A) but also displays normal growth and morphology.

We therefore examined the effect of double mutation. Because both loci are located on the same chromosome, we first selected plants that were homozygous for *pdf2-1* and heterozygous for *atml1-1* in the F2 population of the cross between *pdf2-1* and *atml1-1* mutants and examined segregation of genotypes in their selfed progeny (Table 1). We found that all plants showing severe defects in cotyledon development were homozygous for both *pdf2-1* and *atml1-1* (Fig. 4A). The same result was obtained in the progeny of plants that were

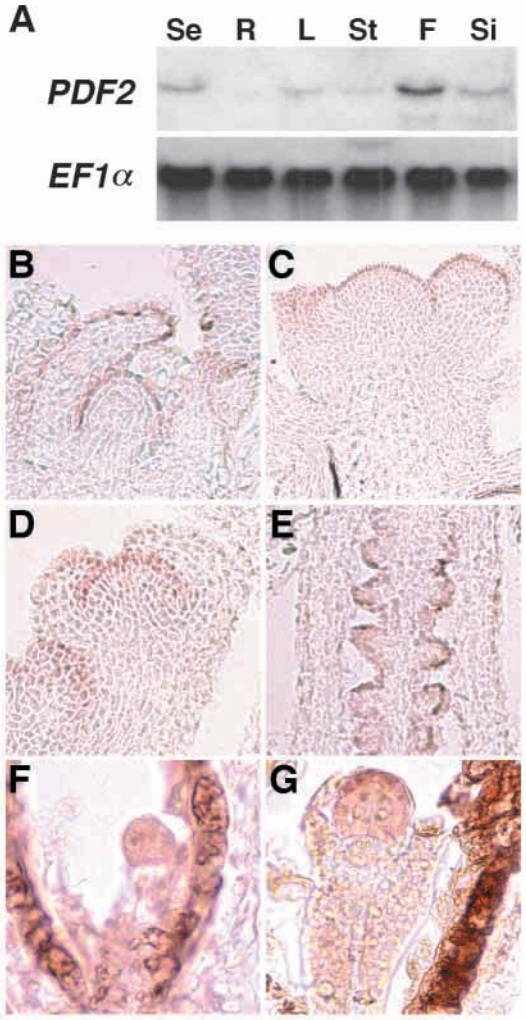

Fig. 2. Expression pattern of *PDF2*. (A) RNA gel blot analysis of *PDF2* mRNA. Total RNA was isolated from whole seedlings (Se), roots (R), leaves (L), stems (St), flower-bud clusters (F) and siliques (Si). Seedlings were grown for 10 days in MS plates. The *EF1α* probe was used as a control for loading and transfer. (B-G) In situ localization of *PDF2* mRNA. Longitudinal sections of a vegetative shoot meristem of a 10-day-old seedling (B), apical inflorescence and floral meristems (C), a flower bud (D), ovule primordia (E), a quadrant-stage embryo (F) and an early globular-stage embryo (G) were hybridized with the antisense *PDF2* RNA probe.

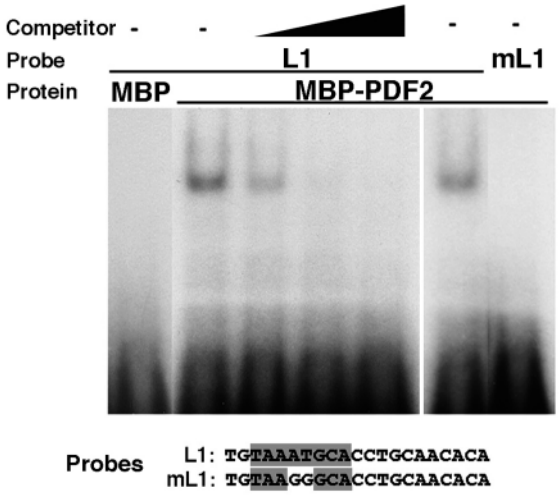

Fig. 3. Interaction of the *PDF2* gene product with the L1 box in vitro. Gel retardation assays were performed using a labeled 21-bp L1 box probe (L1) derived from the *PDF1* promoter, together with the MBP alone or the PDF2 fusion protein (MBP-PDF2) as indicated. The wedge indicates increasing amounts of 100-, 300- and 1000-fold molar excesses of unlabeled L1 probe DNA in competition assays. An assay using a mutated probe (mL1) with MBP-PDF2 is also shown.

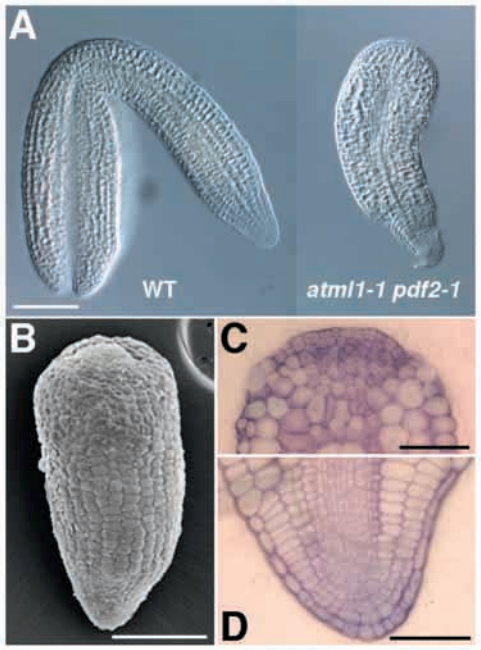

Fig. 4. Phenotype of the *atml1-1 pdf2-1* mature embryo. (A) Wild-type (WT) and double mutant mature embryos dissected from dry seed. Samples were cleared and examined under Nomarski optics. Scale bar: 100μm. (B) SEM view of the double mutant mature embryo. Scale bar: 100 μm. (C,D) Median sections through the double mutant shoot apex (C) and the double mutant root apex (D). Scale bars: 50 μm.

homozygous for *atml1-1* and heterozygous for *pdf2-1* (Table 1). Furthermore, in reciprocal crosses of plants that were homozygous for *pdf2-1* and heterozygous for *atml1-1* with wild-type plants, progeny that was heterozygous for *atml1-1* or homozygous for the wild-type *ATML1* allele segregated at a ratio of 1:1. The whole progeny was heterozygous for *pdf2-1* and showed normal growth. Thus, neither *PDF2* nor *ATML1* are required for germ cell development. SEM showed shoot apical dome-like structures in *pdf2-1 atml1-1* mature embryos (Fig. 4B), but sections revealed an irregular surface of the shoot apex and a lack of distinct cell layers (Fig. 4C). In contrast, the anatomy of the root apical meristem and root growth in *pdf2-1 atml1-1* were indistinguishable from the wild type (Fig. 4D and data not shown).

Although *pdf2-1 atml1-1* double mutants failed to survive after germination under greenhouse conditions, those grown in MS agar supplemented with 3% sucrose produced leaves that appeared moist, glossy and more pointed than wild-type leaves

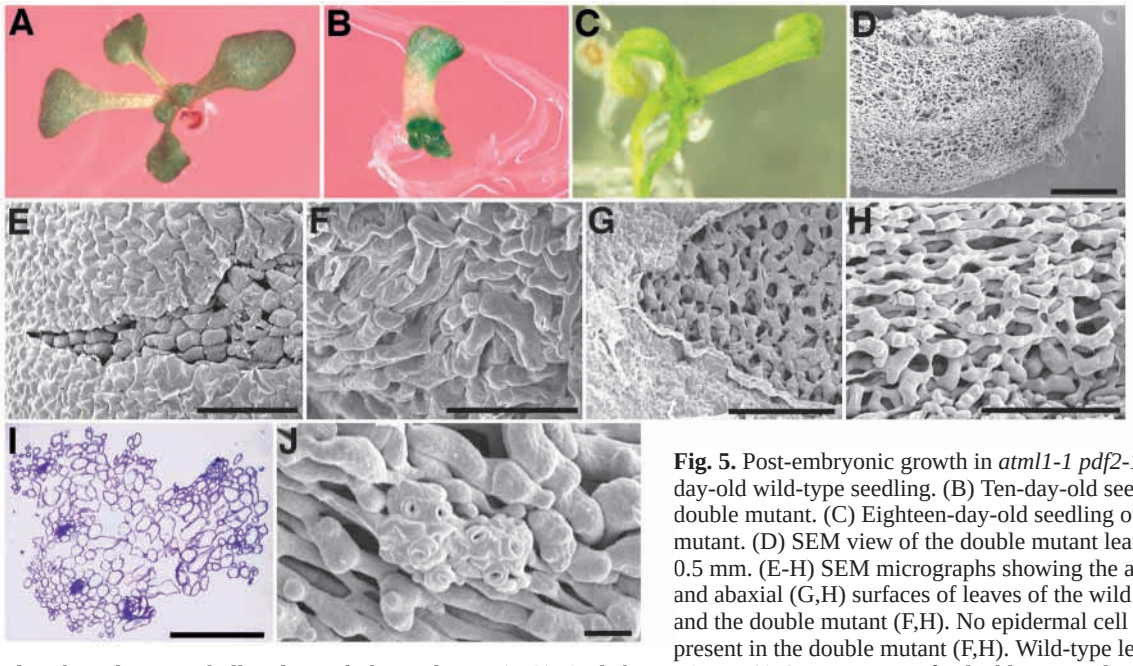

Fig. 5. Post-embryonic growth in *atml1-1 pdf2-1*. (A) Ten-day-old wild-type seedling. (B) Ten-day-old seedling of the double mutant. (C) Eighteen-day-old seedling of the double mutant. (D) SEM view of the double mutant leaf. Scale bar: 0.5 mm. (E-H) SEM micrographs showing the adaxial (E,F) and abaxial (G,H) surfaces of leaves of the wild type (E,G) and the double mutant (F,H). No epidermal cell layer is present in the double mutant (F,H). Wild-type leaves were partially peeled to show the mesophyll underneath the epidermis (E,G). Scale bars: 50 μm. (I) Cross-section of a double mutant leaf. Scale bar: 0.5 mm. (J) A cluster of stomatal guard cells found in a double mutant leaf. Scale bar: 10 μm.

(Fig. 5A-C). The surface of these leaves appeared to lack an epidermis (Fig. 5D). The adaxial and abaxial surfaces of *pdf2-1 atml1-1* leaves consisted of cells that resembled wild-type palisade and spongy mesophyll, respectively (Fig. 5E-H). In cross sections of double mutant leaves, vascular tissue but no epidermal cells were observed (Fig. 5I). However, we occasionally observed abnormal clusters of stomatal guard cells on both adaxial and abaxial leaf surfaces (Fig. 5J). So far, all double mutant plants died without having produced flowers. We also examined whether the *pdf2-1 atml1-1* double mutation affects transcript levels of L1-specific genes. RT-PCR analysis revealed that *pdf2-1 atml1-1* seedlings accumulated no transcripts of *PDF1* and *ACR4* (Tanaka et al., 2002), an *Arabidopsis* homolog of the maize *CRINKLY4* gene that encodes a receptor protein kinase implicated in leaf epidermis differentiation (Becraft et al., 1996) (Fig. 6).

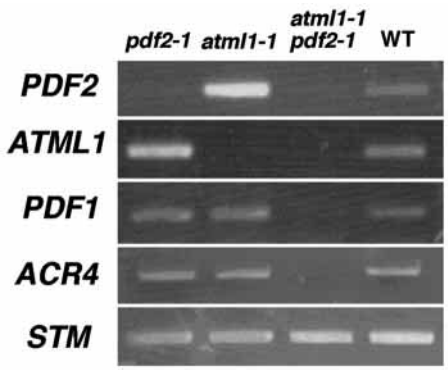

Fig. 6. Effect of *atml1-1 pdf2-1* on the expression of L1 layer-specific genes. RT-PCR analysis of the expression of *PDF1*, *PDF2*, *ATML1* and *ACR4* is shown. Total RNA was prepared from aerial tissues of 10-day-old mutant and wild-type seedlings. Expression of *STM* was also examined as a control.

PDF2 overexpression delays flowering

To examine the effect of *PDF2* overexpression, transgenic *Arabidopsis* plants in which the full-length *PDF2* cDNA is transcribed under the control of the CaMV 35S promoter were generated. We obtained 15 independent lines, and they were classified into two groups based on their phenotypes, one showing delayed flowering and another showing altered flower morphology. RNA gel blot analysis using total RNA from flower bud clusters revealed that the transgenic lines with the late-flowering phenotype accumulated much higher levels of *PDF2* mRNA than did the wild type, whereas the lines with abnormal flowers accumulated reduced levels of *PDF2* mRNA (Fig. 7). These results suggest that the late-flowering phenotype is caused by overexpression of *PDF2* and the abnormal flower development is a consequence of reduced *PDF2* expression.

When grown at 22°C under continuous illumination, the *PDF2* overexpression lines produced approximately 12 extra rosette leaves before bolting compared to wild-type plants (Fig. 8A,B). In situ hybridization revealed that *PDF2* mRNA was ectopically expressed throughout the shoot apex of these overexpression lines (Fig. 8C,D). However, the *PDF2* overexpression had no effect on the mRNA levels of *ATML1* and *PDF1* (Fig. 7).

Morphology of plants with reduced *PDF2* expression

We also generated plants with the antisense *PDF2* construct and found abnormalities in flower development (not shown) which were almost identical to those found in plants carrying the *35S::PDF2* construct but showing reduced *PDF2* mRNA

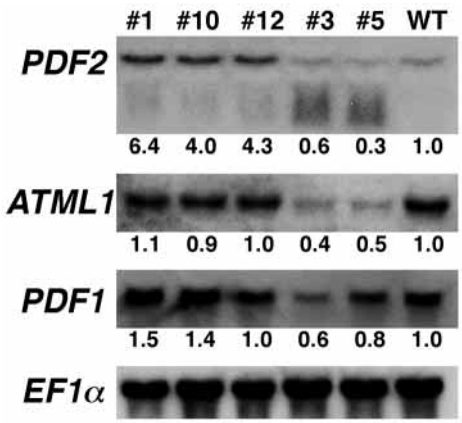

Fig. 7. Northern analysis of mRNA isolated from flower-bud clusters of wild-type and transgenic plants with the *35S::PDF2* construct. Lines 1, 10 and 12 represent *PDF2* overexpression plant lines, and lines #3 and #5 represent co-suppression plant lines. The relative mRNA levels are corrected with the *EF1α* mRNA as a control, compared with wild-type signal levels and indicated below the signals.

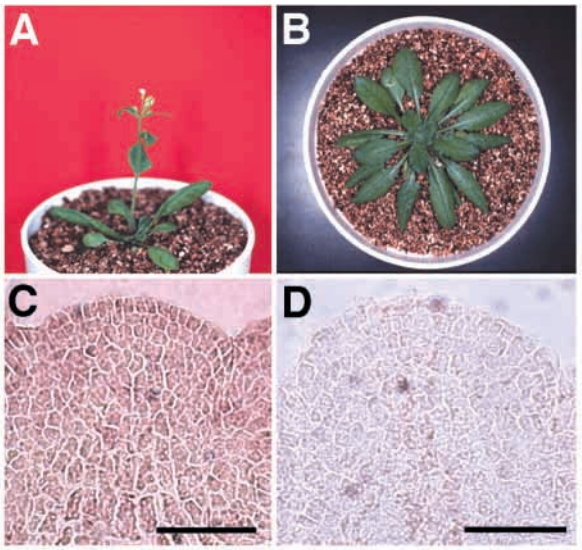

Fig. 8. Phenotypes of *PDF2* overexpression plants. (A) Thirty-day-old wild-type plant. (B) Forty-five-day-old *PDF2* overexpression plant (line 1). (C,D) In situ localization of *PDF2* mRNA in apical inflorescence meristems of *PDF2* overexpression plants (line 1). Longitudinal sections were hybridized with antisense (C) or sense (D) *PDF2* RNA probe.

accumulation (Fig. 9). Therefore, the abnormal flower phenotype observed in these lines with the *35S::PDF2* construct is most probably because of co-suppression of the endogenous gene with the introduced construct (for a review, see Depicker and Van Montagu, 1997). Interestingly, these co-suppression and antisense plants also showed reduction in *ATML1* and *PDF1* mRNA levels (Fig. 7 and not shown). Morphological aberrations were found in sepals and petals. Sepals of these plants were often fused along their edges toward the base, and petals were short and narrow (Fig. 9A,B). Although these flowers did not fully open and usually failed in self-pollination, we confirmed by enforcing crosses that the fertility was normal. SEM revealed that no phenotype was manifested at young bud stages (Fig. 9C,D). Examination of tissue sections revealed no obvious difference between the cell-layered structures of shoot apical meristems in the wild type and these transgenic lines (Fig. 9E,F).

The epidermal surface morphology of each floral organ was further examined by using SEM. At the mid-floral stage (stage nine according to Smyth et al.) (Smyth et al., 1990), petal epidermal cells of wild-type flowers are rounded and some of them are still under division (Fig. 9G). At maturity, the adaxial (interior) epidermal cells become more cone-shaped with straight cuticular ridges, whereas the abaxial (exterior) epidermal cells become cobblestone-like in appearance (Fig. 9I,K). Petal epidermal cells of the lines with reduced *PDF2* expression were noticeably large and rugged at stage nine (Fig. 9H). Later, they became tubular on both adaxial and abaxial sides (Fig. 9J,L). In contrast, wild-type sepals differentiated stomata cells and some extremely elongated cells in the abaxial epidermis (Fig. 9M). Sepals of the lines with reduced *PDF2* expression also contained stomata cells but fewer elongated cells in the abaxial epidermis (Fig. 9N). Epidermal cell morphology in anthers, filaments, carpels and other vegetative organs was indistinguishable between the wild type and these transgenic lines (not shown).

DISCUSSION

PDF2 encodes an L1 box-binding homeodomain protein

Identification of the *Arabidopsis GL2* and *ANL2* genes from their corresponding mutants has highlighted the significance of the HD-GL2 class homeodomain transcription factors in epidermal cell differentiation (Rerie et al., 1994; Di Christina et al., 1996; Masucci et al., 1996; Kubo et al., 1999). Moreover, some genes of this class cloned from *Phalaenopsis* (Nadeau et al., 1996), maize (Ingram et al., 1999; Ingram et al., 2000) and rice (Ito et al., 2002; Yang et al., 2002) have been found to show essentially L1- or protoderm-specific expression. Our data presented here demonstrate that the *PDF2* gene encodes a homeodomain protein with high similarity to ATML1 and shows expression exclusively in the L1 of vegetative, inflorescence and floral meristems. The PDF2 protein binds specifically to the L1 box sequence in vitro (Fig. 3), suggesting a regulatory role for L1 layer-specific gene expression. The presence of an L1 box within the upstream region of the *PDF2* gene itself suggests an autoregulation of *PDF2* expression.

High similarity of the homeodomain among the members of the HD-GL2 class raises the possibility that they may share the same L1 box as a target-binding site and regulate an overlapping set of target genes. Our searches of the GenBank database revealed that the *Arabidopsis* genome contains 16 genes of the HD-GL2 class (not shown). Four maize genes of this class are expressed in distinct regions of the embryonic protoderm during early development (Ingram et al., 2000). Functional specificity of these members in recognition of target genes might be conferred by temporal and spatial expression patterns of each individual and combinatorial interactions with other transcription factors of the same or different class to form a

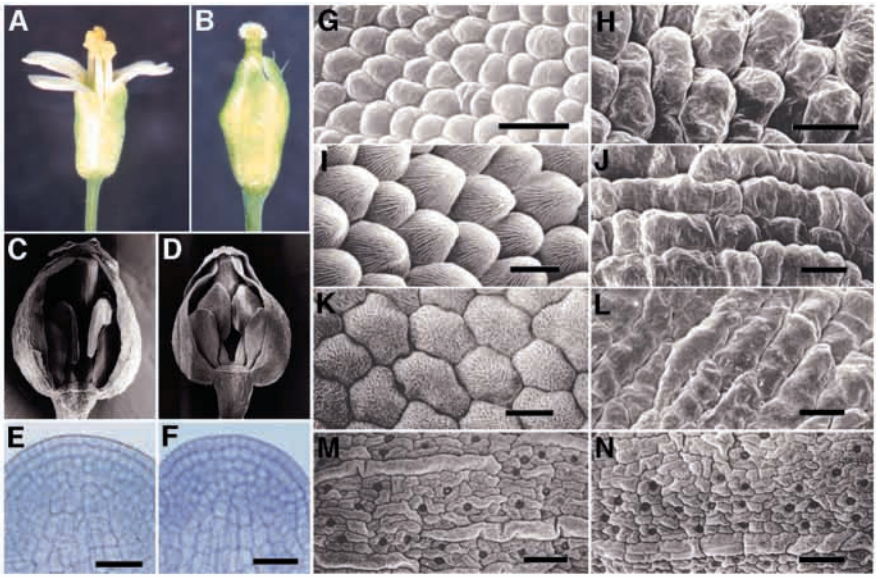

Fig. 9. Phenotypes of *PDF2* co-suppression plants. (A) Wild-type flower. (B) Flower of the *PDF2* co-suppression plant (line 3). (C,D) SEM views of a stage-nine flower of the wild type (C) and the *PDF2* co-suppression plant (D). The adaxial sepal has been removed. (E,F) Longitudinal sections of an apical inflorescence meristem of the wild type (E) and the *PDF2* co-suppression plant (F). (G-N) SEM views of epidermal cells of the wild type (G,I,K,M) and *PDF2* co-suppression plants (H,J,L,N). Epidermal cells of the adaxial side of stage-nine petals (G,H), the adaxial side of stage-13 petals (I,J), the abaxial side of stage-13 petals (K,L) and the adaxial side of stage-nine sepals (M,N) are shown. Scale bars: 25 μm in E,F; 10 μm in G-L; 50 μm in M,N.

transcription complex. The presence of a ZIP motif indicates potential dimer formation (Sessa et al., 1993). In contrast, many studies on *Drosophila* homeodomain proteins have suggested the importance of cofactor interactions in modulating DNA-binding site specificity, transcriptional activity or both (Popperl et al., 1995). The predicted START domain of PDF2 might also serve as a regulatory domain. Alterations in the START domain of *Arabidopsis* PHABULOSA and PHAVOLUTA, both of which belong to an HD-ZIP class distinct from the HD-GL2 class, may render the proteins constitutively active and cause a dominant phenotype of abnormal radial patterning in shoots (McConnell et al., 2001).

The effect of *PDF2* overexpression indicates that PDF2 is insufficient for ectopically activating *PDF1*, which contains an L1 box within the promoter region, suggesting the requirement for another factor(s) for its ectopic expression. It is possible that additional cis-elements other than the L1 box and their binding factors are involved in the activation of target genes. Consistent with this, chimeric promoter constructs consisting of tandemly repeated L1 box-containing fragments (21 bp x 4) of *PDF1* and a minimal 90-bp CaMV 35S promoter with a reporter gene did not activate the reporter (M. A., unpublished). The late-flowering phenotype is reminiscent of the one reported for *fwa* mutants, in which the *FWA* gene encoding a homeodomain protein of the HD-GL2 class is ectopically expressed (Soppe et al., 2000). In wild-type plants, *FWA* is expressed only in developing and germinating seeds (Soppe et al., 2000). It remains unknown whether FWA binds to the L1 box element or not. If so, both PDF2 and FWA might activate or repress a common target gene(s) to delay flowering.

PDF2 and *ATML1* function in shoot epidermal cell differentiation

Based on the phenotype, we conclude that the *pdf2-1 atml1-1* double mutant fails to differentiate epidermal cells. Surprisingly, this has little effect on the development of the mesophyll and the vascular cells, and on the establishment of dorsiventrality. Furthermore, expression of *PDF1* and *ACR4* was found to be downregulated in *pdf2-1 atml1-1*. These results

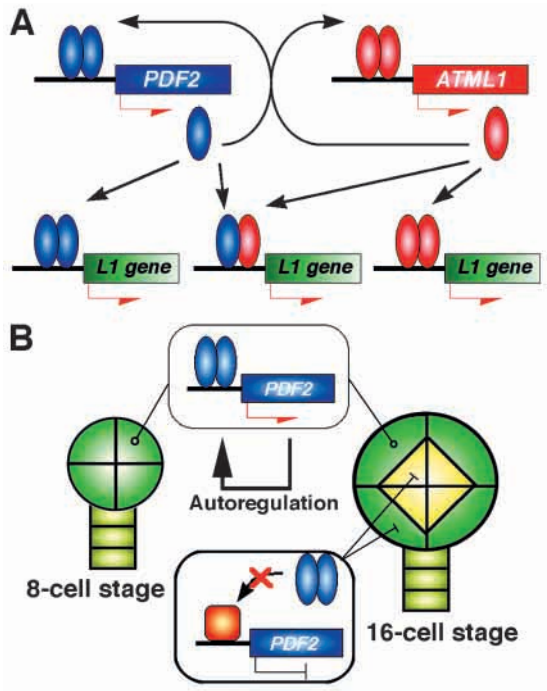

Fig. 10. (A) Schematic summary of regulatory functions of PDF2 (blue) and ATML1 (red) in L1 layer-specific gene expression. The illustration incorporates potential formation of homo- and heterodimers. Red arrows indicate transcriptional activation mediated by the interaction of PDF2 and/or ATML1 with the L1 box located upstream of each gene. (B) A hypothetical model in which the L1 layer is established and maintained in the embryo. Continuation of an autoregulatory loop for the *PDF2* and *ATML1* expressions is maintained in the protodermal layer, and an as-yet-unidentified suppressor(s) functions (orange) in switching off the loop in the basal and inner cells of the 16-cell embryo.

suggest that *PDF2* and *ATML1* activate L1-specific genes, and consequently serve in the differentiation of epidermal cells from the L1 of shoot meristems. The common location of *PDF2* and

ATML1 in a duplicated block of the *Arabidopsis* genome, together with the similarity of the expression pattern and the absence of abnormal phenotypes in single mutants, suggests that *PDF2* and *ATML1* are functionally interchangeable. The double mutant leaves have clusters of guard cells which are normally differentiated from protodermal cells with some spacing and mature basipetally (Pyke et al., 1991; Larkin et al., 1997). This suggests that the competence to form stomatal initials is present in *pdf2-1 atml1-1* plants.

Transgenic lines with reduced *PDF2* expression levels displayed the abnormal flower phenotype. The apparent contradiction between this phenotype and the absence of the abnormal phenotype in *pdf2-1* may be attributable to the difference in the *ATML1* expression level, which is not affected in *pdf2-1* but reduced in the transgenic lines. Given the fact that *PDF2* is not necessarily required for *ATML1* expression (Fig. 6), the reduced *ATML1* expression levels found in these transgenic lines may not be caused by reduced *PDF2* expression but by concurrent co-suppression of *PDF2* and *ATML1* with the *35S::PDF2* construct because of the high sequence similarity between them. The abnormal phenotype became manifest preferentially in the epidermis of sepals and petals, whereas no phenotype was detected in stamens, pistils and other vegetative organs. The total amount of *PDF2* and *ATML1* gene products in the transgenic lines could be still sufficient for normal development of these organs even though some genes, including *PDF1*, would be affected. The critical requirement for the PDF2 or ATML1 function in sepal and petal epidermal cell differentiation might be in agreement with the predominant contribution of L1-derived cells in sepals and petals in *Arabidopsis* (Jenik and Irish, 2000).

How is the L1 layer established and maintained?

In summary, our results suggest that both PDF2 and ATML1 function in activating L1 layer-specific genes through interaction with the L1 box and consequently serve in the differentiation of epidermal cells from the L1 layer of shoot and floral meristems (Fig. 10A). As is the case with *ATML1*, the 5′ promoter region of *PDF2* contains an L1 box (Fig. 1A), which may suggest a positive feedback loop of *PDF2* expression. Because *PDF2* and *ATML1* are expressed in the quadrant-stage embryo (Lu et al., 1996; Sessions et al., 1999) (Fig. 2F), such a feedback loop would seem to necessitate negative regulators that suppress expression in the basal and inner cells at the 16-cell stage (Fig. 10B). Although both of the *PDF2* and *ATML1* promoter regions contain a potential target site of WUS (Fig. 1A) whose expression starts at the four apical inner cells in 16-cell embryos (Mayer et al., 1998), it remains to be determined whether the suppression of the autoregulatory loop of *PDF2* and *ATML1* involves WUS-like transcription factors. Furthermore, the mechanism of the initial onset of *PDF2* and *ATML1* expressions before the eight-cell stage remains unknown. Identification of additional regulatory molecules will be the next important step to elucidate the mechanisms of the establishment and maintenance of the L1 layer in higher plants. Phenotypes similar to that of *pdf2 atml1* have been reported for *gurke* (Torres-Ruiz et al., 1996) and *tumorous shoot development* (Frank et al., 2002) mutants of *Arabidopsis*. Detailed analysis of these mutated genes may provide additional clues.

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