ATML1 promotes epidermal cell differentiation in Arabidopsis shoots

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SUMMARY
Molecular mechanisms that generate distinct tissue layers in plant shoots are not well understood. ATML1, an Arabidopsis homeobox gene, is expressed in the outermost cell layer, beginning at an early stage of development. The promoters of many epidermis-specific genes, including ATML1, contain an ATML1-binding site called an L1 box, suggesting that ATML1 regulates epidermal cell fate. Here, we show that overexpression of ATML1 was sufficient to activate the expression of epidermal genes and to induce epidermis-related traits such as the formation of stomatal guard cells and trichome-like cells in non-epidermal seedling tissues. Detailed observation of the division planes of these ectopic stomatal cells suggested that a near-surface position, as well as epidermal cell identity, were required for regular anticlinal cell division, as seen in wild-type epidermis. Moreover, analyses of a loss-of-function mutant and overexpressors implied that differentiation of epidermal cells was associated with repression of mesophyll cell fate. Collectively, our studies contribute new information about the molecular basis of cell fate determination in different layers of plant aerial organs.

KEY WORDS: Epidermal cell differentiation, Mesophyll cell differentiation, HD-ZIP class IV transcription factor

INTRODUCTION
In plants, cotyledons and leaves consist of different cell layers that include the epidermis, mesophyll and vascular tissues. The epidermis is a single layer of cells that covers the plant body. Leaf epidermal cells produce the cuticle, a hydrophobic barrier composed of lipids and waxes, that reduces water loss and protects plants against entry of pathogens (reviewed by Samuels et al., 2008). There are three types of leaf epidermal cells: jigsaw-puzzle-shaped pavement cells, hair cells (trichomes) and guard cells. These epidermal cells exhibit mostly anticlinal cell divisions, where the plane of division is perpendicular to the organ surface. Most epidermal cells except for guard cells lack chloroplasts, whereas inner green mesophyll cells develop chloroplasts and photosynthesize. Despite their important roles in plant development, the molecular mechanisms that generate distinct tissue layers in plant shoots are not well understood.

ARABIDOPSIS THALIANA MERISTEM LAYER 1 (ATML1) was the first transcription factor whose expression was shown to mark the outermost cell layer of the shoot (Lu et al., 1996; Sessions et al., 1999). ATML1 belongs to the HD-ZIP class IV homeodomain protein family, which is characterized by a StAR-related lipid-transfer (START) domain and a zipper-loop-zipper (ZLZ) motif (Schrick et al., 2004). Mutations in ATML1 and its closest homologue PROTODERMAL FACTOR 2 (PDF2) caused the formation of leaves that lack an epidermis, suggesting that they are required for epidermal cell differentiation (Abe et al., 2003). Importantly, several ATML1 homologues, as well as other epidermis-specific genes involved in cuticle biosynthesis, contained a cis-regulatory element called the L1 box in their promoters. The L1 box was first discovered as an ATML1-binding site, and other HD-ZIP class IV transcription factors were also shown to bind to the L1 box in vitro, suggesting that ATML1 and its homologues may promote epidermis differentiation through the regulation of the L1 box-containing genes (Abe et al., 2001; Nakamura et al., 2006). However, it is unclear whether ATML1 alone is sufficient to confer epidermis identity to non-epidermal cells.

To gain further insight into the mechanisms of ATML1-mediated regulation of epidermal cell fate, we performed gain-of-function experiments in combination with analyses of a loss-of-function mutant and provided evidence that ATML1 is a master regulator for shoot epidermis identity.

MATERIALS AND METHODS

Plant materials and growth conditions

proATML1-nls-3xGFP, atm1-1-pdfl2-1 carrying proATML1-nls-3xGFP, STOMAGEN-GUS, GL2-GUS (CS8851) and KAT1-GUS (CS3763) lines have been described previously (Nakamura et al., 1995; Szymanski et al., 1998; Takada and Jürgens, 2007; Kondo et al., 2010). proATML1-nls-3xGFP was used as the wild type.

For estradiol treatment, plants were germinated and grown on Murashige and Skoog (MS)-Phytagel plates containing 10 µM β-estradiol. β-Estradiol dissolved in dimethyl sulphoxide (DMSO). The same volume of DMSO was added to MS media for control experiments.

Plasmid construction and transgenic plants

The G10 promoter in the pER8 vector was replaced with the promoter region of arabidopsis meristem layer 1 (AtRPS5A) from –1571 to +113 relative to the transcription start site (RUSSA/pER8) (Zuo et al., 2000; Weijers et al., 2001). The ATML1-coding sequence was amplified by PCR using primers 4925 and 4926 (primer sequences are listed in supplementary material Table S1) and inserted into the XhoI and SpeI sites of the RPS5A/pER8 vector (proRPS5A-ATML1/pER8).

proRPS5A-ATML1/pER8 was used to transform a proATML1-nls-3xGFP line (Takada and Jürgens, 2007). Eight of 35 transgenic lines exhibited abnormal phenotypes when grown in the presence of 10 µM estradiol. Four of the eight lines carried T-DNA insertions at a single locus. Three of the four lines, which showed overexpression of ATML1, were used for expression analysis. The three lines chosen for subsequent analyses were designated lines #7, #24 and #35. These transgenic plants did not show an abnormal phenotype in the absence of estradiol.
In situ hybridization

In situ hybridization using digoxigenin-labeled RNA probes was performed as described previously (Lincoln et al., 1994). The templates for RNA probes were constructed by cloning a 598 bp region of ATML1 and a 502 bp region of FDH that were amplified by PCR using primers 6895 and 7021 for ATML1, and primers 7614 and 7615 for FDH into the EcoRV site of pBluescript (Stratagene, La Jolla, USA). RNA probes were synthesized using DIG RNA labeling mix reagent (Roche, Basel, Switzerland). Control experiments using sense probes gave no signal above background (data not shown).

Histological analysis

For histological sections, samples were embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) and cut with a microtome. For the observation of unfixed tissues in sections, seedlings or leaves were embedded in 5% agar, and 40-60 µm sections were prepared on LinearSlicer PRO7 (Dosaka EM, Kyoto, Japan).

Analysis of division planes

Division planes were examined in the mature leaves of 14-day-old line #7 seedlings. Forty-eight sections from seven independent leaves were observed, and division planes of ectopic stomata were categorized as either ‘anticlinal’, ‘periclinal’ or ‘oblique’, judging from their angles relative to the nearest adaxial or abaxial surface of the leaves (anticlinal, 0-30° relative to the surface; periclinal, 60-90°; oblique, 30-60°).

Quantitative RT-PCR analysis

Total RNA was extracted from 7-day-old seedlings using the RNeasy Plant Mini kit (Qiagen, Venlo, The Netherlands). Total RNA was treated with amplification grade DNase I (Invitrogen, Carlsbad, USA) and reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, USA). Real-time PCR reactions were performed using FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, USA). Transcript levels of endogenous ATML1, PDF2, HOMEODOMAIN GLABROUS2 (HDG2), FIDDLEHEAD (FDH) and ECERIFERUM 5 (CER5) were increased with estradiol treatment compared with the wild-type (Fig. 1E; supplementary material Fig. S1C,D). In these three lines, transcript levels of PROTODERMAL FACTOR 1 (PDF1) did not increase significantly in lines #7 and #35 (Abe et al., 1999).

Next, we examined the spatial pattern of ATML1 promoter activity using the proATML1-nls-3xGFP reporter line (Takada and Jürgens, 2007). Ectopic ATML1 promoter activity was detected in the inner cells of cotyledons and hypocotyls of the ATML1-overexpressing seedlings, indicating that ATML1 induced its promoter activity in internal tissues (5 out of 5 seedlings for line #7 and 7 out of 7 for line #35; Fig. 2A,B). In situ hybridization experiments also revealed that epidermis-specific FDH expression expanded to the inner tissues of cotyledons and hypocotyls in ATML1-overexpressing plants (5 out of 7 samples for line #7; Fig. 2E,F). These results showed that constitutive overexpression of ATML1 induced epidermis-specific genes in the inner tissues of the seedlings. Conversely, expression of epidermis-specific genes decreased in the atml1;pdf2 mutant, whereas expression of the central domain-specific ZWILLE (ZLL) was not significantly changed (Mousian et al., 1998; Lynn et al., 1999; supplementary material Fig. S2).

RESULTS AND DISCUSSION

Constitutive expression of ATML1 increased the expression of epidermis-specific genes

To examine the effects of ATML1 expression on epidermal cell specification, we generated transgenic lines for estradiol-inducible overexpression of ATML1 (Fig. 1B,C). We generated seven lines showing arrested seedling development when germinated and grown in the presence of 10 µM β-estradiol (Fig. 1B,C). In these plants, root growth was arrested and shoot apical meristem activity was also impaired with seedlings only producing small leaf primordia (Fig. 1B,C). The majority of the seedlings were small and accumulated anthocyanin. Three representative lines, carrying T-DNA insertions at a single locus, had relatively stronger (lines #7 and #24) or weaker (line #35) expression of ATML1 in the presence of β-estradiol (Fig. 1D; supplementary material Fig. S1A,B). We examined the expression of representative L1 box-containing genes that were uniformly expressed in the shoot epidermis (Fig. 1E; supplementary material Fig. S1C,D).

Fig. 1. Estradiol induced constitutive expression of ATML1. (A-C) Phenotypes of 7-day-old seedlings of the wild type (A), and inducible lines #7 (B) and #35 (C), grown in the presence of 10 µM estradiol. Scale bars: 0.5 mm. (D,E) Real-time RT-PCR analysis of L1 box-containing genes. Values are the mean±s.e.m. of three biological replicates. Data are normalized to the amount of IPT9 (Miyawaki et al., 2004). (D) ATML1 expression in 7-day-old seedlings of estradiol-inducible lines (#7 and #35) and the wild type (WT) grown in the absence (−) or presence (+) of 10 µM estradiol. (E) Expression of endogenous ATML1, PDF2, HDG2, FDH, CER5 and PDF1 in 7-day-old seedlings of estradiol-inducible lines (#7 and #35) and the wild type (WT) grown in the presence of 10 µM estradiol. Expression in the wild type was set to 1. Asterisks indicate a statistically significant increase relative to the wild type (unpaired one-tailed t-test; P<0.05).

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Collectively, our results indicate that ATML1-mediated positive regulation of epidermis-specific genes indeed occurs in planta. Moreover, our results show that ATML1 does not require epidermis-specific co-factors, such as ligands and other transcription factors for its activity.

Ectopic expression of ATML1 induced epidermis-related traits in the inner tissues of the cotyledons and leaves

Next, to examine whether ATML1 can confer epidermis identity to non-epidermal cells, we performed histological analyses. Overexpression of ATML1 induced stomata-like structures in the inner cells of the cotyledons in seven independent lines (Fig. 3B,C; supplementary material Fig. S3C,F,G,H,I). Ectopic guard cell-like cells expressed the guard cell marker KAT1-GUS, suggesting that these cells had guard cell identity (Fig. 3D) (Nakamura et al., 1995).

To determine the effects of overexpression of ATML1 on leaf development, the estradiol-inducible lines were germinated on estradiol-free MS plates and transferred to 10 µM estradiol-containing plates after 5 days. Young leaves that developed after estradiol treatment were narrower and malformed (data not shown). As in the case of cotyledons, ectopic guard cells were formed in the inner tissues of these narrow leaves (Fig. 3G,H,I; supplementary material Fig. S3D,E). Ectopic stomata in leaves were rarely clustered, comparable with the pattern seen for wild-type epidermis (P=0.22, Pearson’s chi-square test), whereas clustering of ectopic stomata was observed in the cotyledons (supplementary material Fig. S3C). Although the typical three-branch trichomes, another characteristic of leaf epidermis, were not found in the inner tissues, large cells expressing the trichome marker GL2-GUS were observed in the inner cells of these leaves (Fig. 3F,H,I; four out of six leaves from independent plants). Surprisingly, overexpression of ATML1 induced ectopic ATML1 promoter activity and ectopic guard-cell marker expression in the roots (supplementary material Fig. S4), suggesting that ATML1 was able to confer a shoot epidermal fate to root cells.

Formation of ectopic stomata and GL2-positive cells indicates that ATML1 is not merely a positive regulator of a specific cell type in the epidermis, in contrast to the cases of overexpression of FAMA, a positive regulator of guard cell fate (Ohashi-Ito and Bergmann, 2006). Rather, ATML1 appeared to induce pluripotent protodermal cells that can produce the several different cell types found in the shoot epidermis.

Epidermal cell fate was not sufficient to induce anticlinal cell division

One of the characteristics of epidermal cells is their anticlinal cell division. Accordingly, epidermis-deficient mutants abolished
development 140 (9)

regular anticlinal cell divisions in the outermost cell layer (Tanaka et al., 2007). This observation led us to expect that overexpression of ATML1 may cause ectopic anticlinal cell divisions in the inner tissues. However, in the ATML1-overexpressing plants, division planes that separate two guard cells were approximately anticlinal (41.0%), periclinal (29.3%) or oblique (29.8%) relative to the adaxial or abaxial surface of the organ, indicating that epidermis identity alone was necessary, but not sufficient, for anticlinal cell division (supplementary material Fig. S5A). By contrast, the cell walls separating the two guard cells of ectopic stomata formed in the subepidermal L2 cell layer were often anticlinal (Fig. 3G; supplementary material Fig. S5B). Interestingly, we noticed that stomatal pores had a tendency to face towards inner air spaces in the mesophyll (Fig. 3H;I; supplementary material Fig. S5C); guard mother cells appeared to divide anticlinally (i.e. perpendicular to the inner surface) to form these stomatal pores. These observations raised the fascinating possibility that ‘surface proximity’ as well as ‘epidermis identity’ is required for anticlinal cell division.

ATML1 negatively influences mesophyll cell differentiation

To investigate whether ectopic ATML1 induction had negative effects on inner cell fate, we examined mesophyll cell differentiation. Mesophyll-specific STOMAGEN-GUS expression was severely depressed in the seedlings of the strong ATML1-overexpressor (Kondo et al., 2010; Fig. 4B). Induction of ATML1 at 5 days after germination resulted in malformed leaves with ectopic patches of transparent cells among the green mesophyll tissues (Fig. 4D; supplementary material Fig. S3D).

The fact that ectopic ATML1 induction decreased mesophyll cell differentiation led us to assume that mesophyll cell fate may be negatively regulated by ATML1 in the outermost cell layer. In support of this idea, atml1;pdf2 produced leaves with mesophyll cells on the surface (Fig. 4E-G) (Abe et al., 2003). In order to determine whether this phenotype is due to a conversion of epidermis into mesophyll cells or due to degradation of the surface epidermis, we carefully observed proATML1-nls-3xGFP expression in the atml1;pdf2 leaves. Our results showed that proATML1-nls-3xGFP was still expressed in some green mesophyll cells near the surface of the atml1;pdf2 leaves (Fig. 4F,H). One interpretation of this observation is that the outermost cells initially specified as epidermis (and hence proATML1-nls-3xGFP positive) partially obtained mesophyll cell identity in the absence of ATML1 and PDF2 activities. In conclusion, these results were consistent with the idea that ATML1 and/or epidermis identity antagonize mesophyll cell differentiation and that loss of epidermal cell identity is associated with ectopic mesophyll cell development in the outermost cell layer.

Perspectives

To our knowledge, this is the first study to report that ectopic epidermis identity can be conferred to the inner tissues of leaves and cotyledons. These ectopic epidermal cells have provided a novel tool to assess the intrinsic properties of epidermal cells, as well as the influence of epidermal cell identity on the development of neighboring mesophyll cells. Future studies should include the identification of the molecular components that govern cell-cell interaction in the same and different cell layers.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.094417/-/DC1

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