Tissue layer and organ specificity of trichome formation are regulated by GLABRA1 and TRIPTYCHON in Arabidopsis

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SUMMARY

In animal development, cellular diversity is generated within tissues which in turn are derived from germ layers. Similar to the germ layers in animals, plants establish three distinct tissue layers early in development which each give rise to a distinct set of cell types. To investigate the role of tissue-layer-specific cues in generating plant cellular diversity we studied the spatial regulation of an epidermal cell type, trichomes (hairs), by the two genes, GLABRA1 (GL1) and TRIPTYCHON (TRY). Ubiquitous expression of the positive regulator GL1 in the absence of the negative regulator TRY leads to ectopic trichome formation not only on additional organs but also in subepidermal tissue layers. Trichomes in inner tissue layers can differentiate the same morphology and show a spacing pattern comparable to trichomes in the epidermis. This clearly shows that cell type specification takes place downstream of tissue-specific cues. We propose a model of how the tissue and organ specificity of trichome induction is regulated in normal development.

Key words: Trichomes, Pattern formation, Tissue layer specificity, Arabidopsis thaliana, GLABRA1, TRIPTYCHON

INTRODUCTION

During the development of multicellular organisms cells become progressively restricted in their developmental potential. The final specification of cells can be considered to be determined by two independent spatial inputs: molecular circuits that generate patterns of cells or cell groups, and tissue-specific cues that dictate the actual differentiation pathway. Well-studied cell differentiation processes include the specification of epidermal sensory organs (Campos-Ortega, 1993; Ghysen et al., 1993; Hinz et al., 1994; Tepass and Hartenstein, 1995; Yan and Jan, 1993), the development of the tracheal system (Wilk et al., 1996), eye development (Hafen and Basler, 1990), and somatic myogenesis (Baylies and Bate, 1996) in Drosophila. In contrast to animal development, little is known about the organ- and tissue-layer-specific developmental constraints on cell type specification in plants. Also plants establish distinct tissue layers that are maintained as separate layers throughout development: the L1 layer that produces the epidermis and the two inner tissue layers, the L2 and the L3, that give rise to the mesophyll and the vascular system (Medford, 1992; Poethig, 1989; Satina et al., 1940; Smith and Hake, 1992). If cells are displaced from the L1 layer to the L2 layer they change fate according to their new position (Dermen, 1953; Stewart and Burk, 1970). This implies that cell-type specification depends on tissue-layer-specific cues.

Numerous studies have focused on epidermal cell type specification because the epidermal surface is readily accessible and therefore easily studied. The epidermis is composed of a few distinct cell types that are specific to this layer, namely epidermal pavement cells, stomatal guard cells and trichome cells. We use trichome development in Arabidopsis as a model system to study epidermal pattern formation. Trichomes are large single-celled epidermal hairs that are regularly distributed on leaves, sepals and stems. Several genes have been identified that act early in trichome initiation and patterning. Two genes, GLABRA1 (GL1) and TRIPTYCHON (TRY), appear to be specifically involved in trichome development (Esch et al., 1994; Hülskamp et al., 1994; Marks and Feldmann, 1989; Oppenheimer et al., 1991), whereas TRANSPARENT TESTA GLABRA (TTG) is also required in other developmental processes, including the regulation of anthocyanin biosynthesis (Koornneef, 1981), the production of seed coat mucilage (Koornneef, 1981) and root hair differentiation (Galway et al., 1994). GL1 and TTG can be considered positive regulators of trichome initiation since homozygous mutant plants are nearly devoid of trichomes (Koornneef et al., 1982). Evidence for the molecular function of the TTG gene in trichome initiation comes from the analysis of Arabidopsis plants that constitutively overexpress the maize R gene. Overexpression of the R gene, which encodes a protein with sequence similarity to myc-related transcription activators, is sufficient to rescue the ttg phenotype (Lloyd et al., 1992). Although the recent cloning of the TTG gene revealed that TTG is not an R gene homolog (A. Walker and J. Gray, personal communication), overexpression of the R gene can still be considered to reflect ectopically provided TTG function. Plants that constitutively express the R gene are...
transheterozygotes were obtained from a cross between homozygous plants and homozygous as F₂ plants that did not segregate. 35S::GL1 was expressed initially at low levels in all cells of the developing epidermis and then high levels of transcripts accumulate in developing trichome cells (Larkin et al., 1993). These data suggest GL1 to be involved in early events of trichome initiation. However, ectopic expression of GL1 is not sufficient to trigger trichome development in cells that normally do not form trichomes (Larkin et al., 1994). TRIPTYCHON (TRY), is thought to act as a negative regulator of trichome fate. In try mutants trichomes are often found in clusters, suggesting that TRY acts to prevent neighboring cells from adopting a trichome fate (Hülskamp et al., 1994).

We reasoned that the trichome specificity of GL1 and TRY reflects that both genes function downstream of most other developmental cues. In this case one would expect that overexpression of the positive regulator GL1 in the absence of the negative regulator TRY would bypass other developmental restrictions of trichome initiation. To test this hypothesis we studied trichome distribution in a homozygous try mutant that constitutively expresses GL1 under the control of the cauliflower mosaic virus 35S RNA promoter (35S::GL1). In addition, the role of TTG in organ- and tissue-specific trichome initiation was analyzed in several double and triple mutant combinations.

**MATERIALS AND METHODS**

**Plants and plant culture**

The wild-type strain used in this work was the Landsberg erecta ecotype. The ttg-1 allele was induced in a Landsberg erecta genetic background and was obtained from M. Koornneef (Agricultural University, Wageningen, Netherlands). The try-EM1 allele was isolated as a recessive mutation in a Landsberg erecta background (Hülskamp et al., 1994). Although partial dominance was observed with respect to the branch number in try heterozygotes (Folkers et al., 1997), none of the three known try alleles show patterning defects in heterogeneous plants (A. Schnittger, unpublished observations).

Therefore, in the following the patterning phenotype of try mutants will be considered as a recessive trait. The transgenic lines containing the 35S::GL1 construct (Larkin et al., 1994) and the pGGE4 construct (Larkin et al., 1993) were obtained from D. Marks. The 35S::R-GR line was obtained from A. Lloyd (Lloyd et al., 1994). The plants were grown under constant illumination as described previously (Mayer et al., 1993). The 35S::GL1 try line was constructed in two steps. Initially plants were selected among the F₂ progeny of a cross between 35S::GL1 homozygotes and try mutants that displayed a new strong cluster phenotype. Plants of the genotype 35S::GL1 were identified as F₂ plants that did not segregate try trichomes among their F₃ progeny. The double mutant line was backcrossed with both parentals to verify the genotype. A similar strategy was used to generate the 35S::R-GR try line. The +35S::GL1 try/tri +pGGE4 plants were obtained from crosses between 35S::GL1 try with pGGE4 try. +35S::GL1 +ttg +try plants were obtained from a cross between 35S::GL1 try and ttg mutants. +35S::GL1 +ttg try/tri plants were generated by crossing a 35S::GL1 try homozygote with a ttg try double mutant. +ttg +35S::GL1 plants were F₁ plants from a cross between homozygous ttg plants and homozygous 35S::GL1 plants. +lttg +try transheterozygotes were obtained from a cross between homozygous ttg and try mutants. +lttg try/tri plants were generated by crossing ttg try double mutants with homozygous try mutants.

**RESULTS**

Overexpression of GL1 in the absence of TRY causes ectopic trichome formation on various organs

In wild-type plants trichome initiation is restricted to leaves, sepals and stems. This organ-specific initiation of trichomes is not affected in try plants. 35S::GL1 plants occasionally have trichomes on cotyledons. 35S::GL1 try plants display a high number of trichomes on the cotyledons (Fig. 1A). In addition, 35S::GL1 try plants display trichomes ectopically on several organs. We found large clusters of trichomes at the base of flower stalks at a position characteristic for the development of bracts in other plant species (Fig. 1B). The pistil is covered densely with branched trichomes which are normally present only on leaves (Fig. 1C). Occasionally we found trichomes on the stamen (Fig. 1D). 35S::GL1 try plants show a marked increase in the size of trichome clusters. While try mutants usually have small clusters of no more than four trichomes, 35S::GL1 try mutants have clusters of up to 8 trichomes. A closer inspection revealed that most of these supernumerary
Subepidermal trichome formation in 35S::GL1 try plants

In wild-type plants trichomes are exclusively initiated in the epidermal cell layer. This cell-layer specificity is lost in 35S::GL1 try mutants which produce trichomes also in subepidermal tissue layers. In mature leaves we observed subepidermal cells that were unusually large and contained a much larger nucleus than the surrounding cells. These cells lacked chloroplasts which are a typical feature of mesophyll cells. Two observations suggest that these large cells have adopted a trichome fate. Many acquire a polarization characteristic for trichome cells. Some cells start to expand towards the leaf surface (Fig. 2A-C,G). Others grow through the epidermal layer and initiate branching (Fig. 2D-F,H,I).

Additional evidence was obtained from studying the expression of a trichome-specific GUS marker construct (pGGE4) in a 35S::GL1 try mutant background. The pGGE4 construct contains a short promoter fragment of the GL1 gene that drives the expression of the GUS gene specifically in trichomes and stipules (Larkin et al., 1993). The GUS histochemical staining in 35S::GL1 try pGGE4 plants revealed abnormally large GUS-positive subepidermal cells in leaves (Fig. 2J,K). Subepidermal trichomes were not only produced on the adaxial side of the leaf but also on the abaxial side (Fig. 3A). No indication of a general transformation of subepidermal tissue layers into epidermal tissue layers was found, e.g. no stomata or other epidermal cell types were found. Except for the subepidermal trichome cells all other cells showed a morphology and organization characteristic of subepidermal leaf tissues (Fig. 2G-I).

Subepidermal trichome formation was also found in other plant organs including the stem and cauline leaves (Fig. 3B). Also cotyledons produced unusually large subepidermal cells. These cells, however, did not show GUS-staining in a pGGE4 background. No subepidermal trichomes were found in flower tissues or in the hypocotyl. Also root hair patterning and differentiation was indistinguishable from wild type.

Subepidermal trichome distribution in 35S::GL1 try plants

Subepidermal trichomes appear to be distributed regularly on the leaf. No correlation of their spatial distribution and other morphological landmarks such as the vascular system was found. The subepidermal trichome pattern appears to be random with respect to the distribution of epidermal trichomes (Fig. 4A). Subepidermal trichomes were found in direct contact with epidermal trichomes as well as at some distance from epidermal trichomes. In order to test whether subepidermal trichome patterning is coupled to the epidermal trichome pattern we compared all distances of subepidermal trichomes to their nearest epidermal trichome and the distances of randomly chosen positions and epidermal trichomes. If subepidermal trichome patterning were independent of the epidermal trichome pattern one would expect a similar distribution of the subepidermal-epidermal distances and the distances between random positions and epidermal trichomes. However, if epidermal and subepidermal trichome patterning depend on each other, one would expect that the two distributions are different. As shown in Fig. 4B the distances between subepidermal and epidermal trichomes show a distribution with a broad peak between 125 μm and 500 μm. A similar distribution was observed for the values for distances between 10 000 randomly chosen positions and epidermal trichomes. The maximum of this distribution, however, was much narrower, with a peak at 250 μm. A difference between the two distributions was found to be statistically significant ($\chi^2=34.37; \alpha=0.01$). These results clearly rule out a preference of subepidermal trichome initiation in the proximity of epidermal trichomes. The larger fraction of more distant subepidermal trichomes may suggests a lateral inhibition of subepidermal and epidermal trichomes. The biological significance of this observation is not clear and remains to be studied in more detail.

Another aspect of trichome patterning, cluster formation as found in try mutants, was also observed for subepidermal trichomes in 35S::GL1 try plants. Approximately 31% of all subepidermal trichome sites contained clusters ($n=219$). The formation of trichome clusters appears to depend on the gene dosage of TRY. This is suggested by the finding that subepidermal trichomes in +/35S::GL1 +/-try plants were never found in clusters ($n=100$).

The finding that trichome patterning in subepidermal tissue layers in 35S::GL1 try plants is comparable to that in the epidermis is remarkable. It suggests that trichome patterning takes place among cell types as different as mesophyll cells and epidermal pavement cells. This suggests that the underlying patterning mechanisms are inherent to the activation of trichome-specific genes.

Subepidermal trichomes lack accessory cells

During trichome maturation all epidermal cells adjacent to the base of a trichome differentiate as accessory cells which are characterized by a rectangular shape. The cell fate of accessory cells appears to be determined by lateral signaling of the central trichome cell rather than by cell lineage since the accessory cells are not clonally related to each other or to the trichome cell (Larkin et al., 1996). In order to assess whether subepidermal trichomes recruit surrounding mesophyll cells to acquire accessory cell fate we inspected optical sections of subepidermal tissue layers by confocal microscopy (Fig. 5). By morphological criteria, subepidermal cells that are in immediate contact with a subepidermal trichome cell are indistinguishable from other mesophyll cells. All surrounding cells contain chloroplasts suggesting that these cells have not changed their normal identity. Also cells in contact with large, branched subepidermal trichomes showed no transformation of mesophyll cells into accessory cells (Fig. 2I). This observation renders it unlikely that differentiation of accessory cells is not initiated simply because trichome development does not reach a critical stage.

Thus, while trichome patterning appears to be normal in subepidermal tissue layers the induction of morphologically recognizable accessory cells is not observed. This suggests that the initiation of accessory cells does not take place because tissue-layer-specific developmental constraints cannot be bypassed. Alternatively, the cell fate of mesophyll cells may
have been irreversibly determined by the time the trichome cell starts lateral signaling.

The role of the TTG/R gene in subepidermal trichome formation

In addition to GL1 and TRY, the TTG gene is thought to be a key factor in the regulation of trichome patterning. The lack of trichome phenotype suggests that the TTG gene acts similarly to the GL1 gene as a positive regulator of trichome initiation. We therefore tested whether overexpression of the R gene alone or in the absence of TRY activity results in subepidermal trichome initiation. For these studies we used transgenic lines overexpressing an inducible form of the R gene that includes a N-terminal fusion of the vertebrate glucocorticoid receptor (35S::R-GR; Lloyd et al., 1994). Neither induced 35S::R-GR nor 35S::R-GR try plants exhibited subepidermal trichomes. Thus, activation of the TTG pathway via overexpression of the R gene is not sufficient to trigger trichome initiation in subepidermal tissue layers. The phenotypic difference between the 35S::GL1 try and 35S::R-GR try plants could be explained by assuming that TTG is normally present in subepidermal tissues while GL1 is specifically expressed in the epidermis and hence is limiting in 35S::R-GR try plants (see Discussion). A second aspect of TTG function during trichome patterning is its apparent negative role during lateral signaling between trichome precursor cells. A reduction of the TTG dosage in 35S::GL1 plants results in a high frequency of clustered trichomes (Larkin et al., 1994). A similar phenotype is produced by weak ttg alleles (Larkin et al., 1994). Both sets of data suggest that the relative concentration of TTG is important during trichome selection. In order to test whether the gene dosage of TTG might play a role in subepidermal trichome formation in try, 35S::GL1 or 35S::GL1 try plants we constructed various double mutants that are heterozygous for ttg. Plants of the genotype +/ttg try/try, +/ttg +/try or 35S::GL1 +/ttg did not produce subepidermal trichomes. We also found no increased cluster frequency in +/35S::GL1 +/ttg try/try plants as compared to 35S::GL1 try plants. Both lines show approximately the same cluster frequency of 35.7% (n=213) and 31.5% (n=219) respectively; no significant difference was found in a Welch test (t’=−0.273, α=0.01). Also the total number of subepidermal trichomes in +/35S::GL1
Subepidermal trichomes in 35S::GL1 try plants was not increased as compared to +/35S::GL1 +/try mutants. Both lines show subepidermal trichomes at a low frequency with 1-5 subepidermal trichomes per mature leaf. These results show that the TTG dosage does not affect subepidermal trichome initiation in 35S::GL1 try plants.

**Regulation of the number of endoreplication cycles by GL1 and TRY**

35S::GL1 try plants are also affected at the cellular level in the regulation of endoreplication. During wild-type development trichomes undergo four rounds of endoreplication. TRY has been described to act as a negative regulator of endoreplication since try trichomes show twice the amount of DNA as wild-type trichomes (Hülskamp et al., 1994). We found that 35S::GL1 trichomes show a similar increase in nuclear DNA content (Fig. 6). This indicates that GL1 is not only required for trichome initiation but also involved in the control of endoreplication. In 35S::GL1 try plants most trichomes had a DNA content equivalent to two or even three additional rounds of endoreplication compared to the two single lines. This result could be explained by considering the 35S::GL1 try phenotype as the addition of two separate effects such that TRY and GL1 function in two independent pathways. Alternatively, TRY could negatively regulate the action of overexpressed GL1 directly.

**DISCUSSION**

The increased DNA content and the ectopic initiation of trichomes in 35S::GL1 try plants support the idea that GL1 and TRY act together downstream of other developmental controls of trichome development. In particular, the initiation of subepidermal trichomes is unexpected. The layered organization and the tissue-layer-specific differentiation of cell types in diverse plant organs (Medford, 1992; Poethig, 1989; Satina et al., 1940; Smith et al., 1992) implies that, similar to germ layers in animals, each tissue layer confers developmental constraints in which context subordinate differentiation processes take place. Our data show that tissue-layer-specific cues can be overridden by activating cell-type-specific pathways. Whether this finding reflects a general difference in the developmental plasticity of plants versus animals is not known.

**Regulation of organ specificity of trichome formation**

A model to explain the organ and tissue layer specific regulation of trichome formation is presented in Figure 7. In this model, trichome formation depends on two pathways that receive different environmental and developmental inputs and both GL1 and TTG functions are required to initiate trichome differentiation. Differences in the presence and density of trichomes on different organs reflect the regulation of trichome formation by organ-specific developmental programs and by
the physiological state or environmental cues. The involvement of organ-specific cues is evident in mutants that have homeotic transformations of cotyledons or flower organs into trichome bearing leaves (Bowman et al., 1991; Meinke et al., 1994). Environmental parameters that modulate trichome distribution include light, day length and the plant hormone gibberellin (Chien and Sussex, 1996; Misera et al., 1994). Two lines of evidence suggest that trichome formation on cotyledons, carpels and stamens depends on the TTG pathway. First, constitutive expression of the R gene is sufficient to induce ectopic trichome formation on these organs (Lloyd et al., 1992). Second, cop1 (fus1) mutant sectors in the epidermis of carpels produce ectopic trichomes (Misera et al., 1994). The COP1 gene is involved in light-induced signal transduction and has been proposed to function as a negative regulator of TTG with respect to the regulation of anthocyanin biosynthesis in subepidermal cells and trichome formation in epidermal cells (Misera et al., 1994). Since GL1 is expressed at low levels in these organs (Larkin et al., 1993), TTG function is limiting for trichome formation. Our finding that 35S::GL1 try plants show ectopic trichome formation reminiscent of R transgenic plants is unexpected. One possible explanation is that the TTG pathway is activated in 35S::GL1 try plants.

**Regulation of tissue layer specificity of trichome formation**

By contrast, tissue layer specificity, the restriction of trichome formation to the epidermal cell layer, appears to be regulated by the tissue-layer specific transcriptional activation of the GL1 gene (Fig. 7). TTG is required as a prerequisite for trichome formation but does not provide spatial information. Activation of the TTG pathway in subepidermal tissue layers is not sufficient to trigger trichome formation. Plants expressing the R gene constitutively under the control of the 35S promoter do not show subepidermal trichomes. Also fus mutants or subepidermal fus sectors in adult plants do not produce subepidermal trichomes (Misera et al., 1994). Although TTG is active in these tissue layers, trichome formation is not promoted in subepidermal tissue layers, presumably because the GL1 gene is not activated. Consistent with this idea, the activation of the GL1 TRY pathway results in subepidermal trichome initiation.

**GL1 and TRY play a dual role in trichome patterning and cell differentiation**

Pattern formation and cell fate determination are, in animal development, usually separate processes. A well-studied example is the regulation of cell type specification by neurogenic and proneural genes in *Drosophila*. The neurogenic genes together with the proneural genes form a gene cassette (Yan et al., 1993) that acts in ectodermal and endodermal patterning. Different cell fates are selected by this system in the respective tissue layers: in the ectoderm, epidermal versus neural precursor cells (Campos-Ortega, 1993; Ghysen et al., 1993), in the endoderm, the segregation of midgut epithelial cells, the midgut precursor cells, and interstitial cell precursors (Tepass and Hartenstein, 1995). By contrast, trichome pattern formation and trichome differentiation are intimately coupled. This is suggested by two lines of evidence. First, 35S::GL1 try plants initiate an apparently normal trichome pattern in a new developmental context, the subepidermal tissue layer. Hence, in this situation not only patterning is initiated but also cell identity is imposed on the selected cells. Second, both genes, GL1 and TRY, play a dual role in cell differentiation (endoreplication) as well as in the spatial control of trichome initiation. Although this could reflect that both genes are involved in two independent regulation events, the same regulatory interactions between GL1 and TRY in both processes, i.e. the suppression of GL1 by TRY, suggests that both processes share the same molecular function of the two genes. This appears to be a paradox since the regulation of cell differentiation processes typically utilize intracellular functions while patterning processes usually involve cell-cell communication processes. Further clarification of the molecular and cell biological function of GL1 and TRY is needed to resolve this paradox.

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