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The homeobox gene *GLABRA 2* is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*

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SUMMARY

The role of the *Arabidopsis* homeobox gene, *GLABRA 2* (*GL2*), in the development of the root epidermis has been investigated. The wild-type epidermis is composed of two cell types, root-hair cells and hairless cells, which are located at distinct positions within the root, implying that positional cues control cell-type differentiation. During the development of the root epidermis, the differentiating root-hair cells (trichoblasts) and the differentiating hairless cells (atrichoblasts) can be distinguished by their cytoplasmic density, vacuole formation, and extent of elongation. We have determined that mutations in the *GL2* gene specifically alter the differentiation of the hairless epidermal cells, causing them to produce root hairs, which indicates that *GL2* affects epidermal cell identity. Detailed analyses of these differentiating cells showed that, despite forming root hairs, they are similar to atrichoblasts of the wild type in their cytoplasmic characteristics, timing of vacuolation, and extent of cell elongation. The results of in situ nucleic acid hybridization and GUS reporter gene fusion studies show that the *GL2* gene is preferentially expressed in the differentiating hairless cells of the wild type, during a period in which epidermal cell identity is believed to be established. These results indicate that the *GL2* homeodomain protein normally regulates a subset of the processes that occur during the differentiation of hairless epidermal cells of the *Arabidopsis* root. Specifically, *GL2* appears to act in a cell-position-dependent manner to suppress hair formation in differentiating hairless cells.

Key words: *Arabidopsis*, homeobox gene, root hairs, cell differentiation, cell fate specification, *GLABRA 2*

INTRODUCTION

The proper growth and development of new organs and tissues requires regulation of cellular differentiation. Relatively little is known about the molecular mechanisms governing cell differentiation in plants, though the presence of rigid cell walls that impede relative cell movement implies that plants may have evolved different strategies than those employed by animal cells. Another distinguishing feature of plant cell differentiation is that it normally occurs throughout the life of the organism, near meristems located at the shoot and root apices. Though the mechanisms that regulate the differentiation of plant cells are poorly understood, the importance of cell position, rather than cell lineage, in determining plant cell fate has been recognized (Stewart, 1978; Barlow and Carr, 1984).

The relatively simple development and accessibility of the root epidermis of *Arabidopsis thaliana* make it an attractive target for molecular genetic studies of cell differentiation (Schiefelbein and Somerville, 1990; Dolan et al., 1994; Scheres et al., 1994; Duckett et al., 1994; Benfey and Schiefelbein, 1994). The root epidermis is composed of only two cell types: cells that bear root hairs (long, tubular outgrowths) and cells that are hairless. The cells are organized into a small number of longitudinal columns or files that arise from a set of initials in the root apical meristem. Regular divisions in these initials generate new epidermal cells which differentiate in a basipetal direction. Thus, the cell files consist of a linear array of cells, each cell being more developmentally advanced than the one before it, which permits the analysis of the complete process of cell differentiation by examining cells along a particular file from the meristem to the mature portion of the root (illustrated in Fig. 1).

The patterning of root-hair and hairless epidermal cells in the *Arabidopsis* root is typical of the family Brassicaceae (Cormack, 1947, 1949; Bunning, 1951, 1952; Dolan et al., 1993; Galway et al., 1994). Each file of epidermal cells is composed entirely of root-hair cells or entirely of hairless cells, with each type of file located in a particular position relative to cells in the underlying cortical layer of the root. Root hairs are produced by epidermal cells in the files that lie over the radial (anticlinal) walls between adjacent, underlying cortical cells. Epidermal cells in the files that lie over a tangential (periclinal) cortical cell wall are hairless. This observed correlation between the location of epidermal cells and their differentiated state implies that cell position influences cell fate.

The hair-bearing and hairless epidermal cells do not differ merely at the relatively late developmental stage of root-hair formation. Studies of differentiating epidermal cells have shown

that developing root-hair cells (trichoblasts) can be distinguished from developing hairless cells (atrichoblasts) prior to the outgrowth of the hair itself, by a variety of biochemical, morphological and histochemical characteristics (discussed by Cutter, 1978; Dolan et al., 1993, 1994; Galway et al., 1994). This implies that the two types of epidermal cells are programmed at an early stage to follow distinct differentiation pathways.

To begin to understand the molecular mechanisms that control cell differentiation in plants, we have analyzed genes that control the development of the root epidermis in *Arabidopsis*. In a previous study, the *TRANSPARENT TESTA GLABRA* (*TTG*) gene was shown to influence the fate of cells in the root epidermis (Galway et al., 1994). Recessive mutations in *ttg* cause differentiating root epidermal cells in all positions to adopt a root-hair cell fate, whereas the strong expression of the maize R gene (a possible *TTG* homolog; Lloyd et al., 1992) in *Arabidopsis* acts in a dominant fashion to cause root epidermal cells in all positions to adopt a hairless cell fate (Galway et al., 1994). The *TTG* gene also acts on the development of the shoot epidermis, where it is required for trichome (leaf hair) formation (Koornneef, 1981; Lloyd et al., 1992; Larkin et al., 1994). Thus, *TTG* may act as a general regulator of epidermal cell patterning.

In the present study, the role of the *GLABRA2* (*GL2*) gene in the differentiation of the *Arabidopsis* root epidermis has been analyzed. The *GL2* gene was previously shown to be required for trichome formation and seed mucilage production, and it encodes a homeodomain-containing protein (Koornneef, 1981; Rerie et al., 1994). In this report, we show that *gl2* mutations cause root hairs to form on cells located in hairless-cell positions, but, surprisingly, *gl2* mutations do not affect the other cellular differences that normally exist between the differentiating root-hair and hairless cells. The analysis of *GL2* expression shows that *GL2* is preferentially expressed in developing hairless epidermal cells, shortly after the cells are generated in the meristematic region. Our results indicate that the *GL2* gene product is normally required to ensure that cells located in particular positions of the *Arabidopsis* root epidermis differentiate into hairless epidermal cells.

MATERIALS AND METHODS

Plant strains

The *gl2-1* mutant (in the Landsberg *erecta* genetic background) was obtained from the Arabidopsis Biological Resource Center (Ohio State University), and the *gl2-2* mutant (in the WS genetic background) was identified in T-DNA transformed lines of *Arabidopsis*, as previously described (Rerie et al., 1994). Plants heterozygous for a *gl2* mutation were generated by crossing homozygous *gl2* mutant plants with Landsberg *erecta* wild-type plants. The *gl2* transgenic lines (in the WS genetic background) used for complementation assays (gl2-B12 and gl2-E9) harbored genomic DNA constructs containing the entire *GL2* transcriptional unit, as described by Rerie et al. (1994). Plants homozygous for the transgenes (assessed by kanamycin resistance) were identified and used in these experiments.

Growth conditions and microscopy

Unless indicated otherwise, seedlings were grown in vertically oriented Petri dishes on agarose-solidified medium containing mineral nutrients (Estelle and Somerville, 1987) as previously described (Schiefelbein and Somerville, 1990). The number of root hairs per mm of root were determined as described by Galway et al. (1994), and the proportion of epidermal cell types were obtained by counting the number of each cell type (root-hair or hairless cells) within 1 mm segments from the differentiated portion of 5 to 10 roots of 4- or 5-day-old seedlings.

Most of the transverse sections of roots were obtained by hand-sectioning seedling roots in 3% agarose blocks and staining with a fluorescent brightener or toluidine blue dye, as previously described (Galway et al., 1994). The position of hair-bearing epidermal cells and the number of cortical and epidermal cell files were determined from similarly prepared hand sections taken from the mature portion of at least 8 roots of 4- or 5-day-old seedlings.

To obtain embedded roots for thin sections, 5-day-old seedlings were fixed for 14 hours on ice in 3% glutaraldehyde and 50 mM $NaPO_4$ (pH 6.8). The seedlings were then washed in the same buffer, followed by distilled water, before being embedded in a minimal amount of 1% low melting-point agarose (Gibco BRL, Gaithersburg, MD). The seedlings were then dehydrated on ice in a graded ethanol series, followed by stepwise infiltrations in 10, 25, 50, 75, and 100% JB4-Plus infiltration solution (Polysciences, Warrington, PA) on ice. The seedlings were given one change of the complete JB4-Plus embedding solution on ice before polymerization at room temperature inside bottle-neck BEEM capsules (Ted Pella, Redding, CA). Transverse sections, 2 μm thick, were dried onto slides and stained with 0.05% Toluidine blue O in sodium citrate buffer (pH 4.4), prior to mounting in distilled water for microscopy.

To examine individual cell files in whole mounts, 4-day-old seedling roots growing along the surface of agarose-solidified media in vertically oriented Petri dishes were immersed in artificial pond water (APW). The selected seedlings and surrounding agarose were transferred onto microscope slides and viewed with DIC optics. Cell files were categorized as displaying either early or late cell vacuolation, and cell lengths were measured on consecutive cells within each file. To compare files from different roots, the first hair-bearing cell within each file was used as the point of reference.

Whole-mount seedling in situ hybridization

RNA and DNA probes used for in situ hybridization were obtained from a 900 bp *GL2* cDNA fragment located 3′ to the homeobox sequence cloned into pBluescript SK+ (pcH1; Rerie et al., 1994). The in vitro transcription reactions were carried out with digoxigenin-labeled UTP as described by Boehringer Mannheim Biochemicals (product manual), using T3 RNA polymerase to generate the antisense strand probe and T7 RNA polymerase to generate the sense strand probe. DNA probes were generated by random priming with digoxigenin-labeled dUTP as described by Boehringer Mannheim Biochemicals (product manual), except that 50 μg of random primers were used in the reaction. Four-day-old *Arabidopsis* seedlings were fixed, hybridized with the RNA or DNA probe, and treated with the digoxigenin antibody essentially as described by de Almeida Engler et al. (1994), with the following modifications. Fixation was in 4% formaldehyde for 15 minutes, proteinase K treatment was carried out for 3-5 minutes, and 4-5 post-antibody washes were carried out for 1 hour each. Following antibody detection, some seedlings were embedded in 3% agarose blocks and hand-sectioned to obtain transverse root sections. Similar results were obtained from both the antisense RNA and the DNA probes.

GUS reporter gene construction and assay

A 4 kb *Xho*I-*Sal*I fragment from the 5′ upstream region of the *GL2* gene was isolated and cloned into the *Sal*I site of pBI101 (Jefferson et al., 1987). The resulting plasmid (pGL2-GUS) produced an N-terminal fusion product between the GL2 protein and β-glucuronidase. Wild-type root explants (ecotype WS) were transformed with *Agrobacterium* (strain EHA105; Hood et al., 1993) carrying pGL2-GUS, using standard transformation and regeneration procedures (Valvekens et al., 1988). Seedlings from the progeny of primary transgenic lines were assayed for β-glucuronidase activity

according to established methods (Gallagher, 1992). Selected stained roots were fixed in 2% gluteraldehyde, 0.05 M $NaPO_4$ (pH 7.0) for 2 hours and then dehydrated in a graded ethanol series. The roots were infiltrated over 2 days with increasing concentrations of Spurr's resin to 100%. Transverse sections (2 μm) were cut and mounted on slides using Geltol (Lipshaw Immunon, Pittsburg, PA) and viewed using DIC microscopy with a Zeiss Axiophot microscope.

RESULTS

Root epidermal cell types in *gl2* mutants

The root epidermis of plants homozygous for recessive *gl2* mutations was examined to determine whether the number and/or arrangement of cell types were affected. Normally, the root epidermis of *Arabidopsis* contains files of cells which differentiate basipetally from the meristem and each file consists entirely of root-hair-bearing cells or hairless cells (Figs 1, 2A). Root-hair cell files are normally present over the radial walls separating files of underlying cortical cells and hairless cell files are present over the tangential cortical cell walls (Fig. 2C; Dolan et al., 1993; Galway et al., 1994). This positional relationship was confirmed in the present study, when two different wild-type *Arabidopsis* ecotypes (Landsberg and WS) were examined (Table 1). In each ecotype, root-hair cells were almost exclusively restricted to cells located over a radial cortical cell wall.

When the roots of homozygous *gl2* mutant plants were examined, excessive root-hair formation was observed (Table 1 and Fig. 2B). The *gl2* mutants examined included *gl2-1*, a fast neutron-induced mutant allele (Koornneef et al., 1982), and *gl2-2*, a T-DNA-induced mutant allele (Rerie et al., 1994), as well as two additional independent *gl2* mutants which exhibited similar phenotypes (data not shown). To determine whether the abnormally high root-hair number present in *gl2* mutants reflected ectopic hair production, transverse sections of roots were analyzed. The *gl2* root hairs form on epidermal cells in all files and are not limited to epidermal cells located over a radial cortical cell wall (Table 1; Fig. 2D). Nearly every epidermal cell in the *gl2* mutant roots produced a root hair (Table 1). The *gl2* root hairs appear morphologically normal and are situated near the apical end of the epidermal cells, the same site where root hairs emerge in epidermal cells of wild-type *Arabidopsis* roots (Schiefelbein and Somerville, 1990). These results show that mutations in *gl2* alter the position-dependent control of cell identity in the root epidermis, causing cells that would normally differentiate into hairless cells to produce root hairs.

It was possible that *gl2* mutations alter root epidermis cell types by affecting the number or organization of cell files in the root. Wild-type *Arabidopsis* primary roots normally possess 8 files of cortical cells and a variable number (between 15 and 25) of epidermal cell files (Dolan et al., 1993; Galway et al., 1994). An analysis of the root cell organization in the two *gl2* mutants demonstrated that the number of cortical and epidermal cell files is indistinguishable from the number in the Landsberg or WS wild-type lines (Table 2). Furthermore, no other abnormalities in root cell organization were identified in mature transverse sections of *gl2* mutants. Therefore, the *gl2* mutations do not alter the structure of the mature root.

To further examine the effect of the *gl2* mutations on the root epidermis, plants heterozygous for the *gl2* mutations were generated and analyzed. In both the *gl2-1*/+ and *gl2-2*/+ heterozygotes, ectopic root-hair formation was observed, though the proportion of ectopic hairs was not as great as that in the *gl2* homozygous mutant roots (Table 1). Thus, the *gl2* mutations appear to act in a semi-dominant fashion to alter the identity of the hairless epidermal cells.

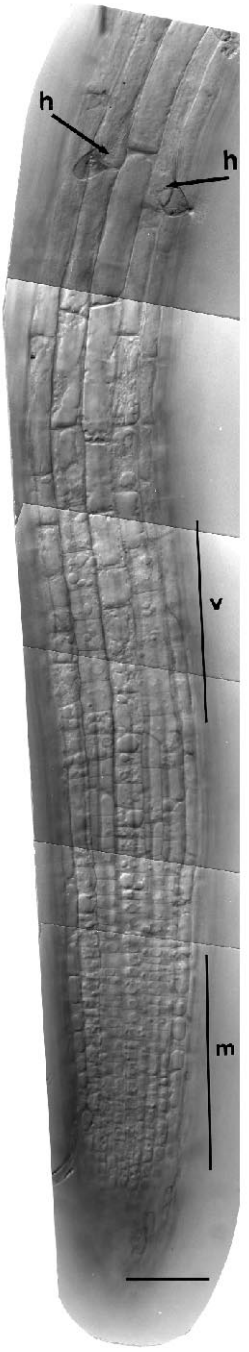

Fig. 1. Epidermal cell differentiation in the *Arabidopsis thaliana* root. An assembly of photomicrographs (using DIC optics) depicting a single wild-type root, showing the progression of epidermis differentiation within cell files from root tip (undifferentiated cells) to base (fully differentiated cells). Cell division in the apical meristematic region (m) generates longitudinal files of epidermal cells. Cell vacuolation differences, indicative of the fates of epidermal cells, are evident near the region of initial cell elongation (v). In all epidermal cell files, cells become progressively more vacuolated distal to the meristem, although vacuolation is delayed in the differentiating root-hair cells, relative to the differentiating hairless cells. Two root-hair cell files are noted (arrows indicate the emerging root hairs in the most-differentiated cells in these files). The file between these two root-hair cell files possesses differentiating hairless cells. Bar, 50 μm.

Differentiation of the root epidermis in *gl2* mutants

To examine whether *GL2* regulates all aspects of hairless cell differentiation or only certain aspects, we analyzed the epidermal cells of the *gl2* mutants at the root tip, where the earliest developmental events occur. In wild-type *Arabidopsis* roots, epidermal cells destined to form root hairs display several characteristics which distinguish them from cells destined to remain hairless (Galway et al., 1994; Dolan et al., 1994). First, at an early stage (within 200 μm of the root apical

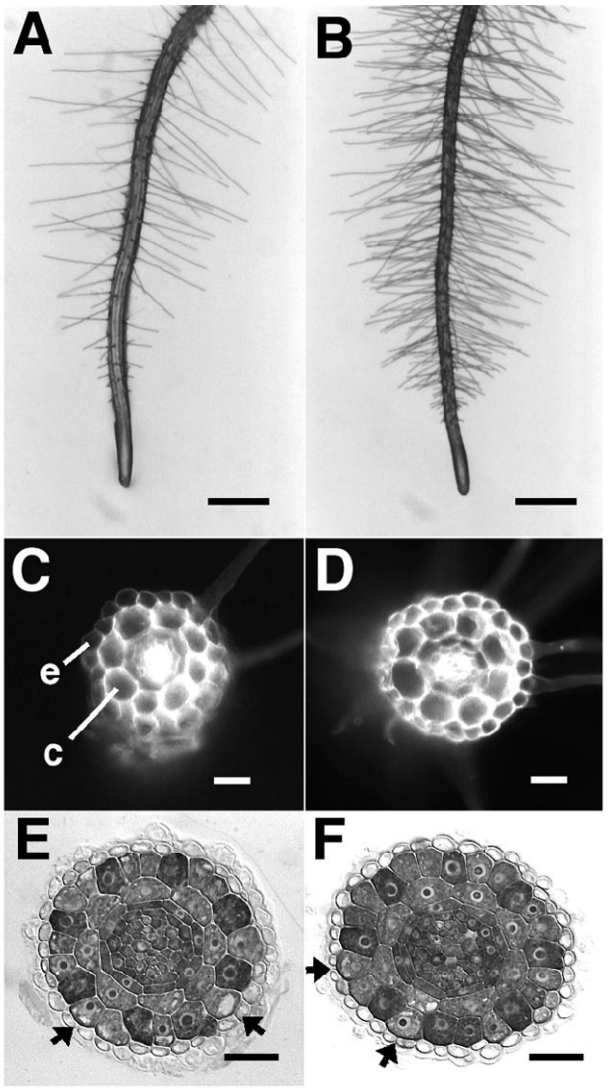

Fig. 2. Seedling roots from wild-type and *gl2* mutant *Arabidopsis* plants. The *gl2* mutant exhibits ectopic root-hair formation. (A,B) Roots of 5-day-old seedlings. (A) Landsberg wild type. (B) *gl2-1* mutant. Bars, 300 μm. (C-D) Transverse sections of 5-day-old roots. (C) Landsberg wild type; e, epidermis, c, cortex. One root-hair cell is visible at a normal position. (D) *gl2-1* mutant. Two hair-bearing epidermal cells are located in abnormal positions. Bars, 20 μm. (E,F) Toluidine-blue-stained transverse sections (2 μm thick) from 4-day-old primary root apices. Sections are from approximately 200 μm above the central cells of the root meristem (equivalent to the distal end of the meristematic region noted in Fig. 1). A single layer of lateral root cap cells surrounds the epidermis in these sections. Arrows indicate epidermal cells developing large vacuoles. Bars, 20 μm. (E) Landsberg wild type. The eight differentiating root-hair cells (trichoblasts) are distinguishable from the remaining epidermal cells (atrichoblasts) by the differential intensity of cytoplasmic staining and early indications of differential cell vacuolation. (F) *gl2-1* mutant. As in wild type, patterns of differential staining and early indications of differential vacuolation are visible in the root epidermal cells.

meristem), the cytoplasm of the differentiating root-hair cells stains more intensely with the dye toluidine blue than does the cytoplasm of the differentiating hairless cells (Fig. 2E), which may reflect differences in the metabolic activity of the cells (discussed in Cutter, 1978). In addition, differentiating root-hair cells display a delay in vacuole formation, relative to differentiating hairless cells. This is readily observed near the onset of epidermal cell elongation (approximately 200-400 μm from the central cells of the apical meristem) in whole mounts of roots, using differential interference contrast microscopy (Fig. 1) or in transverse sections (Fig. 2E). Furthermore, differentiating hairless cells undergo extensive elongation, which causes mature hairless cells to be longer than mature root-hair cells. These cellular differences were used in the present study, as convenient markers to assess the developmental processes/stages that are affected by the *gl2* mutations.

Table 1. Characteristics of root epidermal cells in wild type, *gl2* mutant, and transgenic *Arabidopsis*

Line	Root hair density* (hairs/mm)	Percentage root-hair cells	Percentage root-hair cells in normal position†
Landsberg wild type	63±7	52 (135)‡	96 (115)§
WS wild type	53±6	50 (102)	100 (69)
gl2-1	124±22	100 (121)	50 (159)
gl2-2	108±18	98 (115)	54 (116)
gl2-1/+	67±10	62 (143)	82 (221)
gl2-2/+	72±9	60 (118)	86 (112)
gl2 transformant B12	62±6	48 (75)	100 (43)
gl2 transformant E9	53±7	54 (82)	100 (51)

*Values represent the mean ± standard deviation.
†The normal position for root-hair cells is over a radial (anticlinal) wall that separates adjacent cortical cells.
‡Number of epidermal cells scored in root segments of defined length.
§Number of root-hair cells identified and scored.

Transverse sections were made from fixed and embedded wild-type and *gl2* mutant primary roots in the meristematic and elongation zones. No significant differences were detected in the number, arrangement, or morphology of component cells in the *gl2* root, compared to the wild type (Fig. 2E,F). Furthermore, no abnormal cellular characteristics were observed in the differentiating epidermal cells of the *gl2* mutant. In particular, the developing epidermal cells in the *gl2* mutants displayed the position-dependent differential cytoplasmic staining and differential cell vacuolation that is characteristic of wild-type epidermal cells (Fig. 2E,F). Thus, although the *gl2* epidermal cells present over tangential cortical cell walls produce root hairs, they do not exhibit the characteristics of developing root-hair cells (trichoblasts) in the root apical region.

To consider the developing root epidermis in *gl2* mutants at later developmental stages, the root apex of individual roots was examined in whole mounts to assess the cell lengths and root-hair formation in individual cell files. In the wild type, epidermal cell files can be clearly identified as either root-hair cell files, which exhibit delayed vacuolation and shorter cells, or hairless cell files, which exhibit early vacuolation and longer cells (Fig. 3A). In the *gl2* mutant, two classes of cell files were also identified, with respect to cell vacuolation and cell length. Cell files with delayed vacuolation possessed shorter mature cells, whereas cell files with early vacuolation possessed longer mature cells (Fig. 3B,C). As expected, *gl2* cells in all files produced root hairs, though a difference was detected in the timing of hair formation in different cell files. Cells in files which displayed the characteristics of normal root-hair files (delayed vacuolation and shorter cells) produced root hairs closer to the root apex (i.e. at an earlier developmental time) than cells in files which displayed characteristics of normal

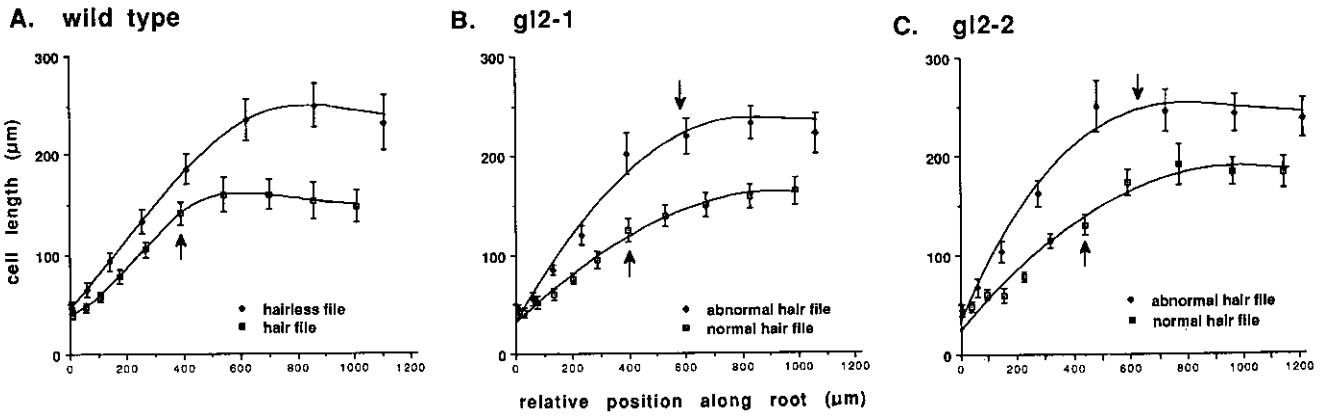

Fig. 3. Cell length and hair formation in files of root epidermal cells of wild-type and *gl2* mutants. Cell files were examined in whole mounts of seedling roots and classified either as displaying early (filled circles) or late (open squares) cell vacuolation. The lengths of the component cells within each file were measured, beginning at the onset of cell elongation (position zero) and continuing through the region of root-hair formation. Data points represent the mean (and standard deviation) of 7-15 independent cell files. Arrows indicate the average position of the first cell within each file to possess a root-hair protrusion. (A) Landsberg *erecta* wild type. (B) *gl2-1* mutant. (C) *gl2-2* mutant.

hairless files (early vacuolation and longer cells) (Fig. 3B,C). These results show that although essentially all epidermal cells in the *gl2* mutants produce root hairs, the mutant roots possess two distinct types of epidermal cell files which exhibit different cellular characteristics during epidermis differentiation. Taken together, these results indicate that *gl2* mutations do not cause a complete conversion of hairless epidermal cells into root-hair cells; rather, they only alter a subset of the hairless cell differentiation processes.

Molecular complementation of the *gl2* mutant root defect

To determine whether the defects associated with the *gl2* mutants were caused by the lesions in the *GL2* gene, molecular complementation tests were conducted. Previously, genomic DNA from the *GL2* gene region was shown to complement the trichome and seed mucilage defects associated with the *gl2* mutant (Rerie et al., 1994). In two of these *gl2* transgenic lines (B12 and E9, which each contain the complete *GL2* transcriptional unit), normal root-hair production was observed, and the position-dependent control of root epidermal cell differentiation was restored (Tables 1 and 2). Thus, the observed abnormalities in the root epidermis of *gl2* mutant plants are due to mutations in the homeobox gene *GL2*.

Expression of the *GL2* gene in roots

Since the *gl2* mutations only affect the developing hairless cells of the root epidermis, the *GL2* gene may only be expressed in these cell files. To analyze the spatial and temporal aspects of *GL2* gene expression in the developing root, two approaches were employed. In one approach, in situ nucleic acid hybridization was used to detect *GL2* RNA in whole mounts of 4-day-old wild-type seedlings. As shown in Fig. 4A, the digoxigenin-labeled probe displayed the strongest hybridization signal in differentiating epidermal cells within individual files. When traced to the mature portion of the root, these cell files were determined to possess differentiating hairless cells. The hybridization signal was not observed in experiments using the sense RNA probe nor in control experiments without a nucleic acid probe. To determine the location of the stained epidermal cells relative to cortical cells, transverse sections were made from stained and embedded roots, and these showed that the staining was limited to those epidermal cells located over tangential cortical cell walls (i.e. the position of differentiating hairless cells; Fig. 4B).

In a second approach to examine *GL2* gene expression, a 4 kb genomic fragment from the 5′ end of the *GL2* gene was fused to the β-glucuronidase (GUS) reporter gene and introduced into wild-type plants. Transgenic plants containing this reporter gene construct were analyzed for GUS expression in the developing root. In each line examined, the highest levels of GUS-specific staining were detected in differentiating epidermal cells within individual cell files near the onset of cell elongation (Fig. 4C,D). Transverse sections made from stained and embedded roots showed that GUS staining was located within epidermal cells situated over tangential cortical cell walls (Fig. 4E). Stained cells were not observed in underlying tissues of the roots, nor in the columella or lateral root caps. These results are consistent with the findings obtained from the in situ hybridization experiments described above, and indicate that the 4 kb *GL2* promoter fragment directs GUS expression in the same pattern as the GL2 product.

Detailed examination of the GUS-expressing plants provided additional clues regarding *GL2* regulation. Stained epidermal cells could be detected not only in the roots of 4-day-old seedlings but also in the emerging radicle of newly germinated seedlings (Fig. 4F) and at early stages of lateral root formation (Fig. 4G). When staining cell files were traced to their origin at

Table 2. Root cell files in wild-type, *gl2* mutant, and transgenic *Arabidopsis* seedlings

Line	Number of cortical cell files	Number of epidermal cell files
Landsberg wild-type	8.0±0.2	19.7±2.1
WS wild-type	8.0±0.0	19.3±1.7
gl2-1	8.0±0.3	20.5±1.8
gl2-2	8.1±0.4	20.3±2.2
gl2 transformant B12	8.1±0.1	17.9±1.1
gl2 transformant E9	8.0±0.0	18.2±1.2

Values represent the mean ± standard deviation.

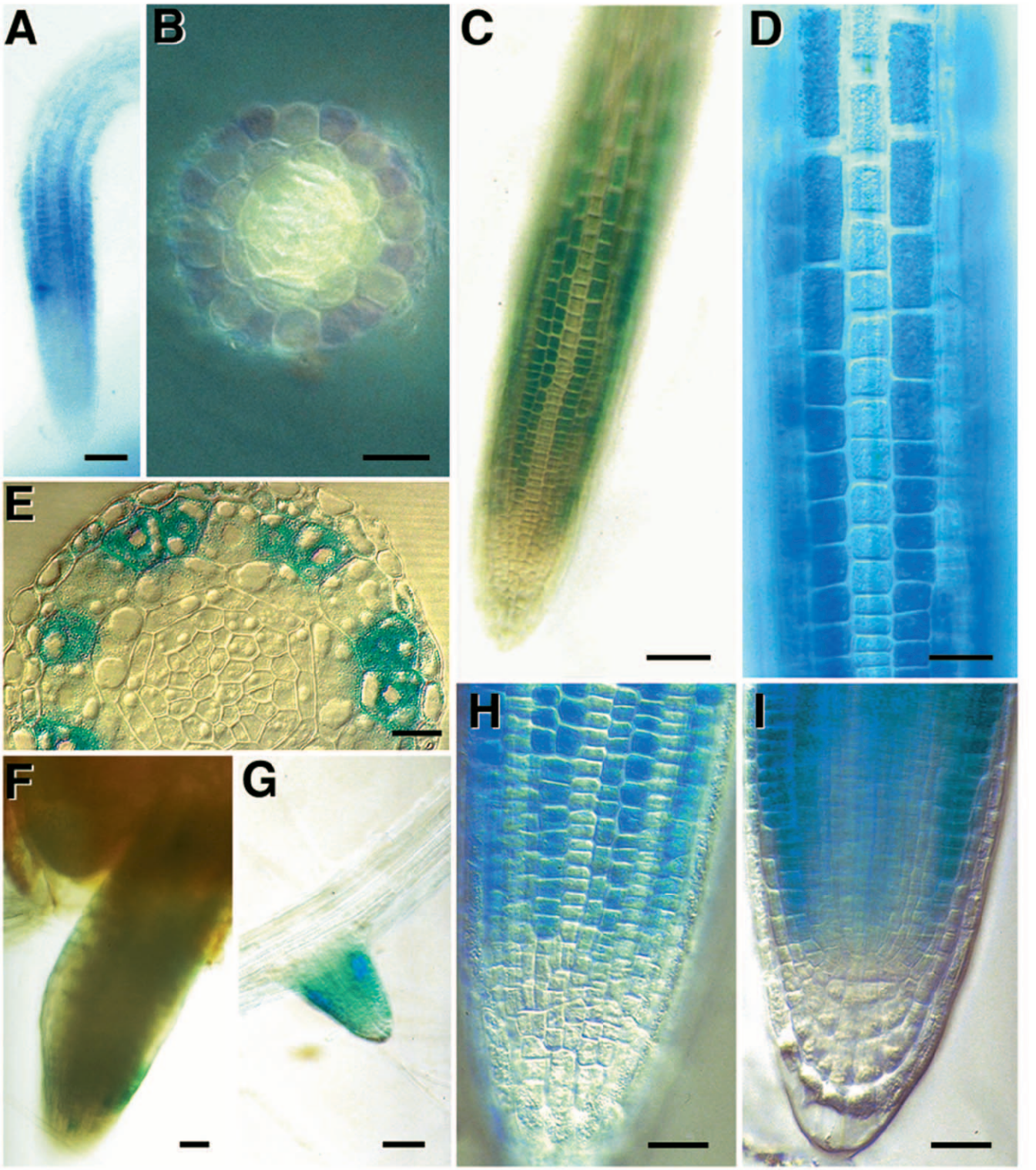

Fig. 4. Analysis of *GL2* gene expression in *Arabidopsis* roots. The differentiating hairless cells of the epidermis display preferential *GL2* expression. (A,B) Whole-mount in situ hybridization of *GL2* mRNA. Four-day-old seedlings were hybridized to a digoxigenin-labeled nucleic acid probe of the *GL2* gene, as described in the Materials and methods. (A) Surface view of root. Bar, 50 μm. (B) Transverse section of root. Bar, 20 μm. (C-H) β-glucuronidase (GUS) assays of transgenic plants harboring a *GL2* promoter-GUS gene fusion. Blue-staining tissue is indicative of GUS expression. (C) Four-day-old primary root. Bar, 50 μm. (D) Portion of root epidermis in region of maximal GUS staining (near onset of cell elongation). Bar, 20 μm. (E) Transverse section of primary root near the onset of cell elongation. Only epidermal cells located over tangential cortical cell walls exhibit GUS staining. A single layer of lateral root cap cells is present. Bar, 10 μm. (F) Two-day-old seedling, with radicle emerging from seed coat. Note staining cells near root apex. Bar, 20 μm. (G) Lateral root emerging from 6-day-old seedling root, stained for GUS activity for an extended period of time. Bar, 50 μm. (H) Epidermis of 4-day-old seedling at the root apex. Bar, 20 μm. (I) Root apex of 4-day-old seedling shown in H, but at a different focal plane to show origin of cell files. Bar, 20 μm.

the root tip, the youngest stained cell was determined to be located near, but not within, the core meristem at the root apex (approximately 2-3 cells from the epidermal/lateral root cap initial; Fig. 4H,I), implying that *GL2* is expressed shortly after a cell is generated in an epidermis-specific file. When the transgenic plants harboring the *GL2*-GUS reporter gene fusions were incubated for an extended period of time in the GUS reaction buffer, staining was detected in all root epidermal cells from the meristematic region through the root-hair formation zone, with the differentiating hairless cells remaining the most intensely staining cells.

In summary, the results of *GL2* expression studies in roots indicate that the *GL2* gene is preferentially expressed in developing hairless cells of the root epidermis. It is not highly expressed in developing root-hair cells, in mature root epidermal cells of either cell type, in the meristem initials of the epidermis/lateral root cap, or in the underlying cell layers of the *Arabidopsis* root.

DISCUSSION

These results show that the homeobox gene *GLABRA 2* is normally required for position-dependent differentiation of hairless cells in the *Arabidopsis* root. Mutations in *GL2* cause cells that would normally differentiate into mature hairless cells to produce root hairs, but they do not alter the differentiation of the normal hair-bearing cells. The *GL2* gene is preferentially expressed in a position-dependent manner, in differentiating cells located over tangential cortical cell walls (a position that is normally occupied by cells destined to be hairless cells), and at a developmental period when epidermal cells are acquiring cell-type-specific characteristics. Therefore, the GL2 homeodomain product is likely to act as a negative regulator of root-hair formation in a cell position-dependent manner to ensure that a subset of differentiating epidermal cells do not form root hairs.

Although the *gl2* mutations cause cells that would normally be hairless to form root hairs, they do not cause a complete conversion of one cell type into another. Interestingly, the *gl2* mutants possess two types of root-hair-bearing cells: one type which is indistinguishable from normal root-hair cells at all stages of development, and a unique type which possesses some of the characteristics of normal hairless cells. Specifically, the unique root-hair-bearing cell type in *gl2* mutants are similar to differentiating hairless cells in their reduced cytoplasmic density, advanced cell vacuolation, and extensive cell elongation. These cells also differ from normal root-hair cells in the timing of root-hair outgrowth; they exhibit a delay in the formation of hairs. The presence of epidermal cells with characteristics of both root-hair and hairless cells indicates that the *gl2* mutations uncouple various aspects of hairless cell differentiation, which implies that the *GL2* gene normally controls only a subset of the hairless cell developmental processes (specifically the inhibition of root-hair formation). Presumably, other regulatory gene(s) exist which specifically control other aspects of hairless cell differentiation.

The spatial and temporal expression of the *GL2* gene provides insight into its action. The *GL2* gene is preferentially expressed in differentiating epidermal cells located over tangential cortical cell walls (hairless cell positions) during a period thought to be critical for cell determination events. The zone of maximal *GL2* gene expression extends from the meristematic region (representing cells recently formed from cell divisions) through the elongating region (but prior to the region of hair formation). As the cells in these positions are the same ones affected by mutations in the *GL2* gene, it is likely that the GL2 product normally acts within these cells to ensure that they differentiate in an appropriate manner. Since the GL2 product contains a putative DNA-binding motif (the homeodomain), it may act to directly inhibit transcription of genes involved in hair formation. Furthermore, the semi-dominant nature of the *gl2* mutations may mean that the concentration of the GL2 product is critical for its action.

Since the *GL2* gene is expressed in a positionally defined set of root epidermal cells during a particular developmental period, it will be important to identify the gene products or factors responsible for the regulation of *GL2* expression. One candidate gene for such a role is *TTG*, since *TTG* normally acts at an early developmental stage to ensure that cells located over a tangential cortical cell wall differentiate into hairless cells (Galway et al., 1994). In *ttg* mutants, hairless cells appear to be completely converted to root-hair cells, and they exhibit intensely staining cytoplasm, delayed vacuolation, and root-hair production (Galway et al., 1994; unpublished observations). Thus, *TTG* may be an early regulator of the hairless cell fate, and *GL2* may be a downstream regulator of hair outgrowth. Plant hormones, such as auxin or ethylene, may also influence the *GL2* expression, since they are known to modify root-hair development in *Arabidopsis* and other species (Cormack 1949, 1962; Dolan et al., 1994; Masucci and Schiefelbein, 1994). The position-dependent expression of the GUS gene in the *GL2*-GUS lines should provide a convenient and sensitive marker for the detection and characterization of genes and factors which may play a role in epidermal cell patterning.

The differentiation of root-hair cells is normally associated with densely cytoplasmic cells, delayed cellular vacuolation and cell elongation. It has been suggested that these cellular characteristics are somehow required for the formation of the hair outgrowth (discussed by Cutter, 1978). The present study of the *gl2* mutant demonstrates that these aspects of cell differentiation can be uncoupled from the process of root-hair outgrowth. Thus, the dense cytoplasm, delayed cell vacuolation, and reduced cell elongation characteristic of developing root-hair cells are not required for the formation of a root hair.

Our research reported here and in an earlier publication (Galway et al., 1994) demonstrates that genes (like *TTG* and *GL2*) exist in *Arabidopsis* to ensure that a subset of epidermal cells do not form root hairs. Thus, an important aspect of root epidermis pattern formation is for certain cells to be 'programmed' to be hairless, which implies that root-hair cell formation may be the result of a 'default' developmental pathway for a root epidermal cell. This notion has been favored by numerous investigators (summarized by Barlow, 1984) and has been supported by physiological experiments. For example, physically isolating developing root epidermal cells from the underlying root tissue leads to the formation of ectopic root hairs, suggesting that a signal from underlying tissue is required for hairless cells to form (Bunning, 1951). In a group of monocotyledonous flowering plants which display a different pattern of epidermal cell types, Ivanov and Filippenko (1976) showed that when cell divisions are inhibited by gamma-irradiation, all epidermal cells form root hairs, indi-

cating that the asymmetric mitoses are essential for the differentiation of the hairless epidermal cells. It should be noted that, although epidermal cell pattern clearly is influenced by genes controlling hairless cell differentiation, it does not exclude the possibility that genes exist which encode positive regulators of the root-hair cell fate.

These results and those of an earlier publication (Galway et al., 1994) show that there is overlap in the genetic control of epidermal cell differentiation in the root and shoot of *Arabidopsis*. Both *GL2* and *TTG* affect trichome and root-hair production. However, it is striking that the differentiation of hair-bearing cells in the root and shoot epidermis is influenced in opposite ways by these genes. Recessive *ttg* mutations lead to the loss of trichomes (Koornneef, 1981) and excess root-hair production, whereas expression of R leads to excess trichomes (Lloyd et al., 1992) and a lack of root hairs over most of the root. Furthermore, *gl2* mutations lead to partial trichome development (Rerie et al., 1994) and partial differentiation of hairless root epidermal cells (this study), implying that the *GL2* gene may act in a subset of the developmental processes in each tissue. The opposite phenotypes in the two tissues may be explained by a difference in the default pathway for the development of the epidermal cells. In shoots, the default pathway may generate a trichome-less cell, whereas the default pathway in the root epidermis may generate a root-hair cell (as previously discussed). It will be important to determine the extent of the genetic overlap in epidermal cell differentiation in the shoot and root. It is clear there is not complete overlap as demonstrated by *GL1* gene function; plants homozygous for a null allele (*gl1-1*) lack trichomes (Oppenheimer et al., 1991; Koornneef et al., 1982) and yet do not show altered root epidermis differentiation (Galway et al., 1994).

In addition to affecting trichome and root-hair production, the *GL2* and *TTG* genes also participate in other epidermis-related pathways. The *TTG* gene has been shown to participate in anthocyanin biosynthesis and seed mucilage production (Koornneef, 1981, 1990), and the *GL2* gene was shown to influence seed mucilage production (Koornneef et al., 1982). It is possible that *TTG* and *GL2* encode general transcription factors in the *Arabidopsis* epidermis that act in concert with other gene products which are specific for each process. Indeed, evidence for interaction between the *TTG* and *GL1* products during trichome development has recently been reported (Larkin et al., 1994). Thus, the identification and analysis of genes, like *GL2* and *TTG*, that regulate root epidermis development should not only lead to further insights into the molecular mechanisms controlling cell differentiation in plants, but also to a more complete understanding of the evolution of developmental gene regulation.

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