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The *TTG* Gene Is Required to Specify Epidermal Cell Fate and Cell Patterning in the *Arabidopsis* Root

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The control of cell fate was investigated in the root epidermis of *Arabidopsis thaliana*. Two distinct types of differentiated epidermal cells are normally present: root-hair-bearing cells and hairless cells. In wild-type *Arabidopsis* roots, epidermal cell fate was found to be correlated with cell position, with root-hair cells located over radial walls between cortical cells, and with hairless cells located directly over cortical cells. This normal positional relationship was absent in *ttg* (*transparent testa glabrous*) mutants (lacking trichomes, anthocyanins, and seed coat mucilage); epidermal cells in all positions differentiate into root-hair cells. The opposite condition was generated in roots of transgenic *Arabidopsis* expressing the maize *R* (*R-Lc*) gene product (a putative *TTG* homologue) under the control of a strong promoter (CaMV35S), which produced hairless epidermal cells in all positions. In both the *ttg* and *R*-expressing roots, epidermal cell differentiation was affected at an early stage, prior to the onset of cell elongation or root-hair formation. The *ttg* mutations were also associated with abnormalities in the morphology and organization of cells within and surrounding the root apical meristem. The results indicate that alterations in *TTG* activity cause developing epidermal cells to misinterpret their position and differentiate into inappropriate cell types. This suggests that, in wild-type roots, *TTG* provides, or responds to, positional signals to cause differentiating epidermal cells that lie over cortical cells to adopt a hairless cell fate. © 1994 Academic Press, Inc.

INTRODUCTION

The growth and development of plants depends on the continuous formation and differentiation of cells in meristematic regions at the root and shoot apices. The mechanisms that specify the fate of newly formed plant cells are poorly understood. To date, most of the evidence concerning cell fate specification in plants has been garnered from studies of genetic mosaics, particularly in maize, and this evidence points to the importance of cell position, rather than cell lineage, in determining cell fate during plant development (Stewart, 1978; Barlow and Carr, 1984; Poethig, 1988; Sinha and Hake, 1990; Becraft *et al.*, 1990).

The myriad forms of specialized epidermal cells in plants such as stomata, trichomes, and root-hair cells have fascinated researchers over the last century (for a review of the older literature see Haberlandt, 1914). Recently, attention has been directed toward understanding the factors controlling the differentiation and spacing of these specialized cells (e.g., Bünning, 1952; Barlow, 1984; Sachs, 1991). During the maturation of the root epidermis, each cell ultimately adopts one of two alternative fates: it may develop into a cell capable of producing a root hair (trichoblast; which we shall hereafter term a root-hair cell) or it may differentiate into a hairless cell (atrichoblast). Root hairs are tip-growing tubular-shaped outgrowths which help anchor roots, interact with soil microorganisms, and assist in the uptake of water and nutrients (Cormack, 1949, 1962; Clarkson, 1985).

Although we lack a comprehensive survey of root-hair ontogeny and spacing among plants, two general mechanisms have been identified for specifying the fate of root epidermal cells. In one mechanism, utilized by a diverse group of species including many monocots, an early asymmetric division of an epidermal cell gives rise to a small densely cytoplasmic cell (which later produces a root hair) and a larger cell (which remains hairless) (Leavitt, 1904, and references therein; Sinnott and Bloch, 1939; Avers, 1963). In the mature root, this yields a pattern of hairless epidermal cells interspersed with root-hair cells throughout the epidermis. A different pattern of epidermal cell differentiation was identified in the Brassicaceae, in which the fate of an epidermal cell is determined by its position relative to cells in the underlying cortical layer of the root (Cormack, 1947; Bünning, 1951; Dolan *et al.*, 1993). This mechanism results in a distinctive pattern of hair-forming and hairless cells

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in the mature root, such that axial files of epidermal cells are composed entirely of hairless cells or entirely of root-hair cells.

The relatively simple and invariant cellular organization of the primary root of *Arabidopsis thaliana* (a member of the Brassicaceae) makes it an attractive target for molecular genetic studies of development (Dolan *et al.*, 1993; Benfey and Schiefelbein, 1994). The cells are largely organized into a small number of longitudinal columns or files that can be traced back to sets of putative initials in the root apical meristem. Regular divisions in these initials generate new cells which differentiate basipetally. Thus, for many cell types, such as those in the root epidermis, it is possible to analyze the complete process of cell differentiation by examining cells along a particular file from the meristem to the mature portion of the root.

To initiate a molecular genetic study of epidermal cell fate in plants, we have analyzed cell differentiation in the root epidermis of wild-type and mutant *Arabidopsis* plants. In previous studies, loci have been identified that affect the morphogenesis of root hairs in *Arabidopsis* (Schiefelbein and Somerville, 1990; Schiefelbein *et al.*, 1993); however, no genes have been reported that affect cell fate in the root epidermis of *Arabidopsis* or any other plant species. In this paper, we detail the position-dependent organization of root-hair-bearing and hairless cells in wild-type *Arabidopsis* roots, and we analyze two *Arabidopsis* lines that exhibit striking alterations in the specification of epidermal cell fate. In each of these two lines, the recessive *ttg* (*transparent testa glabrous*) mutant and transgenic plants expressing the maize *R* (*R-Lc*) cDNA, specific sets of differentiating epidermal cells appear to misinterpret their position and adopt inappropriate cell fates. Our results indicate that the *TTG* gene product is normally required to ensure the position-dependent differentiation of the hairless cells in the *A. thaliana* root epidermis.

MATERIALS AND METHODS

Plant Strains and DNA Constructions

The *ttg* and *ttg-w* mutants (each in the Landsberg erecta genetic background) were obtained from A. R. Kranz of the *Arabidopsis* Information Service and Dr. Maarteen Koornneef, respectively. Two constructs (pAL69 and pAL144) containing the maize *R* cDNA under the transcriptional control of the CaMV35S promoter were made and introduced into *Arabidopsis* by *Agrobacterium*-mediated plant transformation (Lloyd *et al.*, 1992). The 2.4-kb insert in pAL69 includes the entire cloned 5′ untranslated cDNA leader, which contains three upstream AUG codons, as well as the entire *R* coding region. The pAL144 plasmid contains a 2.2-kb fragment from pAL69 that lacks the three upstream AUG codons but retains the native AUG start codon. The upstream open reading frame has been shown to negatively affect the expression of *R* in maize (Damiani and Wessler, 1993). The *R* transformants used in these studies were 1434 and 1436 (*ttg* transformed with pAL144), 1439 (Rschew transformed with pAL144), and 906 (Rschew transformed with pAL69). The *C1* transformant (line 1134) was derived from the Rschew wild type and contains a plasmid (pAL71) with the 2.1-kb cDNA from the maize *C1* gene under transcriptional control of the CaMV35S promoter (Lloyd *et al.*, 1992).

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). Values for root-hair number per millimeter were determined by counting the number of hairs from one side of a 2-mm segment from the differentiated region of at least 10 roots of 5- or 6-day-old plants.

Most of the transverse sections of roots were obtained by placing seedlings into molten 3% agarose, and after solidification, hand-sections of the agarose block were made with a razor blade. For photomicrography, the sections were stained with 0.05% Fluorescent Brightener 28 (Sigma Chemical) and viewed under a Leitz Orthoplan microscope equipped with a Ploempak 2.1 fluorescence vertical illuminator, HBO 200 mercury burner, and Neofluor objectives. Photomicrographs were recorded on Kodak TMAX black and white print film at ASA 400. The number of cortical and epidermal cell files was determined from similarly prepared hand sections taken from the mature portion of 22 to 26 roots of 5-day-old seedlings, and sections were stained with 0.05% toluidine blue O (Fisher Scientific) in 10 m*M* Mes (pH 6.0). The position of root-hair cells was determined from hand sections (briefly stained as above) obtained from the mature portion of at least 10 roots.

For examination by differential interference contrast (DIC) optics, 5-day-old seedlings were placed in APW medium (artificial pond water; Schiefelbein *et al.*, 1992) after staining for 1 min with 0.01% ruthenium red in APW. Photomicrographs were recorded on Kodak TMAX black and white print film at ASA 100 using DIC optics and a green interference filter. Images were obtained from 9 to 14 roots of each genotype.

To observe the cytoplasm in the differentiating root-hair and hairless cells, 5-day-old seedlings were fixed *in situ* in 1% glutaraldehyde in a mineral salts solution (Estelle and Somerville, 1987) for 1 hr. The lower half of each root was severed and transferred to a 2% glutaraldehyde solution for a further 2 hr. The roots were

washed, postfixed in 1% osmium tetroxide for 2 hr, and dehydrated in a graded ethanol series, followed by a graded acetone series. After infiltration and embedding in Spurr's resin, transverse sections (0.4 μm thick) were obtained from the region of differentiating root epidermal cells. The sections were dried on glass slides, stained briefly with 0.5% toluidine blue 0 in 0.1% sodium carbonate (pH 11), washed, and mounted in microscope immersion oil for viewing with a Leitz Laborlux S microscope.

The scanning electron microscopy (SEM) was performed as described (Schiefelbein *et al.*, 1993), using 5- or 6-day-old seedlings examined with an environmental scanning electron microscope (Electroscan Model E3). Conventional SEM was attempted, but it did not preserve the integrity of the root epidermal cells or root hairs.

Transverse (1.5 μm thick) and longitudinal (2 μm thick) serial sections of root apices were taken from Rschew wild type, *ttg* mutant, and *R* transformants embedded in glycol methacrylate (GMA). Five-day-old seedlings were fixed in 2% glutaraldehyde in 25 m*M* sodium phosphate buffer at pH 6.8 for 24 hr at 4°C. After washing for 3 hr, they were embedded in 2% low-melting-point agarose (Gibco BRL, Gaithersburg, MD). The root tips were excised and the surrounding agarose was trimmed to small pieces which were subsequently dehydrated in a graded ethanol series to 100% ethanol. The root tips were then infiltrated through a graded series of ethanol and unpolymerized GMA (kit from Ted Pella, Inc., Redding, CA) over a 12-hr period, followed by a gradual infiltration with partially polymerized GMA over 1.5 days. The specimens were given several changes of 100% partially polymerized GMA over 1 to 2 days and then placed in gelatin capsules and polymerized under a uv light in a cold room at 4°C for 12 to 14 hr. Transverse or longitudinal sections taken from 2 to 8 roots of each genotype were dried down onto glass microscope slides, stained briefly with 0.05% toluidine blue O in sodium acetate buffer (pH 4.4), rinsed, and mounted in distilled water for microscopy. Photomicrographs were recorded on Kodak TMAX black and white film at ASA 100 with or without DIC optics as indicated.

Genetic Analyses

Cosegregation tests were initiated by cross-pollinating the *ttg* mutant with plants of the Landsberg wild type. The F_2 seeds from two independent F_1 plants were germinated on nutrient medium in petri dishes as described (Schiefelbein and Somerville, 1990) and assessed for root-hair phenotype (after 5 days of growth) and trichome phenotype (after 10 days of growth). Heterozygous *ttg*/+ plants used for root-hair counts were generated by crossing homozygous *ttg* mutant plants with the Landsberg wild type.

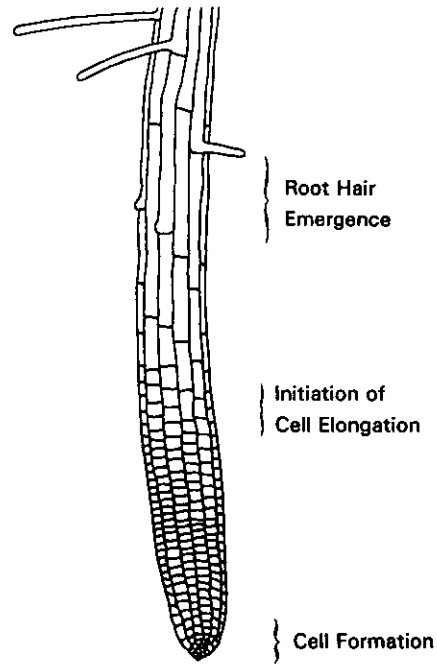

FIG. 1. Epidermal cell development and organization in the root of *Arabidopsis thaliana*. Epidermal cell differentiation progresses from root tip (undifferentiated cells) to base (fully differentiated cells). Cell division in the apical meristem at the root tip generates longitudinal files of epidermal cells. The region of the root where epidermal cell elongation initiates (and where vacuolation differences indicative of the fates of epidermal cells first become evident) is indicated. Also indicated is the region of the root where root hairs emerge. The figure was traced from photographs of a wild-type (Rschew) root, which had a diameter of approximately 100 μm. The root cap, which normally encloses the root tip, is not included in this figure.

For most analyses with the *R* transformants, kanamycin-resistant seedlings from a mixed population (consisting of heterozygous and homozygous individuals) were used. Homozygous *R* lines were obtained by testing selfed seed from individual plants, and heterozygous lines were obtained by crossing verified homozygous plants with Rschew wild-type plants (for Rschew-*R* transformants) or *ttg* mutant plants (for *ttg*-*R* transformants).

RESULTS

Epidermal Cell Differentiation in Wild-Type Roots

The differentiation and arrangement of hair-bearing and hairless root epidermal cells in *Arabidopsis* is typical of the family Brassicaceae (Cormack, 1947, 1949; Bünning, 1951; Dolan *et al.*, 1993). The epidermis is organized into longitudinal files of cells, and the stages of epidermal cell differentiation can be identified along the length of these files (outlined in Figs. 1 and 2A). Each epidermal cell file is composed entirely of root-hair cells or entirely of hairless cells (Figs. 1 and 2D). Transverse sec-

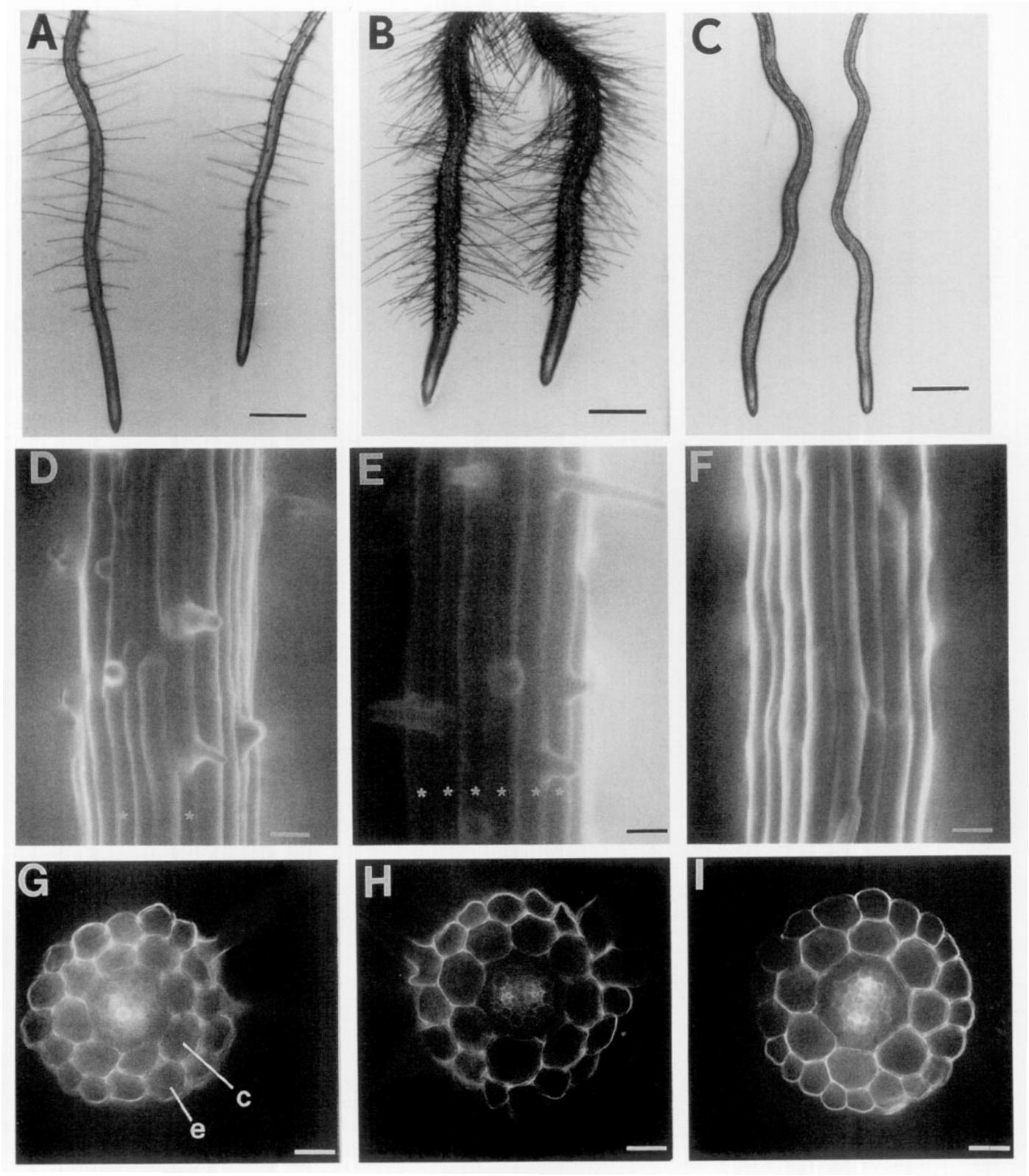

FIG. 2. Seedling roots from wild-type, *ttg* mutant, and *R*-expressing *Arabidopsis* plants. (A–C) Roots of 5-day-old seedlings. (A) Rschew wild type. (B) *ttg* mutant. (C) *ttg-R* transformant 1434. Bars, 300 μm. (D–F) Environmental scanning electron micrographs of 5-day-old seedling roots in the region of root-hair emergence. (D) Wild-type Rschew. (E) *ttg* mutant. (F) *ttg-R* transformant 1434. Asterisks indicate the cell files bearing root hairs. Bars, 20 μm. (G–I) Transverse sections of 5-day-old roots. (G) Rschew wild type; e, epidermis; c, cortex. (H) *ttg* mutant. (I) *ttg-R* transformant 1434. Note that, in wild type and *ttg* mutant, only some of the eight cells located over radial cortical cell walls display root-hair outgrowths. Because root hairs emerge from a small area on the surface of the elongated epidermal cells (note Fig. 1), this position is not uniformly represented in every section. Bars, 20 μm.

TABLE 1
ROOT CELL FILES IN WILD-TYPE, MUTANT, AND TRANSFORMANT *Arabidopsis* SEEDLINGS[a]

Line	Cortical cell files (number)	Epidermal cell files (number)
Rschew wild type	8.0 ± 0.2	17.8 ± 1.4
ttg/ttg	8.1 ± 0.3	18.6 ± 2.1
ttg-R transformant 1434	8.0 ± 0.4	17.5 ± 1.5
Rschew-*R* transformant 1439	8.0 ± 0.2	16.6 ± 0.9

[a] Values represent the mean ± standard deviation.

tions show that wild-type *Arabidopsis* seedling roots (ecotype Rschew) possess 16 to 23 epidermal cell files surrounding a ring of 8 cortical cell files (Table 1, Fig. 2G). Root hairs are produced by epidermal cells in the 8 files that lie over the radial (anticlinal) walls between adjacent, underlying cortical cells. Epidermal cells in the remaining 8 to 15 files, those that lie over a tangential cortical cell wall, are normally hairless (Table 2). This arrangement generates 8 files of root-hair cells and 8 to 15 files of hairless epidermal cells. Typically, the root-hair files are separated by 1 or 2 hairless cell files, although 0 or 3 intervening hairless cell files were occasionally observed. Roots from two other *Arabidopsis* ecotypes (Columbia and Landsberg erecta) were examined, and they possess a similar number of epidermal cell files, ranging from 15 to 25 in number, with epidermal hairs again restricted to the 8 files directly over the radial walls of the underlying cortical cells. This observed correlation between the location of epidermal cells and their differentiated state implies that position may influence cell fate.

Examination of the developing epidermis near the root apex showed that epidermal cells destined to form root hairs could be distinguished from cells destined to remain hairless in two ways. First, differentiating root-hair cells display a delay in vacuolation, relative to cell elongation. This is readily observed near the onset of epidermal cell elongation (approximately 300 μm from the apical meristem), using differential interference contrast microscopy (Fig. 3A). Second, at an earlier stage of differentiation (within 200 μm of the root apical 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. 4), which may reflect differences in the metabolic activity of the cells (discussed in Cutter, 1978). These results show that the two types of epidermal cells are programmed at an early stage to follow distinct differentiation pathways and they do not differ merely at the later stage of root-hair formation.

The ttg Mutation Alters Cell Fate in the Root Epidermis

To identify loci that affect cell fate specification, *Arabidopsis* mutants were screened for their root epidermal cell phenotype. The recessive *ttg* mutant is completely devoid of trichomes (leaf hairs); it also lacks both anthocyanins (the common red, blue, and purple pigments of plants) and seed coat mucilage, and the locus has been mapped to chromosome 5 (Koornneef, 1981, 1990). When the roots of homozygous *ttg* plants were examined, an excessive number of root hairs was observed compared to wild type (Table 3 and Fig. 2B). Roots of plants heterozygous for the *ttg* mutation displayed a normal number of root hairs (Table 3), indicating that the mutation affecting root-hair production is recessive.

To determine whether the *ttg* mutation was responsible for the excess root-hair phenotype, two types of tests were conducted. Cosegregation analysis was performed by cross-pollinating the homozygous *ttg* mutant with the Landsberg erecta wild type, allowing the resulting F_1 plants to self-pollinate and analyzing the F_2 progeny. Among a total of 211 F_2 plants, all 147 plants that produced a normal number of root hairs also produced trichomes on their leaves, and all 64 plants that produced excess root hairs also lacked trichomes. In a second test, root-hair production was examined in an independent *ttg* mutant line (*ttg-w*), which displays a "weak" *ttg* phenotype with respect to trichome formation and anthocyanin production. The roots of the *ttg-w* mutant were found to produce more root hairs than the wild type, but fewer than the original *ttg* mutant (Table 3). The results of these tests show that the excess root-hair phenotype is due to the mutations at the *ttg* locus and is not an effect of the genetic background or another mutation in the *ttg* lines.

Detailed examination of the *ttg* mutant roots showed that root hairs form on epidermal cells in all files and are not limited to epidermal cells located over a radial cortical cell wall (Table 2; Fig. 2E). It appears that although epidermal cells in all files form root hairs, every epidermal cell in the *ttg* root does not form a root hair. The morphology of all root hairs produced by the *ttg* mutant is indistinguishable from that of wild-type hairs. Furthermore, the *ttg* root hairs emerge at 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). Transverse sections from the mature portion of the root show that the number of epidermal and cortical cell files in *ttg* roots is indistinguishable from that of the wild type (Fig. 2H; Table 1). These results indicate that the *ttg* mutation alters the positional control of root-hair cell differentiation, but it does not affect root-hair formation per se nor does it affect the structure of the mature root.

The developing epidermal cells in the *ttg* mutant exhibit the characteristics of differentiating root-hair cells. In the region of initial cell elongation, all epidermal cells display a delay in vacuolation (relative to cell elongation) that is typical of differentiating root-hair

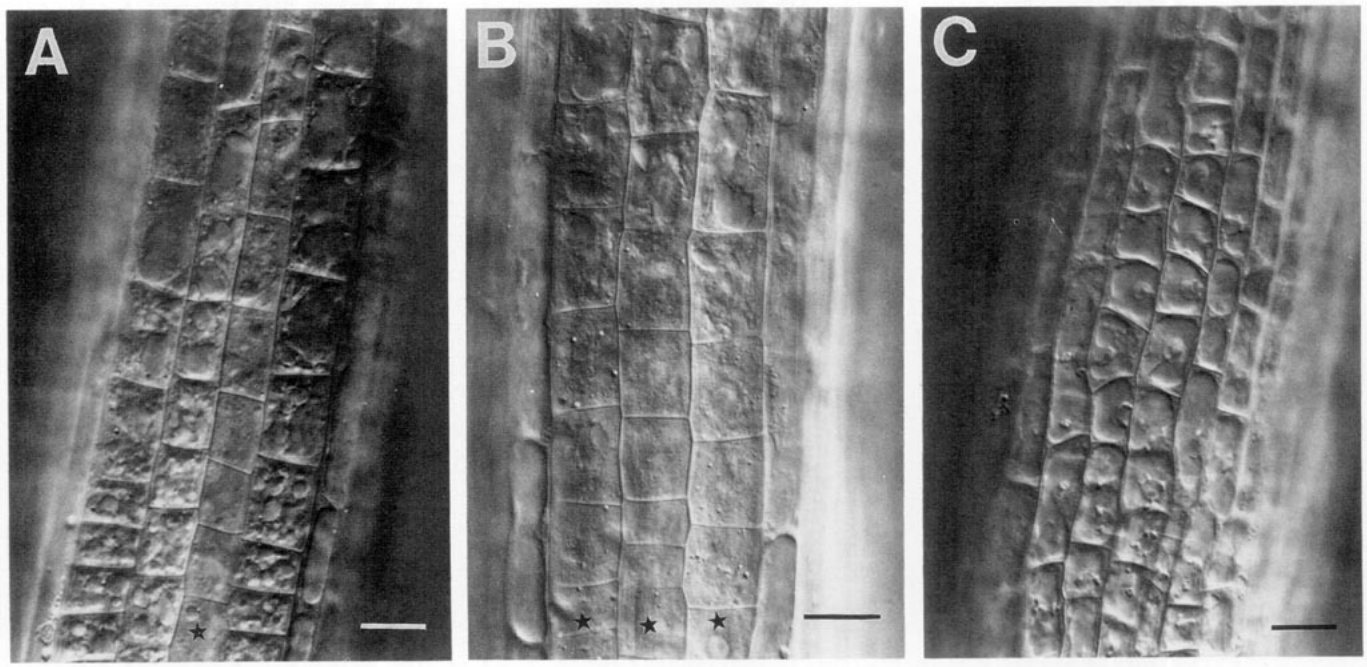

FIG. 3. Vacuole formation in *Arabidopsis* root epidermal cells. The displayed portion of the root encompasses the initial region of epidermal cell elongation, and it is located approximately 0.2 to 0.4 mm distal to the root meristem and approximately 0.3 to 0.5 mm proximal to the position where root hairs emerge. In all epidermal cell files, regardless of genotype, cells become progressively more vacuolated distal to the meristem. (A) Rschew wild type. The star indicates the file that contains differentiating root-hair cells. Differences in the timing of vacuolation between future root-hair and hairless cells are most pronounced at the onset of cell elongation; larger vacuoles form in the future root-hair cells only after cell elongation. (B) *ttg* mutant. Cells in all epidermal files (indicated by stars) are differentiating into root-hair cells. The appearance of large central vacuoles follows the onset of cell elongation. (C) *ttg-R* transformant 1434. Cells in all epidermal files are differentiating into hairless cells. The appearance of large central vacuoles precedes the onset of cell elongation. Bars, 20 μm.

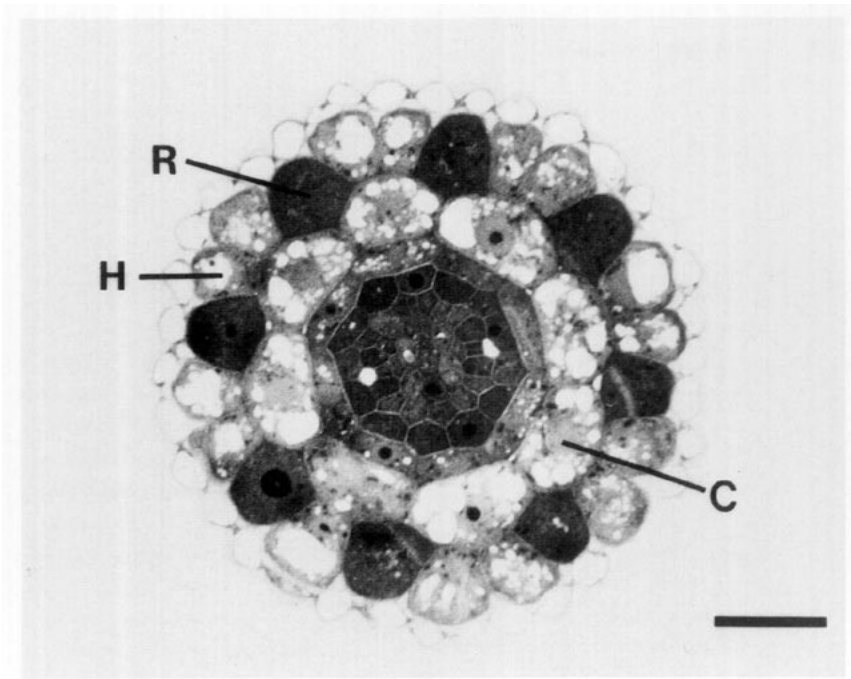

FIG. 4. Transverse section of wild-type *Arabidopsis* root taken prior to the region of initial cell elongation. Note that the differentiating epidermal cells located over the radial cortical cell walls (future root-hair cells) exhibit intense staining, relative to the cells located over the cortical cells (future hairless cells). R, differentiating root-hair cell; H, differentiating hairless cell; C, cortical cell. This section was stained with 0.5% toluidine blue. Bar, 20 μm.

TABLE 2
POSITION OF ROOT-HAIR CELLS IN WILD-TYPE, MUTANT, AND TRANSFORMANT *Arabidopsis* SEEDLING ROOTS

Line (Number[a])	Percent of root-hair cells located in a: Normal position[b]	Abnormal position[c]
Rschew wild-type (32)	100	0
ttg/ttg (61)	51	49
ttg-w/ttg-w (37)	73	27
ttg-R transformant 1434 (65)	98	2
ttg-R transformant 1436 (30)	90	10
Rschew-R transformant 1439 (19)	100	0
Rschew-R transformant 906 (15)	100	0

[a] Number of root-hair cells identified and scored.
[b] The normal position for root-hair cells is over a radial wall that separates adjacent cortical cells.
[c] The abnormal position for root-hair cells is directly over a cortical cell.

cells in wild-type plants (Fig. 3B). Considered together, the suite of *ttg* root phenotypes indicates that the wild-type *TTG* gene normally influences the fate of root epidermal cells but does not affect the cellular organization of the mature root or the morphogenesis of root hairs.

Expression of the Maize R cDNA in Arabidopsis Alters Cell Fate

The maize *R* encodes a protein with an acidic domain and a basic helix-loop-helix domain, two features similar to the *myc*-like family of transcriptional activators (Ludwig *et al.*, 1989). In maize, *R* activates transcription from the promoters of the structural genes in the anthocyanin pathway (Klein *et al.*, 1989; Roth *et al.*, 1991; Bodeau and Walbot, 1992). The expression of the maize *R* cDNA in transgenic *Arabidopsis* plants restores trichome and anthocyanin production in the *ttg* mutant background, suggesting that the *TTG* gene encodes, or activates, an *R* homologue in *Arabidopsis* (Lloyd *et al.*, 1992). Because the *TTG* gene has not been cloned, we examined the effects of maize *R* expression on root epidermal cell fate in *Arabidopsis*. Transgenic *ttg* and wild-type lines were analyzed that express the *R* coding region under the control of the cauliflower mosaic virus 35S promoter. The CaMV35S promoter directs gene expression in most plant cell types, including root cells, although expression levels vary in different cell types (Benfey *et al.*, 1989).

Transformants that possess an *R*-expressing construct in a *ttg* mutant background (designated *ttg-R* transformants) exhibited a dramatic reduction in root-hair production (Figs. 2C and 2F; Table 3). The frequency of root hairs in these lines was reduced more than 17-fold compared to that of the *ttg* mutant (Table 3). The hairless epidermal cells in these lines display no sign of root-hair initiation nor any aborted root hairs (Fig. 2F). Furthermore, nearly all of the differentiating epidermal cells in the region of initial cell elongation in the *ttg-R* roots display an early vacuolation pattern (relative to cell elongation) typical of differentiating hairless cells from wild-type plants (Fig. 3C). The analysis of transverse sections showed that the *ttg-R* roots produce a normal number of cortical and epidermal cell files (Fig. 2i, Table 1). These observations show that the expression of the maize *R* cDNA suppresses the excess root-hair phenotype of the *ttg* mutant and generates an excess number of hairless epidermal cells.

Transformants containing the *R* construct in the Rschew wild-type genetic background (designated *Rschew-R*) displayed the same root phenotype as the *ttg-R* transformants (Tables 1 and 3; other data not shown), indicating that *R* expression (controlled by the CaMV-35S promoter) suppresses the effects of the wild-type *TTG* gene expression with respect to root epidermal cell fate. Taken together, these results indicate that the 35S-promoter-driven expression of *R* in *Arabidopsis* alters the fate of root epidermal cells, such that cells that would normally adopt a root-hair cell fate instead adopt a hairless cell fate.

Root hairs were occasionally produced in each of the four *R*-expressing lines examined (Table 3). The morphology of these root hairs, and the site of root-hair emergence within the epidermal cells, was indistinguishable from the wild type, indicating that *R* expression does not alter root-hair morphogenesis. The location of the epidermal cells that produced these root hairs was determined by examining transverse sections from the various *R* transformants, and the results are summarized in Table 2. As expected, the small number of root hairs in *Rschew-R* transformants emerged from epidermal cells located over a radial wall between adjacent cortical cells (the location of root-hair cells in wild-

TABLE 3
ROOT HAIR PRODUCTION IN WILD-TYPE, MUTANT, AND TRANSFORMANT *Arabidopsis* SEEDLINGS[a]

Line	Root hairs (number/mm)
Rschew wild type	69.1 ± 14.4
Landsberg wild type	61.5 ± 7.7
ttg/ttg	112.7 ± 13.4
ttg/+	62.3 ± 10.5
ttg-w/ttg-w	86.8 ± 8.4
ttg-R transformant 1434	2.3 ± 3.1
ttg-R transformant 1436	6.6 ± 6.2
Rschew-*R* transformant 1439	20.3 ± 10.8
Rschew-*R* transformant 906	18.2 ± 9.3

[a] Values represent the mean ± standard deviation.

type plants). The root hairs in *ttg-R* transformants also preferentially emerged from epidermal cells located in the wild-type position, i.e., over a radial wall between cortical cells. This result implies that, despite the apparent abolition of positional influences in the *ttg* mutant background, differentiating root epidermal cells in the *ttg-R* transformants are not equivalent.

There is considerable variation in the number of root hairs produced by *R*-expressing plants (Table 3). The variation may result from differences in the level of *R* expression, perhaps because of differences in the copy number of the *R* construct or to position effects related to the location of the *R* construct in the *Arabidopsis* genome. To determine whether the number of copies of an *R* construct could affect root-hair cell number, plants that were heterozygous and homozygous for each of the *R* transgenes were generated and analyzed. In each case, the heterozygous plants produced a greater number of root hairs than homozygous plants, as shown in Table 4. Thus, the concentration of the *R* gene product may be an important factor in affecting root epidermal cell fate in these transformants.

At the root–shoot junction, each of the *R* transformants produced a normal number of morphologically wild-type root hairs. This junction represents a unique portion of the seedling root that is formed during embryogenesis (Dolan *et al.*, 1993), and these hairs may therefore be generated by a mechanism(s) different from that operating during postembryonic root development. This conclusion is supported by observations of some root-hair morphology mutants of *Arabidopsis;* abnormal root hairs are present along the major portion of the root, but morphologically normal root hairs are present at the root–shoot junction in these mutants (M. Galway, J. Masucci, and J. Schiefelbein, unpublished results).

Cell Morphology and Organization Is Altered at the Root Apex in ttg and R-Expressing Plants

To determine whether the *TTG* gene affects the earliest stages of root epidermal development, the cellular organization within and surrounding the apical meristem of the primary seedling root was examined in the Rschew wild type, *ttg* mutant, *ttg-R*, and Rschew-*R* lines. Longitudinal and serial transverse sections of the Rschew wild-type root apex showed no significant differences in the number, arrangement, or morphology of component cells (Figs. 5A–5C and 6A), compared to previously described wild-type *Arabidopsis* seedling roots (Columbia ecotype; Dolan *et al.*, 1993). The apical meristem of wild-type *Arabidopsis* primary roots consists of four central cells (the putative quiescent center) surrounded by four groups of cells that appear to act as initials to generate the root-cap columella, the lateral root cap and epidermis, the cortex and endodermis, and the stele (Figs. 5A and 6A; Dolan *et al.*, 1993). The root cap (which encloses the root apex) consists of three orderly tiers of amyloplast-containing columella cells that arise from columella initials situated just below the central cells (Fig. 6A). The columella cells abut three layers of lateral root-cap cells which extend along the side of the root for varying distances. Encircling the four central cells of the meristem are eight cortical/endodermal cell initials (Figs. 5A and 6A). Within 50 μm above the central cells, the cortical cells form a distinct layer consisting of eight cell files, and each cortical cell displays a characteristic oval morphology: a flattened cell wall in contact with the developing endodermis and a rounded cell wall in contact with the epidermis (Fig. 5B). Surrounding the cortical/endodermal initials is a set of cells (the epidermis/lateral root-cap initials) which divides regularly to give rise to both the epidermis and the lateral root cap (Figs. 5A and 6A). As the epidermis develops, the eight cells positioned over the radial cross walls between the underlying cortical cells become distinct from the remaining epidermal cells by virtue of their unique morphology and intense staining by toluidine blue (Figs. 5B and 5C).

Because cell number and cellular organization in the mature portion of the seedling root was not abnormal in the *ttg* and *R*-expressing lines (Fig. 2 and Table 1), we did not expect to find significant differences at the root apex. However, median longitudinal and transverse sections revealed abnormalities in the size, shape, and arrangement of different cell types in and near the root meristems of these seedlings (Figs. 5D–5L and 6B–6D).

Root apices of the *ttg* mutant exhibited the most severe alterations in cell morphology and organization. The degree of severity varied from seedling to seedling and within different portions of the same root. Overall, the diameter of the *ttg* root apex was larger than the wild type, indicating abnormal division planes and/or cell expansion (compare Figs. 5D–5F to Figs. 5A–5C). In *ttg* roots where central cells and/or cell-file initials could be identified, these cells had irregular shapes and sizes. In other *ttg* roots, central cells and cell initials could not

TABLE 4
ROOT-HAIR PRODUCTION IN TRANSFORMANT *Arabidopsis* SEEDLINGS HETEROZYGOUS AND HOMOZYGOUS FOR *R* TRANSGENES[a]

Line	Root-hair number per mm	
	R heterozygotes	*R* homozygotes
ttg-R transformant 1434	3.1 ± 2.8	0.8 ± 1.2
ttg-R transformant 1436	14.4 ± 5.1	4.1 ± 3.3
Rschew-*R* transformant 1439	28.6 ± 9.2	5.5 ± 4.3
Rschew-*R* transformant 906	23.4 ± 8.1	3.2 ± 3.5

[a] Values represent the mean ± standard deviation.

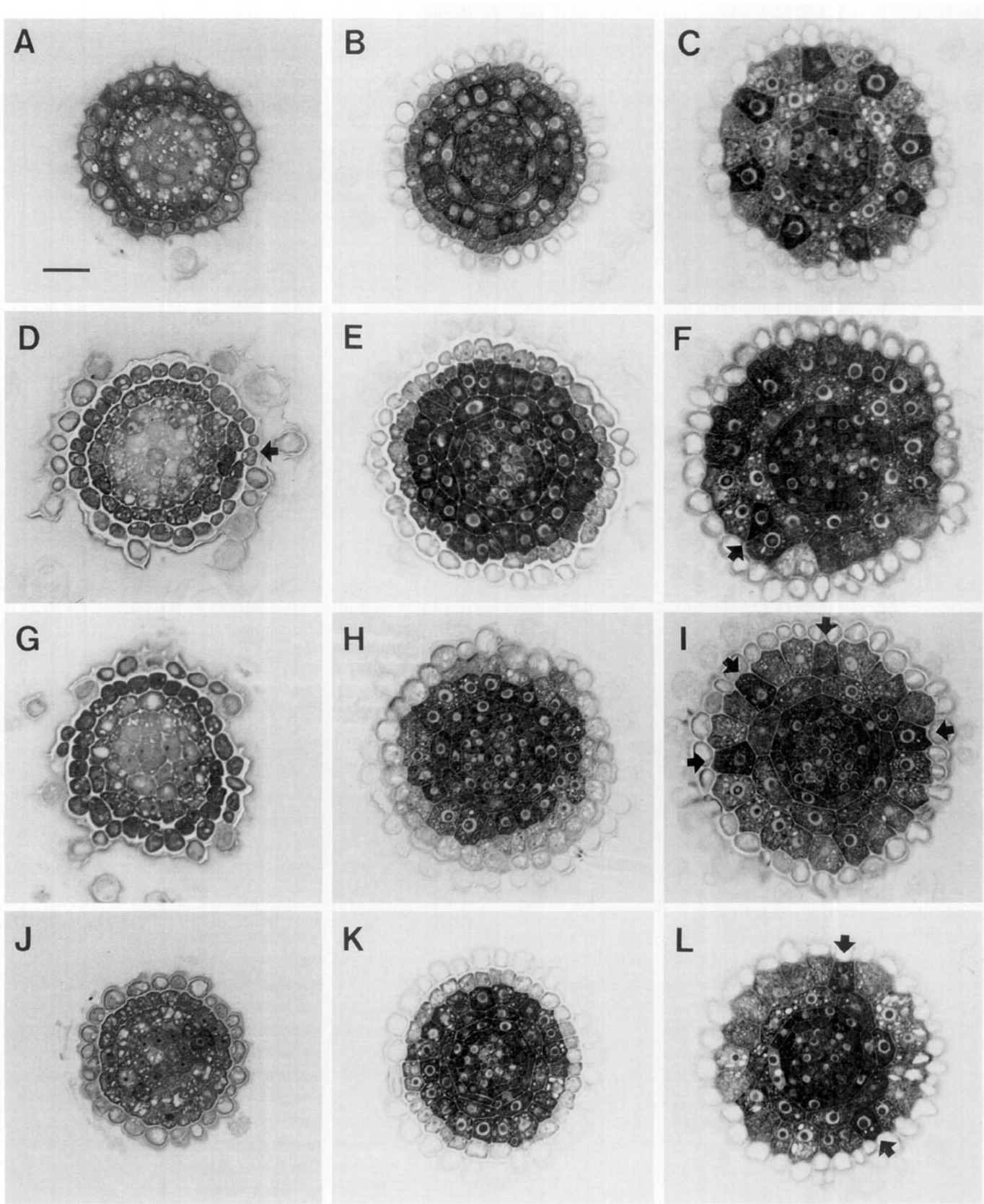

FIG. 5. Series of toluidine blue-stained transverse sections (1.5 μm thick) from four different 5-day-old primary root apices of *Arabidopsis*. All figures are to same scale. Bar in (A), 20 μm. (A–C) Rschew wild type. (A) Central cell region. Four central cells are surrounded by eight putative cortical/endodermal cell initials. Surrounding these eight cells is a layer of putative epidermal/lateral root cap initials, then two layers of lateral root cap cells. (B) Section 50 μm above central cells. Eight differentiating cortical cells (with characteristic oval shape) are surrounded

be positively identified because the organization of cell types into different layers was disrupted by the abnormal sizes and shapes of the cells (Figs. 5D and 6B). The *ttg* root-cap cells were dramatically altered; the columella cells did not form orderly tiers, and in many instances, four tiers of mature amyloplast-containing columella cells were present rather than three (Fig. 6B). Amyloplasts, especially those in the outer columella cells of *ttg* seedling root caps, were not uniformly positioned at the apical ends of the cells as in wild-type columella cells (compare Fig. 6A to Fig. 6B). In several roots, newly divided cells in the columella initials just below the central cell region were separated by oblique cell walls oriented neither parallel nor perpendicular to the apical-basal axis of the roots (not shown). In addition, the outermost columella cells often appeared loosely attached or in the process of separating from the root cap, unlike root-cap integrity in wild type (compare Fig. 5D to Fig. 5A and Fig. 6B to Fig. 6A). The cortical cell layer in the *ttg* mutant often contained more than the normal number of eight cells (typically nine), and it usually included abnormally shaped cells that persisted to at least 250 μm from the central cells (Figs. 5E and 5F). The epidermal cells exhibited abnormal sizes and shapes as well as the oblique placement of new transverse or radial cell walls (Fig. 5E). At 200–250 μm from the central cells, *ttg* epidermal cells generally exhibited uniform staining of low/moderate intensity with toluidine blue, in contrast to the differential staining of the wild-type epidermal cells at this stage (compare Fig. 5F to Fig. 5C).

The cellular organization at the root apex in the *ttg-w* line was also examined, and it displayed similar, but less severe, abnormalities to the *ttg* mutant (data not shown). The alterations at the root apex in these *ttg* mutant lines indicate that the *TTG* gene is normally required to generate or maintain the appropriate organization and morphology of cells within and surrounding the root apical meristem.

The *ttg-R* root apex displayed many of the abnormalities present in the *ttg* mutant, although the defects were generally less pronounced (Figs. 5G–5I and 6C). As in the *ttg* mutant, the *ttg-R* seedling root apex was abnormally large in diameter (compare Figs. 5G–5I with Figs. 5A–5C). Also like *ttg*, the *ttg-R* root cap typically possessed four rather than three tiers of amyloplast-containing columella cells (Figs. 6C), and the outer root-cap cells tend to separate and detach from the root cap proper (compare Fig. 5G with Fig. 5A). When identified, central cells were often enlarged (Fig. 6C) or irregularly shaped (Fig. 5G), compared to the wild-type central cells. Above the central cell region, variations in cell number, size, and shape result in a less orderly arrangement of cell types into distinct layers (Fig. 5H). Within 200 μm above the central cells, the layer of cortical cells in the *ttg-R* roots exhibited distortions similar in shape to those of the *ttg* mutant (Fig. 5I). In this region, some epidermal cells displayed intense staining by toluidine blue (in one root, seven of eight cells over a cortical cell cross wall exhibited intense staining). Serial sectioning showed that this intense staining disappears basipetally a short distance from its first detection, unlike the intense staining of differentiating root-hair cells in the wild-type epidermis (data not shown). Because the *ttg-R* root apex displayed defects similar to those in *ttg*, the 35S-driven expression of *R* appears to be incapable of completely suppressing the *ttg*-associated abnormalities.

The Rschew-*R* transformants generated a relatively normal root apex (Figs. 5J–5L and 6D). Subtle alterations in cell organization and morphology were observed, particularly in root-cap cells and some of the cell files emerging from the central meristem region (Fig. 6D). However, the Rschew-*R* root apex was not abnormally large (like the *ttg* or *ttg-R* roots), nor was root-cap cell separation evident (compare Fig. 5J to Figs. 5D and 5G). Furthermore, the Rschew-*R* transformants generally possessed appropriate numbers of cells. Within 150–

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by a layer of differentiating epidermal cells, then two layers of lateral root-cap cells. (C) Section 200 μm above the central cells. The eight intensely staining differentiating root-hair cells (trichoblasts) are distinguishable from the remaining epidermal cells (atrichoblasts). A single outer layer of small root-cap cells still encloses the root epidermis. (D–F) *ttg* mutant. Note the larger root diameter in these sections compared to wild type. (D) Central cell region. The shape and arrangement of cells is altered, hindering the assignment of cells to specific layers or groups of initials. One region of this section is particularly disrupted (arrow). The lateral root-cap cells are not closely appressed. (E) Section 50 μm above the central cells. The abnormal number and shapes of cells disrupts the usual orderly arrangement of cortical, epidermal, and root-cap cell layers. (F) Section 230 μm above the central cells. Nine cortical cells (instead of the normal eight) are visible, and only one epidermal cell exhibits intense staining (rather than the normal eight; arrow); the remainder exhibit low/moderate staining. (G–I) *ttg-R* transformant 1434. Note the large root diameter in these sections, relative to wild type. (G) Central cell region. Four abnormally shaped central cells are visible, surrounded by disorganized layers of cells of variable size and shape. The lateral root-cap cells are not closely appressed. (H) Section 80 μm above central cells. The putative cortical, epidermal, and lateral root-cap layers consist of cells of variable size and shape. (I) Section 230 μm above central cells. Nine cortical cells are present, and four of the epidermal cells exhibit intense staining (arrows). Note the unusually small cortical cells in the right portion of the section. (J–L) Rschew-*R* transformant 1439. Note the relatively normal root diameter in these sections. (J) Central cell region. The central cells are surrounded by eight putative cortex/endodermis initials. The cell layers containing putative epidermal/lateral root-cap initials and lateral root-cap cells are distinct. (K) Section 50 μm above the central cells. Eight differentiating cortical cells are surrounded by a layer of differentiating epidermal cells, plus two outer root-cap layers, similar in shape and size to the wild type shown in (B). (L) Section 150 μm above the central cells. Two of the epidermal cells exhibit intense staining.

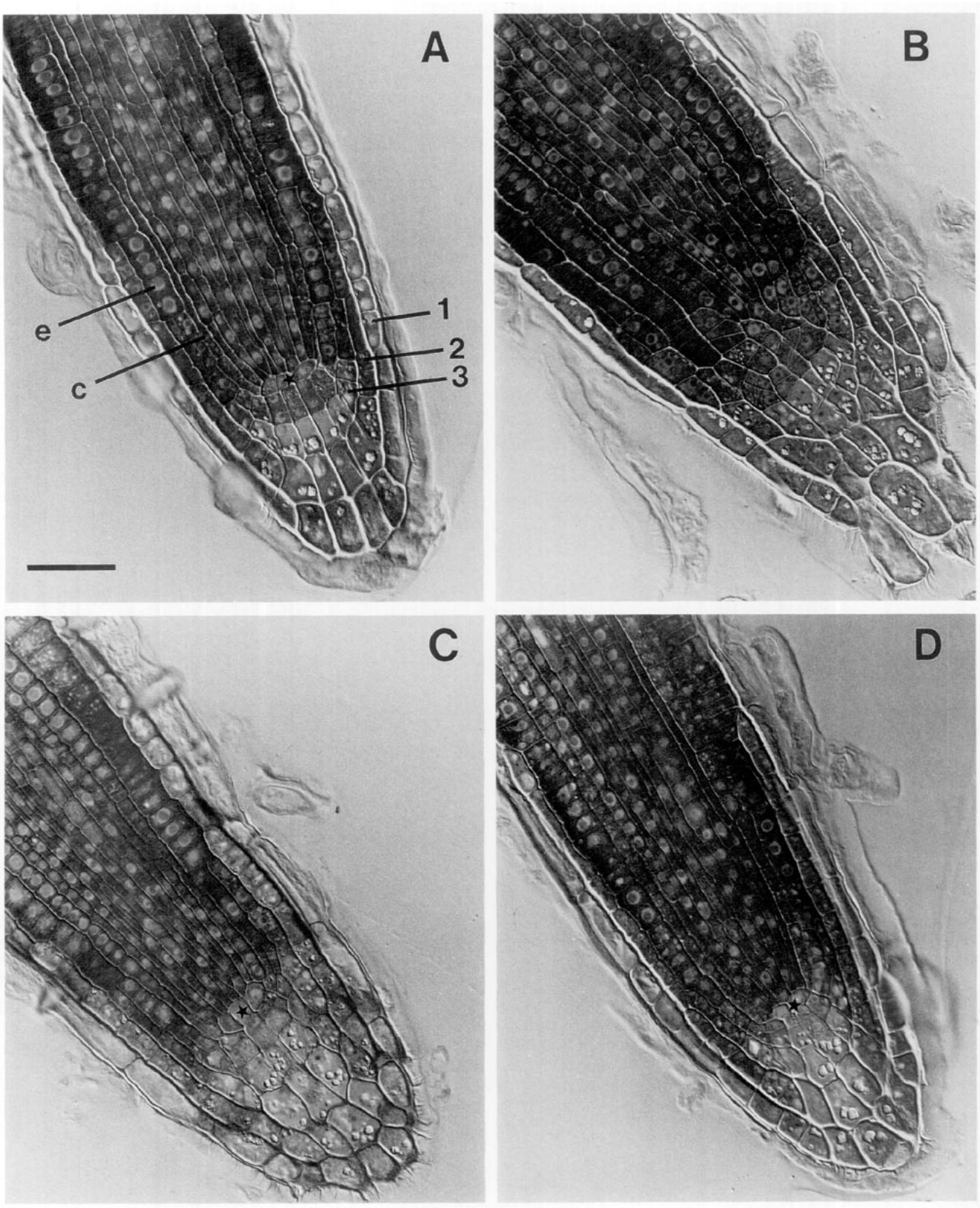

FIG. 6. Toluidine blue-stained, median longitudinal sections (2.0 μm thick) from the primary root apices of four different 5-day-old *Arabidopsis* seedlings. All figures are to same scale. Bar in (A), 30 μm. (A) Rschew wild type. Note central cells (star) and the first (1), second (2), and third (3) layers of lateral root-cap cells. Epidermal cell files (e) terminate at the columella initials below the central cells, whereas the cortical (c) and endodermal cell files terminate at the central cells. (B) *ttg* mutant. The normal organization of different cell types into discrete files and tiers is disrupted, and the cells display variations in size, shape, and division planes. Note the lack of cell organization and integrity in the root cap. (C) *ttg-R* transformant 1434. The cellular organization is similar to *ttg* mutant roots, but somewhat less disrupted. Below the central cells (star), the columella cells and initials clearly exhibit irregular shapes and sizes. (D) Rschew-*R* transformant 1439. Cellular organization is similar to wild type, but subtle differences in columella cell morphology are apparent. Note central cells (star).

250 μm above the central cells, some of the epidermal cells displayed intense toluidine blue staining (Fig. 5L), although, like *ttg-R*, this differential staining did not persist and was not observed in more mature sections of the roots (further from the meristem). The lack of major cellular abnormalities in the Rschew-*R* root apex suggests that *R* expression alone does not cause major changes in the cellular organization of the *Arabidopsis* root apex. Combined with the results from the analyses on wild-type, *ttg*, and *ttg-R* roots from above, it is clear that a simple relationship does not exist between alterations in root apex organization and the pattern of epidermal cell differentiation.

Root Epidermal Cell Fate in Other Arabidopsis Mutants

To determine whether the lack of anthocyanin in the *ttg* mutant could be responsible for the root epidermal cell effects, several *Arabidopsis* mutants with other genetic alterations in the anthocyanin pathway were analyzed. The anthocyanin (*transparent testa;* Koornneef, 1990) mutants *tt3* (deficient in dihydroflavonol 4-reductase; Shirley *et al.*, 1992), *tt4* (deficient in chalcone synthase; Feinbaum and Ausubel, 1988), and *tt5* (deficient in chalcone flavanone isomerase; Shirley *et al.*, 1992) each produced a normal number of root hairs and displayed no noticeable epidermal defects. Likewise, transgenic *Arabidopsis* (Rschew wild type) that express the cDNA of the maize *C1* (a transcriptional activator of the maize anthocyanin pathway that acts in concert with *R* to regulate the structural gene promoters; Paz-Ares *et al.*, 1987) under CaMV35S control (Lloyd *et al.*, 1992) did not exhibit any alterations in the root epidermis. These results indicate that the observed effects of the *ttg* mutation and *R* expression on differentiation of root epidermal cells are not simply the result of alterations in the *Arabidopsis* anthocyanin biosynthetic pathway.

Additional mutants of *Arabidopsis* known to be defective in cell differentiation were studied to determine if these mutations also affect cell fate in the root epidermis. In addition to *TTG*, one other *Arabidopsis* locus, *GL1*, is known to affect trichome initiation (Koornneef *et al.*, 1982). When the roots of *gl1* mutants were examined, a normal number of root hairs was observed and no difference in root epidermal cell patterning was detected. Four root-hair morphology mutants, *rhd1, rhd2, rhd3*, and *rhd4* (Schiefelbein and Somerville, 1990) were also analyzed, but none exhibited alterations in cell fate specification.

DISCUSSION

In this paper, cell differentiation was analyzed in the root epidermis of wild-type, *ttg* mutant, and *R*-expressing *Arabidopsis* plants. Wild-type roots generate a position-dependent pattern of differentiated epidermal cells, with root-hair cells located over radial cortical cell walls and hairless cells located directly over cortical cells. The recessive *ttg* mutant possesses root-hair cells in all epidermal positions, implying that the lack of (or reduced levels of) the *TTG* gene product causes immature epidermal cells that would normally differentiate into hairless cells to instead differentiate into root-hair cells. Plants expressing the maize *R* cDNA (in wild-type or *ttg* mutant backgrounds) produce mature hairless cells in all root epidermal cell positions, indicating that the *R* product causes immature epidermal cells to differentiate into hairless cells rather than root-hair cells. Since *TTG* is likely to encode, or activate, an *R* homologue in *Arabidopsis* (Lloyd *et al.*, 1992; this study), the effect of the CaMV35S-promoter-driven expression of *R* may be similar to the effect of overexpression and/or ectopic expression of *TTG*. Taken together, these results suggest that *TTG* normally is required to cause immature epidermal cells to differentiate into hairless cells.

The *TTG* gene appears to act at an early stage in epidermal cell differentiation. In wild-type roots, differentiating root-hair cells can be distinguished from differentiating hairless cells prior to root-hair formation because they display a delay in vacuolation relative to cell elongation (at approximately 300 μm from the meristem; Fig. 3) and they exhibit intense toluidine blue staining (within 200 μm from the meristem; Fig. 4). In the *ttg* mutant, differentiating epidermal cells in all files exhibit a delay in vacuolation that is characteristic of differentiating root-hair cells. In the *R*-expressing plants (both *ttg-R* and Rschew-*R*), nearly all cells vacuolate at an early stage, typical of differentiating hairless cells of the wild type. Furthermore, the developing epidermis in both *ttg* mutant and *R*-expressing roots displays alterations in the usual pattern of toluidine blue staining; *ttg* epidermal cells exhibit a uniformly low/medium staining and *R*-expressing epidermal cells show variable, weak, and transitory staining. Taken together, these results indicate that *TTG* exerts its effect on epidermal cell fate within 200 μm of the root meristem, prior to the onset of cell vacuolation or differential cytoplasmic staining.

The position-dependent pattern of epidermal cell types in the wild-type *Arabidopsis* root implies that *TTG* is normally able to provide, or respond to, positional signals to cause differentiating epidermal cells located directly over cortical cells to adopt a hairless cell fate. It follows that, in plants with alterations in *TTG* activity (e.g., *ttg* mutant or *R*-expressing plants), epidermal cells may misinterpret their position and adopt inappropriate cell fates. Several possible models for *TTG* action in the wild-type root may be considered that are consistent with our observations (Fig. 7). In one model, a positional signal (perhaps emanating from the central vascular

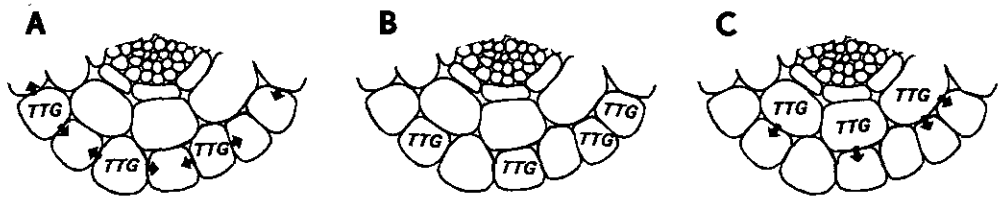

FIG. 7. Possible models for the action of *TTG* in root epidermal cell differentiation. (A) *TTG* is active in differentiating epidermal cells located over a radial cortical cell wall, and it prevents neighboring epidermal cells from adopting a root-hair cell fate. (B) *TTG* is active in differentiating epidermal cells located directly over cortical cells and directs these cells to adopt a hairless cell fate. (C) *TTG* is active in cortical cells and directs differentiating epidermal cells located over cortical cells to adopt a hairless cell fate. See text for a more complete discussion.

tissue) causes *TTG* to be active only in the differentiating epidermal cells that lie over the radial cortical cell walls (the future root-hair cells), and *TTG* acts to prevent neighboring cells from adopting the same fate (Fig. 7A). This mechanism is analogous to lateral inhibition, a process that occurs during animal cell differentiation (Greenwald and Rubin, 1992; Ghysen *et al.*, 1993). In a second model, a positional signal causes *TTG* to be active only in the differentiating epidermal cells lying directly over cortical cells, and *TTG* directs these cells to adopt a hairless cell fate (Fig. 7B). In a third model, *TTG* is active in cortical cells and provides a signal only to the overlying differentiating epidermal cells that directs them to adopt a hairless cell fate (Fig. 7C). Another possibility (not shown) is that *TTG* may act in an internal tissue(s) of the root (e.g., the vascular cylinder) to limit the production or apoplastic transmission of a diffusible, root-hair-promoting signal. In the future, it will be necessary to characterize the normal *TTG* expression pattern to sort out these and other possible models for *TTG* action.

The small number of root-hair cells generated in the *ttg-R* transformants are preferentially located over a radial wall between adjacent cortical cells (the wild-type position for root-hair cells) (Table 2). This implies that the epidermal cells in a *ttg* mutant background are not equivalent and that cells located over radial cortical cell walls possess a greater potential to differentiate into root-hair cells than do their neighbors. Because an increase in the copy number of *R* reduces the number of root-hair cells (Table 4), a higher concentration of the *R* product appears to overcome the tendency of these cells to adopt a root-hair cell fate. The difference in the ability of the two sets of *ttg-R* epidermal cells to adopt a hairless cell fate may reflect residual *TTG* activity in the *ttg* mutant, or it may be the result of other, unknown influences on root epidermal cell identity. For example, it is known that *Drosophila* neurogenesis and vertebrate myogenesis are regulated by several related proteins of the basic helix-loop-helix (bHLH) type that may form a variety of heterodimers and homodimers to affect cell fate (Campuzano and Modolell, 1992; Jan and Jan, 1993; Olsen and Klein, 1994).

A surprising finding from this study was the gross abnormalities in cellular morphology and organization at the root apex associated with the *ttg* mutations. This finding implicates the *TTG* gene in the control of cell division, cell expansion, and/or cell morphogenesis at the root meristem. It is possible that epidermal cell fate is normally influenced by *TTG* at/near the root apical meristem, during or soon after the formation of new epidermal cells. However, we consider it unlikely that the structural defects at the root apex in the *ttg* and *R*-expressing lines are directly responsible for the observed alterations in epidermal cell fate specification, for two reasons. First, the expression of the maize *R* cDNA in a *ttg* background leads to the suppression of the epidermal fate defects associated with *ttg*, but it does not lead to the complete suppression of the root apex defects (compare *ttg* phenotypes to *ttg-R* phenotypes). Second, the expression of the maize *R* cDNA in a Rschew wild-type background causes alterations in epidermal cell fate, but it leaves the root apex largely unaffected (compare Rschew wild-type phenotypes to Rschew-*R* phenotypes).

The complexity of factors controlling plant development is evident in the ability of seedlings of *ttg*, *ttg-R*, and Rschew-*R* lines to overcome the described defects in cellular organization at the root apices and generate a root with a normal structure when fully differentiated. In this respect, the mutant lines illustrate that the pattern of cell divisions and expansion in the root apex does not necessarily determine the final form of the differentiated organ. Cooke and Lu (1992) came to a similar conclusion regarding the independence of cell shape and overall form in their studies of the development of the epidermis in the leaves and thalli of various plant species.

These results show that there is overlap in the genetic control of epidermal cell differentiation in the root and shoot of *Arabidopsis*, since *TTG* affects both trichome and root-hair production. However, it is interesting to note that the differentiation of hair-bearing cells in the root and shoot epidermis is influenced in opposite ways by the *TTG* and *R* gene products. 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. This may be explained by a difference in the nature of the "ground state" of the epidermis in these two organs. In shoots, the ground state may be a nontrichome cell, whereas the ground state for a root epidermal cell may be a root-hair cell. Evidence to support the view that a root-hair cell represents the ground state has been obtained by physically isolating developing root epidermal cells from the underlying root tissue and by suppressing cell divisions with gamma irradiation; each of these treatments led to root-hair formation by most of the epidermal cells (summarized in Barlow, 1984).

The *Arabidopsis TTG* gene has previously been shown to participate in trichome formation, anthocyanin biosynthesis, and seed mucilage production. In the present study, we have shown that *TTG* is also required for cell fate specification in the root epidermis and for controlling/maintaining cellular organization within and surrounding the root apical meristem. It is challenging to envision how the *TTG* product is able to influence these diverse processes. One possibility is that *TTG* encodes a general transcription factor that acts in concert with other gene products which are specific for each process. As an example, the *GL1* gene appears to represent a trichome-specific gene (Koornneef *et al.*, 1982), and it does not affect root epidermis differentiation (this study). The *R* gene product (a *myc*-like protein) is thought to act as a transcriptional activator in maize and to interact with the *C1* product (a *myb*-like protein; Paz-Ares *et al.*, 1987; Roth *et al.*, 1991; Goff *et al.*, 1992). Because the *Arabidopsis GL1* gene also encodes a *myb* homologue (Oppenheimer *et al.*, 1991), it may interact with *TTG* to direct trichome formation.

Another possible explanation for the variety of *TTG* effects is that *TTG* plays a role in a general cellular communication process. Each of the *TTG*-dependent pathways may require appropriate cell–cell signaling (such as that suggested to occur during root epidermis differentiation) in order to be activated. Support for this possibility lies in our finding that the seedling root apices of *ttg* mutants lack the compact and orderly cellular organization typical of wild-type root apices.

Our finding that *TTG* has a key role in the differentiation of root epidermal cells and root pattern formation provides a new means to probe the molecular mechanisms of these processes. Future goals include identifying genes that are specific for root epidermal differentiation, and may interact with *TTG*. The accessibility of the epidermis and the relative simplicity of epidermal cell differentiation in *Arabidopsis* seedling roots make this a useful model for studies of pattern formation and cell fate specification in plants.

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