The bHLH genes *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*) specify epidermal cell fate in the *Arabidopsis* root

Christine Bernhardt[1], Myeong Min Lee[2], Antonio Gonzalez[3], Fan Zhang[3], Alan Lloyd[3] and John Schiefelbein[1,*]

[1]Department of Molecular, Cellular, and Developmental Biology, University of Michigan, 830 North University Avenue, Ann Arbor, MI 48109, USA
[2]Department of Biology, Yonsei University, Sinchon 134, Seoul 120-749, Korea
[3]Institute for Cellular and Molecular Biology, University of Texas, Austin, TX 78712, USA
*Author for correspondence (e-mail: schiefel@umich.edu)

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Summary

The position-dependent specification of the hair and non-hair cell types in the *Arabidopsis* root epidermis provides a simple model for the study of cell fate determination in plants. Several putative transcriptional regulators are known to influence this cell fate decision. Indirect evidence from studies with the maize *R* gene has been used to suggest that a bHLH transcription factor also participates in this process. We show that two *Arabidopsis* genes encoding bHLH proteins, *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*), act in a partially redundant manner to specify root epidermal cell fates. Plants homozygous for mutations in both genes fail to specify the non-hair cell type, whereas plants overexpressing either gene produce ectopic non-hair cells. We also find that these genes are required for appropriate transcription of the non-hair specification gene *GL2* and the hair cell specification gene *CPC*, showing that *GL3* and *EGL3* influence both epidermal cell fates. Furthermore, we show that these bHLH proteins require a functional WER MYB protein for their action, and they physically interact with WER and CPC in the yeast two-hybrid assay. These results suggest a model in which GL3 and EGL3 act together with WER in the N cell position to promote the non-hair cell fate, whereas they interact with the incomplete MYB protein CPC in the H position, which blocks the non-hair pathway and leads to the hair cell fate.

Key words: Epidermis, Pattern formation, Cell differentiation, Root development, Transcriptional regulation

Introduction

The specification and patterning of distinct cell types is a crucial feature of development in multicellular organisms. In plants, the formation of the hair and non-hair cells in the *Arabidopsis* root epidermis has been used extensively as a relatively simple and experimentally tractable model for studying cell fate specification (Dolan and Costa, 2001; Larkin et al., 2003). These cell types are non-essential under laboratory conditions, they are easy to examine, and they arise continuously during root development. Furthermore, the hair and non-hair cell types are patterned in a predictable manner in the *Arabidopsis* root, with the hair cells located in a cleft between two underlying cortical cells (the H position) and the non-hair cells present outside a single cortical cell (the N position) (Dolan et al., 1994; Galway et al., 1994).

Molecular genetic studies have led to the identification of a suite of putative transcription factors that regulate the epidermal cell pattern. These include gene products required for specification of the non-hair cell type, such as the homeodomain protein GLABRA2 (GL2) (Masucci et al., 1996; Rerie et al., 1994), the R2R3 MYB-type transcription factor WEREWOLF (WER) (Lee and Schiefelbein, 1999), and the WD-repeat protein TRANSPARENT TESTA GLABRA (TTG) (Galway et al., 1994; Walker et al., 1999). Other regulators are involved in specifying the hair cell fate, including CAPRICE (CPC) and TRYPTICHON (TRY), which are small one-repeat MYB proteins that lack a transcriptional activation domain and exhibit partially redundant functions (Schellmann et al., 2002; Wada et al., 2002; Wada et al., 1997). The final cell pattern appears to result from positive and negative regulatory interactions between these components (Schiefelbein, 2003). Specifically, WER promotes transcription of *GL2* and *CPC* (and probably *TRY*) in the N position, GL2 inhibits hair cell specification in the N position, and CPC (and probably TRY) act in lateral inhibition by moving to the H cell and repressing transcription of *WER*, *GL2* and its own gene (Lee and Schiefelbein, 2002; Schellmann et al., 2002; Schiefelbein, 2003; Wada et al., 2002).

Several lines of indirect evidence have suggested that a basic helix-loop-helix (bHLH) transcription factor may also be a component of the cell specification pathway in the root epidermis. This evidence includes (1) the ability of the maize R bHLH protein to induce ectopic non-hair cells when expressed in wild type *Arabidopsis* (Galway et al., 1994); (2) the ability of the maize R bHLH protein to restore non-hair cell production when expressed in the hairy *ttg* mutant (Galway et al., 1994); (3) the ability of the maize R bHLH protein to promote *GL2* gene expression (Hung et al., 1998); (4) the

ability of the *wer* mutations to block the effect of the maize R bHLH protein on non-hair cell specification (Lee and Schiefelbein, 1999); and (5) the ability of the R bHLH protein to interact with the WER protein and with the CPC protein in the yeast two-hybrid assay (Lee and Schiefelbein, 1999; Wada et al., 2002). Together, these findings have led to the suggestion that an *Arabidopsis* bHLH protein is likely to exist that interacts with WER to induce cells in the N position to adopt a non-hair fate (Larkin et al., 2003; Lee and Schiefelbein, 1999).

In this study, we confirm this long-standing hypothesis. We find that two *Arabidopsis* bHLH genes, *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*), are important regulators of root epidermal cell specification. These two genes encode bHLH proteins related to the maize R protein and influence trichome development and other TTG-related processes in *Arabidopsis* (Koornneef et al., 1982; Payne et al., 2000; Zhang et al., 2003). We show that *GL3* and *EGL3* have largely redundant functions in the specification of the non-hair cell fate and also participate in specifying the hair cell fate. We propose that the GL3 and EGL3 bHLH proteins act as binding partners for the WER or the CPC MYB proteins and thereby mediate the cell fate decision during root epidermis development.

Materials and methods

Plant materials and growth conditions

The isolation of the mutant alleles used in this study has been described: *cpc-1* (Wada et al., 1997), *egl3-1* (Zhang et al., 2003), *egl3-2* (Zhang et al., 2003), *gl2-1* (Koornneef, 1981), *gl3-1* (Koornneef et al., 1982), *gl3-2* (Hulskamp et al., 1994) and *wer-1* (Lee and Schiefelbein, 1999), and these are likely to represent loss-of-function alleles. The *GL2::GUS*, *CPC::GUS*, *35S::EGL3* and *35S::GL3* constructs and transgenic lines have been previously described (Lee and Schiefelbein, 1999; Masucci et al., 1996; Wada et al., 2002; Zhang et al., 2003). Lines homozygous for multiple mutations and/or transgenes were constructed by crossing single mutant or transgenic plants, examining the F2 progeny for putative double mutant/transgene phenotypes, and confirming the desired genotype in subsequent generations by backcrossing to single mutants, examining reporter gene expression, and/or PCR-based tests.

Arabidopsis seeds were surface sterilized and grown on agarose-solidified nutrient medium in vertically oriented petri plates as previously described (Schiefelbein and Somerville, 1990).

Microscopy

Root hair cell production and cell type pattern analysis were determined from Toluidine Blue-stained roots as previously described (Lee and Schiefelbein, 2002) from at least 24 four-day-old seedling roots for each strain. The upper region of the root was defined as the segment containing 10 epidermal cells whose upper boundary is four cells below the hairy collet region. The lower region of the root is a larger zone representing approximately the lower half of a four-day-old root and occupied by epidermal cells that differentiate during days 3-4. An epidermal cell was scored as a root-hair cell if any protrusion was visible, regardless of its length.

Plastic transverse sections were obtained from four- to five-day-old roots embedded in JB-4 resin and stained with 0.05% Toluidine Blue O, as previously described (Masucci and Schiefelbein, 1996). The relative cell division rate in the H and N cell positions of the epidermis was determined by counting the number of cells in clones derived from rare longitudinal divisions, using a method previously described (Berger et al., 1998a), and by counting the number of cells in adjacent N and H cell files.

The histochemical analysis of plants containing the *GUS* reporter constructs was performed essentially as described (Masucci et al., 1996).

Molecular biology methods

For RT-PCR assays, tissue of wild-type (Columbia) plants was ground in liquid nitrogen and total RNA was extracted as described (Weigel and Glazebrook, 2002). Tissue from roots and hypocotyl/cotyledons was obtained from four-day-old seedling grown on nutrient plates as described above. All other tissues were obtained from soil grown plants. RT-PCR was performed using the Superscript One-Step RT-PCR Kit (Invitrogen) according to manufacturer instructions. Total RNA template (500 ng) was used for each reaction and a total of 40 PCR cycles was performed. *UBQ10* gene-specific primers (Weigel and Glazebrook, 2002) were used in control reactions. The length of the gene-specific products obtained for *GL3*, *EGL3* and *UBQ* was 581 bp, 516 bp and 483 bp, respectively.

Yeast two-hybrid assays were performed essentially as described (Lee and Schiefelbein, 1999). The entire coding regions of the GL3 or EGL3 cDNA were joined as C-terminal fusion to the yeast GAL4 DNA-binding domain in pGBT9 to generate the in-frame protein fusions BD-GL3 and BD-EGL3. The GAL4 transcriptional activation domain in pGAD424 was fused to the full-length WER-coding region to generate AD-WER (Lee and Schiefelbein, 1999) and it was fused to the full-length CPC coding region to generate AD-CPC. After transformation into yeast strain HF7c, the β-galactosidase assays were performed on at least six individual transformants for each combination of constructs.

Results

The *GL3* and *EGL3* genes are expressed during root development

Among the 133 predicted *Arabidopsis* proteins that possess bHLH-like motifs (Heim et al., 2003), the *GL3* and *EGL3* proteins are members of a subfamily with the greatest similarity to the maize R protein. The GL3 bHLH protein (At5g41315) contains 637 amino acids and displays 33% identity with maize R (Payne et al., 2000), whereas the *EGL3* gene (At1g63650) encodes a predicted protein containing 596 amino acids that is 74% identical to GL3 (Zhang et al., 2003). Because the maize *R* is able to influence root epidermis development when expressed in *Arabidopsis* (Galway et al., 1994), the *GL3* and *EGL3* genes were candidates to represent *Arabidopsis* bHLH proteins that participate in root epidermal cell specification.

To determine whether either of these R-like bHLH genes from *Arabidopsis* are normally expressed in the developing root, we conducted RT-PCR analyses using *GL3*- and *EGL3*-specific primers on RNA isolated from roots and other organs. *GL3* and *EGL3* amplified fragments were detected from each RNA sample (Fig. 1), indicating that each bHLH gene is expressed in all of these plant organs. This is consistent with the recent finding that *GL3* and *EGL3* participate in multiple pathways in the above-ground organs (Zhang et al., 2003). Furthermore, the substantial amplification of *GL3* and *EGL3* from root RNA samples (Fig. 1) suggests that these genes are expressed in developing *Arabidopsis* seedling roots.

Overexpression of GL3 and EGL3 promote non-hair cell fate

To examine the possible role of *GL3* and *EGL3* in root epidermis development, we tested their effect when expressed

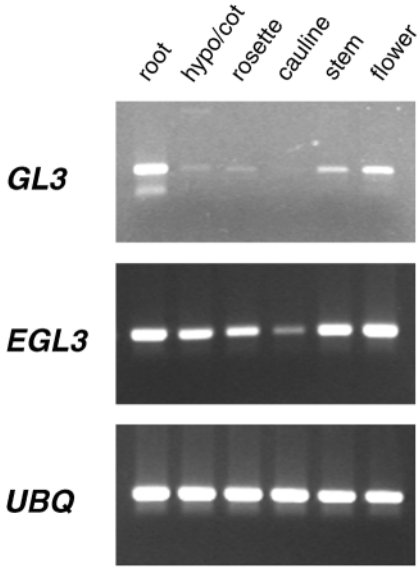

Fig. 1. *GL3* and *EGL3* RNA accumulates in all major *Arabidopsis* organs. Reverse transcriptase (RT)-PCR analysis using *GL3*-, *EGL3*- or *UBQ10*-specific gene primers. Total RNA was isolated from the following tissues of wild-type (Columbia) plants: roots, hypocotyl and cotyledons, rosette leaves, cauline leaves, stems and flowers. 500 ng template RNA was used for each RT-PCR reaction.

under the control of the CaMV35S promoter. Plants bearing either the *35S::GL3* or *35S::EGL3* transgene (Payne et al., 2000; Zhang et al., 2003) produced roots with only a small number of root hairs (Fig. 2), owing to the misspecification of cells in the H position to adopt a non-hair cell fate (Table 1). This result is similar to the effect of the *35S::R* construct in *Arabidopsis* (Galway et al., 1994), and it suggests that expression of a high level of GL3 or EGL3 bHLH protein throughout the epidermis can overcome the effects of the hair cell specification pathway in the H position.

Although the general phenotypic effect of the *35S::GL3* and *35S::EGL3* transgenes is similar, we found that the effect of the *35S::GL3* is not as strong as the *35S::EGL3* (Fig. 2, Table 1). In particular, the *35S::GL3* line produces more hair cells in the lower region (near the apex) of the 4-day-old root than in the upper region (near the root-hypocotyl junction) (Fig. 2; Table 1). These differences in the *35S::GL3* and *35S::EGL3* effects may be due to different transgene expression levels or to differences in the developmental timing or interactions of the two bHLH genes.

To further explore the effect of the *35S::GL3* and *35S::EGL3* transgenes in relation to the previously characterized *35S::R*, we introduced the *35S::GL3* and *35S::EGL3* into the *ttg-1* mutant. The *ttg-1* root specifies hair cells in nearly every root epidermal cell, and this defect can be overcome by the *35S::R* transgene (Galway et al., 1994). We found that the *35S::GL3 ttg-1* and *35S::EGL3 ttg-1* roots exhibited a significant reduction in hair cell production, when compared with the *ttg* mutant (Fig. 2; Table 1). This indicates that the overexpression of either gene can restore non-hair cell production in the *ttg-1* mutant, which is similar to the effect of *35S::R* on *ttg* (Galway et al., 1994). Interestingly, each transgene exhibited a difference in their effect on the upper and lower region of the root, with the *35S::EGL3* having its greatest impact on the lower region and the *35S::GL3* on the upper region, which is similar to their effects in the wild-type background. Because neither of the transgenes was able to induce non-hair cell specification in the *ttg-1* mutant to the same extent as they do in the wild-type background (Table 1), it is likely that TTG is required for the full effect of the *35S::GL3* and *35S::EGL3*. To determine whether this partial TTG dependency can be diminished by expressing both *GL3* and *EGL3* in the *ttg* mutant, we constructed a *35S::GL3 35S::EGL3 ttg-1* line. These roots had an enhanced non-hair cell phenotype when compared with either single transgene (Table 1), suggesting that increased expression of these bHLH genes can overcome the effect of the *ttg-1*. Furthermore, the lack of a synergistic effect implies that the GL3 and EGL3 provide largely similar functions, rather than interdependent functions.

Analysis of *gl3* and *egl3* mutants reveal redundancy in bHLH gene function

To directly assess the involvement of the *GL3* and *EGL3* genes in root epidermis development, we analyzed plants bearing

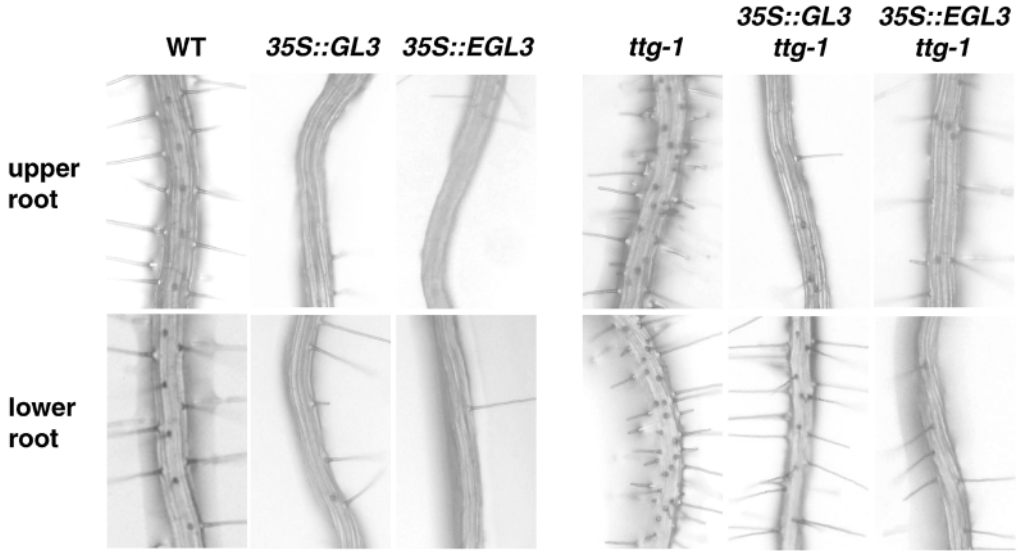

Fig. 2. The *35S::GL3* and *35S::EGL3* transgenes promote non-hair cell specification. Root phenotypes of four-day-old seedlings bearing the *35S::GL3* and *35S::EGL3* transgenes in the wild-type or the *ttg-1* mutant backgrounds. The top set of panels shows upper regions of the roots (near the root-hypocotyl junction), and the bottom panels show lower regions of the roots (near the root apex).

Table 1. Effect of the *35S::GL3* and *35S::EGL3* transgenes on cell type pattern in the root epidermis*

Genotype	Region of root†	Hair cells in epidermis (%)	H cell position		N cell position	
			Hair cells (%)	Non-hair cells (%)	Hair cells (%)	Non-hair cells (%)
Wild type (WS)	UP	44.2±7.4	92.5±9.0	7.5±9.0	1.7±3.1	98.3±3.1
	LP	40.2±7.8	93.3±8.7	6.7±8.7	4.2±9.4	95.8±9.4
35S::GL3	UP	3.5±5.8	10.8±12.3	89.2±12.3	0±0	100±0
	LP	22.2±10.5	65.8±21.2	34.2±21.2	0±0	100±0
35S::EGL3	UP	7.7±7.7	34.2±15.3	65.8±15.3	1.7±3.1	98.3±3.1
	LP	2.2±3.7	22.5±12.3	77.5±12.3	0.8±2.4	99.2±2.4
ttg-1	UP	99.7±13.9	100±0	0±0	99.2±2.4	0.8±2.4
	LP	96.5±6.3	100±0	0±0	91.7±13.7	8.3±13.7
35S::GL3 ttg-1	UP	8.0±10.8	23.3±17.1	76.7±17.1	0±0	100±0
	LP	48.3±15.0	95.0±6.9	5.0±6.9	16.7±14.7	83.3±14.7
35S::EGL3 ttg-1	UP	68.6±21.2	100±0	0±0	45.9±16.1	54.1±16.1
	LP	21.9±18.5	48.9±18.3	51.1±18.3	2.2±6.7	97.8±6.7
35S::GL3 35S::EGL3 ttg-1	UP	8.0±10.1	20.0±18.4	80.0±18.4	4.2±6.2	95.8±6.2
	LP	18.7±18.7	49.6±26.5	50.4±26.5	4.4±9.4	95.6±9.4
gl2-1	UP	99.7±1.1	100±0	0±0	99.2±2.4	0.8±2.4
	LP	100±0	100±0	0±0	100±0	0±0
35S::GL3 gl2-1	UP	96.2±7.5	99.3±2.2	0.7±2.2	91.9±8.7	8.2±8.7
	LP	98.5±3.5	99.3±2.2	0.7±2.2	95.6±6.7	4.4±6.7
35S::EGL3 gl2-1	UP	99.1±2.0	99.4±2.0	0.6±2.0	96.4±5.5	3.6±5.5
	LP	99.2±2.1	99.4±1.9	0.6±1.9	96.7±6.7	3.3±6.7
wer-1	UP	91.8±8.1	90.6±10.0	9.4±10.0	87.2±7.3	12.8±7.3
	LP	98.7±2.8	97.6±4.5	2.4±4.5	99.4±2.0	0.6±2.0
35S::GL3 wer-1	UP	89.6±11.8	87.7±15.8	12.3±15.8	87.2±7.9	12.8±7.9
	LP	94.5±7.8	90.9±10.4	9.1±10.4	95.8±4.5	4.2±4.5
35S::EGL3 wer-1	UP	88.3±10.0	89.2±7.9	10.8±7.9	83.3±8.7	16.7±8.7
	LP	93.1±8.1	93.3±7.1	6.7±7.1	89.2±9.4	10.8±9.4

*Data were obtained from at least 25 five-day-old seedlings from each strain. In all strains, ~40% of epidermal cells are in the H position.
†UP, upper region of root; LP, lower region of root.

homozygous mutations in one or both of these genes. We employed *gl3* mutant lines (*gl3-1* and *gl3-2*) and *egl3* mutant lines (*egl3-1* and *egl3-2*) with mutations that cause premature stop codons and probably represent null alleles (Payne et al., 2000; Zhang et al., 2003). We found that each of the single mutant lines produced a normal number and pattern of epidermal cell types in the lower region of the root, but they show a slight (*egl3-1* and *egl3-2*) or moderate (*gl3-1* and *gl3-2*) increase in hair cell production in the upper region of the root, owing to the misspecification of hair cells in the N (ectopic) position (Fig. 3; Table 2). Thus, *GL3* and *EGL3* are necessary to specify fully the non-hair cell fate and generate the proper epidermal pattern in the upper region, but not the lower region, of the root.

To test the possibility of partial functional redundancy between the *GL3* and *EGL3* genes, we generated and analyzed all four possible *gl3 egl3* double mutants using the *gl3-1*, *gl3-2*, *egl3-1* and *egl3-2* lines. Each double mutant combination produced an extremely hairy root (Fig. 3) because of a dramatic reduction in the frequency of the non-hair cell type throughout the root (Table 2; data not shown). These findings show that *GL3* and

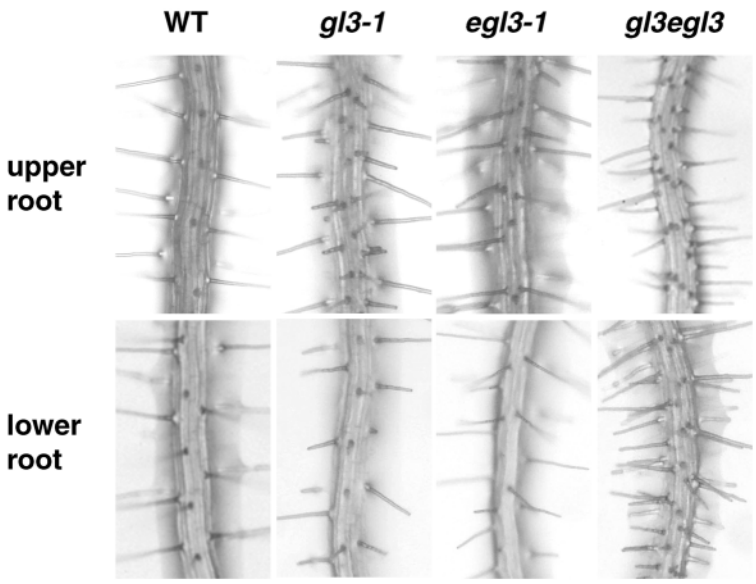

Fig. 3. The *GL3* and *EGL3* genes are required for non-hair cell specification. Root phenotypes of four-day-old seedlings bearing the indicated *gl3* and/or *egl3* mutations. In each composite, the top panels show upper regions of the roots, and the bottom panels show lower regions of the roots.

Table 2. Effect of the *gl3* and *egl3* mutants on cell type pattern in the root epidermis*

Genotype	Region of root†	Hair cells in epidermis (%)	H cell position		N cell position	
			Hair cells (%)	Non-hair cells (%)	Hair cells (%)	Non-hair cells (%)
WT (Ler)	UP	40.2±8.2	97.5±3.5	2.5±3.5	8.3±5.9	91.7±5.9
	LP	44.0±6.9	94.2±5.6	5.8±5.6	3.3±6.2	96.7±6.2
gl3-1	UP	81.6±20.7	99.3±2.1	0.7±2.1	65.3±24.3	34.7±24.3
	LP	45.8±9.5	96.7±5.0	3.3±5.0	6.7±8.7	93.3±8.7
Gl3-2	UP	70.7±19.9	99.2±2.4	0.8±2.4	43.3±13.3	56.7±12.2
	LP	38.7±6.2	95.8±3.5	4.2±3.5	2.5±3.5	97.5±3.5
Egl3-1	UP	57.1±13.9	97.3±4.7	2.7±4.7	24.0±17.6	76.0±17.5
	LP	42.9±7.9	96.7±7.1	3.3±7.1	4.2±5.0	95.8±5.0
Egl3-2	UP	53.1±11.7	92.7±5.6	7.3±5.6	27.3±12.0	72.7±12.0
	LP	38.7±4.1	98.3±4.7	1.7±4.7	1.7±3.1	98.3±3.1
gl3-1 egl3-1	UP	99.7±1.1	98.3±4.7	1.7±4.7	100±0	0±0
	LP	97.8±4.3	100±0	0±0	96.7±5.0	3.3±5.5
gl3-1 egl3-2	UP	96.7±8.1	96.7±9.4	3.3±9.4	93.3±12.9	6.7±12.9
	LP	97.8±6.7	100±0	0±0	99.2±2.4	0.8±2.4

*Data were obtained from at least 25 five-day-old seedlings from each strain. In all strains, ~40% of epidermal cells are in the H position.
†UP, upper region of root; LP, lower portion of root.

EGL3 act in a largely redundant manner to specify the non-hair cell fate.

GL3 and EGL3 act at an early stage in epidermal development

The outgrowth of a root hair from an epidermal cell represents a relatively late event in epidermal cell differentiation. At earlier stages, immature epidermal cells in the H and N positions may be distinguished from one another by their differential vacuolation rate and cytoplasmic density, and these characteristics are controlled by WER and TTG but not GL2 (Galway et al., 1994; Lee and Schiefelbein, 1999; Masucci et al., 1996; Schellmann et al., 2002). To determine whether the altered root hair production in the *gl3 egl3* mutant and the *35S* lines were associated with cell fate abnormalities at an early developmental stage, we examined vacuole formation and cytoplasmic density in developing epidermal cells from transverse sections taken from the meristematic region of the root. In contrast to the wild type, which displayed a greater vacuolation rate and reduced cytoplasmic density in the N cell position relative to the H cell position, all epidermal cells in the *gl3 egl3* exhibit characteristics of developing hair cells, whereas all epidermal cells in *35S::EGL3* exhibit characteristics of developing non-hair cells (Fig. 4).

Another early characteristic of epidermal cell specification is differential cell division rate, whereby the developing hair cells achieve a greater rate of division than developing non-hair cells in the meristematic zone (Berger et al., 1998a). We assessed the relative cell division rate in the mutant and overexpression lines by comparing cell number in the H and N positions. We discovered a significant reduction in the relative division rate in the *gl3 egl3*, the *35S::GL3* and the *35S::EGL3* lines when compared with their respective wild-type lines, but no significant difference was detected in the single mutant lines (Table 3). The reduction was similar to the previously documented effect of the *wer-1* and *ttg-1* mutations on relative cell division rate (Galway et al., 1994; Lee and Schiefelbein, 1999) (Table 3). Together, these results show that alterations in *GL3* and *EGL3* gene function affect cell specification characteristics at an early stage in root epidermis development, similar to the stage affected by *WER* and *TTG*.

GL3 and EGL3 regulate *GL2* transcription

To better define the role of *GL3* and *EGL3* in the specification pathway, we examined the effect of the mutants and transgenes on expression of the *GL2::GUS* reporter construct. The *GL2* gene is required for non-hair cell specification and, accordingly, the *GL2::GUS* reporter is preferentially expressed in the N cell position of the developing root epidermis (Masucci et al., 1996). In the *gl3-1* mutant, *GL2::GUS* expression is reduced, but the appropriate pattern of GUS activity remains (Fig. 5). By contrast, the *egl3-1* mutant has no detectable effect on *GL2::GUS* reporter

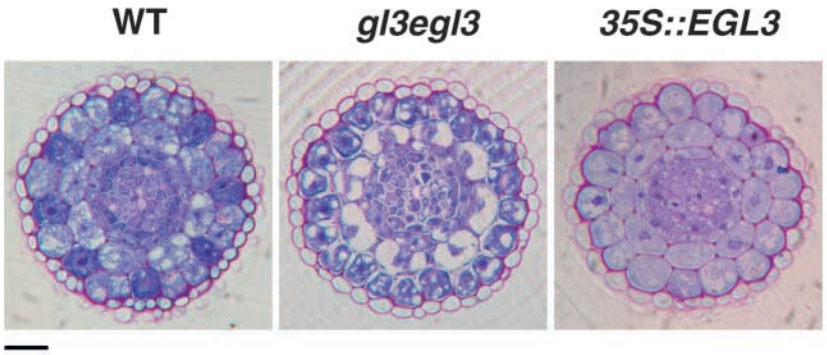

Fig. 4. The *GL3* and *EGL3* genes act early during epidermal cell fate specification. Transverse sections taken from the meristematic region of wild-type, *gl3 egl3* and *35S::EGL3* roots indicate the relative vacuolation and cytoplasmic density in the H and N cell positions. Scale bar: 25 μm.

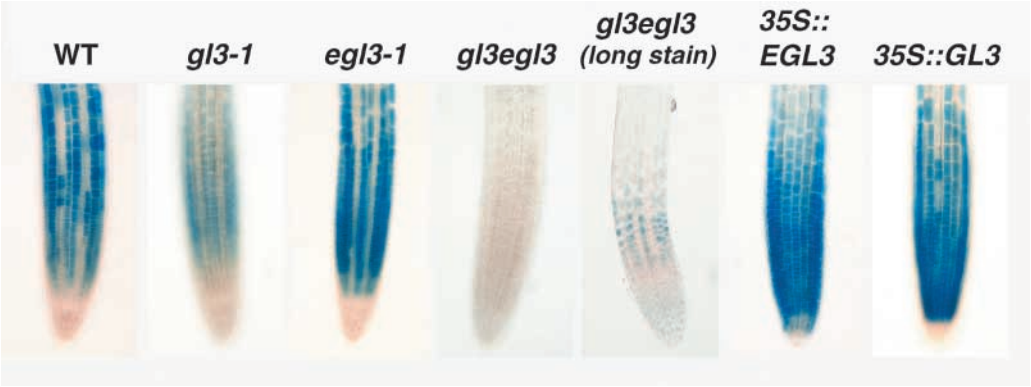

Fig. 5. *GL2* gene expression is regulated by *GL3* and *EGL3*. Four-day-old roots from plants harboring the *GL2::GUS* transgene and the indicated mutation(s) were incubated with X-gluc. The *gl3 egl3* root labeled 'long stain' was incubated for an extended period of time (20 hours rather than 1 hour).

expression (Fig. 5). A strong reduction in *GL2::GUS* expression is present in the *gl3-1 egl3-1* double mutant, although extended incubation time shows that the proper pattern persists (Fig. 5). This indicates that GL3 and EGL3 act redundantly to positively regulate the level, but not the position-dependent pattern, of *GL2* transcription.

In a corresponding fashion, the *35S::EGL3* transgene caused *GL2::GUS* expression to expand throughout the developing epidermis and the surrounding lateral root cap (Fig. 5; data not shown). The *35S::GL3* caused a weaker effect on *GL2::GUS* expression (Fig. 5), consistent with its weaker effect on cell fate specification (Table 1).

Given these results, we wished to investigate the possibility that GL3 and EGL3 promote the non-hair fate by acting through GL2. Therefore, we generated and analyzed *35S::GL3 gl2-1* and *35S::EGL3 gl2-1* plants. Each of these lines possessed a 'hairy' root phenotype that is essentially the same as the *gl2-1* mutant (Table 1). This suggests that a functional *GL2* gene is required for the *35S* overexpression constructs to induce non-hair epidermal cells, and therefore, is consistent with the notion that the *GL3*/*EGL3* genes act through *GL2*.

GL3 and EGL3 regulate CPC transcription

The *CPC* gene is expressed in the N cell position, and it is required for hair cell specification through a lateral inhibition mechanism (Lee and Schiefelbein, 2002; Wada et al., 2002). To determine whether *CPC* is regulated by *GL3* and *EGL3*, we introduced the *CPC::GUS* reporter construct into the various mutant and transgene backgrounds. Like the *GL2::GUS* expression, the *CPC::GUS* expression was reduced in the *gl3-1* mutant, unchanged in the *egl3-1* mutant, and virtually eliminated in the *gl3-1 egl3-1* double mutant (Fig. 6; data not shown). Furthermore, the *CPC::GUS* reporter was expressed throughout the epidermis in the *35S::EGL3*, and to a weaker extent in the *35S::GL3* (Fig. 6). Thus, the *GL3* and *EGL3* genes act in a redundant manner to promote expression of both the non-hair-cell-specification gene *GL2* and the hair-cell-specification gene *CPC* in the N cell position.

Table 3. Relative cell division rate in the root epidermis of *GL3* and *EGL3* mutants and overexpression lines

Genotype	H/N ratio*
Wild type (Ler)	1.64±0.10
gl3-1	1.57±0.10
egl3-1	1.54±0.11
gl3-1 egl3-1	1.31±0.09
WT (Col)	1.62±0.11
wer-1	1.31±0.10
WT (WS)	1.51±0.10
35S::GL3	1.36±0.07
35S::EGL3	1.21±0.07

*Ratio of cell number in the H position to cell number in the N position.

GL3 and EGL3 function relies on WER

Like *GL3* and *EGL3*, *WER* is a positive regulator of *GL2* and *CPC* (Lee and Schiefelbein, 2002). To determine whether *WER* is required for *GL3* or *EGL3* function, we constructed and examined plants bearing the genotype *35S::GL3 wer-1* or *35S::EGL3 wer-1*. Roots from each of these lines produced abundant root hair cells, similar to the *wer-1* mutant (Table 1). Thus, *GL3* or *EGL3* action requires a functional *WER* gene, which implies that *WER* is either acting downstream or at the same step as *GL3*/*EGL3* in the non-hair specification pathway.

GL3 and EGL3 interact with WER and CPC

In prior studies, the maize R bHLH protein has been found to interact with both the WER (Lee and Schiefelbein, 1999) and with the CPC (Wada et al., 2002) MYB proteins. To examine the possibility that the GL3 or EGL3 proteins physically associate with WER or CPC, we employed the yeast two-hybrid assay (Fields and Sternglanz, 1994). First, we found that fusions of the GAL4 DNA binding domain (BD) to either the GL3 or EGL3 protein alone were sufficient to induce a significant level of *lacZ* reporter expression (Table 4). This 'one-hybrid' assay shows that the GL3 and EGL3 proteins possess transcriptional activation domains that are functional in yeast.

Next, we discovered that yeast cells co-expressing either the BD-GL3 or BD-EGL3 fusions together with the AD-WER fusion exhibited a higher level of *lacZ* reporter expression than either single fusion (Table 4). This indicates that the WER protein can physically interact with either the GL3 or EGL3 proteins in yeast cells and is consistent with the possibility that they interact in *Arabidopsis*.

We also discovered that GL3 and EGL3 can each associate with the CPC protein in the yeast two-hybrid assay. Yeast cells expressing an AD-CPC fusion together with the BD-GL3 or the BD-EGL3 produce an increased level of *lacZ* reporter expression (Table 4). This implies that CPC may also interact

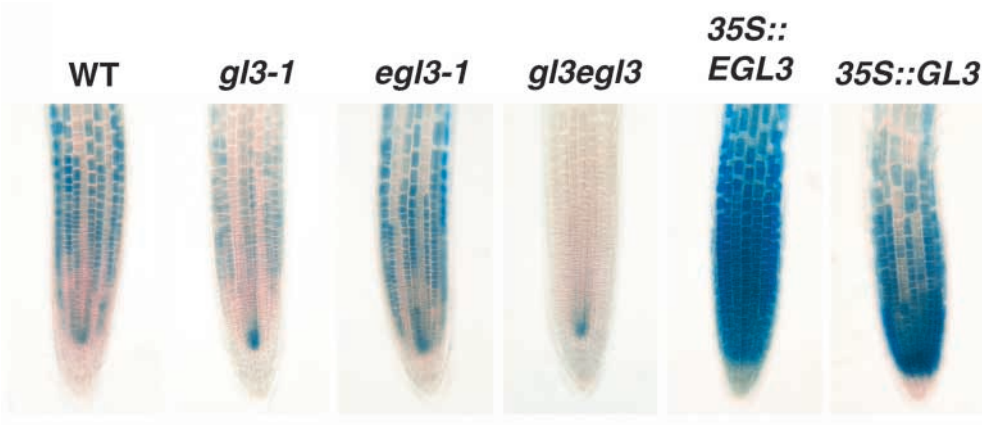

Fig. 6. *CPC* gene expression is regulated by *GL3* and *EGL3*. Seedling roots bearing the *CPC::GUS* reporter and the indicated mutation(s) were exposed to the X-gluc substrate for 12 hours. This reporter is also expressed in the developing stele near the root tip, but this expression is not associated with the role of *CPC* in epidermal development (Wada et al., 2002).

with GL3 or EGL3 in *Arabidopsis*, which suggests a possible competition model for the opposite action of WER and CPC in root epidermis cell specification.

Discussion

In this study, the *Arabidopsis GL3* and *EGL3* bHLH genes have been shown to participate in root epidermis development in a largely redundant manner and consistent with expectations from earlier experiments with the heterologous maize *R* bHLH (Galway et al., 1994). Results from mutant, overexpression and reporter analyses suggest that the major role of *GL3*/*EGL3* is to promote the non-hair cell fate via transcriptional activation of the downstream gene *GL2*. In addition, these genes are required for appropriate expression of the *CPC* gene, which promotes the hair cell fate. Thus, *GL3* and *EGL3* are essential for the specification of both cell fate pathways that generate the normal cell type pattern during root epidermis development.

These results suggest a simple model for the action of the GL3 and EGL3 bHLH proteins in specifying cell fates in the root epidermis (Fig. 7). This model is largely consistent with earlier predictions based on results with the heterologous maize R protein (Galway et al., 1994; Lee and Schiefelbein, 1999; Masucci et al., 1996). First, the GL3 and EGL3 bHLH proteins are likely to act as transcriptional regulators in concert with the WER MYB protein. This proposal is supported by the similar effects of WER and GL3/EGL3 on *GL2* and *CPC* expression, and their similar effects on early stages of cell differentiation. Furthermore, it is consistent with the yeast two-hybrid results showing that GL3 and EGL3 interact with WER, and it is supported by the WER-dependent nature of the *35S::GL3* or *35S::EGL3* induction of non-hair cells. In plants, it is common for bHLH proteins to act in a combinatorial fashion with MYB-related proteins to regulate gene transcription (Singh, 1998). The best characterized example is the control of anthocyanin production in maize, where the tissue-specific activation of the structural genes of the anthocyanin pathway requires the expression of a bHLH protein encoded by the maize R or B loci as well as a MYB-related protein encoded by the C1 or Pl loci (Ludwig and Wessler, 1990; Mol et al., 1998).

Another aspect of our model (Fig. 7), is that the GL3/EGL3 proteins interact with the TTG WD repeat protein and rely on TTG activity, in part, for their function. Although the precise role of TTG is not clear, other WD-repeat proteins are involved in protein-protein interactions (Neer et al., 1994) which implies that TTG may be a component of a signal transduction pathway or may interact with transcription factors (e.g. GL3/EGL3) that specify epidermal cell fate. A close connection between the GL3/EGL3 bHLH proteins and the TTG protein is suggested by several lines of evidence. First, both are essential at an early stage of development, because they each alter all aspects of

Table 4. Interaction between the GL3 or EGL3 proteins and the WER or CPC proteins in the yeast two-hybrid assay

Activation domain construct	DNA-binding domain construct	β-galactosidase activity* (units)
pGAD424 vector	pGBT9 vector	0±0
pGAD424 vector	BD-GL3	217±10
pGAD424 vector	BD-EGL3	26±1
AD-WER	pGBT9 vector	0±0
AD-WER	BD-GL3	284±22
AD-WER	BD-EGL3	93±9
AD-CPC	pGBT9 vector	0±0
AD-CPC	BD-GL3	395±18
AD-CPC	BD-EGL3	430±18

*Activity±s.d.

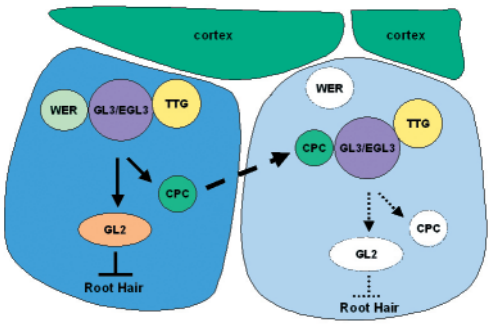

Fig. 7. The proposed role of *GL3* and *EGL3* in epidermal cell specification. In the N cell position, there is a relatively high level of WER relative to CPC, which enables a WER-GL3/EGL3 complex to form and promote GL2 and CPC transcription. In the H position, a relatively high level of CPC leads predominantly to formation of the inactive CPC-GL3/EGL3 complex. Proteins shown in white ovals are at low concentrations. See text for detailed discussion.

non-hair cell differentiation (including early developmental characteristics like cell division rate, cytoplasmic density and vacuolation rate) (Berger et al., 1998a; Galway et al., 1994) (Fig. 4). Second, the *ttg* mutations and the *gl3 egl3* double mutant both exhibit a dramatic reduction in *GL2* expression, but retain its position-dependent pattern, which implies that neither function is essential for the generation of the *GL2* expression pattern but both are required for the proper expression level. In addition, prior studies showed that GL3 and EGL1 can interact with TTG in yeast (Payne et al., 2000; Zhang et al., 2003). Finally, the ability of the *35S::GL3* or *35S::EGL3* to largely complement the N cell defect in the *ttg* mutant suggests that TTG may enhance the abundance, activity or localization of these bHLH proteins, and this influence can be overcome by overexpression of the bHLH genes. However, the *35S::GL3* and *35S::EGL3* were unable to induce non-hair cell production in the *ttg* mutant to the same extent as in the wild type (Table 1), so TTG is likely to be partially required for the function of these transgenes.

Another part of our model (Fig. 7) is that the GL3/EGL3 bHLH proteins interact with the CPC protein in the H position, and this interaction leads to specification of the hair cell fate. This proposal is derived from two lines of evidence. First, CPC physically interacts with GL3 or EGL3 in yeast (Table 4). Second, the ability of the *35S::GL3* and *35S::EGL3* lines to induce *GL2* expression, *CPC* expression and the non-hair cell fate in the H position implies that a high concentration of GL3 or EGL3 is sufficient to alter the fate of the H cells and convert them into non-hair cells. Considering that WER is required for this effect (Table 1) and therefore must be produced/available in the H position, it is possible that, in wild-type roots, the concentration of GL3/EGL3 available for interaction with WER is low because most of it is bound to CPC. The *35S::GL3* and *35S::EGL3* phenotypes may then be explained because the excess supply of these bHLH proteins enables a significant accumulation of the functional WER-bHLH complex even in the presence of the CPC inhibitor. This explanation is consistent with a competition mechanism for epidermal patterning that is essentially similar to one proposed earlier (Lee and Schiefelbein, 1999). Accordingly, the epidermal pattern is determined by the relative concentration of a functional two-repeat MYB (WER) versus an incomplete one-repeat MYB that lacks a transcriptional activation domain (CPC and probably also TRY). Each of these MYBs is envisioned to compete for binding to a limited supply of the GL3/EGL3 bHLH proteins, with the WER-bHLH interaction leading to a functional transcriptional complex that activates *GL2* and *CPC*, whereas the CPC-bHLH interaction generates a non-functional complex that leads to hair cell specification by default (Fig. 7). The cell-type pattern may then result from positional cues and gene regulatory networks that generate a relatively high concentration of WER in the N position and a relatively high concentration of CPC (and probably TRY) in the H position (Fig. 7). We are currently testing various predictions of this model.

Although we have focused our attention on the seedling root, it is likely that the action of the *GL3* and *EGL3* genes is initiated during embryonic root development, because the epidermal pattern is known to be established during embryogenesis and each of the other regulators is active during that period (Costa and Dolan, 2003; Lin and Schiefelbein, 2001). It is also likely that *GL3* and *EGL3* help to establish epidermal cell fate in the hypocotyl because, to date, all of the regulators that have been examined alter epidermal patterning in the root and hypocotyl (Berger et al., 1998b; Hung et al., 1998; Lee and Schiefelbein, 1999). Future studies will be aimed at testing these predictions.

In this study, we detected a significant difference in the epidermal cell pattern in the upper and lower regions of the 4-day-old seedling roots in several of the bHLH mutants and transgenic lines (Tables 1, 2). The cells that comprise the upper region are largely formed during embryogenesis and have been termed the ‘embryonic root’ (Dolan et al., 1994; Lin and Schiefelbein, 2001; Scheres et al., 1994). It is therefore possible that the *GL3* and/or *EGL3* genes or gene products have a different role in epidermal patterning during embryonic versus post-embryonic development. For example, the *GL3* and *EGL3* may differ in their degree of redundancy or their putative partner proteins in a developmentally dependent manner. Alternatively, it is possible that epidermal patterning in this region of the root is generally less ‘tightly regulated’ by the position-dependent mechanism and therefore more sensitive to genetic perturbation, owing to the proximity of this region to the root-hypocotyl junction (collet), where every epidermal cell adopts the hair fate (Dolan et al., 1994; Lin and Schiefelbein, 2001; Scheres et al., 1994).

The patterning of epidermal cells in the root appears to employ a mechanism similar to the one used in the shoot to control trichome distribution (Larkin et al., 2003; Schiefelbein, 2003). This similarity extends to the use of the GL3 and EGL3 proteins, which have been shown to participate in trichome specification in a regulatory network resembling the one described here (Payne et al., 2000; Zhang et al., 2003). Furthermore, these bHLH proteins participate with TTG in seed coat development and anthocyanin production (Zhang et al., 2003), which suggests that a common transcriptional cassette operates in all of these processes and confirms predictions made from studies with the heterologous maize *R* protein in *Arabidopsis* (Galway et al., 1994; Lloyd et al., 1992).

Our work shows that the *GL3* and *EGL3* bHLH genes act in a largely redundant fashion to influence epidermal cell specification in the root. The lack of a major effect of either single homozygous mutant and the lack of a synergistic effect in the *35S::GL3 35S::EGL3* line indicates that the GL3 and EGL3 proteins function in a similar manner. There are two other *Arabidopsis* bHLH genes, *MYC1* (Urao et al., 1996) and *TT8* (Nesi et al., 2000), that are related to the maize *R* and in the same subgroup as *GL3* and *EGL3* (Heim, 2003). In preliminary studies, we have found that at least the *MYC1* gene probably participates in root epidermal patterning (C.B., M. Sridharan and J.S., unpublished). Thus, an unexpectedly large collection of bHLH genes may play a role in the specification of epidermal cell fate in the *Arabidopsis* root. This probably reflects the importance of genetic redundancy and the complex regulatory nature of cell specification in higher plants.

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References

Berger, F., Hung, C. Y., Dolan, L. and Schiefelbein, J. (1998a). Control of cell division in the root epidermis of Arabidopsis thaliana. *Dev. Biol.* **194**, 235-245.

Berger, F., Linstead, P., Dolan, L. and Haseloff, J. (1998b). Stomata patterning on the hypocotyl of Arabidopsis thaliana is controlled by genes involved in the control of root epidermis patterning. *Dev. Biol.* **194**, 226-234.

Costa, S. and Dolan, L. (2003). Epidermal patterning genes are active during embryogenesis in Arabidopsis. *Development* **130**, 2893-2901.

Dolan, L. and Costa, S. (2001). Evolution and genetics of root hair stripes in the root epidermis. *J. Exp. Bot.* **52**, 413-417.

Dolan, L., Duckett, C., Grierson, C., Linstead, P., Schneider, K., Lawson, E., Dean, C., Poethig, R. S. and Roberts, K. (1994). Clonal relations and patterning in the root epidermis of Arabidopsis. *Development* **120**, 2465-2474.

Fields, S. and Sternglanz, R. (1994). The two-hybrid system: an assay for protein-protein interactions. *Trends Genet.* **10**, 286-292.

Galway, M. E., Masucci, J. D., Lloyd, A. M., Walbot, V., Davis, R. W. and Schiefelbein, J. W. (1994). The TTG gene is required to specify epidermal cell fate and cell patterning in the Arabidopsis root. *Dev. Biol.* **166**, 740-754.

Heim, M. A., Jakoby, M., Werber, M., Martin, C., Weisshaar, B., Bailey, P. C. (2003). The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. *Mol. Biol. Evol.* **20**, 735-747.

Hulskamp, M., Misra, S. and Jurgens, G. (1994). Genetic dissection of trichome cell development in Arabidopsis. *Cell* **76**, 555-566.

Hung, C. Y., Lin, Y., Zhang, M., Pollock, S., Marks, M. D. and Schiefelbein, J. (1998). A common position-dependent mechanism controls cell-type patterning and GLABRA2 regulation in the root and hypocotyl epidermis of Arabidopsis. *Plant Physiol.* **117**, 73-84.

Koornneef, M. (1981). The complex syndrome of ttg mutants. *Arabidopsis Inf. Serv.* **18**, 45-51.

Koornneef, M., Dellaert, S. W. M. and van der Veen, J. H. (1982). EMS- and radiation-induced mutation frequencies at individual loci in *Arabidopsis thaliana* (L.) Heynh. *Mutat. Res.* **93**, 109-123.

Larkin, J. C., Brown, M. L. and Schiefelbein, J. (2003). How do cells know what they want to be when they grow up? Lessons from epidermal patterning in Arabidopsis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **54**, 403-430.

Lee, M. M. and Schiefelbein, J. (1999). WEREWOLF, a MYB-related protein in Arabidopsis, is a position-dependent regulator of epidermal cell patterning. *Cell* **99**, 473-483.

Lee, M. M. and Schiefelbein, J. (2002). Cell pattern in the Arabidopsis root epidermis determined by lateral inhibition with feedback. *Plant Cell* **14**, 611-618.

Lin, Y. and Schiefelbein, J. (2001). Embryonic control of epidermal cell patterning in the root and hypocotyl of Arabidopsis. *Development* **128**, 3697-705.

Lloyd, A., Walbot, V. and Davis, R. W. (1992). Arabidopsis and Nicotiana anthocyanin production activated by maize regulators R and C1. *Science* **258**, 1773-1775.

Ludwig, S. R. and Wessler, S. R. (1990). Maize R gene family: tissue-specific helix-loop-helix proteins. *Cell* **62**, 849-851.

Masucci, J. D., Rerie, W. G., Foreman, D. R., Zhang, M., Galway, M. E., Marks, M. D. and Schiefelbein, J. W. (1996). The homeobox gene GLABRA2 is required for position-dependent cell differentiation in the root epidermis of Arabidopsis thaliana. *Development* **122**, 1253-1260.

Masucci, J. D. and Schiefelbein, J. W. (1996). Hormones act downstream of TTG and GL2 to promote root hair outgrowth during epidermis development in the Arabidopsis root. *Plant Cell* **8**, 1505-1517.

Mol, J., Grotewold, E. and Koes, R. (1998). How genes paint flowers and seeds. *Trends Plant Sci.* **3**, 212-217.

Neer, E. J., Schmidt, C. J., Nambudripad, R. and Smith, T. F. (1994). The ancient regulatory protein family of WD-repeat proteins. *Nature* **371**, 297-300.

Nesi, N., Debeaujon, I., Jond, C., Pelletier, G., Caboche, M. and Lepiniec, L. (2000). The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in Arabidopsis siliques. *Plant Cell* **12**, 1863-1878.

Payne, C. T., Zhang, F. and Lloyd, A. M. (2000). GL3 encodes a bHLH protein that regulates trichome development in arabidopsis through interaction with GL1 and TTG1. *Genetics* **156**, 1349-1362.

Rerie, W. G., Feldmann, K. A. and Marks, M. D. (1994). The GLABRA2 gene encodes a homeo domain protein required for normal trichome development in Arabidopsis. *Genes Dev* **8**, 1388-1399.

Schellmann, S., Schnittger, A., Kirik, V., Wada, T., Okada, K., Beermann, A., Thumfahrt, J., Jurgens, G. and Hulskamp, M. (2002). TRIPTYCHON and CAPRICE mediate lateral inhibition during trichome and root hair patterning in Arabidopsis. *EMBO J.* **21**, 5036-5046.

Scheres, B., Wlkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C. and Weisbeek, P. (1994). Embryonic origin of the Arabidopsis primary root and root meristem initials. *Development* **120**, 2475-2487.

Schiefelbein, J. (2003). Cell-fate specification in the epidermis: a common patterning mechanism in the root and shoot. *Curr. Opin. Plant Biol.* **6**, 74-78.

Schiefelbein, J. W. and Somerville, C. (1990). Genetic Control of Root Hair Development in Arabidopsis thaliana. *Plant Cell* **2**, 235-243.

Singh, K. B. (1998). Transcriptional regulation in plants: the importance of combinatorial control. *Plant Physiol.* **118**, 1111-1120.

Urao, T., Yamaguchi-Shinozaki, K., Mitsukawa, N., Shibata, D. and Shinozaki, K. (1996). Molecular cloning and characterization of a gene that encodes a MYC-related protein in Arabidopsis. *Plant Mol. Biol.* **32**, 571-576.

Wada, T., Tachibana, T., Shimura, Y. and Okada, K. (1997). Epidermal cell differentiation in Arabidopsis determined by a Myb homolog, CPC. *Science* **277**, 1113-1116.

Wada, T., Kurata, T., Tominaga, R., Koshino-Kimura, Y., Tachibana, T., Goto, K., Marks, M. D., Shimura, Y. and Okada, K. (2002). Role of a positive regulator of root hair development, CAPRICE, in Arabidopsis root epidermal cell differentiation. *Development* **129**, 5409-5419.

Walker, A. R., Davison, P. A., Bolognesi-Winfield, A. C., James, C. M., Srinivasan, N., Blundell, T. L., Esch, J. J., Marks, M. D. and Gray, J. C. (1999). The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. *Plant Cell* **11**, 1337-1350.

Weigel, D. and Glazebrook, J. (2002). *Arabidopsis: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Zhang, F., Gonzalez, A., Zhao, M., Payne, C. T. and Lloyd, A. M. (2003). A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. *Development* **130**, 4859-4869.