

The bHLH genes *GL3* and *EGL3* participate in an intercellular regulatory circuit that controls cell patterning in the *Arabidopsis* root epidermis

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Summary

The specification of the hair and non-hair cells in the *Arabidopsis* root epidermis provides a useful model for the study of cell fate determination in plants. A network of putative transcriptional regulators, including the related bHLH proteins *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*), is known to influence the patterning of these cell types. Here, we analyze the expression and regulation of *GL3* and *EGL3* during root epidermis development. Although they are thought to act in both the hair and non-hair cell types, we surprisingly found that *GL3* and *EGL3* gene expression and RNA accumulation occurs preferentially in the developing hair cells. By analyzing the expression of *GL3::GUS* and *EGL3::GUS* reporter fusions in various mutant and overexpression lines, we discovered that the expression of both genes is negatively regulated by *WER*, *GL3* and *EGL3* in the

developing non-hair cells, and positively regulated by the *CPC* and *TRY* proteins in the developing hair cells. Further, the analysis of a *GL3*-YFP translational fusion, expressed under the *GL3* promoter, indicates that the *GL3* protein moves from the hair cells to the non-hair cells. These results suggest that *GL3/EGL3* accumulation in the N cells is dependent on specification of the hair cell fate, which itself is known to be influenced (via *CPC*-mediated lateral inhibition) by the non-hair cells. This bi-directional signaling mechanism defines a new regulatory circuit of intercellular communication to specify the epidermal cell types.

Key words: Epidermis, Pattern formation, Root hairs, Gene regulation, Cell differentiation, Embryogenesis, Cell communication, Hypocotyl, *Arabidopsis thaliana*

Introduction

The determination of distinct cell fates in appropriate patterns is a crucial feature of development in multicellular organisms. In plants, the formation of the root hair and non-hair cells in the root epidermis has been used extensively as a simple and experimentally tractable model for studying cell fate patterning (Dolan and Costa, 2001; Larkin et al., 2003). The root epidermal cell types are not essential for growth under laboratory conditions, they are easy to examine, and they arise continuously during development.

In *Arabidopsis*, root hair cells are specified in a position-dependent manner, such that all cells located in a cleft between two underlying cortical cells (designated the H position) develop as hair cells and cells located outside a single cortical cell (designated the N position) adopt the non-hair fate (Dolan et al., 1994; Galway et al., 1994). Molecular genetic studies have shown that a suite of putative transcription factors regulates the patterning of root hair cells in *Arabidopsis*. These factors include a homeodomain protein, *GLABRA2* (*GL2*) (Masucci et al., 1996; Rerie et al., 1994); a WD-repeat protein, *TRANSPARENT TESTA GLABRA* (*TTG*) (Galway et al.,

1994; Walker et al., 1999); an R2R3 MYB-type transcription factor, *WEREWOLF* (*WER*) (Lee and Schiefelbein, 1999); two closely related basic helix-loop-helix proteins, *GLABRA3* and *ENHANCER OF GLABRA3* (Bernhardt et al., 2003); and three small MYB proteins, *CAPRICE* (*CPC*), *TRIPTYCHON* (*TRY*) and *ENHANCER OF TRIPTYCHON AND CAPRICE* (*ETC1*) (Kirik et al., 2004; Schellmann et al., 2002; Wada et al., 2002; Wada et al., 1997). The *GL2*, *TTG*, *WER*, *GL3* and *EGL3* appear to have a primary role in promoting the non-hair fate, whereas the *CPC*, *TRY* and *ETC1* are most important in specifying the hair cell fate. The cell pattern is proposed to result from a lateral inhibition mechanism that is mediated by *CPC*, *TRY* and *ETC1* (Larkin et al., 2003; Schiefelbein, 2003). The transcription of *CPC* (and presumably of *TRY* and *ETC1*) is promoted by a putative complex of *TTG*, *WER*, *GL3* and *EGL3* in the N cell position, and these small MYB proteins inhibit the neighboring H cells from adopting the non-hair fate, possibly by directly moving from cell-to-cell and interfering with the *WER* function (Schellmann et al., 2002; Schiefelbein, 2003; Wada et al., 2002; Wada et al., 1997).

Although the importance of the putative complex containing *GL3*, *EGL3*, *WER* and *TTG* is clear, the mechanisms that

regulate the accumulation of these components is poorly understood. In this study, we sought to analyze the expression and regulation of *GL3* and *EGL3* during root epidermis development. In prior work, we have analyzed mutants and overexpression lines to show that *GL3* and *EGL3* are likely to act redundantly to help specify both the hair and non-hair cell fates (Bernhardt et al., 2003). The *gl3 egl3* double mutant was shown to have excessive root-hair cells, while the overexpression of these genes caused an increased frequency of non-hair cells. Furthermore, these bHLH proteins are required for the positive transcriptional control of *GL2* (which specifies the non-hair fate) and *CPC* (which helps specify the hair cell fate), and they interact with the *WER* and the *CPC* MYB proteins in yeast (Bernhardt et al., 2003). The prediction from this work was that these bHLH genes might be expressed (and their gene products accumulate) in both the H and N cells. Here, we use RNA hybridization, promoter reporter fusions and genetic analyses to show that the *GL3/EGL3* genes are preferentially expressed in the H position, and furthermore, that this expression pattern is controlled by several of the known cell fate regulators, including the *GL3/EGL3* proteins themselves. Using a YFP translational fusion, we find that *GL3* accumulates in the nuclei of the N cells. These results suggest a new feedback loop in the epidermal regulatory network that helps establish and reinforce the cell fate pattern.

Materials and methods

Plant materials and growth conditions

The isolation of the mutant alleles used in this study has been described: *gl3-1* and *gl3-2*, both in the *Ler* background (Koornneef, 1982; Payne et al., 2000); *egl3-1*, in the *Ler* genetic background (Zhang et al., 2003); *wer-1*, in the Columbia ecotype (Lee and Schiefelbein, 1999); *ttg-1*, in the *Ler* genetic background (Galway et al., 1994); *cpc-1*, in the WS genetic background (Wada et al., 1997); *try-82*, in the *Ler* genetic background (Hulskamp et al., 1994); *gl2-1*, in the *Ler* genetic background (Koornneef, 1982); *rh6-1*, in the WS ecotype (Masucci and Schiefelbein, 1994).

The following transgenic lines have been described previously: *GL3::GUS* and *EGL3::GUS* (Zhang et al., 2003), *GL2::GUS* (Masucci et al., 1996), *CPC::GUS* (Wada et al., 2002), *35S::GL3* (Payne et al., 2000), *35S::EGL3* (Zhang et al., 2003), *35S::CPC* (Wada et al., 1997) and *35S::TRY* (Schellmann et al., 2002).

Lines homozygous for multiple mutations and/or transgenes were constructed by crossing single mutant or transgenic plants, examining the F2 progeny for putative mutant phenotypes, and confirming the desired genotype in subsequent generations by backcrossing to single mutants and/or PCR-based tests.

For seedling analysis, *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

The histochemical analysis of plants containing the *GUS* reporter constructs was performed on at least 20 four-day-old root tips for each strain essentially as described (Masucci et al., 1996). Root epidermal cells were deemed to be in the N position if they were located outside a periclinal cortical cell wall, whereas cells in the H position were located outside a radial wall between adjacent cortical cells.

The distribution of epidermal cell types in the hypocotyl was analyzed in 25 nine-day-old seedlings for each strain by determining the number of stomata formed along the hypocotyl in two adjacent

epidermal cell files, one located over anticlinal cortical cell walls and one located over periclinal cortical cell walls.

In situ RNA hybridization

The whole-mount in situ RNA hybridization procedure has been described (de Almeida Engler et al., 1994). The RNA probe was designed to hybridize to both *GL3* and *EGL3* transcripts, so it included bp 550-1120 and bp 1400-1850 downstream from the start site of the *EGL3*-coding sequence corresponding to the most similar region of the *GL3* and *EGL3* proteins but excluding the bHLH signature region to eliminate the possibility of cross-hybridization to other bHLH proteins.

Molecular biology methods

To construct the *GL3::GL3-YFP* translational fusion, pD2L-2 (Payne et al., 2000) was used to provide a *GL3* genomic DNA fragment containing the entire *GL3* gene, including ~1 kb upstream of the start codon and 1 kb downstream of the stop codon. The existing *SacI* and *SalI* sites of pD2L-2 were destroyed and new *SacI* and *SalI* sites were generated by inverse PCR at the 3' end of the *GL3*-coding region. The *EYFP*-coding region was amplified from pEYFP (Clontech) and fused in-frame to the *GL3* 3' end. A *BamHI* fragment from this vector, containing the entire *GL3::EYFP* fusion, was subcloned into the *BglIII* site of the T-DNA vector pAL47 (Lloyd and Davis, 1994). The *GL3-YFP* fusion protein is predicted to be 96 kDa, whereas the predicted size of the *GL3* and *EGL3* proteins is 70 kDa and 66 kDa, respectively. Plant transformation was performed by the floral dip method (Clough and Bent, 1998).

Results

GL3 and *EGL3* are preferentially expressed in root epidermal cells in the H position

Our earlier results led to the suggestion that the *GL3* and *EGL3* genes are expressed in both the developing hair cells and non-hair cells (Bernhardt et al., 2003). To test this, we generated and analyzed promoter-*GUS* fusions for each gene (*GL3::GUS* and *EGL3::GUS*). In each line, the maximum *GUS* activity was observed in the meristematic region of the developing root tip (Fig. 1). This temporal pattern is similar to that previously reported for *WER* and earlier than for *GL2* (Lee and Schiefelbein, 1999; Masucci et al., 1996), which is consistent with the view that the bHLH genes act at the same stage as *WER* and regulate *GL2* expression (Bernhardt et al., 2003). However, the *GL3::GUS* and *EGL3::GUS* showed significantly higher levels of *GUS* activity in cells located in the H position than the N position (Fig. 1), although longer incubation times also caused some *GUS* staining in the N cells. The cells preferentially expressing these reporters were determined to be in the H position because the stained cell files overlie radial cortical cell walls, they show a higher cell division rate than unstained files (see magnified view in Fig. 1), and they can be traced to mature cells that are hair forming. Thus, despite the important role of *GL3* and *EGL3* in specifying the non-hair fate in the N position, these genes are preferentially expressed in the H cell position.

GL3 and *EGL3* RNA is localized to epidermal cells in the H-position

To determine whether the unexpected bHLH promoter activity pattern was associated with a similar bHLH RNA accumulation pattern, we conducted in situ RNA hybridization experiments. Because of the high degree of sequence similarity

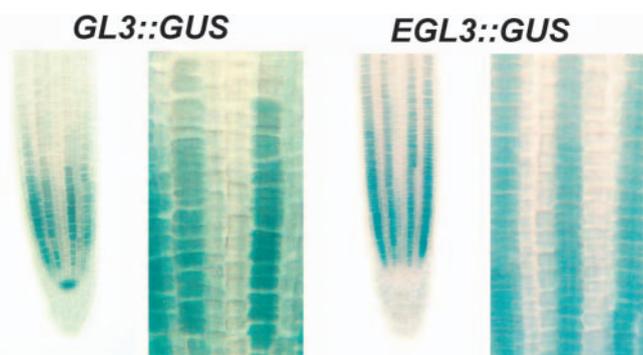


Fig. 1. *GL3* and *EGL3* promoter activity in the developing root. Expression of the *GL3::GUS* and *EGL3::GUS* reporter fusions in the root epidermis of 4-day-old seedlings. The magnified images (right) show the differential cell division rate of N- and H-cell files, indicating that both reporters are preferentially expressed in developing hair cells. The *GL3::GUS* reporter is also expressed in the quiescent center cells in the root meristem, but this does not seem to be associated with the role of *GL3* in epidermal development (C.B. and J.S., unpublished).

throughout the *GL3* and *EGL3* genes, it was not possible to generate viable gene-specific antisense RNA probes. Rather, we designed an antisense RNA probe corresponding to a conserved region in *GL3/EGL3*, so that the probe would recognize both genes, but not other related bHLH genes in *Arabidopsis*. Using whole roots from four-day-old wild-type seedlings, we found that this probe preferentially hybridized to cells in the H position (Fig. 2A). Again, the position of the cell files was determined by their location relative to the underlying cortical cell walls and their differential cell division rate (Fig. 2A). This result was reinforced by the analysis of bHLH RNA accumulation in rare epidermal clones (Fig. 2A, last panel).

These clones form when occasional longitudinal anticlinal divisions in cells in the H position create two cell files; one located over the anticlinal cortical cell wall (the H position) and one overlying a periclinal cortical cell wall (the N position) (Berger et al., 1998a). In these clones, we found that cells in the N position no longer accumulate *GL3/EGL3* RNA (Fig. 2B). As a control, roots hybridized with the sense RNA strand as a probe yielded no signal (Fig. 2A, first panel).

To confirm that the antisense RNA probe indeed recognizes both gene sequences and to determine whether both RNAs accumulate in the same pattern, we also tested roots from the *gl3-2* and *egl3-1* single mutants and the *gl3-2 egl3-1* double mutant. Both single mutants showed a similar but weaker hybridization pattern as the wild-type roots, while *gl3-2 egl3-1* double mutant roots displayed a very low level of signal (Fig. 2B). This suggests that the antisense probe primarily detects *GL3* transcripts in the *egl3* mutant and *EGL3* transcripts in the *gl3* mutant. This conclusion is supported by gene-specific RT-PCR experiments that show a reduced level of *GL3* RNA in the roots of *gl3-2* and *gl3-2 egl3-1* relative to *egl3-1* and a reduced level of *EGL3* RNA in the roots of *egl3-1* and *gl3-2 egl3-1* relative to *gl3-2* (data not shown). Together, these data imply that the *GL3* RNA and *EGL3* RNA exhibit the same pattern of preferential accumulation in the H cell. These results support the reporter fusion results and suggest that the *GL3* and *EGL3* RNAs do not move from cell-to-cell.

***GL3* and *EGL3* function in the embryonic root and during hypocotyl epidermis development**

The position-dependent patterning of root epidermal gene expression is known to be established during early stages of embryogenesis (Costa and Dolan, 2003; Lin and Schiefelbein, 2001). To explore further the unexpected expression pattern of *GL3* and *EGL3*, we examined the *GL3::GUS* and *EGL3::GUS* activity during embryogenesis. We found that GUS activity

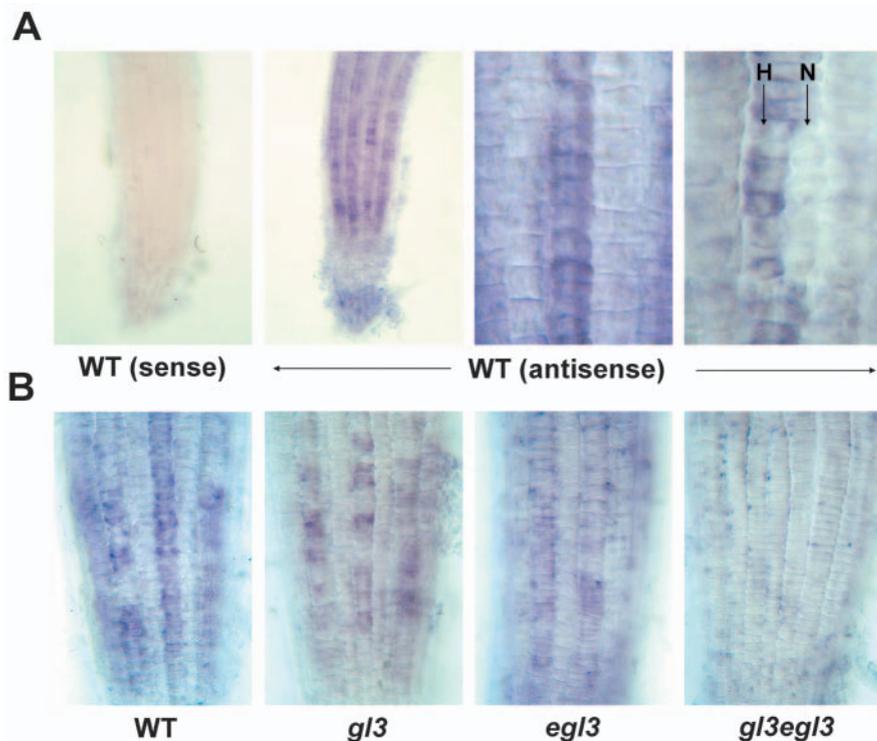


Fig. 2. *GL3* and *EGL3* RNA accumulation in the developing root. Whole-mount in situ RNA hybridization of *GL3* and *EGL3* mRNA in 4-day-old root tips. (A) Hybridization of root tips with *GL3/EGL3* sense and antisense RNA probe in wild-type (WT) seedlings. *GL3/EGL3* RNAs accumulate preferentially in cells in the H position. (The staining occurs in cells with the higher cell division rate.) The panel on the far right shows that this expression pattern is maintained in epidermal clones. The corresponding sense probe does not show any signal in the root tip. (B) Hybridization of root tips with *GL3/EGL3* antisense RNA probe in the wild type, *gl3-2*, *egl3-1* and *gl3-2 egl3-1* lines. This indicates that both *GL3* and *EGL3* RNAs accumulate in the same (H-cell) pattern.

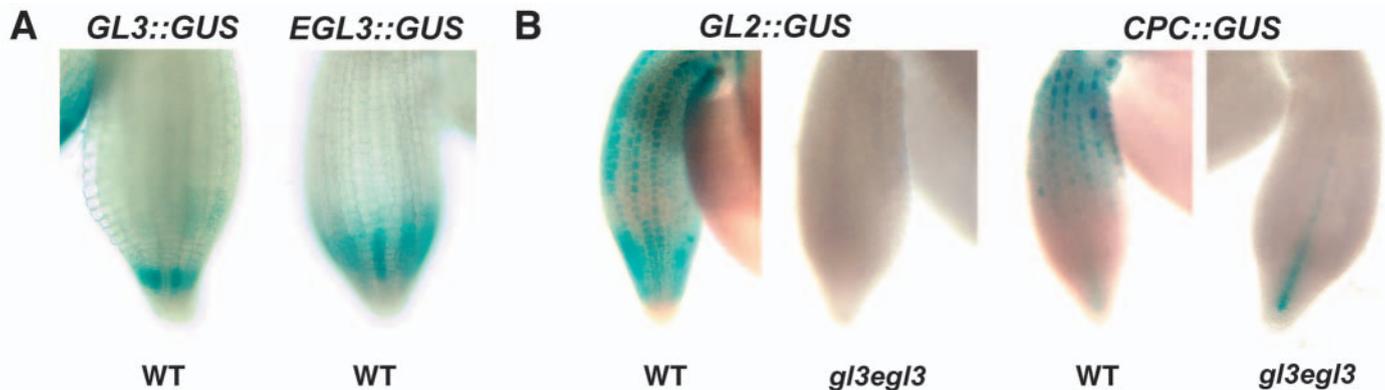


Fig. 3. *GL3* and *EGL3* expression and function in embryos. (A) Analysis of *GL3* and *EGL3* promoter activity in the root of mature wild-type (WT) embryos using *GL3::GUS* and *EGL3::GUS* reporter lines. *GL3* and *EGL3* are expressed in the same H-cell pattern in the embryonic root as in the post-embryonic root. (B) Expression of the *GL2::GUS* and *CPC::GUS* reporters in hypocotyl and root epidermis of mature wild-type and *gl3 egl3* mutant embryos. *GL2* and *CPC* promoter activity is reduced in the *gl3 egl3* mutant in both hypocotyl and root (except that no *CPC::GUS* activity is detected in the embryonic root epidermis in either line).

accumulated in the same H cell pattern in the epidermis of the mature embryonic root as in the postembryonic root (Fig. 3A).

Considering this embryonic expression, we wished to determine whether the *GL3* and *EGL3* genes act in a similar manner in the embryonic and postembryonic root. Specifically, we examined whether embryonic *GL2* and *CPC* gene expression (using the *GL2::GUS* and *CPC::GUS* reporters) is reduced in a *gl3 egl3* mutant, as they are in the seedling root (Bernhardt et al., 2003). Indeed, we observed significantly less GUS activity in the root of the *gl3 egl3 GL2::GUS* mature embryos (Fig. 3B). We were unable to detect any GUS expression in the mutant or wild-type embryonic root bearing the *CPC::GUS* reporter, which prevented an assessment of the role of *GL3/EGL3* on *CPC* (Fig. 3B).

The hypocotyl is known to be patterned by a similar network of transcription factors as the root to define two position-dependent cell types: cells capable of producing stomata and non-stomata cells (Berger et al., 1998b; Hung et al., 1998). In *gl3 egl3* embryos bearing either the *GL2::GUS* or the *CPC::GUS* reporters, we detected a significantly reduced level of GUS activity in the embryonic hypocotyl relative to the wild type (Fig. 3B). This dependence of *GL2* and *CPC* expression on the presence of *GL3/EGL3* in the embryonic hypocotyl suggested a possible role for both proteins during stomatal patterning in the hypocotyl. To explore this further, we analyzed stomata distribution in the hypocotyls of nine-day-old seedlings of wild type and *gl3 egl3* double mutants. In contrast to the wild type, which has stomata predominantly form over a radial cortical cell wall (analogous to the H position of the root epidermis), the *gl3 egl3* hypocotyls have an increased number of stomata and they are present in both positions (Table 1). This shows that *GL3* and *EGL3* are required for proper stomata patterning, and it strengthens the close relationship between the mechanism for patterning the hypocotyl and root epidermis in *Arabidopsis*.

***EGL3* expression is regulated by upstream components of the epidermal patterning pathway**

We wished to determine whether any of the transcription factors in the epidermal cell fate network might regulate

the expression of the *GL3* and *EGL3* genes. Because the *EGL3::GUS* line exhibited a greater expression level than the *GL3::GUS* line, thus allowing for better analysis of weaker effects, we first focused our efforts on studying the control of the *EGL3* expression pattern.

We first examined the possible role of the upstream regulators in this system, *WER* and *TTG*. The *EGL3::GUS* transgene was introduced into the *wer-1* and *ttg-1* mutant backgrounds, and we found that each of the homozygous mutants exhibit ectopic GUS expression in the N cell position (Fig. 4A). This suggests that *WER* and *TTG* negatively regulate *EGL3* expression in the N cells.

Next, we tested the possible effects of the lateral inhibitors *CPC* and *TRY*. We found that neither *cpc* nor *try* mutants significantly altered *EGL3::GUS* expression, but the *cpc try* double mutant exhibited essentially no GUS activity (Fig. 4B). We also introduced the *EGL3::GUS* reporter construct in both the *35S::CPC* and *35S::TRY* background and found that overexpression of each gene induced ectopic *EGL3::GUS* expression in the N cell position (Fig. 4B). As *CPC* and *TRY* are related genes known to act in a partially redundant fashion (Schellmann et al., 2002; Wada et al., 2002), this indicates that the *CPC/TRY* function is required for *EGL3* gene expression in the H position.

We next tested the effect of *gl2* and *rhod6* mutants on *EGL3* expression. Results from mutant and overexpression analyses indicate that *GL3/EGL3* act at an early stage of root epidermis

Table 1. Effect of *gl3* and *egl3* on stomata patterning in the hypocotyl*

Genotype	Number of stomata [†]	Stomata in S position (%) [‡]	Stomata in N position (%)
Wild type (<i>Ler</i>)	5.8±1.8	97.3±6.5	2.7±6.5
<i>gl3 egl3</i>	8.8±3.0	66.7±16.4	33.3±16.4

*At least 25 seedling hypocotyls were examined for each line.

[†]Number of stomata in two adjacent cell files, one located in the S and the other in the N position.

[‡]Stomata in the S position overlie an anticlinal cortical cell wall; stomata in the N position overlie a periclinal cortical cell wall.

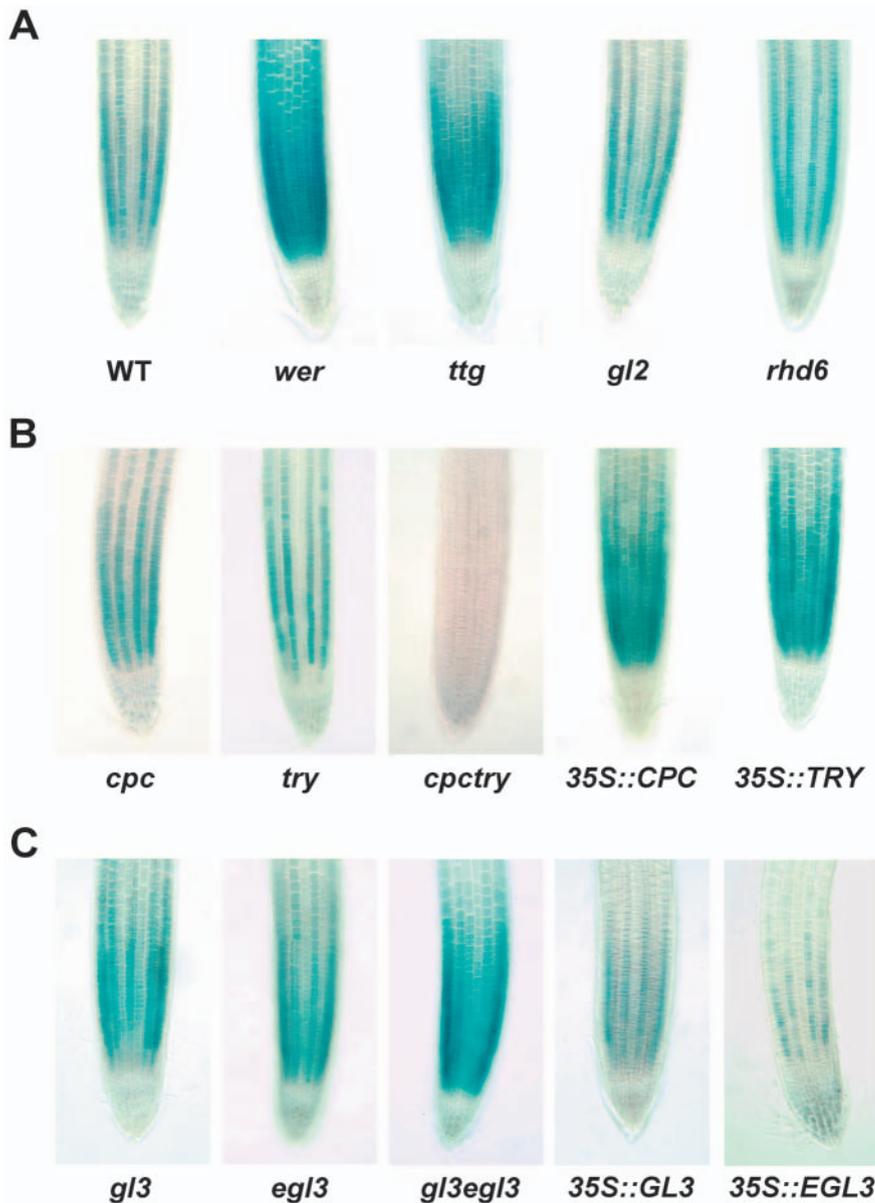


Fig. 4. Regulation of the *EGL3::GUS* expression pattern. Expression of the *EGL3::GUS* reporter in the developing root epidermis of 4-day-old seedlings in mutant backgrounds and transgenic overexpressing lines. (A) *EGL3* promoter activity is expanded in the epidermis of the *wer* and *ttg* lines, but unaffected by the *gl2* and *rhd6* backgrounds. (B) *EGL3* promoter activity is reduced in the *cpc try* line (but not significantly affected in either single mutant), and it is expanded in the epidermis of the *35S::CPC* and *35S::TRY* backgrounds. (C) *EGL3* promoter activity is expanded in the epidermis of the *gl3 egl3* line (but not in either single mutant), and it is reduced in the *35S::GL3* and *35S::EGL3* backgrounds.

development (Bernhardt et al., 2003), so we expected no effect of mutations in the later acting genes *GL2* and *RHD6*. Consistent with this, the *EGL3::GUS* pattern in *gl2* and *rhd6* was indistinguishable from the wild type (Fig. 4A).

Last, we examined the possibility that the *GL3/EGL3* proteins themselves might regulate *EGL3* promoter activity. Although roots of the *gl3 EGL3::GUS* and *egl3 EGL3::GUS* lines exhibit only weak ectopic GUS activity, the *gl3 egl3 EGL3::GUS* double mutant line possessed strong GUS expression in the N position (Fig. 4C). Furthermore,

overexpression of either bHLH gene (via the *35S::GL3* and *35S::EGL3* constructs) caused a weak or modest reduction in *EGL3::GUS* expression. These results suggest an autoregulation of *EGL3* expression: the *EGL3* protein (together with *GL3*) is able to inhibit its own gene's promoter activity.

***GL3* transcription is similarly controlled as *EGL3* transcription**

To determine whether the *GL3* promoter activity is regulated in a similar manner as *EGL3*, we introduced the *GL3::GUS* reporter into selected genetic backgrounds. We found that the *wer* mutant, the *gl3 egl3* double mutant, and the *35S::CPC* overexpression construct caused ectopic *GL3::GUS* expression, whereas the *cpc try* double mutant exhibited a much lower level of *GL3::GUS* expression (Fig. 5). In addition, *GL3::GUS* activity was not effected by the *cpc* single mutant or the *gl2* mutation (data not shown). These results are all consistent with the effects of these factors on *EGL3::GUS*, and it suggests that both genes are regulated similarly.

Accumulation of *GL3/EGL3* RNA in mutants and overexpression lines follows their expression pattern

In addition to assessing the role of the cell fate transcription factors on promoter activity, we wished to examine their effects on *GL3/EGL3* RNA accumulation. Using whole-mount RNA in situ hybridization, we analyzed the hybridization of an antisense probe (which can hybridize to both *GL3* and *EGL3* RNA) to roots from the *wer*, *cpc try* and *35S::CPC*. We found, relative to the wild type, ectopic signal in the *wer* mutant and the *35S::CPC* line, and very low level of signal from the *cpc try* double mutant (Fig. 6). These results provide support for the promoter-reporter fusion studies above, and show that *WER* negatively regulates *GL3/EGL3* RNA

accumulation in the N position and *CPC/TRY* positively regulates *GL3/EGL3* RNA accumulation in the H position.

The *GL3*-YFP translational reporter fusion protein accumulates predominantly in cells in the N position

To determine the location of the *GL3/EGL3* bHLH proteins during epidermis development, we constructed a *GL3-YFP* translational fusion under the control of the *GL3* promoter (*GL3::GL3-YFP*). This construct was introduced into both *gl3-1* and *gl3-2* mutant plants. These transgenic plants showed

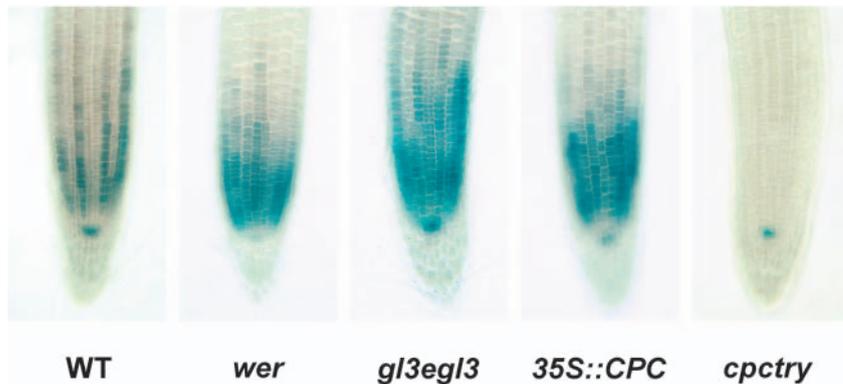


Fig. 5. Regulation of the *GL3::GUS* expression pattern. Expression of the *GL3::GUS* reporter in the developing root epidermis of 4-day-old seedlings in various mutant backgrounds and transgenic overexpressing lines. Ectopic *GL3* promoter activity is found in the *wer*, *gl3 egl3* and *35S::CPC* background. Reduced *GL3* promoter activity is present in the *cpc try* mutant.

normal root hair patterning and trichome formation (data not shown), indicating that the *GL3-YFP* fusion was functional. Transgenic plants expressing this construct displayed YFP expression predominantly in the nuclei of developing epidermal cells in the N position (as determined by their relative cell length, Fig. 7). We also verified this cell location by tracing the cells in the YFP-expressing files back to non-hair cells in the mature portion of the root. This suggests that the *GL3* protein accumulates in the N cell, although the *GL3* promoter activity and *GL3* RNA accumulation is primarily in the H cell.

Discussion

The *GL3* and *EGL3* genes encode bHLH proteins essential for the proper specification of both epidermal cell types in the *Arabidopsis* root. Prior work showed that they probably act together with the *WER* MYB and perhaps *TTG* WD-repeat protein to promote transcription of *GL2* (to specify non-hair fate) and *CPC* in the N cells of the seedling root (Bernhardt et al., 2003). In the H cells, they appear to interact with the *CPC* (and possibly other related small MYBs such as *TRY* and *ETC1*), rather than *WER*, and cause these cells to adopt the hair fate (Bernhardt et al., 2003). Here, we extend the role of *GL3* and *EGL3* by showing that they are also required for proper stomata patterning in the hypocotyl, because the *gl3 egl3* mutant lacks an appropriate stomata distribution. Furthermore, we find that *GL3* and *EGL3* probably begin to act during embryogenesis, by inducing *GL2* and *CPC*

expression in the N cell position to help establish the cell pattern.

Despite their important role in specifying both epidermal cell types, we used promoter reporter fusions to show that *GL3* and *EGL3* are preferentially expressed in the developing hair cells. How might *GL3/EGL3* affect specification of the N cells while being expressed predominantly in the H cells? One possibility is that the *GL3* and *EGL3* proteins may not directly function in the N cells but rather act through another yet unidentified factor involved in a lateral signaling from H to N. However, this possibility is not supported by yeast two-hybrid data, which suggest a direct interaction between the *WER* and *GL3/EGL3* (Bernhardt et al., 2003). In a second scenario, *GL3* and *EGL3* could act in a non-cell autonomous manner in that either the proteins themselves or their RNA is moving from cells in the H position to cells in the N position. Our results support this second scenario. Although in situ hybridization analysis indicated that *GL3* and *EGL3* RNAs accumulate preferentially in cells in the H position (similar to results from the promoter reporter fusion analysis), a *GL3-YFP* construct was detected predominantly in the N cell position. Thus, the requirement for *GL3/EGL3* activity in the developing N cells may be fulfilled by the movement of these proteins from H cells to N cells.

It is likely that the H-cell specific expression pattern of the *GL3* and *EGL3* genes is due, at least in part, to negative autoregulation at the transcriptional level. We found that functional *GL3* and *EGL3* genes are required to inhibit *GL3/EGL3* gene transcription and RNA accumulation in the N

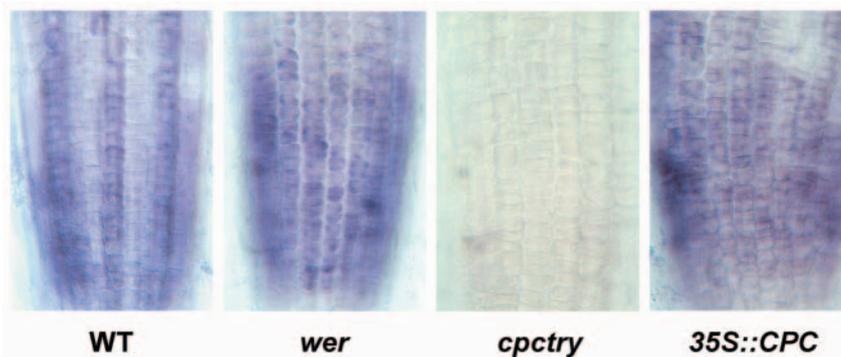


Fig. 6. *GL3* and *EGL3* RNA accumulation in mutant and overexpression lines. Whole-mount in situ RNA hybridization of *GL3* and *EGL3* mRNA in 4-day-old root tips in wild-type (WT), *wer*, *cpc try* and *35S::CPC* background. *GL3/EGL3* RNAs accumulate throughout the epidermis in *wer* and *35S::CPC* seedling roots, while no *GL3/EGL3* RNAs were detected in *cpc try* root epidermis.

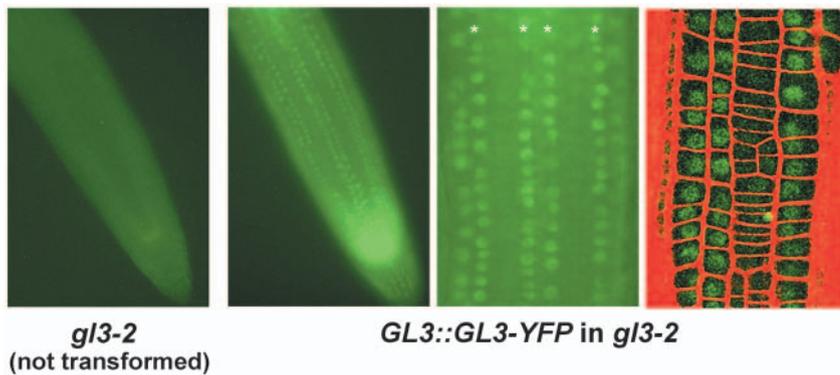


Fig. 7. Localization of a GL3-YFP translation fusion in the root epidermis. Expression of a *GL3::GL3-YFP* construct in the developing root epidermis of 4-day-old *g/3-2* mutant seedlings. First three panels: wide-field fluorescence microscope images. Stars indicate cells files composed of developing non-hair cells, as determined by tracing the files to the mature region of the root. Right-most panel: confocal microscope image (cell walls were counterstained with propidium iodide (red signal) to enhance visualization). The GL3 fusion protein accumulates predominantly in the nuclei of the developing non-hair cells, as apparent from their lower cell division rate and paired nature of these cell files. Consistent with the *GL3::GUS* expression pattern (Fig. 1), the GL3 fusion protein also accumulates in the quiescent center cells of the root meristem.

position. We also found that overexpression of *GL3* or *EGL3* causes modest reduction in *EGL3* transcription in the H position. Furthermore, we show that the putative partners of GL3/EGL3 action, WER and TTG, are also required to inhibit *GL3/EGL3* in the N cells. Taken together, these data indicate that the *GL3* and *EGL3* gene transcription is negatively regulated by the putative WER/GL3/EGL3/TTG complex, which is likely to be most abundant in the N cell position.

However, the *GL3* and *EGL3* genes were found to be positively regulated by the CPC and TRY proteins, which act in the H cell. Functional *CPC/TRY* genes are necessary for *GL3/EGL3* expression in the H position, and overexpression of the *CPC/TRY* genes cause ectopic *GL3/EGL3* promoter activity and RNA accumulation in the N position. This may be due, in part, to the ability of CPC (and possibly TRY) to inhibit *WER* gene expression in the H position (Lee and Schiefelbein, 2002), which would reduce the abundance of the WER complex and thereby indirectly increase GL3 and EGL3 transcription.

Together, these results lead us to make the following proposal (Fig. 8). In the N cell, the abundant WER protein accumulation (presumably owing to the action of positional cues) leads to the formation of sufficient WER/GL3/EGL3/TTG complex to induce expression of the N-

cell fate-promoting factor *GL2* and the lateral inhibitor *CPC* (Bernhardt et al., 2003; Lee and Schiefelbein, 2002). The CPC protein (and possibly TRY) moves to neighboring cells in the H position leading to the formation of an inactive CPC/GL3/EGL3/TTG complex that prevents activation of *GL2*, thus allowing for the specification of the hair cell fate (Bernhardt et al., 2003; Lee and Schiefelbein, 2002; Wada et al., 2002). At the same time, while the accumulation of CPC (and possibly TRY) in H cells leads to a reduction in *WER* (and *CPC*) expression, it also leads to an increase in *GL3* and *EGL3* expression (this study). The GL3 (and likely EGL3) protein then acts in a lateral feedback loop by moving to the neighboring N cells (possibly through plasmodesmata). This process is likely to be efficient (perhaps driven by the constant removal of free bHLH protein out of the equilibrium by binding to WER), as the GL3-YFP fusion protein is found predominantly in N cells rather than being distributed in equal intensity in all cell files. The additional GL3/EGL3 protein would generate more of the WER/GL3/EGL3/TTG complex in the N cells, inducing additional *GL2* and *CPC* expression and also repression of *GL3* and *EGL3* expression. In the end, this would mean that essentially all of the GL3/EGL3 protein used in the N-cell complex formation would come from the H cells.

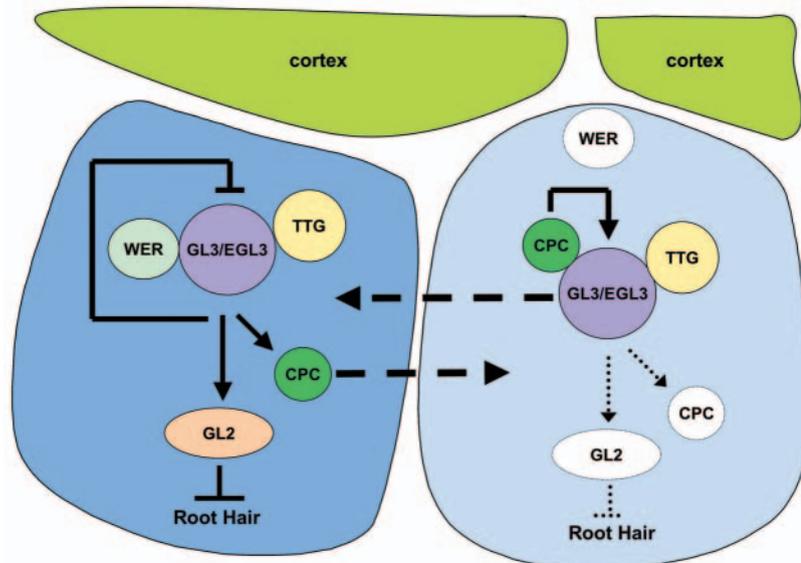


Fig. 8. Proposed model for the involvement of GL3/EGL3 in a novel intercellular regulatory circuit. A WER/GL3/EGL3/TTG complex forms in cells in the N position and promotes expression of *GL2* and *CPC*. Accumulation of GL2 in the N-position leads to the specification of the non-hair cell fate, while CPC/(TRY) moves laterally to the neighboring cell in the H position to form the inactive complex CPC/GL3/EGL3/TTG, which prevents expression of *GL2* in the future hair cell. The presence of CPC/(TRY) in the H position also leads to activation of *GL3* and *EGL3* expression. In a lateral feedback loop, GL3/(EGL3) protein moves to the N cell to participate in the WER/GL3/EGL3/TTG complex, which activates *GL2* and *CPC* and inhibits expression of *GL3* and *EGL3*. See text for further discussion. Unbroken lines indicate gene transcription regulation; broken lines indicate protein movement; dotted lines indicate little/no transcription regulation. Proteins shown in white are at a low concentration.

An important aspect of the proposal above is that CPC (and probably TRY) effectively acts as a positive regulator of *GL3/EGL3* transcription in the H position. Prior work has shown that *CPC* gene expression requires *GL3/EGL3* action in the N position (Bernhardt et al., 2003). Taken together, this implies that expression of *GL3/EGL3* and *CPC* is regulated by a reciprocal positive feedback circuit across adjacent cells. This mechanism of mutual positive feedback is likely to reinforce their gene expression patterns and help enhance the distinction between the N and H cells during epidermal development.

The explanation above suggests that bi-directional signaling, from the N to the H cell (via CPC and possibly also TRY/ETC1) and from the H to the N cells (via GL3 and possibly EGL3), is required for appropriate accumulation of *GL3/EGL3* in the N cell position during root epidermis development. The 'back and forth' signaling between cells proposed here is conceptually similar to the kinds of bi-directional signaling identified in other cell specification models, including the forespore/mother cell fate decision in *Bacillus*, embryonic midgut and larval wing patterning in *Drosophila*, and vulval cell specification in *C. elegans* (Bondos and Tan, 2001; Losick and Dworkin, 1999; Yoo et al., 2004). However, the signaling mechanism used in the *Arabidopsis* root epidermis differs from these others because it involves the intercellular movement of transcription factors, rather than receptor-mediated signaling, thereby directly influencing gene expression and cell fates.

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