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Embryonic control of epidermal cell patterning in the root and hypocotyl of *Arabidopsis*

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SUMMARY

A position-dependent pattern of epidermal cell types is produced during the development of the *Arabidopsis* seedling root and hypocotyl. To understand the origin and regulation of this patterning mechanism, we have examined the embryonic expression of the *GLABRA2* (*GL2*) gene, which encodes a cell-type-specific transcription factor. Using in situ RNA hybridization and a sensitive *GL2::GFP* reporter, we discovered that a position-dependent pattern of *GL2* expression is established within protodermal cells at the heart stage and is maintained throughout the remainder of embryogenesis. In addition, we show that an exceptional *GL2* expression character and epidermal cell pattern arises during development of the root-hypocotyl junction, which represents an anatomical transition zone. Furthermore, we find that two of the genes regulating seedling epidermal patterning, *TRANSPARENT TESTA GLABRA* (*TTG*) and *WEREWOLF* (*WER*), also control the embryonic *GL2* pattern, whereas the *CAPRICE* (*CPC*) and *GL2* genes are not required to establish this pattern. These results indicate that position-dependent patterning of epidermal cell types begins at an early stage of embryogenesis, before formation of the apical meristems and shortly after the cellular anatomy of the protoderm and outer ground tissue layer is established. Thus, epidermal cell specification in the *Arabidopsis* seedling relies on the embryonic establishment of a patterning mechanism that is perpetuated postembryonically.

Key words: Cell specification, Epidermis, Embryogenesis, *GLABRA2*, *Arabidopsis thaliana*

INTRODUCTION

A fundamental feature of development in multicellular organisms is the specification and patterning of distinct cell types. An important issue in cell specification relates to the developmental origin of the mechanisms that establish cell-type patterns. Of particular interest is the relationship between the origin of cell patterning mechanisms and the development of the anatomy and identity of the resident tissues and organs during embryogenesis (Davidson et al., 1998; Simpson et al., 1999).

The development of a position-dependent pattern of epidermal cell types in the *Arabidopsis* root and hypocotyl provides a simple model system to explore the control of cell patterning in plants. Epidermal cells in contact with two underlying cortical cells (i.e. cells over an anticlinal cortical cell wall; the 'H' cell position) preferentially differentiate as root hair cells in the root and stomata in the hypocotyl, whereas cells in contact with a single cortical cell (i.e. cells over a periclinal cortical cell wall; the 'N' cell position) differentiate into non-hair cells in the root and non-stomatal cells in the hypocotyl (Dolan et al., 1993; Dolan et al., 1994; Galway et al., 1994; Berger et al., 1998b; Hung et al., 1998). This cell type pattern implies that the underlying cortical cells provide, or influence the presentation of, an inductive signal that determines epidermal cell fate.

Several genes have been identified that influence the specification of the epidermal cell types in the root and hypocotyl of *Arabidopsis*. The *TRANSPARENT TESTA GLABRA* (*TTG*), *WEREWOLF* (*WER*) and *GLABRA2* (*GL2*) genes are each required for proper specification of the N cell (non-hair or non-stomatal cell) fate (Galway et al., 1994; Masucci et al., 1996; Hung et al., 1998; Lee and Schiefelbein, 1999). The WD40 protein encoded by *TTG* (Walker et al., 1999) and the myb transcription factor encoded by *WER* (Lee and Schiefelbein, 1999) are positive regulators of the *GL2* gene (Hung et al., 1998; Lee and Schiefelbein, 1999). The *GL2* gene encodes a homeodomain-leucine zipper (HD-Zip) protein (Rerie et al., 1994; DiCristina et al., 1996) and is preferentially expressed in the N-cell position in the developing epidermis of both the root and hypocotyl (Masucci et al., 1996; Hung et al., 1998). In contrast to these three genes, the *CAPRICE* (*CPC*) gene is required to specify the hair cell fate in the root epidermis and encodes a small myb protein that negatively regulates *GL2* (Wada et al., 1997). Thus, epidermal cell fate appears to be largely determined by the regulated expression of the downstream N-cell-specific transcription factor *GL2*.

The similar pattern of epidermal cell types in the root and hypocotyl implies that the patterning mechanism originates during embryogenesis, because the epidermal tissue of the root and hypocotyl is derived from the same set of embryonic protodermal cells (Scheres et al., 1994). To define the

embryonic origin of the cell patterning mechanism, we have assessed *GL2* gene expression throughout embryogenesis. In a prior study, N-cell-specific expression of an enhancer-trap marker gene was found to initiate in the torpedo stage embryo (Berger et al., 1998a). However, the identity of the presumed gene responsible for this expression pattern is unknown, the possible role of this gene in the epidermal cell fate pathway is unclear, and the embryonic N-cell expression is limited to the root (Berger et al., 1998a). Thus, the reporter gene expression from this enhancer trap line may not accurately reflect the activity of the fundamental patterning mechanism that is shared by the root and hypocotyl.

In the present study, we have employed in situ RNA hybridization and reporter fusions to show that *GL2* expression becomes established in a cell-position-dependent pattern during the heart stage of embryogenesis. Further, we find a progressive reduction in *GL2* expression during embryonic development of an anatomical transition zone between the root and hypocotyl, which is associated with a unique pattern of epidermal cell differentiation in the seedling. We also show that the *TTG* and *WER* genes, but not *CPC* or *GL2*, are required for proper establishment of the *GL2* expression pattern during embryogenesis.

MATERIALS AND METHODS

Arabidopsis strains and growth conditions

The *ttg-1*, *wer-1*, *cpc* and *gl2-1* mutant alleles used in this study have been described previously (Koornneef, 1981; Lee and Schiefelbein, 1999; Wada et al., 1997; Koornneef et al., 1982). Plants harboring the *GL2::GUS* reporter construct have been described previously (Masucci et al., 1996). The *GL2::GFP* and *GL2::GUS* reporter constructs were introduced into the various mutant backgrounds by genetic crosses. The growth and analysis of *Arabidopsis* seedlings in vertically oriented agarose-solidified plates has been described (Schiefelbein and Somerville, 1990).

Gene constructs and plant transformation

The *GL2::GFP* translational reporter construct was generated by fusing a 2032 bp 5′ promoter fragment from the *GL2* gene (including the first four codons of *GL2*) to the mGFP5 (ER-localized GFP variant; Haseloff et al., 1997) coding sequence. Details of the transgene construction are available upon request.

Plant transformation was achieved by electroporating constructs (in the binary vector pBIN19) into the *Agrobacterium* strain GV3101 followed by introduction into *Arabidopsis* using the floral dip method (Clough and Bent, 1998). T_1 seeds were collected in separate pools and transgenic plants selected by plating on medium containing kanamycin (50 μg/ml). More than ten lines were generated and all had similar phenotypes, and three independent lines were chosen for detailed characterization.

Microscopy

The number of cells in the ground and epidermal tissue layers was determined from transverse sections from 3-day-old seedlings. Hand sections were obtained from at least 15 independent agarose-embedded seedlings and stained with Fluorescent Brightener 28 (Sigma Chemical) as described previously (Galway et al., 1994). Sections were designated as either root (if no ectopic root hair cells were observed), root-hypocotyl junction (if ectopic root hair cells were present), or hypocotyl (if no root hairs were visible) regions.

Histochemical analysis of whole seedlings containing the *GL2::GUS* reporter was performed essentially as described (Masucci et al., 1996). The GFP expression in the *GL2::GFP* embryos and seedlings was examined with a Zeiss LSM510 confocal microscope, with a 488 nm excitation mirror and a 505-530 nm and 530-560 nm emission filter to record images. Seedling roots and some mature embryos were also stained with propidium iodide (10 μg/ml) to visualize cell boundaries.

In situ RNA hybridization

The in situ localization of *GL2* RNA in embryo sections was performed as described by Long et al. (Long et al., 1996). A detailed protocol is available electronically (http://www.wisc.edu/genetics/CATG/barton/protocols.html). Sense and antisense gene-specific *GL2* probes (Lee and Schiefelbein, 1999) were synthesized by in vitro transcription reactions with digoxigenin-labeled UTP as previously described (Masucci et al., 1996).

RESULTS

Position-dependent protodermal expression of *GL2* is established early in embryogenesis

As a first step toward defining the developmental origin of the position-dependent *GL2* expression pattern in the seedling epidermis, we analyzed *GL2* transcript accumulation during embryogenesis using in situ RNA hybridization. The stages of *Arabidopsis* embryogenesis that we refer to here have been extensively described (Jürgens and Mayer, 1994). Furthermore, our analysis was aided by the previously determined embryonic fate map for the *Arabidopsis* root and hypocotyl (Scheres et al., 1994), which depicts the likely developmental fate of cells and cell groups at various stages of embryogenesis.

Using in situ RNA hybridization, we were unable to consistently detect *GL2*-specific transcripts from embryo sections taken before the heart stage (Fig. 1A). Occasionally, we observed a *GL2* hybridization signal in patches of protodermal cells in embryos at the triangular/early-heart stage (unpublished data). At the heart stage, we consistently observed a hybridization signal within protodermal cells in the basal portion of the embryo (Fig. 1B). These *GL2*-expressing cells are located in the lower lower tier (llt) region of the heart stage embryo, which is the region destined to give rise to the root and hypocotyl in the postembryonic plant (Scheres et al., 1994). The same basic pattern of *GL2* RNA accumulation was detected in embryo sections taken at all later stages of embrogenesis, including torpedo stage embryos (Fig. 1C) and mature embryos (Fig. 1D). These results show that *GL2* expression is initiated by the heart stage of embryogenesis within protodermal cells that generate the future root and hypocotyl epidermis.

To assess the embryonic pattern of *GL2* gene expression in greater detail, we wished to employ a sensitive *GL2* reporter gene fusion. An available GUS reporter fusion (*GL2::GUS*; Masucci et al., 1996) was deemed unsuitable for this purpose because of diffusion problems associated with the GUS system in *Arabidopsis* embryos (Y. L. and J. S., unpublished observations). Therefore, we generated a green fluorescent protein (GFP) reporter construct by translationally fusing a 2 kb fragment from the 5′ end of the *GL2* gene to the coding region of *mGFP5*. When employed in complementation constructs, this 5′ *GL2* fragment is sufficient to rescue the *gl2* root defect (unpublished data). Transgenic plants bearing this *GL2::GFP* construct exhibited reporter gene expression within the seedling root and hypocotyl in the same cell-specific

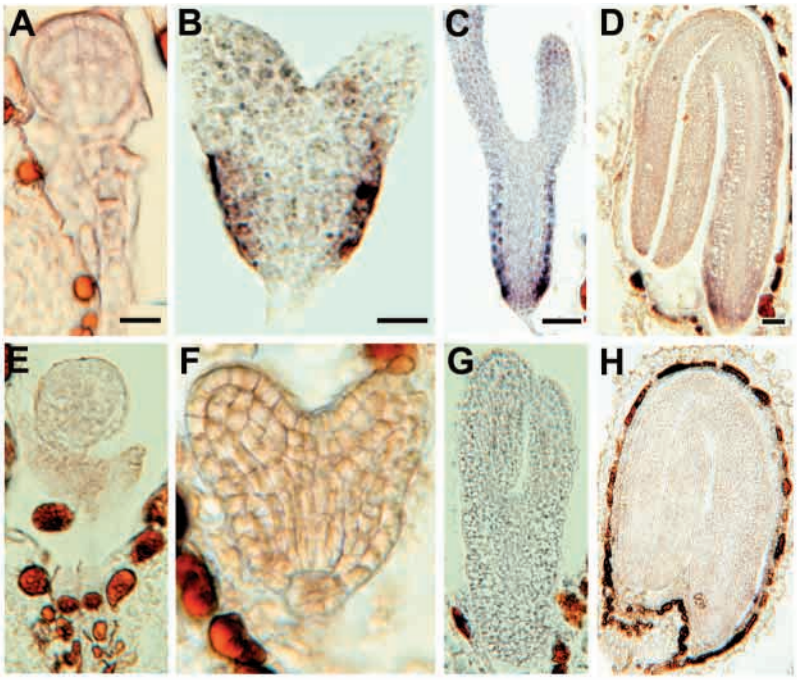

Fig. 1. Accumulation of *GL2* transcripts during embryo development. Median longitudinal sections of embryos were hybridized to *GL2* antisense (A-D) or sense (E-H) RNA probes labeled with digoxigenin-UTP. The low *GL2* hybridization signal in D does not necessarily reflect a reduction in RNA abundance, because this in situ technique is less sensitive with sections from mature embryos (Y. L. and J. S., unpublished observations). (A,E) Globular stage embryos. Bar, 5 μm. (B,F) Heart stage embryos. Bar, 10 μm. (C,G) Torpedo stage embryos. Bar, 20 μm. (D,H) Mature embryos. Bar, 25 μm.

manner as previously identified for *GL2* RNA accumulation and GUS expression in *GL2::GUS* plants (Fig. 2; Masucci et al., 1996). In particular, GFP was preferentially detected in epidermal cells located outside periclinal cortical cell walls (the N position; Fig. 2D). This indicates that GFP accumulation in the *GL2::GFP* plants accurately reflects the transcription pattern of the *GL2* gene.

Embryos produced by homozygous *GL2::GFP* plants were collected at all developmental stages and examined for GFP accumulation. We also examined embryos from non-transformed plants at each stage and employed them as negative controls for these experiments. No GFP signal was detected in *GL2::GFP* embryos examined at the globular or early triangular stages (Fig. 3A; data not shown). The earliest group of embryos in which green fluorescence could be detected were those at the late-triangular or early-heart stage. Most embryos in this group lacked any detectable *GL2::GFP* expression (like embryos from non-transformed plants), but approximately one-third of them (12 embryos out of 31 examined) exhibited a low level of GFP either within a few scattered cells (Fig. 3B) or within a larger collection of cells (Fig. 3C). Although generally patchy in their distribution, the GFP-expressing cells sometimes appeared to be arranged in columns (Fig. 3C). Further, the vast majority of the GFP-expressing cells were located in the basal portion of the embryo (the lower tier region; Scheres et al., 1994), although fluorescing cells were occasionally found in the apical portion (Fig. 3C). In all GFP-expressing embryos, the GFP was restricted to cells in the protoderm (Fig. 3D).

In *GL2::GFP* embryos at the heart stage, a large number of GFP-expressing cells were present in most of the embryos examined (21 of 24). The GFP was exclusively protodermal and principally located in the basal portion of the embryo (Fig. 3E,F) that is destined to give rise to the epidermis of the seedling root and hypocotyl (the lower lower tier region; Scheres et al., 1994). GFP expression is evident within cells at the base of the protoderm layer (destined to form

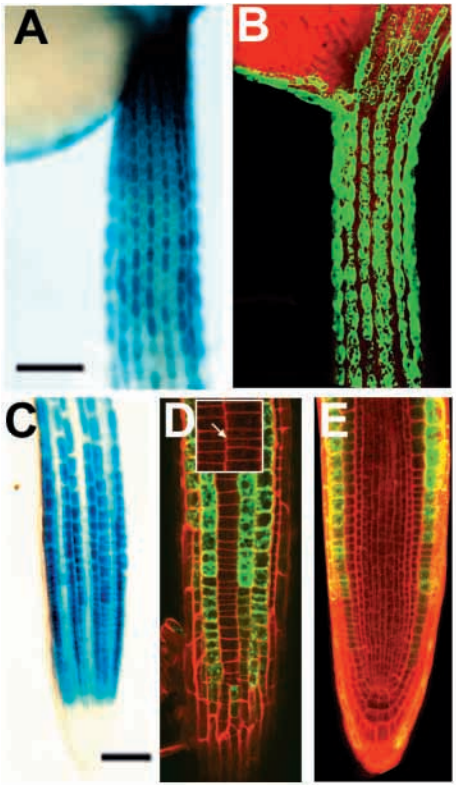

Fig. 2. *GL2* gene expression in the root and hypocotyl of 3-day-old plants bearing the *GL2::GUS* or *GL2::GFP* reporter constructs. Postembryonic expression of *GL2::GUS* (blue staining) and *GL2::GFP* (green fluorescence) occurs preferentially in epidermal cells located outside periclinal cortical cell walls. Propidium iodide staining (red) was performed on plants in D and E to visualize cell boundaries. (A) Hypocotyl bearing the *GL2::GUS* transgene, stained for GUS activity (blue). Bar, 100 μm for A and B. (B) Hypocotyl bearing the *GL2::GFP* transgene, viewed by fluorescence microscopy (green). (C) Surface view of root tip from seedling with the *GL2::GUS* transgene, stained for GUS activity. Bar, 50 μm for C and D. (D) Surface view of root tip from seedling with *GL2::GFP*, viewed by fluorescence microscopy. The inset shows the arrangement of the underlying cortical cells, with an arrow marking the anticlinal cortical cell wall. (E) Median longitudinal optical section of root tip with the *GL2::GFP* transgene, viewed by fluorescence microscopy. Bar, 50 μm.

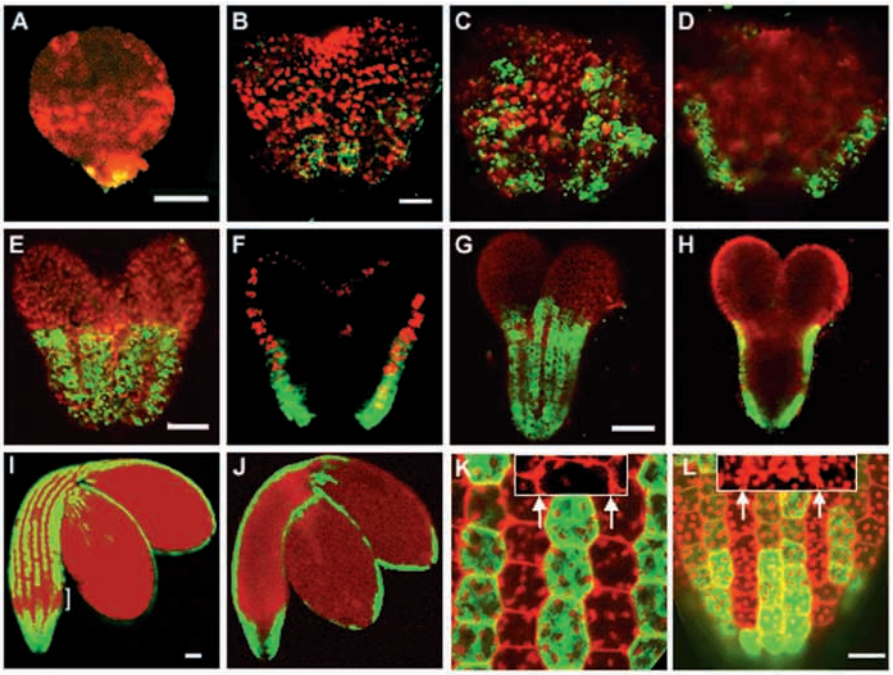

Fig. 3. The pattern of GFP accumulation (green) in embryos from plants bearing the *GL2::GFP* transgene viewed by fluorescence microscopy. The red background in the images is due to autofluorescence. Owing to the progressive increase in the level of *GL2::GFP* expression during embryogenesis, the GFP signal was amplified in embryos at earlier stages (e.g. B and C) relative to later stages (e.g. I and J), so accurate comparisons of signal intensity between the stages cannot be made with these images. Propidium iodide staining (red) was performed with the mature embryos shown in K and L to visualize cell boundaries. (A) Median longitudinal optical section from a globular stage embryo. Bar, 50 μm. (B,C) Surface view of two different embryos from the late-triangular/early-heart stage. Bar, 50 μm for B-D. (D) Median longitudinal optical section from a late-triangular/early-heart stage embryo. (E) Surface view of a heart stage embryo. Bar, 50 μm for E and F. (F) Median longitudinal optical section from a heart stage embryo. (G) Surface view of a torpedo stage embryo. Bar, 50 μm for G and H. (H) Median longitudinal optical section from a torpedo stage embryo. (I) Surface view of a mature embryo. Bracket indicates the location of the root-hypocotyl junction region. Bar, 40 μm for I and J. (J) Median longitudinal optical section from a mature embryo. (K) Magnified view of the hypocotyl region of a mature embryo. (L) Magnified view of the root region of a mature embryo. The insets in K and L show optical sections of the underlying cortical cell layer, revealing that epidermal cells outside the anticlinal cortical cell walls (marked by arrows) lack GFP expression. Bar, 15 μm for K and L.

epidermal/lateral root cap initials) but is notably absent from derivatives of the hypophyseal cell (Fig. 3F), which give rise to the columella root cap and quiescent center within the lower portion of the root apical meristem (Scheres et al., 1994). In each of the 21 embryos exhibiting GFP, the GFP-expressing cells were arranged in particular columns that were separated by columns of non-GFP-expressing cells (Fig. 3E). By examining and comparing optical sections of the protoderm and underlying ground meristem layer, we determined that the non-GFP-expressing cells were located over an anticlinal wall between ground meristem cells (the H cell position; data not shown).

Embryos at later stages displayed a pattern of *GL2::GFP* expression similar to the heart stage embryos. At the torpedo stage, embryos exhibit a higher level of GFP expression and a clearer distinction between the GFP-expressing and non-GFP-expressing files of cells (Fig. 3G). The GFP retains its protoderm-specific expression and remains localized in the future root and hypocotyl region (Fig. 3H). In the mature embryo, GFP expression is present in distinct files of cells in the root-hypocotyl axis that mirrors its postembryonic pattern (Fig. 3I). GFP-expressing cells are restricted to the N position and non-GFP-expressing cells are in the H position of both the embryonic hypocotyl (Fig. 3K) and embryonic root (Fig. 3L). In addition to epidermal expression in the root and hypocotyl, GFP is present in epidermal cells on the cotyledon margins (Fig. 3I,J), which mirrors the GL2 reporter expression observed in the corresponding cells of the seedling cotyledon (Fig. 2A,B; Masucci et al., 1996).

Taken together, our analysis of *GL2* transcript accumulation and *GL2::GFP* reporter expression indicates that epidermal cell patterning is initiated early in embryogenesis and establishes position-dependent *GL2* expression by the heart stage.

GL2 expression and epidermal cell specification is altered during development of the root-hypocotyl junction

During the course of our analysis of *GL2::GFP* embryos, we discovered that *GL2::GFP* is not uniformly expressed along the length of the epidermal cell files at all embryonic stages. Specifically, there is significantly less GFP accumulation within an apical-basal segment containing four to seven cells per file near the junction of the root and hypocotyl segments in the mature embryo (Fig. 3I). This difference in *GL2::GFP* expression is first detected in embryos at the torpedo stage (Fig. 4A) and it becomes more apparent as the embryo matures (Fig. 4B,C).

Because of the unusual *GL2::GFP* expression in this root-hypocotyl junction region during embryogenesis, we analyzed postembryonic *GL2* expression and epidermis development in this region. We discovered that this region of sharply diminished *GL2* expression persists in the young seedling, and it precisely coincides with an apical-basal segment of approximately four to seven cells that exhibits abnormal epidermal cell patterning (Fig. 4D,E). This unusual region of the *Arabidopsis* seedling had been previously noted as consisting of shortened epidermal cells that exclusively differentiate as root hair cells (Dolan et al., 1993; Scheres et al., 1994; Cheng et al., 1995). Considering that GL2 acts as a negative regulator of root-hair cell differentiation (Masucci et al., 1996), our finding of diminished *GL2* expression within this region is consistent with the unusual epidermal cell phenotype. We also observed that the root hairs formed in this

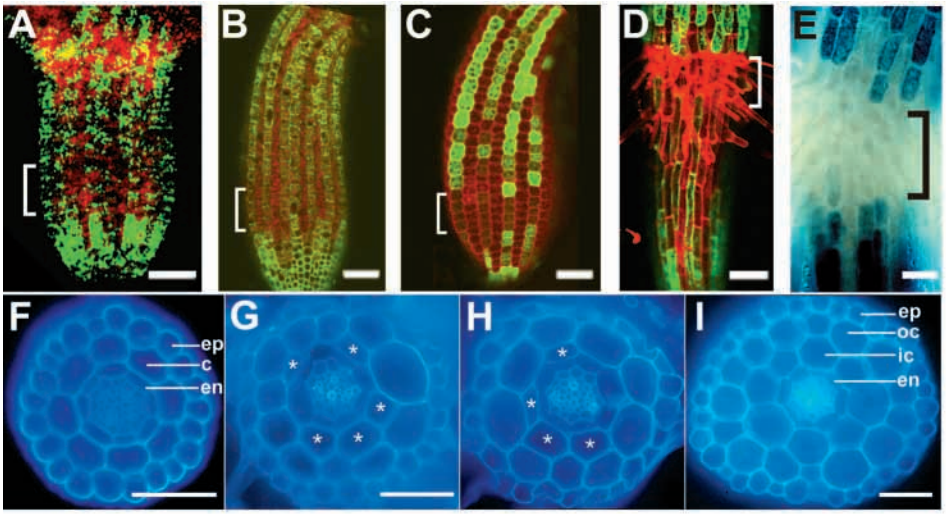

Fig. 4. *GL2* gene expression and cell organization in the root-hypocotyl junction region of *Arabidopsis* embryos and seedlings. Brackets in A-E mark the region of the root-hypocotyl junction. Propidium iodide staining (red) was performed with the mature embryo in C and the seedling in D to visualize cell boundaries. (A-D) Root-hypocotyl junction region from torpedo stage embryo (A), curled cotyledon stage embryo (B), mature embryo (C) and 2-day-old seedling (D) bearing the *GL2::GFP* transgene, viewed by fluorescence microscopy. Note the numerous root hairs (red) visible in D. Bars in A and D, 50 μm; in B, 45 μm; in C, 20 μm. (E) Root-hypocotyl junction region from 2-day-old seedling bearing the *GL2::GUS* transgene and stained for GUS activity. Numerous root hairs are visible in the junction region. Bar, 50 μm. (F-I) Transverse sections from the root (F), root-hypocotyl junction region (G,H) and hypocotyl (I) from 3-day-old seedlings. Sections were stained with a fluorescent cell wall dye and viewed by fluorescence microscopy. The epidermis (ep), cortex (c), outer cortex (oc), inner cortex (ic), and endodermis (en) layers of the root and hypocotyl sections are indicated. Note the unusual cell anatomy in the ground tissues in G and H, where cells occupying the incomplete middle-ground-tissue layer are indicated by asterisks. Bars in F,G,I, 50 μm; bar in G also applies to H.

region tend to emerge closer to the center of the cell, rather than the basal end as is typical for hairs in the root proper (Schiefelbein and Somerville, 1990). Thus, the epidermis of the root-hypocotyl junction region is unique in lacking position-dependent *GL2* expression and cell-type patterning.

Although the root and hypocotyl exhibit a similar position-dependent pattern of epidermal cell types, they differ in their cellular anatomy. We considered the possibility that the unique *GL2* expression and epidermal patterning characteristics of the root-hypocotyl junction region may be related to an anatomical transition within this region. To test this, we analyzed the cell organization of the ground and epidermal tissues within the root, hypocotyl, and root-hypocotyl junction regions from transverse sections of 3-day-old seedlings. As expected from prior studies (Dolan et al., 1993; Scheres et al., 1994), the root sections possessed two concentric layers of ground tissue (one layer of endodermis and one layer of cortex), whereas the hypocotyl sections contained three layers of ground tissue (one layer of endodermis and two layers of cortex; Fig. 4F,I). Sections from the root-hypocotyl junction region were identified by the presence of ectopic root-hair cells (root-hair bearing epidermal cells located outside a periclinal cortical cell wall). Most of these sections (32/38) exhibited an arrangement of ground tissue layers not found in the hypocotyl or root (Fig. 4G,H). Because the identity of individual ground tissue cells in this region is unclear, we employed the terms inner, middle, and outer layer of ground tissue to describe the set of cells that contact the vascular tissue (inner), contact neither vascular or epidermal tissue (middle), or contact the epidermal tissue (outer). Using these criteria, we interpret the root-hypocotyl junction sections as typically containing two complete ground tissue layers (inner and outer layers) and one incomplete layer (middle layer) (Table 1). Considering that the middle layer of ground tissue represents the defining difference between the root and hypocotyl, the presence of an incomplete middle layer in the junction region indicates that a transition in ground tissue anatomy does indeed occur in this region.

We also analyzed the number of cells within the inner and outer ground tissue layers and the epidermal layer in these transverse sections. We found that the inner layer of the root-hypocotyl junction region invariably contains the same number of cells (8) as the inner layer of the root and the inner layer of the hypocotyl (Table 1). However, the outer layer of ground tissue in the root-hypocotyl junction sections contains a variable and intermediate number of cells (range of 8-15 cells), relative to the root and hypocotyl (Fig. 4G,H; Table 1). Because the number of cells in this outer ground tissue layer determines the number of epidermal cells in the H position, the differences in cell number in this layer in the junction region means that there are corresponding differences in the number of H cell files in the epidermis of this root-hypocotyl junction. In addition, we determined that the total number of epidermal cell files in the root-hypocotyl junction region is intermediate to that in the root and hypocotyl (range of 21-35 cells; Table 1). Together, this anatomical analysis shows that the ground and epidermal tissue of the root-hypocotyl junction region is

Table 1. Number of cells in the ground and epidermal tissue layers of *Arabidopsis* seedlings

Apical-basal region	Ground tissue			Epidermis
	Inner layer*	Middle layer‡	Outer layer§	
Hypocotyl	8.0±0.0	8.0±0.0	14.6±0.9	32.9±2.4
Hypocotyl-root junction¶	8.0±0.0	5.0±2.2	11.5±2.7	27.0±4.3
Root	8.0±0.0	0.0±0.0	8.0±0.0	19.6±1.3

Values represent mean±s.d.

*Defined as the ring of cells surrounding, and in contact with, the vascular tissue. This is the endodermis in the root and hypocotyl.

‡Defined as all ground tissue cells not represented in either the inner or outer layer. This is the inner cortex in the hypocotyl.

§Defined as the ring of cells surrounded by, and in contact with, the epidermis. This is the outer cortex of the hypocotyl and the cortex of the root.

¶Defined as the apical-basal seedling region containing ectopic root-hair cells.

distinct from the root or hypocotyl and effectively represents an anatomical transition zone between these apical-basal segments.

Identification of genes regulating the embryonic *GL2* expression pattern

Following the discovery of an embryonic origin for epidermal cell patterning, we wished to define genes that control this process. The *TTG*, *WER* and *CPC* genes are known regulators of postembryonic patterning of the epidermal cell types (for review, see Schiefelbein, 2000). To determine whether these might also regulate the embryonic pattern, we examined the effect of mutations in each of these, as well as mutations in *gl*2, on the position-dependent *GL2::GFP* expression during embryogenesis.

ttg mutations cause a significant reduction in the level, but no change in the position-dependent pattern, of *GL2* expression in the seedling root and hypocotyl (Fig. 5E,F, compare with Fig. 2D; Hung et al., 1998). In an analogous manner, we found that *ttg-1* inhibits the level, but not the basic pattern, of *GL2::GFP* expression during embryogenesis. In *ttg GL2::GFP* embryos, GFP expression is generally too weak to be detected until the torpedo stage (Fig. 5A,B). However, the expression pattern is the same as the wild type, with GFP-expressing cells confined to epidermal cells outside periclinal cortical cell walls (the N position) in the future root and hypocotyl regions (Fig. 5B,C; data not shown). This normal pattern persists and intensifies during embryogenesis, but the GFP expression level is significantly lower than wild type throughout all stages, including the mature embryo (Fig. 5C,D).

The *wer* mutations dramatically alter the postembryonic expression of *GL2*, eliminating expression in most of the cells and abolishing the position-dependent pattern of the few *GL2*-expressing cells in the root and hypocotyl (Fig. 5K,L, compare with Fig. 2D; Lee and Schiefelbein, 1999). We discovered that the *wer-1* mutation has essentially the same effect on the embryonic expression of *GL2*. GFP expression was generally undetectable in the *wer-1 GL2::GFP* embryos, although some embryos (particularly at the mature stage) possessed a few scattered protodermal cells with a low level of GFP (Fig. 5G-J). These GFP-expressing cells were not restricted to the N position, but were located in both the N and H positions (data not shown).

In contrast to *TTG* and *WER*, the *CPC* gene is proposed to act as a negative regulator of *GL2* expression (Wada et al., 1997). Accordingly, the *cpc* mutation reduces the frequency of root-hair cells, generates a large number of ectopic non-hair cells, and causes *GL2* expression in the H-cell position during postembryonic development of the root epidermis (Wada et al., 1997; Lee and Schiefelbein, 1999; Fig. 5Q). Unexpectedly, we found that the *cpc* mutation does not alter *GL2::GFP* expression during embryogenesis. Like the wild type, GFP expression in *cpc GL2::GFP* embryos is first detected at the late-triangular stage, is established in a position-dependent pattern by the heart stage, and is maintained and enhanced in the same pattern during the rest of embryogenesis (Fig. 5M-P). Further, we did not detect any GFP accumulation in *cpc GL2::GFP* embryos at the globular and pre-globular stages, which indicates that CPC does not act to restrict *GL2* expression during early embryogenesis (data not shown).

We also examined the effect of the *gl2-1* mutation on *GL2::GFP* expression to determine whether the GL2 homeodomain transcription factor may regulate the expression of its own gene during embryogenesis. In prior studies, *gl2* mutations did not significantly alter the postembryonic expression of the *GL2* (Hung et al., 1998), and we have

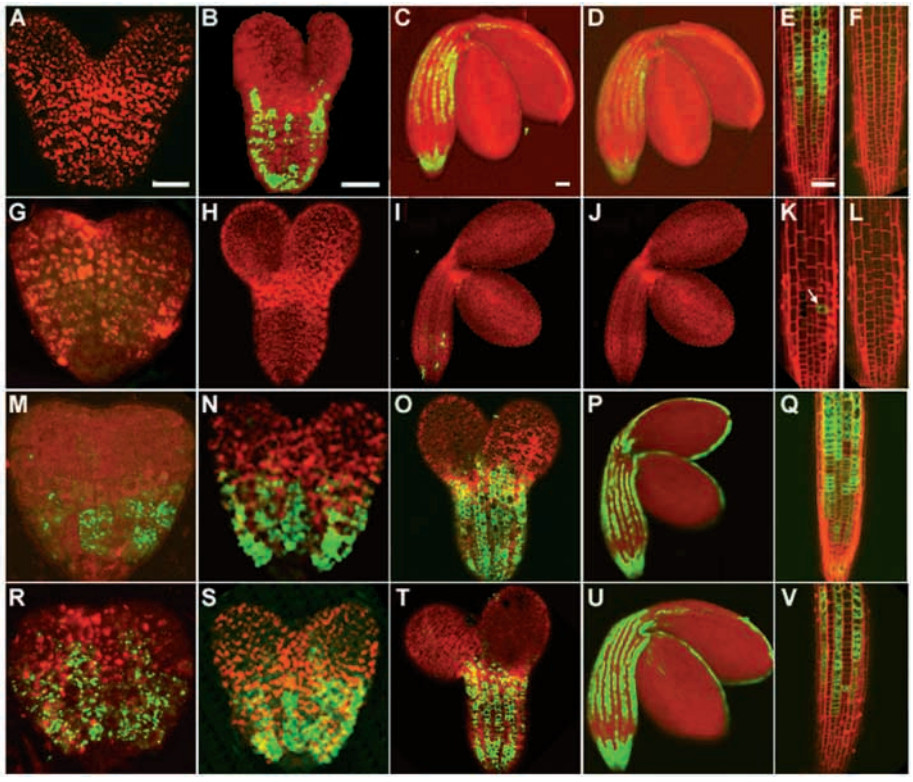

Fig. 5. The pattern of GFP accumulation in mutant embryos and seedlings bearing the *GL2::GFP* transgene viewed by fluorescence microscopy. Propidium iodide staining (red) was performed on the seedling roots in E, F, K, L, Q and V to visualize cell boundaries. (A-F) The *ttg-1* mutant. (G-L) The *wer-1* mutant. Surface view of heart-stage embryo (A,G), torpedo stage embryo (B,H), mature embryo with the fluorescence signal amplified (C,I), mature embryo without amplification of the signal (D,J) (compare to Fig. 3I), seedling root tip with fluorescence signal amplified (E,K), and seedling root tip without amplification of the signal (F,L) (compare with Fig. 2D). A GFP-expressing cell is indicated by an arrow in K. (M-Q) The *cpc* mutant. (R-V) The *gl2-1* mutant. Surface view of late-triangular/early-heart stage embryo (M,R), heart stage embryo (N,S), torpedo stage embryo (O,T), mature embryo (P,U) and seedling root tip (Q,V). Bar in A (G,M,N,R,S) 15 μm; in B (H,O,T) 50 μm; in C (D,I,J,P,U) 40 μm; in E (F,K,L,Q,V) 50 μm.

confirmed this here by showing that position-dependent expression of *GL2::GFP* in the seedling root epidermis is not altered in the *gl2-1* mutant background (Fig. 5V). Similarly, we did not detect any difference in embryonic *GL2::GFP* expression in *gl2-1*, as compared with the wild type (Fig. 5R-U). Throughout embryogenesis, a normal level and position-dependent pattern of *GL2* expression was observed in the *gl2-1 GL2::GFP* embryos.

Together, these findings show that the embryonic establishment of the *GL2* expression pattern is dependent on the TTG and WER products but does not require the CPC or GL2 proteins.

DISCUSSION

Origin and establishment of epidermal cell pattern during embryogenesis

The basic organization of the plant body plan is accomplished during embryogenesis, including establishment of apical-basal and radial patterns of tissues, organs and regions (Laux and Jürgens, 1997). In this paper, we examined the possible embryonic origin and regulation of the circumferential pattern of cell types that arises in the *Arabidopsis* seedling epidermis. We employed a cell-type-specific transcription factor gene, *GL2*, that is expressed postembryonically in a position-dependent fashion during epidermal differentiation in the seedling root and hypocotyl. We discovered that *GL2* gene expression initiates within the protoderm during the late-triangular and early-heart stage of embryogenesis. Although this initial expression is weak and sporadic, it rapidly assumes a position-dependent pattern that is clearly visible by the mid-heart stage and matches the postembryonic pattern. This demonstrates that the mechanism responsible for establishing a specific pattern of cell types in the postembryonic plant is already in place by the early-heart stage of embryogenesis.

Many of the basic patterning elements and anatomical features of the *Arabidopsis* embryo have already been established by the early-heart stage of embryogenesis. In particular, the apical-basal and radial axes are already defined by this stage (Scheres et al., 1994; Laux and Jürgens, 1997), meaning that groups of cells destined to give rise to hypocotyl, root, or cotyledon segments have been defined, as have the cell layers generating the epidermis, ground tissue and vascular tissue. Notably, it is at the early-heart stage when the final number of cell files in the embryonic epidermis and outer ground tissue layer is established in the future root/hypocotyl region. A ring of eight ground meristem cells and a ring of 16 protoderm cells are initially generated in the lower tier region (which gives rise to root and hypocotyl) by the globular stage (Scheres et al., 1994). However, it is not until the early-heart stage that an additional ground meristem layer (destined to be the outer cortical cell layer) is formed and the final number of cells in the protoderm and ground meristem is achieved within the future hypocotyl region (Scheres et al., 1994). Thus, the final cellular anatomy of the future epidermis and outer ground tissue is established at approximately the same embryonic stage that position-dependent *GL2* expression is initiated. Considering the established connection between epidermal cell fate and cell position relative to the underlying cortical cells (Berger et al., 1998a), it may be that initiation of the epidermal cell patterning program is in some way triggered by completion of the divisions generating the final anatomy of the protoderm and outer ground tissue layer.

Our finding of the initiation of the epidermal cell patterning mechanism during the early-heart stage of embryogenesis has additional implications. This timing is much earlier than the N-cell expression pattern of the J2301 enhancer-trap line, which first arises at the mid-torpedo stage (Berger et al., 1998a). It is therefore likely that the reporter gene transcription in this line is responding to later-acting factors associated with N-cell specification. It is also interesting to note that the early-heart stage initiation of *GL2* expression precedes the establishment of the root and shoot apical meristems in the *Arabidopsis* embryo (Dolan et al., 1993; Scheres et al., 1994; Laux and Jürgens, 1997). Thus, despite the general postembryonic connection between meristem activity and cell specification in plants, the present study demonstrates that patterning of cell types within a tissue need not require a functional meristem.

One of the key features of the embryonic *GL2* expression pattern uncovered by our study is that *GL2* largely initiates its expression in a distinct positionally defined set of cells, rather than within a broader set of cells or tissues and subsequent refinement of the expression domain. This feature is reminiscent of the embryonic cell-specific expression of some transcriptional regulators in animals, including the selector genes associated with bristle patterning in *Drosophila*. In this system, the proneural genes (including members of the achaete-scute complex) act in larger clusters of cells and cause the selector genes (including the homeobox gene *cut*) to become initially expressed in a particular set of cells (Simpson, 1996; Simpson et al., 1999). In an analogous manner, the precise initiation of position-dependent expression we have found for the *GL2* homeobox gene in the early *Arabidopsis* embryo may be dictated by regulators that exhibit broader domains of expression.

Epidermal cell specification at the root-hypocotyl junction

In this study, we identified an apical-basal segment of the *Arabidopsis* embryo with a unique *GL2* expression character. This segment, located near the root-hypocotyl junction, exhibits a progressive reduction in *GL2* expression during embryogenesis that culminates in an undetectable level of *GL2* expression in newly germinated seedlings (Fig. 4D,E). This region coincides with an apical-basal segment of the seedling previously characterized as producing root hairs from every epidermal cell, and termed the collet or upper part of the embryonic root (Dolan et al., 1993; Scheres et al., 1994; Cheng et al., 1995; Schneider et al., 1997). Although this region has been considered to possess root identity because of the presence of root-hair cells and lack of pigmentation, we discovered that it possesses an intermediate cellular anatomy and therefore, anatomically, represents a transition zone between the root and hypocotyl segments. Further, the lack of *GL2* expression and the formation of ectopic root-hair cells in this root-hypocotyl junction region implies that the epidermal patterning mechanism is not employed in this region.

It is notable that the reduction in *GL2* expression in this region is first detected at the torpedo stage of embryogenesis (Fig. 4). This is the stage when a set of periclinal divisions generate two layers of ground tissue in the root and three layers

in the hypocotyl (Scheres et al., 1994; Scheres et al., 1995), probably creating the incomplete middle layer of ground tissue in the future root-hypocotyl junction (Table 1). Further, although the differences in cell file number in the epidermal and outer ground tissue layer of the future root and hypocotyl is established during the early-heart stage, the apical-basal segment of cells comprising the root-hypocotyl junction arise later, in part by divisions during the torpedo stage and later in embryogenesis. Taken together, our findings show that a close correlation exists between the unique cellular anatomy that develops in this root-hypocotyl region and its unusual *GL2* expression and epidermal cell specification features.

At present, it is unclear whether the correlation between the cellular anatomy and cell specification characteristics of this region reflect a cause-effect relationship. It is possible that the unusual epidermal and ground tissue anatomy in this region prevents cell signaling events that are required for epidermal specification. For example, the incomplete middle layer of ground tissue (Table 1) may disrupt the identity and thereby the signaling capacity of the cells in the ground tissue layers, and/or the variation in cell file number in the epidermal (from 21 to 35) and outer ground tissue (from 8 to 15) layers in this short region may inhibit signaling between cells within individual files. Alternatively, it may be that the correlation we observe reflects the influence of a regional regulator that independently controls both cellular organization and epidermal cell specification within this root-hypocotyl junction region.

Genetic regulation of embryonic patterning of the epidermis

In addition to defining the embryonic *GL2* expression pattern, we examined the role of known postembryonic regulators on embryonic *GL2* expression. We discovered that two genes, *TTG* and *WER*, are required for the proper establishment of the embryonic *GL2* expression pattern. Mutations in *ttg* significantly reduce the level of *GL2* expression throughout embryogenesis, whereas *wer* mutations effectively abolish embryonic *GL2* expression entirely. These embryonic effects of *ttg* and *wer* are similar to their postembryonic effects (Hung et al., 1998; Lee and Schiefelbein, 1999), indicating that these genes' products act in a similar manner during embryonic and postembryonic epidermis development. The disruption of embryonic *GL2* expression in the *wer-1* mutant is particularly notable because it suggests that the WER protein is a critical regulator of the establishment of the position-dependent patterning mechanism during embryogenesis.

Mutations in two genes, *gl2* and *cpc*, had no detectable effect on the embryonic *GL2* expression pattern. The *gl2* mutation was previously shown to have no effect on postembryonic *GL2* expression (Hung et al., 1998), which together with the findings reported here, implies that the GL2 homeodomain protein does not regulate the transcription of its own gene. The inability of the *cpc* mutation to alter embryonic *GL2* expression was unexpected, because *CPC* acts genetically as a negative regulator of *GL2* and the *cpc* mutation causes ectopic *GL2* expression in the seedling root epidermis (Wada et al., 1997; Lee and Schiefelbein, 1999; Fig. 5Q). This suggests a difference in the molecular control of embryonic vs. postembryonic epidermal patterning, which may be due to the postembryonic restriction of CPC action or to the presence of a redundant factor acting during embryogenesis.

The reason for activation of the epidermal patterning mechanism during early embryogenesis is not clear. We were not able to detect any cytological difference in the epidermal cells in the N and H positions at any stage of embryogenesis (Y. L. and J. S., unpublished observations), which implies that their final differentiation programs (Dolan et al., 1993; Galway et al., 1994) are not engaged before seed germination. Furthermore, successful epidermal patterning is not essential for embryo development or epidermal tissue identity, because *gl2* mutants do not exhibit any detectable abnormalities in cell organization or cytological features during embryogenesis or early postembryonic epidermal development (Masucci et al., 1996; Y. L. and J. S., unpublished observations). One possibility is that position-dependent patterning during early embryogenesis helps to ensure the correct specification of cells generated later in embryogenesis and during postembryonic development. In a conceptually similar mechanism, the activity of meristem initial cells is influenced by signaling from more mature cells during postembryonic root development (van den Berg et al., 1995).

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REFERENCES

Berger, F., Haseloff, J., Schiefelbein, J. and Dolan, L. (1998a). Positional information in root epidermis is defined during embryogenesis and acts in domains with strict boundaries. *Curr. Biol.* **8**, 421-430.

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.

Cheng, J.-C., Seeley, K. A. and Sung, Z. R. (1995). *RML1* and *RML2*, *Arabidopsis* genes required for cell proliferation at the root tip. *Plant Physiol.* **107**, 365-376.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* **16**, 735-743.

Davidson, E. H., Cameron, R. A. and Ransick, A. (1998). Specification of cell fate in the sea urchin embryo: summary and some proposed mechanisms. *Development* **125**, 3269-3290.

DiCristina, M. D., Sessa, G., Dolan, L., Linstead, P., Baima, S., Ruberti, I. and Morelli, G. (1996). The *Arabidopsis Athb-10* (*GLABRA2*) is an HD-Zip protein required for regulation of root hair development. *Plant J.* **10**, 393-402.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. and Scheres, B. (1993). Cellular organisation of the *Arabidopsis thaliana* root. *Development* **119**, 71-84.

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

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.

Haseloff, J., Siemering, K. R., Prasher, D. C. and Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. *Proc. Natl. Acad. Sci. USA* **94**, 2122-2127.

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.

Jürgens, G. and Mayer, U. (1994). *Arabidopsis*. In *A Colour Atlas of Developing Embryos* (ed. J. Bard), pp. 7-21. London: Wolfe Publishing.

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

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

Laux, T. and Jürgens, G. (1997). Embryogenesis – a new start in life. *Plant Cell* **9**, 989-1000.

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.

Long, J. A., Moan, E. I., Medford, J. and Barton, M. K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the SHOOTMERISTEMLESS gene of Arabidopsis. *Nature* **379**, 66-69.

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.

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

Scheres, B., Wolkenfelt, 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.

Scheres, B., Di Laurenzio, L., Willemsen, V., Hauser, M.-T., Janmaat, K., Weisbeek, P. and Benfey, P. N. (1995). Mutations affecting the radial organization of the *Arabidopsis* root display specific defects throughout the embryonic axis. *Development* **121**, 53-62.

Schiefelbein, J. W. (2000). Constructing a plant cell: the genetic control of root hair development. *Plant Physiol.* **124**, 1525-1531.

Schiefelbein, J. W. and Somerville, C. (1990). Genetic control of root hair development in *Arabidopsis thaliana*. *Plant Cell* **2**, 235-243.

Schneider, K., Wells, B., Dolan, L. and Roberts, K. (1997). Structural and genetic analysis of epidermal cell differentiation in *Arabidopsis* primary roots. *Development* **124**, 1789-1798.

Simpson, P. (1996). A prepattern for sensory organs. *Curr. Biol.* **6**, 948-950.

Simpson, P., Woehl., R. and Usui, K. (1999). The development and evolution of bristle patterns in Diptera. *Development* **126**, 1349-1364.

van den Berg, C., Willemsen, V., Hage, W., Weisbeek, P. and Scheres, B. (1995). Cell fate in the *Arabidopsis* root meristem determined by directional signalling. *Nature* **378**, 62-65.

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.

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**, 1337-1349.