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Epidermal patterning genes are active during embryogenesis in *Arabidopsis*

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SUMMARY

Epidermal cells in the root of *Arabidopsis* seedling differentiate either as hair or non-hair cells, while in the hypocotyl they become either stomatal or elongated cells. *WEREWOLF* (*WER*) and *GLABRA2* (*GL2*) are positive regulators of non-hair and elongated cell development. *CAPRICE* (*CPC*) is a positive regulator of hair cell development in the root. We show that *WER*, *GL2* and *CPC* are expressed and active during the stages of embryogenesis when the pattern of cells in the epidermis of the root-hypocotyl axis forms. *GL2* is first expressed in the future epidermis in the heart stage embryo and its expression is progressively restricted to those cells that will acquire a non-hair identity in the transition between torpedo and mature stage. The expression of *GL2* at the heart stage requires *WER* function. *WER* and *CPC* are transiently expressed throughout the root epidermal layer in the torpedo stage embryo when the cell-specific pattern of *GL2* expression is being established in the epidermis. We also show that WER positively regulates *CPC* transcription and GL2 negatively regulates *WER* transcription in the mature embryo. We propose that the restriction of *GL2* to the future non-hair cells in the root epidermis can be correlated with the activities of *WER* and *CPC* during torpedo stage. In the embryonic hypocotyl we show that *WER* controls *GL2* expression. We also provide evidence indicating that *CPC* may also regulate *GL2* expression in the hypocotyl.

Key words: *Arabidopsis* embryos, Epidermis, *CAPRICE*, *WEREWOLF*, *GLABRA2*

INTRODUCTION

Epidermal cells enclose the cortex and differentiate in a position-dependent manner along the root-hypocotyl axis of *Arabidopsis*. In the root, epidermal cells located over two cortical cell files develop as hair cells while those overlying a single cortical cell file develop as non-hair cells (Dolan et al., 1994; Galway et al., 1994). In the hypocotyl, epidermal cells lying over two files of cortical cells develop as stomatal complexes while those epidermal cells overlying a single cortical cell file develop as elongated cells (Gendreau et al., 1997; Berger et al., 1998a). The root meristem, from which the seedling root is derived, forms at the basal end of the root-hypocotyl axis and the cellular organisation of the meristem is in place by the late heart stage of embryogenesis (Scheres et al., 1994). A combination of genetic determinants and positional signals specifies the identity of epidermal cells in the seedling root (Galway et al., 1994; Lee and Schiefelbein, 1999; Masucci et al., 1996; Wada et al., 1997; Berger et al., 1998b). Enhancer-trap and promoter-reporter gene studies indicate that the root epidermal pattern is established during embryogenesis (Berger et al., 1998b; Lin and Schiefelbein, 2001).

Characterisation of mutants with defects in the specification of epidermal cell types has defined a pathway for epidermal patterning in the root and in the hypocotyl of the seedling. GLABRA2 is a homeodomain protein required for non-hair fate in the root, and elongated epidermal cell fate in the hypocotyl (Masucci et al., 1996; Di Cristina et al., 1996; Berger et al., 1998a; Hung et al., 1998). Positive regulators of *GL2* transcription in seedlings include *WEREWOLF* (*WER*), which encodes a MYB protein (Lee and Schiefelbein, 1999) and *TRANSPARENT TESTA GLABRA* (*TTG*), which encodes a WD40 protein (Galway et al., 1994; Walker et al., 1999). *GL2* and *WER* are expressed in non-hair cells in the root and elongated cells in the hypocotyl of the seedling (Masucci et al., 1996; Di Cristina et al., 1996; Lee and Schiefelbein, 1999) and the accumulation of *GL2* transcript in these cells requires *WER* function (Lee and Schiefelbein, 1999; Lee and Schiefelbein, 2002). CAPRICE (CPC) is a MYB protein and is required for the specification of root hair cell identity (Wada et al., 1997), yet it is expressed in non-hair cells (Lee and Schiefelbein, 2002; Wada et al., 2002). In *cpc* mutants only a few hairs are formed on the root (Wada et al., 1997), but no mutant phenotype has been described for the hypocotyl. *TRIPTYCHON* (*TRY*) encodes a CPC-related MYB protein and acts in a partially redundant manner with *CPC* to specify root hair cell identity (Schellmann et al., 2002).

It has been shown, on the basis of double mutant phenotypes and promoter-reporter gene studies, that a reciprocal transcriptional control between *WER* and *CPC* contributes to the establishment of the position-dependent expression of *GL2* in the seedling root (Lee and Schiefelbein, 2002). WER positively regulates the expression of both *GL2* and *CPC* in non-hair cells, while CPC moves into hair cells and represses the transcription of *WER* and *GL2* in these cells (Lee and

Schiefelbein, 2002; Wada et al., 2002). Lee and Schiefelbein (Lee and Schiefelbein, 1999) proposed that a WER:CPC protein ratio could determine the fate of epidermal cells in the root. A high WER:CPC ratio would result in the specification of a non-hair cell while a low ratio would result in the formation of a hair cell. In the seedling, positional cues may be responsible for the expression of *WER* in non-hair cells and a high WER/CPC ratio, which initiates the cascade of molecular events that leads to position-dependent cell type specification in the root epidermis (Lee and Schiefelbein, 2002). The *WER*, *CPC* and *GL2* genes have been shown to be required for the maintenance of pattern in the post-embryonic seedling (Berger, 1998a; Masucci et al., 1996; Di Cristina et al., 1996; Lee and Schiefelbein, 1999; Lee and Schiefelbein, 2002). Lin and Schiefelbein (Lin and Schiefelbein, 2001) have shown that *GL2* is expressed during embryogenesis and is controlled by *WER* and *TTG*. Here we show the embryonic expression of *WER*, *CPC* and *GL2* and describe how the complex transcriptional interaction between these genes starts in the embryo and defines the specific expression of *GL2*, which leads to epidermal cell differentiation in the root-hypocotyl axis of the seedling.

MATERIALS AND METHODS

Plant material

Wild-type Landsberg *erecta* (L*er*) ecotype and *gl2-1* (in L*er*) were obtained form the Nottingham Stock Centre, *cpc* (in WS) was kindly provided by K. Okada (Kyoto University, Japan), and *wer-1* (in Col-o) was kindly provided from J. Schiefelbein (University of Michigan, USA).

RNA in situ hybridisation

Tissue fixation and wax embedding were performed as described by Long and Barton (Long and Barton, 1998). Tissue sectioning and in situ hybridisation of digoxigenin-UTP-labelled RNA probes were performed as described by Coen et al. (Coen et al., 1990). Antisense *GL2* probe was generated using T7 RNA polymerase to transcribe the pGL5 cDNA clone that spans exon 5 of the *GL2* gene after linearising with *Bam*HI. Sense *GL2* probe was transcribed with T3 RNA polymerase from the same clone linearised with *Eco*RI. Antisense *WER* probe was generated using T7 RNA polymerase to transcribe the pWER1 clone, containing a 450 bp *WER* cDNA fragment that spans exons 2 and 3, after linearisation with *Bam*HI. Sense *WER* probe was synthesised with T3 RNA polymerase from the same clone linearised with *Eco*RI. Antisense *CPC* probe was generated using T7 RNA polymerase, from the full-length *CPC* cDNA clone linearised with *Eco*RI, and the *CPC* sense probe was generated by transcription with T3 RNA polymerase, of the PCR amplified insert of the same clone. Antisense *GFP* probe was generated using T7 RNA polymerase to transcribe the clone containing the *GFP5* cDNA, after linearising with *Xho*I, and the sense *GFP* probe was transcribed with T3 RNA polymerase from the same clone, but linearised with *Bam*HI. All the clones are in pBluscript SK+ (Stratagene).

For each gene, serial sections through embryos at different developmental stages were probed by in situ hybridisation and the experiments were repeated on independent samples.

Digital images were captured with a Nikon Cool Pix 995 using differential interference contrast (DIC) optics and assembled using Photoshop 5.

Confocal analysis of embryos

Embryos at different developmental stages were dissected from the seed coats with a hypodermic needle and aligned on the surface of a thin layer of Murashige and Skoog medium supplemented with 0.5% (w/v) Phytagel and 1% (w/v) sucrose to avoid embryo desiccation. Embryos were then imaged with a MRC600 Biorad confocal microscope using a 488 nm excitation line, and a 523 nm short-pass filter to monitor the expression of GFP. Optical sections of 2.5-3 μm were collected, processed with the NIH Image program (http://rsb.info.nih.gov/nih-image/) and assembled using Photoshop 5. At least ten embryos for each of the heart, torpedo and mature developmental stages of the J2301 marker line and of the homozygous *gl2*/*gl2* J2301/J2301, *cpc*/*cpc* J2301/J2301, and *wer*/*wer* J2301/J2301 lines were analysed.

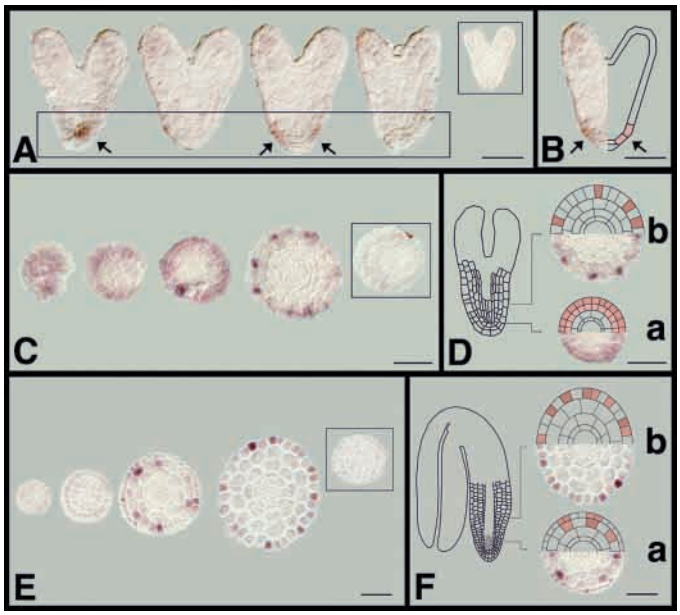

Fig. 1. In situ hybridisation of *GL2* mRNA in developing wild-type embryos. In the schematics in B, D and F, RNA localisation is indicated in orange. (A) Serial longitudinal sections through a heart stage embryo. Weak expression of *GL2* mRNA is detected at the basal end of the embryo, in the protodermal layer, in alternate sections (arrows). Inset: a longitudinal section hybridised with *GL2* sense probe shows no signal. (B) Schematic representation of the *GL2* expression pattern detected in one of the sections in A. (C) Transverse sections through the torpedo stage embryo. From the left to the right of the panel the sections displayed were taken from: the root pole at the base of the embryo, the root close to the root pole, the root close to the hypocotyl, the hypocotyl. At the root pole, *GL2* mRNA is present in the root cap, lateral root cap and throughout the epidermis. In the hypocotyl, *GL2* mRNA is present in epidermal cells that overlie a single cortical cell. Inset: section hybridised with *GL2* sense probe. (D) A schematic representation of the longitudinal organisation of the embryo at torpedo stage and the radial organisation in the embryonic root (a) and hypocotyl (b) as indicated. (E) Transverse sections through the mature stage embryo. From the left to the right of the panel the sections displayed were taken from: the root pole at the base of the embryo, the root close to the root pole, the root close to the hypocotyl, the hypocotyl. In the root, *GL2* is expressed only in those cells that overlie the walls of single cortical cells that will develop into non-hair epidermal cells in the seedling. In the hypocotyl *GL2* is only expressed in epidermal cells that will become elongated, non-stomatal cells overlying the walls of single cortical cells. Inset: section hybridised with *GL2* sense probe. (F) A schematic representation of the longitudinal organisation of the embryo at the mature stage and the radial organisation of the root (a) and the hypocotyl (b) as indicated. Scale bars: 40 μm.

RESULTS

GL2 expression is initiated in the heart stage embryo

In the seedling *GL2* is expressed in non-hair cells of the root and elongated cells of the hypocotyl (Hung et al., 1998; Berger et al., 1998a). These two cell types overlie the wall of single cortical cells and *GL2* is required for their position-dependent specification (Masucci et al., 1996). To investigate the earlier temporal and spatial pattern of *GL2* expression during embryogenesis, *GL2* mRNA was detected by in situ hybridisation on transverse and longitudinal sections of the root and hypocotyl of wild-type embryos at different developmental stages, as classified by Jürgens and Mayer (Jürgens and Mayer, 1994). We consistently detected *GL2* expression in heart stage embryos (Fig. 1). Series of consecutive longitudinal sections through wild-type heart stage embryos were hybridised with the *GL2* antisense probe and alternate sections displayed a faint *GL2* expression in protodermal cells at the base of the embryo (arrows in Fig. 1A,B). This suggests that protodermal cells, which are not in contact with each other, first express *GL2* at the basal end of the embryo during the heart stage. During the transition between heart and torpedo stage, periclinal divisions in the protodermal cells give rise to an outer lateral root cap layer of cells and an inner epidermal layer (Scheres et al., 1994). At the torpedo stage *GL2* transcript is detectable throughout root cap, lateral root cap and epidermal layer in transverse sections of the root (Fig. 1C,Da). In the hypocotyl, *GL2* is detected in most of the epidermal cells that overlie a single cortical cell when viewed in transverse section (Fig. 1C,Db). At the mature stage, in both root and hypocotyl, *GL2* is expressed in epidermal cells that overlie a single cortical cell and will become non-hair cells in the root and elongated cells in the hypocotyl (Fig. 1E,F). These data suggest that *GL2* expression is first established in isolated protodermal cells at the base of the heart stage embryo, and by the torpedo stage the expression spreads to every cell of the root cap and epidermis of the root. Between the torpedo and the mature stage *GL2* expression is restricted to those cells that will become non-hair cells in the seedling. In contrast, in the hypocotyl, *GL2* expression is already established in an alternate pattern by the torpedo stage, which is then maintained throughout the subsequent stages. The temporal difference in the establishment of the cell-specific expression pattern of *GL2* between the root and hypocotyl suggests that during embryogensis the two epidermal cell types are defined earlier in the hypocotyl than in the root.

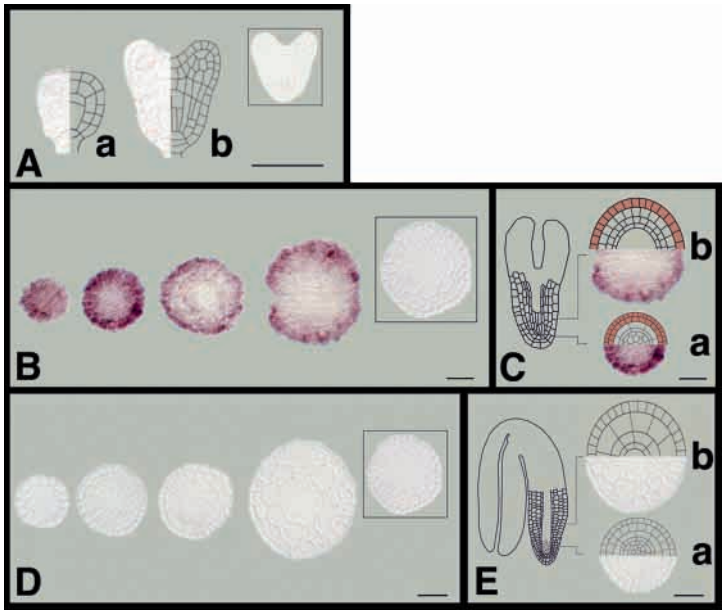

Fig. 2. Localisation of *WER* mRNA in developing wild-type embryos. (A) Longitudinal sections through a globular (a) and a heart (b) stage embryo and schematic cellular organisation. No expression of *GL2* mRNA is detected in either embryonic stage. Inset: a longitudinal section hybridised with *WER* sense probe shows no signal. (B-E) Transverse sections through embryos at different developmental stages hybridised with *WER* antisense RNA probe, and schematic representations of the cellular organisation of root and hypocotyl. The localisation of mRNA is indicated in orange on these schematics. From the left to the right of B and D the sections displayed were taken from: the root pole at the base of the embryo, the root close to the root pole, the root close to the hypocotyl, the hypocotyl. (B) Torpedo stage. *WER* mRNA is detected in lateral root cap and epidermis at the root pole. In the hypocotyl *WER* expression is detected throughout the epidermal layer. Inset: section hybridised with *WER* sense probe. (C) A schematic representation of the longitudinal organisation of the embryo at torpedo stage and the radial organisation in the embryonic root (a) and hypocotyl (b) as indicated. (D) Mature stage. *WER* expression was not detected in either the root or the hypocotyl. Inset: section hybridised with a *WER* sense probe. (E) A schematic representation of the longitudinal organisation of the embryo at the mature stage and the radial organisation of the embryonic root (a) and hypocotyl (b) as indicated. Scale bars: 40 μm.

WER and *CPC* are transiently expressed in the epidermis of the torpedo stage embryo

WER is required to repress the formation of hairs and stomatal complexes in those cells that contact a single underlying cortical cell in the root and the hypocotyl, respectively (Lee and Schiefelbein, 1999). To define when *WER* expression is established during embryonic development, in situ hybridisation studies were performed on wild-type embryos at different developmental stages. *WER* expression was not detected in globular or heart stage embryos (Fig. 2Aa,Ab), but was first detected in torpedo stage embryos. At this stage *WER* mRNA is consistently present in consecutive transverse sections throughout the root cap, the lateral root cap and the epidermal layer in the root (Fig. 2B,Ca) and throughout the epidermal layer in the hypocotyl (Fig. 2B,Cb). Later in embryogenesis, in mature embryos, no *WER* expression is detected in any cell type of either the root or the hypocotyl (Fig. 2D,E). Therefore, high levels of *WER* expression may be required for only a short period during embryogenesis.

CPC is required for hair cell specification in the root (Wada et al., 1997) and so far its function has not been characterised in the hypocotyl. Based on the role and expression of the other genes involved in epidermal patterning in the root and the hypocotyl we suspected that *CPC* might also be expressed during embryogenesis. *CPC* expression was first detected in the torpedo stage embryo and no expression was detected in earlier embryonic stages (Fig. 3Aa,Ab,Ac). At the torpedo

stage, *CPC* mRNA was detected throughout the root in sections close to the basal end (Fig. 3B,Ca). *CPC* expression was also detected in epidermal and cortical cell layers of the hypocotyl (Fig. 3B,Cb). In the mature embryo *CPC* mRNA was present in the root cap and lateral root cap of the root and was also detectable at a low level throughout the root (Fig. 3D,Ea). In the hypocotyl *CPC* mRNA was only present in the vascular tissues (Fig. 3D,Eb).

WER and *GL2* regulate each others transcription

It has previously been shown that *WER* positively regulates *GL2* expression in seedlings (Lee and Schiefelbein, 1999). To test if *WER* controls *GL2* in the embryo, the expression of each gene was determined in *wer* and *gl2* mutant embryos by in situ hybridisation. No *GL2* mRNA was detected in the root or hypocotyl of mature *wer* embryos (Fig. 4A,B) but we found that in wild-type embryos *GL2* is expressed in epidermal cells that overlie single cortical cells (Fig. 1F). This indicates that *WER* is required for the establishment and maintenance of *GL2* expression in the embryo. Surprisingly, *WER* expression was detected throughout the epidermal cell layer of *gl2* mutant mature embryos in both root and hypocotyl (Fig. 4C,D), whereas no expression of *WER* was previously detected in mature wild-type embryos (Fig. 2D,E). This suggests that *GL2* is required to repress *WER* transcription in the mature embryo. Even though *WER* expression was not detectable in early embryonic stages, i.e. globular and heart stage (Fig. 2A), we wanted to test if *WER* is active and required for the onset of *GL2* expression at the heart stage. No *GL2* mRNA was detected in *wer* heart stage embryos (Fig. 4E,F). This data indicate that at the heart stage the level of *WER* expression is below detection but that the gene is active and promotes *GL2* expression.

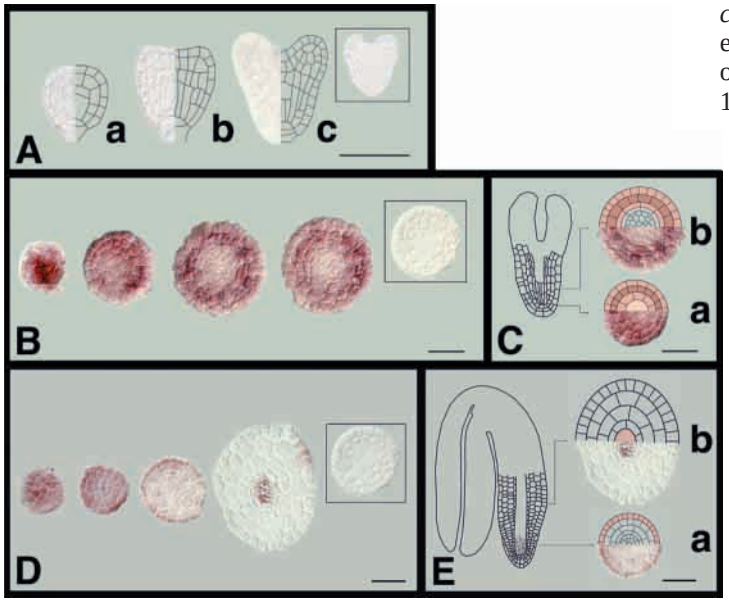

Fig. 3. Pattern of *CPC* mRNA localisation in wild-type embryos. (A) Longitudinal sections through a globular (a), an early-heart (b) and a mid-heart (c) stage embryo and schematic cellular organisation. No expression of *CPC* mRNA is detected in any embryonic stage. Inset: a longitudinal section hybridised with *CPC* sense probe shows no signal. (B-E) Transverse sections through embryos at different developmental stages probed with *CPC* antisense RNA and schematic representation of the cellular organisation of root and hypocotyl. The localisation of mRNA is indicated in orange on these schematics. From the left to the right of B and D the sections displayed were taken from: the root pole at the base of the embryo, the root close to the root pole, the root close to the hypocotyl, the hypocotyl. (B) Torpedo stage. *CPC* mRNA is present throughout the root. In the hypocotyl *CPC* mRNA is present throughout the epidermal and cortical layers. Inset: a transverse section hybridised with *CPC* sense probe shows no signal. (C) A schematic representation of the longitudinal organisation of the embryo at torpedo stage and the radial organisation in the embryonic root (a) and hypocotyl (b) as indicated. (D) Mature stage. *CPC* is preferentially expressed in root cap and lateral root cap cells in the root. In the hypocotyl *CPC* expression is confined to the vascular tissues. Inset: transverse section hybridised with *CPC* sense probe. (E) A schematic representation of the longitudinal organisation of the embryo at mature stage and the radial organisation in the embryonic root (a) and hypocotyl (b) as indicated. Scale bars: 40 μm.

CPC controls *GL2* expression

To determine if *CPC* regulates *GL2* expression in the developing embryo we examined the expression of *GL2* in *cpc* mutant embryos. In *cpc* heart stage embryos *GL2* expression is detected in protodermal cells at the basal end of the embryo, as observed in wild type (Fig. 4G,H; Fig. 1A,B) and in some protodermal cells in the central region (future hypocotyl) of the embryo. In mature *cpc* mutant embryos *GL2* is expressed at a low level in the lateral root cap and in every epidermal cell of the root (Fig. 4I,Ja). This contrasts with the pattern of *GL2* expression in wild-type in which epidermal *GL2* expression is restricted to the future non-hair cells (Fig. 1Fa). To determine if *GL2* can control *CPC*, the expression of *CPC* was examined in mature *gl2* mutant embryos. The pattern of *CPC* mRNA localisation in mature *gl2* mutant embryos is very similar to that found in wild-type embryos, i.e. it is located in the lateral root cap (Fig. 4K,La; Fig. 3D,E). Therefore, *GL2* does not control the accumulation of *CPC* mRNA whereas *CPC* is required for the establishment of the specific pattern of *GL2* expression in the root of the mature embryo but not for the initial expression of *GL2* at the heart stage.

In the hypocotyl of mature *cpc* embryos *GL2* mRNA was present in some epidermal cells that were located over the wall of single cortical cells (Fig. 4I,Jb), as in wild type (Fig. 1Fb). In *gl2* embryos, *CPC* expression was present in the vascular precursor cells as in wild-type embryos (Fig. 4K,Lb; Fig. 1Fb). This indicates that *GL2* does not require the activity of *CPC* for the maintenance of its expression in the hypocotyl of the mature embryo. Furthermore, as it is found in the root, *GL2* does not control *CPC* expression in the hypocotyl.

CPC transcription requires WER activity

To determine if *WER* controls *CPC* we examined the expression of *CPC* in the root and in the hypocotyl of *wer* embryos. In *wer* torpedo stage embryos, the same *CPC* expression was detected as in wild-type mature stage embryos (Fig. 4M,N; Fig. 3D,E). In *wer* mature stage no *CPC* mRNA was detectable in any sections through *wer* mutant embryos hybridised with the *CPC* antisense probe (Fig. 4O), in contrast to wild-type mature embryos where *CPC* is expressed in the lateral root cap and in the provascular tissue in the hypocotyl (Fig. 3E) and *WER* is

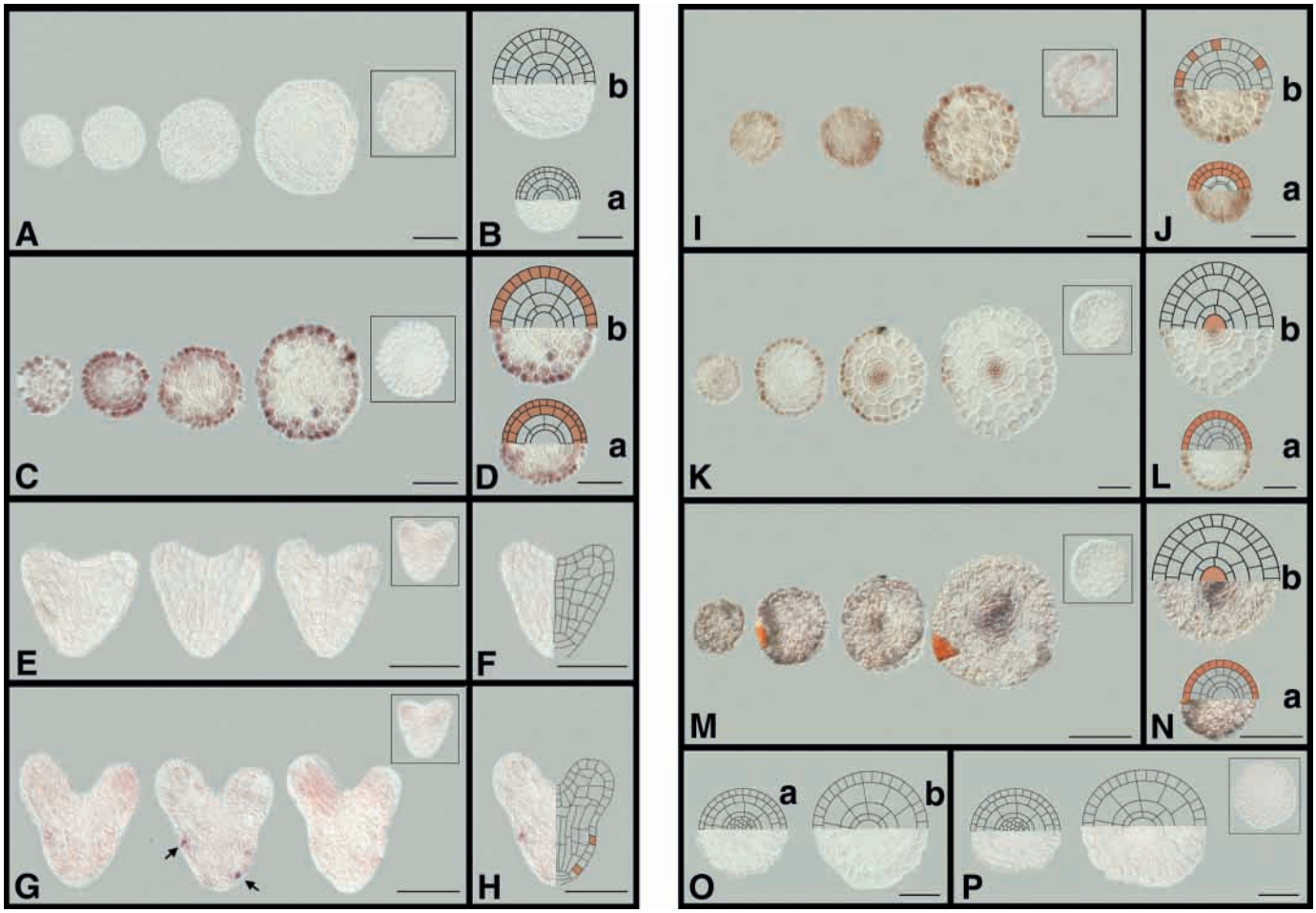

Fig. 4. Localisation of *GL2, WER* and *CPC* mRNA in transverse sections through mutant embryos at different developmental stages. From the left to the right of A,C,K,M the sections displayed were taken from: the root pole at the base of the embryo, the root close to the root pole, the root close to the hypocotyl, the hypocotyl. E and G are consecutive sections throughout heart stage embryos. (A) *GL2* expression in *wer* mature embryo. No *GL2* mRNA was detected in either the root or the hypocotyl. Inset: transverse section hybridised with *GL2* sense probe. (B) Schematic representations of the cellular organisation in transverse sections of the root (a) and the hypocotyl (b). (C) *WER* expression in *gl2* mature embryo. *WER* mRNA is detected in every epidermal and lateral root cap cell in the root and throughout the epidermal layer in the hypocotyl. Inset: transverse section hybridised with *WER* sense probe. (D) Schematic representations of the cellular organisation in transverse sections of the root (a) and the hypocotyl (b) and the localisation of *WER* mRNA (orange). (E) *GL2* expression in *wer* heart stage embryo. No expression of *GL2* mRNA is detected. Inset: a longitudinal section hybridised with *GL2* sense probe shows no signal. (F) Schematic representation of the cellular organisation of one of the sections in E. (G) *GL2* expression in *cpc* heart stage embryo. *GL2* mRNA is detected in protodermal cells both in the centre and at the basal end of the embryo (arrows). Inset: transverse section hybridised with *GL2* sense probe. (H) Schematic representation of the *GL2* expression pattern (orange) detected in one of the sections in G. (I) *GL2* expression in *cpc* mature embryo. From the left to the right of the panel the sections displayed were taken from: the root pole at the base of the embryo, the root close to the root pole, the hypocotyl. *GL2* mRNA is detected throughout the root cap, lateral root cap and epidermal layers in the zone above the initials. In the hypocotyl *GL2* mRNA is detected in epidermal cells that overlie single cortical cells, as it is in the wild-type embryo. Inset: transverse section hybridised with *GL2* sense probe. (J) Schematic representations of the cellular organisation in transverse sections of the root (a) and the hypocotyl (b) and the localisation of *GL2* mRNA (in orange). (K) *CPC* expression in *gl2* mature embryo. *CPC* mRNA is present in lateral root cap of the root and in the vascular precursor cells in the hypocotyl. Inset: transverse section hybridised with *CPC* sense probe. (L) Schematic representations of the cellular organisation in transverse sections of the root (a) and the hypocotyl (b) and the localisation of *CPC* mRNA (in orange). (M) *CPC* expression in *gl2* torpedo stage embryo. *CPC* mRNA is present in lateral root cap of the root and in the vascular precursor cells in the hypocotyl. Inset: transverse section hybridised with *CPC* sense probe. (N) Schematic representations of the cellular organisation in transverse sections of the root (a) and the hypocotyl (b) and the localisation of *CPC* mRNA (in orange). (O) *CPC* expression in *wer* embryo. No *CPC* mRNA is detected in transverse sections in the root (a) and hypocotyl (b). (P) *WER* expression in *cpc* embryo. No *WER* mRNA is detected in transverse sections in the root (a) and hypocotyl (b); inset, section hybridised with *WER* sense probe. Scale bars: 40 μm.

not expressed (Fig. 2E). These data indicate that WER positively regulates *CPC* transcription. To examine if there is a reciprocal control between WER and CPC we analysed the expression of *WER* in *cpc* mature embryos; no *WER* mRNA was detectable, either in the root or in the hypocotyl (Fig. 4P). As *WER* was also not expressed in the wild-type mature embryo (Fig. 2E), this suggests that *CPC* does not negatively regulate *WER* transcription at this stage.

GL2, *CPC* and *WER* are active in the embryo

We have shown that *GL2*, *CPC* and *WER* are expressed and active in the embryo. To further investigate their role during embryogenesis and to confirm that they are functional in the wild-type embryo, we analysed the GFP expression pattern of the root-hypocotyl epidermal marker line J2301 in *gl2*, *cpc* and *wer* mutants.

In seedlings the GFP enhancer trap line J2301 (http://www.plantsci.cam.ac.uk/haseloff/Home.html) expresses GFP in non-hair cells of the root and in elongated cells of the hypocotyl (Berger et al., 1998a). Berger et al. (Berger et al., 1998b) indicated that GFP is expressed at the basal end of the mid-heart stage embryo in the derivatives of the hypophysis in J2301 enhancer trap embryos. Later, from the mid-torpedo stage onwards, GFP is expressed in the root epidermis and root cap cells. We repeated this analysis by confocal microscopy, but consistently detected no GFP expression in any embryonic stage prior to the torpedo stage (Fig. 5Aa). Instead we consistently found that isolated cells expressed GFP at the torpedo stage (Fig. 5Ab,Ad). It was difficult to determine if these GFP-expressing cells were located in the base of the hypocotyl or in the upper part of the root (Fig. 5Ab,Ac). By the end of the torpedo stage, GFP was expressed in alternate longitudinal files of two to three cells (Fig. 5Ad). At the root end of mature stage embryos every cell expressed GFP and above this zone cells expressed GFP in an alternative pattern (Fig. 5Ae).

It was not possible by confocal analysis to determine with certainty the identity of the cells that expressed GFP along the radial axis. It was also difficult to resolve the root from the hypocotyl. Therefore, we analysed the expression pattern of *GFP* by in situ hybridisation. Consecutive transverse sections from the root tip to the hypocotyl of late torpedo stage embryos were hybridised using an antisense *GFP* mRNA probe. In the root we detected *GFP* mRNA exclusively in the lateral root cap (Fig. 5B,Ca), while in the hypocotyl we detected *GFP* mRNA in those epidermal cells that overlay single cortical cells (Fig. 5B,Cb). These results indicate that the enhancer trap J2301 is first expressed in the embryo at the torpedo stage in isolated cells, and later, GFP expression continues in the lateral root

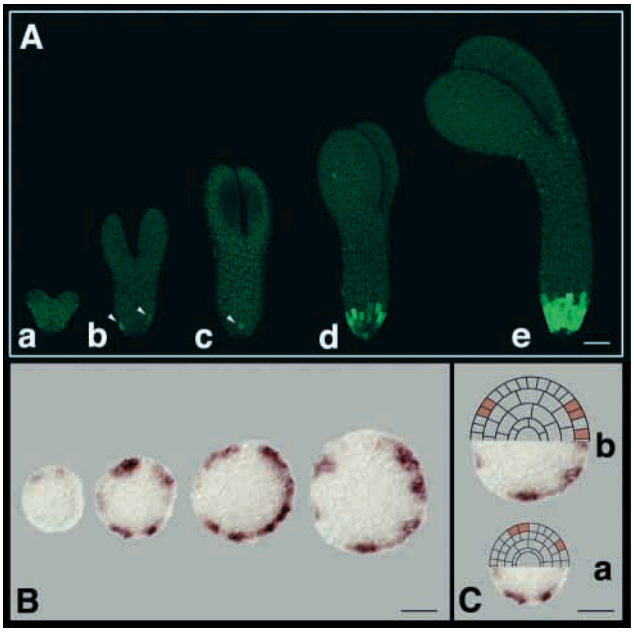

Fig. 5. Pattern of GFP expression in J2301 enhancer trap embryos. (A) Confocal images showing the GFP expression in J2301 embryos dissected from the seed coats at different developmental stages. From left to right: heart stage (a), early, mid and late torpedo stage (b,c,d) and bent cotyledons stage (e). Arrowheads point to cells expressing GFP. (B) In situ hybridisation of *GFP* mRNA in transverse sections of a late torpedo stage embryo of the J2301 enhancer trap line. From the left to the right the sections displayed were taken from: the root pole at the base of the embryo, the root close to the root pole, the root close to the hypocotyl, the hypocotyl. *GFP* is expressed in lateral root cap cells in the root and in epidermal cells in the hypocotyl. (C) Schematic representations of the cellular organisation in transverse sections of the root (a) and the hypocotyl (b) and the localisation of mRNA (in orange). Scale bars: 40 μm.

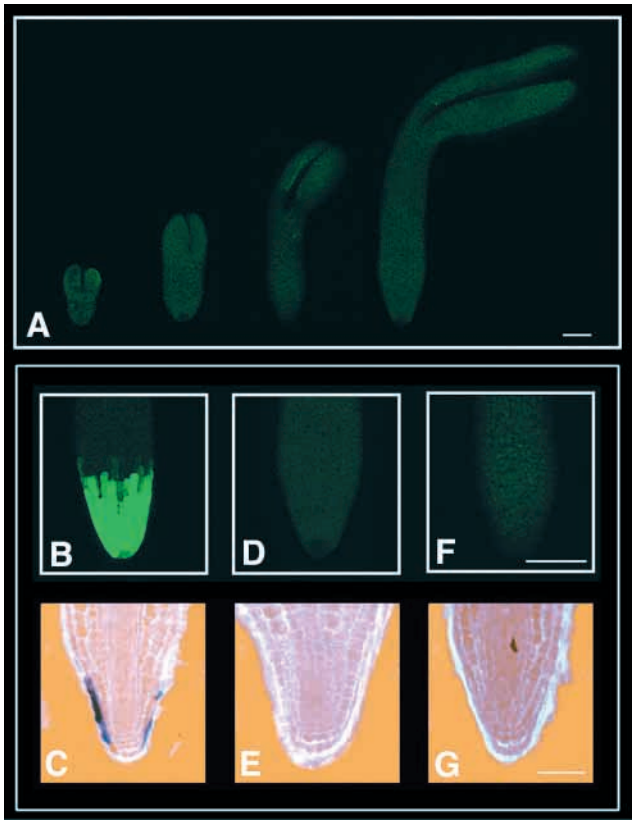

Fig. 6. Embryonic expression of the J2301 enhancer trap in wild-type, *cpc*, *gl2* and *wer* mutants. (A) Confocal images showing the absence of *GFP* expression in *gl2* mutant embryos carrying the J2301 enhancer trap dissected from the seed coat at different developmental stages. Scale bar, 60 μm. The dark green colour in whole embryos does not represent *GFP* expression. The image was obtained using and open iris and high gain during the collection of the optical sections with the confocal microscope in order to visualise the embryos. (B-G) Detection of *GFP* expression at the basal pole of mature wild-type, *cpc* and *gl2* mutant embryos carrying the J2301 enhancer trap using confocal microscopy (B,D,F) and by in situ hybridisation (C,E,G). (B) In wild type, strong *GFP* expression was detected in cells at the basal end of the embryo, which in median longitudinal section (C) was shown to be precisely located in lateral root cap cells. In *cpc* embryos (D,E) and *gl2* embryos (F,G) *GFP* expression was not detected. Scale bars: 60 μm.

cap cells of the root and in those cells of the hypocotyl that overlie the anticlinal cortical cell walls.

After characterising the GFP expression pattern of the J2301 enhancer trap we generated plants that carried the enhancer trap in *gl2*, *cpc* and *wer* mutant backgrounds. No GFP expression was detected by confocal microscopy in any embryonic stage of *gl2* mutants (Fig. 6A), similarly, no GFP expression was detected in *cpc* and *wer* mutants (data not shown). To confirm these results we analysed the *GFP* expression by in situ hybridisation on longitudinal sections of mature embryos of GFP marker line J2301 in wild type, *gl2* and *cpc* mutants. *GFP* mRNA was detected in the lateral root cap and hypocotyl of wild type (Fig. 6B,C), but none was detected in *gl2* (Fig. 6D,E) or *cpc* mutants (Fig. 6F,G). These data indicate that *GL2*, *CPC* and *WER* genes are required to establish the expression of the *GFP* in the J2301 enhancer trap in both the root and the hypocotyl of the mature embryo. Therefore, these genes not only control their reciprocal expression in both the root and the hypocotyl, but also the expression of the epidermal marker line J2301. The results further prove that *GL2*, *CPC* and *WER* are functional during embryogenesis in the epidermis and lateral root cap of the root and in the epidermis of the hypocotyl.

DISCUSSION

We show that the *WER*, *CPC* and *GL2* genes, which are required for the patterned differentiation of cells along the root-hypocotyl axis in the seedling (Galway et al., 1994; Masucci et al., 1996; Di Cristina et al., 1996; Wada et al., 1997; Berger et al., 1998a; Hung et al., 1998; Lee and Schiefelbein, 1999; Walker et al., 1999; Wada et al., 2002), are expressed and active during embryogenesis. We show that these genes are expressed along the root-hypocotyl axis of the developing embryo and propose that a complex interaction of transcriptional regulation exists among *WER*, *CPC* and *GL2* genes during the formation of epidermal pattern in the embryo.

Model for the establishment of *GL2* position-dependent expression during embryogenesis

We propose a model based on the regulatory interactions between *WER*, *CPC* and *GL2* that occur during embryogenesis (Fig. 7). We have shown that *WER* positively regulates *GL2* expression at the heart stage. Then, by the torpedo stage, *GL2* expression has spread to all cells in the future epidermis. *WER* and *CPC* expression is then detectable and *WER* promotes *GL2* expression throughout the epidermis. *CPC* is in turn required for the preferential accumulation of *GL2* transcript in future non-hair cell, perhaps by negatively regulating *GL2* transcription in hair cells position. In the mature embryo *GL2* negatively regulates *WER* transcription and *WER* positively regulates *CPC* expression from the torpedo to mature stages. These events result in *GL2* being expressed at high levels in the future non-hair cells and absent from the future hair cells in the mature embryo. The pattern of *GL2* expression is then maintained in the root of the seedling and accounts for the pattern of hair cells and non-hair cells in the root epidermis, where *GL2* negatively regulates hair formation in cells located in the non-hair position. The demonstration that *WER* activity is required for the transcription of *GL2* at the heart and mature

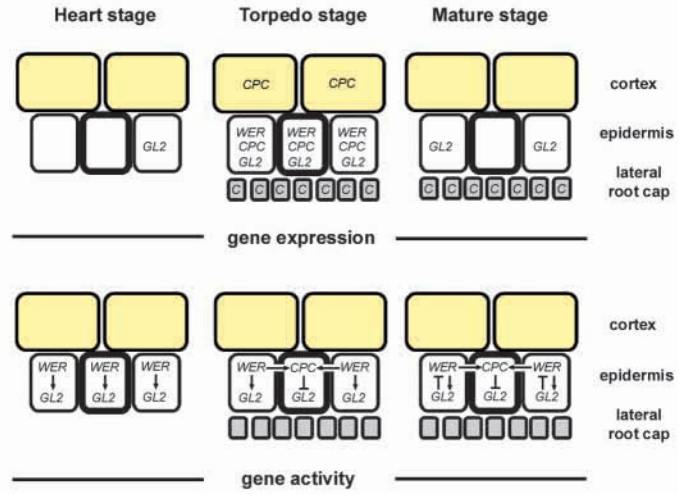

Fig. 7. Schematic representation of the expression of patterning genes in the developing root during embryogenesis (upper row) and the proposed model for their transcriptional regulation (lower row). Cortex is shown in yellow, epidermis in white and lateral root cap in grey. (Upper row) *GL2* is first expressed in the heart stage embryo in a subset of cells in the protoderm. By the torpedo stage, *GL2* and *WER* are expressed in all cells of the epidermis and *CPC* is expressed in the cortex, epidermis and lateral root cap (C). In mature stage embryos *GL2* is expressed in the future non-hair cells and *CPC* is expressed in the root cap. (Lower row) *WER* positively regulates *GL2* transcription in heart stage embryos. At the torpedo stage *WER* positively regulates *GL2* transcription while *CPC* negatively regulates *GL2* transcription in future hair cells, which overlie the cleft between two cortical cells. In the mature embryo *GL2* negatively regulates *WER* transcription that in turn positively regulates *CPC* transcription. *CPC* is required for preferential accumulation of *GL2* in future non-hair cells.

stage and for the expression of *CPC* at the mature stage is at odds with the fact that we cannot detect *WER* mRNA at either stage by in situ hybridisation. It is possible that *WER* transcript accumulates at levels that are too low to be detected by in situ hybridisation or that the protein is stable and persists despite the fact that the *WER* mRNA has disappeared. Further experiments are required to explain this observation.

In the hypocotyl, *GL2* is required to specify the fate of elongated epidermal cells (Berger et al., 1998a; Hung et al., 1998; Lin and Schiefelbein, 2001) and from the early stages of embryogenesis, *GL2* expression is restricted to those epidermal cells that will become elongated cells (this study). *WER* is required for the control of *GL2* expression in the hypocotyl of the embryo (Lin and Schiefelbein, 2001) (this study) and of the seedling (Lee and Schiefelbein, 1999). We show that *CPC* may also regulate *GL2* transcription at the heart stage (Fig. 4G,H). Further support for this role of *CPC* comes from the observation that the J2301 enhancer trap is not expressed in the *cpc* hypocotyls in the mature embryo (Fig. 6F,G). This indicates that during embryogenesis, both *WER* and *GL2* are required for the specification of epidermal cell identity throughout the entire length of the root-hypocotyl axis. In contrast *CPC* does not control the position-dependent expression of *GL2* expression in the embryonic hypocotyl, as it does in the root. It is therefore likely that other MYB-related proteins are required for the restriction of *GL2* expression in

the hypocotyl and that *CPC* may have other functions not related to epidermal cell specification in the hypocotyl since it is also expressed in the provascular region.

Different timing of establishment of epidermal patterning along the root-hypocotyl axis

During embryogenesis the different domains of the embryo are progressively laid down and by the late heart stage, the cellular organisation of the future root is in place (Scheres et al., 1994) and *GL2* mRNA is detectable. Tissues are concentrically organised in the root (Dolan et al., 1993) and it is likely that the radial organisation of the tissue layers is laid down first, and then later, the epidermal patterning is superimposed on the pre-existing radial elements. The epidermal pattern is then completed when *GL2* expression is restricted to the cells in the non-hair cell position of the root at the mature stage and to the cells in the elongated cell position of the hypocotyl at the torpedo stage. In the seedling, the pattern established during embryogenesis is then propagated in a position-dependent manner, under the control of information that might be located in the cell wall of the underlying layer of cortical cells (Berger et al., 1998b). The model that we propose for the establishment of the root and the hypocotyl epidermal pattern differs from those reported by Lin and Schiefelbein (Lin and Schiefelbein, 2001) and Berger et al. (Berger et al., 1998b). Based on *GL2* promoter-reporter gene expression studies, Lin and Schiefelbein proposed that the epidermal cell patterning mechanism in the root initiates during the early heart stage and it occurs before the establishment of a functional meristem. This interpretation is clearly correct for the patterning of the hypocotyls but contentious for the patterning of the root because it is impossible to resolve the precise patterns of expression in the root pole from the published images. Furthermore, Berger et al. (Berger et al., 1998b) proposed that domains of positional information in the root epidermis are established by the torpedo stage. This study was based on a confocal microscope analysis of the GFP expression pattern of the J2301 line, an enhancer trap that is expressed in non-hair cells of the root and elongated cells of the hypocotyl in the seedling. We have now shown that in the mature stage embryo the enhancer trap J2301 expresses GFP in the lateral root cap and epidermis of the hypocotyl. Hence, the gene expression pattern observed by Berger et al. (Berger et al., 1998b) was probably in the epidermis of the basal end of the hypocotyl and could not have been in the root epidermis. This is consistent with our view that the epidermal cells of the hypocotyl are patterned by the torpedo stage and the root pattern is established later.

We can conclude from the results of these two previous studies and this one that in the hypocotyl the initiation of epidermal pattern occurs in the heart stage and is completed by the torpedo stage. The development of cell patterning in the root epidermis is also initiated in the heart stage embryo, but it is not completed until the embryo reaches the mature stage. The difference between root and hypocotyl in the timing of the establishment of epidermal pattern may reflect the presence of a gradient of positional information along the apical-basal axis of the embryo. Alternatively, it may reflect the presence of an equivalent source of positional information along the apical-basal axis that it is interpreted at different developmental stages in the root and in the hypocotyl.

The dynamic development of epidermal pattern is revealed by *GL2* expression

GL2 mRNA is first present in isolated epidermal cells at the root pole in the heart stage embryo. By the torpedo stage *GL2* is present in every epidermal cell and by the end of the mature stage it is restricted to cells that will develop as non-hair epidermal cells. This restriction of *GL2* expression to future non-hair cells is regulated by genes whose transcripts do not accumulate in the same cell-specific manner as *GL2*, i.e. in non-hair cells. For example *CPC* transcripts are found in a number of different cell layers and in every cell of the future epidermis. This suggests the spatial distribution of *WER* and *CPC* transcript alone cannot account for the pattern of *GL2* transcript accumulation and suggests that cell to cell movement of a signal that is controlled by *CPC* or *WER* may play an important role in the establishment of epidermal pattern. Since the cell to cell movement of *CPC* protein in the epidermis of seedling roots has been demonstrated, *CPC* is a strong candidate for such a mobile signal (Wada et al., 2002).

The restriction of *GL2* transcript to non-hair cells indicates that the development of the pattern in the epidermis is progressive – there is early widespread *GL2* expression throughout the epidermis that is later restricted to cells in the future non-hair location. This progressive restriction of gene expression is analogous to the process that occurs during the development of sensory bristles in *Drosophila*. Early in development the proneural genes are expressed in large fields of cells only to become restricted to individual cells later (Simpson et al., 1999). The restriction of proneural expression to individual cells is mediated by cell-cell interactions. It is therefore likely that cell-signalling events may act in the *Arabidopsis* embryo between the heart and torpedo stages. The molecular basis of these interactions remains to be elucidated.

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