Stomata Patterning on the Hypocotyl of *Arabidopsis thaliana* Is Controlled by Genes Involved in the Control of Root Epidermis Patterning

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Stomata complexes are epidermal specialized structures typical of the upper aerial part of plants (shoot). In the model plant *Arabidopsis thaliana,* we show that in the hypocotyl (the junction between the shoot and the root), stomata are organized according to a clear pattern reminiscent of the root epidermis pattern. Although stomata complexes are typical of the shoot epidermis, their pattern on the hypocotyl is under the control of genes involved in root epidermis patterning. Moreover, we have isolated a GFP marker line for the hypocotyl epidermal cells which do not differentiate stomata complexes. In this line the root and the hypocotyl epidermal patterns are similar. Our data support the existence of interactions between developmental mechanisms involved in the control of the apical/basal polarity and the radial symmetry of the plant body.
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Key Words: epidermis; hypocotyl; stomata; *Arabidopsis thaliana*; TTG; GL2.

INTRODUCTION

The epidermis in animal and in plants presents many specialized structures. These are usually patterned and have proven to be very useful biological models as shown for example by studies of the *Drosophila* eye (Basler and Hafen, 1989) and the *Caenorhabditis elegans* vulval epidermis (Sundaram and Han, 1996). As in animals, the epidermis of plants represents the interface between the plant and its environment. In *Arabidopsis,* the development of the three most abundant specialized epidermal cells, root hair cells, trichomes and stomata has been the focus of considerable attention (Dolan and Roberts, 1995, Larkin *et al.,* 1997; Schiefelbein *et al.,* 1997). It has been shown that specialized epidermal structures are not randomly distributed in plants and a number of processes controlling their development have been characterized.

The root epidermis is composed of only two cell types,

root-hair cells which differentiate tubular, elongated, tip-growing appendages and non-root-hair cells (Dolan *et al.,* 1994; Dolan, 1996). Root-hair cells are organized in cell files which are interspersed with one or two cell files of the other cell type. Root-hair cells are always positioned over an anticlinal cell wall between underlying files of cortex cells (ACCW) (Dolan *et al.,* 1993). This pattern appears to be under the control of at least the three genes *CPC* (Wada *et al.,* 1997), *GL2* and *TTG* (Masucci *et al.,* 1996; Galway *et al.,* 1994). The overall organization of the shoot epidermis is more complex than the organization at the basal pole (root). Despite the absence of obvious morphological cues, the distribution of trichomes is not random (Hülskamp *et al.,* 1994, Larkin *et al.,* 1996). The control of trichome cell fate and pattern at least depends on the genes *TRIP-TYCHON* (Hülskamp *et al.,* 1994), *TTG* (Korneef, 1981) and *GL1* (Larkin *et al.,* 1994). Unlike trichomes and root hairs, stomata are multicellular structures which develop from precursor cells called meristemoids (Sachs, 1991). They consist of accessory cells and of guard cells which surround a pore, the stoma. Hence small lineages of cells are established and prevent the formation of stomata side by side

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(Serna and Fenoll, 1997; Larkin *et al.,* 1996; Sachs, 1991).This is altered in two mutants *four lips* (*flp*) *and too many mouths* (*tmm*) (Yang *et al.,* 1995) and under environmental stress (Serna and Fenoll, 1997).

Although the cytology and functions of the epidermal layer vary dramatically along the apical-basal axis of the plant, its development is under the control of common factors as shown by mutants *ttg* which do not have trichomes and develop ectopic root-hair cells. The root and the hypocotyl derive from the basal pole of the embryo (Scheres *et al.,* 1994) but nevertheless the hypocotyl epidermis differentiates stomata. We have studied the pattern of stomata in this special developmental compartment of the plant body and have investigated potential interactions between mechanisms controlling the development of the shoot epidermis and of the root epidermis. This led to the description of a new epidermal pattern of stomata and to the demonstration of the control of its development by genes involved in root patterning. We moreover report that an enhancer trap line which expresses Green Fluorescent Proteins (GFP) specifically in non-root-hair cells and trichomes presents an epidermal pattern of GFP expression in the hypocotyl which overlaps the pattern of stomata. This pattern appears to be controlled by *TTG.*

MATERIALS AND METHODS

Plant Material

The following lines were obtained from the Notthingam *Arabidopsis* Stock Centre: wild types, ecotype Columbia, ecotype Landsberg *erecta,* and ecotype WS; *GL1, GL2,* and *TTG* in Landsberg background. The 35::*R* transformants were obtained from Alain Tissier, Sainsbury Laboratory (Norwich, UK). The line *Triptychon* in Landsberg *erecta* background was a kind gift from Martin Hülskamp. The line J2301 was obtained from a screen of a population of enhancer trap lines (Haseloff, unpublished) which carry the modified GFP 5 (mGFP5) reporter gene (Haseloff *et al.,* 1997).

Plant Growth Conditions

Seeds were sterilized in 5% sodium hypochlorite and stratified on growth medium at 4°C in the dark for 2-3 days. Growth medium was 0.3% gelrite, 1% sucrose in half strength Murashige and Skoog medium at pH 5.8. Measurements were made at 3 to 4 days after germination. Plants were grown in Petri dishes, horizontally under light, under sterile conditions.

Microscopy

For confocal microscopy, Seedlings were incubated for 60 min in 10 μg/ml propidium iodide (Sigma) solution in growth medium to stain the cell wall. Optical sections were obtained on living roots using a confocal microscope (Bio-Rad, MRC 1000) with 488 nm excitation line. Monitoring of the propidium iodide fluorescence was with a 580 nm long pass emission filter and a 523 nm short pass filter was used to monitor GFP expression. Images were pro-
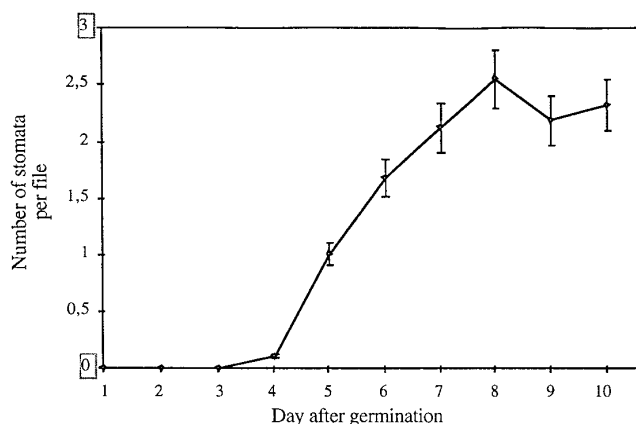
FIG. 1. Time scale of stomatal development in the hypocotyl epidermis after germination. Stomata are not present at germination and begin to differentiate after 4 days. A maximal density of stomata per file is reached after 8 days postgermination. Error bars represent SEM.

cessed using the softwares Confocal Assistant and Photoshop 3.0 (Adobe).

Fluorescence microscopy was performed on a microscope equipped with a FITC filter set (Axiophot, Zeiss). Live seedling were imbedded in growth medium between a slide and a coverslip during observation. Images were processed with Photoshop 3.0 and the red chlorophyll autofluorescence was subtracted from the RGB images.

Light transmission microscopy with Nomarski optics (Nikon optiphot microscope) was used for the analysis of the number and distribution of stomata complexes on live hypocotyls from 7-day-old old seedlings. Two- to 10-day-old seedlings were observed to determine developmental sequences of stomata complexes. The position of epidermal files relative to the anticlinal cortical cell wall could be easily localized by focusing alternatively on different focal planes.

Scanning electron microscopy was used to examine cellular arrangements on 1 week-old seedling hypocotyls. Those were placed directly on a cold stage (−180°C) of a Phillips scanning electron microscope fitted with a cryostage.
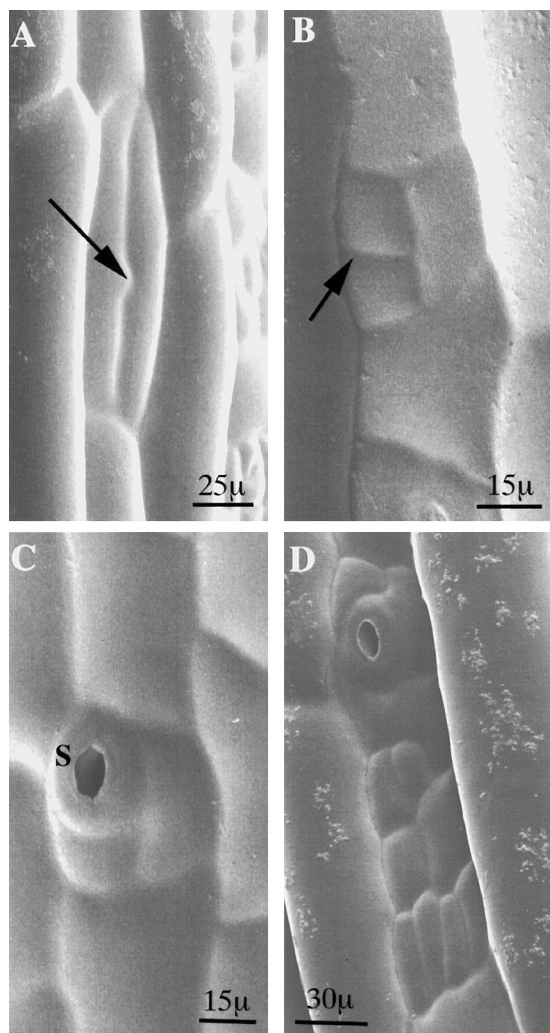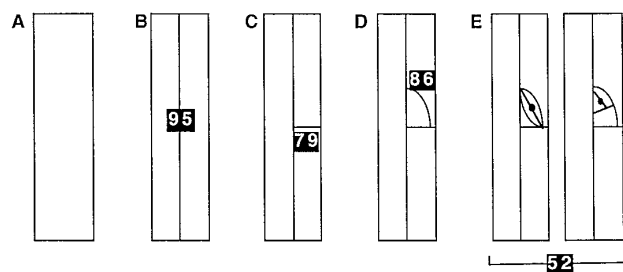
RESULTS

Stomata Development in the Hypocotyl

We have analyzed stomatal development in the wild-type Landsberg *erecta* by scoring the number of stomata complexes after germination (Fig. 1). First signs of stomatal development were observed at the apical pole of the hypocotyl, 3 days after germination. Fully differentiated stomata were first observed at 4 days after germination and their number increased for up to 7 days to reach a maximum density of 2.1 to 2.2 stomata per file. Hence stomata development starts when hypocotyl elongation is reaching com-

pletion as reported by others (Gendreau *et al.,* 1997). We have noticed that stomata complexes development progressed from the apical to the basal pole of the hypocotyl though usually no guard cells were found in the basal third of the organ.

Stomata complexes were drawn schematically (*n* = 125) and their development was observed in 3- and 4-day-old hypocotyls to analyze sequences of cell divisions. In young hypocotyls, few cells at the apical pole of the hypocotyl (at the junction with the cotyledons) were observed to divide along the longitudinal axis (Fig. 2A). Those divisions were followed by at least one transverse division in one of the


FIG. 3. Model of stomata development and sequence of stereotyped divisions. The number associated with each division corresponds to the percentage of stomatal complex where such division was observed. The pattern of cell division is thus probabilistic. Most stomatal complex initial (A) undergo a transverse division (B), which is followed by a transverse division in one of the two cells (C). In most cases an oblique division defines the mother of the guard cells (D), though this division may be followed by a second oblique division (E). However, only half of the stomata complexes undergo strictly this sequence of division.

FIG. 2. Patterns of cell division during stomata ontogeny in the hypocotyl epidermis. An initial longitudinal division (A) is usually followed by a transverse division (B). One of the two small cells eventually divides into two guard cells which separate around a stoma in the mature stomatal complex (s) (C). Multiple series of this pattern can reiterate to form larger stomatal complexes (D).

cells resulting from the initial longitudinal division (Fig. 2B). In many complexes, guard cells differentiated from one of the two small cells defined by the transverse division (Fig. 2C). This series of three divisions was recognized in 52% of the mature stomata complexes observed and represents probably the basic sequence of development of stomata complexes in the hypocotyl (Fig. 3). However, more intricate patterns of cell division of the initial epidermal cell were observed (Fig. 2D). In mature complexes, each couple of guard cells were surrounded by a variable number of cells from which they clonally derive. This may be sufficient to prevent the side-by-side development of guard cells within clusters and indeed, guard cell clusters were found in 0.5% of stomata observed.

A stereotypical pattern of division is thus difficult to define since successions of longitudinal and transverse divisions can give rise to rather large structures which contain more than one pair of guard cells. Hence, 27% of complexes contained 2 pairs of guard cells and 27% contained 3 to 4 pairs of guard cells. We propose a probabilistic model of development where an initial longitudinal division is followed by a transverse division in the cell where the guard cells will differentiate (Fig. 3).

Stomata Complexes Develop in the Hypocotyl According to a Pattern akin to the Root Epidermis Pattern

Observations using scanning electron microscopy showed that two cell types were readily distinguishable by their protruding or non-protruding morphology (Fig. 4A). This had been reported by Gendreau *et al.* (1997). We observed that most guard cells developed from non-protruding epidermal cells (Fig. 4A). Those cells have been reported to lie
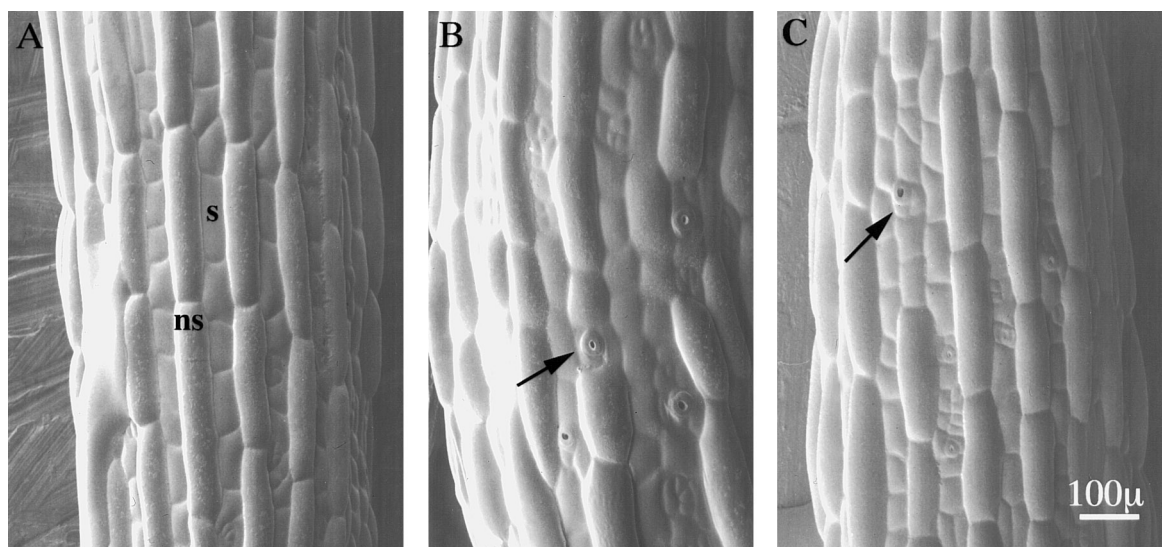
FIG. 4. Stomatal pattern in the hypocotyl in wild-type Landsberg *erecta* (A) and in the mutants *ttg* (B) and *gl2* (C) in Landsberg *erecta* background. Scanning electron micrographs were taken from 7-day-old seedlings and show files of protruding cells where stomatal complexes do not develop (ns) interspersed with files of flat cells where guard cells are present (s). Ectopic stomatal complexes develop in files of protruding cells in the mutants *ttg* and *gl2* (arrows).

over the ACCW (Gendreau *et al.,* 1997). This prompted us to analyze more thoroughly stomata distribution in respect to the position of the ACCW. In all wild-type ecotypes analyzed (Landsberg *erecta,* WS and Columbia) more than 98% guard cells complexes developed from cells overlying ACCWs (Table 1, Fig. 5A). Hence the hypocotyl epidermis possesses two cell types which are organized into clearly patterned cell files. Stomata complexes develop only in cell files overlying ACCWs. The pattern of stomata in the hypocotyl mirrors the pattern of root-hairs observed in the root epidermis (Dolan *et al.,* 1993) with stomata complexes developing at positions equivalent to positions of root-hair

TABLE 1

Stomatal Unit Distribution in WT and Various Mutant Backgrounds

Genotype	Total number of cell files observed	Total number of stomatal units scored	Stomatal units density per file	% of ectopic stomatal units
Wild-type				
(Landsberg *erecta*)	405	609	1.50	1.9
(SEM)			(0.60)	
ttg/ttg	180	268	1.48	16.8
(SEM)			(0.42)	
35S::*R*/35S::*R*	180	65	0.36	18.5
(SEM)			(0.31)	
gl2/gl2	150	214	1.43	11.7
(SEM)			(0.43)	
gl1/gl1	60	137	2.28	4.2
(SEM)			(0.56)	
try/try	165	229	1.39	1.7
(SEM)			(0.60)	

Note. Distribution of stomatal complexes relative to the anticlinal cortical cell wall in wild-type and mutants affected for epidermal patterning in roots (*ttg,* 35S::*R, gl2*) and shoot (*ttg,* 35S::*R, gl1, try*). Stomatal complexes mostly originate from epidermal cells which overlie the anticlinal cortical cell wall. This distribution is perturbed in mutants affected for root epidermal patterning but not for epidermal patterning in shoot.

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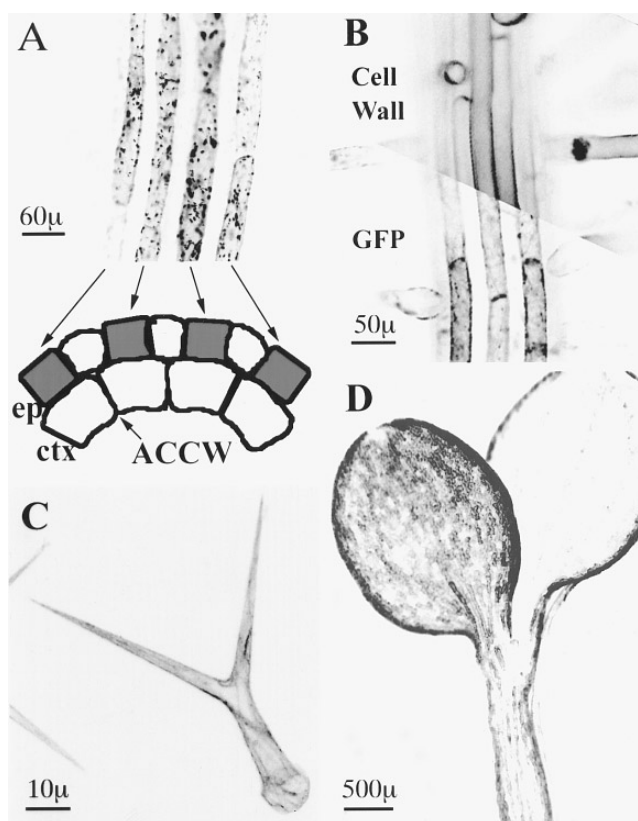
FIG. 5. Pattern of GFP expression in the enhancer trap line J2301. Confocal sections visualizing GFP and propidium iodide (marker for cell walls) were obtained from the epidermis in various parts of *Arabidopsis* seedlings. (A) The hypocotyl presents a striking stripped pattern of GFP expression in epidermal cells (ep) which do not overlie the anticlinal cortical wall (ACCW). The position of such walls is localized on the diagram on the bottom half of the figure. (B) GFP expression pattern in the root epidermis is very similar to the pattern in the hypocotyl and GFP is only expressed in nonhair cell files. Hair cell cross-sections can be observed in the section for the cell wall in the top half. (C) Trichomes are the only epidermal cells which express GFP in the mature leaf. (D) A general view of the upper part of a 3-day-old seedling shows how the stripped pattern of GFP in the hypocotyl epidermis is converted into a different pattern in the cotyledon epidermis. Only the abaxial epidermis and the marginal cells express GFP in cotyledons and stomata do not show GFP expression.

cells. We therefore examined the role played by some genes previously known to regulate epidermal cell patterning in *Arabidopsis* (Dolan and Roberts, 1995), namely *TTG, GL1, GL2,* and *TRY.*

The Development of Stomata Complexes in the Hypocotyl is under the Control of Genes Which Control Root Epidermis Patterning

Mutations in *TRY* and *GL1* known to affect specifically the spatial pattern of trichomes (Hülskamp *et al.,* 1994;

Larkin *et al.,* 1994) did not cause the development of ectopic stomata complexes on the hypocotyl (Table 1) indicating that stomata patterning does require neither *TRY* nor *GL1* activities. In contrast, more ectopic stomata complexes were found in *gl2* and *ttg* mutants (Figs. 4B, C, Table 1) which are known to affect the pattern of root epidermis (Galway *et al.,* 1994; Masucci *et al.,* 1996). This increase did not correlate with an increase of the density of stomata per file. We did not observe any obvious effect of *ttg* and *gl2* mutations of stomata distribution in cotyledons and leaves (not shown). Constitutive expression of the maize *R* gene is known to restore the ability of *ttg* mutants to form trichomes on leaf epidermis (Lloyd *et al.,* 1992) and to develop non-root-hair cells in the root epidermis (Galway *et al.,* 1994). Consequently we examined the pattern of hypocotyl epidermis development in 35S::*R* plants. The percentage of ectopic stomata increased in the homozygous 35S::*R* background. Moreover in the latter background, the density of stomatal complexes decreased to the fifth of the density observed in the wild type.

The GFP Marker Line J2301 Suggests a Common Origin for Root and Hypocotyl Epidermis Patterns

Amongst a group of enhancer trap lines which express GFPs in various cell types we have isolated the line J2301 which is characterized by the specific expression of GFPs in root epidermal cells which do not form root hairs (atrichoblasts) (Fig. 5B). In the hypocotyl epidermis a stripped pattern of GFP expressing and GFP nonexpressing cells was observed (Fig. 5A). Cells in files which overlie the ACCWs, i.e., the files where stomata complexes develop, do not express the reporter gene whereas GFP expressing cells never overlie ACCWs (Fig. 5A; Table 2). The hypocotyl epidermis thus presents a pattern of GFP expression which mirrors the pattern observed in the root. The intensity of GFP expression was not the same in all areas of the hypocotyl (Fig. 6; Table 2). The expression was the strongest at the base of the hypocotyl close to the root pole and remained constant up to a third of the hypocotyl length. Virtually no expression was observed in the upper half of most hypocotyls where stomatal development is predominant. Unlike the epidermal pattern of expression in the root, the pattern observed on the hypocotyl was irregular since GFP was not expressed in every cell which do not overly the ACCW (Fig. 5A; 6A, C). Homozygous plants obtained from the F2 of the cross J2301 × *ttg* showed a clear disruption of the pattern of GFP expression (Figs. 6B, D). No GFP expression was detected in any epidermal cells in all seedlings observed (*n* = 52) which showed that the pattern characteristic of J2301 was under the control of the gene *TTG.*

Moreover we observed the pattern of GFP expression in other parts of the plant epidermis. In the leaf epidermis only trichomes (Fig. 5C) and trichome precursor cells (not shown) express the marker. In cotyledons, GFPs expression is restricted to most epidermal cells present on the abaxial face

TABLE 2

Description of the GFP Expression Pattern in the Hypocotyl

GFP expression relative to radial positional markers ($n = 69$)		GFP expression pattern along the basal–apical axis of the hypocotyl ($n = 69$)		
% of patterns where GFP is expressed in cells which do not overlie the ACCW	% of patterns where GFP expression is correlated with the absence of stomatal development	% of patterns present in the basal segment	% of patterns present in the middle segment	% of patterns present in the apical segment
100.0	98.6	100.0	47.8	8.7

Note. The pattern of GFP expression was scored together with the position of epidermal cells relative to the underlying anticlinal cortical cell wall and to the apical–basal axis. GFP appears to be expressed only in cells which do not overlie the anticlinal cortical cell wall and are present in the basal part of the hypocotyl, close to the root. This correlation is paralleled by the fact that cells which express GFP do not form stomatal complexes.

and to elongated marginal cells and no GFP expression was detected in stomata (Fig. 5D). GFP expression was observed as well in marginal cells bordering the valves of the immature silique and in cells presents at the abscission zone of the silique petiole (not shown). Thus the line J2301 is characterized by the expression of GFP in most specialized epidermal derivatives to the exception of cells belonging to stomata complexes and root-hair cells.

DISCUSSION

In plants the epidermis is defined early during embryogenesis, e.g. in *Arabidopsis* after the fifth round of embryonic divisions (Jürgens, 1995). This takes place after the partition between the apical and basal poles from which differentiate on the one hand the cotyledons and the shoot and on the other hand the root and the hypocotyl. Thus the root and hypocotyl epidermis share a common set of embryonic initials. However, the hypocotyl epidermis does not differentiate root-hair cells but stomata complexes typical of the apical part of the plant. Our results show that despite this apparent acquisition of an apical identity, the hypocotyl epidermis is patterned according to mechanisms specific of the basal pole of the plant.

### Stomata Ontogenesis and Pattern in the Hypocotyl

The development of stomata complexes appears to be similar in the hypocotyl and in the cotyledons (Serna and Fenoll, 1997; Yang *et al.,* 1995) although there are some noticeable differences. Previous to stomata ontogenesis, the initial cells also called meristemoids have stopped their elongation in the hypocotyl and not in cotyledons. Hypocotyl meristemoids are not triangular but rectangular in cross-section and are much larger than the mature guard cells. As reported for stomata development in leaves and cotyledons (Serna and Fenoll, 1997; Yang *et al.,* 1995), the pattern of divisions involved in the formation of stomata

in the hypocotyl is not invariant. Meristemoids undergo a probabilistic sequence of cell divisions which resembles the one described for stomata in cotyledons. In the hypocotyl the repetition of this basic pattern of cell division leads to stomatal complexes which contain more than one stomata. Such sequences of events have been reported as well in leaves of plants grown under environmental stress (Serna and Fenoll, 1997). Although multiple guard cell pairs develop in those complexes found in the hypocotyl epidermis, they are very rarely contiguous which suggests the existence of inhibition of stomata development in closest neighbors of meristemoids as has been shown in the cotyledon epidermis (Yang *et al.,* 1995).

In contrast to the cotyledon and leaf epidermis, meristemoids all originate from specific cell files in the hypocotyl epidermis. Two cell types have been already identified in the hypocotyl epidermis on a morphological basis (Gendreau *et al.,* 1997). We show that non-protruding cells are present at the position where stomata develop. This position corresponds to the position of the underlying ACCW. This position is typical for the development of root-hair cells (Dolan *et al.,* 1993) which suggests the existence of common mechanisms for epidermis patterning between the root and the hypocotyl.

Stomata Patterning in the Hypocotyl Is Controlled by Genes Involved in Root Epidermis Patterning.

We observed stomata development in hypocotyls of mutants defective in patterning and fate specification of trichomes and root hairs. Stomatal pattern did not appear perturbed in mutants which affect specifically trichome pattern (*try*) and identity (*gl1*) but in mutants which affect root epidermis organization, i.e., *ttg* and *gl2*. Both mutants *ttg* and *gl2* are characterized by an increase in the number of root-hair cells and the development of ectopic root-hair cells. Those mutations cause the development of ectopic stomata complexes in the hypocotyl. *TTG* is epistatic to *GL2* (Koorneeff, 1981) and appears to affect
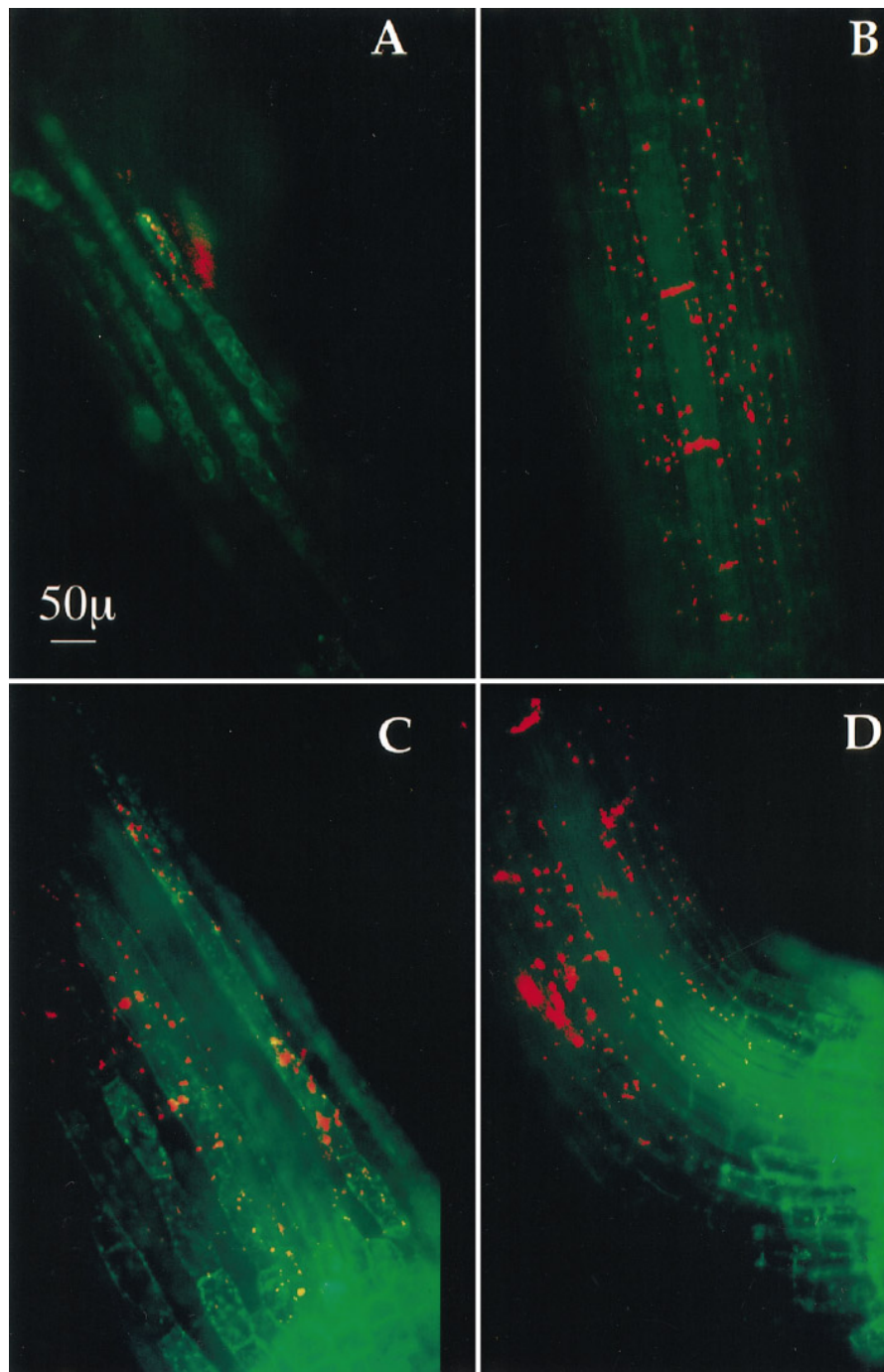
FIG. 6. Pattern of GFP expression in the hypocotyl of the line J2301 × *ttg/ttg*. The pattern of GFP expression observed in fluorescence microscopy in the hypocotyl of the line J2301 (A, C) is compared with the line homozygous for the marker J2301 and the mutation *ttg* (B, D). The background red fluorescence of the chlorophyll was subtracted and alternating files of epidermal cells expressing and nonexpressing green fluorescent GFP are visible in the wild-type background. Note that the expression is stronger and the pattern is more clearly defined in cells present at the basal pole of the hypocotyl (C) than in the apical part (A). The hypocotyl epidermal cells are distinguished from the colar cells at the junction with the root which all strongly express GFP (C). This strong and uniform expression is not altered by the mutation *ttg* (D). However, the introduction of *ttg* causes the absence of GFP expressing cells both at the base (D) and in the apical pole of the hypocotyl (B).

cell fate in the root epidermis earlier than *GL2* (Galway *et al.,* 1994; Masucci *et al.,* 1996). Our results showing a stronger effect of *TTG* on stomata complexes development supported this view.

Arabidopsis plants which constitutively overexpress the *R* gene from maize have been characterized by a strong reduction in the root hair density which appears to be the opposite phenotype to the one displayed by *ttg* mutant and this has been interpreted as a sign of the overexpression of *TTG* function (Galway *et al.,* 1994). We observed in such plants a characteristic reduction in the number of stomata complexes similar to the root phenotype. This suggests that an *R*-like molecule is a negative regulator of stomata development in the hypocotyl. It was surprising not to find an increase in the density of stomata complexes in *ttg* mutants. Moreover, in 35S::*R* transformants, many remaining stomata complexes were in ectopic position which was not been reported for root-hair cells probably as a consequence of the very low number of remaining root hairs. In conclusion, stomata development in the hypocotyl is controlled by genes controlling root-hair cell fate and development.

This conclusion was supported by the marker line J2301 which displays a similar expression pattern of GFP in root epidermis and in hypocotyl epidermis. This pattern was abolished when *ttg* homozygous background was introduced showing that all epidermal cell switch fate to become stomata-forming cells which typically do not express the marker. *TTG* controls as well the specification of root-hair versus non-root-hair cell fate (Galway *et al.,* 1994) and appears to control the function of the gene *GLABRA2* (Hülskamp *et al.,* 1994). It has been reported that *GLABRA2* expression monitored by a GUS marker displays a characteristic pattern of alternating cell files in the hypocotyl (John Schiefelbein, personal communication). It is likely that *GL2* is preferentially expressed in non-stomata forming cells, the position of which is parallel to atrichoblast position in the root (Masucci *et al.,* 1996). The parallel between root hair and stomata development can be developed further. Both cell types occupy the position over the ACCW. They both differentiate after the arrest of elongation. Moreover their differentiation is sensitive to environmental signals. Light is necessary for stomata development and ethylene fluctuations in response to stress affect root hair differentiation (Tanimoto *et al.,* 1995) and may affect stomata development as well (Serna and Fenoll, 1997). In conclusion, although the final epidermal differentiated products are different, the epidermal patterns of both root and hypocotyl are identical and under the control of the same set of genes. This is in agreement with the fact that both structures originate from the basal pole of the embryo and thus can be considered as a single developmental unit (Scheres *et al.,* 1994).

It is remarkable that a structure typical from the apical pole of the plant body is patterned as a result of processes specifically active in the basal pole. The choice of the identity of the epidermal specialized structures, i.e. root-hair cell versus stomata meristemoid, is likely to depend on cues

linked to the apical-basal axis which is set up very early during plant embryogenesis (Jürgens, 1995). We have observed that stomatal development takes place along a gradient in the hypocotyl with a higher density of stomata at the apical pole. This gradient was inversely correlated with the gradient of expression of GFP in the marker line. This suggests that the hypocotyl is the site of conflicting influences from the apical and the basal poles. The patterning mechanisms of both root and hypocotyl epidermis obviously involves a control of radial symmetries which are considered to be another early embryonic feature. The results reported in this study thus indicated that there are interactions between apical/basal and radial patterning mechanisms during plant development.

ACKNOWLEDGMENTS

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