**INTRODUCTION**

The development of plant organs requires the establishment of symmetry. Radial symmetry in roots is set up during embryogenesis and maintained during postembryonic growth of the seedling. Surgical experiments on developing roots and shoot meristems have led to a partial understanding of the mechanism of the patterning of cells within organs and of the patterning of lateral organs derived from the shoot meristem (e.g. Sussex, 1955; van den Berg et al., 1995; Berger et al., 1998). It is clear for example that fields of positional information direct development but the molecules that constitute this positional information have not been identified. Genetic dissection of the process of tissue and organ formation has identified genes required for the establishment of symmetry in leaves, flowers and roots (Scheres et al., 1995; Di Lorenzo et al., 1996; Waites and Hudson 1995; Luo et al., 1996; Timmermans et al., 1998; Schneeberger et al., 1998).

The Arabidopsis root is a tractable system to study the development of pattern in plants because of its small size, simple tissue organization and genetic resources (Dolan et al., 1993). The root comprises concentric rings of tissue with lateral root cap outside the epidermis, which surrounds the ground tissue. The development of radial pattern in the ground tissues requires the products of the _SHORT ROOT_ ( _SHR_ ) and _SCARECROW_ ( _SCR_ ) genes (Scheres et al., 1995; Di Lorenzo et al., 1996). The cells that make up these tissue layers are arranged in files that converge on a ring of initials (lateral root cap/epidermis initial) from which the epidermal and lateral root cap tissues of the seedling are derived, once root growth is initiated after germination. Each initial gives rise to a clone of epidermal cells and a clone of lateral root cap cells. These initial divisions in the epidermal/lateral root cap initial are defective in _tornado1_ ( _trn1_ ) and _trn2_ plants indicating a requirement for _TRN1_ and _TRN2_ for initial cell function. Furthermore, lateral root cap cells develop in the epidermal position in _trn1_ and _trn2_ roots indicating that _TRN1_ and _TRN2_ are required for the maintenance of the radial pattern of cell specification in the root. The death of these ectopic lateral root cap cells in the elongation zone (where lateral root cap cells normally die) results in the development of gaps in the epidermis. These observations indicate that _TRN1_ and _TRN2_ are required to maintain the distinction between the lateral root cap and epidermis and suggest that lateral root cap fate is the default state. It also suggests that _TRN1_ and _TRN2_ repress lateral root cap fate in cells in the epidermal location. Furthermore, the position-dependent pattern of root hair and non-root hair cell differentiation in the epidermis is defective in _trn1_ and _trn2_ mutants. Together these results indicate that _TRN1_ and _TRN2_ are required for the maintenance of both the radial pattern of tissue differentiation in the root and for the subsequent circumferential pattern within the epidermis.

**SUMMARY**

The cell layers of the Arabidopsis primary root are arranged in a simple radial pattern. The outermost layer is the lateral root cap and lies outside the epidermis that surrounds the ground tissue. The files of epidermal and lateral root cap cells converge on a ring of initials (lateral root cap/epidermis initial) from which the epidermal and lateral root cap tissues of the seedling are derived, once root growth is initiated after germination. Each initial gives rise to a clone of epidermal cells and a clone of lateral root cap cells. These initial divisions in the epidermal/lateral root cap initial are defective in _tornado1_ ( _trn1_ ) and _trn2_ plants indicating a requirement for _TRN1_ and _TRN2_ for initial cell function. Furthermore, lateral root cap cells develop in the epidermal position in _trn1_ and _trn2_ roots indicating that _TRN1_ and _TRN2_ are required for the maintenance of the radial pattern of cell specification in the root. The death of these ectopic lateral root cap cells in the elongation zone (where lateral root cap cells normally die) results in the development of gaps in the epidermis. These observations indicate that _TRN1_ and _TRN2_ are required to maintain the distinction between the lateral root cap and epidermis and suggest that lateral root cap fate is the default state. It also suggests that _TRN1_ and _TRN2_ repress lateral root cap fate in cells in the epidermal location. Furthermore, the position-dependent pattern of root hair and non-root hair cell differentiation in the epidermis is defective in _trn1_ and _trn2_ mutants. Together these results indicate that _TRN1_ and _TRN2_ are required for the maintenance of both the radial pattern of tissue differentiation in the root and for the subsequent circumferential pattern within the epidermis.

Key words: Root epidermis, Lateral root cap, Radial pattern, Cell specification, Root hair formation, _tornado_ mutants, _Arabidopsis thaliana_
which are arranged in a ring at the root tip. The initials divide periclinally (new wall parallel to the root surface) to form a pair of cells. The outermost cell undergoes a series of divisions to form a clone of lateral root cap cells. The inner cell undergoes a number of anticlinal divisions (new wall perpendicular to the root surface) to form a clone of epidermal cells, the basal most of which forms a new initial. Epidermal and lateral root cap cells divide in the meristematic zone but upon transition to the elongation zone the lateral root cap cells die. To our knowledge no mutations have been described that define genes involved in the definition of epidermis from root cap.

The mature epidermis is composed of two cell types whose fate is determined by domains of positional information with strict boundaries (Berger et al., 1998). Trichoblasts form root hairs and are located at the junction between two underlying cortical cells. Atrichoblasts form non-root hair epidermal cells and are located over the outer wall of a single cortical cell (Dolan et al., 1993, 1994). Genetic analysis of the development of this circumferential pattern has revealed that the homeodomain protein GLABRA2 (GL2) is a positive regulator of atrichoblast fate, which are positively regulated by TRANSPARANT TESTA GLABRA (TTG) and WEREWOLF (WER) (Masucci et al., 1994; Tanimoto et al., 1998). Trichoblasts form root hairs and are located at the junction between two underlying cortical cells. Atrichoblasts form non-root hair epidermal cells and are located over the outer wall of a single cortical cell (Dolan et al., 1993, 1994). Genetic analysis of the development of this circumferential pattern has revealed that the homeodomain protein GLABRA2 (GL2) is a positive regulator of atrichoblast fate, which are positively regulated by TRANSPARANT TESTA GLABRA (TTG) and WEREWOLF (WER) (Masucci et al., 1994; Tanimoto et al., 1998). GL2 is negatively regulated by CAPRICE (CPC) a putative transcriptional repressor (Wada et al., 1997). Ethylene and auxin positively regulate the processes of root hair initiation and elongation (Wilson et al., 1990; Dolan et al., 1994; Masucci et al., 1994; Tanimoto et al., 1995; Leyser et al., 1996; Dolan et al., 1997; Pitts et al., 1998).

We previously described the gross morphology of the tornado1 mutation and used an AFLP strategy to position the TRN1 locus at the bottom of chromosome 5 (Cnopps et al., 1996). In this paper we describe the genetic characterisation of 9 trn mutations that constitute two complementation groups. Phenotypic characterisation of the primary roots in trnl and trn2 mutants reveals that TRN1 and TRN2 are required to differentiate between lateral root cap and epidermis tissues in the radial dimension and for the establishment of epidermal cell pattern in the circumferential dimension.

MATERIALS AND METHODS

Genetic analysis

The isolation and mapping of trnl-1 is described by Van Liisebettens et al. (1996) and Cnopps et al. (1996). trn2-1 was isolated from an F3 population of EMS-mutagenised Col seeds obtained from Lehle Seeds, Arizona, USA. F2 progeny of a back-cross of trn2-1 to Col segregated for 1862 wild-type and 618 mutant seedlings, showing that this mutation is nuclear recessive ($\chi^2 (3:1) = 0.011, P > 0.5$).

Heterozygous, kanamycin resistant (KmR) trnl-1 and heterozygous (or wild-type) trn2-1 plants were used to pollinate heterozygous trn plants to test for allelism and approximately 50-200 F1 progeny were analysed for the presence of mutant individuals. These complementation tests identified three further trnl alleles and 6 trn2 alleles.

Plant material

Other trn mutants were kindly provided by the following persons; lop1 (trn1-2) by F. Carlund and N. McHale (Department of Biochemistry and Genetics, The Connecticut Agricultural Experiment Station, New Haven, USA); trnl-3 by L. Corben (Laboratorium Voor Genetica/VIB, University of Gent, Gent, Belgium); trn2-2 by B. Scheres (Department of Molecular cell Biology, Utrecht University, The Netherlands); trn2-3 by H. Adler (Department of Botany, University of Washington, Seattle, USA), trn2-4 by J. Traas (INRA, Laboratoire de Biology Cellulaire, Versailles, France), trn2-5 by J. Goodrich (ICMB, Edinburgh, UK) and trn2-6 by S. De Block (Laboratorium Voor Genetica/VIB, University of Gent, Gent, Belgium). trtg, axr2 and the enhancer trap line J3411, in which GFP is expressed in the lateral root cap in young seedlings, were obtained from the Nottingham Arabidopsis Stock Centre. axr3 was obtained from O. Leyser (University of York, UK) and the enhancer trap line J2301 was obtained from J. Haseloff (MRC Laboratory of Molecular Biology, Cambridge, UK).

Plant growth conditions

Seeds were surface sterilised in 5% sodium hypochlorite and sown onto half strength Murashige and Skoog (Duchefa, Haarlem, The Netherlands) medium (pH 5.8). 1% sucrose and 0.8% phytage. The plants were stratified for 2 days and grown in the light at an angle of 45°. For the analysis of meristems, expression of the enhancer trap lines and examination of double mutant phenotype, plants were grown on phytage-solidified modified Hoagland solution with the following composition: 1% sucrose, 0.8% phytage, 4 mM KNO3, 1 mM Ca(NO3)2, 0.3 mM MgSO4, 2 mM KH2PO4, 90 µM Fe-EDTA, 46 µM H3BO3, 9 µM MnCl2, 0.77 µM ZnSO4, 0.31 µM CuSO4 and 0.11 µM NaMoO4 (pH 5.8).

Confocal microscopy

5- to 7-day-old seedlings were stained with 10 µg/ml propidium iodide solution for 5-60 minutes. Propidium iodide-stained roots were imaged with an MRC600 Biorad confocal microscope using 568 nm excitation line and a YHS filter block or a Leica TCS SP confocal microscope using the 568 nm excitation and 498-551 nm emission lines or a Zeiss LSM510 confocal microscope using the 543 nm excitation and 505-530 nm emission lines for PI. The 488 nm excitation and 580-700 nm emission lines on the Leica or LP560 filter on the Zeiss microscope were used to image GFP expression in the enhancer trap lines (J2301 and J4311). Images were processed using NIH Image (http://rsb.info.nih.gov/nih-image) or LSM-image, assembled using Adobe Photoshop 4 or 5 and printed on a Fujix Pictography 3000 printer.

Tissue fixation and embedding

5- to 7-day-old roots were fixed for 1 hour in 2% glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2. For electron microscopy, roots were post-fixed in 1% osmium tetroxide (Agar Scientific, Stansted, Essex, UK) in phosphate buffer, pH 6.8, on ice for 1 hour. The roots were arranged on a thin slab of 1% LMP agarose (Sigma Aldrich Chemie GMBH, Steinheim, Germany) and covered with further agarose to make a ‘root-agarose sandwich’ for ease of handling. The sandwich was refixed in glutaraldehyde overnight, washed twice in water for 5 minutes, dehydrated in 25%, 50%, 75% and 95% ethanol for 10 minutes each and infiltrated with 50% LR white (medium grade plus 0.5% benzoin methyl ether): 50% ethanol and twice in 100% resin for at least 2 hours each. The samples were transferred to resin-filled capsules and polymerised at 60°C for 24 hours. The sections were cut on a Reichert Jung Ultracut Microtome. For light microscopy 0.5 μm sections were collected onto glass slides and stained with 0.05% toluidine blue and viewed on a Nikon E800 microscope. For transmission electron microscopy, 0.1 μm thick sections were collected onto carbon-coated copper grids and stained with 2% uranyl acetate and 1% lead citrate and the sections were examined in a Jeol 1200 EX electron microscope (Jeol, Tokyo, Japan).
RESULTS

Genetic analysis of tornado mutants

tornado (trn) mutations are recessive and fall into two complementation groups (Table 1 and Cnops et al., 1996). No significant phenotypic variation was detected between the 9 different tornado mutations at the gross morphological level. We therefore only characterised trn1-1, trn1-3 and trn2-1 in more detail (Fig. 1). lopped1 (lop1) (Carland and McHale, 1996) is allelic to trn1. trn roots are shorter than wild-type and are hairy and twisted (Fig. 1). Root twisting around its longitudinal axis begins approximately 3 days after germination. The twisting coincides with a retardation of longitudinal root extension (Fig. 1E,F). Organ twisting occurs in the expanding first leaf pair, while cotyledons and hypocotyl exhibit no such defects, suggesting that these genes act postembryonically. Apical dominance is severely reduced in trn mutants. Many rosette-like structures develop extremely twisted inflorescences containing twisted and abnormally shaped flowers, resulting in reduced fertility (Fig. 1C).

To examine the phenotypes of trn1 trn2 double mutants, trn1-1 (C24) was introgressed into the Col ecotype before crossing to trn2-1. The F2 progeny of the cross between trn1 and trn2, consisting of 201 wild-type and 169 mutant \((\chi^2(9:7)=0.56, P>0.5)\) revealed no novel phenotype. These data indicate that trn1-1 trn2-1 double mutants are indistinguishable from the single mutants, suggesting that TRN1 and TRN2 genes may act in the same morphogenetic pathway.

The TRN1 gene maps to the bottom half of chromosome 5, close to the visible marker tz and the RFLP marker m233 and is located on the CIC YAC clones 6C5 and 8H1 (Cnops et al., 1996). Analysis of the F2 segregation of a trn2-1 cross to Ler indicated genetic linkage between the TRN2 locus and the DFR, AthPHYC and Ath50191 markers, indicating that TRN2 is located on the bottom half of chromosome 5, most likely above TRN1.

Table 1. Genetic segregation\(^a\) of trn mutants

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>Trn(^a)</th>
<th>Trn(^b)</th>
<th>(\chi^2)</th>
<th>Ecotype</th>
<th>Mutagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>trn1-1/+</td>
<td>C24</td>
<td>5</td>
<td>26</td>
<td>0.65</td>
<td>WS</td>
<td>Agro</td>
</tr>
<tr>
<td>trn2-1/+</td>
<td>trn1-1/+</td>
<td>79</td>
<td>250</td>
<td>0.17</td>
<td>C24</td>
<td>EMS</td>
</tr>
<tr>
<td>trn2-1/+</td>
<td>trn1-1/+</td>
<td>334</td>
<td>406</td>
<td>0.58(^c)</td>
<td>Col4</td>
<td>EMS</td>
</tr>
<tr>
<td>trn2-1/+</td>
<td>trn2-1/+</td>
<td>27</td>
<td>65</td>
<td>0.12</td>
<td>Col4</td>
<td>EMS</td>
</tr>
<tr>
<td>trn2-1/+</td>
<td>trn2-1/+</td>
<td>6</td>
<td>37</td>
<td>3</td>
<td>No</td>
<td>Agro</td>
</tr>
<tr>
<td>trn2-1/+</td>
<td>trn2-1/+</td>
<td>18</td>
<td>44</td>
<td>0.53</td>
<td>Ws</td>
<td>Agro</td>
</tr>
<tr>
<td>trn2-1/+</td>
<td>trn2-1/+</td>
<td>40</td>
<td>118</td>
<td>0.01</td>
<td>Ws</td>
<td>Agro</td>
</tr>
</tbody>
</table>

\(^a\)F2 segregation data of F1 plants heterozygote for both mutations; \(^b\)\(\chi^2\) data for 1:3 segregation; \(^c\)\(\chi^2\) data for 7:9 segregation; \(^d\)All mutations induced by Agrobacterium are unlinked with the T-DNA insertion; \(^e\)transactivated Ds population; \(^f\)Cnops et al. 1996; \(^g\)Carland and McHale, 1996; \(^h\)Corben, L.; \(^i\)Scheres, B.; \(^j\)Adler, H.; \(^k\)Traas, J.; \(^l\)Goodrich, J.; \(^m\)De Block, S. and Wang, X.
Meristem organisation is altered in \textit{trn} mutants

Cellular organisation in the vicinity of the central cells is defective in \textit{trn} roots. 5- to 7-day-old meristems of \textit{trn1-1} (n=17), \textit{trn1-3} (n=9) and \textit{trn2-1} (n=9) primary roots were analysed and compared with \textit{C24} (n=19) and \textit{Col} (n=9) wild-type meristems. The majority of \textit{trn1} and \textit{trn2} mutants displayed one or more phenotypic defects in the meristem. Most irregularities are seen in the epidermal/lateral root cap cell files and are often restricted to only one side of the meristem. Most irregularities are seen in the epidermal/lateral root cap cell files and are often restricted to only one side of the meristem. The protoderm initials in wild-type roots are situated around the columella initials and divide periclinally to give rise to the epidermis and the lateral root cap (Dolan et al., 1993) (Fig. 2A,G). \textit{trn1-1} (n=6), \textit{trn1-3} (n=3) and \textit{trn2-1} (n=3) primary roots had abnormal cell divisions in either the initial (Fig. 2C,E) or the daughter cell (Fig. 2B) of the protodermal initial. Cell divisions in the adjacent initials of columella and protoderm do not occur synchronously in \textit{trn1} (n=6) and \textit{trn2} (n=2) meristems. This results either in non-continuous cell files between lateral root cap and columella (Fig. 2H) or a large protodermal initial neighbouring not only the columella initial but also one or two daughter cells (Fig. 2I). Such large initial cells were not observed in wild-type control roots (n=19). The initials of the columella and protoderm divide synchronously so that when viewed in longitudinal sections each lateral root cap cell abuts one columella cell. Irregular divisions in \textit{trn} meristems are sometimes found in cells of other tissue types. Fig. 2C,D show the displacement of a subset of cells in the lateral root cap. The cortical/endodermal initial is occasionally misplaced relative to the central cells as depicted in Fig. 2E. Rarely, alignments of cell walls on one side of the root is disorganized (Fig. 2D,F). Furthermore, at least one of the four central cells is observed to divide in \textit{trn1-1} (n=8) and \textit{trn1-3} (n=6) roots while none were observed in wild-type (C24 n=3). These data indicate that functional \textit{TRN} genes are required to maintain the spatial organisation of the cells surrounding the central cells in the root, especially the protoderm.

Protodermal specification is defective in \textit{trn} mutant roots

The analysis of \textit{trn1-1}, \textit{trn1-3} and \textit{trn2-1} root tips reveals that a subset of epidermal cell files contains \textit{long-thin} cells, exhibiting lateral root cap like features. These \textit{long-thin} cells die in the elongation zone, where lateral root cap cells are normally programmed to die. The death of these, mis-specified epidermal cells results in the formation of gaps that run along the length of the root (Fig. 3A,B). Examination of transverse sections of \textit{trn1-1} indicates that the \textit{long-thin} cells display characteristics of lateral root cap cells. Their walls are thicker and their cytoplasm more diffuse, with a greater degree of vacuolation than epidermal cells at the same stage of development (Fig. 4C,G). In addition these \textit{long-thin} cells are found in both RH and NRH positions (Figs 3A, 4H).

To investigate the mis-specification of the epidermis in
molecular detail, we examined the expression of a GFP marker in the enhancer trap line J3411 in a trn1-1 background. This marker line is expressed in cells of the columella and the lateral root cap (http://www.plantsci.cam.ac.uk/Haseloff/Home.html) (Fig. 3C). The analysis of 5- to 7-day-old trn1-1 roots \((n=17)\) revealed that all lateral root cap-like cells in the epidermis display the J3411 expression pattern, indicating that these long-thin cells exhibit lateral root cap fate (Fig. 3D). The mis-specification of these cells is an early event since the protodermal initial expresses the J3411 marker in some trn1-1 root meristems (Fig. 3F). The morphology of long-thin cells in epidermal layers of the meristematic region and their expression of J3411 together with the initiation of the gaps in the elongation zone and the observation that cells die at the beginning of the gap suggest that a sub-population of epidermal cells is mis-specified as lateral root cap cells. Since loss-of-function alleles of \(TRN\) genes result in the formation of lateral root cap cells in the epidermis, they can be formally described as negative regulators of lateral root cap identity in the epidermis.

Phenotypic characterisation of the embryonic root indicates that \(TRN1\) is required for the postembryonic reinforcement of epidermal fate. Examination of approximately 60 fully grown embryos, derived from a trn1-1 segregating population, were analysed and no altered protoderm phenotype was observed (data not shown). Furthermore, progressively more epidermal cells acquire lateral root cap fate as the root grows. 7-day-old roots have many more lateral root cap cells in the epidermis than 4-day-old roots (Fig. 4C,E). 7-day-old roots also have many gaps in the epidermis in the differentiation zone while 3-day roots had no gaps. This suggests that specification of epidermal cells laid down in the embryo that give rise to the uppermost regions of the root is normal. Only a subset of cells in any particular file are mis-specified (Fig. 4G).

**Epidermal sub-specification is abnormal in trn mutants**

Morphological characterisation of trn1-1, trn1-3 and trn2-1 primary roots reveals defects in epidermal sub-specification in trn roots. Root hairs develop over the anticlinal walls between two cortical cells (ACCW) and non root hair cells develop in the epidermis overlying the outer periclinal wall of individual cortical cells (PCCW). Plants homozygous for the trn1 and trn2 mutations develop ectopic root hairs, i.e. root hairs develop over PCCWs (Figs 1H, 4D,F and 5B). Cell fate and position of cells in RH (root hair) and NRH (non root hair) files in relation to the underlying cortex, was determined for C24 (wild-type) and trn1-1 roots. All cells overlying ACCWs in the
differentiation zone of C24 formed root hairs while 7% of cells (3 out of 43) overlying PCCW produced a root hair. In trn1-1 roots, 37% of cells in a RH file were hairless and 54% of cells in a NRH position produced a root hair (Table 2). Furthermore, cells in both files are not only reduced in size in trn1-1, trn1-3 and trn2-1, but also the size difference between cells in RH and NRH files is reduced (Table 3 and Fig. 5B). trn meristems also lack the regular pattern of alternating trichoblasts and atrichoblasts as seen in wild-type where more cytoplasmically dense cells (trichoblasts) are positioned over the ACCW and differentiate into RH cells. In trn roots however, cytoplasmically dense, and less dense cells were found over both PCCW and ACCW (data not shown). These data suggest that epidermal sub-specification is defective in trn from the meristematic through mature zones.

To investigate this mis-specification in more detail, we examined the expression of J2301 in plants homozygous for trn1-1. J2301 is expressed in NRH files and not expressed in RH files in wild-type (Fig. 5A,C). J2301 expression pattern is altered in trn1-1. A subset of cells in both epidermal positions expresses the marker (Fig. 5D). This altered expression pattern persists through the differentiation zone where non root hair cells, independent of their position, express the J2301 marker and root hair cells do not express the marker (Fig. 5B). This is in agreement with the J2301 marker being a marker line for cell identity rather than cell position per se. Therefore, quantitative analysis of the epidermal cell morphology and marker gene expression indicate that subsets of cells in both positions acquire an incorrect fate in trn1-1 mutant roots. This indicates that TRN1 activity is required for the specification of fate in the epidermis.

Double mutant combinations indicate that TRN1 activity is required throughout the process of specification of epidermal cell fate

To determine where TRN1 acts in the genetic pathway regulating cell specification in the root epidermis, double mutants were made combining trn1-1 with ttg, cpc1, axr2, axr3-1 mutations. TTG is a negative regulator of RH fate in NRH cells and is one of the first genes to act in the specification of identity in the root epidermis (Galway et al., 1994). The fates and positions of 400 epidermal cells of 8 roots from plants homozygous for the ttg mutation (C24 background) were scored (Table 2). All 216 cells overlying in the ACCW and most of the cells overlying the PCCW (195 out of 205) acquired root hair fate. Almost all epidermal cells in ttg trn1-1 double mutants (n=8) differentiated as root hairs (Table 2; Fig. 6B). This suggests that ttg is epistatic to trn1 since the phenotype of the ttg trn1-1 double mutants resembles ttg with respect for cell specification of the root epidermis.

In contrast to TTG, CPC promotes root hair fate possibly through the negative regulation of the GL2 gene expression, since a recessive cpc mutation produces fewer root hairs than wild-type (Wada et al., 1997). cpc trn1-1 double mutants display a novel phenotype. The roots develop fewer root hairs than the cpc single mutant (Fig. 6H,I). The analysis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cells in RH file</th>
<th>Cells in NRH file</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RH</td>
<td>% NRH overlying</td>
</tr>
<tr>
<td>C24</td>
<td>54</td>
<td>54</td>
</tr>
<tr>
<td>trn1-1</td>
<td>156</td>
<td>98</td>
</tr>
<tr>
<td>ttg</td>
<td>216</td>
<td>216</td>
</tr>
<tr>
<td>ttg trn1-1</td>
<td>244</td>
<td>243</td>
</tr>
</tbody>
</table>

RH, root hair; NRH, non root hair; ACCW, anticlinal wall between two cortical cells; PCCW, periclinal wall of individual cortical cells.

Fig. 4. Epidermal cells are mis-specified in trn1-1 roots. Transverse sections through 7-day-old C24 (A,B), and 4- (C,D) and 7- (E,F) day-old trn1-1 primary roots. Sections through the meristem are on the left, sections through the differentiation zone at the right. (G) Longitudinal section through a 4-day-old trn1-1 root. (H) TEM sections through a 7-day-old trn1-1 root. In C-H arrowheads indicate lateral root cap like cells in an epidermal position. (D,F) Ectopic root hairs are indicated by an asterisk and a gap in the epidermis is indicated by a double arrow. Ep, epidermis; c, cortex and lrc, lateral root cap. Bar 20 μm in A-G.
Table 3. Epidermal cell length in trn mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell length in a RH file (μm)</th>
<th>n of cells</th>
<th>Cell length in an RH file (μm)</th>
<th>n of cells</th>
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<tbody>
<tr>
<td>C24</td>
<td>165</td>
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<td>259</td>
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<td>trn1-3</td>
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<td>66</td>
<td>21</td>
</tr>
<tr>
<td>Col</td>
<td>185</td>
<td>31</td>
<td>305</td>
<td>59</td>
</tr>
<tr>
<td>trn2-1</td>
<td>135</td>
<td>29</td>
<td>174</td>
<td>46</td>
</tr>
</tbody>
</table>

RH, root hair; NRH, non root hair.

TRN1 and TRN2 are required to maintain the radial pattern of tissues in the root by negatively regulating root cap fate among cells in the epidermal position. Furthermore, TRN1 and TRN2 are required for patterned cell differentiation in the epidermis – in the absence of TRN activity hair cells and non hair cells develop but their pattern is defective with similar numbers of each cell type developing in each epidermal position. This suggests that these genes are required firstly to maintain the radial pattern of the root and secondly for the specification of cell pattern in the developing epidermis.

TRN1 and TRN2 are required for the development of radial pattern in the root

Radial patterning is defective in trn seedling roots. The division of the epidermal initial (the cell that divides periclinally to form an outer root cap cell and an inner epidermal cell) is altered in trn roots indicating that the TRN1 and TRN2 are necessary for the execution of asymmetric cell divisions in this cell. Later in development lateral root cap cells develop in the place of epidermal cells in plants homozygous for the trn mutations. These ‘ectopic’, lateral root cap cells vacuolate earlier than normal epidermal cells, have thicker cell walls, express a lateral root cap molecular marker and die at the beginning of the elongation zone resulting in the formation of gaps in the epidermis – all characteristics of lateral root cap cells. Since a subset of inner protoderm cells in trn mutants are mis-specified as lateral root cap cells it is possible that lateral root cap fate is the default state for cells derived from the embryonic protoderm. TRN genes are then directly or indirectly involved in either the positive regulation of epidermal fate or the negative regulation of lateral root cap determinants in the inner protoderm derivatives. We await the isolation of the TRN genes to distinguish between these alternatives.

SCR and SHR are required for the development of the radial pattern in the ground tissue (cortex and endodermis) of the root. The division of the cortical initial daughter cell does not occur in scr and shr roots, and the resulting cell file develops as a cortex in shr and is of mixed identity in scr roots (Scheres et al., 1995; Di Laurenzio et al., 1996). Furthermore the embryonic divisions that give rise to the cortex and endodermis (during pattern formation) are also defective in scr roots indicating that scr participates both in the early stages of pattern formation in the embryo and the later stages of pattern maintenance in the seedling meristem. It is as yet unclear what the precise roles of SCR and SHR are in these processes but their products are required for the division of the initial. The TRN genes play a similar role in the epidermal initial, since the division of this cell is defective in plants homozygous for the trn1 or trn2. Nevertheless, trn roots display no obvious embryonic defects, suggesting that the activity of TRN1 and TRN2 is not required in the embryo. It is therefore likely that TRN1 and TRN2 activities are required post embryonically in the seedling meristem.

TRN 1 is required for maintenance of epidermal cell pattern in the root

Evidence presented here indicates that TRN1 is required for the development of the distinctive circumferential pattern found in Arabidopsis. Hair cells are equally frequent in the RH and NRH locations in trn1 roots. Likewise, non root hair cells are equally frequent in RH positions. This suggests that TRN1 is required of 22 root hairs derived from seven double mutants showed that two root hairs were located over PCCWs; i.e. were in an ectopic position.

Plants homozygous for the axr3-1 mutation form no root hairs (Leyser et al., 1996). axr3-1 trn1-1 double mutants are completely hairless (Fig. 6F), suggesting that axr3 is epistatic to trn1. 27% of axr2 epidermal cells bear root hairs and they are all positioned over the ACCW (Masucci et al., 1996). The position, altered elongation and number of root hairs in axr2-trn1-1 double mutants is similar to axr2 single mutants (C24 background) (Fig. 6C,D). However, nine out of 30 root hairs analysed in 6 different axr2 trn1-1 double mutants were situated over the PCCWs, i.e. in an ectopic position. This means that a functional AXR2 gene product is necessary for initiation and elongation of excess root hairs in trn1-1, and that TRN1 is required for correct positioning of root hairs.

Ethylene is a positive regulator of RH fate and blocking ethylene synthesis by aminoethoxyvinyl glycine (AVG) results in a reduction of root hairs in both C24 and trn1-1. Neither C24 nor trn1-1 form ectopic root hairs after the addition of 2.5 μM AVG. The root hairs in C24 however are reduced in length but almost all the cells overlying ACCWs initiate a very short root hair. Cells in a RH file in trn1-1 mutants react differently. Approximately 70% of these cells develop into non root hair cells. These data suggest that trn1-1 is more sensitive to AVG than C24 with respect to root hair formation.

Mis-specification of a subset of epidermal cells into lateral root cap is independent of twisting

ttg supresses the twisting phenotype characteristic of trn1 roots. Ten out of twelve ttg-trn1-1 double mutants completely lacked twisting (Fig. 6B). Gaps formed in the differentiation zone of these straight roots, again indicating that twisting and mis-specification of the trn1-1 epidermis are two distinct aspects of the trn1-1 phenotype and are not coupled. Mutations in AXR2 and AXR3 enhance twisting in trn1-1 (Fig. 6). Enhancement is extreme in the lateral roots of axr3 trn1-1 double mutants, which look like overtwisted ropes (Fig. 6G).

DISCUSSION

Patterned groups of cells are progressively organized during the formation of the embryonic root (radicle) (Scheres et al., 1994). Upon germination, mechanisms exist for the maintenance and perpetuation of such patterns. TRN1 and TRN2 are required for the maintenance of pattern and cell specification in cells derived from the protoderm in the seedling root meristem. The mutant phenotypes indicate that
Fig. 5. Altered J2301 expression pattern in *trn1-1* roots.
(A) Differentiation zone of C24 primary root. Only cells in a non root hair file express GFP from the J2301 enhancer trap. (B) J2301 pattern is not position, but fate, dependent in the differentiation zone of *trn1-1*, i.e. J2301 is only expressed in non root hair cells. (C) Trichoblasts express the J2301 marker in the meristematic/elongation zone of C24. (D) J2301 mis-specification in meristematic (bottom) and elongation (top) zones of *trn1-1*. N, file overlying 1 cortical cell file; R, cell file overlying 2 cortical cell files. Bar, 3.5 μm in A; 4 μm in B; 5 μm in C.D.

Fig. 6. Effect of other mutations on twisting and altered root hair phenotype in *trn1-1* roots.
(A) Differentiation zone in *trn1-1* root. (B) Degree of twisting is reduced in *ttg trn1-1* double mutants. (C) *axr2* primary root. (D) Enhanced twisting in *axr2 trn1-1* primary root. (E) *axr3* primary root. (F-G) Enhanced twisted in *axr3-1 trn1-1* primary (F) and secondary (G) roots. The lateral roots of *axr3-1 trn1-1* double mutants show an increased degree of twisting. The double mutant is hairless too. (H) *cpc* mutants bear few root hairs. (I) *cpc trn1-1* primary roots have only a few to no root hairs at all. Bar, 50 μm in A,B; 25 μm in C,D,E; 20 μm in F; 125 μm in G, 500 μm in H; 1 mm in I.
for the establishment of pattern and if pattern is a prerequisite for cell differentiation, it might be predicted that TRN1 would act upstream of TTG. Evidence that TTG and TRN1 may act in the same pathway, comes from the observation that ttg trn1 double mutants develop hairs on all cells (ttg phenotype) although other aspects of the phenotype are clearly intermediate. This phenotype of the double mutant is also consistent with TTG acting downstream of TRN1. Nevertheless, we cannot rule out the possibility that TTG acts before TRN1. Furthermore, the trn1 cpc double mutant displays an intermediate phenotype indicating that TRN1 and CPC act independently. This is consistent with previous observations in which it was shown that cpc and ttg also act independently (Wada et al., 1997). A working model is that TRN1 is required for the establishment of epidermal cell pattern in the root meristem and acts before TTG (but in the same pathway) which positively regulates GL2. CPC functions independently of TRN1 and TTG, perhaps through negative regulation of GL2. Nevertheless, as mentioned above the complex phenotypes of the various double mutant combinations reported here do not preclude the existence of alternative hypotheses.

Is the disruption in cell pattern in trn1 caused by altered auxin perception or transport?

Recent experiments with the auxin responsive reporter gene, DR5::GUS, confirmed the existence and the significance for root patterning of an auxin ‘maximum’ [sic] in the root tip (Sabatini et al., 1999). Disruption of this maximum with drugs or mutation results in the development of defective cellular organization around the root initials consistent with a role for auxin in the maintenance of cellular organization (Sabatini et al., 1999). Given that auxin transport is defective in stem sections derived from plants homozygous for the lopped1 mutation (lopl is allelic to trn1) (Carland and McHale, 1996) it is formally possible that phenotypes of plants homozygous for trn mutations are due to defects in auxin transport. Mis-specification of epidermis cells into lateral root cap cells in trn roots is accompanied by abnormal cell division in the protodermal initial and might thus be related to a defect in the formation of perception of an auxin maximum. This would suggest that defective polar auxin transport may be involved in the maintenance of the distinction between epidermis and lateral root cap and between the different epidermis cell types in seedling roots.

Auxin has previously been implicated in the development of the hair cells in the epidermis, where double mutant combinations suggested that auxin acts late in the pathway (Masucci and Schiefelbein, 1996). The data presented might also be interpreted to indicate that auxin acts early in the maintenance of the distinction between the two cell types in the root epidermis. The complex phenotypes of the atrx trn1 and atrx trn2 doublets could be interpreted as indicating that TRN1, AXR2 and AXR3 act in different pathways. However the observation that atrx2 and atrx3 suppress hair formation suggests that these genes may act downstream of TRN1. A working model involves regulated auxin fluxes as being important to maintain the differences between the two cell types in the epidermis and between epidermis and lateral root cap in the seedling root meristem.

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