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# *TORNADO1* and *TORNADO2* are required for the specification of radial and circumferential pattern in the *Arabidopsis* root

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## 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*

## 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 has lead 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 Lorenzio 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 the radial pattern in the ground tissues requires the products of the *SHORT ROOT* (*SHR)* and *SCARECROW* (*SCR*) genes (Scheres et al., 1995; Di Lorenzio et al., 1996). The cells that make up these tissue layers are arranged in files that converge on a population of initials at the root tip. Initials divide slowly, adding to the population of rapidly dividing cells (meristem). When cells stop dividing they continue to grow in the ‘elongation zone’ before acquiring their mature shape and size in the differentiation zone.

The lateral root cap and epidermis are derived from the outermost tissue layer of the embryo, the protoderm (Dolan et al., 1993; Scheres et al., 1995). In growing seedling roots these tissues are derived from 16 ‘lateral root cap/epidermis initials’,

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 and Schiefelbein, 1996; Lee and Schiefelbein, 1999). 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 (Cnops 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 *trn1* 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 *trn1-1* is described by Van Lijsebettens et al. (1996) and Cnops et al. (1996). *trn2-1* was isolated from an $F_3$ population of EMS-mutagenised Col seeds obtained from Lehle Seeds, Arizona, USA. $F_2$ 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 ($Km^R$) *trn1-1* and heterozygous (or wild-type) *trn2-1* plants were used to pollinate heterozygous *trn* plants to test for allelism and approximately 50-200 $F_1$ progeny were analysed for the presence of mutant individuals. These complementation tests identified three further *trn1* alleles and 6 *trn2* alleles.

#### Plant material

Other *trn* mutants were kindly provided by the following persons; *lop1* (*trn1-2*) by F. Carland and N. McHale (Department of Biochemistry and Genetics, The Connecticut Agricultural Experiment Station, New Haven, USA); *trn1-3* 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 (Depatment of Botanty, University of Washington, Seatle, 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). *ttg*, *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% phytagel. 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 phytagel-solidified modified Hoagland solution with the following composition: 1% sucrose, 0.8% phytagel, 4 mM $KNO_3$, 1 mM $Ca(NO3)_2$, 0.3 mM $MgSO_4$, 2 mM $KH_2PO_4$, 90 μM Fe-EDTA, 46 μM $H_3BO_3$, 9 μM $MnCl_2$, 0.77 μM $ZnSO_4$, 0.31 μM $CuSO_4$ and 0.11 μM $NaMoO_4$ (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 TC5 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 on 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).

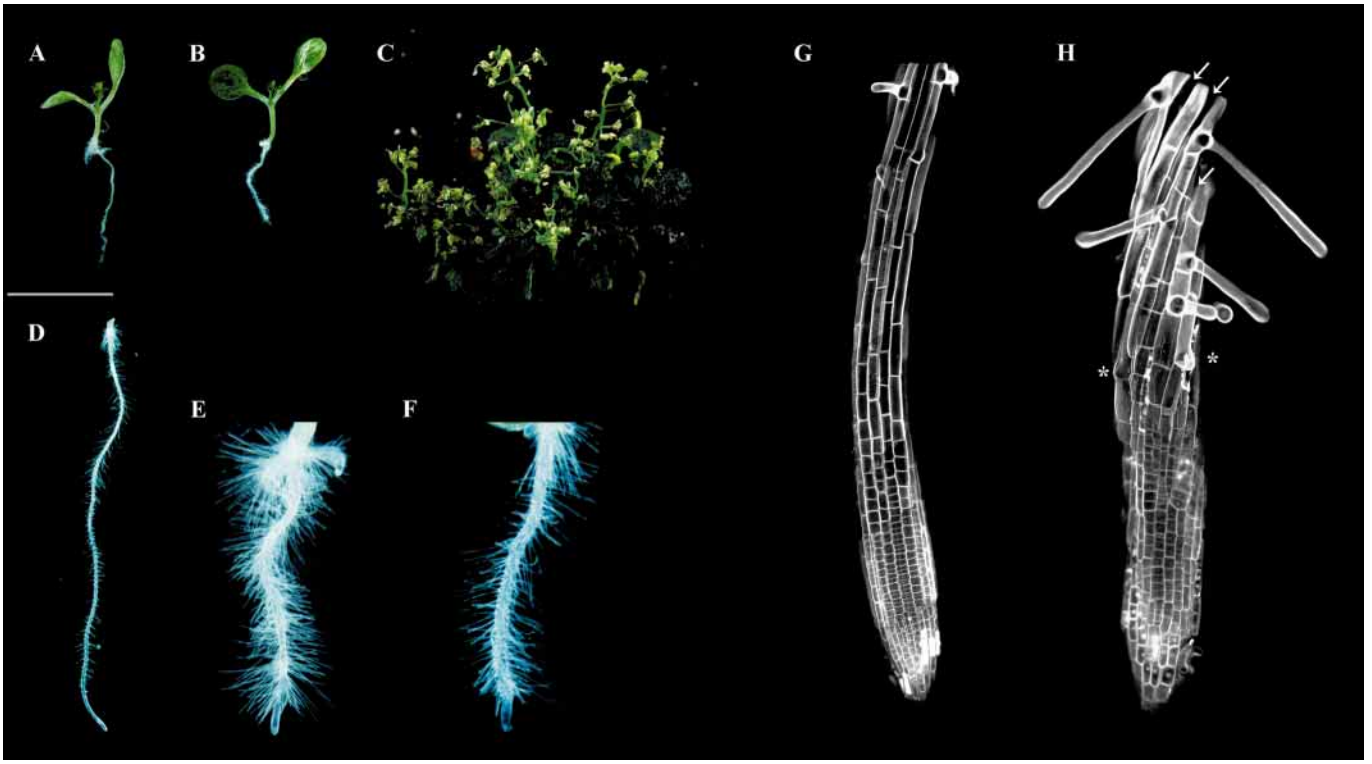


**Fig. 1.** Morphology of *trn* mutants. 1-week-old C24 (A) and *trn1-1* (B) seedlings. (C) 3-month-old flowering *trn1-1* plant. Stem and flower organs are heavily twisted and apical dominance is severely reduced. (D-F) Detail of the primary root of 1-week-old C24, (D) wild-type, (E) *trn1-1* (F) and *trn1-3* plants. The hairy primary roots of *trn1* mutants are twisted and shortened compared to wild-type. (G,H) Montage of propidium iodide stained confocal sections showing the meristem, elongation zone and beginning of the differentiation zone of a Col (G) and a *trn2-1* (H) 5-day-old primary root. The arrows indicate gaps in the epidermis. Ectopic root hairs are indicated with an asterisk. Bar, 5 mm in A,B; 3 mm in D;1 mm in E,F.

## 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 $F_2$ 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 $F_2$ 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**

| Female | Male | Trn$^-$ | Trn$^+$ | $\chi^{2b}$ | Ecotype | Mutagen |
|---|---|---|---|---|---|---|
| *trn1-1*/+[c] | | | | | C24 | Agro[d] |
| *trn1-2=lopped*/+[f] | *trn1-1*/+ | 5 | 26 | 0.65 | WS | Agro |
| *trn1-3*/+[g] | *trn1-1*/+ | 79 | 250 | 0.17 | C24 | EMS |
| *trn2-1*/+[h] | *trn1-1*/+ | 334 | 406 | 0.58[c] | Col4 | EMS |
| *trn2-2*/+[i] | *trn2-1*/+ | 27 | 65 | 0.12 | Col4 | EMS |
| *trn2-3*/+[j] | *trn2-1*/+ | 6 | 37 | 3 | No | Agro |
| *trn2-4*/+[k] | *trn2-1*/+ | 18 | 44 | 0.53 | Ws | Agro |
| *trn2-5*/+[l] | *trn2-1*/+ | 40 | 118 | 0.01 | Ws | Agro |
| *trn2-6*/+[m] | *trn2-1*/+ | 20 | 53 | 0.22 | No | tDs[e] |

[a]$F_2$ segregation data of $F_1$ 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; [c]Cnops et al. 1996; [f]Carland and McHale, 1996; [g]Corben, L.; [h]Clarke, J. and Cnops, G.; [i]Scheres, B.; [j]Adler, H.; [k]Traas, J.; [l]Goodrich, J.; [m]De Block, S. and Wang, X.

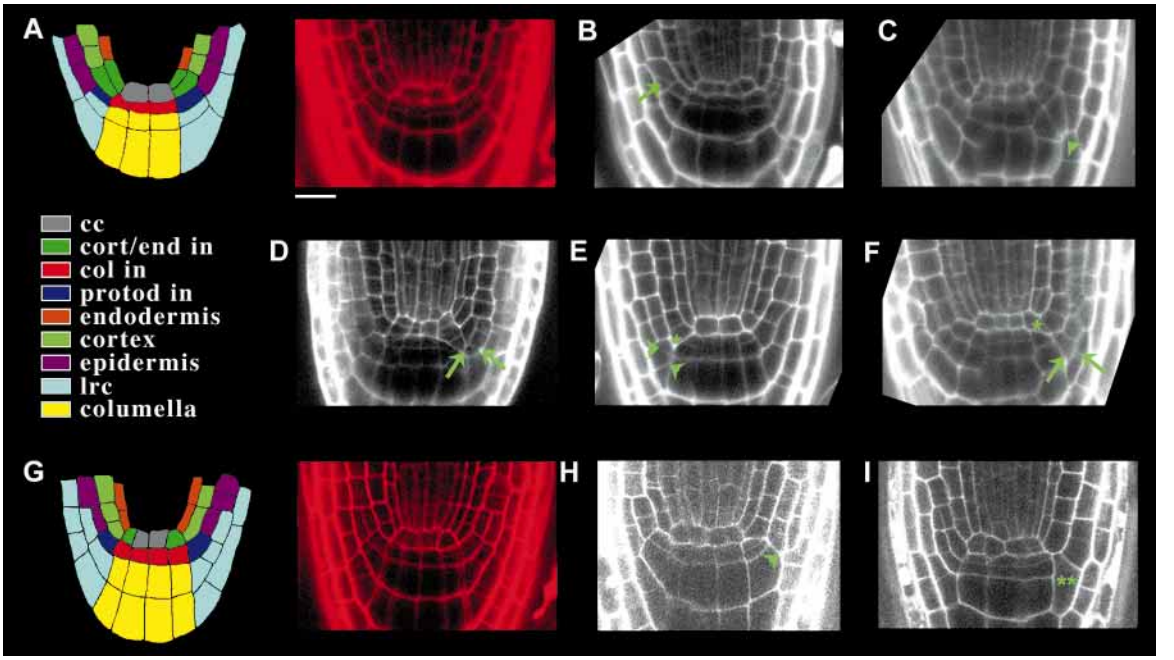


**Fig. 2.** Defects in the cellular organisation of *trn* meristems. Longitudinal sections of propidium iodide-stained 5- to 7-day-old meristems of C24 (A), *trn1-1* (B-D), *trn1-3* (E-F), Col (G) and *trn2-1* (H-I). The wild-types, C24 (A) and Col (G) are schematised on the left. The cortex/endodermis initials surround the central cells on the left and right. In C24 roots, a periclinal cell plate is already present in most of these initials. Columella initials are situated below the central cells. Columella and protoderm initials divide synchronously. The latter undergo a periclinal division giving rise to epidermis and lateral root cap cells. (B) Altered division plane in the protoderm daughter cell of the root of a plant homozygous for the *trn1-1* mutation (arrow). (C,E) Abnormal divisions in protoderm initial in a plant homozygous for the *trn1-1* mutation are indicated by an arrowhead. (D,F) The cellular organisation of the meristem is defective at one side of the root (double arrows). (E,F) A cortical/endodermal initial is misplaced relative to the central cells and protodermal initial (asterisk). (H) Lateral root cap and columella files are not continous (arrowhead). (I) Abnormal sized protodermal initial (double asterisk) abutting the columella and two columella cells. Bar, 10 μm.

## Meristem organisation is altered in *trn* mutants

Cellular organisation in the vicinity of the central cells is defective in *trn* roots. 5- to 7-day-old meristems of *trn1-1* (*n*=17), *trn1-3* (*n*=9) and *trn2-1* (*n*=9) primary roots were analysed and compared with C24 (*n*=19) and Col (*n*=9) wild-type meristems. The majority of *trn1* and *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. 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). *trn1-1* (*n*=6), *trn1-3* (*n*=3) and *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 *trn1* (*n*=6) and *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 *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 *trn1-1* (*n*=8) and *trn1-3* (*n*=6) roots while none were observed in wild-type (C24 *n*=3). These data indicate that functional *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 *trn* mutant roots

The analysis of *trn1-1*, *trn1-3* and *trn2-1* root tips reveals that a subset of epidermal cell files contains *long-thin* cells, exhibiting lateral root cap like features. These *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 *trn1-1* indicates that the *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 *long-thin* cells are found in both RH and NRH positions (Figs 3A, 4H).

To investigate the mis-specification of the epidermis in

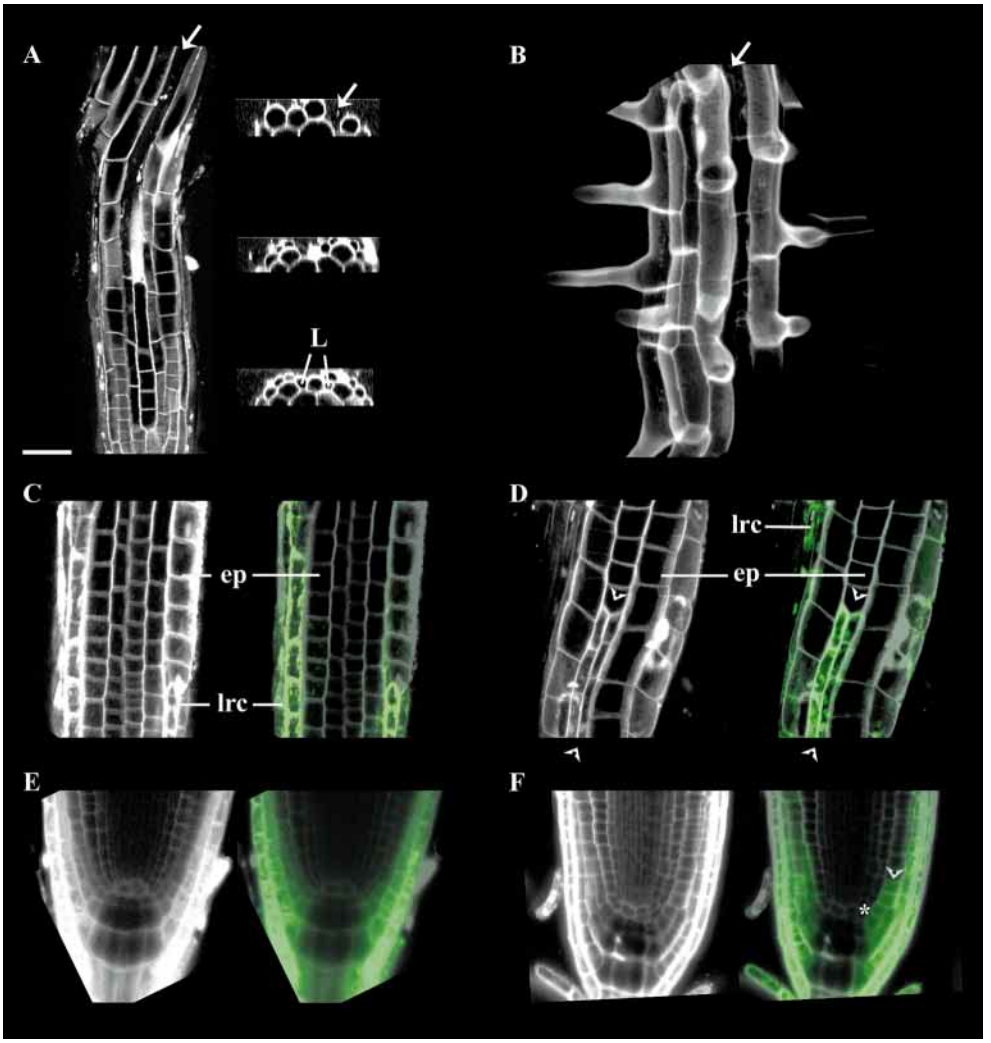


**Fig. 3.** Disrupted protodermal specification leads to gap formation in the differentiation zone of *trn* roots. (A) Projection through a *trn2-1* primary root showing the location of a gap in a RH and a NRH file. Transverse sections through the same root are presented on the right. Bottom section shows two *long-thin* 'epidermal' cells (L) in the meristematic region surrounding a normal sized trichoblast. A few cells above this trichoblast are two lateral root cap like cells, the uppermost of which is dead (propidium iodide stained cell in the middle section). Gaps are induced in both the RH and the NRH cell file (top). (B) Gap formation in the differentiation zone of a 7-day-old *trn1-1* root (arrow). (C,D) End of the meristematic/beginning of the elongation zone of a C24 (C) and a *trn1-1* (D) primary root. Propidium iodide stained optical sections are shown in gray (left); the J3411 expression pattern (green) is superimposed on the propidium iodide stained section (right). The ectopic lateral root cap cells (arrowheads) express the J3411 marker. (E,F) Longitudinal sections through the root tip of C24 (E) and *trn1-1* (F). Ectopic J3411 expression in *trn1-1* epidermis cells is indicated with an arrowhead, and in the initial with an asterisk. Bar, 10 μm in A; 5 μm in B; 30 μm in C,E; 25 μm in E; 35 μm in F.

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 2. Epidermal specification in *trn1-1***

| | Cells in RH file | | | | Cells in NRH file | | | |
|---|---|---|---|---|---|---|---|---|
| Strain | *n* | RH | NRH | % NRH overlying ACCW | *n* | NRH | RH | % RH overlying PCCW |
| C24 | 54 | 54 | 0 | 0 | 43 | 40 | 3 | 6.9 |
| *trn1-1* | 156 | 98 | 58 | 37.2 | 175 | 80 | 95 | 54.3 |
| *ttg* | 216 | 216 | 0 | 0 | 205 | 10 | 195 | 95.1 |
| *ttg trn1-1* | 244 | 243 | 1 | 0.5 | 267 | 7 | 260 | 97.4 |

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

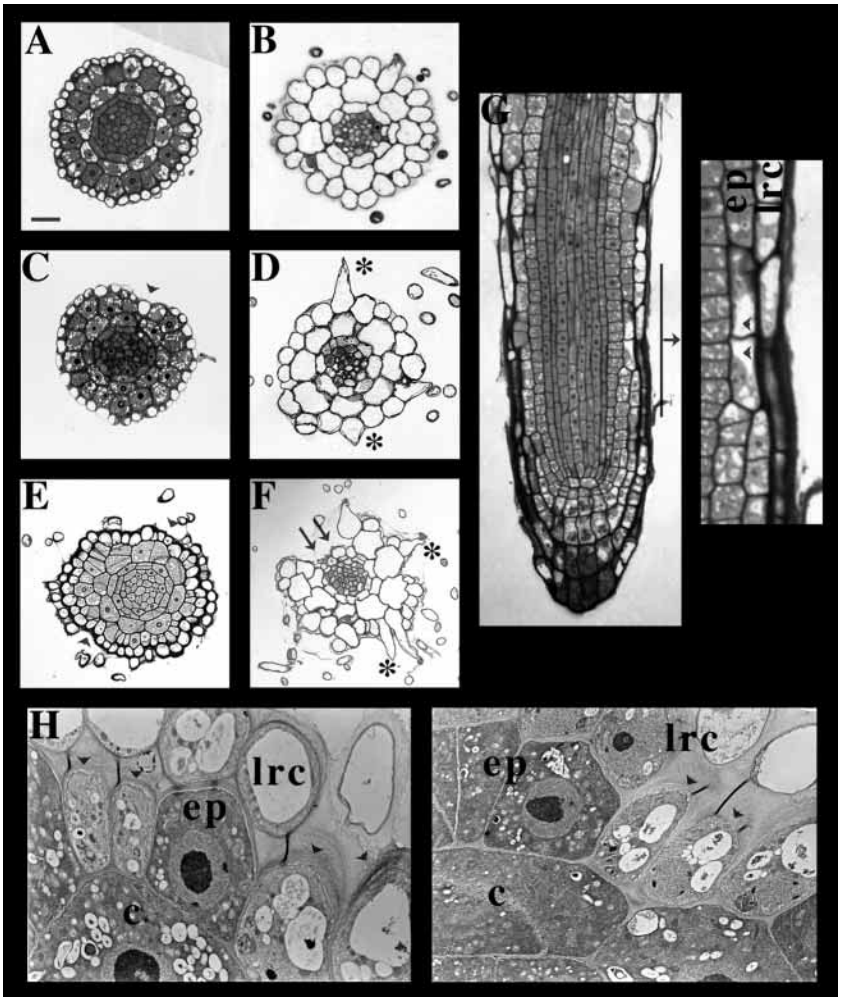


**Fig. 4.** Epidermal cells are mis-specified in *trn1-1* roots. Transverse sections through 7-day-old C24 (A,B), and 4- (C,D) and 7- (E,F) day-old *trn1-1* primary roots. Sections through the meristem are on the left, sections through the differentiation zone at the right. (G) Longitudinal section through 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**

| Strain | Cell length in a RH file (μm) | *n* of cells | Cell length in an RH file (μm) | *n* of cells |
|---|---|---|---|---|
| C24 | 165 | 22 | 259 | 35 |
| *trn1-1* | 56 | 24 | 68 | 28 |
| *trn1-3* | 63 | 17 | 66 | 21 |
| Col | 185 | 31 | 305 | 59 |
| *trn2-1* | 135 | 29 | 174 | 46 |

RH, root hair; NRH, non root hair.

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-specifcation 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 *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

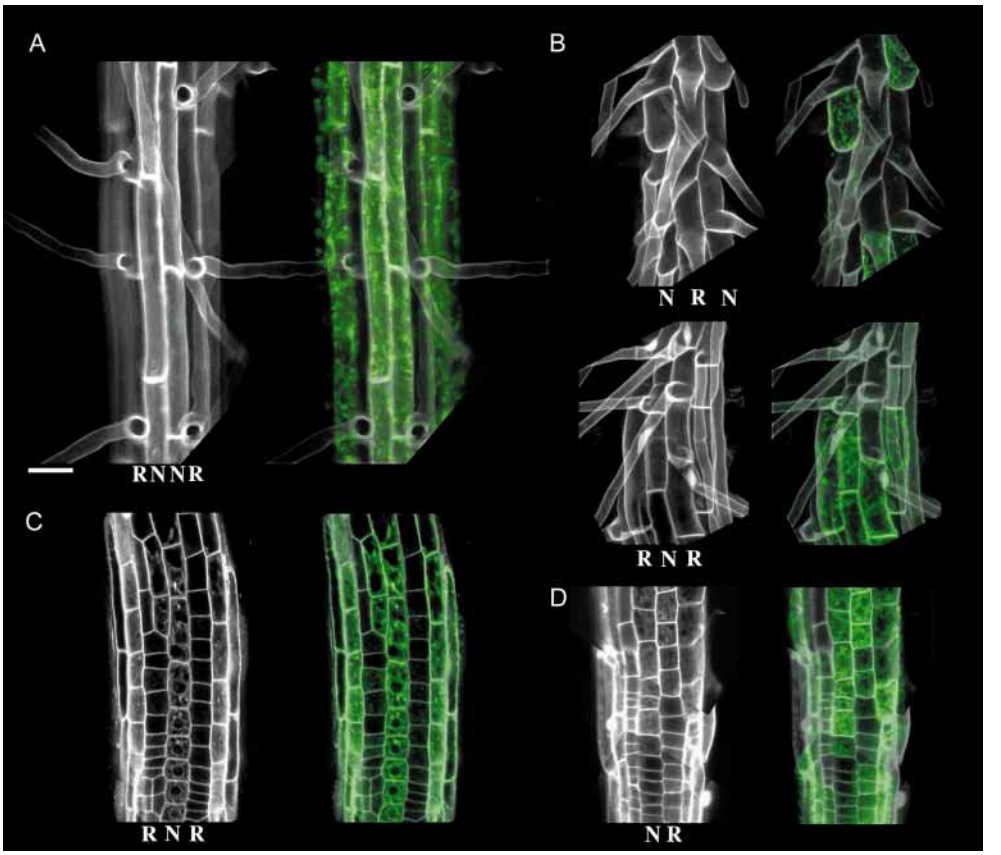


**Fig. 5.** Altered J2301 expression pattern in *trn1-1* roots. (A) Differentiation zone of C24 primary root. Only cells in a non root hair file express GFP from the J2301 enhancer trap. (B) J2301 pattern is not position, but fate, dependent in the differentiation zone of *trn1-1*, i. e. J2301 is only expressed in non root hair cells. (C) Trichoblasts express the J2301 marker in the meristematic/elongation zone of C24. (D) J2301mis-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.

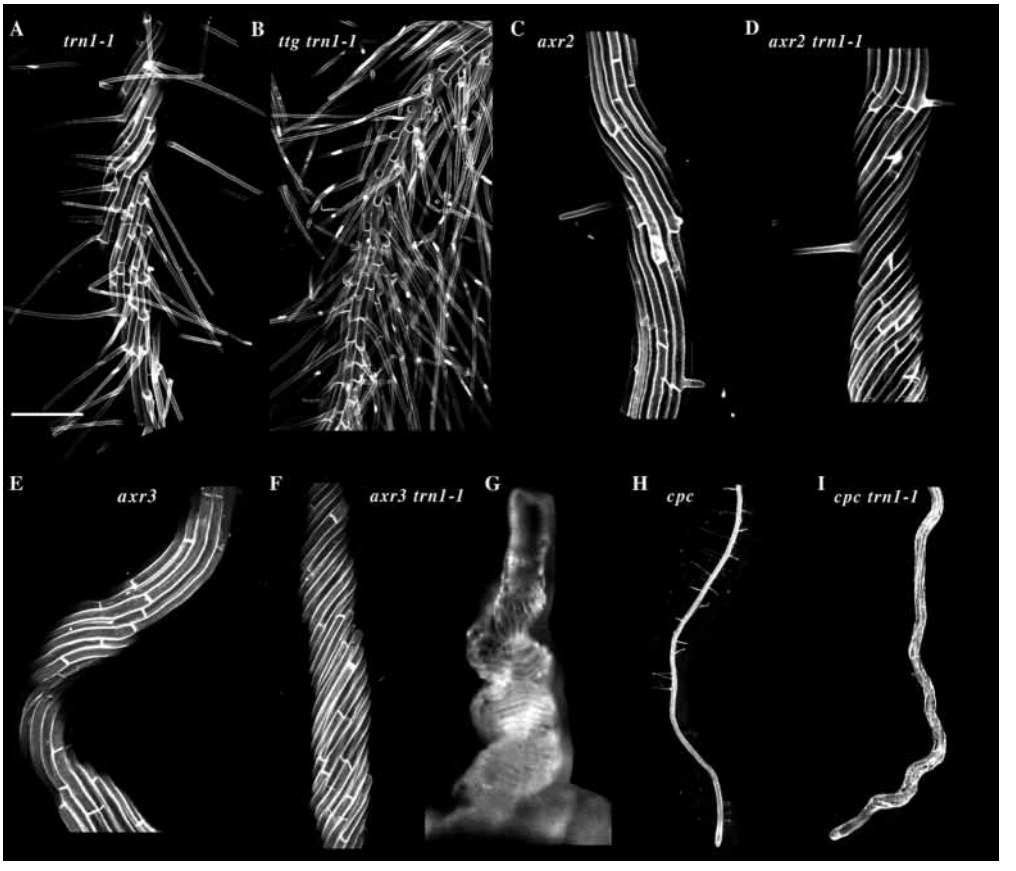


**Fig. 6.** Effect of other mutations on twisting and altered root hair phenotype in *trn1-1* roots. (A) Differentiation zone in *trn1-1* root. (B) Degree of twisting is reduced in *ttg trn1-1* double mutants. (C) *axr2* primary root. (D) Enhanced twisting in *axr2 trn1-1* primary root. (E) *axr3* primary 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 (*lop1* 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 *axr3 trn1* and *axr2 trn1* doubles could be interpreted as indicting that *TRN1*, *AXR2* and *AXR3* act in different pathways. However the observation that *axr2* and *axr3* 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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