

Transformation of shoots into roots in *Arabidopsis* embryos mutant at the **TOPLESS** locus

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SUMMARY

We describe a novel phenotype in *Arabidopsis* embryos homozygous for the temperature-sensitive *topless-1* mutation. This mutation causes the transformation of the shoot pole into a root. Developing *topless* embryos fail to express markers for the shoot apical meristem (*SHOOT MERISTEMLESS* and *UNUSUAL FLORAL ORGANS*) and the hypocotyl (*KNATI*). By contrast, the pattern of expression of root markers is either duplicated (*LENNY*, *J1092*) or expanded (*SCARECROW*). Shifts of developing *topless* embryos between permissive and restrictive temperatures show that apical fates (cotyledons plus shoot apical meristem) can be transformed to basal fates (root) as late as transition stage. As the apical pole of transition stage embryos shows both morphological and molecular

characteristics of shoot development, this demonstrates that the *topless 1* mutation is capable of causing structures specified as shoot to be respecified as root. Finally, our experiments fail to show a clear link between auxin signal transduction and *topless-1* mutant activity: the development of the apical root in *topless* mutant individuals is not dependent on the activity of the predicted auxin response factor *MONOPTEROS* nor is the expression of *DR5*, a proposed 'auxin maximum reporter', expanded in the apical domain of *topless* embryos.

Key words: *Arabidopsis*, *TOPLESS*, Shoot transformation, Embryogenesis, Polarity

INTRODUCTION

Shoot and root systems of land plants carry out complementary functions fundamental to plant survival. These systems form from meristems established at opposite poles of the embryo. Morphological indications of differences along the shoot-root axis are most clearly manifested after the globular stage of embryogenesis. Globular stage embryos consist of two parts: a spherical mass of cells (the embryo proper) that gives rise to the seedling plant, and a suspensor, often filamentous, that anchors the embryo proper to the ovule and may act as a conduit for nutrients from the mother plant. In *Arabidopsis*, the suspensor consists of a single row of cells giving the globular stage embryo the appearance of a lollipop. In *Arabidopsis*, as in many other dicotyledonous plant species, the future shoot-root axis of the globular embryo proper is aligned parallel to the long axis of the suspensor. The root pole forms from the side of the globular embryo closest to the suspensor, while the shoot pole forms from the side directly opposite. As the embryo makes the transition from the globular to the heart stage of embryogenesis, growth occurs more rapidly in the apical-basal dimension than in the radial dimension. Concomitant with this, morphological features of the shoot

(cotyledons and shoot apical meristem) and root poles (organization of cell files and root cap) develop.

Polarity along the long axis of the globular stage embryo is foreshadowed in the polar organization of the egg cell and the zygote (Mansfield and Briarty, 1991; Mansfield et al., 1991). The egg cell is positioned in the embryo sac with the basal end facing the micropyle (the opening through which the pollen tube enters) and the apical end facing the central cell. The egg cell itself is polarized with the nucleus located closer to the apical end and the vacuole located closer to the basal end. The first division of the zygote is unequal, generating a large basal cell and a smaller terminal cell. The larger basal cell gives rise to the suspensor, while the smaller apical cell generates the embryo proper. The basal cell also gives rise to the hypophysis. The hypophysis is the only descendent of the basal cell to generate a portion of the seedling; it gives rise to portions of the root meristem and root cap (Mansfield and Briarty, 1991; Dolan et al., 1993).

Mutations in the *GNOM/EMB30* gene disrupt both the polarity of the zygote and of the developing embryo proper (Mayer et al., 1993). The orientation of the first zygotic cell division is randomized in *gn* mutant embryos and the most severely affected embryos develop as green balls that lack an axis. Polarity appears to be abolished at the cellular level in *gn*

mutants. Consistent with this, the PIN1 molecule, which is normally localized to the basal pole of the cell, is found around the circumference of the cell or localized to a side of the cell that bears no relationship to the apical-basal axis (Steinmann et al., 1999). The *GN/EMB30* gene encodes a GEF/ARF-like molecule that may mediate its effects through targeted secretion (Shevell et al., 1994; Busch et al., 1996).

While polar cues embedded in the egg cell or early embryo may be important for early development of the embryo and for the proper alignment of the embryonic axis with that of the embryo sac, such cues are not absolutely required for the development of an embryo with a shoot-root axis. Ectopic embryos form from cells of the suspensor in some mutants (Vernon and Meinke, 1994). Embryos that form in this way may be oriented in the same or in the opposite direction to the embryo from which they derive. In addition, somatic embryos with a normal shoot-root axis can develop from a variety of tissue types.

Several mutants exist in which the development of the shoot or the root pole is disrupted, but in which a polar axis is nonetheless generated. For example, *monopteros*, *bodenlos* and *axr6* mutations all interfere with the development of the root pole (Hardtke and Berleth, 1998; Hamann et al., 1999; Hobbie et al., 2000). Conversely, *cup-shaped cotyledon* (*cuc*) and *shoot meristemless* (*stm*) mutations interfere with the development of the cotyledons and meristem (Barton and Poethig, 1993; Aida et al., 1997). Such genes presumably act downstream of a signal that distinguishes cells of the future root pole from cells of the future shoot pole.

In cultured cells, ratios of the plant growth regulators cytokinin and auxin have been shown to play a role in the process of specifying cells as root or shoot (Skoog and Miller, 1957). High auxin to cytokinin ratios promote root development, while high cytokinin to auxin ratios promote shoot development. It is still unknown whether such ratios play a role in the embryo. However, the observation that *axr6* mutants fail to develop a root and also fail to respond normally to auxin suggests they may (Hobbie et al., 2000).

One mutation, *doppelwurzel*, may cause a polar duplication of pattern during embryogenesis as it causes two complete seedlings to develop from a single ovule in a head to head (cotyledon to cotyledon) configuration (Jürgens et al., 1991). However, little is known about embryonic development in this mutant and it is possible that the apparent polar duplication is actually the result of the development of twin embryos from a single zygote.

We describe here a novel phenotype caused by the *topless-1* (*tpl-1*) mutation. In homozygous *tpl-1* mutants the apical pole of the embryo develops as a root instead of as a shoot. The result is a seedling with two roots joined at the base and no hypocotyl, cotyledons or shoot apical meristem. The novel *tpl-1* mutation should provide an entrance to molecular genetic study of this important, but previously experimentally intractable, decision in plant development.

MATERIALS AND METHODS

Genetics

The *tpl-1* mutation was isolated in the Landsberg erecta genetic background after EMS mutagenesis. Three backcrosses to Landsberg

erecta were performed prior to phenotypic analysis. The *tpl-1* mutation was mapped using CAPS markers as described by Konieczny and Ausubel (Konieczny and Ausubel, 1993). *tpl-1* mutant individuals were crossed to wild-type Columbia individuals. The F₁ progeny were allowed to self-fertilize at 24°C. DNA was prepared from F₂ seedlings exhibiting strong *topless* mutant phenotypes (monocot or more extreme). Three out of 268 chromosomes were recombinant between *tpl-1* and CAPS marker g2395 (recombinant frequency=1.1%). Two out of 268 chromosomes were recombinant between *tpl-1* and CAPS marker m59 (recombinant frequency=0.8%). In all cases, chromosomes recombinant between one marker and *tpl-1* were non-recombinant for the other marker, indicating that *tpl-1* lies between them.

In situ hybridization experiments

In situ hybridization and probe preparation were performed as in Long and Barton (Long and Barton, 1998). The *KNATI*, *SCR*, *STM*, *ANT* and *UFO* probes have been described by Lincoln et al. (Lincoln et al., 1995), DiLaurenzio et al. (DiLaurenzio et al., 1996), Long et al. (Long et al., 1996), Long and Barton (Long and Barton, 1998) and Lee et al. (Lee et al., 1997), respectively.

Histology

Staining of β -glucuronidase (GUS) activity was performed by incubating embryos dissected from their ovules overnight at 37°C in 2 mM 5-bromo-4-chloro-3-indolyl b-D-glucuronic acid, 100 mM NaPo₄, pH 7, 0.1% Triton X-100, 1mM FeCN and 10 mM EDTA.

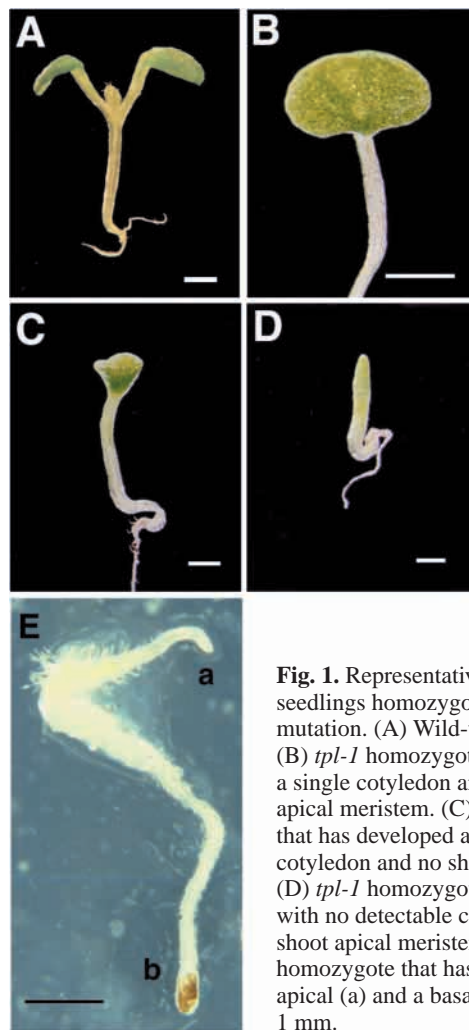


Fig. 1. Representative phenotypes of seedlings homozygous for the *tpl-1* mutation. (A) Wild-type seedling. (B) *tpl-1* homozygote that has developed a single cotyledon and lacks a shoot apical meristem. (C) *tpl-1* homozygotes that has developed a cup-shaped cotyledon and no shoot apical meristem. (D) *tpl-1* homozygote that has developed with no detectable cotyledon tissue or shoot apical meristem. (E) *tpl-1* homozygote that has developed both an apical (a) and a basal (b) root. Scale bars: 1 mm.

Table 1. The *topless-1* allele is temperature sensitive

Temperature	One or two cotyledons, no SAM*	Tube-shaped cotyledon, no SAM	SAM and cotyledons absent	Shoot replaced by a root
24°C	57% (n=254)	36% (n=158)	4% (n=17)	3% (n=12)
28°C	7% (n=25)	16% (n=61)	13% (n=49)	64% (n=246)

*Absence of SAM refers to SAM formed during embryogenesis, not to postembryonic SAM formation. Nearly all seedlings of this class are able to form a SAM within 2 weeks of germination.

Temperature shift experiments

Homozygous *tpl-1* plants were grown at 24°C until they began to flower. For each time point, 10–12 *tpl-1* flowers were emasculated and allowed to mature at 24°C for 2 days. Pollen from *tpl-1/tpl-1* plants was then crossed onto the carpels and the plants were immediately placed at either 29°C or 17°C. To determine the developmental stage of the embryos being shifted, embryos from one hand pollination were dissected and examined microscopically at the time of each temperature shift.

RESULTS

topless mutant phenotype – seedling stage

Plants carrying the *topless-1* (*tpl-1*) mutation were identified in a screen for seedlings defective in shoot apical meristem (SAM) formation (Barton and Poethig, 1993). The *tpl-1* mutation maps to the upper arm of chromosome 1 between markers g2395 and m59 (see Materials and Methods for data).

When homozygous, the *tpl-1* mutation causes either loss of apical structures or transformation of the apical pole of the embryo into a root. The *tpl-1* mutation is temperature sensitive (Table 1). Lower temperatures increase the fraction of seedlings with cotyledon tissue and decrease the fraction of double-root seedlings. Conversely, higher temperatures increase the fraction of severely mutant seedlings, especially those with two roots.

Fig. 1B–D shows homozygous *tpl-1* seedlings that fall into the ‘loss’ category. The mildest phenotype is a seedling with two cotyledons (fused to varying extents) and no SAM (not shown). Homozygous *tpl-1* seedlings may also develop a single cotyledon and no shoot apical meristem (Fig. 1B, Fig. 2B). More severely affected seedlings form a radially symmetric, cup-shaped cotyledon and lack a shoot apical meristem (Fig. 1C, Fig. 2C). Yet others appear to lack cotyledons and SAM completely, and only make a hypocotyl (Fig. 1D, Fig. 2D). (It is because of this last phenotype that the gene was named *topless*.)

Fig. 1E and Fig. 2E–F show homozygous *tpl-1* seedlings that fall into the ‘transformation’ category. In these seedlings, an apical root replaces the shoot. The apical root has a root cap and exhibits normal gravitropism. The cellular organization of the apical root is roughly similar to a wild-type root with long files of cells converging near the root tip (Fig. 3). However, the apical root is often broader and appears to incorporate more cells than the wild-type basal root (Fig. 2F, Fig. 3).

Rarely, homozygous *tpl-1* seedlings show simultaneous expression of apical and basal

characteristics. Fig. 2G shows a homozygous *tpl-1* seedling in which root hairs grow from a reduced cotyledon.

A fraction of *topless* mutant individuals are able to form a SAM by a few weeks after germination. These SAMs form at the adaxial base of the cotyledon. Such shoot apical meristems go on to form fairly normal, fertile plants which serve as a source of homozygous *tpl-1* seed. The most penetrant abnormality detected in such *tpl-1* ‘escape’ plants is a tendency for the carpels to be unfused (not shown). Nearly all *tpl* seedlings with one or two cotyledons are able to form SAMs postembryonically. *topless* mutants with cup-shaped cotyledons do so only rarely, while *tpl-1* mutants in which the apical pole is transformed into a root have never been observed to form a SAM.

Seedlings heterozygous for *tpl-1* sometimes exhibit mild fusion of the cotyledons. To determine whether this is due to a dominant, zygotically effect of *tpl-1* or to a maternal effect of

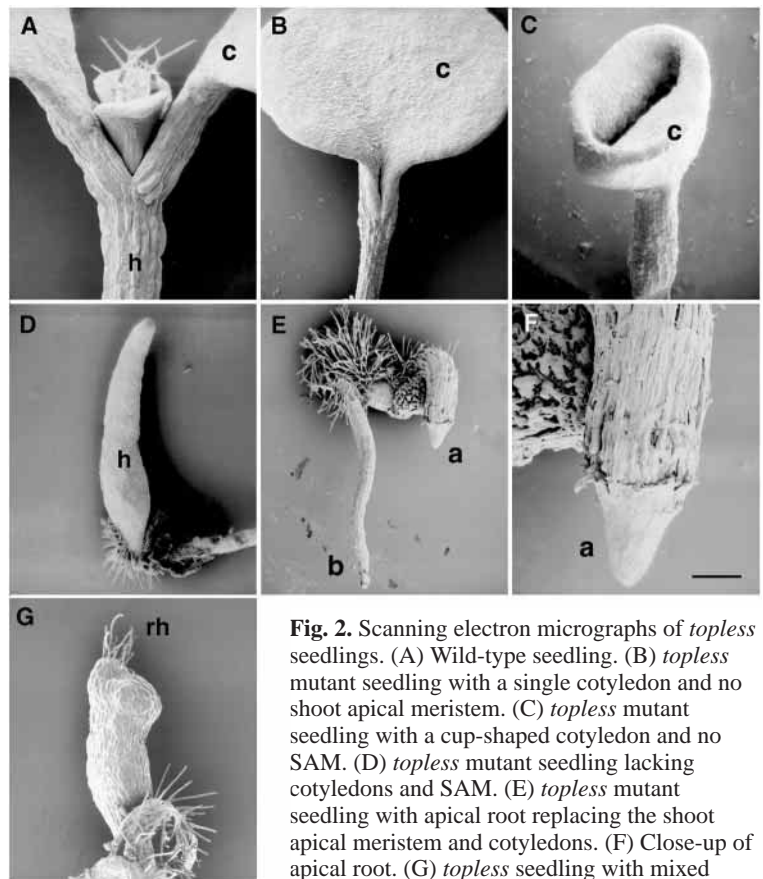


Fig. 2. Scanning electron micrographs of *topless* seedlings. (A) Wild-type seedling. (B) *topless* mutant seedling with a single cotyledon and no shoot apical meristem. (C) *topless* mutant seedling with a cup-shaped cotyledon and no SAM. (D) *topless* mutant seedling lacking cotyledons and SAM. (E) *topless* mutant seedling with apical root replacing the shoot apical meristem and cotyledons. (F) Close-up of apical root. (G) *topless* seedling with mixed fates. Root hairs are present on a reduced

cotyledon. h, hypocotyl; c, cotyledon; a, apical root; b, basal root; rh, root hairs. Scale bar: 0.2 mm in A and C; 0.37 mm in B and E; 0.43 mm in D and G; 0.1 mm in F.

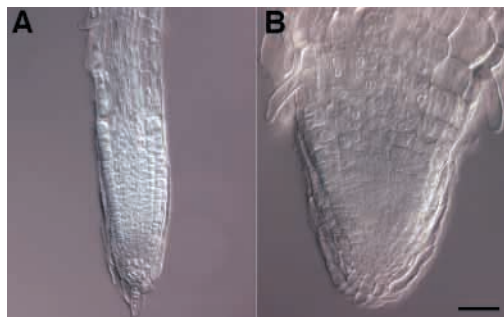


Fig. 3. DIC images of apical and basal *topless* roots. (A) Basal root. (B) Apical root. Scale bar: 0.1 mm.

the *tpl-1* mutation, reciprocal crosses were carried out. The fraction of heterozygotes showing a cotyledon fusion defect was the same whether the maternal or paternal parent was homozygous for *tpl-1* (Table 2). Thus, there is no indication of a maternal effect in these experiments. Instead, we conclude the weak cotyledon fusion defect in *tpl-1* heterozygotes is dependent on the genotype of the zygote and thus due to weak dominance of the *tpl-1* allele.

Development of *topless* mutant embryos

tpl-1 mutant embryos are initially morphologically identical to wild-type embryos. At the globular stage they are similar to wild-type embryos in size, shape and cellular organization (see Fig. 5J). By the heart stage, *tpl-1* mutant embryos differ from wild-type embryos. They are oblong and lack apparent cotyledon primordia (Fig. 4). The oblong *tpl* embryos continue to elongate during embryogenesis, accumulate storage proteins and mature.

To further determine the extent to which *tpl* mutants deviate from wild-type embryos in early development, a panel of markers for apical and basal fates was examined in mutant embryos.

Markers associated with apical fates

Mutants homozygous for *tpl-1* fail to form a SAM and frequently also lack cotyledons. These defects could be due to a failure to specify cells as SAM or cotyledon cells in the apical region of the globular embryo. Alternatively, they could be due to a failure of properly specified cells to develop into recognizable structures. To distinguish between these possibilities, we analyzed markers normally expressed in early SAM and cotyledon development in *topless* mutant embryos.

The *SHOOTMERISTEMLESS* (*STM*) gene is required for

Table 2. The *tpl1* mutation is incompletely dominant

Temperature (°C)	Female parent	Male parent	Number of progeny with fused cotyledons
29	<i>TPL/TPL</i>	<i>tpl/tpl</i>	86/390 (22%)
	<i>tpl/tpl</i>	<i>TPL/TPL</i>	100/429 (23%)
24	<i>TPL/TPL</i>	<i>tpl/tpl</i>	4/195 (2.1%)
	<i>tpl/tpl</i>	<i>TPL/TPL</i>	3/178 (1.7%)
17	<i>TPL/TPL</i>	<i>tpl/tpl</i>	2/144 (1.4%)
	<i>tpl/tpl</i>	<i>TPL/TPL</i>	3/135 (2.2%)

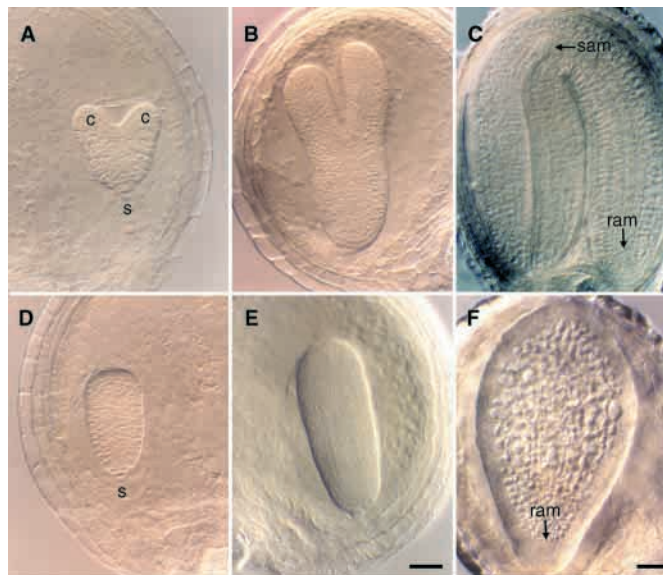


Fig. 4. DIC images of developing wild-type and *topless* embryos. All seedlings developed at 29°C. (A) Wild-type heart stage embryo. (B) Wild-type torpedo stage embryo. (C) Wild-type mature embryo. (D) *topless* heart stage embryo. (E) *topless* torpedo stage embryo. (F) *topless* mature embryo. c, cotyledon; s, suspensor; sam, shoot apical meristem; ram, root apical meristem. Scale bars: in E, 25 µm for A,B,D,E; in F for 25 µm C,F.

initiation and maintenance of the SAM (Barton and Poethig, 1993; Long and Barton, 1998). In wild-type embryos, *STM* RNA marks the developing SAM and is first detected at the late globular stage, before there is morphological evidence of a shoot apical meristem. In homozygous *tpl-1* embryos, the *STM* transcript is not detected ($n>50$; Fig. 5B). Wild-type siblings segregating in the same silique and processed along with the *tpl-1* embryos exhibited normal *STM* expression (Fig. 5A). *tpl-1* embryos accumulate transcripts related to cellular functions, such as the *UBIQUITIN* transcript, which could be detected throughout the *tpl-1* embryo (Fig. 5D).

UFO is also expressed in the SAM during embryogenesis. Accumulation of the *UFO* transcript is dependent on wild-type *STM* function (Long and Barton, 1998). Given the failure of *STM* expression in *tpl-1* mutants, we would expect *UFO* transcript also to be missing. Indeed, *UFO* mRNA fails to accumulate in *tpl-1* mutants ($n>50$; Fig. 5C). As in the *STM* experiment, control wild-type embryos processed simultaneously showed normal *UFO* expression (not shown).

Finally, the distribution of a transcript ordinarily found in the developing hypocotyl was examined. *KNATI* is normally expressed in peripheral regions of the hypocotyl (Fig. 5G). In *tpl-1* mutants, *KNATI* transcript is either missing or only faintly expressed (Fig. 5H). Loss of *KNATI* transcript accumulation is somewhat surprising as *tpl-1* mutant seedlings grown at 24°C usually make a structure recognizable as a hypocotyl. The expression data suggest that the program for hypocotyl development may be somewhat impaired even at this relatively permissive temperature.

The above experiments were done at 24°C, a temperature that does not cause a particularly large number of double root seedlings (Table 1). The experiments have not been carried out

at 29°C. However, given that there is even less development of apical structures at 29°C, it is unlikely that expression of *STM*, *UFO* or *KNATI* would be restored at the higher temperature.

The experiments with the *UFO*, *STM* and *KNATI* markers indicate that the failure of apical structures to develop in *tpl-1* mutants is due to a failure to specify apical (shoot) fates, rather than a failure of properly specified domains to grow and develop into morphologically recognizable structures.

The *AINTEGUMENTA* (*ANT*) RNA marks developing leaf, floral organ and cotyledon primordia (Elliott et al., 1996; Long and Barton, 1998). In the wild-type embryo *ANT* is first expressed in the globular stage embryo prior to any morphological evidence of cotyledon initiation. The two cotyledon primordia appear to be specified initially as a single donut-shaped primordium encircling the apical half of the globular embryo (Fig. 5E). In *tpl-1* mutants, by contrast, *ANT* transcript is found in the center of the apical pole of the embryo in addition to the peripheral ring ($n > 200$; Fig. 5F). *ANT* transcript was also found in *topless* embryos developing at the most restrictive temperature (29°C). However, at this temperature, it is often confined to a more apical region: the outermost (protodermal) layer (not shown).

The experiments with the *ANT* marker indicate that some cells are specified as cotyledon in *tpl-1* mutants, even at the most restrictive temperature. However, the pattern of cells specified is altered: the altered pattern shows changes both in the apical/basal dimension of the embryo as well as in the radial (or central/peripheral) dimension.

Markers associated with basal fates

Homozygous *tpl-1* mutant embryos developing at 29°C show a high frequency of apparent shoot to root transformation. To further investigate the development of such double root embryos, we analyzed the expression of three markers for root cell fates. The *SCARECROW* transcript accumulates in the lenticular cell and in the cortical cell files (Fig. 5I) (DiLaurenzio et al., 1996). In the wild-type embryo, *SCR* expression is limited to the region below the O' boundary. (The O' boundary divides the globular stage embryo into apical and basal halves. It is created when the four cells of the quadrant stage embryo divide to generate the octant stage embryo.) In *tpl-1* mutant embryos, *scr* extends above the O' boundary, indicating that root and/or hypocotyl fates extend further apically in *topless* mutant embryos (Fig. 5J).

The *LENNY* reporter was isolated in a screen of enhancer trap lines. [The approach used has been described by Sundaresan et al. (Sundaresan et al., 1995).] The *LENNY* reporter expresses GUS activity in the root pole of the developing embryo and not in the shoot pole (Fig. 6A,B). *LENNY* is initially expressed in the lenticular cell (a descendent of the hypophysis) beginning at the heart stage. Expression later spreads to encompass surrounding cells in the root apical meristem.

Twenty-one percent (16/76) of homozygous *topless* mutant embryos developing at 29°C show expression of the *LENNY* reporter at both poles of the embryo by 168 hours postfertilization (Fig. 6D-F). (Wild-type embryos grown at 29°C are typically at bent cotyledon

stage by 168 hours.) The extent to which embryos express the *LENNY* reporter varies. Some embryos show only a weak area of *LENNY* expression at the apical pole (Fig. 6D). More commonly, both poles express *LENNY* with near equal intensity (Fig. 6E,F).

Note that the apical root primordium does not have the same cellular organization as the normal, basal, root primordium. For example, the files of cells that make up the developing vasculature are not apparent in the *tpl* apical root (Fig. 6E,F). In addition, the apical root primordium is not tapered as in a normal root but instead is much wider.

A small fraction (less than 5%) of *tpl* embryos developing at 29°C arrest during embryogenesis. The arrested embryos express *LENNY* throughout a greater fraction of the embryo than do the non-arrested embryos (Fig. 6G,H). One interpretation of this arrested phenotype is that it reflects an early and more complete transformation of apical to basal fates and that such a transformation results in the arrest of embryonic growth. However, it is also possible that a second, unrelated mutation is responsible for the arrest or that the *topless* mutation interferes with the viability of early embryos in a way that is unrelated to the specification of apical or basal fates.

The J1092 marker is expressed in the embryo in presumptive root cap cells (Fig. 6C) (described by J. Haseloff, <http://www.plantsci.cam.ac.uk/Haseloff/Home.html>). Thirty seven percent (15/41) of homozygous *topless* mutant embryos developing at 29°C show expression of the J1092 reporter at both poles of the embryo by 168 hours postfertilization (Fig. 6J,K).

In summary, markers for basal root fates were often found expressed in the region of the developing apical root of *topless* mutants. Taken together with the experiments with apical

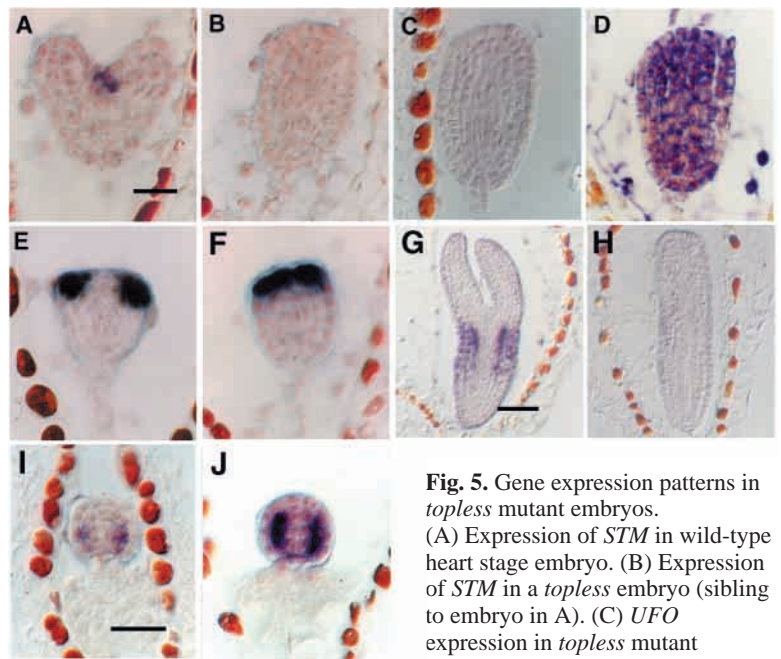


Fig. 5. Gene expression patterns in *topless* mutant embryos. (A) Expression of *STM* in wild-type heart stage embryo. (B) Expression of *STM* in a *topless* embryo (sibling to embryo in A). (C) *UFO* expression in *topless* mutant embryo. (D) *UBIQUITIN*

expression in *topless* mutant embryo. (E) *ANT* expression in wild-type heart stage embryo. (F) *ANT* expression in *topless* mutant. (G) *KNATI* expression in wild-type walking stick stage embryo. (H) *KNATI* expression in *topless* embryo. (I) *SCARECROW* expression in wild-type globular embryos. (J) *SCARECROW* expression in *topless* mutant embryos. Scale bars: in A, 25 μ m for A-F; in G, 50 μ m for G,H; in I, 25 μ m for I,J.

markers, we conclude that there is a transformation of the apical region of the embryo from a shoot 'primordium' into a root 'primordium' in *topless* mutant embryos.

Expression of the DR5 auxin reporter in *topless* mutants

A high auxin to cytokinin ratio will promote root development over shoot development in cultured cells (Skoog and Miller, 1957). One simple explanation for the mechanism of action of the *topless* mutation is that it increases the auxin concentration in apical regions of the embryo.

The activity of the DR5::GUS construct appears to reflect high levels of free auxin in the root (Sabatini et al., 1999). This synthetic promoter consists of seven tandem repeats of an element found in auxin responsive promoters (Ulmasov et al., 1997a). This sequence binds to a family of AUXIN RESPONSE FACTORS (ARF) thought to be involved in transducing the auxin signal. Sabatini et al. (Sabatini et al., 1999) have shown that this reporter is active in the presumptive root in the late heart stage embryo.

If the *tpl-1* mutation acts to increase auxin levels in the apical region of the embryo and this, in turn, causes the apical pole to develop root fate, we might expect to see an early and broad accumulation of GUS activity in the apical regions of *topless1*; DR5::GUS embryos. To test this hypothesis, we allowed *topless1*; DR5::GUS embryos to develop at restrictive temperature (29°C) and assayed them for GUS expression at 168 hours post-fertilization. While all embryos expressed the reporter in the basal root, only two out of 100 embryos expressed GUS in the developing apical root of the *topless* mutant embryos at this timepoint. Recall that a much larger proportion of *topless* embryos express the J1092 (rootcap) and LENNY reporters by this point (see above). Hundreds of *topless*; DR5::GUS embryos younger than this stage were examined and none were found to express GUS in the developing apical root. When expression was observed in the apical root, it was found only in small, randomly located, patches (Fig. 6I). Because the DR5::GUS transgene is normally active in the tips of cotyledons later in embryogenesis, we do not know if this patchy accumulation marks cells because they are presumptive root cells or because they are presumptive cotyledon tip cells. In either case, this finding argues against the simple hypothesis that the *tpl-1* mutation mediates apical root development by increasing auxin levels in the apical pole of the embryo.

The *topless-1* mutation partially suppresses the monopteros mutant phenotype

The *MONOPTEROS* gene encodes a member of the ARF family of transcription factors and is thought to be responsible for transducing auxin signals crucial for root development (Hardtke and Berleth, 1998). *monopteros* mutants fail to develop a root and most of the hypocotyl during embryogenesis.

MONOPTEROS encodes a protein that has a DNA-binding domain at its N terminus, and a dimerization domain at its C terminus (Hardtke and Berleth, 1998). Between these two domains is a predicted activation domain. The *rootless* mutation (Barton and Poethig, 1993) is an EMS-induced allele of *MONOPTEROS*. PCR amplification of the *MONOPTEROS*-coding sequence in *rootless* mutants showed that these mutants carry a stop codon at position 594 of the polypeptide [Q594

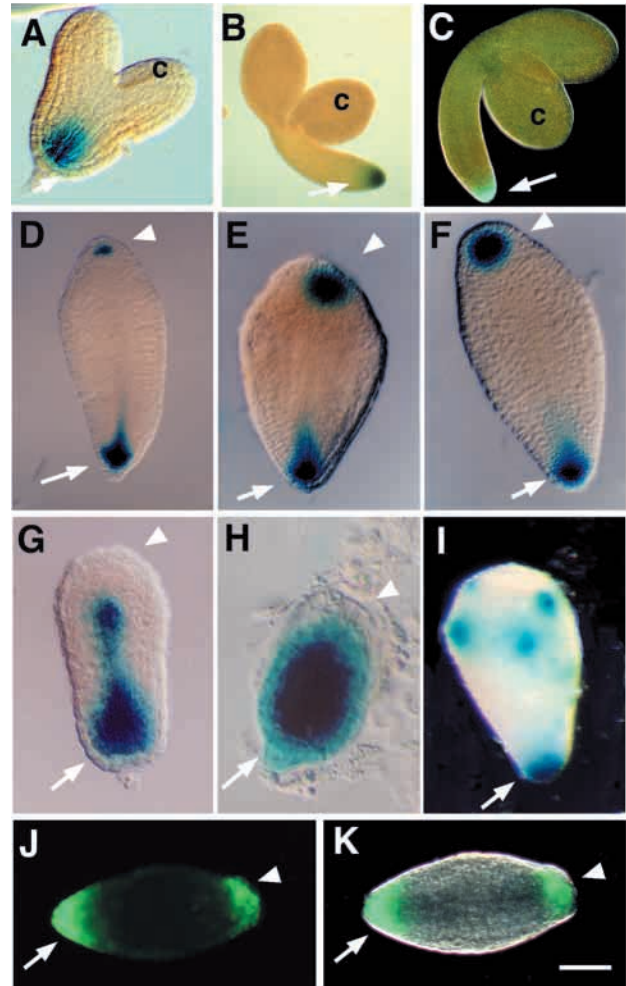


Fig. 6. Expression of the *LENNY* (lenticular cell), *DR5* (auxin maximum) and *J1092* (root cap) markers in wild-type and *topless* mutant embryos. (A) Wild-type heart stage embryo expressing the *LENNY* reporter in the lenticular cell and adjacent cells. (B) Wild-type mature stage embryo expressing the *LENNY* reporter. (C) Wild-type mature stage embryo expressing the *J1092* reporter expressed in the root cap. (D) *topless* embryo showing a weak signal for the *LENNY* reporter at the apical pole. (E) *topless* embryo with *LENNY* expression at both poles. Note that the apical pole is wider and does not yet show root-like cellular organization. (F) *topless* embryo with *LENNY* expression at both poles. (G) *topless* embryo arrested as an elongated embryo. Notice the two foci of *LENNY* expression connected to each other. (H) *topless* embryo arrested at globular stage. *LENNY* reporter is expressed throughout. (I) A rare *tpl-1* embryo that expresses the *DR5* reporter in the apical region of the embryo. In these rare individuals, expression is typically patchy. (J) Expression of the *J1092* root cap marker in a homozygous *tpl-1* mutant embryo. (K) Image in J overlaid on Nomarski image of embryo. Scale bars: 25 μ m in A,G,H; 65 μ m in B,C; 50 μ m in D-F,I-K.

(CAG) to STOP (TAG)]. This is downstream of the DNA-binding domain and upstream of the C-terminal dimerization domain. We have renamed this allele *monopteros-7* (*mp-7*).

To determine if the *topless-1* mutation acts through the monopteros pathway, we created mutants homozygous for both *tpl-1* and *mp-7* (Fig. 7). Self progeny from a plant of genotype *tpl-1 mp-7/tpl-1 MP⁺* were scored. (Both genes are located on

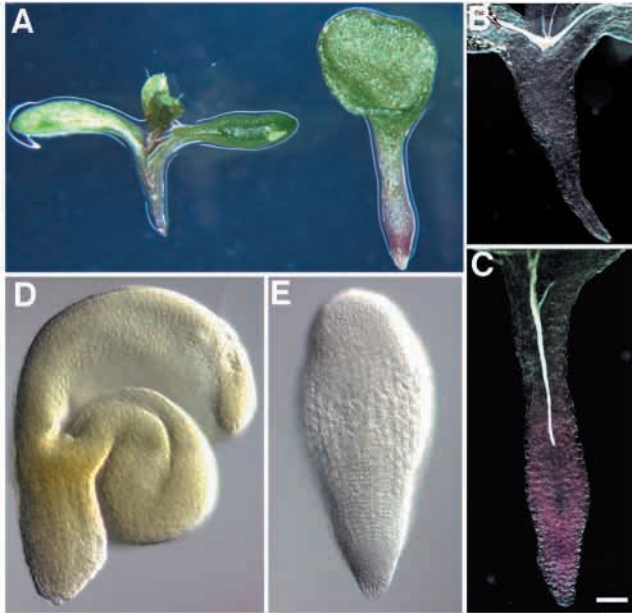


Fig. 7. Phenotypes of mutants homozygous for both *tpl-1* and *mp-7*. (A) Seedling to left is *mp-7* homozygote. Seedling to right is *mp-7 tpl-1* homozygote. Both seedlings developed at 24°C. The double mutant has a more well-developed hypocotyl than the *mp-7* single mutant. (B) Cleared *mp-7* single mutant (grown at 24°C) showing lack of vasculature in the hypocotyl 'stub'. (C) Cleared *mp-7 tpl-1* double mutant (grown at 24°C) showing vasculature extending down into the more well developed hypocotyl. (D) Homozygous *mp-7* single mutant grown at 29°C. (E) Homozygous *mp-7 tpl-1* double mutant grown at 29°C. The double mutant is able to develop an apical root. Scale bar: 200 µm in A; 100 µm in B,C; 50 µm in D,E.

chromosome 1.) At both 24 and 29°C, a quarter of the self-progeny lacked roots and were assumed to be the *tpl-1 mp-7/tpl-1 mp-7* double mutants. Thus, the *tpl-1* mutation did not restore the ability to make a basal root to *monopteros* mutants. By contrast, the *tpl-1 mp-7* double mutants made apical roots similar to *tpl-1* single mutants (at 29°C). Thus, basal root formation is dependent on wild-type *MONOPTEROS* activity in *topless* mutants but apical root formation is not.

The *tpl-1* mutation partially suppresses the hypocotyl defect seen in *monopteros* mutants: *tpl-1 mp-7* double mutants were able to form much more hypocotyl tissue than *mp-7* single mutants (Fig. 7). Whereas only 9% (8/86) of *monopteros* single mutants made any detectable vasculature in the basal 'stub' typically found in *mp* mutants, 97% (102/105) of *tpl-1 mp-7* mutants showed the presence of hypocotyl-associated vascular development (Fig. 7; this experiment was performed at 24°C). The partial suppression of the *monopteros* hypocotyl defect by *tpl-1* shows that the *tpl-1* mutation can have effects on basal portions of the developing embryo and that mutant *tpl-1* activity can partially bypass the requirement for *MONOPTEROS* activity in the development of the hypocotyl and its vasculature.

Temperature shift experiments show that the *tpl* mutation can mediate its effects late in embryogenesis

The temperature sensitivity of the *tpl-1* mutation allows for

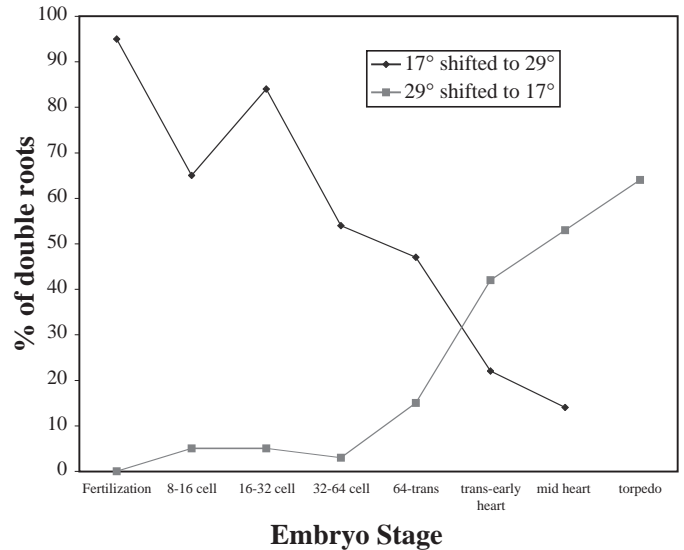


Fig. 8. Effect of temperature shift on *topless* embryo development. Number of embryos that developed an apical root when shifted from 29°C to 17°C at fertilization, 0/126; at 8-16 cells, 6/127; at 16-32 cells, 8/154; at 32-64 cells, 4/126; at 64 cells to transition stage, 26/175; at transition to early heart stage, 43/103; at midheart stage, 48/91; at torpedo stage, 74/116. Number of embryos that developed an apical root when shifted from 17°C to 29°C at fertilization, 142/149; at 8-16 cells, 93/143; at 16-32 cells, 113/134; at 32-64 cells, 46/85; at 64 cells to transition stage, 92/195; at transition to early heart stage, 31/143; at midheart stage, 18/127.

temperature shift experiments to determine at what point the *tpl* mutation affects the development of the apical pole of the embryo. For this experiment, homozygous *tpl-1* plants were grown at 24°C. They were then pollinated and placed at 29°C or 17°C. Embryonic stages at the time of shift were determined by dissecting embryos from siliques pollinated and shifted at the same time as the experimental cohort.

Embryos allowed to develop at 17°C from fertilization onwards did not develop apical roots (Fig. 8). Embryos that experienced high temperature early in embryogenesis, but developed at 17°C from the 32-cell stage and onwards, developed a small fraction of double root seedlings. This indicates that *tpl* mutant activity, or synthesis of mutant *TOPLESS* product, during early stages of embryogenesis has the potential to affect development in an irreversible way. A majority of embryos shifted from 29°C to 17°C at the torpedo stage developed apical roots, indicating that the shift to permissive temperature was often not sufficient to restore normal shoot development after this point.

The experiment in which embryos were shifted from 17°C to 29°C at varying time points gave largely reciprocal results. Nearly all of the embryos that developed at 29°C from fertilization and on developed as double root embryos (Fig. 8). Shifting to 29°C during the 16-32 cell stage also resulted in a majority of double root seedlings, indicating that *topless* mutant activity or synthesis could alter fates at the globular stage. Surprisingly, shifting embryos from 17°C to 29°C as late as the transition to midheart stage could cause many of the embryos to make apical roots. This is surprising because transition and heart stage embryos express normal apical

(shoot) fates not only at the molecular level but also in the development of structures such as cotyledon primordia.

While the results are largely complementary, there are nevertheless differences in the two shift experiments that suggest the embryos are biased toward normal development. For example, an embryo shifted from 29°C to 17°C from midheart onwards has a roughly 50% chance of developing cotyledon or SAM fates at the apical pole. However, an embryo shifted from 17°C to 29°C from midheart on has an 85% chance of developing cotyledon or SAM fates at the apical pole.

DISCUSSION

Homozygous *topless* mutant embryos exhibit transformation of shoot to root at the apical pole

The *tpl-1* mutation causes dramatic changes in the development of the *Arabidopsis* embryo. The most dramatic and novel *topless* phenotype is seen at high temperature where embryos develop apical roots in place of shoot apical meristem and cotyledons. The apical root that develops has a recognizable root cap, exhibits normal gravitropism, has root hairs and shows generally normal morphology, although it may be wider than the wild-type root. In agreement with these gross characters, developing *tpl-1* embryos show changes in pattern formation as detected with molecular markers for regional identities.

To our knowledge, this is the first example of a mutation that causes the transformation of apical into basal fates in the embryo of any plant species. The double root phenotype indicates that the *tpl-1* mutation can bypass the requirement for any basal, root promoting, positional cues laid down in the egg cell or in the early zygote. Analogous mutations that cause transformation of basal into apical fates have not yet been discovered but the existence of the *tpl-1* mutation suggests the existence of a master regulatory network that can switch relatively cleanly between apical and basal fates.

At lower temperatures, the seedlings are missing the shoot apical meristem and/or the cotyledons normally found at the apical pole. The SAM is more likely to be missing than are the cotyledons. Consistent with this, expression of the SAM markers *STM* and *UFO* was completely absent in all mutants, even at 24°C. Thus, *tpl* mutants are unable to specify SAM identity during embryogenesis. By contrast, *topless* mutant embryos appear to be able to specify cotyledon identity as *ANT* expression, although somewhat abnormal in pattern, is present. Surprisingly, *ANT* was even found to be present in embryos developing at 29°C, even though most of these embryos develop apical roots in place of cotyledons.

The abnormal pattern of *ANT* expression in *topless* mutant embryos, expression throughout the apical dome instead of only in a peripheral ring, indicates that pattern formation is defective not only in the apical-basal dimension but also in the radial dimension.

As loss of *STM* function is known to cause cotyledon fusion, the cotyledon defects may in part be a result of failure of homozygous *tpl-1* seedlings to accumulate *STM* product. However, the severity of cotyledon fusion in *tpl-1* seedlings can go well beyond what is seen in *stm* mutants, indicating that the regulation of other genes must be affected. Candidates include

the *CUP-SHAPED COTYLEDON 1* and *CUP-SHAPED COTYLEDON 2* genes; when simultaneously mutant, *cuc1* and *cuc2* cause the development of a cup-shaped cotyledon, failure to express *STM* and failure to form a SAM (Aida et al., 1997; Aida et al., 1999). This phenotype is seen in some *tpl-1* mutants. It will be interesting to find out whether *tpl-1* mutants are affected in *CUC* expression.

The effect of *tpl-1* on the development of the hypocotyl is more subtle. With the exception of the double root seedlings, *tpl-1* mutant hypocotyls appear superficially normal. However, developing *topless* embryos show greatly reduced *KNAT1* transcript, a transcript normally specific to the developing hypocotyl. (Failure to express *KNAT1* by *tpl-1* mutant embryos is not a secondary consequence of failure to form a SAM, as *stm* mutants are able to express *KNAT1* (J. L. and M. K. Barton, unpublished). Additional evidence for action of the *tpl-1* mutation in the region of the developing hypocotyl is seen in the ability of the *tpl-1* mutation to partially restore hypocotyl development in *monopteros* mutants.

Polarity in the embryo remains susceptible to *tpl* mutant activity until the heart stage

Temperature-shift experiments of developing *topless* embryos show that the probability of apical root development can be influenced by temperature even after cotyledons have begun to develop. Thus, a significant number (20%) of seedlings will develop apical roots when *topless* embryos are shifted to restrictive temperature at the transition to early heart stage, a stage when the presumptive cotyledons are beginning to grow out. This result strongly suggests that cells initially developing with apical fates are redirected into root fates in *tpl* embryos. Several other observations are consistent with this as well. First, *ANT*, a cotyledon marker, is expressed in all *topless* embryos even in conditions in which the embryos will develop apical roots. Second, the development of the apical root seems to occur later than that of the primary root in many *topless* embryos. This is indicated by our finding that despite the fact that a majority of *topless* embryos develop into double-root seedlings, a minority of *topless* embryos show expression of root specific molecular markers by 168 hours (7 days) post-fertilization. Finally, the *tpl-1* apical root appears to derive from a larger group of cells than does a normal root. In the embryo, the *tpl* apical root is morphologically broader than a normal root and lacks the cellular organization of a normal root. Development from a larger group of cells is expected if the apical root develops from the more expanded apical region of a transition stage embryo.

Given the apparent respecification of shoot structures into root structures at the apical pole of at least some *topless* embryos, it will be interesting to learn whether the shoot to root transformation requires first dedifferentiation followed by redifferentiation, or whether apical cells switch fates directly, without undergoing an intervening stage of dedifferentiation.

One can speculate that the reason the double root embryos survive to be recognized at all is, in fact, because they undergo such a re-specification. If the apical pole of the globular embryo is mis-specified as 'basal' from the outset, the resulting 'double-basal' embryo may fail to develop sufficiently to complete embryogenesis. For example, failure to establish polarity along the apical/basal axis correctly might prevent growth of the embryo. The observation of arrested embryos

expressing root markers throughout the embryo is one line of evidence for such an effect. However, it will be crucial to determine that this arrest is due to the *tpl-1* mutation and not to a second unrelated mutation in this genetic background.

Experiments fail to link the *topless 1* mutation to alterations in auxin signaling

It has long been recognized that a high auxin to cytokinin ratio will promote root development over shoot development in cultured cells (Skoog and Miller, 1957). One possibility is therefore that *TPL* interferes with the perception of auxin to cytokinin ratios. *tpl* mutant embryos could have altered distribution patterns of these hormones, increased sensitivity to auxin or reduced sensitivity to cytokinins.

Our results do not support the simple hypothesis that the *topless-1* mutation increases auxin levels in the apical pole of the embryo and thereby causes apical root development. If this were true, we would have expected to see early and ectopic expression of the *DR5::GUS* reporter; expression of this reporter is believed to reflect high auxin concentrations in the root (Sabatini et al., 1999). Instead, while a significant fraction of *topless 1* embryos had begun to differentiate root fates by 168 hours postfertilization (as determined by the *J1092 GFP* and *LENNY* reporters), only a small fraction had begun to express the *DR5::GUS* promoter. One problem with the interpretation of this experiment is that the *DR5::GUS* promoter may not function as a reliable reporter for high free auxin levels in apical regions of the embryo.

The *MONOPTEROS* gene product has been implicated in auxin action in the developing root (Hardtke and Berleth, 1999). *MONOPTEROS* is required for the proper development of vasculature in the embryo and for the development of basal (hypocotyl and root) fates during embryogenesis. Many classical studies have indicated that auxin promotes both vascular and root development when exogenously applied. The finding that the *MONOPTEROS* gene product is a transcription factor that is similar to transcription factors believed to transduce auxin signals is further evidence that *MONOPTEROS* promotes vascular and root development through an auxin-regulated mechanism.

The *MONOPTEROS* gene product was not necessary for the formation of the apical root in *topless* mutant embryos. This is similar to the finding that *MONOPTEROS* is not required for ectopic root formation from the hypocotyl and cotyledons in *superroot* mutants (M. K. B., unpublished), the difference being that the apical root in *topless* mutants forms during embryogenesis, while the ectopic roots in *superroot* mutants form postembryonically. As there are many ARF-like genes in the *Arabidopsis* genome, it is possible that one or more homologous genes substitute for *MONOPTEROS* function when roots are formed from apical regions of the plant. Alternatively, *topless* may bypass the requirement for *ARF-LIKE* genes in apical root formation. By contrast, *topless* could not bypass the requirement for *MONOPTEROS* in the formation of the basal root.

topless monopteros double mutant individuals make more hypocotyl tissue than *monopteros* single mutants, indicating that the *topless 1* mutation partially suppresses the monopteros phenotype. This could indicate that the *tpl-1* mutation activates a developmental pathway that can substitute in part for *MONOPTEROS* activity in hypocotyl development. Alternatively, the *topless* mutation may boost the activity of

whatever residual *MONOPTEROS* activity exists in the *monopteros-7* mutant.

In summary, the relationship of *topless* mutant activity to auxin signaling in root development remains unclear. Preliminary observations of *axr1 topless* double mutants show only additive interactions (J. L., unpublished), indicating that the two processes may indeed be independent of one another. Consistent with the notion of independent action of *topless* and auxin signaling, treatment of developing Indian mustard embryos with auxin transport inhibitors resulted in ball- or tube-shaped seedlings, but not in double rooted seedlings, as seen in *topless* mutants (Liu et al., 1993).

An additive action of *topless* and auxin signaling could also explain the temperature sensitive nature of the *tpl-1* mutation. Gray and co-workers have described auxin-dependent processes that are enhanced by high temperature (Gray et al., 1998). In addition, they measured higher levels of auxin in plants grown under high temperatures than under low temperatures. Thus, a combination of a mutant *tpl* gene product plus enhanced sensitivity to auxins and/or higher auxin levels at high temperatures might act in combination to explain the temperature sensitivity of the *topless* phenotype.

The role of *TOPLESS* in wild-type embryo development

The *TOPLESS* locus is currently represented by only one mutant allele. This allele is temperature sensitive and also shows a weak dominant effect. Additional screens for *topless*-like mutants have failed to turn up more alleles of the *TPL* locus, indicating that the change in *tpl-1* is likely to be a rare type of mutation (J. L. and M. K. B., unpublished).

Given the unique nature of this allele, it is difficult to determine the wild-type function of *TPL*. Several roles are possible, depending on the nature of the *tpl-1* mutation. If *tpl-1* is a loss-of-function or dominant negative mutation, the wild-type role of *tpl-1* is to confer shoot identity (or repress root identity) to the apical pole. However, if the mutation elevates wild-type *TOPLESS* activity or makes *TOPLESS* activity spatially or temporally constitutive, the wild-type role would be to confer root fate to the basal pole. Given the difficulty in obtaining new alleles, the molecular cloning of the *TOPLESS* locus is likely to be the most direct route to determining its wild-type function.

Rare dominant alleles of genes are difficult to obtain except through the use of efficient point mutagens such as EMS. Such alleles provide important clues to gene function that are easily missed in standard reverse genetic studies that rely on 'knock-out' or overexpression strategies. Examples of rare dominant mutations that disrupt regulation of protein products are those in the *ETR1* (ethylene resistant 1) and *PHABULOSA* genes (Bleecker et al., 1988; Chang et al., 1993; McConnell and Barton, 1998). Interestingly, both of these gene products appear to act as receptors. Whatever role *TOPLESS* plays in wild-type development, understanding and manipulating the *tpl-1* gene should lead to insights in embryonic pattern formation, as well as the ability to manipulate the generation of new root apical meristems from shoot tissue.

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