An expanded role for the *TWN*1 gene in embryogenesis: defects in cotyledon pattern and morphology in the *twn*1 mutant of *Arabidopsis* (Brassicaceae)[1]

Daniel M. Vernon,[2] Michael J. Hannon, MyPhuong Le, and Nancy R. Forsthoefel

Department of Biology, Whitman College, Walla Walla, Washington 99362 USA

The suspensor is a specialized basal structure that differentiates early in plant embryogenesis to support development of the embryo proper. Suspensor differentiation in *Arabidopsis* is maintained in part by the *TWIN*1 (*TWN*1) gene, which suppresses embryogenic development in suspensor cells: *twn*1 mutants produce supernumerary embryos via suspensor transformation. To better understand mechanisms of suspensor development and further investigate the function of *TWN*1, we have characterized late-embryo and post-embryonic development in the *twn*1 mutant, using seedling culture, microscopy, and genetics. We report here that the *twn*1 mutation disrupts cotyledon number, arrangement, and morphology and occasionally causes partial conversion of cotyledons into leaves. These defects are not a consequence of suspensor transformation. Thus, in addition to its basal role in suspensor differentiation, *TWN*1 influences apical pattern and morphology in the embryo proper. To determine whether other genes can similarly affect both suspensor and cotyledon development, we looked for twinning in *Arabidopsis* mutants previously identified by their abnormal cotyledon phenotypes. One such mutant, *amp*1, produced a low frequency of twin embryos by suspensor transformation. Our results suggest that mechanisms that maintain suspensor identity also function later in development to influence organ formation at the embryonic shoot apex. We propose that *TWN*1 functions in cell communication pathways that convey local positional information in both the apical and basal regions of the *Arabidopsis* embryo.

Key words: *Arabidopsis*; cotyledon; embryogenesis; morphogenesis; pattern formation; shoot apical meristem; suspensor; twinning.

The suspensor is a basal structure formed early in angiosperm embryogenesis. Although it is zygotically derived and thus part of the plant embryo, it is usually distinguished from the "embryo proper" (EP), the apical structure that gives rise to most of the mature embryo and adult plant. Long thought to be important primarily for physically positioning the EP within the seed, the suspensor is now known to play a more active role in early plant development, supporting growth of the embryo proper through absorption and transport of nutrients and by synthesis of growth regulators (reviewed by Yeung and Meinke, 1993; Schwartz, Vernon, and Meinke, 1997).

Suspensor formation involves processes of central importance to embryogenesis, including the establishment of embryo polarity, early cell differentiation, and programmed cell death; it is therefore interesting from a developmental biology perspective. In most species, the suspensor develops from the basal cell produced after the first zygotic division, and its specification likely reflects morphogenetic apical–basal polarity present in the zygote itself (Schwartz, Vernon, and Meinke, 1997). The suspensor rapidly completes development to carry out its physiological functions and is the first specialized structure to differentiate in most plant embryos. Later in develop-

ment, suspensor degeneration provides the earliest example of developmentally programmed cell death in plant development.

Arabidopsis thaliana has become an important system for genetic studies of plant development, including many aspects of plant embryogenesis (Goldberg, de Pavia, and Yadegari, 1994; Meinke, 1995). In *Arabidopsis* the suspensor is specified in the two-cell proembryo, after the zygote divides asymmetrically to form an elongate basal cell and a smaller apical terminal cell (Mansfield and Briarty, 1991; West and Harada, 1993). Soon after its formation, the basal cell undergoes a rapid series of divisions to produce a suspensor consisting of a single column of six to eight cells. The suspensor is fully formed by the early globular stage of embryogenesis and maintains its structure until it begins degenerating during the linear stage (West and Harada, 1993; Yeung and Meinke, 1993).

Characterization of *Arabidopsis* developmental mutants has established that interaction between the suspensor and EP is of central importance for suspensor development: many genetic defects that disrupt the embryo-proper trigger abnormal development in the suspensor. Three classes of *Arabidopsis* mutants with abnormal suspensors have been described in detail. In one class, consisting of the *sus* and *rsp* mutants, aberrant EP development is followed by suspensor cell proliferation and at least partial transformation, resulting in development of inviable cell masses that resemble the mutant embryo proper (Schwartz, Yeung, and Meinke, 1994; Yadegari et al., 1994). The second class of suspensor mutants, the *twin* (*twn*) mutants, can produce viable secondary embryos via embryogenic transformation of the suspensor and thus give rise to an abnormally high percentage of seeds containing twin embryos (Vernon and Meinke, 1994; Zhang and Somerville,

1997). One such mutant, *twn*2, has been characterized at the molecular level: the phenotype is caused by a unique regulatory mutation that eliminates expression of an essential valyl-tRNA synthase gene in the EP, but not in the suspensor. As a result, the *twn*2 EP degenerates early in development, but suspensor cells survive, enter into embryogenic development, and form one or more embryos (Zhang and Somerville, 1997). It is clear from the *sus, rsp,* and *twn*2 phenotypes that cells of the suspensor have embryogenic potential, and that this potential is normally suppressed by interaction with the EP. This interpretation is consistent with classical studies of EP–suspensor interaction in a variety of species (Haccius, 1955, 1963; Yeung and Meinke, 1993). A third class of embryo-defective mutants, the late embryo mutants, exhibit belated suspensor degeneration relative to wild type, due to delayed or impaired development in the embryo proper (Vernon and Meinke, 1995). Thus, many aspects of suspensor development, including cell proliferation, embryogenic potential, and even the onset of cell death, appear to be influenced by the embryo proper.

Despite the importance of EP–suspensor interactions, little is known about the nature of cell communication between these two parts of the plant embryo. One mutant that may provide clues is *twn*1. In ~8% of homozygous mutant seeds, *twn*1 produces twin embryos by embryogenic transformation of suspensor cells, while exhibiting only occasional and variable defects in the early EP (Vernon and Meinke, 1994). In contrast to *twn*2, suspensor transformation in *twn*1 is not triggered by death of the embryo proper. Rather, twinning occurs in the presence of a viable, and often morphologically normal, EP. Thus, in *twn*1, suspensor transformation does not appear to be an indirect consequence of arrested development in the EP, as is the case with other suspensor mutants. Based on these observations, Schwartz, Yeung, and Meinke (1994) proposed that *TWN*1 functions directly in EP–suspensor cell communication.

Three general models of *TWN*1 activity have been proposed to explain twinning and the occasional morphological defects observed in *twn*1 embryos (Vernon and Meinke, 1994). In all three models, suspensor transformation occurs in the mutant because EP–suspensor communication is compromised and suspensor development is not properly inhibited. In the first model, *TWN*1 is active in the suspensor and is required for proper response to growth-inhibiting signal(s) from the embryo proper. In the second model, *TWN*1 is proposed to act in the embryo proper to suppress (directly or indirectly) embryogenic development in the neighboring suspensor. The third model proposes that *TWN*1 may have more than one developmental function and act in different embryo regions, affecting suspensor and EP development separately. One prediction of this third model is that the *twn*1 EP should show developmental defects that cannot be attributed to defects in the suspensor and that occur separately of twinning in *twn*1 homozygotes.

To better define *TWN*1 gene function and distinguish between the models described above, we have further characterized the *twn*1 mutant phenotype, with a focus on late embryo and post-embryonic morphology. We report here that the *twn*1 mutation alters cotyledon patterning, thus disrupting the establishment of bilateral symmetry and subsequent morphogenesis in the *Arabidopsis* embryo proper. The observed cotyledon defects suggest that *TWN*1 helps specify the location, size, and boundaries of cotyledon-forming fields in the wild-type embryonic shoot apex. Comparison of the *twn*1 phenotype to

those of other cotyledon mutants indicates that *twn*1 is a unique *Arabidopsis* cotyledon mutant, with defects restricted to embryonic and primary leaf development. In addition to characterizing *twn*1, we determined that another polycotyledonous mutant, *amp*1, also produces twins by suspensor transformation, confirming that there is overlap between the genetic mechanisms underlying suspensor and cotyledon development. Our results suggest that *TWN*1 acts in pathways that impart or maintain positional information in both the suspensor and shoot apex of the developing *Arabidopsis* embryo.

MATERIALS AND METHODS

Plant material and seedling culture—*Twn*1 seeds were obtained from selfed *twn*1 homozygotes (Vernon and Meinke, 1994). Seeds for *amp*1 (*amp*1-1 allele), *pin*1 (*pin*1–1 allele), and *pid*1 (*pid*1–2 allele) were obtained from the *Arabidopsis* stock center at Ohio State University. Seeds for *cuc*1 and *cuc*2 were kindly provided by Dr. M. Tasaka (Kyoto University). Plants grown for genetic crosses, embryo microscopy, or characterization of vegetative development were seeded in soil, chilled at 4°C for 2–3 d, and grown to maturity at 22°C under 16 h light/8 h dark cycles in a controlled climate growth chamber. Seedlings grown for phenotype analyses were sterilized and germinated in culture in petri dishes containing germination medium as previously described (Vernon and Meinke, 1995). For comparison of the dark response in *twn*1, *amp*1, and wild type seedlings, plates were exposed to light for 12–16 h to stimulate germination, then wrapped in foil and set vertically in the dark. Seedlings germinated in culture that were chosen for further use in genetic crosses, heritability studies, or seed collection were transplanted to soil after 7–10 d in culture and grown to maturity under the conditions described above.

Phenotypic analysis and microscopy—Phenotypes of seedlings germinated in culture were scored and characterized by observation under a Wild Heerbrugg M8 dissection microscope. For characterization of cotyledon veination patterns, seedlings or excised cotyledons were cleared in ethanol prior to viewing. Dark response of wild type, *amp*1, and *twn*1 seedlings was gauged by manual measurement under a dissection microscope of hypocotyls and roots of seedlings following 5 d growth on plates in the dark. To normalize for the generally reduced size of *amp*1 seedlings, hypocotyl : root length ratios, rather than simply hypocotyl length, were measured.

Scanning electron microscopy was carried out essentially as described by McConnell and Barton (1995). Seedlings were selected from plates and fixed on ice in 2% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4; PBS) for 6 h. Seedlings were washed with cold PBS and dehydrated by 10-min incubations in increasing amounts of ethanol in PBS, starting with 5% ethanol and increasing in 10% increments to 95%. Final dehydration was accomplished by an additional 30-min incubation in 95% ethanol followed by 5 30-min incubations in 100% ethanol. Seedlings were subjected to critical point drying and sputter coating immediately following ethanol dehydration and viewed with a Jeol JSM-T300 scanning electron microscope.

For observation of developing embryos, seeds at various stages of development were excised from siliques and cleared in Hoyer's solution as previously described (Vernon and Meinke, 1994). Cleared seeds were viewed as whole mount preparations on an Olympus BX-60 compound microscope equipped with Nomarski differential interference contrast optics.

RESULTS

*Twn*1 was originally identified as a polyembryonic embryo-defective mutant that produced supernumerary embryos via suspensor transformation (Vernon and Meinke, 1994). The polyembryonic phenotype showed ~8% penetrance; that is, most mutant seeds germinated to produce a single seedling. In addition to twinning, variable defects in the EP were occasionally observed, and one model of *TWN*1 activity proposed that the gene may have other developmental functions affecting the EP, as well as the suspensor. The identification of *twn*1

EP defects that are independent of suspensor transformation would support this model and rule out alternative models in which *TWN*1 acts solely in the early EP or suspensor to maintain suspensor identity (Schwartz, Yeung, and Meinke, 1994; Vernon and Meinke, 1994). To further investigate *TWN*1 gene function and determine if *TWN*1 has developmental functions specific to the embryo proper, we characterized late embryo and seedling morphology in *twn*1 homozygotes.

Diverse cotyledon defects in twn1 mutant seedlings—Mutant seeds produced by selfed *twn*1 homozygotes were germinated in culture and examined by dissection microscope. Consistent with previous studies (Vernon and Meinke, 1994), ~8% of seeds yielded two seedlings and most seedlings resembled wild type. However, ~10% of mutant seedlings (N = 1277) displayed dramatic defects in cotyledon number, arrangement, and/or morphology. Similar cotyledon defects were not observed on wild-type seedlings ($N > 300$). Cotyledon defects were observed on seedlings germinated from monoembryonic as well as polyembryonic seeds, indicating that the defects were not a consequence of twinning. A wild-type seedling and representative examples of *twn*1 seedlings with abnormal cotyledons are shown in Figs. 1–6. Defects in cotyledon number included apparent single cotyledons (Fig. 2) as well as extra cotyledons (Figs. 4 and 5). Fused cotyledons were also observed. Fusion phenotypes ranged from almost complete fusion to subtle connections at the base of clearly distinct cotyledons (Figs. 3 and 4). In all cases of fusion, cotyledons were connected from the base, indicating that fusion occurred during cotyledon formation and not postgerminatively. Defects in cotyledon number often occurred in combination with fusions of varying severity, making strict categorization of cotyledon abnormalities difficult. A small percentage of *twn*1 homozygotes ($<0.5\%$) had cotyledons with trichomes (Fig. 6). In *Arabidopsis,* trichomes are a characteristic of vegetative organs and are not found on wild-type cotyledons. When present on *twn*1 cotyledons, trichomes were far less numerous and irregularly distributed than on leaves. Nevertheless, their occasional presence on cotyledons suggests that postembryonic developmental programs were precociously deployed to some extent during *twn*1 cotyledon development.

Cotyledon numbers on mutant seedlings could not always be determined by routine observation. For example, *twn*1 seedlings with apparently single cotyledons could be truly "monocotyledonous," or they could have severely fused pairs of cotyledons that resemble single cotyledons. To further investigate the cotyledons on *twn*1 seedlings with apparent single or abnormally large cotyledons, we viewed the vasculature of cleared mutant seedlings. Cotyledon venation patterns from wild type and representative mutant seedlings are shown in Figs. 7–10. We observed a range of venation patterns in abnormal cotyledons. In some cases venation patterns helped define organ number on mutant seedlings. For example, some seedlings had organs resembling a truly single cotyledon (Fig. 8), while others had double venation patterns that clearly indicated the fusion of two adjacent organs (as in the larger cotyledon shown in Fig. 10). Some apparently monocotyledonous seedlings, however, had venation of intermediate complexity (Fig. 9). Such vascular patterns could be interpreted in two ways: they could be a result of extensive organ fusion, or they could reflect plasticity in vascular development, such that abnormally large cotyledons develop more complex and extended venation patterns.

Polycotyledonous *twn*1 phenotypes included seedlings with fused supernumerary cotyledons (e.g., Fig. 4) as well as those with three completely separate organs (Fig. 5). Polycotyly was limited to one extra cotyledon; no more than three cotyledons were present on any of the thousands of *twn*1 seedlings observed in the course of this study. Conway and Poethig (1997) recently determined that supernumerary cotyledons on *Arabidopsis xtc* mutants are actually precocious leaves that initiate prematurely from the embryonic shoot apical meristem. However, all cotyledons observed on *twn*1 tricots were true cotyledons, based on the following criteria: (1) all arose from the first node; (2) they consistently had the simple veination pattern characteristic of cotyledons; and (3) precocious embryonic leaf initiation and abnormally large shoot meristems have not been observed on developing *twn*1 embryos, as is the case with mutants that produce cotyledon-like precocious leaves (Vernon and Meinke, 1994; Conway and Poethig, 1997; and see below).

Both cotyledon patterning and organogenesis are disrupted in twn1—The different cotyledon defects in *twn*1 seedlings could possibly be due to initiation of cotyledons at abnormal positions, or to improper recruitment of apex cells during cotyledon initiation at proper locations. The occurrence of truly tricotyledonous seedlings indicated that *twn*1 at least in some cases affects the position (patterning) of cotyledon initiation. Organ fusion phenotypes, however, could conceivably arise through two different developmental mechanisms: (1) cotyledons could initiate normally on opposing sides of the apex, but cells between initiation sites could be improperly recruited into cotyledon primordia, creating a single organ with a wide base; or (2) cotyledons could incorrectly initiate adjacent to each other on one side of the embryonic axis, and fusion could somehow result as a consequence of the abnormal proximity of developing organs. To determine how cotyledon fusions were occurring, we used scanning electron microscopy to view the apices of *twn*1 seedlings with cotyledon fusions of different severity. Figure 11 shows the apex of a representative "monocotyledonous" seedling similar to that shown at lower magnification in Fig. 2. A "collar" of cotyledon tissue encircled much of the shoot apex on such seedlings, spanning the region between normal cotyledon initiation sites on opposite sides of the apex. Thus, at least in some cases, the *twn*1 mutation appears to extend the field of cells recruited for cotyledon formation, as suggested in the first scenario described above. However, the more subtle organ fusions observed on some seedlings may arise through the second mechanism. Such a fusion is visible in Fig. 12, which shows the apex of a representative tricot seedling similar to the one shown at lower magnification in Fig. 4. Three distinct cotyledons are positioned around the axis. The bases do not appear to extend abnormally around the apex, but two of the organs are slightly fused near the base. Thus, cotyledon fusions on different seedlings may arise by either of the mechanisms described above, and the *twn*1 mutation can disrupt both the pattern of organ initiation (as in tricots), or the recruitment of cells into cotyledon primordia during organogenesis (as in seedlings with single large organs or fusions).

Cotyledon defects are not a consequence of other defects in twn1 morphology—Germination studies indicated that *twn*1 cotyledon defects were not an indirect consequence of twinning, because seedlings from polyembryonic seeds often fea-
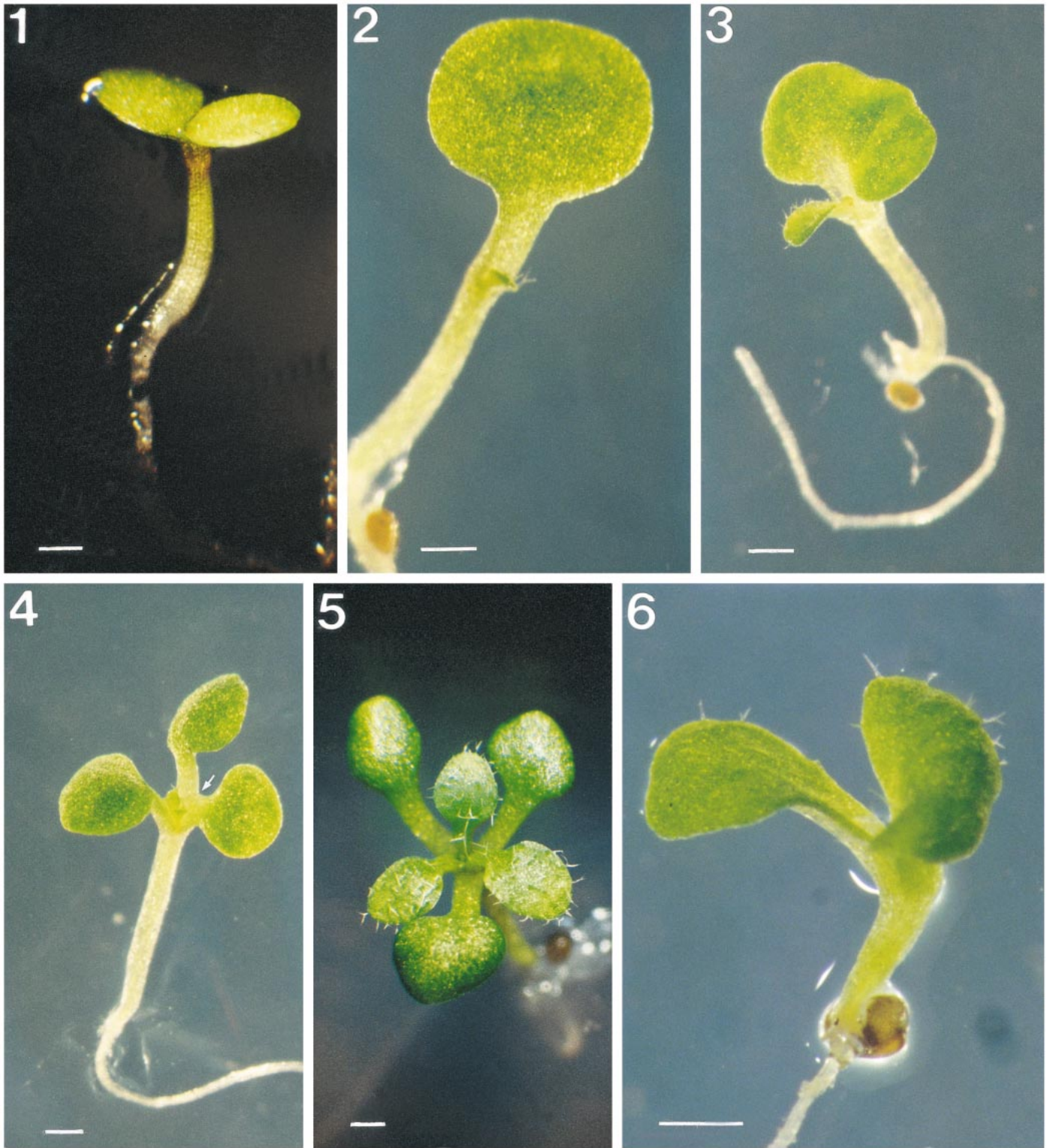
Figs. 1–6.   Wild-type and *twn*1 seedlings germinated in culture. **1.** Wild-type seedling. **2–4.** *Twn*1 seedlings with representative cotyledon defects: a single large cotyledon, severely fused cotyledons, and an extra cotyledon, respectively. Arrow (Fig. 4) indicates subtle cotyledon fusion. Note the presence of only two primary leaves on this tricotyledonous seedling. **5.** A fully tricotyledonous *twn*1 seedling with three primary leaves. **6.** A *twn*1 seedling with sparse trichomes on both cotyledons. The larger cotyledon is a likely a fusion. All seedlings shown in Figs. 2–6 germinated as single seedlings from monoembryonic *twn*1 seeds. Scale bars = 0.5 mm.
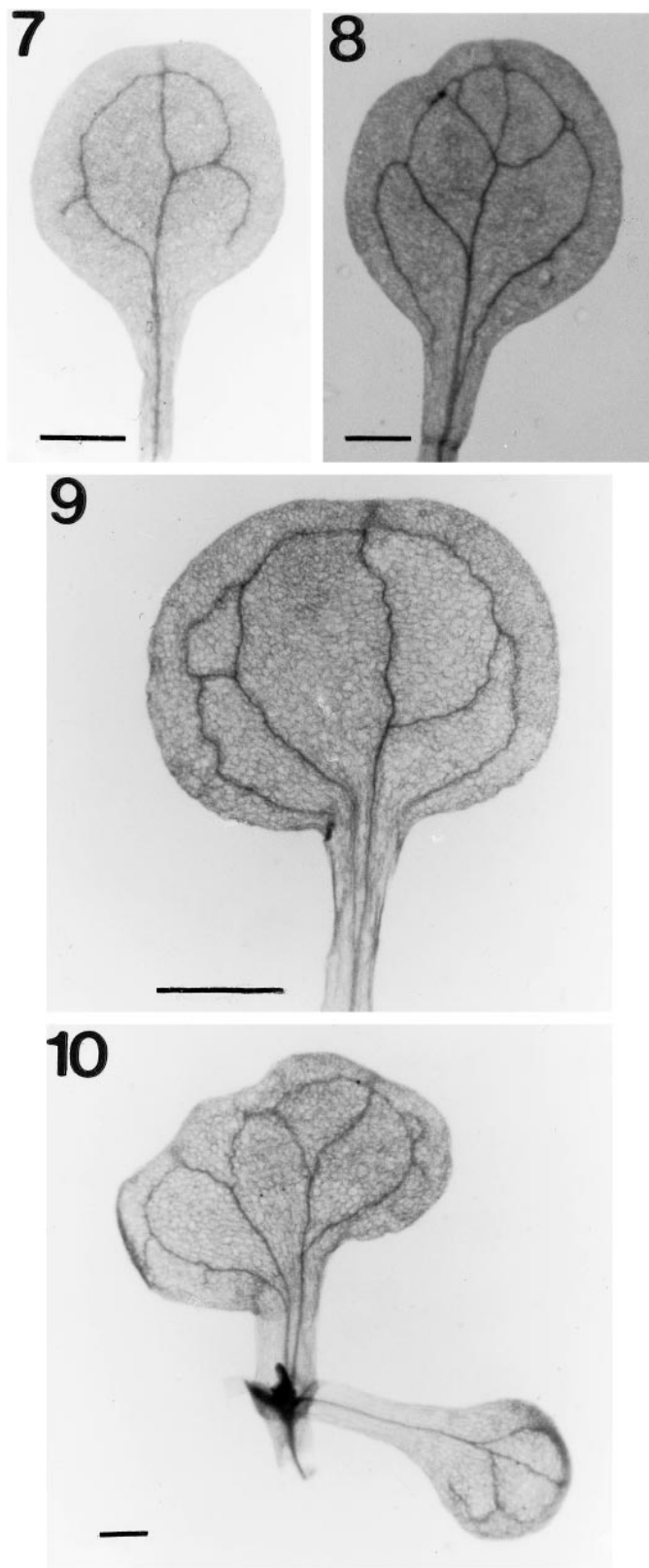
Figs. 7–10. Venation patterns in cleared cotyledons from wild-type and *twn*1 seedlings. **7.** Wild-type cotyledon. **8.** A cotyledon from an apparently monocotyledonous *twn*1 seedling. The vascular pattern is only slightly more complex than that of wild type. **9.** A cotyledon from another apparently monocotyledonous *twn*1 seedling. The venation pattern reveals that this organ may

tured normal cotyledons, and (more importantly) most seedlings with cotyledon defects germinated from monoembryonic seeds. To further investigate whether cotyledon abnormalities appeared in the absence of other embryonic defects, we used Nomarski microscopy to view *twn*1 embryos in cleared whole-mount seeds. Figures 13–16 show a linear-stage wild-type embryo and mutant embryos with representative cotyledon defects. As expected from germination studies, embryos with the full range of cotyledon defects were observed as single embryos in monoembryonic seeds (Figs. 14–16), and suspensors of such embryos typically had normal cell number and morphology (best seen in Fig. 14). Even mutant embryos with dramatic cotyledon defects had EP morphology that otherwise resembled wild type (Figs. 14–16). Of particular note was the absence of clearly enlarged or domed shoot apical meristems or precocious leaves on polycotyledonous *twn*1 embryos. Enlarged meristems are frequently observed on developing embryos of the other polycotyledonous mutants *xtc* and *amp*1 (Chaudhury et al., 1993; Conway and Poethig, 1997). Thus, *twn*1 cotyledon defects do not result from suspensor defects, obvious defects in embryo morphology, or premature vegetative meristem activity. Rather, the *twn*1 mutation appears able to specifically affect cotyledon patterning and morphology.

The twn1 cotyledon phenotypes are inherited as a single recessive trait that displays incomplete penetrance—The germination studies with *twn*1 homozygotes, as well as microscopy of developing embryos, suggested that cotyledon defects are expressed with ~10% penetrance. All *twn*1 homozygotes, regardless of their cotyledon morphology, consistently produced progeny with the full range of cotyledon defects at a frequency of ~10% following self-fertilization. In addition, cotyledon defects and polyembryony appeared to be separate consequences of the *twn*1 mutation that could occur separately in populations of *twn*1 seedlings: all *twn*1 homozygotes, whether from polyembryonic or monoembryonic seeds, produced progeny with the full range of cotyledon phenotypes, as well as twins. We carried out additional genetic analyses to confirm these aspects of phenotype heritability.

To establish the heritability and incomplete penetrance of the diverse cotyledon defects in *twn*1 homozygotes, we tested two specific predictions: (1) ~90% of the progeny of selfed *twn*1 homozygotes with dramatic cotyledon defects should have cotyledons that resemble wild type; and (2) the full range of cotyledon defects should be observed among progeny of such homozygotes. To test these predictions, we collected seeds produced by five tricotyledonous *twn*1 seedlings, germinated them in culture, and scored progeny phenotypes. As shown in Table 1, ~90% of progeny had cotyledons resembling wild type, and the total frequency of cotyledon defects in progeny from each tricot was ~10%. The progeny populations from each parent exhibited the full range of cotyledon defects, including single, fused, and extra cotyledons, and combinations of these. Thus, cotyledon defects are co-inherited and consistently expressed with ~10% penetrance in
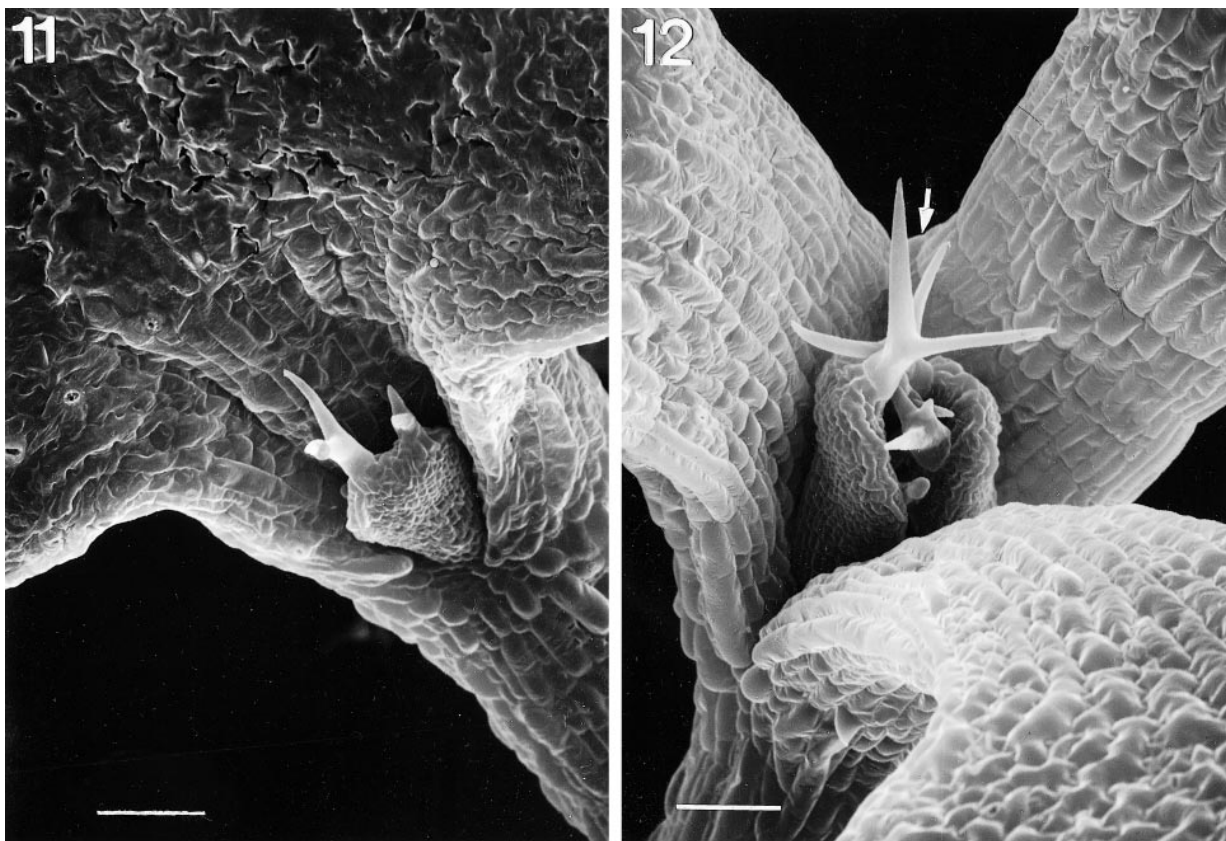
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actually consist of two thoroughly fused cotyledons. **10.** Cotyledons from a *twn*1 seedling with one normal and one abnormally large cotyledon. The latter contains two separate, parallel midribs and a double veination pattern, clearly indicating the presence of two fused organs. Primary leaves had been removed from this seedling for photograph clarity. Scale bar = 0.5 mm.

Figs. 11–12. Scanning electron microscopy of the apices of *twn*1 seedlings featuring fused cotyledons. **11.** Apex of a seedling with a single large cotyledon, similar to that shown at smaller magnification in Fig. 2. The base of the organ encircles one entire side of the apex, suggesting an abnormally broad field of cells had been recruited for incorporation into the cotyledon (see text). A single primary leaf is emerging. **12.** Apex of a seedling with three distinct cotyledons, similar to that shown at lower magnification in Fig. 4. The cotyledon in the foreground resembles wild type with regard to its base and its separation from its neighbors. There is a subtle fusion between the two other cotyledons on the opposite side of the apex (marked by arrow). Such small organ fusions could arise as an indirect consequence of the abnormally close position of cotyledon initiation sites (see text). Two primary leaves resembling those of wild type are emerging. Scale bar = 0.1 mm.

progeny of selfed *twn*1 homozygotes, regardless of parent plant morphology.

To further confirm the consistent penetrance and heritability of *twn*1 cotyledon defects, we crossed phenotypically normal *twn*1 homozygotes (i.e., homozygous mutants that did not exhibit altered cotyledon or polyembryonic phenotypes) to wild-type *Arabidopsis* (ecotype Wassilewskija) and scored F2 progeny for cotyledon abnormalities. Seven independent crosses to wild type were performed. All F1 progeny resembled wild type, as expected for plants heterozygous for a recessive mutation. From each cross F2 progeny displayed the full range of cotyledon defects, including monocotyly, fusions, and polycotyly. As shown in Table 2, cotyledon defects in F2 progeny from each cross were expressed at the frequency predicted for a recessive defect with penetrance of ~10%. All F2 families also produced twins at the predicted frequency. Thus, cotyledon defects exhibited a consistent pattern of inheritance following reintroduction of the *twn*1 mutation into a wild type background, regardless of the phenotype of the mutant parent used in the cross.

Heritability and penetrance remained consistent in the subsequent (F3) generation derived from the crosses to wild type. Because of the incomplete penetrance of both polyembryonic and cotyledon defects in *twn*1, we restricted our F3 to analysis progeny produced by F2 parents that could be identified by

their phenotypes as homozygous mutants. Four to six *twn*1 homozygotes from each F2 family (35 F2 seedlings in total) were identified on the basis of their cotyledon phenotype or their germination from polyembryonic seeds. These selected mutant F2s were grown to maturity, selfed, and their progeny scored for cotyledon and polyembryonic phenotypes. Regardless of whether the F2 parent had originated from a mono- or polyembryonic seed and regardless of the cotyledon phenotype of the F2 parent, each of the 35 F3 families exhibited cotyledon defects at the predicted frequency (10%; $N > 100$ for each family; >3500 in total), and each produced twins. Thus *twn*1 plants consistently produced progeny with cotyledon defects, and the expression of cotyledon defects remained consistent for three generations following reintroduction of *twn*1 into wild type.

Taken together, these genetic studies demonstrate that cotyledon defects in *twn*1 follow a pattern of inheritance characteristic of a single recessive mutation that is phenotypically expressed with a penetrance of ~10%. Indeed, it is difficult to conceive of an equally straightforward alternative model that can account for the observed inheritance of cotyledon defects in this mutant. Along with the demonstration of twinning in another *Arabidopsis* cotyledon mutant (see below), these results also support the view that cotyledon defects and polyembryony are indeed pleiotropic consequences of the *twn*1
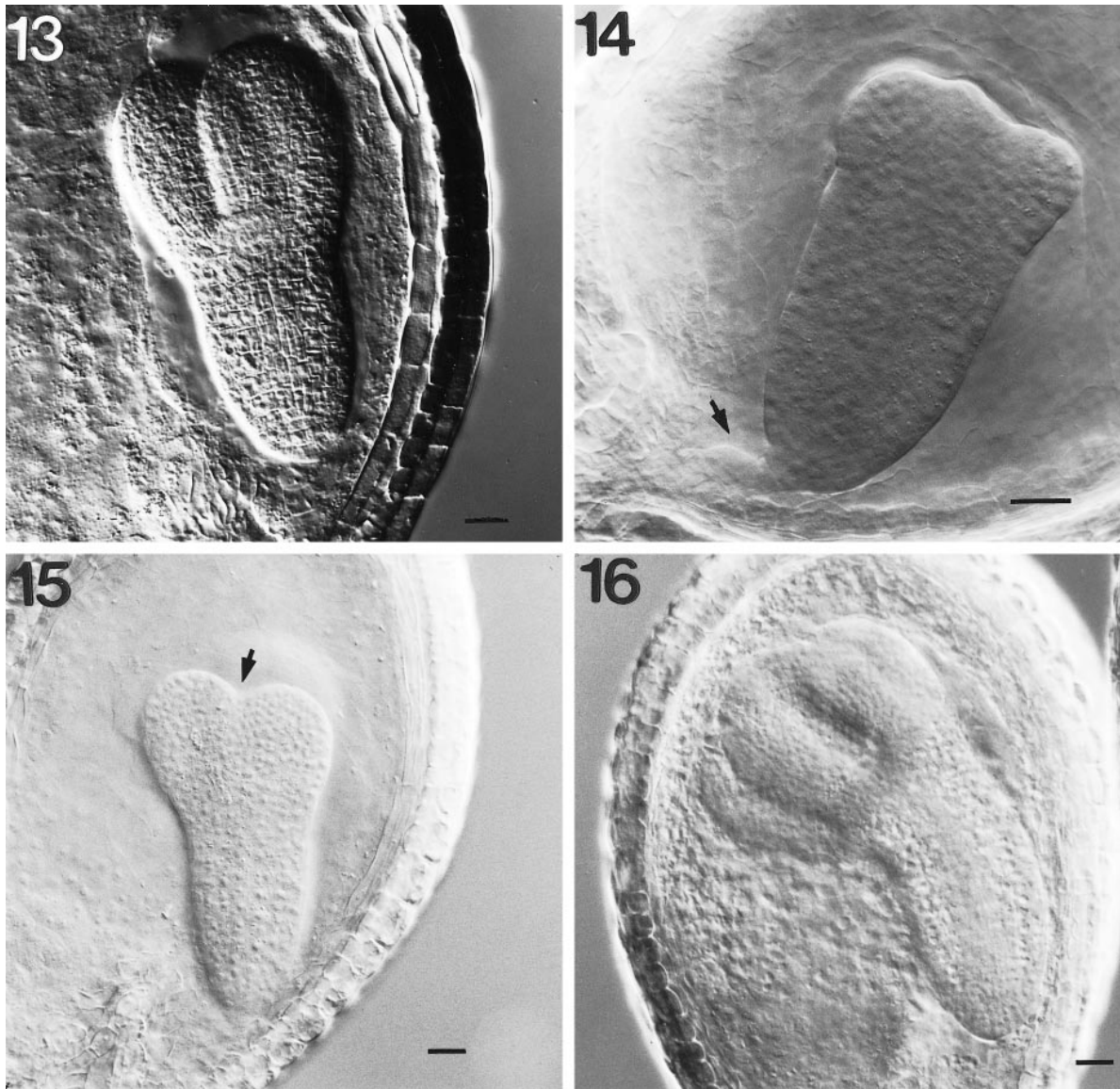
Figs. 13–16. Wild type and representative *twn*1 embryos at the linear stage, viewed by Nomarski differential interference contrast microscopy. **13.** Wild-type embryo at early linear stage, showing two distinct developing cotyledons. **14.** Linear-stage *twn*1 embryo with greatly reduced, or delayed, cotyledons. Hypocotyl size and morphology resemble wild type, as does the suspensor (arrow), the cells of which appear slightly bloated at this late stage of embryogenesis. **15.** An early linear-stage *twn*1 embryo with adjacent fused cotyledons. The arrow marks the interface of the fused organs. A separate third cotyledon is present on the opposite side of this embryo but is not entirely visible in the focal plane shown. **16.** A *twn*1 seedling with three distinct cotyledons. Note that, apart from their cotyledons, the mutant embryos shown in Figs. 14–16 do not have other obvious morphological defects; nor are they accompanied by suspensor-derived secondary embryos. Scale bar = 20 μm.

mutation. Absolute proof of the single-gene basis for these disparate developmental defects will ultimately require molecular complementation with a cloned copy of the *TWN*1 gene.

The twn1 mutant is not allelic to other Arabidopsis cotyledon mutants—Several other *Arabidopsis* mutants have been identified that exhibit at least some of the cotyledon defects observed in *twn*1: *pin*1, *pid*1, *amp*1, *cuc*1, and *cuc*2 (Okada et al., 1991; Chaudhury et al., 1993; Bennett et al., 1995; Aida et al., 1997). Except for *cuc*2, all of these mutations have been localized to different linkage groups than *twn*1, which has been assigned to the lower arm of *Arabidopsis* chromosome five (Vernon and Meinke, 1994). Because

*cuc*2 can cause fused cotyledons and has also been mapped to chromosome 5, we tested for allelism between it and *twn*1. As a single mutation, *cuc*2 causes limited cotyledon fusion (with a penetrance of only 0.5%), whereas *cuc*1,*cuc*2 double mutants have a unique, fully penetrant cup-shaped cotyledon phenotype (Aida et al., 1997). Therefore, rather than carrying out standard complementation crosses between the *twn*1 and *cuc*2, we crossed *twn*1 with *cuc*1 and scored F2 seedlings for the fully penetrant *cuc*1:*cuc*2 double mutant phenotype. If *twn*1 were allelic to *cuc*2, ~6.25% of the F2 progeny from such a cross would be predicted to have a single ''cup-shaped'' cotyledon. Phenotypes of F2 progeny clearly indicated that *twn*1 was not allelic to *cuc*2 (Table 3).

TABLE 1. Incomplete penetrance of cotyledon defects in progeny of *twn*1 homozygotes with abnormal cotyledons.

Tricot parent	Total progeny scored[a]	Progeny with cotyledon defects[b]	Progeny with normal cotyledons (%)[c]
1	135	15	120 (89%)
2	170	16	154 (91%)
3	160	22	138 (86%)
4	230	24	206 (90%)
5	260	23	237 (91%)

[a] Seeds were collected from selfed tricotyledonous *twn*1 homozygotes, germinated in culture, and scored for cotyledon defects.

[b] Total cotyledon defects included single, fused, and extra cotyledons, and combinations of these. The full continuum of such defects was observed in progeny of each tricot parent.

[c] Approximately 90% of progeny from selfed *twn*1 homozygotes should resemble wild type, if the penetrance of cotyledon defects is ~10%.

Suspensor polyembryony in the polycotyledonous amp1 mutant—The independent expression of the cotyledon and polyembryonic phenotypes in *twn*1 embryos suggested that *TWN*1 may influence seemingly unrelated developmental processes in the suspensor and embryonic apex. To further support this unexpected link between genetic control of suspensor and cotyledon development, we looked for polyembryony in *Arabidopsis* mutants previously shown to have defects in cotyledon development. Homozygous mutant seeds obtained from *amp*1, *cuc*1, and *cuc*2 homozygotes and from *pin*1 and *pid*1 heterozygotes were germinated and scored for twinning. Also, developing embryos from each mutant line were examined with Nomarski microscopy. Twinning was not observed in mutant seeds from the *pin*1 and *pid*1 lines. However, a low frequency ($\geq$0.2%, $N = 1500$) of twin seedlings germinated from homozygous *amp*1 seeds in culture (Fig. 17). This polyembryony rate is far below that of *twn*1, but is still approximately ten times the twinning frequency observed in wild-type *Arabidopsis* (Akhundova, Schevchenko, and Grinikh, 1979; Vernon and Meinke, 1994). Nomarski analysis of developing *amp*1 embryos revealed that *amp*1 twins develop from suspensor cells via a mechanism resembling that previously described for *twn*1 (Fig. 18). Thus, two *Arabidopsis* developmental mutants independently reveal that common mechanisms influence suspensor and cotyledon development.

Comparison of post-embryonic development in twn1 and amp1—In addition to suspensor polyembryony, the *amp*1 mutant exhibits a high frequency of cotyledon defects similar in some respects to those we observed in *twn*1 (Chaudhury et al., 1993). Because of the phenotypic similarities between these mutants, we further compared their development. In addition to its embryonic defects, *amp*1 also displays fully penetrant, dramatic defects in postembryonic development, including cytokinin accumulation, rapid and disorganized leaf production, abnormally high leaf number, small size, multiple bolts, and photomorphogenic development in the dark (Chaudhury et al., 1993; Chin-Atkins et al., 1996). To determine if *twn*1 exhibited any of the vegetative defects observed in *amp*1, we further characterized postembryonic development in *twn*1. Wild-type *Arabidopsis* have two primary leaves that arise at opposite positions around the seedling axis. The *twn*1 seedlings with abnormal cotyledon numbers occasionally had one or three primary leaves (e.g., see Figs. 2 and 5). Also, leaf

TABLE 2. Heritability of *twn*1 phenotypes following crossing to wild type.

Cross[a]	Total F2 seedlings scored[b]	Predicted cotyledon defects[c]	Observed cotyledon defects[d]	Predicted twins[e]	Observed twins
1	507	~13	18	~10	12
2	460	~12	16	~9	7
3	423	~11	11	~8	9
4	395	~10	11	~8	2
5	389	~10	12	~8	9
6	409	~10	12	~8	4
7	319	~8	9	~6	6

[a] Seven different *twn*1 homozygotes were crossed to wild type. To more rigorously establish the heritability of *twn*1's incompletely penetrant developmental defects, homozygotes that resembled wild type were chosen for these crosses (i.e., parental plants did not exhibit either polyembryonic or abnormal cotyledon phenotypes).

[b] F2 seeds were harvested from selfed F1 plants, plated in culture, and scored for cotyledon and polyembryonic phenotypes.

[c] F1 plants were heterozygous for the *twn*1 mutation, and only ~25% of F2 progeny were *twn*1 homozygotes. Therefore, based on an estimated phenotype penetrance of 10% in *twn*1 homozygotes, ~2.5% of F2 seedlings were predicted to have cotyledon defects.

[d] Observed cotyledon defects included single, fused, and extra cotyledons, and combinations of these. The full continuum of cotyledon defects was observed in each of the F2 populations.

[e] F1 plants were heterozygous for the *twn*1 mutation and only ~25% of F2 progeny were *twn*1 homozygotes. Therefore, ~2% of F2 seeds were predicted to yield twins, based on an estimated 8% penetrance of the polyembryonic phenotype in *twn*1 homozygotes (Vernon and Meinke, 1994).

fusion was observed between *twn*1 primary leaves at a very low frequency (<0.5%). These phenotypes demonstrated that the *twn*1 mutation can have a limited effect on development after embryogenesis. However, in contrast to *amp*1, discernable developmental defects did not persist beyond the first leaf pair in *twn*1 homozygotes: even on seedlings with obvious defects in cotyledon or primary leaf pattern, the leaf number, rate, and arrangement beyond the primary leaf(s) consistently resembled wild type. Results of a representative experiment quantifying rosette leaf rate and number are shown in Fig. 19.

TABLE 3. Results of *twn*1 $\times$ *cuc*1 cross to test allelism of *twn*1 and *cuc*2[a].

Phenotype	Predicted if *twn*1 is allelic to *cuc*2[b]	Observed in F2	χ^2
Cup-shaped cotyledon	21 (6.25%)	1 (0.3%)	19.36
Wild-type cotyledons	—	320	NA
Fused or extra cotyledons	—	18[d]	NA

[a] *twn*1 and *cuc*1 homozygotes were crossed and F2 progeny were scored for the fully penetrant *cuc*1:*cuc*2 double-mutant (cup-shape cotyledon) phenotype. *cuc*1 alone does not produce seedlings with this phenotype (Aida et al., 1997).

[b] If *twn*1 was allelic to *cuc*2, 6.25% of F2 progeny from a *twn*1 $\times$ *cuc*1 cross would be predicted to have the *cuc*1:*cuc*2 double mutant phenotype. In making this prediction, *twn*1 allele strength was assumed to be equal to or greater than that of *cuc*2. This assumption was justified because the frequency of cotyledon defects in *twn*1 is ~20 times that of *cuc*2.

[c] High χ^2 value indicates the number of cup-shaped cotyledon F2 seedlings deviated significantly from the predicted value ($P \ll 0.05$), indicating that *twn*1 and *cuc*2 are not allelic. NA, not applicable.

[d] This total should include both *twn*1 homozygotes and *cuc*1 homozygotes with fused cotyledons segregating within the F2 population.
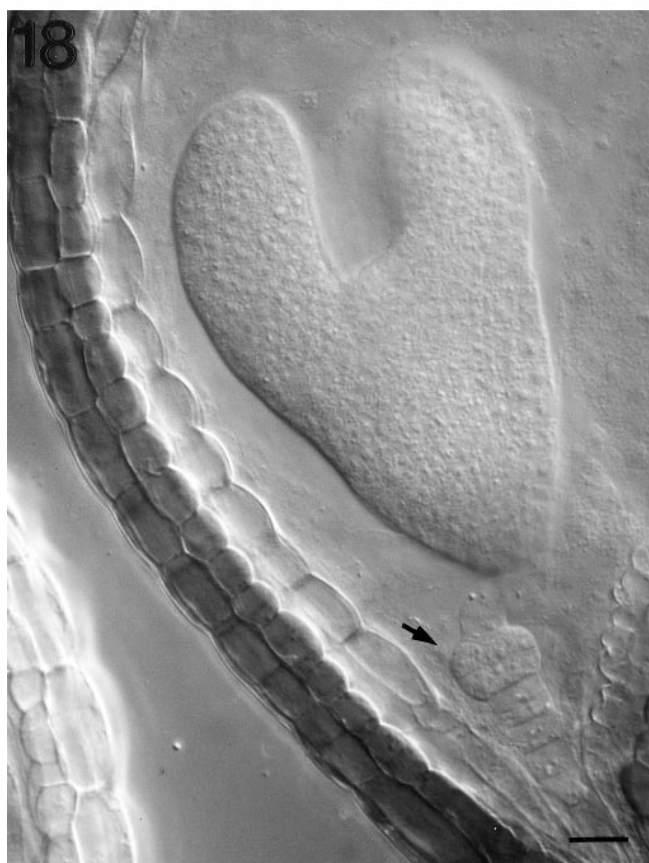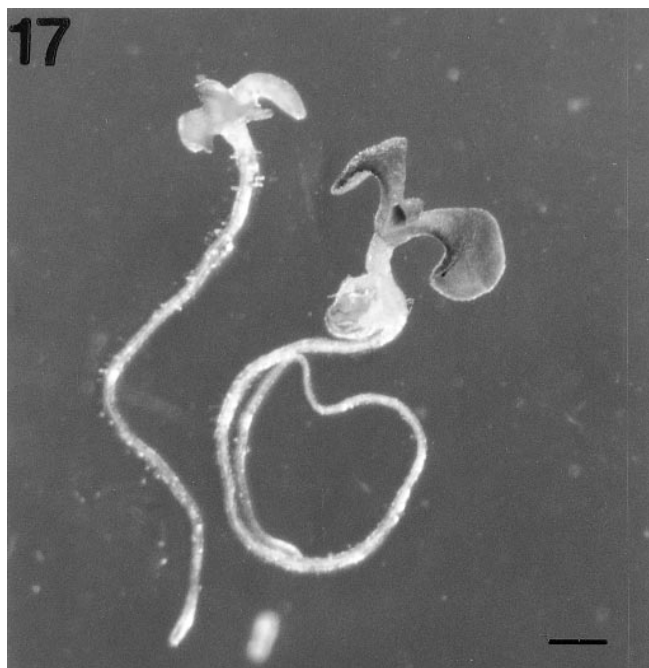
Figs. 17–18. Suspensor polyembryony in the *amp*1 mutant. **17.** Twin *amp*1 seedlings germinated in culture from a single seed. Scale bar = 0.5 mm. **18.** A cleared *amp*1 seed viewed with Nomarski optics. A globular stage secondary embryo is developing within the suspensor (arrow). Note the short hypocotyl and the abnormally broad meristem region between the cotyledons, consistent characteristics of *amp*1 embryos. Scale bar = 20 μm.

Furthermore, no obvious alterations in bolt number, timing, or flower morphology were observed on *twn*1 under our growth conditions; *twn*1 also differed from *amp*1 in its response to growth in the dark, which resembled that of wild type (Fig. 19b). Thus, although *twn*1 and *amp*1 share some cotyledon defects and can produce suspensor-derived twins, *twn*1 does not appear to have long-term influence on the vegetative apical meristem or apical organ production. Rather, *TWN*1 appears to act in an embryonic pathway that impacts only the earliest phase of apical organ patterning in *Arabidopsis*. This observation is consistent with the view that genetic control of cotyledon formation differs at least in part from that of postembryonic leaves (Barton, 1998; Lenhard and Laux, 1999).

DISCUSSION

Twn1 is a new member of a class of cotyledon mutants— We have described a variety of cotyledon abnormalities caused by mutation of the *TWN*1 gene. Our results demonstrate that *twn*1 is one of a growing class of *Arabidopsis* mutants with altered cotyledon pattern and number. The cotyledons are a dominant feature of the developing embryo in dicotyledonous plants and their formation serves as an important milepost in *Arabidopsis* embryo morphogenesis. Cotyledon initiation indicates the beginning of organ production from the shoot apex, marks the embryo's switch from radial to bilateral symmetry, and defines the transition from the globular to heart stage of development (West and Harada, 1993; Kaplan and Cooke, 1997; Kerstetter and Hake, 1997). For these reasons, cotyledons have served as markers for assessing genetic control of various aspects of embryogenesis. For example, *Arabidopsis* mutants with severe defects in cotyledon formation, such as *gurke,* have helped identify putative "patterning genes" essential for proper development of the apical region of the embryo (Mayer et al., 1991; Torrez-Ruiz, Lohner, and Jurgens, 1996). Mutations resulting in polycotyly, such as *xtc* and *amp*1, have led to insights about the control of shoot meristem activity and timing of organ initiation (Chaudhury et al., 1993; Conway and Poethig, 1997). Another group of *Arabidopsis* cotyledon mutants, the *leafy cotyledon* (*lec*) mutants, have allowed identification of key regulatory genes that control embryo-specific programs during seed development (Meinke, 1992; Meinke et al., 1994; West et al., 1994; Lotan et al., 1998).

Other *Arabidopsis* mutations have been identified that cause cotyledon defects resembling those we report here for *twn*1. Interestingly, like *twn*1, these mutations are pleiotropic, cause variable defects and have incomplete penetrance with respect to their cotyledon phenotypes. The *amp*1 mutation results in embryos with deformed or extra cotyledons, as well as occasional precocious leaves that resemble extra cotyledons (Chaudhury et al., 1993; Conway and Poethig, 1997). The *pin*1 and *pid* mutations, like *twn*1, cause variable phenotypes, causing cotyledon fusion, deformities, or formation of a third cotyledon on some mutant individuals (Okada et al., 1991; Bennett et al., 1995). Another group, the *cuc* mutations, result in a low frequency of fused cotyledons when present as single mutations, although *cuc*1, *cuc*2 double homozygotes consistently produce a single, cup-shaped cotyledon that encircles the embryonic apex (Aida et al., 1997). The incomplete penetrance of each of these mutations with respect to cotyledons likely reflects redundancy in the genetic control of cotyledon formation (Taylor, 1997; Barton, 1998). Such redundancy could
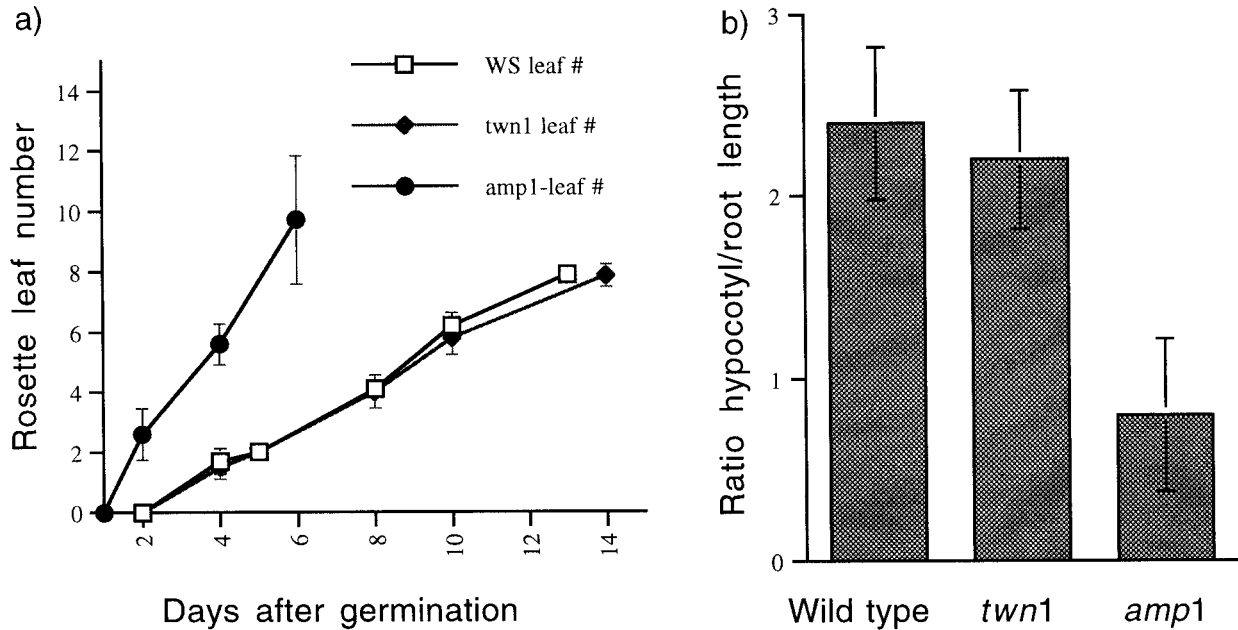
Fig. 19. Vegetative development in *twn*1 resembles wild type and differs from *amp*1. (a) Rosette leaf number and timing in *twn*1, *amp*1, and wild type. Plants were grown in soil and leaf number was determined at the time points indicated. (b) Dark response of *twn*1, *amp*1, and wild type, as gauged by hypocotyl : root length ratio. Plants were germinated in culture and measured as described in MATERIALS AND METHODS.

be due to the existence of genes with overlapping function and/or to the presence of multiple, distinct physiological mechanisms that act in parallel on cotyledon patterning.

Although the *amp*1, *pin, pid,* and *cuc* mutations can affect cotyledon development similarly to *twn*1, all also trigger dramatic developmental defects not observed in *twn*1. Many of these defects are in postembryonic development. *Amp*1 overaccumulates cytokinins and features a greatly enlarged shoot apical meristem, bushy, disorganized vegetative growth, and abnormal seedling response to growth in the dark (Chaudhury et al., 1993; Chin-Atkins et al., 1996). *Pin*1 and *pid*1 are defective in auxin-related processes, and they have severe defects on the inflorescence, including spike-like bolts with few or no cauline leaves or flowers (Okada et al., 1991; Bennett et al., 1995). *Pin*1 can also have fully fused primary leaves that encircle the apex. The *cuc*1 and 2 mutations also affect postembryonic development as single mutations, causing a low percentage of fused floral organs (Aida et al., 1997). Thus, *twn*1 is a unique *Arabidopsis* cotyledon mutant that exhibits a much narrower range of defects in shoot apical development than other mutants in its class.

TWN1's role in the embryonic apex—The cotyledon defects observed in *twn*1 embryos expand the known role for *TWN*1, indicating that the gene is active in the EP during later stages of embryogenesis and that it influences embryo (and seedling) symmetry through its effects on cotyledon patterning. The diversity of *twn*1 cotyledon defects, including fusions and deformities as well as extra cotyledons, indicates that *TWN*1 does not strictly affect cotyledon number. Rather, the gene appears to have a broader impact on cell fate at the embryo apex. We propose that *TWN*1 helps define both the location and size of cotyledon-forming fields, such that organs initiate at the proper positions around the embryonic axis, and apex cells are subsequently recruited correctly into cotyledon

primordia. This interpretation is supported by microscopy of mutant seedlings, which revealed defects in both the spatial arrangement of cotyledons (patterning) and the incorporation of apex cells into cotyledon tissue. All of the cotyledon deformities we observed, including abnormal number, asymmetry, fusions, and complex venation patterns, could result from mislocalization of cotyledon-forming fields, and/or misallocation of apical cells into those fields.

We suggest two general models for how *TWN*1 could influence cell fate in the embryonic apex. These models take into account various aspects of the *twn*1 phenotype and those of other cotyledon mutants; they are summarized below and in Fig. 20. The first possibility is that *TWN*1 could influence cotyledon development as part of a hormone-mediated mechanism. Hormones are prime candidates for mediating cell interactions in the plant embryo, especially between the EP and suspensor and within the shoot apex (Goldberg, de Pavia, and Yadegari, 1994). Both auxins and cytokinins are known to affect embryo symmetry and cotyledon pattern (Schiavone and Cooke, 1987; Cooke, Racusen, and Cohen, 1993; Lui, Xu, and Chua, 1993; Faure, Jullien, and Caboche, 1994). Also, the other *Arabidopsis* cotyldeon mutants *amp*1, *pin*1, and *pid* are defective in growth regulator physiology: *amp*1 overaccumulates cytokinin (Chaudhury et al., 1993), *pin*1 is defective in auxin transport, and *pid* in auxin signaling (Okada, 1991; Galweiler et al., 1998; Christensen *et al.*, 2000). However, it is unlikely that *TWN*1 has a broad role in growth-regulator accumulation or transport like *AMP*1 and *PIN*1, because *twn*1 lacks most of the other severe developmental defects seen in these other cotyledon mutants (e.g., the enlarged meristem, short hypocotyl, and disorganized vegetative growth of *amp*1). *TWN*1 could instead encode a downstream component of hormone-regulated developmental pathways, with localized roles restricted to the embryonic apex and suspensor (Fig. 20). Such a scenario would explain how *twn*1 exhibits some of the cotyledon de-
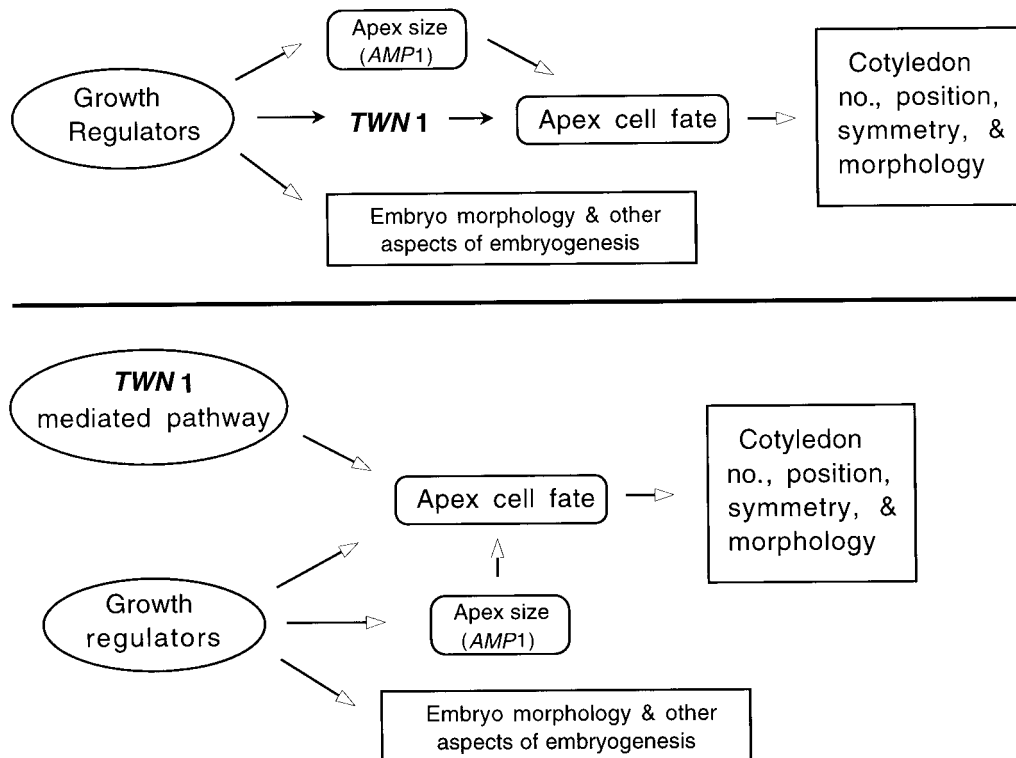
Fig. 20. General models for *TWN*1 activity at the embryonic shoot apex. In the top scenario, *TWN*1 influences cotyledon development as a downstream component of a hormone-mediated pathway. Hormones also affect other developmental processes, via mechanisms that do not involve *TWN*1. In the bottom scenario, *TWN*1 affects cotyledon development through a hormone-independent mechanism that acts in parallel to hormone-mediated pathways. Both scenarios account for phenotypic differences between *twn*1 and other *Arabidopsis* mutants with similar cotyledon defects (see DISCUSSION).

fects observed in *amp*1, *pin*1, and *pid*1, without many of the other defects caused by widespread imbalances in hormone accumulation, sensitivity, or transport in these mutants.

An alternative model for *TWN*1 function in the apex is that the gene affects a hormone-independent pathway that acts separately and in parallel to hormone-mediated mechanism(s). Like the previous model, this too could account for the phenotypic differences between *twn*1 and the hormone mutants discussed above. In addition, it is consistent with the view that multiple, partially redundant mechanisms control organ formation at the shoot apex (Taylor, 1997) and thus can explain the incomplete penetrance of the *Arabidopsis* cotyledon mutations. Within the context of both these models, *TWN*1 is proposed to act on or within the embryonic apex to affect the size, number, and location of cotyledon-forming fields. Thus, *TWN*1 might be predicted to act upstream of genes that directly define or separate cotyledon primordia, such as *CUC*1 and *CUC*2. We do not propose that *TWN*1 influences vegetative shoot meristem establishment, architecture, or maintenance, because *twn*1 individuals do not exhibit persistent defects in postembryonic organ formation. Therefore, genes that function throughout development in fundamental meristem maintenance and organization, such as *STM, PINHEAD/ZLL,* and *CLV* (Barton and Poethig, 1993; McConnell and Barton, 1995; Clark, 1997; Moussian et al., 1998; Lynn, Fernandez, and Barton, 1999), are predicted to be unaffected by *TWN*1 activity. To discern between the models proposed in Fig. 20, molecular characterization of *twn*1 will be necessary. Physiological measurements of hormone status in *twn*1 are difficult because of the embryo-specificity and incomplete penetrance

of the mutation, and genetic strategies (such as double mutant studies with hormone mutants) cannot be interpreted meaningfully without knowledge of the molecular nature of the *twn*1 mutant allele.

In addition to influencing the size and location of cotyledon primordia, *TWN*1 appears to occasionally affect the timing of cotyledon formation. This conclusion is based on our observation of delayed cotyledon formation in some *twn*1 embryos (e.g., Fig. 14), as well as the infrequent presence of trichomes on *twn*1 cotyledons. In wild-type *Arabidopsis,* trichomes form on leaves but not cotyledons, and they can be considered an indicator of postembryonic development (Meinke, 1992). Their presence on *twn*1 cotyledons indicates that mutant cotyledons are partially transformed into leaves in some individuals; this transformation is probably due to delayed development. Cotyledons and leaves are homologous organs: cotyledons are modified leaves that have evolved specialized embryonic functions (Kaplan and Cooke, 1997). Consistent with this, there is ample genetic evidence that disrupted regulation of specialized embryonic developmental programs in cotyledons can cause cotyledons to resemble leaves, and vice versa. Dramatic conversion of cotyledons to leaf-like organs with trichomes has been observed in the *Arabidopsis lec* mutants, in which regulators of embryo-specific programs have been disrupted (Meinke, 1992; Meinke et al., 1994; West et al., 1994; Lotan et al., 1998). A converse scenario is observed in the *xtc* mutant, in which leaves initiate prematurely from the embryonic shoot meristem during seed development. As a result of this early initiation, embryo-specific programs are active in early *xtc* leaves, and the leaves exhibit cotyledon-like

features such as late-embryo storage reserves and an absence of trichomes (Conway and Poethig, 1997). The *xtc* phenotype indicates that the timing of apical organ development can affect organ identity. Analogously, the presence of trichomes on *twn*1 cotyledons is likely an infrequent and indirect consequence of delayed cotyledon initiation in some *twn*1 embryos, such that cotyledons occasionally complete their development in a context in which there is decreased expression of embryo-specific genetic programs.

Distinct developmental functions for TWN1 in embryogenesis—Pleiotropic developmental mutations can be difficult to interpret, but they can also be informative, for they can reveal common genetic components of seemingly unrelated developmental processes and can indicate multiple roles for a gene in development. Given the pleiotropic effects of the *twn*1 mutation on cotyledon and suspensor development, overall models of *TWN*1 gene function must be revised to account for the role of *TWN*1 in the embryo apex as well as its previously defined role in suspensor cell maintenance. Previous models suggested that *TWN*1 was essential for proper communication between the embryo proper and suspensor, and three general scenarios had been proposed (Schwartz, Yeung, and Meinke, 1994; Vernon and Meinke, 1994; summarized in the introduction of this manuscript). Models in which *TWN*1 is active solely in EP–suspensor communication can now be ruled out, as they fail to account for the cotyledon and primary leaf defects we observed. Our results are most consistent with an alternative model in which *TWN*1 has separate developmental functions affecting the suspensor and embryo proper (Vernon and Meinke, 1994). Within the context of this general model, the most straightforward scenario is one in which *TWN*1 functions in a cell communication mechanism that affects both these embryonic regions. Cell interaction is known to be important throughout plant development to establish positional information, especially in the shoot apex (Barton, 1998; Lenhard and Laux, 1999). During embryogenesis it is thought to be of particular importance both for control of suspensor fate and cotyledon patterning (Yeung and Meinke, 1993; Schwartz, Vernon, and Meinke, 1997; Fernandez, 1997; Harada, 1999). Therefore, a role in cell communication is consistent with *TWN*1's previously proposed role in EP–suspensor interaction and with its newfound role in cotyledon patterning.

Our finding that a second cotyledon mutant, *amp*1, also produces twins via suspensor transformation demonstrates that the pleiotropic link between suspensor and cotyledon development is not restricted to *twn*1, and it further supports the interpretation that common genetic mechanisms influence development in these different embryo regions. How can individual genes such as *TWN*1 or *AMP*1 influence such different processes as suspensor maintenance and apical organ pattern? Actually, such a scenario jibes well with our proposed role for *TWN*1 in cell communication. In animal systems as diverse as *Drosophila* and vertebrates, specific cellular signals or signal perception pathways can trigger distinct cellular responses at different times and in different regions of the developing embryo. This allows a single pathway (and its component gene products) to function in multiple, separate developmental events (Gilbert, 1997). As complex multicellular eukaryotes, plants, like animals, have only a limited molecular ''toolkit'' with which to carry out the numerous cellular processes required for development, such as imparting or perceiving positional information. Therefore, it perhaps is not surprising that

there is overlap between the mechanisms operating during suspensor and cotyledon development in the *Arabidopsis* embryo.

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