The Plant Cell, Vol. 5, 621–630, June 1993 © 1993 American Society of Plant Physiologists

Auxin Polar Transport Is Essential for the Establishment of Bilateral Symmetry during Early Plant Embryogenesis

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We used an in vitro culture system to investigate the effects of three auxin polar transport inhibitors (9-hydroxyfluorene-9-carboxylic acid, *trans*-cinnamic acid, and 2,3,5-triiodobenzoic acid) on the development of early globular to heart-shaped stage embryos of Indian mustard (*Brassica juncea*) plants. Although the effective concentrations vary with the different inhibitors, all of them induced the formation of fused cotyledons in globular (≤60 μm) but not heart-shaped embryos. Inhibitor-treated Brassica embryos phenocopy embryos of the Arabidopsis *pin-formed* mutant *pin1-1*, which has reduced auxin polar transport activity in inflorescence axes, as well as embryos of the Arabidopsis *emb30* (*gnom*) mutant. These results indicate that the polar transport of auxin in early globular embryos is essential for the establishment of bilateral symmetry during plant embryogenesis. Based on these observations, we propose two possible models for the action of auxin during cotyledon formation.

INTRODUCTION

In dicotyledonous plants, the early embryo is globular in shape, whereas the mature embryo is a bilaterally symmetrical structure along the apical–basal axis and has two cotyledons. The attainment of bilateral symmetry from axial symmetry in plant embryogeny occurs at the time of the transition from the globular to the heart-shaped stage (Tykarska, 1979). The mechanism underlying this change in symmetry is unknown. Experimental embryology, which has been extremely valuable in elucidating certain mechanisms in animal morphogenesis, has only played a limited role in our understanding of plant morphogenesis (Steeves and Sussex, 1989). A major reason for the limited contribution of experimental embryology to plant development is the relative inaccessibility of the plant embryo at an early stage (Raghavan, 1986). Although there has been recent progress from the genetic dissection of embryo development by the isolation and characterization of mutants (Mayer et al., 1991; Meinke, 1991), the study of plant embryo ontogeny still depends on traditional reconstructions from serial sections of fixed materials.

Somatic embryogenesis has long been considered a model system for the understanding of plant embryo development since it was first reported in carrot more than 30 years ago (Steward, 1958). Owing to the totipotency of plant cells, somatic embryogenesis can be induced from differentiated tissues and organs of many species, including pollen, mesophyll, stem, root, and even isolated protoplasts (Carman, 1990). Results from cell culture studies indicate that this process is elicited by growth regulators, especially auxin and cytokinin. However, the fact that different plant species have different requirements for exogenous hormones makes interpreting the mode of hormone action difficult (Ammirato, 1983; Terzi and Lo Schiavo, 1990). Because plant hormones are small molecules and exist in low concentrations in the plant cell, it is very difficult to ascertain their site of synthesis and to determine their sites of action during plant development. In the case of auxin, an indirect approach to studying this problem, which has proven to be effective in seedlings (Okada et al., 1991) and shoot apices (Schwabe, 1984), is the use of auxin transport inhibitors or antagonists. Unfortunately, no such work has been performed on zygotic embryos thus far because of the difficulty in culturing proembryos in vitro (Raghavan, 1986).

We have recently described an in vitro system for culturing proembryos (≥35 μm) of Indian mustard (*Brassica juncea*) (Liu et al., 1993). In this culture system, early globular stage zygotic embryos can develop into normal, mature embryos with high efficiency (~75%). In this study, we have used the in vitro culture system to show that inhibitors of auxin polar transport can cause the formation of fused cotyledons in embryos of Indian mustard. These Brassica embryos with fused cotyledons are phenocopies of the *pin-formed pin1-1* mutants of Arabidopsis, which are impaired in auxin polar transport (Okada et al., 1991), and of the Arabidopsis *emb30* (*gnom*) mutant embryos (Meinke, 1985; Mayer et al., 1993).

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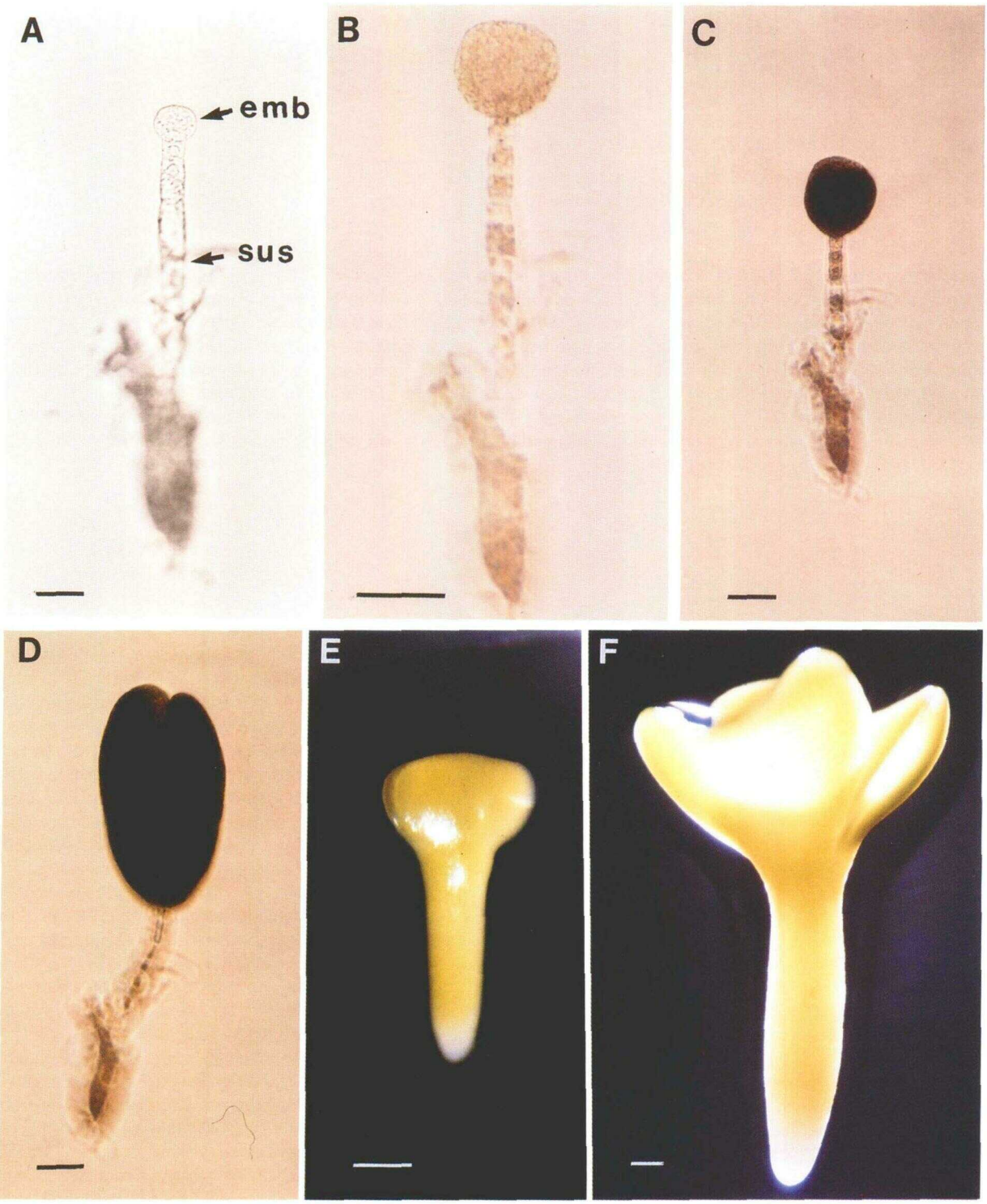

Figure 1. Normal Development of in Vitro–Cultured Embryos of Indian Mustard.

(A) A freshly dissected early globular-stage embryo (emb; 38 μm) with intact suspensor (sus) from an ovule 5 days after anthesis.
(B) to **(F)** The same embryo cultured in ECM medium for 1 day, **(B)**; 2 days, **(C)**; 4 days, **(D)**; 7 days, **(E)**; 12 days, **(F)**.
Bars in **(A)** to **(D)** = 50 μm, and bars in **(E)** and **(F)** = 200 μm.

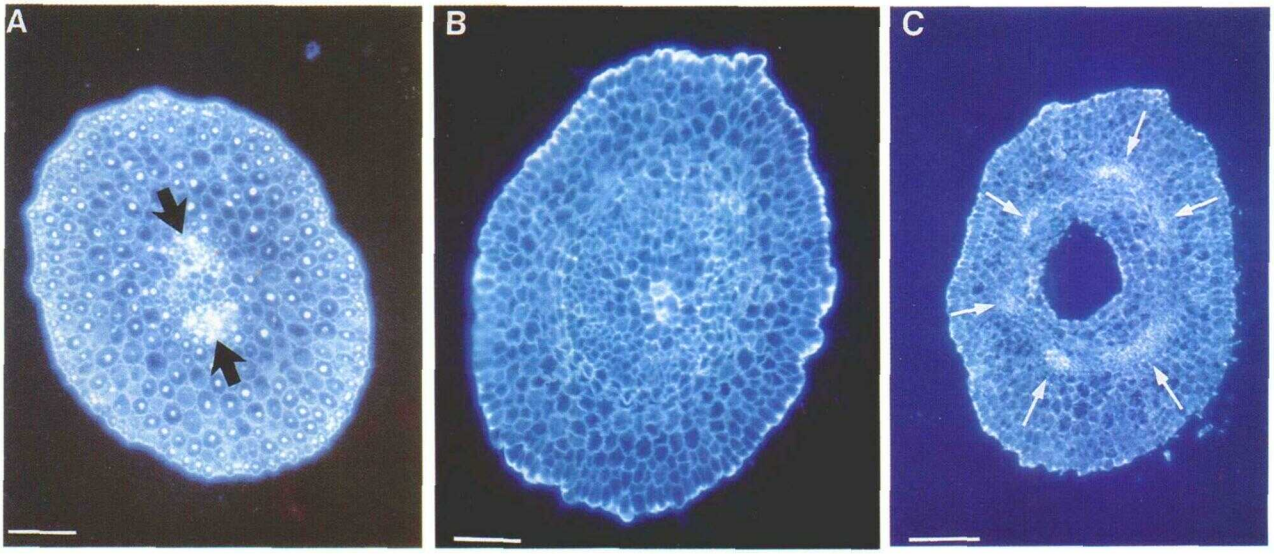

Figure 2. Transverse Section of in Vitro–Developed Embryo of Indian Mustard with or without Auxin Transport Inhibitor.

(A) Hypocotyl of normal mature embryo developed in vitro and stained with both fluorescent brightener 28 and 4',6-diamidino-2-phenylindole.
(B) Hypocotyl of cylindrical embryo obtained by treatment with 10 μM HFCA and stained with fluorescent brightener 28 only.
(C) Fused cotyledon obtained by treatment with 10 μM HFCA and stained with fluorescent brightener 28 only.
Arrows in **(A)** and **(C)** indicate the vascular bundles. Bars = 50 μm.

RESULTS

Normal Development of Indian Mustard Embryos in Vitro

When cultured in a double-layer culture system with the embryo culture medium (ECM) previously developed by Liu et al. (1993), Indian mustard proembryos developed to the normal cotyledonary stage within 12 to 15 days. The transition from globular to heart-shaped stage required ~2 days, and an additional 2 to 3 days were needed to reach the torpedo-shaped stage, as shown in Figures 1A to 1E. More than 75% of the cultured globular embryos achieved maturity within 12 to 15 days, by which time the two expanded cotyledons and an elongated hypocotyl could be distinguished (Figure 1F). Tissue sections showed that there were two vascular bundles in the hypocotyl, as shown in Figure 2A, which are connected to the main veins of the cotyledons. The cultured embryos showed the same differentiation of organs and tissues as embryos grown in vivo. Because the top-layer medium was quite thin, the embedded embryos developed within the medium in the first 4 to 5 days, after which time the embryos (normally at torpedo-shaped stage) continued to grow on the surface of the medium. Mature embryos were germinated in a low-sucrose B5 (Gamborg et al., 1968) medium to produce seedlings (Liu et al., 1993).

Effects of Auxin Polar Transport Inhibitors on Cultured Embryos of Indian Mustard

Three different auxin transport inhibitors, 9-hydroxyfluorene-9-carboxylic acid (HFCA), *trans*-cinnamic acid, and 2,3,5-triiodobenzoic acid (TIBA), were added at various concentrations to both the bottom and top layers of the ECM to determine their effects on embryo development. At the range of concentrations tested (see Methods), there was no apparent effect of the chemical on embryo growth rate. Both treated and control embryos reached the same developmental stage at approximately the same time. Whereas the normal Indian mustard embryo has two separate cotyledons, all of the three inhibitors induced the fusion of cotyledons. The fused cotyledon appeared like a collar around the apical meristem, resulting in an embryo with a cylindrical structure above the apical meristem, as shown in Figures 3D and 3E. The fused cotyledon initiated at the late-globular stage, which was 2 days after the globular-stage embryos were placed in culture with the inhibitors (Figure 3C). Instead of two cotyledon primordia emerging from the top of the globular embryo, cotyledon initiation occurred simultaneously all around the apical promeristem, forming a collarlike structure (Figures 3D and 3E). For this reason, there was no real heart- or torpedo-shaped embryo formed in the presence of the inhibitors. The morphology of the cylindrical embryo can be clearly seen in the scanning electron micrograph in Figure 4A.

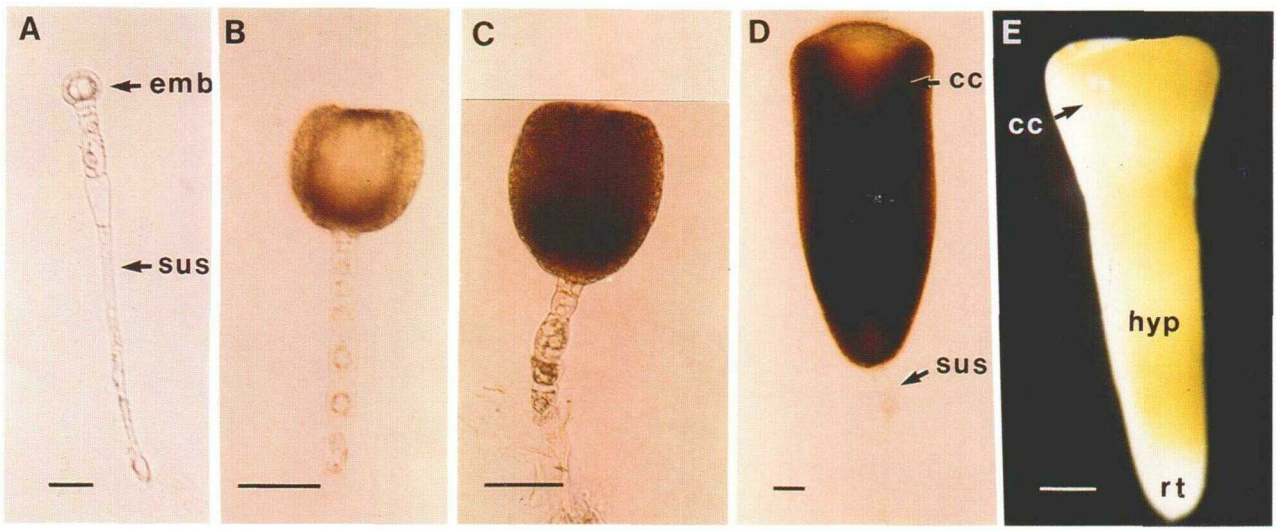

Figure 3. In Vitro–Developed Embryo of Indian Mustard in the Presence of TIBA.

(A) Embryo at 0 days after culture. emb, embryo proper; sus, suspensor.
(B) Embryo at 1 day after culture.
(C) Embryo at 2 days after culture.
(D) Embryo at 4 days after culture. cc, cylindrical cotyledon; sus, suspensor.
(E) Embryo at 7 days after culture. cc, cylindrical cotyledon; hyp, hypocotyl; rt, root.
Early globular stage embryos (45 μm) of Indian mustard were developed in the presence of 2 μM TIBA. As shown in **(C)**, note that the fused cotyledon started to form at the late-globular stage. Similar results were obtained with 10 μM of HFCA and 80 μM of *trans*-cinnamic acid (data not shown). Bars in **(A)** to **(D)** = 50 μm, and the bar in **(E)** = 200 μm.

The minimal concentration at which 100% of the globular-stage embryos developed into mature embryos with fused cotyledons varied with the different inhibitors used, as shown in Figure 5. TIBA was the most effective of the three chemicals tested; only 2 μM was required to induce cotyledon fusion. For HFCA, 10 μM was required, and for *trans*-cinnamic acid, 80 μM was required. There was no apparent morphological difference among the cylindrical embryos induced by the different inhibitors (Figure 3 and data not shown). At suboptimal concentrations of auxin transport inhibitor, for example 5 μM of HFCA, embryos with half-fused cotyledons could be induced sometimes, i.e., one side of the two cotyledons would remain separated but the other would be fused, as shown in Figure 6. In most cases, the fused side was in contact with the medium. Increasing the concentration of the inhibitors above the optimal concentration by 5 to 10 times did not produce any obvious morphological effect on development. For instance, TIBA at 5 or 10 μM still induced 100% of the globular embryos to form collar cotyledons.

The induction of cotyledon fusion by auxin transport inhibitors only occurred when globular-stage embryos were treated. When heart-shaped embryos (60 to 120 μm) were cultured in the same medium in the presence of these inhibitors, all the embryos continued to develop two normal cotyledons (data not shown). The morphology of the two cotyledons appeared the same as those of normal, mature embryos. Fused cotyledons were never observed in control experiments (without auxin transport inhibitor; Figure 1) with globular or heart-shaped embryos.

Histological Observation of the Cylindrical Embryos

A detailed histological examination was performed to compare the collar cotyledon embryos with normal embryos. Because the walls of phloem and xylem cells are thicker than those of parenchyma cells, the vascular bundles could be easily detected by staining with a cell wall–specific fluorescent dye (fluorescent brightener 28). Transverse sections of the embryos through the hypocotyl regions, just below the cotyledonary node, showed two vascular bundles in the hypocotyl of normal embryos; these bundles were connected to the main vein of the cotyledons (Figure 2A). In the inhibitor-treated embryos, it was difficult to determine the number of vascular bundles in the hypocotyl region because this region was shorter and also less developed than that of the normal embryo (Figure 2B). In the collar cotyledon region, there were six vascular bundles of similar size (Figure 2C), in contrast to the normal

cotyledon in which there was only one main vein and several minor veins. Longitudinal sections of the inhibitor-treated embryos indicated that most of the tissues, such as epidermis, cortex, and root meristem, were perfectly developed, as shown in Figure 7. The only notable difference was that the apical meristem appeared more juvenile than that of the normal embryos; at this stage, the apical meristem normally forms a convex dome (see Figure 5H of Liu et al., 1993), whereas in the cylindrical embryo only densely stained meristematic cells were observed (Figure 7A).

Germination of Cylindrical Embryos

The embryos with fused cotyledons were germinated by transferring them onto B5 medium (Gamborg et al., 1968) with 1% sucrose, which has been shown previously to be suitable for the germination of embryos grown in vitro (Liu et al., 1993). Two days after transfer to this medium, the collar cotyledon and the radicle began to elongate. The length of the hypocotyl plus cotyledon reached 1.5 to 2.0 cm after 5 days of culture, as shown in Figure 8A. There was no obvious change over the next 10 days, but subsequently, a new shoot emerged, splitting the tubular cotyledon in two. The shoot originated from inside the base of the tubular structure (Figure 8B), indicating that most of this structure (Figure 8A) is part of the tubular cotyledon and not the hypocotyl. This was confirmed by transverse section of the cylindrical embryos. Further observation showed that the seedlings were morphologically normal and also fertile after transplantation into soil in pots.

Embryo Development in the *pin1-1* Mutant of Arabidopsis

To investigate whether the function of auxin polar transport in embryo development is a more widespread phenomenon, we investigated embryo development of the Arabidopsis *pin1-1* mutant that has reduced auxin polar transport activity (14% of normal plant) along the inflorescence axis (Okada et al.,

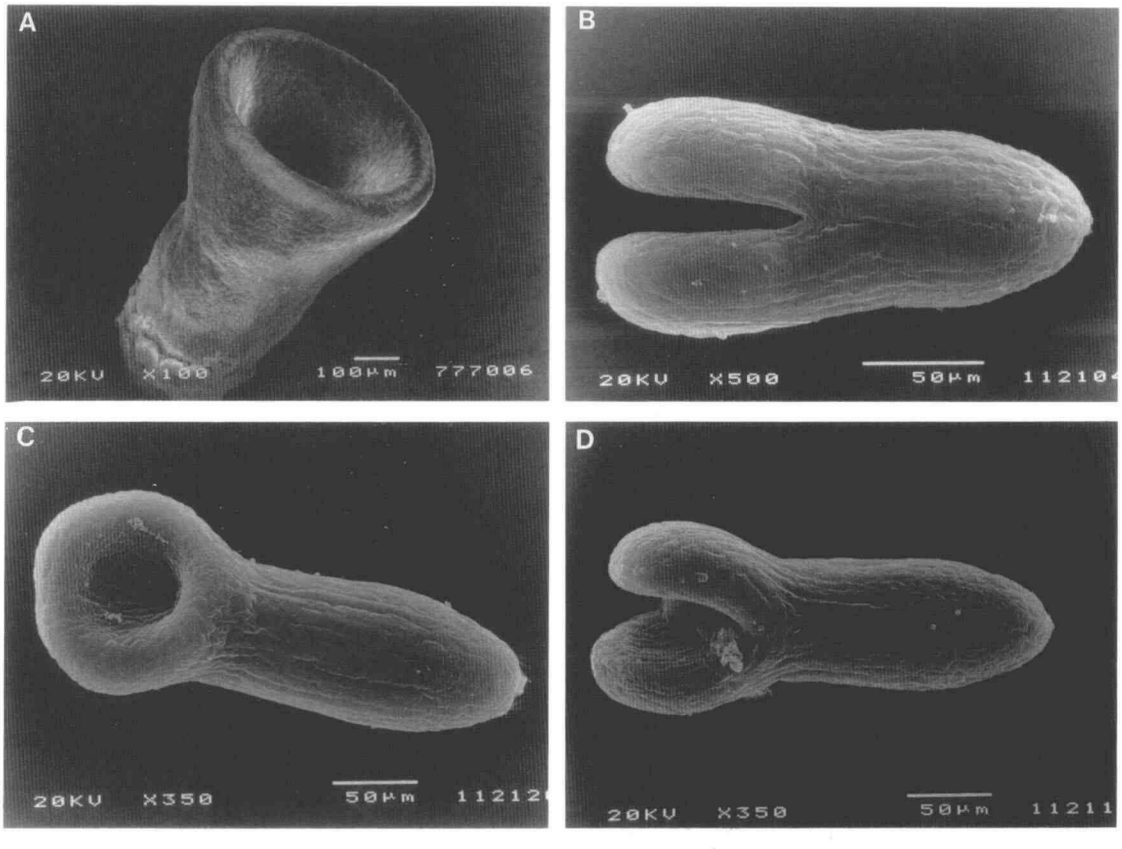

Figure 4. Scanning Electron Micrographs of Indian Mustard and Arabidopsis Embryos.

(A) Indian mustard embryo with fused cotyledon induced by 80 µM of *trans*-cinnamic acid after 10 days of culture in vitro.
(B) to **(D)** Morphology of Arabidopsis embryos dissected from selfed heterozygous plants of a *pin1-1* mutant. The three different phenotypes that were found are as follows: normal embryo in **(B)**, 79%; fused cotyledon in **(C)**, 5%; intermediate type with partially fused cotyledon in **(D)**, 16%.

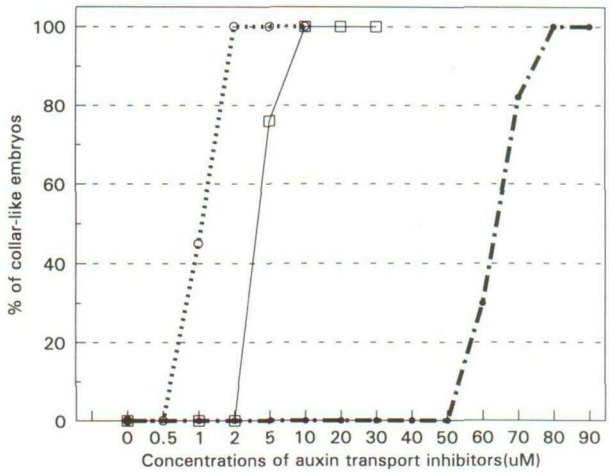

Figure 5. Effect of Different Concentrations of Three Auxin Polar Transport Inhibitors on the Induction of Embryos with Fused Cotyledon.

Dotted line, with TIBA; solid line, with HFCA; dashed line, with *trans*-cinnamic acid. The minimal concentrations required to convert 100% of the globular embryos to cylindrical mature embryos are 2 μM for TIBA, 10 μM for HFCA, and 80 μM for *trans*-cinnamic acid. Only globular embryos were used in this experiment. Data were collected from 10 independent experiments with 12 to 24 embryos for each treatment. No effect of the auxin transport inhibitors on heart-shaped stage embryos was observed.

1991). Embryos at different stages from siliques of selfed heterozygous plants were dissected under a microscope. The best time to observe the mutant embryo phenotype was at the torpedo-shaped stage, when any changes could be detected easily. Three to four siliques were examined on each plant. Each silique contained 20 to 30 ovules. There were three different types of embryos produced by the selfed *pin1-1* heterozygous plant: normal, with two separated cotyledons (79%; Figure 4B); cylindrical, with a fused cotyledon (5%; Figure 4C); and phenotype intermediate between the two (16%; Figure 4D). In the intermediate type, the length of the two separated cotyledons appeared shorter than normal but the hypocotyl appeared longer, and there was a deep depression between the two cotyledons. In other words, the upper portion of the cotyledons remained separate but the lower portion was connected (Figure 4D). The unexpected ratio of 4:1 between normal embryos and embryos with partially or completely fused cotyledon might be caused by the difficulty in distinguishing the intermediate type embryos from the normal embryos, resulting in an underestimation of the former category.

DISCUSSION

The transition from the globular to heart-shaped stage during embryogenesis is a marker for the attainment of bilateral symmetry, which is characterized by the cessation of uniform growth and the initiation of the two cotyledons. At this point, cell division occurs predominantly at the cotyledonary parts, and the cells at the cleft of the cotyledons cease to divide, thus bringing about a heart-shaped embryo (Tykarska, 1979; Raghavan, 1986). The physiological and biochemical basis for these processes are poorly understood. Wardlaw (1955) suggested that the transition from axial symmetry to bilateral symmetry might be explained by the presence of bilateral gradients in the embryo sac. Results from somatic embryogenesis in plant tissue culture, however, indicate that the change in symmetry seems to be caused by purely endogenous factors (Halperin, 1966). In this paper, we have presented evidence that the change in symmetry can be blocked by inhibitors of auxin polar transport. Thus, auxin polar transport in the embryo is important for the formation of two cotyledons.

Of all the known plant hormones, auxin is the only one that exhibits polar transport (Kaldewey, 1984). The direction of movement is predominantly basipetal (toward the bottom of the plant) in the shoot and acropetal (toward the top of the plant) in the root, and this has been recognized since auxin was discovered more than 60 years ago (Went, 1928). The polarity of auxin transport has long been considered to be associated

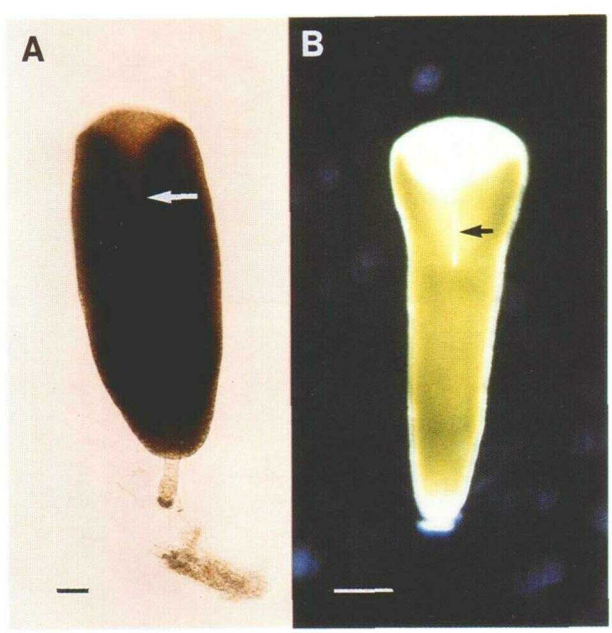

Figure 6. In Vitro Development of an Early Globular-Stage Embryo (40 μm) of Indian Mustard in the Presence of 5 μm of HFCA in the Medium.

(A) Four days after culture. Bar = 50 μm.
(B) Seven days after culture. Bar = 20 μm.
Note the formation of partially fused cotyledons (arrows indicate the fused side of the cotyledons) when the concentration of the auxin polar transport inhibitor was slightly lower than the optimal concentration.

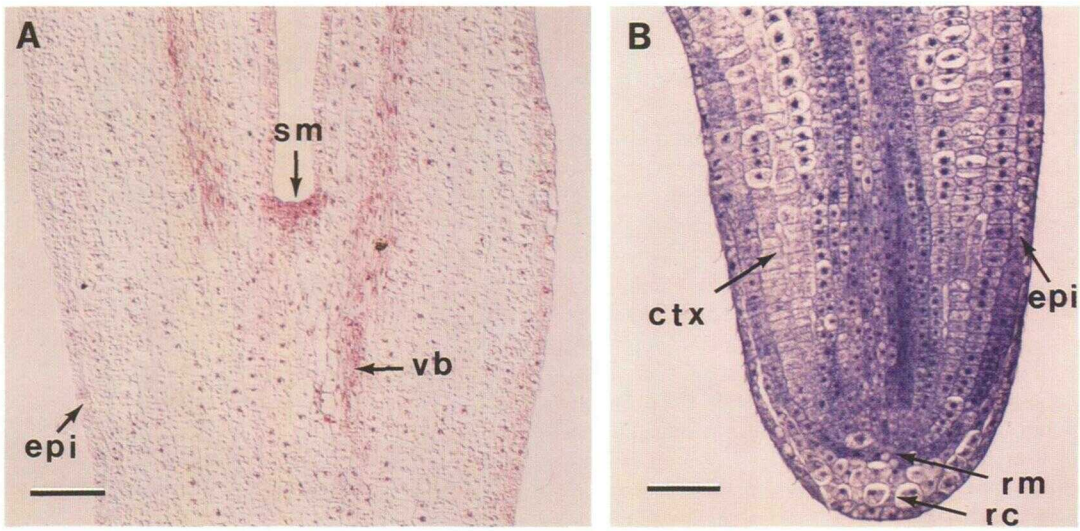

Figure 7. Longitudinal Sections of a Cylindrical Embryo of Indian Mustard Obtained by Treatment with 10 μM of HFCA.

(A) Region around the apical meristem. sm, shoot meristem; epi, epidermis; vb, vascular bundle.
(B) Region around the root meristem. ctx, cortex; rm, root meristem; rc, root cap; epi, epidermis.
Sections were stained with hematoxylin. Note that the apical meristem in **(A)** appears juvenile compared to that of normal mature embryos grown in vitro (Liu et al., 1993). Bars = 50 μm.

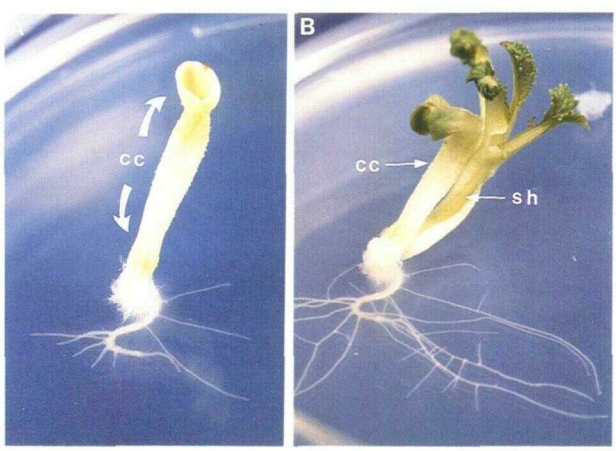

Figure 8. Germination of an Indian Mustard Embryo with Fused Cotyledons on Hormone-Free B5 Medium Containing 1% Sucrose.

(A) and **(B)** show the same embryo cultured on the medium for 5 and 20 days, respectively.
(A) The main root and lateral roots were formed within the first 5 days; the tubular cotyledon also elongated during this time. cc, cylindrical cotyledon.
(B) As the new shoot germinated, the tubular cotyledon was split apart. cc, cylindrical cotyledon; sh, shoot.

with a number of developmental and growth phenomena, such as phototropism, apical dominance, and adventitious root formation (Jacobs, 1979; Rubery, 1987). Although various types of embryo development mutants have been isolated in Arabidopsis (Meinke, 1985; Mayer et al., 1991) and a number of abnormalities in somatic embryogenesis have been reported in some plant species (Ammirato, 1983), there is as yet no evidence that auxin polar transport is involved in embryo morphogenesis. Recent advances in zygotic proembryo culture provide a precise approach to investigate embryo development in vitro (Liu et al., 1993). The advantage of this system is that embryos comprising only 8 to 32 cells can be cultured to maturity at high efficiency. Using this method, we have cultured early globular embryos in the presence of auxin polar transport inhibitors (HFCA, TIBA, and *trans*-cinnamic acid) and have shown that these inhibitors can induce the formation of fused cotyledon in Indian mustard. The minimal concentrations for the different inhibitors varied: 2 μM for TIBA, 10 μM for HFCA, and 80 μM for *trans*-cinnamic acid. The number of vascular bundles in the cotyledon also changed from two to six. Using the word "fused" may be inappropriate because, in fact, two distinct cotyledons were never formed even at a very early stage. It appears that a circular layer of cells on top of the embryo divided parallel to the surface and developed into a collarlike cotyledon. A similar phenomenon was observed by

Schwabe (1984) on the position of leaf primordia. He applied TIBA to the shoot apex of Epilobium plants and found that the leaf primordia phyllotaxis was disrupted, producing a collar-shaped apex.

There is little difference between the normal mature embryo and the cylindrical embryo in longitudinal section, except that the apical meristem appears juvenile. These results therefore suggest that either tissue differentiation and organogenesis along the longitudinal axis are not affected by reduced auxin polar transport or that the structural organization and functional differentiation of root, hypocotyl, and apical meristem are already determined at the globular stage of development.

Because auxin can be transported in plant tissue, it is difficult to distinguish the site of synthesis from the site of action of this hormone. A major difficulty in obtaining reliable results is that auxin is highly soluble in both polar and nonpolar solvents, which are commonly used in conventional methods to prepare specimens for microscopy. In this case, an auxin polar transport inhibitor provides a useful tool to explore this problem.

It is known that auxin transport inhibitors, such as TIBA, can effectively block auxin transport by binding to the regulatory site of an auxin efflux carrier complex (Depta et al., 1983; Venis, 1985). Unfortunately, little is known about auxin synthesis and transport in the plant embryo. From our experiments, we speculate that auxin is not evenly synthesized in wild-type globular-stage embryos. When the embryo was treated with these inhibitors, auxin would accumulate at the site of synthesis; therefore, the target site would receive insufficient amounts of auxin to bring about stimulation of growth and development. Based on this hypothesis, we propose two possible models, shown in Figure 9, for the action of auxin in proembryos to explain our experimental results. Auxin could either be synthesized in the shoot primordium, as shown in Figure 9A, or in a circular layer of cells around the apical meristem, as shown in Figure 9B.

In model A, if auxin is synthesized in the shoot primordium, there must be an excess amount of auxin in these primordial cells to inhibit their growth. At the same time, auxin is transported polarly in opposite directions to the two areas on top of the globular embryo to initiate the formation of two cotyledons. When polar transport is blocked, auxin could diffuse randomly from the central region to the surrounding cells. This would stimulate the formation of a collar cotyledon (Figure 9A). Another possible model (Figure 9B), which is consistent with the formation of a cylindrical structure, is that auxin is synthesized at a low level in a circular layer of cells on top of the globular embryo and then transported to the two cotyledon-forming regions to initiate cotyledon primordia. A prerequisite of this hypothesis is that in the embryo auxin is present at a concentration sufficient to stimulate cell division and elongation rather than to inhibit these processes. When auxin polar transport is blocked, local accumulation of auxin in this circular area would lead to the formation of a collarlike cotyledon (Figure 9B). Because auxin transport inhibitors only induced cotyledon fusion in globular-stage embryos, but not in heart-shaped ones, it is reasonable to suggest that auxin transport

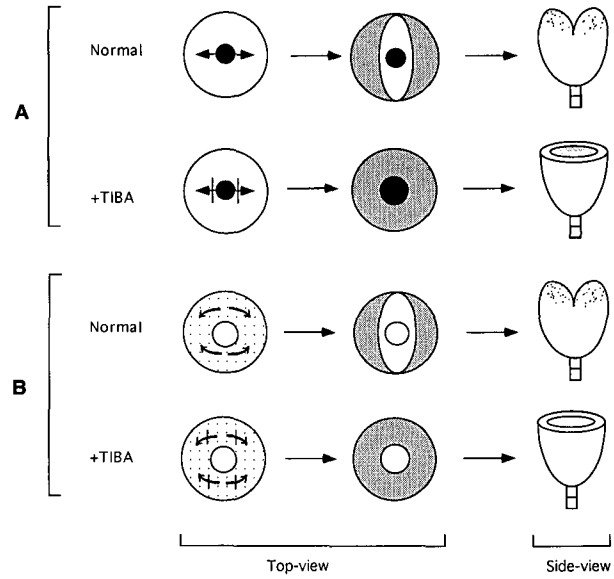

Figure 9. Diagrams of Two Possible Models of Auxin Synthesis and Transport during Cotyledon Initiation.

Because the cylindrical structured embryo was formed when auxin transport was blocked, it is not possible that auxin is evenly synthesized in the entire globular embryo. Otherwise, the central depression would not be formed when auxin polar transport was blocked. Auxin could either be synthesized in the shoot primordium **(A)** or the area surrounding the shoot primordium **(B).** The top view shows the site of auxin synthesis and direction of polar transport (indicated by arrows) in globular embryo. The side view shows the shapes of embryo produced in the absence or presence of auxin polar transport inhibitors. **(A)** If auxin is synthesized in the shoot primordium (black area) and then transported in opposite directions (shown by arrows) to the two cotyledon-forming areas (dotted area), an appropriate amount of auxin would stimulate cells of these two regions to divide, thus leading to the formation of two cotyledons. If this is true, the concentration of auxin in the shoot primordium must inhibit cell division so that a heart-shaped embryo would be formed. When auxin polar transport is blocked, diffusion of the auxin from the shoot primordium to the surrounding cells would result in the formation of a collar cotyledon. **(B)** If auxin is synthesized in a circular layer of cells outside the shoot primordium (lightly dotted area) and then transported along this circle (shown by arrows) to the two cotyledon-forming regions (densely dotted areas), cell division would be stimulated in these two regions and a heart-shaped embryo would ensue. In this case, when auxin polar transport is blocked, accumulation of auxin would occur in the circle area, and a collarlike cotyledon would be induced as well. In this case, auxin is present in the embryo at a level sufficient to stimulate but not inhibit cell division.

for the initiation of the two cotyledons is needed only for a short period during the globular stage. Further growth of the cotyledons does not depend on a normal rate of auxin polar transport.

The requirement of auxin polar transport for the establishment of bilateral symmetry during embryogenesis is further suggested by observations of a mutant of Arabidopsis (*pin1-1*). The *pin1-1* mutant, which has abnormal flower and

inflorescence axis development, was first isolated by Goto et al. (1987). Okada et al. (1991) showed that auxin polar transport activity in the inflorescence axis of *pin1-1* is decreased to 14% of the wild type. We found that ~20% of the embryos derived from selfing *pin1-1* heterozygous plants had abnormal cotyledons; the cotyledons were either partially or completely fused. This result indicates that in wild-type Arabidopsis the auxin polar transport system operates not only in inflorescence and floral bud development but also in embryogenesis. It is particularly striking that *pin1-1* embryos with completely fused cotyledons show more or less the same morphology as Indian mustard embryos treated with auxin polar transport inhibitors. The partial fusion of the cotyledons might be caused by the residual auxin transport (14%) existing in *pin1-1* (Okada et al., 1991). Another mutant, *emb30* (*gnom*), also produces embryos with partially or completely fused cotyledons (Meinke, 1985; Mayer et al., 1993), suggesting that the *emb30* gene product may play a direct or indirect role in auxin polar transport.

In conclusion, our data show that auxin polar transport plays an important role in cotyledon formation during embryo development. Interference with this transport causes a failure in the transition from axial to bilateral symmetry and results in the formation of embryos with fused cotyledons.

METHODS

Chemicals

The three auxin transport inhibitors used in this study were 9-hydroxyfluorene-9-carboxylic acid (HFCA; Sigma H8507), *trans*-cinnamic acid (Sigma C6004), and 2,3,5-triiodobenzoic acid (TIBA; Sigma T7267). The composition of the embryo culture medium (ECM) was reported previously (Liu et al., 1993).

Plant Materials and Embryo Culture

Indian mustard (*Brassica juncea*) plants were grown in a growth chamber under 14-hr light/10-hr dark conditions at 24°C day/20°C night. The light intensity was ~4000 lux and the relative humidity was 70%. After surface sterilization with a 10% solution (v/v) of a commercial bleach (containing 5.25% sodium hypochlorite) for 10 min, pods were washed with sterile distilled water before the ovules were collected. Globular-stage zygotic embryos (35 to 60 μm) were dissected from the ovules 5 to 6 days after pollination, and heart-shaped embryos (60 to 120 μm) were collected from the ovules 7 to 8 days after pollination. All the dissection steps were performed in a 9% (w/v) glucose solution, which was required to prevent osmotic shock of the embryos. Details of the dissection procedure have been described previously (Liu et al., 1993). Stock solutions of TIBA, HFCA, and *trans*-cinnamic acid were prepared in water at a concentration of 1 or 10 mM, sterilized by filtration, and stored at 4°C. To test the effects of auxin transport inhibitors on embryo development, 0.5 to 10 μM final concentration of TIBA, 1 to 50 μM HFCA, and 1 to 90 μM *trans*-cinnamic acid were added to the ECM (Liu et al., 1993), with the same concentrations in both the bottom and top layers. Six or 12 embryos were used for each treatment in 24-well Multiplates (Nunc, Life Technologies, Gaithersburg, MD). Twenty-four or 48 embryos were dissected for each experiment. Embryo growth and development were observed and photographed periodically using an inverted phase-contrast microscope (Zeiss, Thornwood, NY).

To germinate cultured embryos, matured embryos (after 12 to 15 days of culture in the ECM) were transferred onto B5 (Gamborg et al., 1968) agarose medium with 1% (w/v) sucrose. For optimal germination, it was necessary to insert the radicle part of the embryo into the agarose medium and avoid contact of the cotyledons with the latter.

Embryo Development in the Arabidopsis *pin1-1* Mutant

To observe embryo development in the *Arabidopsis thaliana pin-formed* mutant *pin1-1*, heterozygous seeds were sown in a growth chamber at 19°C under a 12-hr photoperiod. Siliques were collected at 12 days after anthesis, and embryos at torpedo-shaped stage were dissected from ovules under a microscope. Normal and abnormal embryos were counted and collected for scanning electron microscopy.

Histological Analyses

Cultured or dissected embryos were fixed for 2 hr in a modified FAA fixative (containing 50% ethanol, 5% formaldehyde, 6% acetic acid, and 5% glycerol). Fixed specimens were dehydrated in a graded ethanol series, cleared with xylene, and embedded with paraplast (Oxford, St. Louis, MO). The embedded specimens were then sectioned (6 to 7 μm) with a microtome (model 2050; Reichert Jung, Buffalo, NY). Dewaxed sections were stained either with Delafield's premixed aluminum hematoxylin (Schneider, 1981) for tissue structure or 4',6-diamidino-2-phenylindole (1 μg/mL; Sigma, D1388) for nuclei, and fluorescent brightener 28 (10 μg/mL; Sigma, F6259) was used for cell walls. Photographs were taken with an Axiophot microscope (Zeiss) using either a fluorescence or a differential-interference contrast device.

Scanning Electron Microscopy

Specimens were fixed in 4% glutaraldehyde in PBS overnight at 4°C and then dehydrated through a graded ethanol series and critical-point dried in liquid carbon dioxide on Samdri-780A (Tousimis Research Corporation, Rockville, MD). Specimens were mounted on stubs, sputter coated with gold, and examined with a scanning electron microscope (model JSM-T220A; JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV.

ACKNOWLEDGEMENTS

We thank Dr. Kiyotaka Okada (National Institute for Basic Biology, Okazaki, Japan) for seeds of Arabidopsis *pin1-1* mutant, Grek-Luen Loy (Dept. of Zoology, National University of Singapore) for assistance in electron microscopy, Drs. Gunther Neuhaus and Ben Kost (ETH, Zurich) for useful discussions, and Drs. Diane Shevell (The Rockefeller University) and Trevor L. Wang (John Innes Institute) for critical comments on the manuscript.

Received April 15, 1993; accepted April 29, 1993.

REFERENCES

Ammirato, P.V. (1983). The regulation of somatic embryo development in plant cell culture: Suspension culture techniques and hormones requirement. Biotechnology **1,** 68–74.

Carman, J.G. (1990). Embryogenic cells in plant tissue cultures: Occurrence and behaviour. In Vitro Cell. Dev. Biol. **26,** 746–753.

Depta, H., Elisele, K.-H., and Hertel, R. (1983). Specific inhibitors of auxin transport: Action on tissue segments and in vitro binding to membranes from maize coleoptiles. Plant Sci. Lett. **31,** 181–192.

Gamborg, O.L., Miller, R.A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50,** 151–158.

Goto, N., Starke, M., and Kranz, A.R. (1987). Effect of gibberellins on flower development of the *pin*-formed mutant of *Arabidopsis thaliana*. Arabidopsis Inform. Serv. **23,** 66–71.

Halperin, W. (1966). Alternative morphogenetic events in cell suspensions. Am. J. Bot. **53,** 443–453.

Jacobs, W.P. (1979). Plant Hormones and Plant Development. (Cambridge, UK: Cambridge University Press).

Kaldewey, H. (1984). Transport and other modes of movement of hormones (mainly auxins). In Hormonal Regulation of Development II, T.K. Scott, ed (Heidelberg: Springer-Verlag), pp. 80–148.

Liu, C.-M., Xu, Z.-H., and Chua, N.-H. (1993). Proembryo culture: *In vitro* development of early globular-stage zygotic embryos from *Brassica juncea*. Plant J. **3,** 291–300.

Mayer, U., Ruiz, R.A.T., Berleth, T., Misera, S., and Jurgens, G. (1991). Mutations affecting body organisation in the *Arabidopsis* embryo. Nature **532,** 402–407.

Mayer, U., Buttner, G., and Jurgens, G. (1993). Apical-basal pattern formation in the *Arabidopsis* embryo: Studies on the role of the *gnom* gene. Development **117,** 149–162.

Meinke, D.W. (1985). Embryo-lethal mutants of *Arabidopsis thaliana*: Analysis of mutants with a wide range of lethal phases. Theor. Appl. Genet. **69,** 543–552.

Meinke, D.W. (1991). Perspectives on genetic analysis of plant embryogenesis. Plant Cell **3,** 857–866.

Okada, K., Ueda, J., Komaki, M.K., Bell, C.J., and Shimura, Y. (1991). Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. Plant Cell **3,** 677–684.

Raghavan, V. (1986). Embryogenesis in Angiosperms: A Developmental and Experimental Study. (Cambridge, UK: Cambridge University Press).

Rubery, P.H. (1987). Auxin transport. In Plant Hormones and Their Role in Plant Growth and Development, P.J. Davies, ed (Dordrecht: Martinus Nijhoff Publishers), pp. 341–362.

Schneider, H. (1981). Plant anatomy and general botany. In The Staining Procedures, 4th ed., G. Clark, ed (Baltimore: Williams & Wilkins), pp. 315–333.

Schwabe, W.W. (1984). Phyllotaxis. In Positional Controls in Plant Development, P.W. Barlow and D.J. Carr, eds (Cambridge, UK: Cambridge University Press), pp. 403–440.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development. (Cambridge, UK: Cambridge University Press).

Steward, F.C. (1958). Growth and development of cultivated cells. III. Interpretations of the growth from free cell to carrot plant. Am. J. Bot. **45,** 709–713.

Terzi, M., and Lo Schiavo, F. (1990). Somatic embryogenesis. In Plant Tissue Culture: Applications and Limitations, S.S. Bhajwani, ed (Amsterdam: Elsevier), pp. 54–101.

Tykarska, T. (1979). Rape embryogenesis. II. Development of embryo proper. Acta Soc. Bot. Pol. **48,** 391–421.

Venis, M. (1985). Hormone Binding Sites in Plants. (New York: Longman).

Wardlaw, C.W. (1955). Embryogenesis in Plants. (New York: John Wiley and Sons).

Went, F.W. (1928). Wuchsstoff und Wachstum. Recl. Trav. Bot. Neerl. **25,** 1–116.