Development 125, 879-887 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV0143

Auxin-induced developmental patterns in *Brassica juncea* embryos

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Accepted 18 December 1997: published on WWW 4 February 1998

SUMMARY

To investigate the mechanism of auxin action during pattern formation in dicot embryos, we tested the effects of the natural auxin indole-3-acetic acid (IAA), the auxin transport inhibitor *N*-(1-naphthyl)thalamic acid (NPA) and the antiauxin p-chlorophenoxyisobutyric acid (PCIB). In vitro treatments of isolated zygotic *Brassica juncea* embryos with these substances led to a wide range of morphogenetic alterations. Treatment of globular embryos with exogenous auxin (10-40 μM) either completely inhibited morphogenesis, resulting in ball-shaped embryos, or caused the development of egg- and cucumber-shaped embryos, which only consisted of a shortened hypocotyl without any apical structures. Axis duplication was observed sometimes after inhibition of auxin transport in globular embryos, and led to the development of twin embryos. During the transition from globular to heart stage, changes in auxin distribution or activity frequently caused the development of either split-collar or collar-cotyledons. Antiauxin inhibited cotyledon growth, leading to embryos with single or no cotyledons, or inhibited the development of the hypocotyl and the radicle. Inhibition of auxin transport in transition embryos sometimes led to axis broadening, which resulted in the development of two radicles. The described changes in embryo shapes represent arrests in different auxin-regulated developmental steps and phenocopy some *Arabidopsis* morphogenetic mutants.

Key words: *Brassica juncea*, Embryogenesis, Pattern formation, Auxin polar transport, Pattern mutants

INTRODUCTION

A basic question in pattern formation is how undifferentiated cells obtain specific fates. In plants, pattern formation is not confined to embryogenesis, because morphogenesis continously occurs during postembryonic development. The primary meristem of the shoot and the root, which are the two pattern elements that produce the structures of the adult plant, are generated in the embryo (Steeves and Sussex, 1989). A fruitful strategy to study pattern formation is the analysis of the response of the morphogenetic system to perturbation. Perturbation in plant development can be induced by physiological factors (e.g., light), by the application of hormones or other substances, or by genetic mutation (Green, 1996). To identify genetic factors regulating pattern formation in plant embryos, *Arabidopsis thaliana* mutants with defects in embryogenesis have been isolated (Jürgens et al., 1991; Mayer et al., 1991; Meinke, 1991, 1995). The first isolated embryogenesis-related genes seem to have basic cellular functions rather than resembling the regulatory genes identified through homeotic mutants in animals (Busch et al., 1996; Long et al., 1996; Lukowitz et al., 1996; Shevell et al., 1994).

New insights into embryonic pattern formation have been achieved over the last decade by perturbing the in vitro development of isolated zygotic or somatic embryos with auxin and related substances (Chasan, 1993). Fry and Wangermann (1976) first proposed that polarized auxin transport in the globular embryo initiates the establishment of morphological axiality. In agreement with this hypothesis, Schiavone and Cooke (1987) found that auxin-treated somatic carrot embryos tend to revert to undifferentiated callus. In addition, treatments with the auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and NPA caused the formation of enlarged globular and oblong embryos and, in heart embryos, the development of additional growth axes on the hypocotyl. Fischer and Neuhaus (1996) reported abnormal development of wheat embryos after treatments with auxins or related substances, indicating that auxin effects on embryo development are not limited to dicots.

Whereas TIBA and NPA blocked morphological transitions to the subsequent stage in somatic carrot embryos (Schiavone and Cooke, 1987), the transport inhibitors TIBA, 9-hydroxyfluorene-9-carboxylic acid (HFCA) and *trans*-cinnamic acid (TCA) did not cause developmental arrest in in vitro-cultured globular zygotic embryos of *Brassica juncea* but rather the growth of collar like cotyledons (Liu et al., 1993). However, the latter study was limited to the treatment of globular embryos and involved the use of a medium containing coconut milk. To our knowledge, there have not been any studies in which a broad range of developmental stages of zygotic embryos have been exposed to auxin and regulating substances using a fully defined medium.

In this study we address the importance of auxin and auxin transport during dicot embryogenesis. We cultured several stages of *Brassica juncea* embryos ranging from early globular to late transition stages on a defined medium. We tested the effects of the auxin transport inhibitor NPA, the natural auxin

IAA, and the antiauxin (PCIB) on the in vitro morphogenesis of isolated zygotic embryos. We propose that auxin-mediated processes are not limited to cotyledon separation but regulate many aspects of embryonic pattern formation.

MATERIALS AND METHODS

Plant material, embryo isolation and culture

Seeds of Indian mustard, *Brassica juncea* (L.) Czernova, were obtained from Sunrise Enterprises, Newington, CT, USA. Plants were grown in soil in a growth chamber under 16 hours light (13000 lux) at 20°C, and 8 hours dark at 16°C. Harvested siliques were surface-sterilised for 10 minutes in 1% (w/v) calcium hypochlorite solution supplemented with 0.1% (v/v) Tween 80 (Merck, Darmstadt, Germany), and subsequently rinsed twice with sterile water. Siliques were opened aseptically under a dissection microscope (Stemi SV 11; Zeiss, Oberkochen, Germany) and excised embryos with intact suspensors were collected in a filter-sterilised 12% (w/v) glucose solution buffered at pH 5.8 with 0.1% (w/v) 2-(N-morpholino)-ethanesulfonic acid (MES). Isolated embryos were transferred in a drop of glucose solution to the embryo culture medium and placed on the top of the agar using micropipettes.

The embryo culture medium contained B5 salts and vitamins (Gamborg et al. 1968), 400 mg/l glutamine, 10% (w/v) sucrose and 0.6% (w/v) agarose (Sea Plaque; FMC Bioproducts, Rockland, Maine, USA) in 3.5 cm diameter Petri dishes (Greiner, Nürtingen, Germany). Globular (length between 30-60 μm, cell number approximately between 16-64) and transition (length between 70-110 μm) embryos were cultured until maturation in the presence of IAA (1, 3, 10, 20 and 40 μM; Sigma), NPA (0.1, 0.25, 0.5, 1, 2.5, 5, and 10 μM; Chem. service, West Chester, USA) or PCIB (10, 25, 50 and 100 μM; Sigma), respectively. The development of each embryo was monitored individually. After 14-16 days of in vitro cultivation, embryos were either fixed for light or electron microscopy, or transferred to germination medium for further development. Germination medium contained half-concentrated B5 salts and vitamins with 1% (w/v) sucrose and 1.2% (w/v) agarose (Sea Plaque; FMC Bioproducts, Rockland, Maine, USA) in 305-ml culture containers (Greiner, Nürtingen, Germany). All cultures were kept at 21°C, in 16 hours light (2500 lux) / 8 hours dark.

Electron microscopy and light microscopy

For scanning electron microscopy, embryos were fixed in 2.5% glutaraldehyde overnight and in 2% osmium tetroxide for 2 hours. After dehydration, the samples were transferred to acetone and critical point dried (Polaron CPD E 3000). The samples were sputter-coated with gold palladium (Polaron sputter coater E5400) and examined in a Hitachi S-520 SEM at 30 kV.

For paraffin embedding, samples were fixed over night in FAA solution (3.5% (v/v) formaldehyde, 5% (v/v) acetic acid, 50% (v/v) ethanol). Afterwards, the embryos were dehydrated through a graded series of ethanol (10 to 100% v/v). Samples were embedded in Paraplast (Sherwood Medical Co., St. Louis, Mo. USA), cut into 5 μm serial sections and stained with toluidine blue (Merck, Darmstadt, Germany). Photographs were recorded with an Axiovert 135 TV microscope (Zeiss, Oberkochen, Germany) using a Contax 167 MT camera with a Kodak Ektachrome 64T film.

RESULTS

Normal in vitro development of *Brassica juncea* zygotic embryos

In all experiments, globular or transition zygotic *Brassica juncea* embryos were used (Fig. 1A,B). These embryos completed their development in vitro without applied hormones. Approximately 20% of globular and 15% of transition embryos died immediately or after 1 to 2 days of cultivation. Surviving embryos required 3-4 days to develop from one embryonic stage to the next. As observed at a low frequency in planta, 3-5% of the embryos in culture formed an additional cotyledon, the other embryos appeared normal. Mature embryos were observed after 14-16 days of culture (Fig. 1D). After transfer of the mature embryos to germination medium, the petioles and hypocotyl elongated, the apical meristem formed leaves, and the primary root elongated.

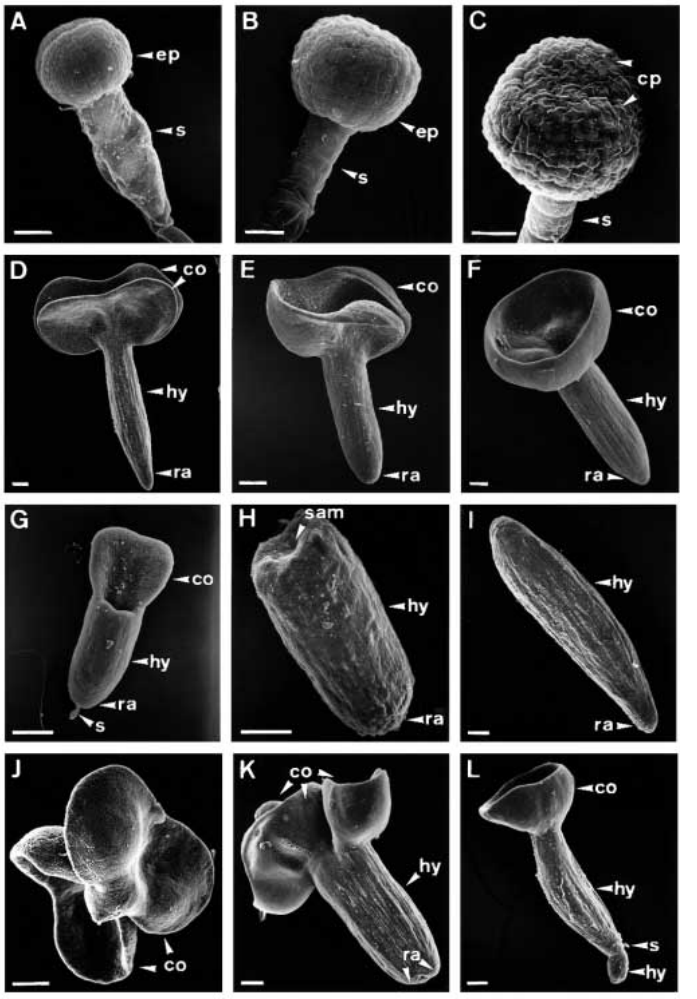
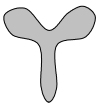
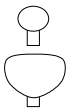
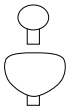
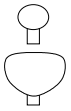
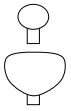
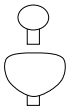


Fig. 1. Scanning electron micrographs of *Brassica juncea* embryos. Freshly isolated embryos in (A) early globular stage; (B) early transition stage; (C) early heart stage. (D) Control embryo, matured in vitro without growth regulators. (E) Split collar-cotyledon embryo (25 μM PCIB). (F) Collar-cotyledon embryo (1 μM NPA). (G) Single cotyledon embryo (100 μM PCIB). (H) No cotyledons embryo (50 μM PCIB). (I) Cucumber-shaped embryo (3 μM IAA). (J) No hypocotyl or radicle embryo (25 μM PCIB). (K) Double radicle embryo (0.5 μM NPA). (L) Twins: embryo with collar-cotyledon fused in the radicle with an egg-shaped embryo (0.5 μM NPA). co, cotyledon; cp, cotyledon primordia; ep, embryo proper; hy, hypocotyl; ra, radicle; s, suspensor; sam, shoot apical meristem. The scale bars represent 25 μm (A-C), and 250 μm (D-L).

Table 1. Effect of IAA on the morphogenesis of globular and transition *Brassica juncea* embryos

Observed embryo shapes	Globular / transition stage at beginning of treatment	IAA concentration (μM)					
		0	1	3	10	20	40
 normal		**100**	**69**	10	0	0	0
		100	**77**	**53**	9	0	0
 split collar-cotyledon		0	19	25	0	0	0
		0	17	22	6	0	7
 collar-cotyledon		0	12	**50**	3	5	3
		0	6	19	**65**	**57**	38
 cucumber		0	0	5	3	5	6
		0	0	0	0	0	0
 ball / egg		0	0	10	**94**	**90**	**91**
		0	0	6	20	43	**55**

The frequency (%) of embryo shapes observed after IAA-treatment is expressed relative to the number of survivor embryos (100%). The most frequent phenotypes are indicated in bold numbers. Controls include 3-5% embryos with three cotyledons. A total of 66-74 embryos (survivor) were used for each concentration in three independent experiments.

Effects of IAA, NPA and PCIB treatments on pattern formation

IAA, NPA, and PCIB affected the morphogenesis of *Brassica* embryos in specific ways, depending upon their concentration and the developmental stage of the isolated embryos. The position of the morphogenetic defects were not influenced by the orientation of the embryos on the media. The percentage of embryos that died during cultivation was not altered by the applied substances. Rare control embryos with three cotyledons (3-5%, see above) were included in the 'normal' shape.

Auxin-induced developmental aberrations

Inhibition of morphogenesis

Of the substances tested, IAA had the most extreme effect on embryo development. About 90% of globular embryos treated with 10 μM and higher concentrations of IAA were not able to undergo morphogenesis and formed a ball or egg-shaped body. These embryo shapes were less frequent after NPA or PCIB treatments (Tables 1-3; Fig. 2B,D). Ball-shaped embryos formed as a result of radial growth, which was arrested when the embryos reached a diameter of 200-400 μm. Histological analysis showed that ball-shaped embryos consisted of morphologically different cell layers (Fig. 2B).

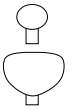
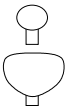
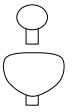
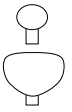
In egg-shaped embryos, provascular tissue had developed along the apical-basal axis (Fig. 2D). The upper part of the egg-shaped embryo body greened, suggesting that it represented the hypocotyl, and the basal part was glossy and appeared similar to the radicle in mature control embryos. On germination medium egg-shaped embryos slightly elongated and developed primary roots. Further development resulted in de-differentiation of the embryos, which became colourless and formed hairs that appeared similar to root hairs on the surface (data not shown).

Cucumber-shaped embryos developed most frequently when globular embryos were treated with IAA (Table 1). Cucumber-shaped embryos lacked cotyledons and apical meristem, but had either a normal or a slightly shortened hypocotyl and radicle (Fig. 1I). In histological sections, provascular tissue was detected in the hypocotyl of cucumber-shaped embryos (Fig. 2E). The apical region was round and the small cells typical for shoot apical meristems were absent (Fig. 2E). After transfer of cucumber-shaped embryos to germination medium, the primary root elongated but no further development of the shoot was observed.

Defective cotyledon separation and cell differentiation

Globular embryos treated with 1 or 3 μM IAA frequently developed split collar- or collar-cotyledons, similar to NPA-treated embryos (Fig. 1E,F, Table 1). About half of the transition embryos that were treated with 20 or 40 μM IAA also developed narrow, collar-cotyledons, while the other half

Table 2. Effect of NPA on the morphogenesis of globular and transition *Brassica juncea* embryos

Observed embryo shapes	Globular / transition stage at beginning of treatment	NPA concentration (μM) 0	0.1	0.25	0.5	1	2.5	5	10
 normal		**100**	2	1	0	1	0	0	0
		100	14	13	14	15	0	7	7
 split collar-cotyledon		0	6	4	2	1	0	0	0
		0	25	12	13	9	0	18	7
 collar-cotyledon		0	**84***	**92***	**93***	**92**	**90**	**97**	**93**
		0	**55**	**73**	**70****	**71**	**100**	**75**	**86**
 ball / egg		0	8	3	5	6	10	3	7
		0	6	2	3	5	0	0	0

The frequency (%) of embryo shapes observed after NPA-treatment is expressed relative to the number of survivor embryos (100%). The most frequent phenotypes are indicated in bold numbers. Controls include 3-5% embryos with three cotyledons. A total of 60-150 embryos (survivor) were used for each concentration in three independent experiments (*including 2% twins, **including 6% double radicle).

of the embryos were egg-shaped (Table 1). None of these embryos showed further development on germination medium.

After IAA treatment the apical meristem of mature embryos was concave instead of convex and, in contrast to the control, no leaf primordia were visible (Fig. 2A,C). Neverthless, on germination medium the apical meristem developed leaves, but only if the IAA concentration in the embryo culture medium was below 10 μM. In the case of collar-cotyledons, these leaves were not able to grow out of the funnel-shaped structure (data not shown). Histological analysis showed that cell shape and size were irregular in the epidermis and ground tissues (Fig. 2C). In contrast to the control, neither palisade nor spongy mesophyll differentiation was detectable in the cotyledons. The cells of the provascular tissue were extremely small and not elongated as in control embryos, and did not form distinct bundles (Fig. 2C).

Developmental aberrations induced by inhibition of auxin transport

Defective cotyledon separation and cell differentiation

The most frequent morphological defect after treatments with NPA was the development of collar-cotyledons. Globular embryos developed collar-cotyledons with high frequency even with relatively low concentrations of NPA (Table 2). The frequency of collar-cotyledon development by transition embryos increased in proportion to the concentration of NPA added (Table 2).

The size and shape of cells in the epidermis and ground tissues of the hypocotyl were not affected by the addition of NPA. Vascularization of the hypocotyl and collar-cotyledon was increased by higher concentrations of NPA. However, cells of the provascular tissue were rather small and not elongated as in control embryos. At 5 μM NPA, a continuous layer of vascular tissue developed instead of individual vascular bundles (data not shown). Mesophyll tissue did not differentiate in the cotyledons. In contrast to the control embryos, no leaf primordia were formed on shoot apical meristems of NPA-treated mature embryos. Nevertheless, these embryos formed leaves after transfer to germination medium, but these were not able to grow out of the collar, similar to the embryos observed after IAA treatment (data not shown). Embryos treated with low concentrations of NPA showed poorly differentiated radicles; however, the primary root could develop on germination medium. A very short root (1-2 cm) developed when 5 or 10 μM NPA-treated embryos were transferred to germination medium; this root showed no further growth and lateral roots later developed at the base of the hypocotyl.

Axis duplication

Two percent of the embryos that were treated with 0.1-0.5 μM NPA developed as twins (Table 2). This type of development was only observed if embryos were treated at a very early globular stage (16-32 cells). The first visible step in twin formation was the development of a double globular structure with one central suspensor. Twins did not originate from the suspensor. This development either led to twins with a common radicle and partially fused or separate hypocotyls (Figs 1L, 2E), or to twins which had no common organs but were superficially connected by other cell layers of the hypocotyl. Twins with fused hypocotyls had separate central vascular bundles, which were fused in the radicle. Twins that

were superficially connected in the hypocotyl had no common organs (data not shown). In most cases one of the twins was less differentiated than the other. While one partner formed an egg-shaped (Fig. 1L) or cucumber-like body (Fig. 2E), the other partner developed either normal or collar-cotyledons.

Axis broadening

Six percent of transition embryos treated with 0.5 μM NPA developed a broadened hypocotyl; however, this embryo shape was sporadically also observed on media with other NPA concentrations (Fig. 1K). The size of the shoot apical meristem also increased, and these embryos developed either a collar-cotyledon, two cotyledons or three separate cotyledons. During development, the broadened hypocotyl separated into two parts on both sides of the suspensor, which led to the formation of two new, distinct radicles (Fig. 2F). Both new primary roots elongated when these embryos were placed on germination medium.

Developmental aberrations induced by decreased auxin activity

Inhibition of cotyledon or hypocotyl and radicle growth

PCIB treatments induced strong developmental aberrations in embryos. The size of the cotyledon primordia were inversely correlated with PCIB concentrations. After transfer of the embryos to germination medium, the resulting cotyledon stumps elongated but did not develop to normal size. In extreme cases, one or both cotyledons were lacking completely (Fig. 1G,H). The position of the missing cotyledon was not dependent on the orientation of the embryo on the medium. Embryos with a single cotyledon were observed after culture on medium with 25 μM or higher PCIB concentration (Fig. 1G; Table 3). This morphological alteration was more frequent if the PCIB treatment started at the globular stage (12-24%) than at the transition stage (2-11%; Table 3). Histological sections showed only small primordia cells in the second cotyledon, which did not complete its development when the embryo was transferred to germination medium. After treatment of globular embryos with 50 or 100 μM PCIB, 3% and 4% of embryos lacked both cotyledons, respectively (Table 3; Fig. 1H). In embryos without cotyledons a ring of cotyledon primordia surrounded the apical meristem. The shoot meristem of embryos that lacked both

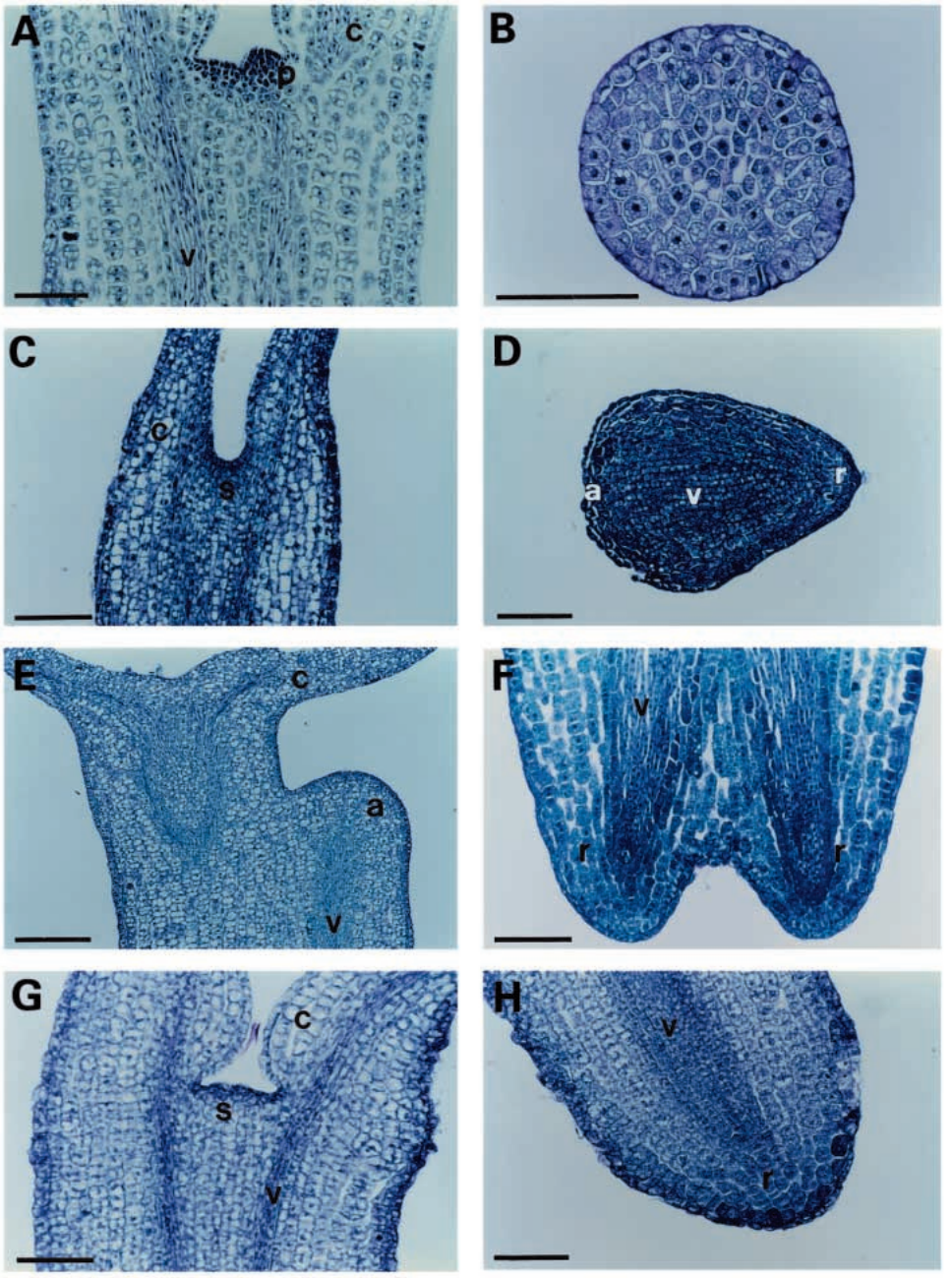
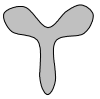
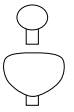
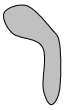
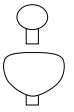
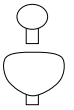
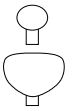
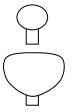
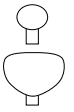


Fig. 2. Histological sections of *Brassica juncea* embryos. The paraffin sections were stained with toluidin blue. All sections are longitudinal unless otherwise indicated. (A) The shoot apex of a control embryo. (B) Median section through a ball-shaped embryo (1 μM NPA). (C) The shoot apex of an embryo with two cotyledons (3 μM IAA). (D) An egg-shaped embryo (10 μM IAA). (E) Twins (embryo with collar-cotyledon fused with a cucumber-shaped embryo, 0.5 μM NPA). (F) The root apex of an embryo with double radicle (0.5 μM NPA). (G) The shoot apex (50 μM PCIB). (H) Longitudinal section through the root apex (50 μM PCIB). a, apex; c, cotyledon; p, leaf primordium; r, radicle; s, shoot meristem; v, developing vascular tissue. The scale bars represent 100 μm.

Table 3. Effect of PCIB on the morphogenesis of globular and transition *Brassica juncea* embryos

Observed embryo shapes	Globular / transition stage at beginning of treatment	PCIB concentration (μM) 0	10	25	50	100
 normal		**100**	32	27	21	14
		100	**57**	**47**	**43**	17
 single cotyledon		0	0	12	21*	28**
		0	0	4	2	11
 split collar-cotyledon		0	**62**	**48**	**46**	**31**
		0	36	43	38	**41**
 collar-cotyledon		0	4	4	2	13
		0	0	4	4	15
 no hypocotyl or radicle†		0	0	0	0	0
		0	0	0	25	25
 ball / egg		0	2	9	11	14***
		0	7	2	13	16

The frequency (%) of embryo shapes observed after PCIB-treatment is expressed relative to the number of survivor embryos (100%). The most frequent phenotypes are indicated in bold numbers. Controls include 3-5% embryos with three cotyledons. †The frequency of no hypocotyl or radicle phenotype is expressed relative to the number of embryos which developed hypocotyls. A total of 62-76 embryos (survivor) were used for each concentration in three independent experiments (*including 3% without cotyledons, **including 4% without cotyledons, ***including 4% cucumber-shaped).

cotyledons and that were grown on 50 μM PCIB developed only some reduced, finger like leaves on germination medium (data not shown).

Twenty-five percent of transition embryos treated with 50 or 100 μM PCIB failed to develop a hypocotyl and radicle (Fig. 1J). These embryos had either two cotyledons or (split) collar-cotyledons. After transfer of these embryos to germination medium, neither the hypocotyl nor the radicle developed, and the apical meristem produced aberrant, or no, leaves.

PCIB-treatment led frequently to the development of split collar-cotyledons (about 50% and 40% of globular and transition embryos, respectively), but seldom to collar-cotyledons (about 4% of globular or transition embryos, Table 3).

Defective cell differentiation

PCIB treatments of 50 or 100 μM reduced the size of the embryos to about 1/3 that of the control. The development of the shoot and root apical meristems were strongly affected (see above). No leaf primordia were seen at the shoot apex of PCIB-treated mature embryos (Fig. 2G). After transfer of these embryos to germination medium, the shoot apical meristems produced some reduced, finger-like leaves, or, for embryos treated with 100 μM PCIB, no primary leaves at all. The radicle differentiation was inhibited when PCIB was given in concentrations of 25 μM or higher (Fig. 2H). On germination medium, embryos developed only very short primary roots. PCIB also affected the differentiation of the mesophyll and vascular tissues. In contrast to the control embryos, mesophyll tissues did not differentiate in the cotyledons. The provascular tissue consisted of very small cells (Fig. 2G), which formed a tissue layer as in NPA-treated embryos. No provascular cells could be seen in embryos treated with 100 μM PCIB (data not shown).

DISCUSSION

During the transition from globular to heart stage the body of the plant embryo becomes partitioned into four regions: cotyledons, future shoot meristem, hypocotyl and radicle (Mansfield and Briarty, 1991). The IAA-, NPA-, and PCIB-

treated *Brassica* embryos reveal that these morphogenetic changes occur in separate steps, because the aberrant embryo shapes represent blockage or perturbation in distinct growth processes (summarised in Fig. 3). The phenotypic alterations described in this study are representative of specific effects of each class of substances, because we observed the same alterations using other transport inhibitors (TIBA, HFCA and TCA), the antiauxin 2-(p-chlorophenoxy)-2-methylpropionic acid, and the auxin 2,4,5-trichloro-phenoxyacetic acid (Boge and Neuhaus, unpublished data).

Establishment of the apical-basal axis in globular embryos

The development of twin and ball-shaped embryos indicated that the apical-basal axis is established even in globular embryos (Fig. 3, a,b,c). Inhibition of auxin transport by NPA in early globular *Brassica* embryos occasionally led to the development of twins. NPA inhibits the efflux of auxin and leads to auxin accumulation in the cell (Lomax et al., 1995). The resulting altered auxin distribution caused axis duplication, which occurred partially or completely. Twins did not originate from the suspensor, in contrast to the suspensor-derived twinning in *Arabidopsis* characterized by Vernon and Meinke (1994) and Zhang Somerville (1997). When the twins shared common organs (Fig. 3, b), they were fused in the radicle or in the radicle and a part of the hypocotyl, which indicates that the polarity of the apical-basal axis is fixed first at the base. Although the direction of the polar auxin transport in early globular embryos is not known precisely, its function appears to be the establishment of a single embryonic axis. Inhibition of auxin transport promotes the establishment of multiple axes in monocot embryos as well, and leads to the development of polyembryos (Fischer et al., 1997).

Auxin flooding of globular embryos either completely inhibited or interfered with morphogenesis and axialisation, resulting in the development of ball-, egg- or cucumber-shaped embryos, probably by the inhibition of establishment of auxin gradients. Although egg- and cucumber-shaped embryos developed a hypocotyl and radicle, they never developed cotyledons or shoot meristems, suggesting that they did not enter into the transition stage (Fig. 3, b). This indicates that the elongation of the hypocotyl is independent of the apex morphogenesis, and that without cotyledon initiation no shoot meristem develops. How the exogenous auxin acts is not clear. It either acts directly in the embryo or, as it was recently demonstrated on IAA-treated hypocotyl segments of carrot (Ribnicky et al., 1996), it may activate auxin conjugation leading to lower concentrations of active IAA in the embryo. Although active auxin concentrations in auxin-treated embryos were not measured, we assume that the aberrant development was caused by an increased total amount of active auxin, because the effects of lowered IAA activity should be similar to the PCIB effects.

It is not known when and where auxin synthesis begins and how auxin is distributed in embryos. The only data about the auxin content of embryos were obtained by Michalczuk et al. (1992) in somatic carrot embryos. The authors suggested that the lower level of free and total auxin in postglobular embryos, in comparison to 2,4-D-treated embryonic callus, allow the establishment of internal auxin gradients. Our results are in agreement with this and other reports that suggest that internal auxin gradients, which trigger specific steps in morphogenesis, already exist in globular embryos (Fischer and Neuhaus, 1996; Fry and Wangermann, 1976; Michalczuk et al.,1992; Schiavone and Cooke, 1987).

Our observations that the establishment of the apical-basal axis can be disturbed even during the globular stage are also in accordance with the fact that procambium cell files appear first in the globular stage of the dicot embryo, indicating histologically the beginning of the development of the apical-basal axis (Mansfield and Brierty, 1991).

Auxin-induced morphogenetic changes in transition embryos

Partitioning of the apex in future shoot meristematic and cotyledonary regions

In the early phase of transition the embryo body still shows radial symmetry around the apical-basal axis. A depression indicates the region of the future shoot meristem, and the surrounding tissue ring corresponds to the emerging cotyledon primordia. Two observed embryo shapes represent blockage immediately after this phase of transition: embryos without cotyledons (but with a ring of cotyledon primordia) and embryos with collar-cotyledons, because they have shoot

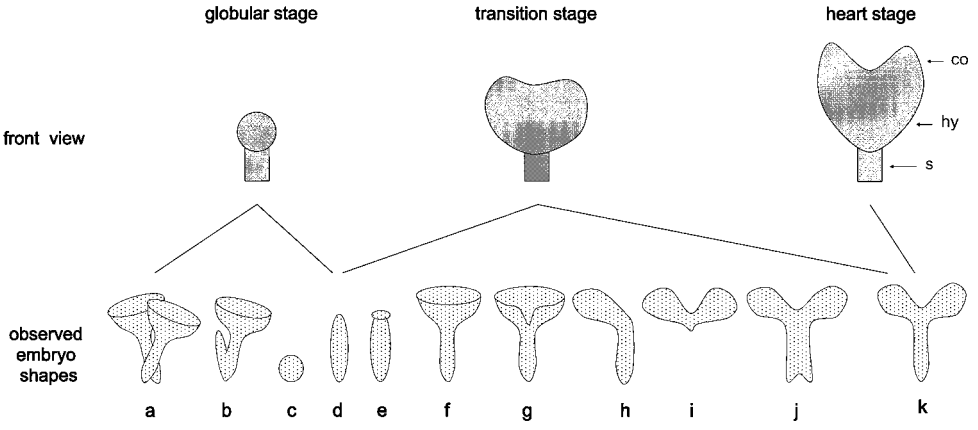


Fig. 3. Auxin-induced developmental patterns in *Brassica* embryos. At the top of the figure are the different embryonic stages: globular, transition and heart. At the bottom of the figure are the observed embryo shapes arranged after the developmental stage in which their defects occur: a, twins resulting from complete axis duplication; b, twins resulting from partial axis duplication; c, ball-, and egg-shaped embryo; d, cucumber-shaped embryo; e, embryo without cotyledons, but with shoot meristem; f, embryo with collar-cotyledon; g, embryo with split collar-cotyledon; h, embryo with a single cotyledon; i, embryo without hypocotyl and radicle; j, embryo with broadened axis and two radicles; k, normal-shaped embryo. co, cotyledon; hy, hypocotyl; s, suspensor.

apical meristems but their cotyledon primordia are not separated (Fig. 3, e,f)

Cotyledon separation

During transition the emerging cotyledonary primordial ring separates into two parts, and by this process bilateral symmetry around the apical-basal axis is established (Fig. 1C). Polar auxin transport plays a main role in this process, as shown by Liu et al. (1993) and in this study. The most frequent developmental aberrations during this step are the split collar- and collar-cotyledons after IAA, NPA or PCIB treatments. There are two possibilities how auxin transport could affect the cotyledon separation. Auxin transport may either lead to the accumulation or to the removal of auxin in the separation area (area of the future shoot meristem), and the resulting too high or too low concentrations of auxin might be responsible for the lack of cotyledon tissue growth. In the first case, additional auxin should not affect the establishment of bilateral symmetry. However, the appearance of collar-cotyledons after IAA treatment suggests that the first assumption is incorrect. In the second case, a disturbance of cotyledon separation could be predicted by auxin application as well as by transport inhibition and our results verified both predictions. Therefore, we conclude that polar transport removes auxin from the area between the two emerging cotyledon primordia. Furthermore, a continuous auxin transport is required until the morphologically visible separation of the cotyledon primordia occurs, because short pulses of NPA treatment did not lead to the development of collar-cotyledons (data not shown).

The frequently observed partial separation of the cotyledons, which leads to split collar-cotyledons, indicates that the process of separation occurs asymmetrically, with one side separating before the other. This interpretation is in accordance with our microscopic observations that the central apical depression expands asymmetrically across the top of the embryo. Therefore, we conclude that auxin removal starts in the central apical region of the globular or early transition embryo, and expands asymmetrically across the apex of the embryo. We suggest that the auxin transport is directed to the cotyledon region, as Liu et al. (1993) also proposed.

Partially separated cotyledons are frequent after PCIB treatment. PCIB is described as an antiauxin or an auxin antagonist, because it interferes with auxin action (Evans and Hokanson, 1969; Foster et al., 1955; Heupel and Stange, 1995). How the decrease of auxin activity by PCIB interferes with auxin transport is not clear. One possibility is that PCIB weakly inhibits auxin transport, which would be consistent with earlier observations by Niedergang-Kamien and Leopold (1959) and Hertel et al. (1969).

Cotyledon and hypocotyl growth

Reduced auxin activity during the transition stage inhibited the growth of the cotyledon primordia, leading to the lack of one or both cotyledons. These altered embryo shapes reflect that the outgrowth of cotyledons can be inhibited by PCIB before and after separation of the primordia, and indicate that separated cotyledons did not develop simultaneously (Fig. 3, e,h).

PCIB treatment of late-transition embryos leads to a loss of hypocotyl and radicle development (Fig. 3, i). Embryos with cotyledons but without hypocotyl and radicle indicate that the initiation of cotyledon growth and hypocotyl growth by auxin occurs independently and in different phases of the transition stage.

Inhibition of auxin transport in this phase can lead to broadening of the apical-basal axis, and as a secondary effect to the development of two radicles at the base of the broadened hypocotyl (Fig. 3, j), which shows that transport supports the normal axis development.

Cell differentiation

Changes in auxin distribution pattern or activity affect not only the shape of embryos but also cell differentiation and meristem development. IAA or PCIB treatments strongly inhibited the development of shoot and root meristems. Treatment of adult plants with transport inhibitors or antiauxin has similar effects (Lyndon, 1994). The described alterations in the vascular tissue development of *Brassica* embryos after IAA, NPA and PCIB treatments are in accordance with the fact that auxin seems to be the major signal involved in the control of plant vascular differentiation (Aloni, 1987).

Auxin-induced developmental patterns

Pharmacological experiments and genetic evidence support our assumption, that auxin-mediated processes which trigger pattern formation in the embryo are closely related to pattern formation in shoot apical meristem. Similar to collar-cotyledons in embryos, addition of auxins or transport inhibitors to the shoot apex cause the formation of collar-like leaf primordia in several plant species (Lyndon, 1994). Genetic defects in flower development, as observed in *Arabidopsis* mutants such as *pin-formed*, *pinoid*, *altered meristem program 1* and *cuc*, are often coupled with defects in cotyledon separation or initiation, indicating that a similar mechanism operates in flowers and embryos (Aida et al., 1997; Bennet et al., 1993; Chaudhury et al., 1995; Okada et al., 1991). The mutants *pin-formed* and *pinoid* exhibit deficiencies in the polar transport of auxin (Bennet et al., 1993; Okada et al., 1991). The aberrant *Brassica* embryos described in this study phenocopy some *Arabidopsis* embryonic pattern mutants such as *gnom*, *gurke*, *monopteros*, *laterne*, and *shoot meristemless* (Endrizzi et al., 1996; Jürgens et al., 1991; Lukowitz et al., 1996; Mayer et al., 1991, 1993; Torres-Ruiz et al., 1996). The *monopteros* mutant has already been shown to have strongly reduced polar auxin transport capability (Przemeck et al., 1996).

To our knowledge, this paper presents the first detailed description of a broad range of developmental aberrations in zygotic dicot embryos induced by auxin and related substances at different embryonic stages. The embryo shapes obtained by disturbing the auxin balance during embryogenesis demonstrate that auxin-mediated processes in the developing *Brassica* embryo are involved in the morphogenetic changes, which occur during transition from globular to heart stages and which are a prerequisite for the basic body formation.

We thank Rainer Hertel, Alan Jones, Catharina Coenen and Elke Duwenig for many helpful discussions, Sonja Eberenz and Klara Ronai for excellent technical assistance, and Chris Lundberg for editorial help.

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