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# Developmental basis of homeosis in precociously germinating *Brassica napus* embryos: phase change at the shoot apex

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## SUMMARY

**Precociously germinating *Brassica napus* (oilseed rape) embryos produce extra cotyledons or chimeric organs with sectors of cotyledon and leaf tissue, rather than leaves, at the shoot apex. To investigate this phenomenon in more detail, scanning electron microscopy was used to examine the development of organ primordia at the shoot apex. In situ hybridizations with molecular markers of the embryonic phase were used to assess the status of individual cells in the shoot apex with regard to the transition between embryonic and vegetative phases. The results indicate that, under conditions that support precocious germination, primordia develop at the shoot apex in the mode characteristic of postgerminative growth, i.e. they arise sequentially in a spiral phyllotaxy. Cells in the rest of the embryo, however, can continue to express molecular markers of the embryonic phase for several weeks after the start of culture. When patterns of gene expression and the fate of individual primordia were compared, a strong correlation was found between organ identity and the status of cells in the vicinity of the meristem with regard to phase. Primordia that develop in situations where neighboring cells are in the embryonic phase always produce organs with cotyledon morphology. Primordia that develop in situations where neighboring cells have exited the embryonic phase produce leaves. Based on an examination of situations where chimeric organs are produced, I propose that short range interactions or signalling are likely to be involved in communicating information about phase to developing primordia.**

Key words: oilseed rape, *Brassica napus*, seed development, embryogenesis, germination, cotyledon, heterochrony, storage protein

## INTRODUCTION

The life cycle of a flowering plant consists of several distinct developmental phases. Individuals start out in the embryonic phase and then pass through one or more phases of vegetative growth before entering the reproductive phase. Each phase is marked by a characteristic set of growth patterns, physiological processes and patterns of gene expression. During the transition between phases, referred to as a 'phase change' (Poethig, 1990), features that are characteristic of one phase are eliminated and replaced by features characteristic of the next phase. Although multiple changes occur, they generally take place within a fairly narrow window of developmental time. This implies that there is some means of signaling or otherwise coordinating these changes. The mechanism or mechanisms for coordination and the level at which these mechanisms operate is unknown.

To assess when a phase change has occurred, investigators have typically relied on changes in the identity of the organs produced at or near the shoot apex. During the embryonic phase, cotyledons, which are homologous to leaves but are modified for storage purposes, are produced. During vegetative phases, leaves with juvenile or adult traits are produced. During the reproductive phase, various floral organs, which are also homologous to leaves but are modified for reproductive purposes, are produced. An apical meristem generally only produces organs indicative of one phase at any given time; however, other meristems on the same plant may produce different organs.

By focusing on changes in the pattern and morphology of structures produced at the shoot apex, investigators have been able to identify at least some genetic and environmental factors that affect the transition between phases. The shift from the vegetative to reproductive phase has been most extensively studied in this regard (reviewed in Bernier, 1988). The other major phase change in the life cycle, which occurs as individuals exit the embryo phase, has been less intensively studied. This is partly due to experimental difficulties. Because the transition occurs at the end of seed development, when water contents are low and the meristems are quiescent, reprogramming of the shoot apex is not always immediately evident. In addition, embryos that make the transition early or fail to make it properly are generally desiccation intolerant and die as the seeds dry.

To overcome some of the experimental limitations, I have studied the transition from the embryo phase to the vegetative phase in a culture system that supports precocious germination. When excised *Brassica napus* embryos are placed in culture, growth resumes at the shoot apex. An unusual series of organs, including extra cotyledons and chimeric organs, is produced

(Finkelstein and Crouch, 1987). In this study, I have examined the relationship between the identity of the organs produced at the shoot apex and phase change, as indicated by molecular markers, in the rest of the embryo. The results of this study suggest that fate is determined in developing primordia on the basis of information provided by neighboring cells in the shoot apex.

## MATERIALS AND METHODS

### Plant material

*Brassica napus* L. (cv. Tower) plants were grown to maturity in a environmental chamber with 16-hour days (370 μE/$m^2$/second, 15˚C) and 8-hour nights (10˚C). Flowers were hand-pollinated on the day they opened (0 d.a.p., days after pollination) and developing embryos were isolated by removing the seed coats with tungsten knives. For culture experiments, embryos were removed aseptically, placed in glass jars on Monnier's embryo medium (Monnier, 1976) containing 1% (w/v) sucrose, and allowed to develop at 20˚C under constant cool white fluorescent lights.

### Tissue preparation

Embryos and seedling parts were fixed by infiltrating with freshly prepared 4% (w/v) paraformaldehyde in 50 mM potassium phosphate buffer (pH 7) for 3 hours at room temperature under a light vacuum. Fixation was continued overnight without vacuum at 4˚C. Fixed embryos were subsequently dehydrated in an ethanol series. For in situ hybridization experiments, embryos were embedded in Paraplast Plus (Sherwood Lancer, St. Louis, MO, USA) and sectioned at 7 μm. Sections were mounted on ProbeOn Plus slides (Fisher Scientific, USA). For scanning electron microscopy, fixed embryos were dried in a Pelco Model H critical point dryer (Ted Pella Co., Tustin, CA, USA), coated with gold-palladium (60:40), and examined in a Cambridge Stereoscan electron microscope (Cambridge Instruments Ltd., Cambridge, UK) operated at 20 kV.

### In situ hybridizations

Labelled sense and antisense RNA probes were generated using T3 and T7 RNA polymerases, the Bluescribe/Bluescript vector system (Stratagene, La Jolla, CA, USA) and digoxigenin-11-UTP according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN, USA). The sequence of the storage protein cDNA clones (napin: pN2, 739 bp; cruciferin: *Hin*dIII-*Pst*I restriction fragment of pC1, 771 bp) used to generate probes have been reported previously (Crouch et al., 1983; Simon et al., 1985). AX92 probes were generated from the 0.28 kb *Pst*I restriction fragment of pAX92 (Harada et al., 1988).

Slides were processed using a combination of several procedures that have been described previously. Prehybridization treatments and hybridizations were performed according to Jackson (1991) with the modifications described by Lincoln et al. (1994). Proteinase K was used at a final concentration of 1 μg/ml. Hybridizations were performed overnight at 50˚C using a probe concentration of 0.2 ng/μl per kb of sequence complexity. After hybridization, slides were treated as described by Cox et al. (1984). The slides were washed at moderate stringency (0.1× SSC, 55˚C) after treatment with RNase A. Digoxigenin was detected using the procedure described by Coen et al. (1990). Tissue sections were incubated for 5 hours with a 1:1500 dilution of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN, USA). The color reaction was stopped after an overnight incubation in the presence of substrate. Slides were examined on a Zeiss Axioskop and photographed using Kodak Ektachrome 160T color slide film. Slides were scanned using a Kodak Professional RFS 2035 film scanner and plates were assembled using Adobe Photoshop 3.0.

## RESULTS

### Primordium development during embryo maturation in the seed

Scanning electron microscopy was used to examine the development of organ primordia at the shoot apex during later stages of embryogenesis in *Brassica napus*. Organization of the shoot apical meristem into L1, L2 and L3 layers was typically complete by 28-30 d.a.p. (Fernandez et al., 1991). In embryos excised at 38 d.a.p., which corresponds to mid-maturation stage, the shoot apex consisted of a small raised dome of cells between the bases of the two embryonic cotyledons (Fig. 1A). The primordium that gives rise to the first organ of the epicotyl (hereafter referred to as the first primordium) was visible as a small buttress on one side of the apical dome. By 44 d.a.p., the apical dome was broader and the first primordium was more distinct (Fig. 1B). By 55 d.a.p., the first primordium had enlarged but no signs of cellular differentiation in the form of trichome or stomatal initials were visible (Fig. 1C). The primordium that gives rise to the second organ of the epicotyl (second primordium) was also visible by this point and consisted of a small buttress of cells on the side of the apical dome (Fig. 1C). By 60-65 d.a.p., the seed coats were brown, indicating that seed development was complete. No further changes were seen at the shoot apex. I conclude that the primordia for the first two organs of the epicotyl arise during the course of embryo maturation in *Brassica napus* but undergo only limited development before germination.

### Morphology of lateral organs produced during precocious germination

When maturing embryos were removed from their seed coats and placed in culture on a basal medium with no added hormones, the shoot apices showed signs of active growth within the first few days. By 7-10 days after the start of culture,

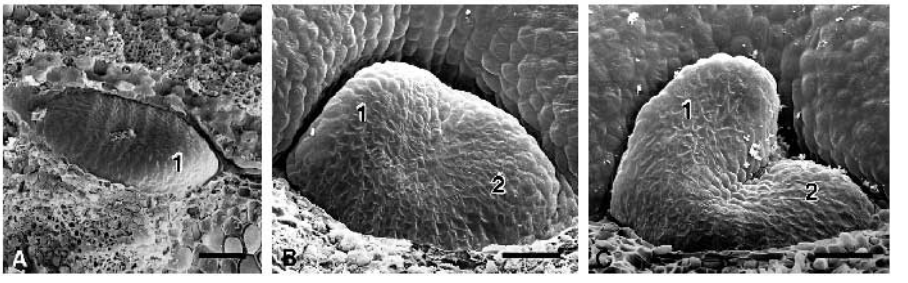


**Fig. 1.** Scanning electron micrographs of shoot apices in maturing *Brassica napus* embryos. The cotyledons of the embryo have been removed and the area between the cotyledon bases is shown. Numbers indicate the primordia that will become the first (1) and second (2) leaves. (A) Shoot apex at 38 d.a.p. (B) Shoot apex at 44 d.a.p. (C) Shoot apex at 55 d.a.p. Bars, 50 μm.

primordia that arose prior to excision had developed into large expanding lateral organs. Additional lateral organs appeared over the next 20-30 days, indicating that primordia continued to arise at the shoot apex. In older embryos, the primordia developed into leaves; however, in younger embryos, the primordia often developed into unusual organs with cotyledon features. To establish the structural basis for this apparent switch in organ identity, the development of organs at the shoot apex was analyzed in greater detail using scanning electron microscopy.

When rehydrated dry seed or embryos excised at 60 d.a.p. were placed in culture, leaves were produced at the shoot apex (Fig. 2A). Primordia arose sequentially and in a spiral phyllotaxy. As the primordia elongated and expanded, features such as dentate margins and large, unbranched trichomes, which are characteristic of leaves in this species, appeared. A pair of small stipule primordia (arrows, Fig. 2A) was associated with the base of each leaf.

When embryos excised during the middle of maturation stage (38 d.a.p.) were placed in culture, organs resembling cotyledons were produced at the shoot apex (Fig. 2B). These organs lacked trichomes and had smooth margins. Unlike the cotyledons produced at the heart stage during embryo morphogenesis however, these organs appeared sequentially and in a spiral phyllotaxy. Small stipule primordia (arrows) were associated with the base of each developing organ. Embryos excised at 38 d.a.p. continued to produce organs of this type for at least 6-8 weeks. Eventually, axillary meristems at the cotyledon nodes were activated and these produced leaves.

When embryos excised during later stages of maturation (47-50 d.a.p.) were placed in culture, three kinds of organs were produced at the shoot apex (Fig. 2C,D). Some embryos only produced cotyledons and others only produced leaves, but most embryos produced a sequence of organ types. In the most common sequence, the first primordium developed into an organ that resembled a cotyledon (Fig. 2C). The second primordium developed into a leaf, with trichomes covering the entire surface of the blade (Fig. 2C). The third primordium, which could not be detected at the time of excision and presumably arose while the embryo was in culture, developed either into a leaf or into an organ with separate sectors of cotyledon tissue and leaf tissue (Fig. 2D). In some experiments, up to 50% of the cultured embryos produced chimeric, or mosaic, organs (data not shown). The sectors on these organs were large and coherent, i.e. there was generally only one sector of each type per organ rather than multiple small patches scattered over the surface of the blade. The proportion of the blade that a given sector occupied varied widely between organs; however, the relative position of the sectors did not. Cotyledon sectors on the third organ were located proximal to the site where the first organ of the epicotyl (a cotyledon in this case) arose and leaf sectors were distal. Primordia that arose after the third primordium developed into leaves. Regardless of organ morphology, all of the primordia arose sequentially, in a spiral phyllotaxy, and were flanked by stipule primordia (data not shown). Therefore, in precociously germinating embryos, determination of organ identity is clearly separate from the process of pattern formation at the apical meristem.

### Molecular markers for embryonic identity

To assess the status of individual cells in the shoot apex with regard to identity and/or developmental phase, in situ hybridizations were performed. Previous studies showed that sequences encoding the storage proteins napin and cruciferin begin to accumulate in the cotyledons and axes of the embryo

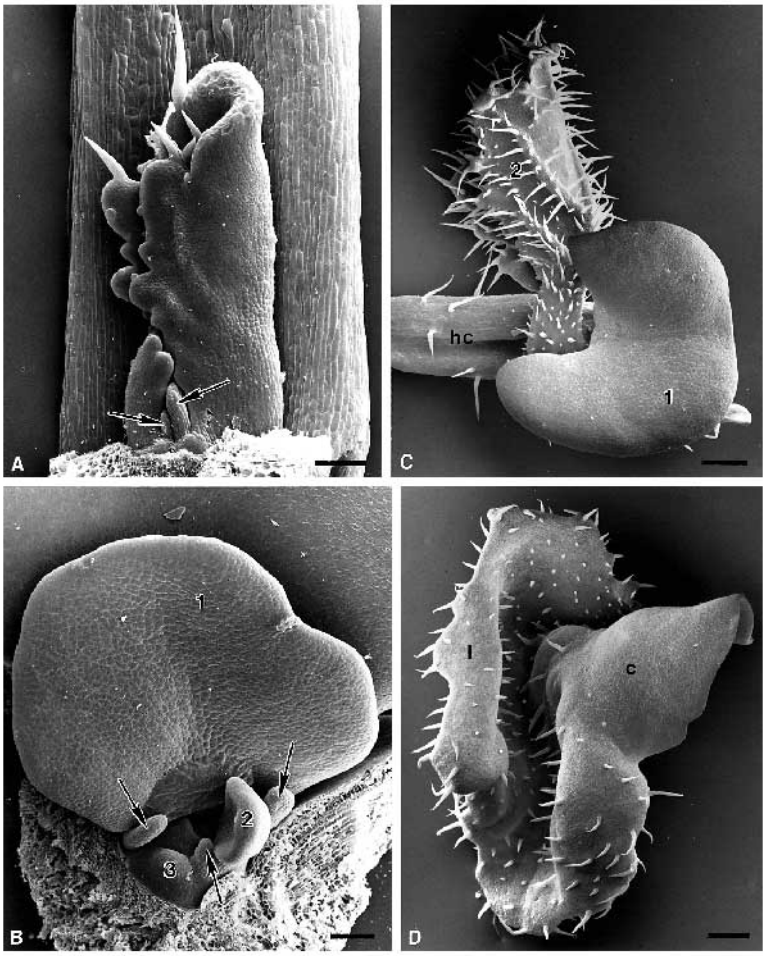


**Fig. 2.** Scanning electron micrographs of organs produced at the shoot apex by cultured embryos. One or more embryonic cotyledons were removed to show the shoot apex. Numbers in B and C indicate order of initiation. (A) Shoot apex of seedling resulting from germination of dry seed, after 6 days in culture. Several leaves with dentate margins, trichomes, and stipule primordia (arrows) have formed. Bar, 200 μm. (B) Shoot apex of embryo excised at 38 d.a.p., after 9 days in culture. A series of extra cotyledons, each flanked by a pair of stipule primordia (arrows), were produced sequentially and in a spiral phyllotaxy. Bar, 200 μm. (C) Shoot apex of embryo excised at 50 d.a.p., after 33 days in culture. The hypocotyl (hc) is shown but both embryonic cotyledons have been removed. The first primordium of the epicotyl developed into an extra cotyledon. The second primordium developed into a leaf. Bar, 500 μm. (D) Chimeric organ removed from the shoot apex of an embryo excised at 47 d.a.p., after 33 days in culture. One half of the blade has the morphology and surface characteristics of a cotyledon (c), the other half displays leaf (l) features. Bar, 500 μm.

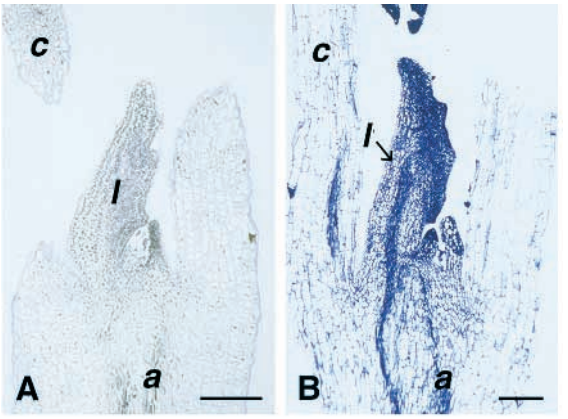


**Fig. 3.** Localization of mRNAs in the shoot apex of seedlings derived from culture of embryos excised at 60 d.a.p. (A) Napin mRNAs could not be detected in the shoot apex, new leaves, or organs formed during embryogenesis, after 5 days in culture. (B) AX92 antisense probes hybridized to mRNAs in different regions of the shoot apex, including the developing vascular tissue, leaf margins, and stipule primordia of young leaves, after 7 days in culture. Bars, 200 μm. *a*, embryo axis; *c*, cotyledon of embryo; *l*, leaf.

during heart stage, reach high levels during maturation stage, and decline to low levels as the seeds dry (Fernandez et al., 1991; Bisgrove et al., 1995). AX92 sequences are preferentially expressed during early stages of postgerminative growth (Harada et al., 1988). RNA probes corresponding to sense and antisense transcripts of napin, cruciferin and AX92 were generated. Because no colored product was detected when sense transcripts were used as probes, the results of those hybridizations are not shown.

When sections of young seedlings derived from embryos excised at 60 d.a.p. were hybridized with napin antisense probes, no accumulation of napin mRNAs could be detected in the primordia, young expanding leaves, or meristem region of the shoot apex (Fig. 3A). Similar results were obtained with cruciferin antisense probes (data not shown). To demonstrate that cells in the shoot apex are accessible to probes and enzyme substrates, sections of young seedlings were also hybridized with AX92 sequences. AX92 mRNAs accumulated to relatively high levels in the shoot apex and in young leaves (Fig. 3B). However, because AX92 or related sequences also accumulate in the cotyledons in young embryos (Fernandez, unpublished observation), they could not be used as molecular markers of leaf identity for subsequent experiments. On the other hand, the accumulation pattern of napin and cruciferin sequences indicated that they could be used in two ways: (1) to distinguish between phases (late embryonic and vegetative) in the cotyledons and axes of the embryo, and (2) to distinguish developing cotyledons, which accumulate these mRNAs as part of their cellular differentiation program, from developing leaves, which do not.

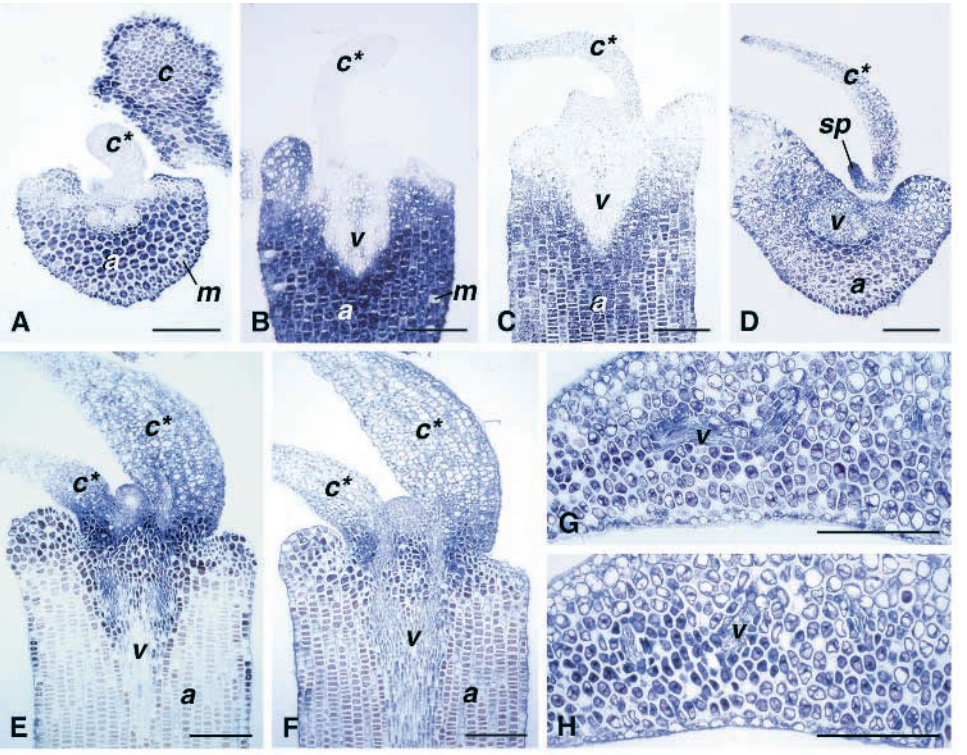


**Fig. 4.** Localization of napin and cruciferin mRNAs in cultured embryos (excised at 38 d.a.p.) producing extra cotyledons. (A) Shoot apex after 3 days in culture. Napin mRNAs were present at relatively high levels in the axis and cotyledons of the embryo but could not be detected in the new organs developing at the shoot apex. (B) Shoot apex after 5 days in culture. Napin mRNAs could not be detected in the immediate vicinity of the shoot apical meristem. Napin mRNAs were present in the cortical and epidermal cells in the axis, but not in provascular (v) or myrosin (m) cells. (C) Shoot apex after 7 days in culture. Accumulation of napin mRNAs could first be detected in the extra cotyledons and shoot apex at the start of the second week in culture. (D) Shoot apex after 10 days in culture, oblique section. Napin mRNAs were present at relatively high levels in stipule primordia and enlarging extra cotyledons. Napin mRNAs accumulated in provascular tissue throughout the embryo. (E) Shoot apex after 20 days in culture. Napin mRNA levels were reduced in the axis but levels remained relatively high in the vicinity of the shoot apical meristem and in the extra cotyledons. (F) Shoot apex after 20 days in culture. Cruciferin mRNAs accumulated in the same spatial pattern as napin mRNAs. (G) Napin mRNAs were present at relatively high levels in all tissues in the cotyledons formed during embryogenesis after 20 days in culture. (H) Cruciferin mRNAs were also present at relatively high levels in all tissues in the cotyledons formed during embryogenesis after 20 days in culture. Bars, 200 μm. *a*, embryo axis; *c*, cotyledon of embryo; *c**, extra cotyledon; *m*, myrosin cell; *sp*, stipule primordium; *v*, vascular or provascular tissue.

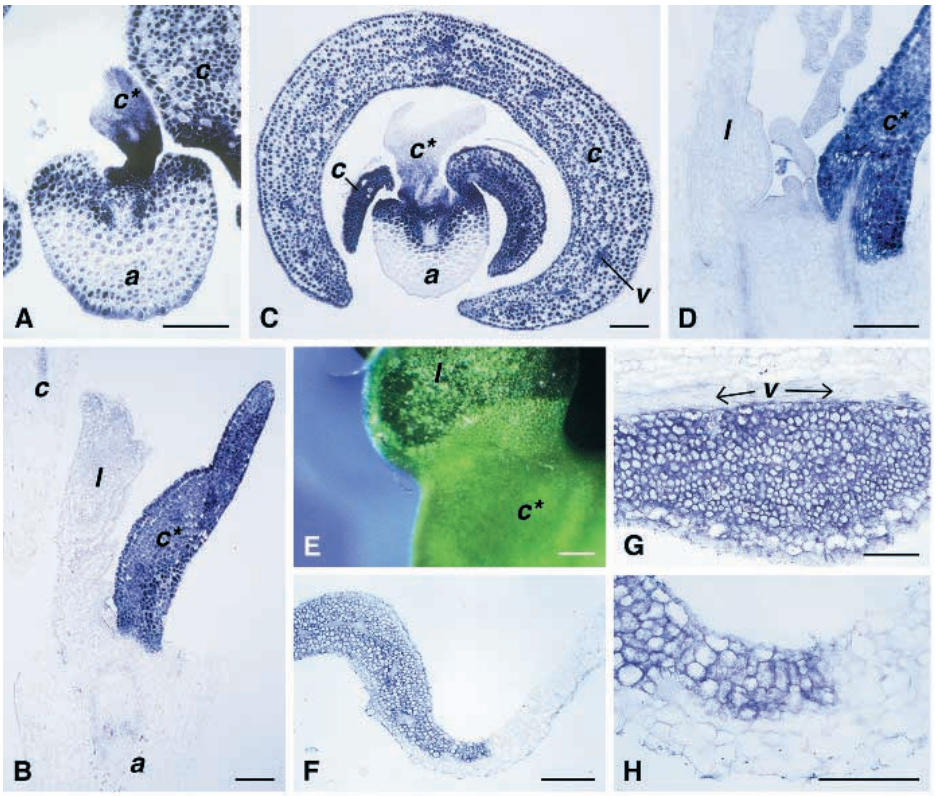


**Fig. 5.** Localization of napin mRNAs in cultured embryos (excised at 47 d.a.p.) producing a mixture of extra cotyledons and leaves. (A) Shoot apex after 7 days in culture. Napin mRNA levels were relatively low in the axis and relatively high in the shoot apex, in the cotyledons formed during embryogenesis and in the extra cotyledon developing from the first primordium. (B) Shoot apex after 20 days in culture. Napin mRNA accumulation was restricted to newly formed organs displaying cotyledon morphology. (C) View of whole embryo, after 10 days in culture. Napin mRNAs were undetectable in the axis below the cotyledonary node. Napin mRNA levels were relatively high in the shoot apex, on the abaxial face of the extra cotyledon (abaxial face at base, adaxial face at tip), and throughout the inner and outer cotyledons of the embryo, which are still folded in this case. (D) Shoot apex after 20 days in culture. Napin mRNAs could not be detected in the shoot apical meristem or in second lateral organ (leaf) of the epicotyl. (E) Sector boundary on a chimeric organ removed from an embryo cultured for 35 days. The dark green sector corresponds to leaf tissue and the light green sector corresponds to cotyledon tissue. (F) Chimeric organ from embryo cultured for 30 days. Napin mRNAs accumulated in a large coherent sector on one half of the organ. (G) Chimeric organ from embryo cultured for 30 days. High magnification view of a sector boundary that was coincident with a vein. (H) High magnification view of the sector boundary in the chimeric organ shown in 5F. Bars, (A- F) 200 μm; (G-H) 100 μm. *a*, embryo axis; *c*, cotyledon of embryo; *c**, extra cotyledon; *l*, leaf; *v*, vascular or provascular tissue.

## Gene expression in embryos producing extra cotyledons

To compare the development of cotyledons at the shoot apex during precocious germination with the development of cotyledons during embryogeny, in situ hybridizations were performed on sections of embryos excised at 38 d.a.p. Napin mRNAs could not be detected in the primordia or young expanding organs during the first week in culture (Fig. 4A,B). However, by 7 days after the start of culture, cells in the new organs began to accumulate napin mRNAs (Fig. 4C). By day 10, levels of accumulation in cells on the abaxial side of new organs and in the stipule primordia approached or exceeded the levels in cells of the embryo axis (Fig. 4D). The results with cruciferin probes were similar (data not shown), except that accumulation was delayed by several days relative to accumulation of napin mRNAs. By 20 days after the start of culture, several new organs had developed at the shoot apex. Napin (Fig. 4E) and cruciferin (Fig. 4F) mRNAs accumulated throughout these organs.

The patterns of mRNA accumulation also changed over time in tissues that were part of the excised embryo. Napin and cruciferin mRNAs were present at high levels in cells in the cotyledons and axes at the time of excision (Fernandez et al., 1991). Although levels declined thereafter (cf. Bisgrove et al., 1995), no changes in the spatial pattern of expression were seen during the first week in culture. At the start of the second week however, napin mRNAs began to accumulate in the provascular tissue of the cotyledons and axes (Fig. 4D,G,H) and in many cells in the vicinity of the shoot apical meristem (not shown). This pattern is characteristic of late maturation and appears around 45 d.a.p. in embryos developing in the seed environment (Fernandez et al., 1991). By 20 days after the start of culture, levels of napin (Fig. 4E) and cruciferin (Fig. 4F) mRNAs had declined in cells in the lower part of the embryonic axes, indicating that these cells had exited the embryonic phase, as they would have in the seed. In contrast, cells elsewhere in the embryo behaved differently than they would have in the seed. Instead of declining, high levels of napin and cruciferin mRNAs were maintained throughout the culture period in cells in the shoot apex (Fig. 4E,F) and in the cotyledons of the embryo (Fig. 4G,H).

## Gene expression in embryos producing organs of different identities

Sections of precociously germinating embryos that produce a mixture of cotyledons and leaves at the shoot apex (excised at 47 d.a.p.) were also hybridized with napin antisense probes (Fig. 5). By 7 days after the start of culture, napin mRNAs had accumulated to high levels in the organs with cotyledon morphology that developed from the first primordium, as well as in associated stipule primordia (Fig. 5A). High levels of napin mRNAs were maintained as these organs enlarged and expanded (Fig. 5B). Napin mRNAs could not be detected in the organs with leaf morphology that developed from the

second primordia (Fig. 5B). The mRNA accumulation patterns were consistent with the assignment of 'cotyledon' and 'leaf' identities to the first and second organs, respectively.

Levels of napin mRNA accumulation changed over time in organs that were part of the excised embryo. At 47 d.a.p., napin mRNAs were present at relatively high levels, in the late maturation pattern, throughout the embryonic cotyledons, axes and shoot apices (Fernandez et al., 1991). At 10 days after excision (Fig. 5C), napin mRNAs were still found at high levels in cells of the cotyledons and shoot apices. However, no accumulation of napin mRNA could be detected in cells of the axes below the cotyledonary nodes. By 20 days after the start of culture, napin mRNAs could not be detected in any cell that had been part of the excised embryo. At the shoot apex, napin mRNAs accumulated in the first new organ, which displayed cotyledon features, but not in other new organs, developing primordia, or the meristem itself (Fig. 5D). At the base of the first new organ, cells that accumulated high levels of napin mRNAs were found immediately adjacent to cells that showed no sign of accumulation.

### Gene expression in organs of mixed identity

To further investigate the nature of the interface between tissues with different identities corresponding to different developmental phases, napin mRNA accumulation was also examined in chimeric organs. Most of these organs contained one large sector of tissue with leaf features and another large sector of tissue with cotyledon features. Based on visible markers such as trichome density in the L1 layer (Fig. 2D) and/or chlorophyll content in the L2 and L3 layers (Fig. 5E), the boundaries between sectors appeared to be sharply defined. Large sectors of cells with different mRNA accumulation patterns could also be recognized on sections hybridized with napin antisense probes (Fig. 5F). Sector boundaries were marked by abrupt changes in the pattern of mRNA accumulation: cells that accumulated napin mRNAs at high levels were found immediately adjacent to cells that showed no sign of napin mRNA accumulation. In some cases, the sector boundaries corresponded to morphogenetic landmarks like major and minor veins (Fig. 5G). In other cases, they appeared to be more arbitrarily placed (Fig. 5F,H). Although cells that accumulated napin mRNAs had at least one neighbor that also accumulated these mRNAs, cells in one layer did not always show the same expression pattern as the neighboring cells in a different layer. Differences could also be seen across the thickness of an organ, i.e. cells on the adaxial side did not always accumulate the same mRNAs as cells on the abaxial side (Fig. 5H).

## DISCUSSION

### Extra cotyledons are produced via homeotic conversions of leaf primordia

The unusual organs produced at the shoot apex during precocious germination of immature *Brassica napus* embryos could be distinguished from leaves on the basis of their overall morphology, surface characteristics and patterns of gene expression. Leaves in this species have expanded lamina, trichomes, and dentate margins. The organs produced at the shoot apex during precocious germination are glabrous, with smooth margins and undergo more limited expansion, i.e. they assume the morphology of cotyledons. Cotyledons accumulate mRNAs, such as those encoding storage proteins, that are related to their roles as storage organs. The organs produced at the shoot apex also accumulate these sequences, in the same spatial and temporal pattern seen in embryos. Thus, the development of these organs is likely to involve a 'replay' of the programs expressed during embryo morphogenesis and maturation.

Unlike the cotyledons of the embryo however, the 'extra' cotyledons are produced by primordia that arise sequentially and in a spiral phyllotaxy, i.e. in a pattern characteristic of post-germinative development. A pair of stipule primordia, which have no structural equivalent in embryogenesis, is also associated with the base of each extra cotyledon or chimeric organ. Based on this unique combination of features, I conclude that these organs are not adventitious (because they arise directly from the shoot apical meristem of the embryo), but are products of presumptive leaf primordia that assume an embryonic identity as they develop.

Homeotic conversions from 'leaf' to 'cotyledon' are not restricted to *Brassica napus* or precocious germination systems. In *Arabidopsis*, embryos that are homozygous for *amp1-1* (*altered meristem program*), *pt-1* (*primordia timing*), *xtc1-1* (*extra cotyledon*), or *xtc2-1* mutations often produce seedlings that have one or more extra cotyledon-like organs (Chaudhury et al., 1993; Conway and Poethig, 1993). Recent reports indicate that *amp1-1* and *pt-1* are alleles of the *COP2* (*constitutive photomorphogenic)* locus (Lehman et al., 1996). The penetrance of the extra cotyledon trait varies with the locus and genetic background but rarely approaches 100%. In *amp1-1* mutants for instance, only about 20% of the seedlings have 3 or 4 cotyledons (Chaudhury et al., 1993). The phenotype is associated with changes in the extent of shoot apex development during embryogeny. Although primordium development is generally quite limited in wild-type embryos, mutant embryos often have large primordia at maturity (Conway and Poethig, 1993).

### Homeosis occurs as a consequence of heterochrony

Although transformations from leaves to cotyledons occur in different environments and under different circumstances in *Brassica napus* and *Arabidopsis*, they appear to have a common physiological basis. In mutants of *Arabidopsis* that produce extra cotyledons, defects introduced by mutation allow primordia to develop prematurely during the later part of embryogeny. In *Brassica napus*, which has non-dormant embryos, primordium development can be activated by placing excised embryos in an environment with a high water content. Our previous work (Bisgrove et al. 1995) showed that excised *Brassica* embryos continue to mature in culture in much the same way they would mature in the seed. Therefore, cultured embryos of *Brassica napus* are physiologically equivalent to the *Arabidopsis* mutants during maturation stage. In both *Brassica napus* and *Arabidopsis* then, it appears that homeotic transformations are a consequence of a heterochronic event: specifically, a change in the onset of rapid growth in the primordia relative to some critical parameter. Organ identity changes with embryo age in *Brassica napus*; therefore, the critical parameter is not the germination process, but rather,

other changes or events that occur in the course of embryo maturation.

One implication of this work is that cues in the external environment play only an indirect role in specification of organ identity during this period, at least in *Brassica napus*. An increase in water availability is necessary to stimulate and support growth at the shoot apex; however, embryos excised at different ages can and will produce very different organs in the same culture flask. A very different conclusion about the role of environmental cues was reached from studies of primordium determination during floral reversion in *Impatiens balsamina* (Battey and Lyndon, 1986, 1988). This plant is responsive to photoperiod and requires a constant input of the floral stimulus to maintain the reproductive phase. A change in photoperiod from inductive to noninductive conditions is sufficient to cause a change in identity from 'petal' to 'leaf', sometimes even within a single organ.

### Role of other factors

What other factors or processes are likely to play a role in specifying organ identity during precocious germination? If important extrinsic factors are supplied by maternal tissues, primordia that arise after excision should assume different identities than primordia that arise before excision. This is not the case. The first primordium arises before excision and can produce either cotyledons (in embryos excised at 47 d.a.p.) or leaves (in embryos excised at 60 d.a.p.). Primordia that arise after excision generally produce leaves but can also produce cotyledons (in embryos excised at 38 d.a.p.) or chimeric organs (in embryos excised at 47 d.a.p.). Organ identity also does not appear to depend on any intrinsic property of an individual primordium at the time of excision, such as its size or chronological age. Primordia present in embryos excised at 38 d.a.p. are small and 'young' at the time of excision and give rise to cotyledons. Primordia present in embryos excised at 60 d.a.p. are larger and 'older' and give rise to leaves. However, in embryos excised at 47 d.a.p., the relationship between age/size and fate is reversed. The larger, 'older' primordia now produce cotyledons while the smaller, 'younger' primordia produce leaves. Because there is no correlation between the age of primordia at the time of excision and the identity they assume, developmental progressions in the primordia themselves may not be as important for specifying organ identity as developmental progressions elsewhere in the embryo.

### Relationship between organ fate and gene expression in the shoot apex

Storage protein mRNAs can be used as indicators of developmental progressions in the cotyledons and axes of cultured embryos. The pattern of accumulation of napin mRNAs changes approximately halfway through maturation stage and again when embryos exit the embryonic phase. The results of in situ hybridization experiments with napin antisense probes were consistent with the results of previous RNA blot analyses (Bisgrove et al., 1995), which indicated that the cotyledons and axes of cultured embryos exit the embryonic phase at different times. During precocious germination in *Brassica*, embryo development and postgerminative growth occur simultaneously in individual embryos and in chimeric organs. The apparent overlap between the two developmental phases has been used to argue against the idea that the phase transition involves a molecular switch (reviewed by Kermode, 1990). Analysis of the system at a cellular level eliminates the possibility of extensive overlap and this argument. The results of in situ hybridization experiments indicate that the programs characteristic of embryo development and postgerminative growth operate in spatially distinct domains. Therefore, the behavior of these embryos is entirely consistent and compatible with models where the developmental programs are regulated by molecular switches operating at the cellular level.

Analysis of in situ hybridization results also indicated that there is a strong correlation between the production of extra cotyledons in cultured embryos and expression of molecular markers of the embryonic phase in cells in the cotyledons and upper axis. Embryos excised at 47 d.a.p. represent the most dramatic illustration of this relationship. The first primordium develops during the first week in culture, during a period when cells in the region of the shoot apical meristem accumulate storage protein mRNAs at high levels. This primordium assumes an embryonic identity and develops into an extra cotyledon. Levels of storage protein mRNAs decline over the course of the next 2 weeks in culture, indicating that the cells in the vicinity of the meristem are exiting or have exited the embryonic phase. The second primordium develops during this period and assumes a 'non-embryo' (leaf) identity. In embryos excised at 38 d.a.p., cells in the vicinity of the meristem progress from mid- to late maturation but do not appear to exit the embryonic phase. The primordia that develop in these embryos always produce extra cotyledons.

### Primordium determination in chimeric organs

Chimeric organs represent a more complex situation. They are produced at a stage when the cells in the cotyledons and axes of the embryo have exited the embryonic phase; however, some cells assume an embryonic identity. The nature of the sectors on the chimeric organs indicates several things about the specification of organ identity under these conditions. First of all, organ identity need not be specified identically in every part of a primordium. The work on floral reversion in *Impatiens balsimina*, where chimeric organs with sectors of leaf and petal tissue are produced (Battey and Lyndon, 1988), indicates that this is also true for primordia in other developmental contexts. Secondly, the sectors in *Brassica* are large and include several cell layers, which suggests that specification involves fields of cells rather than single cells. The sector boundaries are irregular but sharp. I interprete this as indicating that embryo identity is an 'all-or-nothing' feature at the cellular level, and that determination of identity (as well as the transition between phases) is likely to involve a molecular switch. If identity is determined at an early stage and then stably propagated, irregular boundaries can arise from differences in the growth rate of individual clones of cells. Finally, although the relative size of sectors is variable in both *Impatiens* and *Brassica*, their placement is not. In *Impatiens*, identity changes along the length of the blade of an organ and reflects a gradient in organ maturation (Battey and Lyndon, 1988). In *Brassica*, identity can change across the width of the blade. Sectors of cotyledon tissue on the third organ cover the portions of the blade closest to the extra cotyledon (first organ). Although the bulk of the

cells in the cultured embryos have exited the embryonic phase by this stage, the nearest neighbors of the cells in this portion of the third primordium are either undetermined (other cells in the primordium and/or meristem) or are in the embryonic phase (first organ). Similarly, the nearest neighbors of cells on the opposite side of the third primordium are undetermined or have exited the embryonic phase (second organ= leaf) and they assume a leaf identity. This pattern suggests that, in *Brassica*, cell position is important and the identity/phase of neighboring cells has some influence on the identity of a developing primordium.

The observation that neighboring cells influence development in the primordia implies that a system exists in the vicinity of the meristem for signalling information about developmental phase and/or identity. In embryos producing extra cotyledons, cells expressing molecular markers of the embryonic phase would control the local environment where the primordia develop. In the case of chimeric organs, signals corresponding to the embryonic phase must be either differentially supplied or differentially interpreted within a relatively small field of cells.

## Conclusions

The overall picture that emerges from this analysis is that primordia that arise at the shoot apex in embryos are not immediately committed to any particular fate. This implies that primordia pass through an intermediate stage, after initiation and before determination of organ identity, when groups of cells are receptive to various factors involved in specifying identity. In *Brassica napus*, primordia appear to assume an embryonic identity whenever they develop in the immediate vicinity of cells that express markers of the embryonic phase (summarized in Fig. 6). In embryos excised at 38 d.a.p., cells adjacent to the developing primordia are in the embryonic phase more or less continuously, and the primordia that arise at the shoot apex produce extra cotyledons. In embryos excised at 47 d.a.p., cells near the meristem are in the embryonic phase when the first primordium develops but are exiting the embryonic phase when the second primordium develops. In this situation, an extra cotyledon and a leaf are produced in sequence. In embryos excised at 60 d.a.p., all of the cells in the vicinity of the meristem have exited the embryonic phase and the primordia develop into leaves.

The production of chimeric organs can also be accounted for on the basis of a relationship between identity and the expression of phase-specific markers in neighboring cells. By the time the third primordium develops in embryos excised at 47 d.a.p., cells in the first organ (extra cotyledon) have accumulated high levels of napin mRNAs. The section of the third primordium nearest that organ assumes an embryonic identity. The fact that the distal section of the primordium assumes a different identity suggests that the process of specification involves short-range signals or interactions that operate in the context of relatively small groups of cells.

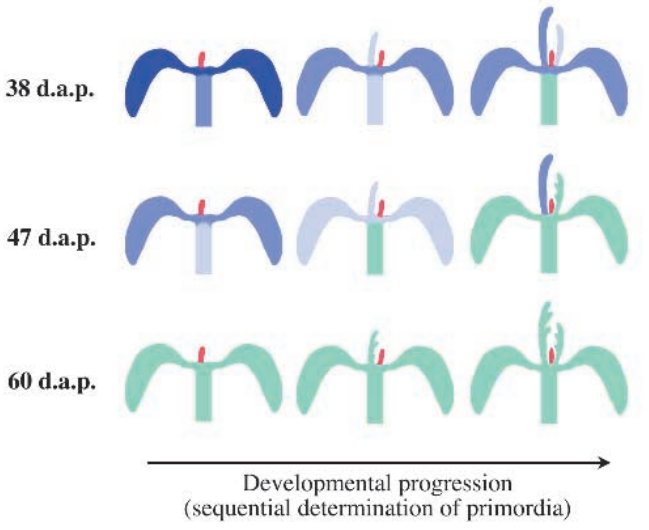


**Fig. 6.** Diagram summarizing the relationship between patterns of gene expression and the specification of organ identity in precociously germinating *Brassica napus* embryos excised at different times (d.a.p.). Red indicates the primordium being determined at each stage. The intensity of the blue color reflects the relative level of napin mRNA accumulation. The tissues that have exited the embryonic phase are indicated with light green. Extra cotyledons form in situations where cells in the vicinity of the meristem accumulate intermediate to high levels of napin mRNAs.

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