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In situ localization of storage protein mRNAs in developing meristems of *Brassica napus* embryos

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Summary

Probes derived from cDNA clones of napin and cruciferin, the major storage proteins of *Brassica napus*, and *in situ* hybridization techniques were used to examine changes in the spatial and temporal distribution of storage protein messages during the course of embryogeny, with a special emphasis on the developing apical meristems. Napin mRNAs begin to accumulate in the cortex of the axis during late heart stage, in the outer faces of the cotyledons during torpedo stage and in the inner faces of the cotyledons during cotyledon stage. Cruciferin mRNAs accumulate in a similar pattern but approximately 5 days later. Cells in the apical regions where root and shoot meristems develop do not accumulate storage protein messages during early stages of embryogeny. In the upper axis, the boundary between these apical cells and immediately adjacent cells that accumulate napin and cruciferin mRNAs is particularly distinct. Our analysis indicates that this boundary is not related to differences in tissue or cell type, but appears instead to be coincident with the site of a particular set of early cell divisions. A major change in the mRNA accumulation patterns occurs halfway through embryogeny, as the embryos enter maturation stage and start drying down. Final maturation of the shoot apical meristem is associated with the development of leaf primordia and the accumulation of napin mRNAs in the meristem, associated leaf primordia and vascular tissue. Cruciferin mRNAs accumulate only in certain zones of the shoot apical meristem and on the flanks of leaf primordia. Neither type of mRNA accumulates in the root apical meristem at any stage.

Key words: cruciferin, embryogenesis, *in situ* hybridization, mRNA localization, napin, rapeseed, seed development.

Introduction

All of the organs in a mature plant are products of postembryonic cell divisions in organized meristematic regions in the root and shoot. These special regions, located at the apices of a growing plant, are established during the course of embryogeny. One root apical meristem and one shoot apical meristem are carried as organized structures in the seed; other apical meristems are secondarily derived from these two primary meristems at some point after germination. Cells in the meristems are apparently determined at an early stage of embryogeny (Christianson, 1986; Poethig *et al.* 1986) and exist for much of the time in a relatively quiescent, largely undifferentiated state at opposite poles of the developing embryo. Despite their developmental significance, few careful studies have been done on these special cells and on the initial establishment of apical meristems in the embryo. We have been able to learn about early events in the establishment of apical meristems by studying temporal and spatial patterns of storage protein mRNA accumulation in rapeseed embryos.

Storage proteins accumulate to high levels in seeds and are degraded rapidly after germination to support seedling growth and development (review: Higgins, 1984). Expression of the genes encoding these proteins is developmentally regulated (review: Goldberg *et al.* 1989). Although storage protein mRNAs accumulate to high levels during particular stages of embryogeny, they are generally not present at other stages of the life cycle. Recent studies have shown that these mRNAs may accumulate preferentially in specific cells, tissues (Raikhel *et al.* 1988; Perez-Grau and Goldberg, 1989) or organs of the embryo (Ladin *et al.* 1987; Guerche *et al.* 1990). However, cells in the developing apical meristems have not been carefully examined in this regard.

In this study, we have used storage protein cDNA clones and *in situ* hybridization techniques to look at mRNA accumulation patterns in developing *Brassica napus* embryos. Previous histocytochemical studies (Kirk and Pyliotis, 1976; Werker and Vaughan, 1974) in the related plant *Sinapis alba* led us to predict that nearly all of the cells in *Brassica napus* embryos should accumulate storage proteins. We find that the napin and cruciferin mRNAs do accumulate in many different cell

types, but at distinctly different times in different regions of the embryo. The patterns of storage protein mRNA accumulation at the apices of the embryo are particularly interesting. Although storage proteins are generally expressed at later stages of embryogeny, the patterns we see appear to reflect early determinative events related to the establishment of apical meristems at these sites.

Materials and methods

Plants

Brassica napus L. cv. Tower plants were grown from seed (provided by Dr W. D. Beversdorf, University of Guelph, Ontario, Canada) in a temperature-regulated (10°C nights, 15°C days) growth chamber (Controlled Environments Inc., Pembina, ND, USA). Light was provided 16 h per day by a combination of fluorescent (cool white, very high output, Sylvania, GTE Products Corp., Danvers, ME, USA) and incandescent (40 W, Sylvania) lamps. Flowers were hand-pollinated and tagged on the day they opened (day of anthesis), and embryos were collected and staged according to the criteria outlined in Crouch and Sussex (1981).

Tissue fixation

Embryos were dissected out of developing seeds using tungsten knives (Cutter, 1967) and fixed by placing whole embryos or parts of embryos under a light vacuum and infiltrating for 3 h with freshly prepared 4 % (w/v) paraformaldehyde in 50 mM potassium phosphate buffer (pH 7.0) at room temperature. The fixation was continued overnight, without vacuum, at 4°C. Young embryos (20–25 DPA, days post-anthesis) are quite small (150 μm at the transition between globular and heart stages) and difficult to handle. These embryos were fixed as described above, embedded in hi-gelling-temperature agarose (FMC BioProducts, Rockland, ME, USA) and then refixed overnight. Fixed embryos were subsequently dehydrated in an ethanol series and embedded in Paraplast Plus (Sherwood Lancer, St Louis, MO, USA) for sectioning.

Scanning electron microscopy

Globular and early heart stage embryos (approximately 18 DPA) were washed briefly with 1 % Tween-20, incubated 20–30 min in 5 % acetic acid, and fixed with FAA (4 % (w/v) paraformaldehyde, 5 % acetic acid, 45 % ethanol). Older embryos were fixed as described in the previous section. Embryos of all ages were dehydrated in an ethanol series, 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, England).

In situ *hybridizations*

Embryos embedded in Paraplast Plus were sectioned (7 μm) with a steel knife and mounted on glass slides coated with 100 μg ml^{-1} poly-L-lysine in 10 mM Tris (pH 8.0). *In situ* hybridization experiments were performed using ^{35}S-labeled RNA probes essentially as described by Cox *et al.* (1984), except the probes were not sheared. Sense and antisense probes were generated using the Bluescribe vector system (Stratagene Cloning Systems, La Jolla, CA, USA), T3 and T7 RNA polymerases, and [^{35}S]UTP (Amersham Corporation, Arlington Heights, IL, USA). The sequences of the storage protein cDNA clones (napin: pN2, insert size=739 bp; cruciferin: pC1, insert size=1585 bp) used to generate probes have been reported previously (Crouch *et al.* 1983; Simon *et al.* 1985). Sections on glass slides were cleared with xylene, blocked with bovine serum albumin, treated with proteinase K and acetylated to block non-specific charge interactions. Hybridizations were performed overnight at 42°C and the slides were treated with RNAase A and washed at moderate stringency (0.1×SSC, 55°C). The slides were then coated with autoradiography emulsion (type NTB2, Eastman Kodak Co., Rochester, NY, USA), dried and exposed for 8–22 days at 4°C. Slides were developed using D-19 developer (Eastman Kodak Co.) and counterstained very briefly with toluidine blue (0.05 % (w/v) aqueous). Photomicrographs were taken using Panatomic X film (Eastman Kodak Co.) and a Zeiss photomicroscope equipped with bright- and dark-field condensors and phase contrast.

Results

Morphogenetic changes during embryogeny

Plant embryos undergo major changes in morphology during the early stages of embryogeny. If *Brassica napus* plants are grown under controlled conditions in an environmental chamber, the morphological changes occur at roughly predictable times after flower opening or anthesis. The time course for these changes under our culture conditions was established using scanning electron microscopy. Most of the major morphological changes occur during early stages of embryogeny, i.e. the first 35 DPA (days post-anthesis). Around 18 DPA, the radially symmetric globular embryos become bilaterally symmetric (Fig. 1A). Cells in the axis and in the developing cotyledon buttresses divide over the next few days and a heart stage embryo is formed (20 DPA, Fig. 1B). During heart stage, cells in the top and bottom halves of the embryo begin to divide at very different rates (Tykarska, 1979). The axis cells divide rapidly and by 25 DPA a torpedo stage embryo (Fig. 1C) is formed. Cotyledon cells also divide, although at first more slowly than axis cells. By early cotyledon stage (30 DPA, Fig. 1D), the width of the cotyledons greatly exceeds the width of the axis and the axis begins to curve because of the constraints of the seed coat. The cotyledons enlarge throughout cotyledon stage and finally wrap around the embryo axis (38 DPA, Fig. 1E).

The majority of cells in the embryo stop dividing during cotyledon stage (Tykarska, 1979), and few morphological changes take place during the second 35 days of embryogeny (late stages of embryogeny). During this time (maturation stage, 40–60 DPA), cells in both the axes and cotyledons accumulate storage materials and enlarge until they fill the entire space within the seed coat. The embryos gradually dry down and reach their final mature dry state at approximately 60–70 DPA.

In situ *hybridization conditions*

Previous studies in this laboratory have shown that the mRNAs for the major storage proteins in *Brassica napus* are expressed during embryogenesis and that

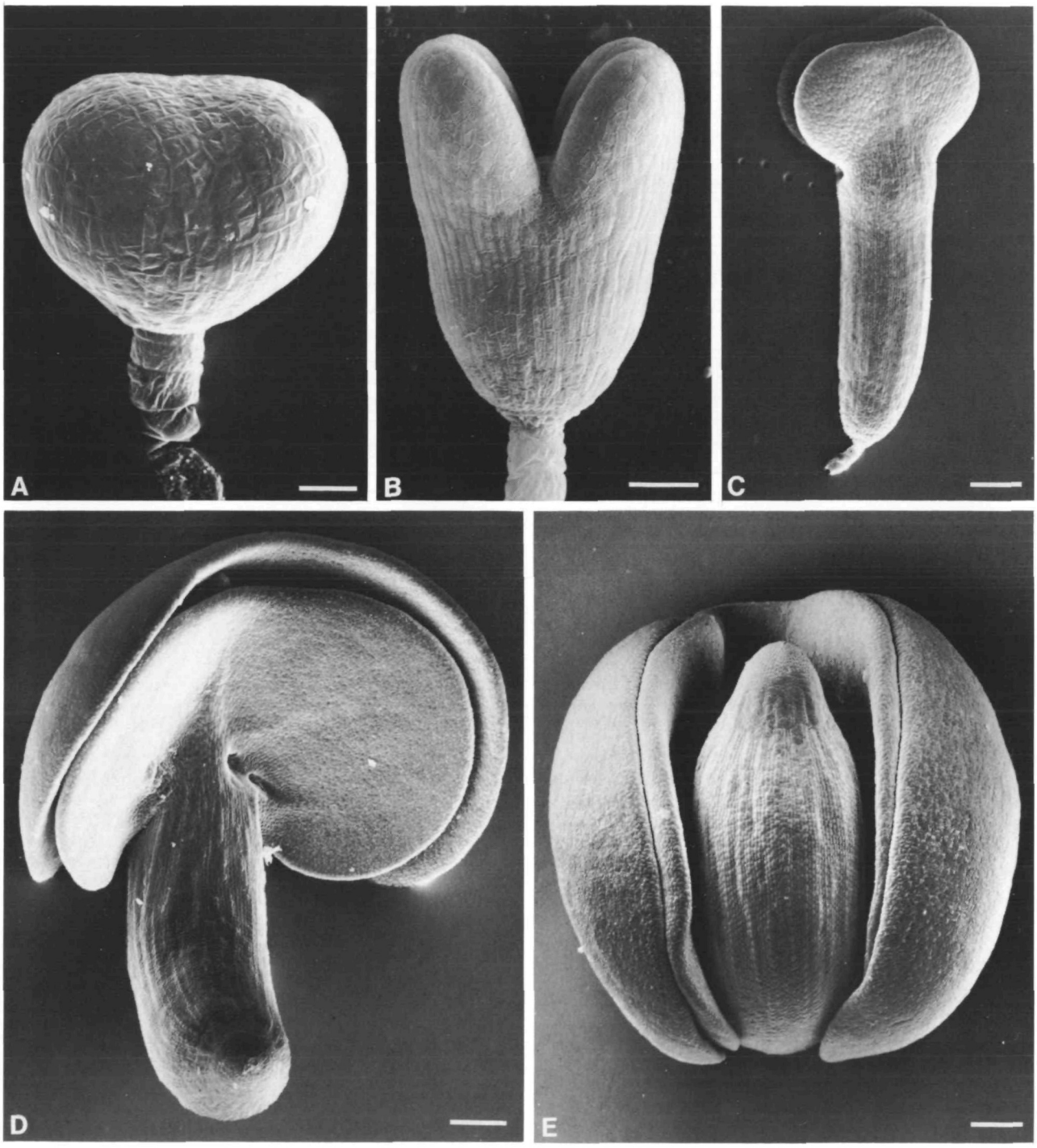


Fig. 1. Scanning electron micrographs of morphological changes that occur during the first 35 DPA in developing *Brassica napus* embryos. (A) Embryos change from radial to bilateral symmetry during transition stage, 18 DPA. ×375, bar=25 μm. (B) Cell divisions in the cotyledon buttresses generate heart stage embryos, 20 DPA. ×280, bar=40 μm. (C) Axes elongate rapidly during torpedo stage, 25 DPA. ×75, bar=100 μm. (D) Cotyledons expand in width during early cotyledon stage, 30 DPA, and the axes begin to bend. ×45, bar=200 μm. (E) Cotyledons continue expanding and wrap around the axes during mid-cotyledon stage, 35 DPA. ×40, bar=200 μm.

each class of mRNAs has a specific temporal pattern of accumulation (Finkelstein *et al.* 1985). In this study, we have used *in situ* hybridization techniques to document changes in the spatial patterns of mRNA accumulation. ^{35}S-labelled sense and antisense RNA probes were generated using cDNA clones for napin, a 1.7S albumin, and cruciferin, a 12S globulin. Although napin and cruciferin genes exist as multigene families in *Brassica napus*, probes generated from the cDNA clones will hybridize to all members under moderately

stringent conditions (Scofield and Crouch, 1987; Breen, 1990). Analysis of genomic clones of the five-member cruciferin gene family indicated that the coding sequences are 90–99% identical for all members (Breen, 1990). The napin cDNA clone hybridizes to at least 16 different members in Southern analyses (Scofield and Crouch, 1987). Three napin genomic clones have been isolated and sequenced (Josefson *et al.* 1987; Scofield and Crouch, 1987; Baszczynski and Fallis, 1990), and the coding sequences are at least 90% identical. Under the moderately stringent conditions used in this investigation then, we expect that the probes generated from the cDNA clones will hybridize to mRNAs derived from any member of the gene family under consideration. Therefore, the tissue distribution patterns should reflect total expression of either the napin or cruciferin gene family. Hybridizations with sense probes were used to define background levels and are not shown; hybridizations performed with antisense probes produced signals significantly above background levels.

Localization of napin mRNAs during early stages of embryogeny

Napin mRNA accumulation begins relatively early in embryo development. Although no accumulation is apparent in early heart stage embryos or suspensors (short, filamentous organs of zygotic tissue which form at the basal end of embryos) (Fig. 2A,B), napin mRNAs are clearly detectable in the embryo at the transition between late heart and early torpedo stages. Messages initially accumulate in the cortex of the axis (Fig. 2C,D), and are particularly abundant in a part of the upper axis immediately beneath the region where the shoot apical meristem will form later in development (Fig. 2E,F, arrow). Accumulation in cotyledon cells lags slightly behind accumulation in the axis cells. By early torpedo stage, low levels of napin mRNA have accumulated in the cotyledons (Fig. 2E,F, arrowhead); increasing amounts of mRNA accumulate in the cotyledons as torpedo stage progresses. By late torpedo stage, napin mRNAs are very abundant in the epidermis and ground tissue of the axis (Fig. 2G,H), and are slightly less abundant in the ground tissue of the cotyledons (Fig. 2G,H). Napin mRNAs do not accumulate in the provascular tissue, nor do they accumulate to equal levels in all the cells in the cotyledons. The distribution of mRNA accumulation is in fact quite asymmetric, and when cross-sectional views of cotyledons in early cotyledon stage embryos are examined (Fig. 2I), it is clear that only cells in the outer faces (facing away from the central axis) of the cotyledons accumulate mRNAs at this stage. Napin message cannot be detected in cells in the inner faces of the cotyledons except in the region adjacent to the vascular system. This asymmetric pattern is very pronounced during early cotyledon stage, but as cotyledon stage progresses, increasing amounts of napin mRNA accumulate in the inner faces of the cotyledons (not shown). By mid-cotyledon stage (approx. 35 DPA, Fig. 2J,K), napin mRNAs are evenly distributed throughout the cotyledons and are found in both the epidermis and ground tissue. In contrast, napin mRNAs do not appear to accumulate in the provascular tissue at any stage during early embryogeny (Fig. 2C–K).

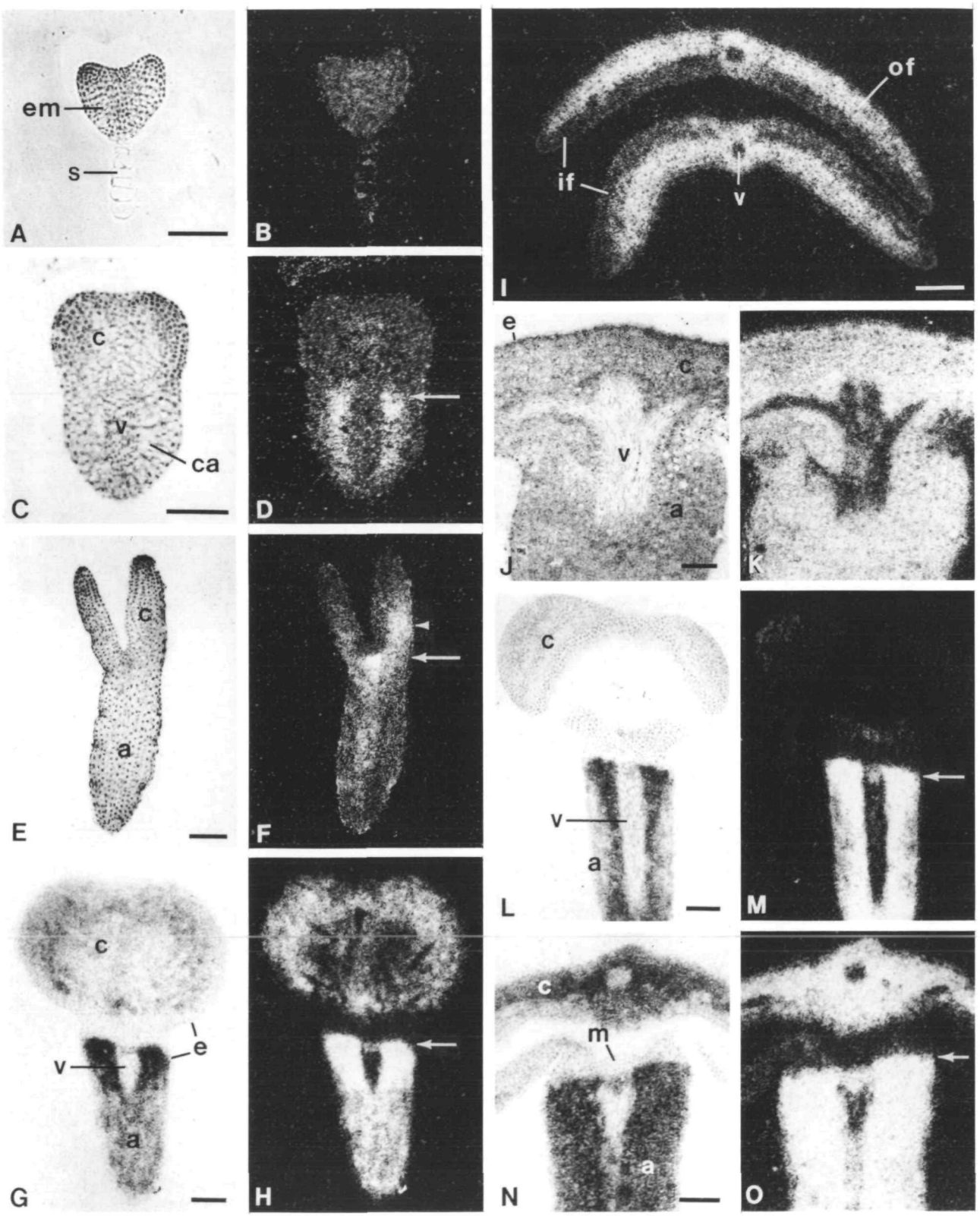
When the upper part of the axis of the embryo was examined in greater detail (i.e. the region where the shoot apical meristem will develop), an intriguing pattern of mRNA accumulation was observed. By late torpedo stage, a dramatic discontinuity in napin mRNA accumulation is visible (Fig. 2G,H, sectioned along outer face of the cotyledons; Fig. 2L,M, sectioned along inner face of the cotyledons). Although mRNA levels in neighboring cells can be quite high, cells in a restricted part of the upper axis do not appear to accumulate napin mRNAs. The upper boundary of this region is somewhat indistinct, but the lower boundary is very sharply defined (arrows). Cells beneath this boundary accumulate napin mRNA while immediately adjacent cells above this boundary do not (Fig. 2L,M). The boundary is visible at the very earliest stages of napin mRNA accumulation (Fig. 2C,D, arrow) and apparently persists throughout early development, since it is easily distinguished in both torpedo and early cotyledon stage embryos. There is no obvious morphological differentiation within this region to account for this pattern of differential gene expression. However, we have noted that the lower boundary is consistently

Fig. 2. Localization of napin mRNAs during early stages of embryogeny. Bright-field and dark-field images of the same section, hybridized with napin antisense RNA probes, are shown in pairs. Bars=100 μm. (A,B) Napin mRNAs do not accumulate in either the embryo (em) or suspensor (s) during early heart stage, 18 DPA. Longitudinal section, A: phase contrast, ×100. (C,D) Napin mRNAs accumulate within the cortex of the axis (ca) during late heart stage, 20 DPA. Longitudinal section, ×105. (E,F) Napin mRNAs accumulate in the axis (a), particularly immediately below the future shoot apex (arrow), and in the outer face (arrowhead) of the cotyledons (c) during torpedo stage, 25 DPA. Longitudinal section, ×65. (G,H) Napin mRNAs do not accumulate in the provascular tissue (v) or in a particular set of cells in the upper axis (above arrow) during torpedo stage, 25 DPA. Longitudinal section through outer face of cotyledons, ×60. (I) Napin mRNAs accumulate within the outer faces (of) of the cotyledons during early cotyledon stage, 30 DPA), but not within the inner faces (if) or provascular tissue (v). Transverse section, ×80. (J,K) During mid-cotyledon stage, 38 DPA, napin mRNAs are present in the epidermis (e) and throughout the ground tissue of the cotyledons (c) and axis (a), but do not accumulate in the provascular tissue (v). Longitudinal section, ×65. (L,M) The boundary in the upper axis between cells that do or do not accumulate napin mRNAs (arrow) is well-defined during torpedo stage, 25 DPA. Cells in the inner face of the cotyledons (c) do not accumulate napin mRNAs at this stage. Longitudinal section through inner face of cotyledons, ×60. (N,O) The mRNA accumulation boundary (arrow) in the upper axis is located 2–3 cell layers below the shoot apical meristem (m) at early cotyledon stage, 30 DPA. Longitudinal section, ×70.

situated in close proximity to the developing shoot apical meristem (Fig. 2N,O).

Localization of cruciferin mRNA during early stages of embryogeny

Cruciferin mRNAs accumulate later in development than napin mRNAs. Previous studies in this laboratory using mRNAs isolated from whole *Brassica napus* embryos (Finkelstein *et al.* 1985) indicated that the onset of cruciferin mRNA accumulation lags behind the onset of napin mRNA accumulation by approximately 5 days. Our studies using *in situ* hybridization confirm this



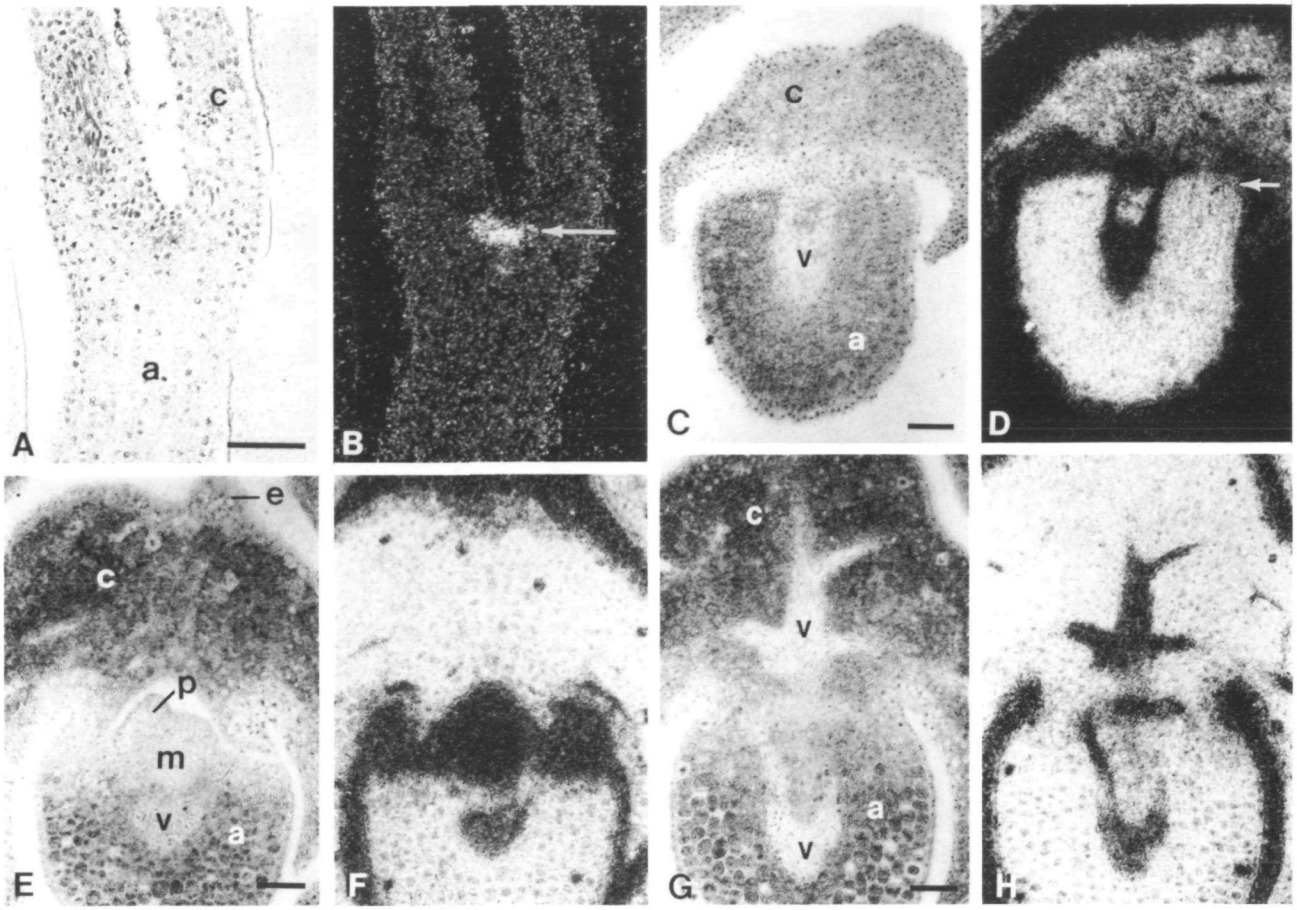


Fig. 3. Localization of cruciferin mRNAs during early stages of embryogeny. Bright-field and dark-field images of the same section, hybridized with cruciferin antisense RNA probes, are shown in pairs. Bars=100 μm. (A,B) Cruciferin mRNAs accumulate in the upper axis immediately below the future shoot apex (arrow) during late torpedo stage, 25 DPA. Longitudinal section, A: phase contrast, ×100. (C,D) Cruciferin mRNAs accumulate within the ground tissue of the axis (a) and in the cotyledons (c) during early cotyledon stage, 30 DPA. A distinct boundary between cells that do or do not accumulate message (arrow) is present in the upper axis. Longitudinal section, ×60. (E,F) The mRNA accumulation boundary in the upper axis is located several cell layers below the shoot apical meristem (m) and associated leaf primordia (p). Cruciferin mRNAs are present in lower levels in the epidermis (e) than in the ground tissue in mid-cotyledon stage embryos, 38 DPA. Oblique section, ×60. (G,H) Cruciferin mRNAs do not accumulate within the provascular tissue (v) in either the cotyledons (c) or axis (a) in mid-cotyledon stage embryos, 38 DPA. Oblique section, ×60.

result. Although napin mRNA accumulation can be detected at the transition between heart and torpedo stages, cruciferin mRNA accumulation is not detected until late torpedo stage, when the embryo reaches approximately 1.5 mm in length. The spatial distributions of cruciferin and napin mRNAs are similar in most respects. Cruciferin mRNAs accumulate initially in the upper axis, particularly immediately below the presumptive meristem region (Fig. 3A,B, arrow). The mRNAs accumulate at a slightly later period in the cotyledons, again in an asymmetric manner (i.e. in the outer faces before the inner faces) (not shown); however, this phase is neither as distinct nor as prolonged as in the case of napin mRNAs. During early cotyledon stage (30 DPA), cruciferin mRNAs are present in higher levels in the axis than in the cotyledons (Fig. 3C,D); but by mid-cotyledon stage (38 DPA), the levels are approximately equal (Fig. 3E,F). Cruciferin mRNAs accumulate in both the epidermis and ground tissue of the cotyledons and axes but, unlike napin mRNAs, accumulate to different levels in the two tissues. Throughout early embryogeny, cruciferin mRNA levels in the epidermal cells are markedly lower than levels in the ground tissue (Fig. 3E,F). As with napin, cruciferin mRNAs do not accumulate in the provascular tissue (Fig. 3G,H) of either the cotyledons or axes.

The spatial distribution of cruciferin mRNAs in the upper axis also matches the pattern obtained with napin probes. As with napin mRNAs, cruciferin mRNAs do not accumulate within a restricted group of cells in the upper axis. Again, cells that accumulate message appear to be immediately adjacent to those that do not and a distinct boundary is formed. The site at which this boundary forms is apparent as soon as cruciferin mRNA accumulation begins (late torpedo stage, Fig. 3A,B, arrow). The boundary is well defined by early cotyledon stage (30 DPA, Fig. 3C,D) and persists

into mid-cotyledon stage. Again, the only structural feature that the boundary can be consistently related to is the developing shoot apical meristem. In every embryo that has been examined, the boundary has been located several cell layers below the site where the first leaf primordia develop (Fig. 3E,F).

Localization of storage protein mRNAs in the shoot apical meristem region

The observation of a group of cells in the upper axis that differ from the rest of the cells in the embryo in terms of storage protein mRNA accumulation was unexpected. It was particularly intriguing that the boundaries between cells that do or do not accumulate message were so distinct, and appeared to delineate a particular region of the embryo. In order to define the extent of this region, torpedo or early cotyledon stage embryos were serially sectioned in different orientations and probed with napin and cruciferin antisense sequences.

Regardless of which storage protein probe is used, the boundaries of the region in the upper axis containing cells that do not accumulate message are the same. In medial longitudinal sections cut on the plane passing between the cotyledons, the lower boundary is distinct and sharply defined, and is located approximately 2–3 cell layers (at early cotyledon stage) below the shoot apical meristem with both napin (Fig. 4A,B) and cruciferin (Fig. 4C,D) probes. In embryo sections probed with napin antisense sequences, it is clear that the boundary extends completely across the axis, crossing both the epidermis and ground tissue (Fig. 4A,B; cf. Fig. 2N,O). Therefore, the difference in gene expression appears, in this case, to transcend tissue boundaries. In medial longitudinal sections that bisect both cotyledons, non-vascular cells that do not accumulate storage protein message are restricted to the center of the upper axis. In sections of late torpedo stage embryos, obvious boundaries delineate a narrow pocket that contains the shoot apical meristem and is subtended by a V-shaped region defined by the branches of the vascular system extending into the cotyledons (napin probe, Fig. 4E,F; the pattern with cruciferin probe is identical, not shown). Hence the apical region containing cells that do not accumulate storage protein mRNAs appears to be highly asymmetric. The pattern is most clearly seen when storage protein mRNAs are localized on transverse sections of the upper part of the axis. In transverse sections cut at the level of the shoot apical meristem (Fig. 4F, top arrowhead), the region clearly extends from one side of the embryo to the other in one direction and is restricted to the center of the axis in the other direction (cruciferin probe, Fig. 4G,H). All of the cells in the region lie between two branches of the vascular system. One branch enters each cotyledon; therefore, the cells of interest must lie in a narrow groove between the bases of the cotyledons. Not every cell between the branches contributes to the region however. In transverse sections cut more than 3–4 cell layers below the level of the meristem (Fig. 4F, bottom arrowhead), cells between the two vascular branches accumulate both napin (not shown) and cruciferin mRNAs (Fig. 4I,J).

Localization of storage protein mRNAs during late stages of embryogeny

During the transition from early to late stages of embryogeny, changes occur in the spatial distribution of storage protein mRNAs. Beginning around 35–40 DPA, napin message accumulates in cells that did not accumulate storage protein message during early embryogeny. By 45 DPA, napin mRNAs are found in most cells and tissues in the embryo, including the cells in the shoot apical meristem and provascular tissue in both the cotyledons and axes (Fig. 5A,B). Although previous studies (Finkelstein *et al.* 1985) indicated the level of napin mRNAs in the embryo as whole drops gradually after 40–45 DPA, napin antisense probes continue to hybridize intensely to certain subpopulations of cells in the embryo until at least 55 DPA. In particular, significant napin mRNA accumulation is prolonged until well into maturation stage in cells that did not accumulate storage protein message during early development. In the upper axis, this results in a pattern at 50 DPA (Fig. 5C,D) that is the reverse of the pattern seen in early cotyledon and torpedo stage embryos (cf. Fig. 2E). Cells in the ground tissue and epidermis immediately subjacent to the shoot apical meristem accumulate higher levels of napin mRNA than neighboring cells lower in the axis (Fig. 5C,D, asterisks). As during early stages of embryogeny, a boundary seems to separate these two populations of axis cells. Napin mRNAs also accumulate, at 50–55 DPA, in the shoot apical meristem and associated leaf primordia (Fig. 5C–F), as well as throughout the provascular system (Fig. 5E,F). The levels of napin mRNA apparently drop soon thereafter however. We were unable to detect accumulation in embryos at 60 DPA (not shown) even with prolonged exposure times.

The spatial distribution of cruciferin mRNAs also changes during the transition from early to late stages of embryogeny, but at a slightly later time (40–45 DPA) than that of napin mRNAs. In general, only a subset of the cells that accumulate napin mRNAs during this period also accumulate cruciferin mRNAs. By 45 DPA, cruciferin mRNAs have accumulated to detectable levels within cells subjacent to the shoot apical meristem (Fig. 5G,H), and levels remain relatively high for at least the next 10 days (Fig. 5I–L). At some point, the differential accumulation of cruciferin mRNAs in the epidermis and ground tissue that was seen at mid-cotyledon stage (cf. Fig. 3E,F) disappears: at 50 and 55 DPA, cruciferin probes hybridize with approximately equal intensity to both tissues (Fig. 5I–L). On the other hand, cells in the provascular system do not accumulate cruciferin mRNAs at any time (Fig. 5G–L) and, while napin mRNAs accumulate throughout the shoot apical meristem, cruciferin mRNAs accumulate only in certain areas. In particular, cruciferin mRNAs accumulate in cells near the edges of the meristem and on the flanks of developing leaf primordia but not in the center of the meristem and other portions of the leaf primordia

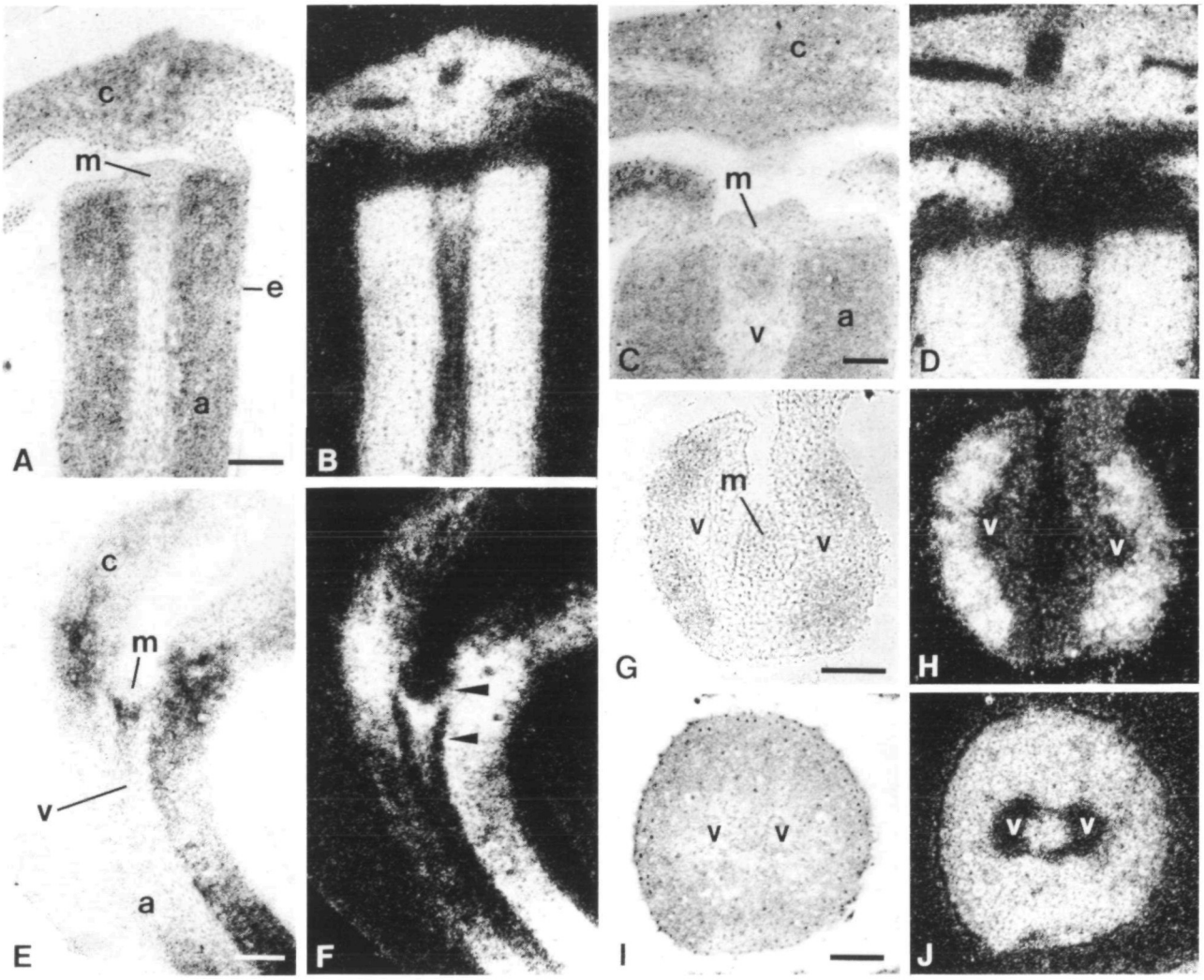


Fig. 4. Localization of storage protein mRNAs in the shoot apical meristem region. Bright-field and dark-field images of the same section, hybridized with either napin (A,B,E,F) or cruciferin (C,D,G–J) antisense RNA probes, are shown in pairs. Bars=100 μm. (A,B) The boundary in the upper axis (a) between cells that do or do not accumulate napin mRNAs crosses both the epidermis (e) and ground tissue and is located 2–3 cell layers below the apical meristem (m) during early cotyledon stage, 30 DPA. Longitudinal section, ×75. (C,D) The boundary in the upper axis between cells that do or do not accumulate cruciferin mRNAs is also located 2–3 cell layers below the shoot apical meristem (m) at mid-cotyledon stage, 38 DPA. As with napin probes, the boundary extends the entire width of the embryo. Longitudinal section, ×60. (E,F) In medial longitudinal sections perpendicular to views in 4A–D, non-vascular cells that do not accumulate napin mRNAs are restricted to the center of the axis in the meristem (m) region during late torpedo stage, 25 DPA. Longitudinal section, ×70. (G,H) In transverse sections cut at the level of the top arrowhead in 4F, cruciferin mRNAs do not accumulate in an elongate region containing the meristem (m) and bordered by branches of provascular tissue (v). Early cotyledon stage, 30 DPA, G: phase contrast, ×90. (I,J) In transverse sections cut at the level of the bottom arrowhead in 4F, cruciferin mRNAs accumulate in the ground tissue of the axis and between the branches of provascular tissue (v). Early cotyledon stage, 30 DPA, ×75.

(Fig. 5I–L). As maturation proceeds, message levels drop gradually and, by 60 DPA, little or no mRNA accumulation can be detected even with prolonged exposure times.

Localization of storage protein mRNAs in root apical meristems

During the course of embryogeny, changes also occur in the distribution of storage protein mRNAs in the region of the embryo containing the root apical meristem, but they are less dramatic than those that occur at the shoot apex. Napin mRNAs begin to accumulate in the lower axis during early torpedo stage and are confined initially to cells in the cortex (ground tissue) and the outermost root cap cells (Fig. 6A,B). At somewhat later stages, napin mRNAs accumulate within the epidermis as well, at levels approximately equal to those in the cortex (not shown). Cells belonging to the root apical meristem, unlike those in the shoot apical meristem, have a characteristic organization even at early stages and can

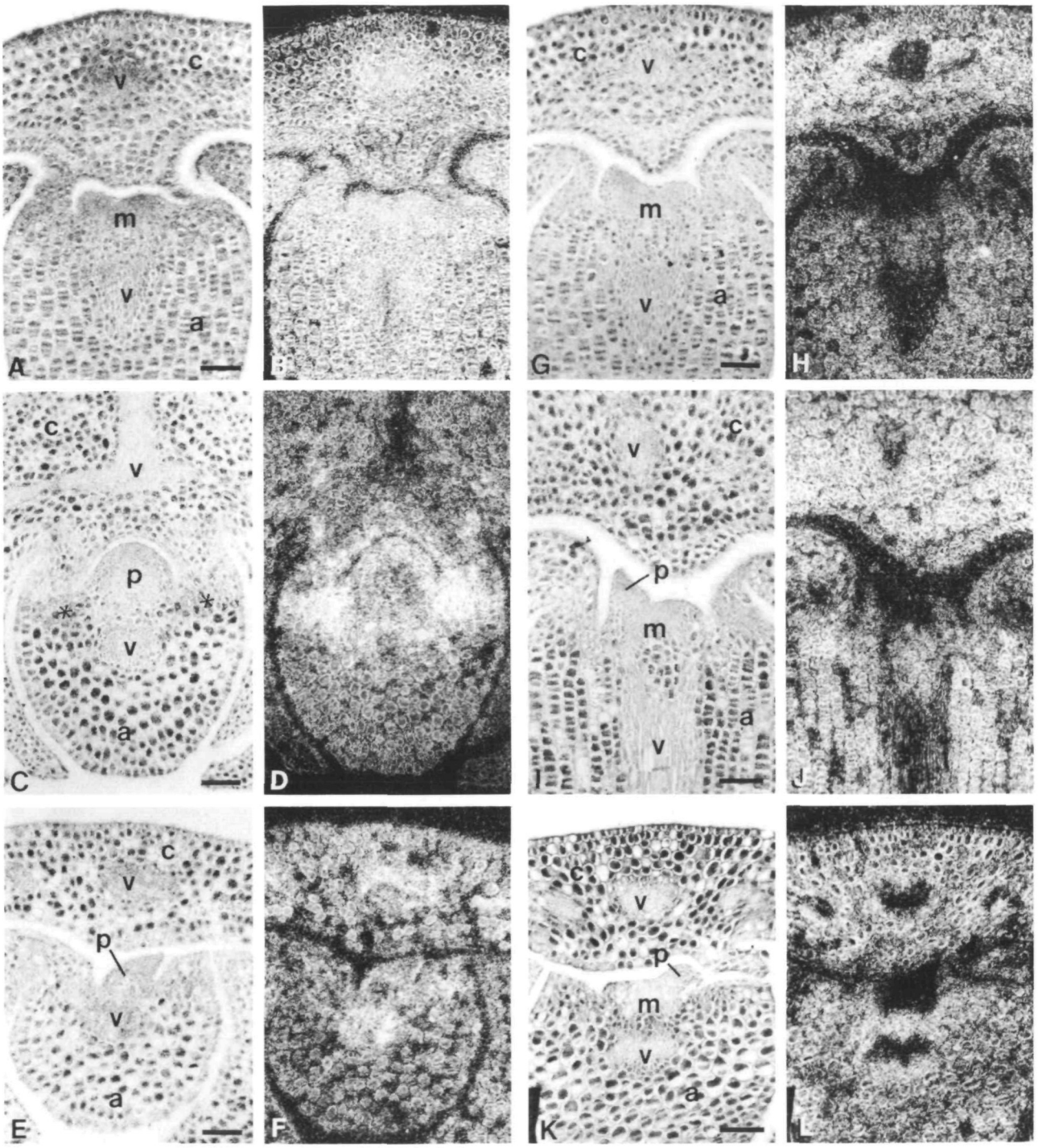


Fig. 5. Localization of storage protein mRNAs in the shoot apical meristem region during late stages of embryogeny. Bright-field and dark-field images of the same section, hybridized with either napin (A–F) or cruciferin (G–L) antisense RNA probes, are shown in pairs. Bars=100 μm. (A,B) Napin mRNAs accumulate throughout the meristem region (m) and provascular tissue (v) in early maturation stage, 45 DPA, embryos. Longitudinal section, ×65. (C,D) Napin mRNAs accumulate to higher levels in cells (asterisks) subjacent to the apical meristem (m, with associated primordium, p) than in neighboring axis cells at 50 DPA. Oblique section, ×65. (E,F) Napin mRNAs are present in developing leaf primordia (p) and provascular tissue (v) during late maturation stage, 55 DPA. Oblique section, ×65. (G,H) Cruciferin mRNAs accumulate in cells subjacent to the apical meristem (m) but not in provascular tissue (v) during early maturation stage, 45 DPA. Longitudinal section, ×65. (I,J) Cruciferin mRNAs accumulate on the flanks of leaf primordia (p) associated with the apical meristem (m) at 50 DPA. Longitudinal section, ×70. (K,L) Cruciferin mRNAs are present in the peripheral zone but not in the central zone of apical meristems during late maturation stage, 55 DPA. Oblique section, ×70.

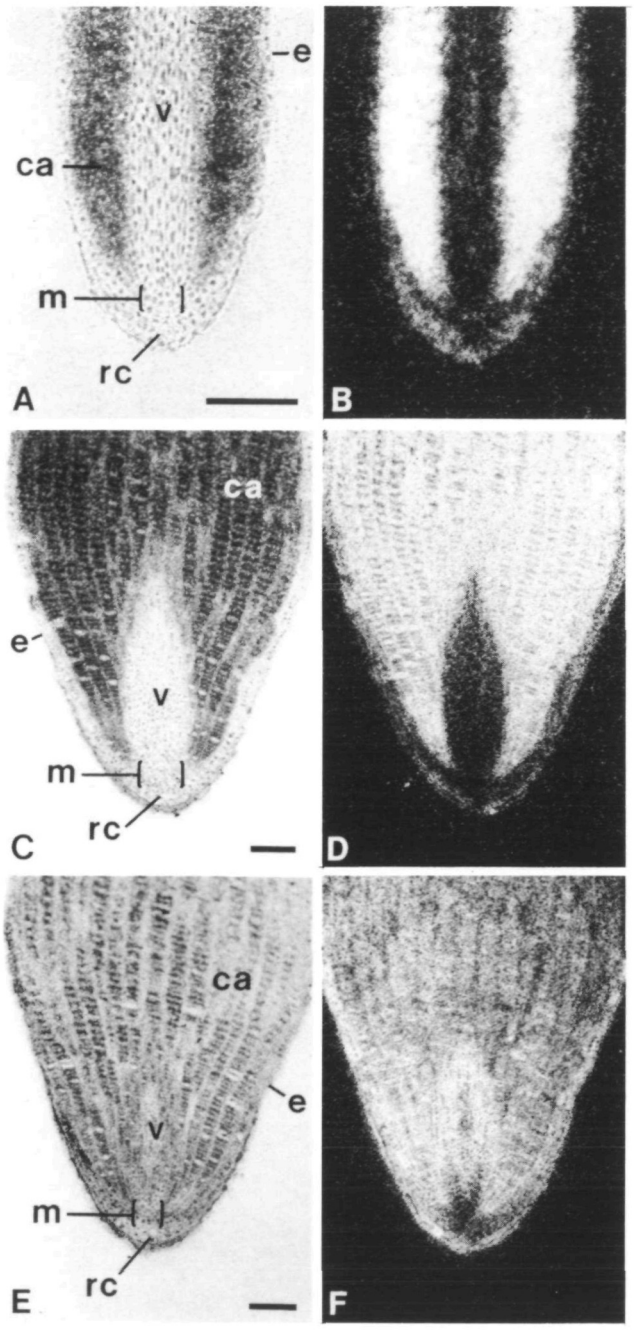


Fig. 6. Localization of storage protein mRNAs in root apical meristems. Bright-field and dark-field images of the same section, hybridized with either napin (A,B,E,F) or cruciferin (C,D) antisense RNA probes, are shown in pairs. Bars=100 μm. (A,B) Napin mRNAs accumulate in the cortex of the axis (ca) and outer cells of the root cap (rc) during torpedo stage, 25 DPA. mRNAs do not accumulate in the provascular tissue (v) or root apical meristem (m, brackets). Longitudinal section, A: phase contrast, ×115. (C,D) Cruciferin mRNAs accumulate at high levels in the cortex of the axis (ca) and at lower levels in the epidermis (e), but not in the provascular tissue (v) or apical meristem (m, brackets) at mid-cotyledon stage, 38 DPA. Longitudinal section, ×60. (E,F) At late stages of embryogeny, napin mRNAs accumulate throughout the axis and radicle tip except in the apical meristem (m, brackets) and columella cells in the central portion of the root cap (rc). Longitudinal section, 38 DPA, ×60.

be easily distinguished from adjacent cells. Napin mRNAs clearly do not accumulate in either the quiescent center of the apical meristem (centrally located cells immediately above the root cap, indicated with brackets) or in the innermost cells of the root cap (Fig. 6A,B) during the early stages of embryogeny. In addition, as is the case in other regions of the embryo, napin message apparently does not accumulate within cells of the provascular tissue. Cruciferin mRNAs accumulate within the same cells that accumulate napin mRNAs, but at slightly later times (late torpedo and early cotyledon stages). The overall distribution of cruciferin message is virtually identical to that of napin message except that the level of cruciferin message in the epidermis generally remains lower than the level in the ground tissue (Fig. 6C,D).

During the transition between early and late stages of embryogeny, the spatial distribution of napin mRNAs in the lower axis changes. Starting around 35–40 DPA, napin mRNAs accumulate within the central vascular cylinder in the lower axis (Fig. 6E,F). Napin mRNAs may also accumulate in a few additional cells at the periphery of the root cap, but do not accumulate in the apical meristem of the root or adjacent cells in the central portion of the root cap (Fig. 6E,F). Thus the overall pattern is quite different from that found at late stages in the shoot apex, where every cell in and around the apical meristem accumulates napin message. The distribution of cruciferin mRNAs does not appear to change during this time period. Cruciferin mRNAs do not accumulate in the vascular system of the lower axis, and the pattern in root tips after 38 DPA (not shown) is essentially identical to that seen during early stages of embryogeny.

Discussion

Linear boundaries in mRNA accumulation patterns

Careful analysis of patterns of storage protein mRNA accumulation in developing *Brassica napus* embryos indicates that boundaries of gene expression exist that may be more closely related to boundaries set up by early divisions in the embryo than to cell type differences. Because cells divide regularly in *Brassica* embryos and do not migrate during the course of embryogenesis, it is possible to look at medial longitudinal sections and use the alignment of cell walls and cytohistological features to trace the ontogeny of particular regions of the embryo with some accuracy. Tykarska (1976, 1979) used these characteristics to distinguish groups of cells of common origin in an exhaustive histological study of *Brassica napus* embryo development. In particular she notes a major boundary, the O′ boundary, which lies 2–3 cell layers below the shoot apical meristem in torpedo and cotyledon stage embryos, and apparently marks the site of the first transverse divisions in the embryo proper (Fig. 7). Using *in situ* mRNA hybridization techniques, we have identified a striking boundary that extends across the embryo at this same level, where cells that accumulate

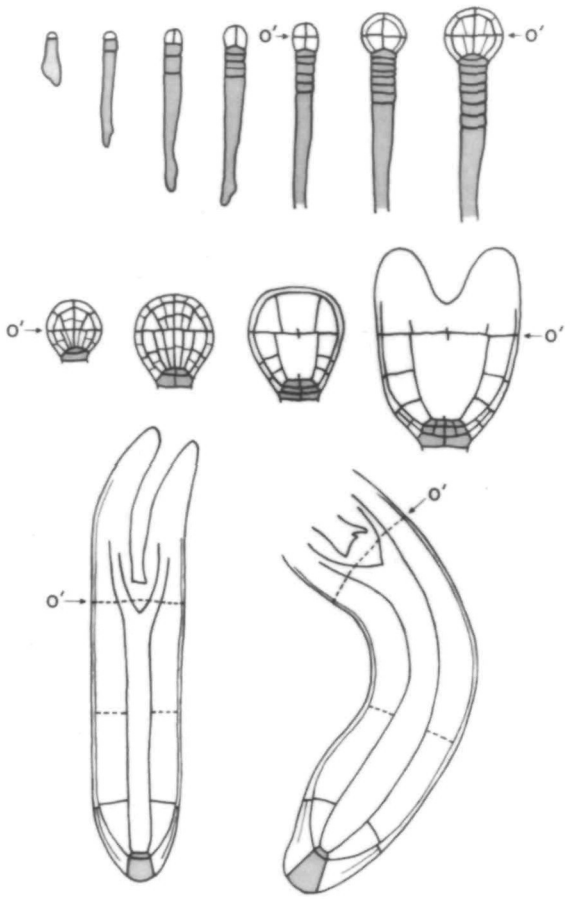


Fig. 7. Summary diagram of *Brassica napus* embryo development (adapted from Tykarska, 1979). Cells contributed by the suspensor lineage are shaded gray. The cell walls marking the O′ boundary are initiated during the first transverse division of the embryo proper (unshaded cells) and are located approximately 3–4 cell layers below the shoot apical meristem in mature embryos.

storage protein mRNAs are immediately adjacent to cells that do not. Since the set of divisions that establishes the O′ boundary immediately precedes the establishment of the protoderm (precursor of the epidermis), it is also the only major transverse boundary in the embryo proper that would be expected to cross tissue types. We find that the boundary of napin mRNA accumulation extends through the ground tissue and into the epidermis. Because of this distribution, we are fairly confident that the boundary we have observed and the O′ boundary coincide, although more definitive cell lineage studies will, of course, be necessary to unequivocally establish the origin of this boundary.

The differences in patterns of storage protein mRNA accumulation that we see may reflect differences in developmental fate established at some point after the first set of transverse divisions. The divisions that give rise to the O′ boundary divide the embryo into 2 tiers (Fig. 7), which, in *Brassica napus*, have distinctly different fates. Cells derived from the upper tier form cotyledons and epicotyledonary parts, while cells derived from the lower tier give rise to the hypocotyl and roots (Tykarska, 1976). We find that cells derived from the lower tier accumulate high levels of storage protein message during early development. Certain cells derived from the upper tier do not accumulate napin and cruciferin mRNAs during this period; however, other cells derived from the upper tier, such as those in the cotyledons, clearly do. Therefore, it appears that patterns of storage protein mRNA accumulation do not correspond in any simple way to the O′ boundary but are likely to be influenced by additional determinative events involving cells derived from the upper tier.

Delayed storage protein mRNA accumulation in the epiphysis

Using mRNA hybridization techniques, we have been able to visualize a distinct population of cells derived from the upper tier that do not accumulate storage protein mRNAs during early development and which we believe constitute the epiphysis of the embryo. The epiphysis consists of a group of small cells located at the apex of the embryo opposite the suspensor. Cells in the epiphysis can be recognized during very early stages of embryogeny by their densely staining cytoplasm and are thought to contribute to the development of the shoot apical meristem (Steeves and Sussex, 1989). Their most consistent characteristic is a decreased frequency of cell division relative to cotyledon cells (Swamy and Krishnamurthy, 1977), and once the rate of cell division slows in the cotyledons, epiphysis cells are difficult to distinguish from neighboring cells. The epiphysis is derived from centrally located cells near the apex, and, in many species, arises soon after the formation of tiers in the embryo. In these cases, parts of the walls separating the tiers become the lower walls of epiphysis cells (Swamy and Krishnamurthy, 1977; Fig. 8). In *Brassica napus*, these walls would correspond to the O′ boundary, which should be located at the level where we observe the boundary in storage protein mRNA accumulation during early embryogeny. During the transition to heart stage, the balance between rapid cell divisions in the cotyledonary loci and the relative quiescence of epiphysis cells results in the formation of an elongated epiphysis region extending completely across the embryo in one direction and flanked by masses of cotyledon tissue in the other (Swamy and Krishnamurthy, 1977; Fig. 8). The region we have visualized at cotyledon stage has the same conformation and location. Thus, we believe that we have been able to define the previously indistinct boundaries of the epiphysis using mRNA localization techniques that do not depend on morphology or frequencies of cell division. The inherent difference between epiphysis cells and those belonging to the cotyledons is one of developmental fate. As we might expect, this difference is expressed as a change in the pattern of gene expression.

Our studies indicate that epiphysis cells do accumulate storage protein mRNAs, but at a very different time than neighboring cells. Storage protein mRNAs cannot be detected within cells of the epiphysis during

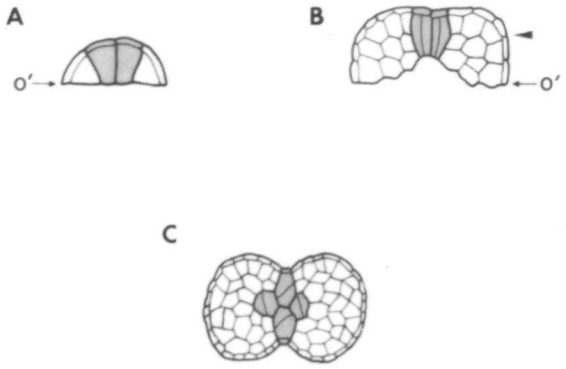


Fig. 8. Summary diagram of the ontogeny of the epiphysis (adapted from Swamy and Krishnamurthy, 1977). Only the upper tier of the embryo is shown and cells contributing to the epiphysis are shaded gray. (A) Medial longitudinal section of the embryo at the stage when the upper tier contains 16 cells. The epiphysis is derived from a group of cells in the central portion of the upper tier. The O′ boundary forms the lower wall of the epiphysis cells. (B) Medial longitudinal section (bisecting both cotyledon buttresses) at transition stage (between globular and heart stages). Cells in peripheral positions in the upper tier divide rapidly to form the cotyledon buttresses. Epiphysis cells divide more slowly and are largely confined to the center of the upper tier. (C) Transverse section of the embryo in 8B cut at the level of the arrowhead. The balance between the two populations of cells dividing at different rates in the upper tier generates an epiphysis region which extends completely across the embryo in one direction and is flanked by masses of cotyledon tissue in the other.

early stages of embryogeny and do not begin to accumulate until after the shoot apical meristem is organized. The cell divisions that establish the shoot apical meristem start during late torpedo stage and continue into cotyledon stage in *Brassica napus* (Tykarska, 1979). We have shown that, after this point (approximately 35 DPA), storage protein mRNAs accumulate in the cells derived from the epiphysis (shoot apical meristem, axis cells subjacent to the meristem, leaf primordia) in a sequence similar to that observed during early embryogeny. Napin mRNAs accumulate first, and approximately five days later, cruciferin mRNAs begin to accumulate. Thus, our data suggest that epiphysis cells contain the factors necessary to activate embryo-specific genes and accumulate embryo-specific messages in the normal sequential fashion, but the program appears to be delayed relative to neighboring cells.

Ontogeny and developmental restriction in the epiphysis

It appears highly likely that positional cues play a role in the establishment of the epiphysis. Cells in the epiphysis do not belong exclusively to one cell lineage since they are not derived from a single 'ancestoral' cell, but from a minimum of four precursor cells in the upper tier of the embryo (Swamy and Krishnamurthy, 1977). Only a subset of the progeny of the original four cells in the upper tier, those in the centrally located portions closest to the apex (cf. Fig. 8), will give rise to the epiphysis. Although the epiphysis encompasses all of the cells that could potentially contribute to an apical structure, i.e. all the cells between the cotyledons, only a subset of the cells in the epiphysis will actually manifest a difference in developmental fate and become part of the meristem. We suggest that differentiation of the meristem proper is the consequence of a second, later developmental segregation that occurs among the progeny of this first group of cells, and may also depend on positional cues.

The early developmental segregation that establishes the epiphysis appears to define the developmental compartment within which the shoot apical meristem develops. Work in insect development (Garcia-Bellido and Merriam, 1971; Garcia-Bellido *et al.* 1976) indicated that compartmentalization affects groups of cells, and that, once segregated, cells in compartments display behavior that is distinct from neighboring cells. Epiphysis cells fit this description in that they are derived from a group of centrally located cells, and divide more slowly and at later stages than immediately adjacent cells. We have shown that cells of the epiphysis also function as a distinct group in terms of storage protein mRNA accumulation during both early and late stages of embryogeny. *In situ* RNA hybridization techniques have allowed us to identify a demarcation line or boundary that represents a possible compartment border. This line in the upper axis is not correlated with any obvious linear morphological feature or tissue boundary, and cannot be attributed to either cell death, intercalary cell growth, biased cell division patterns, or secondary fusion events. In addition, because the cells do not migrate during embryogenesis, the line necessarily separates cells that lay side by side when the developmental segregation occurred.

Segregation of the epiphysis lineage appears to occur at an early stage in embryogenesis. The time at which developmental segregations or restrictions occur can be inferred from the position of the demarcation lines (Garcia-Bellido *et al.* 1976). Our analysis suggests that the position of the demarcation line in the upper axis coincides with the position of the O′ boundary. Thus, the developmental segregation that establishes the epiphysis may occur soon after the first transverse divisions in the embryo proper and around the time that the protoderm is first delineated in *Brassica napus*. Clonal analysis studies in other plant species also indicate that the cells whose progeny form the shoot apical meristem are present in very young embryos. Christianson (1986) has analyzed chimeric seedlings derived from a 'semigametic' strain of cotton and concludes that distinct 'compartments' for the shoot apical initials, first leaf, second leaf and cotyledons all exist at the time when the protoderm is established, i.e. at the transition between proembryo and globular embryo stages. In maize, the shoot apical meristem appears to be determined gradually. Poethig *et al.* (1986) have observed that twinning, which they suggest results from aberrations in the initiation of the shoot

apical meristem, is especially prevalent in embryos irradiated during mid- to late proembryo stage. Determination of the shoot apical meristem in maize appears to be complete by transition stage, 8–10 DPP (days post-pollination).

Ontogeny and developmental restriction in the hypophysis

The apical meristem of the root is derived from the hypophysis, a well-defined group of cells at the suspensor end of the embryo. In *Brassica napus*, cells of the hypophysis are descendants of a single suspensor cell immediately below the embryo; subsequent growth pushes these cells inside the mass of embryo cells (Tykarska, 1976). They are the only cells in the mature embryo (shaded gray, Fig. 7) derived from the large basal cell that is produced by the asymmetric first division of the zygote.

Our mRNA localization studies indicate that cells of the hypophysis display a pattern of storage protein mRNA accumulation that is qualititatively different from that in all other cells in the embryo. Unlike cells derived from the epiphysis, which accumulate storage protein mRNAs during late stages of embryogeny, cells derived from the hypophysis (root apical meristem and central part of the root cap; Fig. 7, shaded areas) do not accumulate storage protein mRNAs at any point during embryogeny (cf. Fig. 6E,F). Differences in developmental potential (including the ability to accumulate embryo-specific mRNAs) between the hypophysis and other cells in the embryo could arise as early as the first cell division in the zygote, when the embryo and suspensor lineages are set apart. If the ability to produce embryo-specific factors segregates with the smaller apical cell in the first division of the fertilized egg, hypophysis cells and other cells derived from the suspensor lineage may not contain the factors necessary to express storage protein genes.

Shoot apical meristem zonation

The shoot apical meristems of actively growing, mature seed plants have a characteristic structural organization that is not apparent in most embryos. At least two major zones exist which can be distinguished using cytohistological techniques. The central zone near the top of the apex contains cells that are relatively quiescent, while the peripheral zone nearer the bottom and edges of the meristem contains cells that divide more frequently and have a higher total RNA content (Steeves and Sussex, 1989). In mature embryos, all of the cells are quiescent, and pronounced cytohistological zonation generally cannot be observed. Thus, zonation is generally thought to arise during germination and subsequent seedling growth when the shoot apex assumes its mature form (Steeves and Sussex, 1989).

The results presented in this paper provide clear evidence for the existence of zonation in shoot apical meristems of *Brassica napus* embryos. Napin mRNAs accumulate throughout the meristem during late stages of embryogeny, but cruciferin mRNAs accumulate only in cells at the edges and bottom of the meristem region (cf. Fig. 5I–L). The cruciferin mRNA accumulation pattern matches that obtained with tritiated thymidine in apices of mature sunflower plants (Steeves *et al.* 1969), hence cells that accumulate cruciferin mRNAs appear to be confined to the peripheral zone. Thus, it appears that at least two zones exist even in relatively undeveloped meristems like those in *Brassica napus* embryos (no pronounced apical dome and only 1 small leaf primordium) and persist throughout the period when embryos are drying down. These results suggest that zonation is a feature that arises early, apparently as soon as the meristem is established. In addition, the results confirm the notion that, while the cytohistological differences in zones may appear slight, zonation may actually reflect fundamental differences in gene expression in terms of individual gene products.

Implications for models of gene regulation and patterning during embryogeny

Models for the regulation of expression of storage protein genes in *Brassica napus* embryos must account for dramatic changes in the pattern of mRNA accumulation during the course of embryogeny (summarized in Fig. 9). Previous studies in this laboratory using mRNAs isolated from whole embryos indicated that napin and cruciferin mRNAs accumulate to high levels during late torpedo, cotyledon and early maturation stages (Finkelstein *et al.* 1985). Our mRNA localization results show that the high levels are actually the sum of at least three separate waves of message accumulation and that, during each wave, mRNAs accumulate within a different set of cells. Some qualitative differences exist: cells in the provascular system, for example, only accumulate napin mRNAs. The most striking differences, however, are temporal in nature. Most of the cells in the embryo appear to progress through a similar maturation program, since storage protein mRNAs appear in the same sequence and with similar relative timing, but the program can apparently 'start' at different times in different parts of the embryo. We have shown that napin mRNAs begin to accumulate during heart stage in cells in the axis, during torpedo stage in cells in the outer face of the cotyledons, during cotyledon stage in cells in the inner face of the cotyledons, and during mid-cotyledon stage in cells in the epiphysis. Some of the same individual genes of the gene families may be expressed in each set of cells. In the closely related plant *Arabidopsis thaliana*, only one of the four 2S albumin (napin equivalent) genes showed any organ specificity (Guerche *et al.* 1990). Each set of cells in the embryo of *Brassica napus* appears to have a different internal 'clock' that determines when the program starts. We suggest that a consideration of the developmental history of the cells involved will provide insights into the 'clock-setting' mechanism.

A major temporal difference in the onset of mRNA accumulation exists between cells in the epiphysis and their immediate neighbors. Setting or resetting of an internal 'clock' may actually be one aspect of the segregation of developmental capacity that establishes

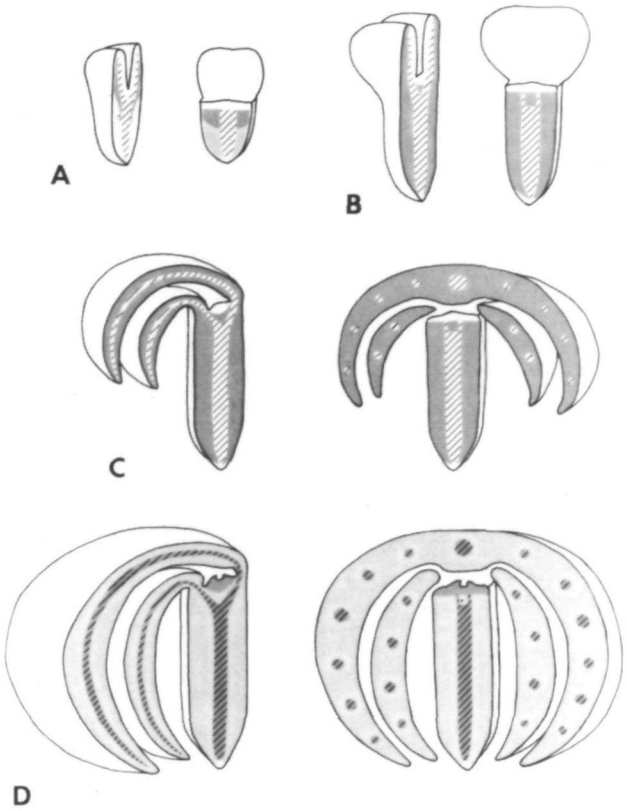


Fig. 9. Summary diagram of changes in the distribution of napin mRNAs during the course of embryogeny in *Brassica napus*. Two embryos are shown for each stage: the first embryo is sectioned on the medial longitudinal plane bisecting both cotyledons and the second embryo on the medial longitudinal plane passing between the cotyledons. Provascular tissue is found in the hatched areas and regions where napin mRNAs accumulate are indicated by gray shading. Levels of accumulation are highest in the regions shaded dark gray. Embryos are not drawn to scale and the curvature of the axis in mature stages is not illustrated. (A) During heart stage, napin mRNAs accumulate in the cortex of the axis and, at lower levels, in the outer face of the cotyledons. (B) During torpedo stage, napin mRNAs accumulate to high levels in both the axis and outer faces of the cotyledons. (C) During early cotyledon stage, napin mRNAs accumulate to high levels in the axis and in both faces of the cotyledons. (D) During maturation stage, napin mRNAs accumulate throughout the embryo except in cells at the root tip. The highest levels of message are found in the vascular tissue and at the shoot apex.

the epiphysis. As we have seen, the epiphysis is apparently established soon after the protoderm is delineated, in early globular stage embryos. The embryos are radially symmetrical up to this point, but then cells in different regions begin dividing at different rates and generate the asymmetries seen in the mature embryo. Cells in the lower tier form the axis; cells in the upper tier divide to form either the outer face of the cotyledons, the inner face of the cotyledons or the epiphysis, depending on how far they are from the apex. Temporal differences in mRNA accumulation define the same spatial gradient relative to the apex. The time that elapses before the onset of the maturation program is relatively short in cells in the axis and outer face of the cotyledons, intermediate in length in cells in the inner face of the cotyledons, and long in cells in the epiphysis. It is possible then that internal clocks may be set in response to one or more factors that exist in a gradient centered on the apex. According to this model, cells exposed to high levels of the factor would begin the maturation program late in development; cells exposed to lower levels would begin earlier.

The time at which the initial segregations of developmental capacity occur appears to vary from species to species and may give rise to mRNA accumulation patterns that are different from that in *Brassica napus*. The first clearly visible sign of developmental restriction in plant embryos is the delineation of the protoderm. In crucifers like *Brassica napus*, the protoderm is delineated at a very early stage, when there are 8 cells in the embryo (Tykarska, 1976); in other groups of plants, the delineation may occur later (e.g. approximately 50 cells in cotton, Christianson, 1986). The time at which other important developmental segregations occur, such as that involved in the establishment of the epiphysis, may vary as well. Since restriction borders reflect the time of the segregation event, we would not expect to see the border between the epiphysis and neighboring cells in the same position in every species. The coincidence that we have observed between the O′ boundary and a possible restriction border may be rather unusual and probably reflects both the regularity of early cell divisions and the fact that the segregation occurs early in *Brassica napus* embryos.

Species-to-species variation in the time at which the important developmental segregations occur may affect 'clock-setting' as well. We have shown that storage protein mRNAs begin to accumulate at distinctly different times in the inner and outer faces of the cotyledons in *Brassica napus*. Perez-Grau and Goldberg (1989) report that, in soybean, the onset of seed protein mRNA accumulation moves in a wave-like pattern from the outer face of the cotyledons to the inner. According to our 'clock-setting' model then, the internal clocks become progressively slower as you move across the cotyledon in both species, but change more gradually in soybean embryos than in *Brassica napus* embryos. We have suggested that internal clocks are set in response to a spatial gradient of some factor centered at the apex at a critical time in early development. If this critical time occurs later in soybean than in *Brassica napus*, there will be more cells in the embryo when the gradient is 'read'. If the average level of factor that a cell is exposed to determines how its clock is set and the gradient is the same in both cases, the final pattern will be quite different in embryos with large numbers of cells (critical time at later stages) and embryos with small numbers of cells (critical time at early stages). In embryos with small numbers of cells, only a small number of clocks will be set, resulting in few and fairly discrete steps in the pattern of gene expression later in development. In contrast, in

embryos with large numbers of cells, a large number of settings are made, resulting in a more gradual, wave-like pattern of expression. According to our model then, the particular pattern of temporal differences seen in different species would reflect the developmental history of the embryos.

Models that invoke spatial gradients emphasize the importance of spatial or positional cues in establishing overall patterning. It may be particularly important to think in terms of such models for events in early stages of plant embryogenesis. In some plant species, patterns of early cell division appear to be very irregular. Early cleavage patterns also differ substantially from species to species (Natesh and Rau, 1984). Therefore, it is difficult in many cases to invoke cell-lineage-dependent mechanisms for determining fate. However, if patterning is position-dependent and is imposed at some later period, variation in early division patterns could be easily accommodated.

In summary, we have documented intriguing patterns of storage protein mRNA accumulation in developing *Brassica napus* embryos using *in situ* localization techniques. When we analyze the temporal and spatial patterns that we see in the context of developmental history, we find indications of both early patterning events and developmental compartmentalization related to meristem development.

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