

## Microamputation of somatic embryos of the domestic carrot reveals apical control of axis elongation and root regeneration

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### Summary

Somatic heart- and torpedo-stage embryos of the domesticated carrot, *Daucus carota* L., were severed at their midlengths to produce two halves termed apical and basal pieces. These pieces may be grafted or kept separate. Grafted embryos developed normally, with the exception that they tended to mature earlier than uncut control embryos. If kept separate, the apices grew at rates similar to grafted apices, while the basal ends, behaving as if they had been released from an inhibition of growth, rapidly elongated and matured (e.g. produced root hairs and a root cap) 3–4 days earlier than uncut controls. Grafted embryos treated with the transport inhibitor TIBA (2,3,5-

triiodobenzoic acid) had basal sections that behaved as if the sections had been kept separate. Additionally, resupplying IAA (indole-3-acetic acid) via a novel wick-bridge forced isolated basal pieces to behave as if the embryo apex were present. This apparent inhibition of root growth by the apex appears to be controlled by either the polar transport of auxin, and/or the accumulation of auxin at the root end. These experiments suggest that polar auxin transport has a greater influence on root, rather than on apex, development in these embryos.

Key words: somatic embryogenesis, carrot, polar auxin transport, pattern formation.

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### Introduction

Experimental embryology traces its origin to experiments in which cells or tissues of a developing organism were mechanically separated in an attempt to determine the nature by which portions of an embryo act in concert to regulate the development of the whole. Work using eggs from frog by Roux (1888) and sea urchin by Driesch (1892) provided the groundwork for later experiments which have ultimately given rise to the concepts of mosaic and regulative development and pattern formation in animals. Since these pioneering studies, technical advancements have permitted more refined manipulations such as nuclear transplantations, *in situ* localization of gene transcripts and their products, and gene insertions, which, in turn, provide greater access to more discrete components of development. The value of results obtained from techniques of greater precision, of course, depends on the relative state of knowledge about a system; thus, for developmental phenomena that are characterized by descriptive changes in form, surgical removal or the separation of

component parts provides a simple expedient with which one may characterize the intercellular communication in an organism. These observations can then help to formulate more astute questions, leading to the design of more probing and detailed experiments.

In plants, little experimental manipulation of developing embryos has occurred for several reasons. Until recently, there has been little opportunity to overcome the problem of accessibility of zygotic (seed-borne) embryos. Moreover, plant workers tend to perceive that plant development is sufficiently different from animals as to preclude the use of experimental approaches from the classical embryological literature.

Advances in plant tissue culture alleviated the problem of accessibility to embryos in a number of plant species. Perhaps the best known example comes from the work of Reinert (1958), who was the first to derive somatic embryos from callus cultures of the wild carrot, *Daucus carota*. These embryos are initiated whenever the growth regulator auxin (or an analogue) is removed from the medium. Although

somatic embryos often lack a smooth protoderm in the earlier stages, and they fail to undergo a period of dormancy like their zygotic counterparts, they faithfully recapitulate the stages traversed by embryos developing *in ovulo*. The obvious advantages to using somatic embryos include the ability to raise large numbers of embryos in a small volume, and the ease with which they are manually manipulated.

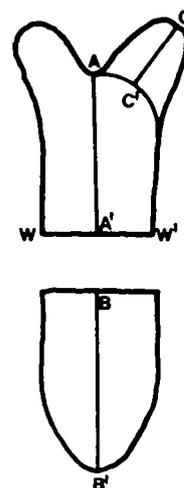
The most distinctive morphological feature of many adult angiosperms is an organization which is centred around a series of repetitive units; e.g. the repeating pattern of leaves, associated nodes and internode segments, along the stem. This feature is a result of meristem formation which is apparent after the plant embryo has matured; thus, development in plant embryos is limited to the specification of the root-shoot axis and the initial differentiation of vascular tissue. Since it is felt by some researchers that the mature plant body plan is oriented around the vascular system, the period in embryogenesis where the vascular system is initially laid down represents an important point in plant development. Light-microscope examination of somatic embryos reveals that the early vascular system may have its origins in the transition from the globular to oblong stages (Schiavone & Cooke, 1985). This transition marks the first outward sign of the axial polarity from which the plant will permanently orient development. During this time the vascular system begins to form at the embryo's base and develops acropetally to include the embryo apex and cotyledons in the heart and torpedo stage (Schiavone & Cooke, 1985). Thus the heart- and torpedo-stage embryo represents a phase in the development where the plant is clearly polarized, yet lacks repetitive structures.

Because the axial polarization of the root and shoot are the major pattern-generating elements in heart- and torpedo-stage embryos, these stages were selected for experiments involving surgical separation of apical and basal halves of an embryo. With paths of communication thus severed, grafting experiments were conducted to assess the importance that each half of an embryo plays in orienting overall development and the maintenance of pattern. The effects of the topical application of the plant growth regulator auxin and of an inhibitor of polar auxin transport were also evaluated in the grafted and ungrafted embryo halves.

## Materials and methods

### Cell culture

Cells of the domesticated carrot were cultured in Murashige & Skoog (1962) medium containing  $5 \mu\text{M}$  2,4-D to maintain callus growth, or lacking 2,4-D (MSE) to elicit embryo formation. All culture methods and procedures for the



**Fig. 1.** Diagrammatic representation of the lines used to define measurements of embryos in this study. Lines A-A' and B-B' represent the axial lengths of the apical (apex) and basal (base) pieces, respectively (in grafted or uncut embryos, this value is derived from line A-B'). Line W-W' is the width of the embryo at the cut surface, while line C-C' (ending on a smooth arc between the apex, A, and the base of the cotyledon), represents the cotyledon length.

initiation of embryos have been previously described (Schiavone & Cooke, 1985).

### Microinstrument manufacture

Microscalpels were manufactured following methods modified from Lowry & Passonneau (1972). Briefly, double-edged razors were trimmed producing two thin strips, approximately 2 mm wide, with the sharp edge of the razor as the long side of the strip. This strip was then cut into smaller pieces (approx. 2 mm square), and a nylon toothbrush bristle was cemented to the side opposite the sharp edge of each piece. The free end of the bristle was cemented to the tip of a wooden dissecting probe. As mentioned in Lowry & Passonneau (1972), the presence of this nylon bristle is important to dampen the motion of the hand while using the microscalpel. Microprobes were made by cementing a 1.5 cm long tungsten wire (0.0127 cm diam.) to a wooden dissecting probe. All instruments were alcohol-sterilized prior to use.

### Microsurgery

The following steps were conducted in a sterile transfer hood. Embryos (heart or torpedo stage) that were between 10 and 14 days old were selected for amputation. To accomplish this, 5 ml of MSE containing embryos were transferred to a sterile 100 mm diam. Petri dish containing 20 ml fresh MSE. While being viewed under a  $\times 10$  dissecting microscope, individual embryos were selected using a Pasteur pipette and were transferred to a second Petri dish containing 10–15 ml fresh MSE. This step was necessary to dilute the number of non-embryogenic cell clusters and abnormal embryos found in all embryogenic cultures, enabling further selection of a single embryo with no

**Table 1.** Onset of root hair formation for amputated and uncut embryos\*

Embryo number	Initial length ( $\mu\text{m}$ )	Time of root hair formation (days)	Final length ( $\mu\text{m}$ )†	
			Base	Total
<i>Cut and separated embryos</i>				
51C	800	7	1700	3500
52-7C	400	4	1000	2000
53-D6B	1200	2	3000	3800
53-CC	600	4	1500	2300
57-D5B	500	6	2800	3200
53-CB	600	4	1200	1900
<i>Uncut embryos</i>				
51-5	1000	7		4000
51-9	1000	7		3000
59-2	800	9		4000
59-4	900	9		7500
59-5	600	10		3500

\* The time of root hair formation is expressed as days post-surgery for amputated embryos. For uncut control embryos, it is expressed as the number of days between embryo selection and root hair formation.

† Final lengths refer to the lengths of the base, or base and apex (total length) at the time of root hair formation.

extraneous material. Using a Pasteur pipette, a single embryo was selected from this dish, along with 5 to 10  $\mu\text{l}$  of solution, and transferred to a sterile, dry, plastic Petri dish. Using the same pipette, the solution was withdrawn and deposited 1 or 2 cm away, within the field of vision, leaving the embryo moist and adhering tightly to the dish. A microscalpel was used to cut the embryo in half, by first ensuring that the blade was parallel to the dish bottom and then by positioning the blade over the embryo's midlength and normal to the longitudinal axis of growth. The blade was then brought downward and the embryo cut in one motion. Once complete, the 5–10  $\mu\text{l}$  of media were again drawn into a pipette and the embryo pieces picked up by using a pumping motion with the pipette bulb. The embryo pieces were then transferred to the experimental set-up containing either MSE solidified with 1.2% agar, or this mixture supplemented with 2,3,5-triiodobenzoic acid (TIBA; Sigma, St Louis, MO, USA). Final positioning of embryo pieces was accomplished with a microprobe.

#### Data collection

Data were gathered on either a 3- or 4-day schedule, depending upon the experiment. Generally, Petri dishes containing embryos were placed under a high-power dissecting microscope ( $\times 50$ ) and the lengths of the apical and basal pieces, and the longest cotyledon, as well as the width of the pieces at the cut surface, were determined. Fig. 1 is an explanation of the lines used for these measurements. Photomicrographs were taken with a Wild MES11/15 semiphotomat using ASA 400 film.

## Results

Somatic heart or torpedo embryos that are cut in half can easily be manipulated to recombine the cut pieces (Fig. 2), here referred to as grafted embryos. When performing grafts, it was not always possible to align perfectly the cut surfaces, and gaps of approx. 5–15  $\mu\text{m}$  sometimes remained after butting the sections. In these cases, the cells along the cut edges produced enough callus to fill such gaps and restored these embryos to their preoperation profiles. During the course of this healing response, callus was not observed to extend beyond the edge of the embryos. In cases where the two pieces were realigned such that there were no visible gaps, there was also no visible formation of a zone of callus.

It had been expected that cut embryos would show some sign of injury response, for example retardation or cessation of growth. To the contrary, the total length of these embryos increased at a rate which was nearly threefold greater than uncut controls (Fig. 3). While the rate of elongation was greater than that for the control embryos, both apical and basal sections of grafted embryos tended to elongate at the same rate (Fig. 4A). Here, the axial lengths of both apical and basal pieces were measured starting at the graft, which remained clearly visible throughout the duration of an experiment. When embryos were cut in half and the resulting sections kept separate (here termed cut and separated embryos), the apical ends elongated at rates which were similar to those for grafted apices, while the basal ends elongated at rates roughly twofold greater than their grafted counterparts (Fig. 4B). Using root hair and root cap formation as indices of root maturation, the separated basal sections also tended to mature 3–4 days earlier than uncut embryos. Table 1 lists the time of maturation for separated basal ends and uncut control embryos. In all treatments, the lengths of cotyledons in cut and separated or grafted embryos did not differ appreciably from those of uncut control embryos, so that parameter was not considered further.

An unexpected outcome of these experiments was the observation that a new basal end (ultimately becoming a new root) would occasionally form from the cut end of an apical piece. Basal ends arose either from a smooth-surfaced dome of tissue which formed across the cut surface or, more rarely, as a mature root which pushed its way through the cells of the cut surface, apparently having formed from within the apical piece. Consistent with work by Greenwood & Goldsmith (1970) where mature *Pinus* embryos were excised from seed and severed at the hypocotyl, TIBA-treated embryos did not display root regeneration. Thus, from the data that follow, TIBA-treated embryos are omitted. From a pool of nearly 100

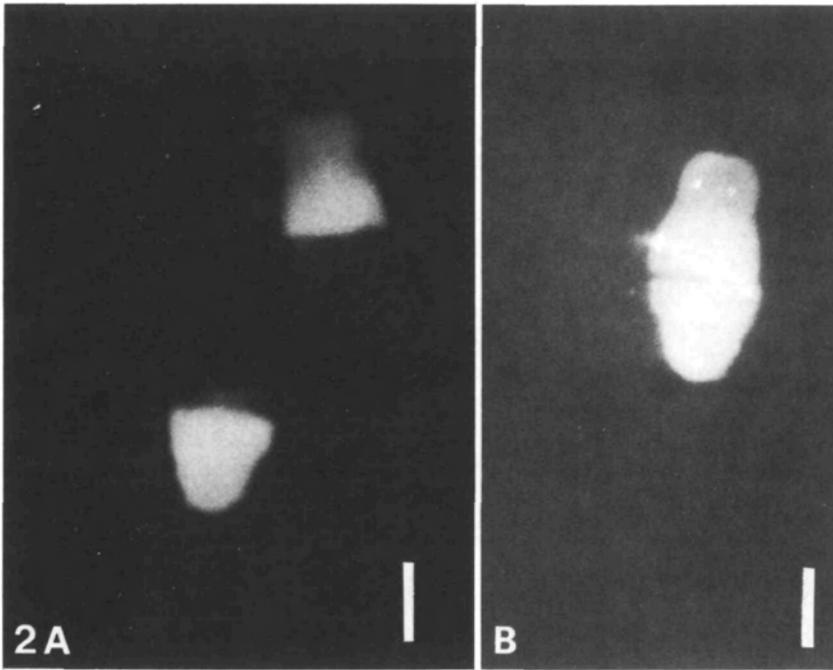


Fig. 2. Photomicrographs of a heart-stage embryo immediately after severing into two halves (A) and after positioning the two pieces in preparation for graft formation (B). Bars, 200  $\mu\text{m}$ .

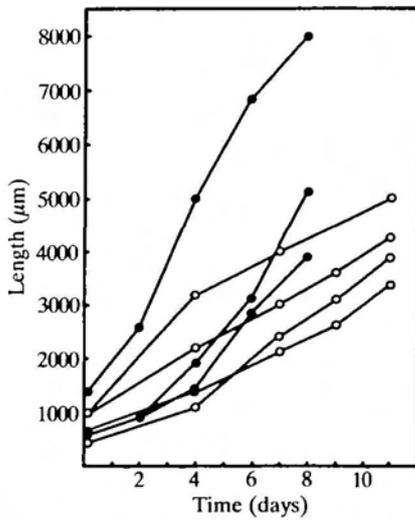


Fig. 3. Plot of the embryo length *versus* time for cut and grafted (●) and control (○) embryos. The length of a grafted embryo is the sum of the lengths of the apical and basal pieces, while the length of a control embryo is the line running between points A and B' in Fig. 1.

amputated embryos, 15–20% of the apical pieces regenerated a new basal half (determined by dividing the number of root-forming apices by the number of surviving same-age apices). Growth rate data from cut apices indicate that although all apices, including those that did not form roots (herein referred to as incompetent apices), had roughly the same axial length (approx. 300  $\mu\text{m}$ ), the growth rates of apices that formed roots were faster than those for apices incompetent to form roots. By 5 days post-surgery

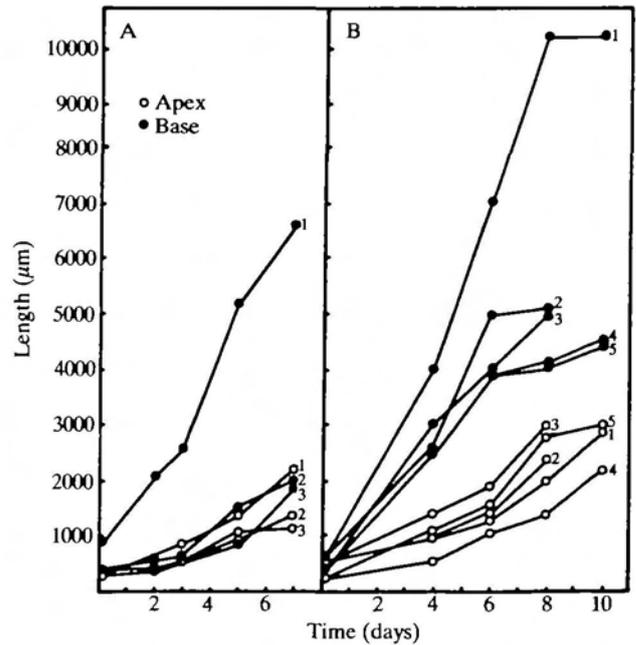
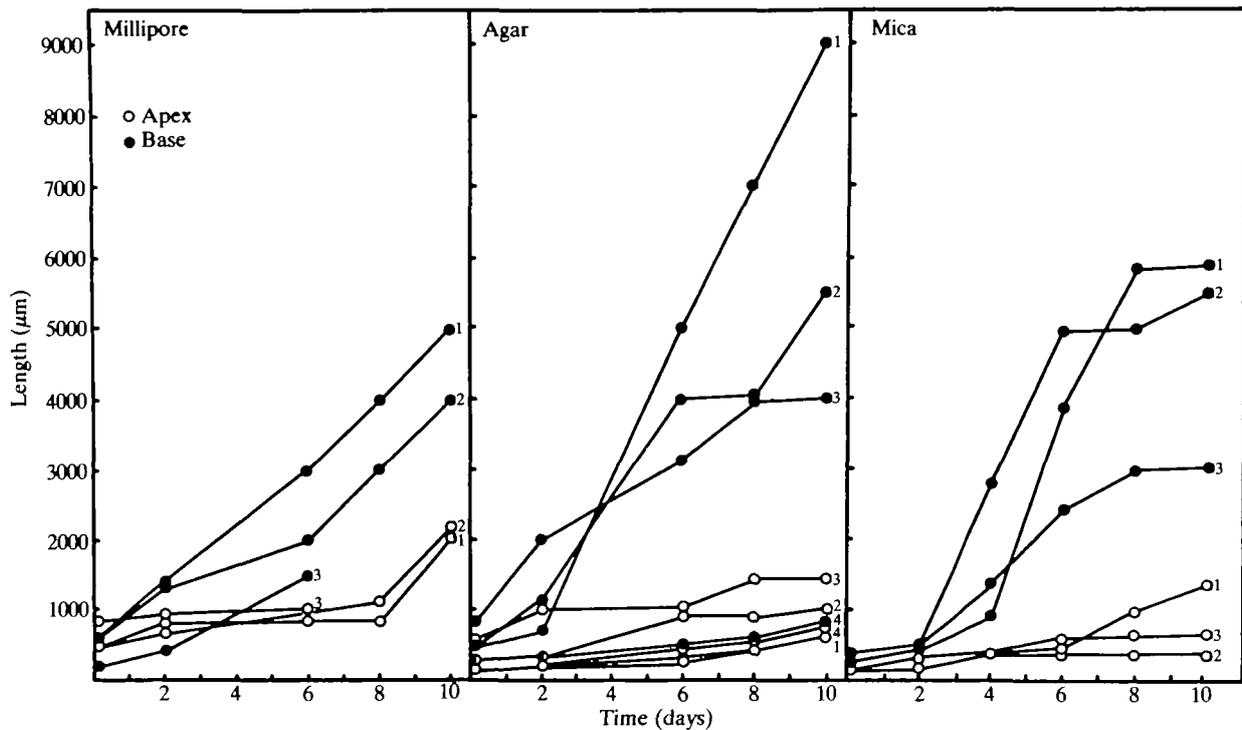


Fig. 4. Growth rates following grafting (A) or separating (B) the apical and basal pieces of an embryo. Notice that the apical sections increase in length at similar rates in both treatments, while basal sections elongate more rapidly if kept separate from their apices (B). The numbers in each panel refer to the embryo identification number for that experiment. Thus, base 1 and apex 1 were derived from embryo 1. The base of embryo no. 1 in panel A is elongating at a rate which suggest that a complete graft did not form.



**Fig. 5.** The effect of inserting a fragment of a millipore filter, block of MSE+agar, or a mica fragment between an apical and basal piece. Although the apical pieces tended to elongate at similar rates between treatments, basal sections elongated most slowly in the millipore treatment, suggesting that a diffusible substance from the apex was inhibiting their elongation. Agar blocks (which allow a substance to diffuse into the solid media) and mica fragments both produced basal sections which elongated as if the apex were absent. The pieces of embryo no. 4 were separated by an agar block that was approximately one quarter the volume of those used in other treatments; it, alone, behaved as if the two sections were grafted. Embryo pieces grafted without any intervening material (controls) grew in a manner similar to the grafted embryos presented in Fig. 3.

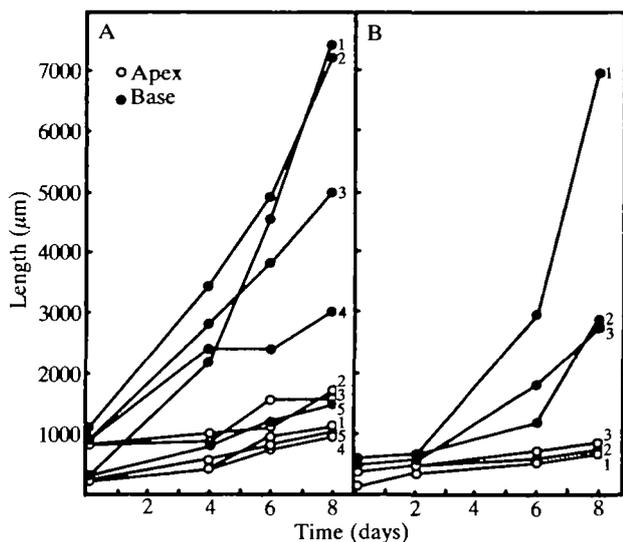
(the average time of the onset of root regeneration), the average competent apex was approx.  $650\ \mu\text{m}$  long, while it took the average incompetent apex 11 days to achieve an axial length of  $600\ \mu\text{m}$  (data not shown).

The time dependency of the ability to form successful grafts was examined by cutting embryos and attempting to reposition the pieces at 1-day intervals. It was determined that satisfactory grafts resulted only when the cut pieces were positioned together on the day of surgery. Attempts to graft pieces 1–4 days post-surgery failed to produce the callus necessary for a functional graft. Often, the two pieces would typically separate from one another (usually due to growth of the basal end). In these cases, as well as those where the pieces remained in physical contact, the basal ends grew in a manner similar to basal pieces deliberately kept separate from their apical counterparts (data not shown).

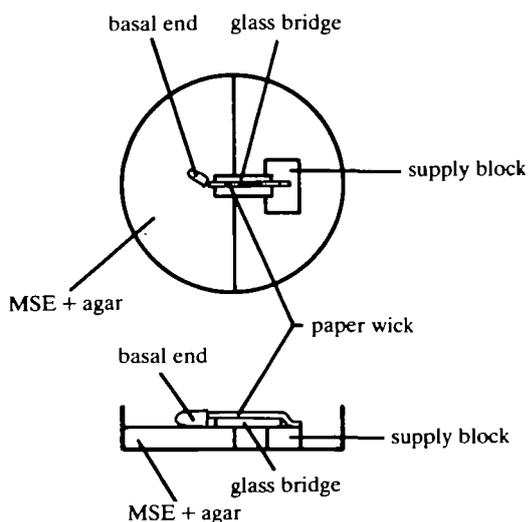
These experiments suggested that the basal end of an embryo is subject to an inhibitory control by the apical end. To provide insight into the nature of this control, severed embryos were positioned with either mica fragments, agar blocks, or sections of millipore

filters between apical and basal halves. The millipore filters ( $0.22\ \mu\text{m}$  pore size) allow for the diffusion of compounds between the cut halves, and when two pieces were placed on either side of a filter, they grew at rates somewhat similar to those for grafted embryos (Fig. 5). As might be predicted, the use of agar blocks (approximately  $400\ \mu\text{m}$  on each side) increased the length of the diffusive pathway and the rates of elongation resembled those for cut and separated pieces (Fig. 5). The single exception was an embryo which was separated by a much smaller block (approx.  $200\ \mu\text{m}$  on each side). In this case (embryo no. 4 in Fig. 5), the basal end elongated at a rate similar to that for the base of a grafted embryo. When the conductive path was blocked altogether using mica fragments (Fig. 5), the sections behaved like their cut and separated counterparts.

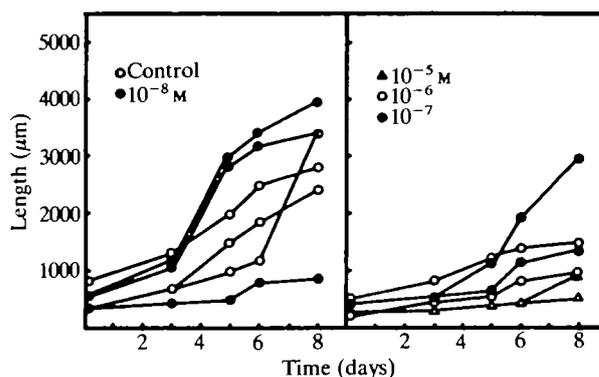
Since earlier work (Schivone & Cooke, 1987) suggested that the polar transport of auxin is vital for the successful development of an embryo, the polar auxin transport inhibitor TIBA was used to determine whether auxin plays a role in regulating embryo elongation. For these experiments, cut and separated, or grafted embryos were placed on solid MSE



**Fig. 6.** Plot of the length of apical and basal pieces versus time for embryos treated with 100 nM-TIBA. Panel A represents cut and separated embryos while those that were grafted appear in panel B. Although the grafted basal ends elongated at a rate slightly less than their separated counterparts, they show growth rates which are greater than that for untreated and grafted bases (see Fig. 4A).



**Fig. 7.** Schematic of the wick-bridge devised to resupply IAA to the cut surface of a basal piece. Briefly, one half of the MSE+agar was removed from a 55 mm diam. Petri dish. A block of agar (roughly 1 cm<sup>3</sup>) was positioned on the dry section of the Petri dish, within 1 cm of the edge of the MSE+agar. This dry space was bridged with a coverslip fragment and a strip of Whatman no. 1 filter paper (moistened in a solution identical to that which made up the supply block, but without the agar). Approximately 50–100 µm of the paper was allowed to overhand the glass bridge on the MSE+agar side. Against this edge, an embryo's basal piece was positioned such that its cut surface contacted the paper. All construction was conducted under sterile conditions.



**Fig. 8.** The effect of resupplying IAA (via a wick-bridge) to basal sections of embryos. IAA was resupplied at concentrations of 10<sup>-5</sup>, 10<sup>-6</sup>, 10<sup>-7</sup> and 10<sup>-8</sup> M. Concentrations higher than 10<sup>-7</sup> produced growth rates similar to those for grafted bases.

media containing 100 nM-TIBA. This treatment produced results suggesting that the polar transport of auxin (or its basal accumulation) may be responsible for the inhibition of rapid growth seen by grafted basal sections. Fig. 6A illustrates how cut and separated pieces responded to the presence of TIBA. Here, the sections grew at rates similar to untreated, separated sections (for comparison see Fig. 4A). When similar embryos were cut and grafted (Fig. 6B), the basal ends continued to elongate at rates similar to those of cut and separated pieces. Since the grafts appeared to form normally, this suggests that blocking polar transport of auxin causes basal sections to behave as if the apex was absent. As mentioned earlier, separated apical sections failed to regenerate new roots in the presence of TIBA.

Since the basal ends elongated rapidly following a cessation of the apically-derived supply of IAA, the reapplication of IAA to these pieces should elicit growth responses similar to those for grafted basal ends. Using the wick-bridge apparatus shown in Fig. 7, IAA at concentrations ranging from 10<sup>-8</sup> to 10<sup>-5</sup> M was resupplied to the cut surface of a number of basal ends. As shown in Fig. 8, the growth rates of these basal sections progressively increased for decreasing IAA concentrations, from a low rate of approximately 50 µm day<sup>-1</sup> at 10<sup>-5</sup> M-IAA to a rate of about 400 µm day<sup>-1</sup> at 10<sup>-8</sup> M-IAA. In addition, after 8 days post-treatment, only the control sections exhibited root hair formation. None of the IAA-treated basal sections showed any sign of precocious root maturation as was typical for the separated, fast-growing basal sections.

### Discussion

The importance of polar auxin transport to plant

development has been extensively reviewed (Goldsmith, 1977; Sachs, 1984). Almost all work on polar auxin transport has centred around mature plants, i.e. tissues where the polarity of individual cells has already been clearly established. In most plants investigated, auxin is produced at the apical end (usually in the leaves) and is actively transported basipetally, toward the root end, where it is presumably metabolized and/or conjugated into inactive forms. During this transport, auxin is able to control the differentiation of new vascular strands. For example, Sachs (1975a) applied IAA to bean hypocotyl and found that vascular tissue formed, connecting the site of application to existing vascular tissue. Additional experiments indicated that new vascular tissue in hypocotyl always formed in a basipetal manner and appears to be coupled to polar auxin transport (Sachs, 1975b; Gersani & Sachs, 1984). Thus auxin, in this utility, is considered to be profoundly important in maintaining the polarity of the vascular system, and hence of the polarity of the mature plant.

The influence of polar auxin transport on the development of an embryo is less well established. Greenwood & Goldsmith (1970) and Fry & Wangermann (1976) both removed mature embryos from seed and, using agar blocks containing  $^{14}\text{C}$ -IAA, determined that the embryo was capable of polar auxin transport. In the cases where mature embryos were severed at the hypocotyl, polar auxin transport continued unabated in the apical end (Greenwood & Goldsmith, 1970). By using the polar auxin transport inhibitors TIBA and *N*-(1-naphthyl)phthalamic acid (NPA), Schiavone & Cooke (1987) provided indirect evidence that this transport occurs as early as the globular and oblong stages. These embryos, when treated with either  $1\ \mu\text{M}$ -TIBA or NPA, failed to correctly continue development, remaining instead in their present stage and growing into giant globular or oblong embryos. The present paper provides indirect evidence that polar auxin transport occurs in heart- and torpedo-stage embryos, and that the mechanism of transport, or alternatively the accumulation of IAA in the embryo's basal end, controls the rate at which the future root elongates and matures.

It is unlikely that auxin is acting alone in regulating the rate of root development in these embryos; root maturation probably involves a complex series of cellular processes, ultimately producing such specialized structures as root hairs, a root cap and an apical meristem. These events probably require a series of closely timed physiological steps. Whether the cessation of auxin transport or of its depletion triggers a series of sequential developmental events, or acts *via* a cascade-type mechanism is presently unknown. Whatever the physiological complexities of the devel-

oping root, it was observed that a supply of auxin, delivered at the cut surface of the base, causes the basal sections to behave as if they had been successfully grafted to an apical piece. This observation implies that auxin plays a major role in the regulation of growth in these embryos and, thus, appears to act as a diffusible morphogen. Consistent with this view is the observation that apical sections, regions considered to be a source of auxin, which displayed root regeneration always displayed stable pattern regeneration by the production of a single root, which formed at the centre of the cut surface beneath the vascular cylinder. This is in contrast to findings by Greenwood & Goldsmith (1970), where the apical halves of excised, mature *Pinus* embryos often regenerated several roots at the cut surface. Thus, in the case of the younger carrot embryos, this phenomenon may be better viewed as pattern reestablishment rather than *de novo* proliferation of root structures.

If auxin can act as a diffusible morphogen in carrot embryos, the question arises as to whether it acts singularly or in concert with other morphogens in a manner similar to the double-gradient theory proposed by Sander (1960). Sachs (1986) has suggested that this process may occur *via* a bidirectional flow of compounds allowing the apex to control root formation and *vice versa*. In the case of the results reported here for carrot somatic embryos, it is unlikely that a second morphogen, emanating from cells in the basal region, acts in conjunction with IAA because apical portions of embryos appeared to grow at rates similar to grafted pieces, even in the absence of their basal halves. Thus, on the bases of this and earlier work (Schiavone & Cooke, 1987), it appears that the polar transport of auxin begins at least by the heart stage, and possibly is initiated as early as the globular stage in somatic carrot embryos. These findings also indicate that the apical end of a developing embryo exerts an inhibitory influence on the rate of growth of the basal end of the structure and that this restraint appears to be related to some aspect of the supply or distribution of auxin.

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