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Temporal and spatial regulation of symplastic trafficking during development in *Arabidopsis thaliana* apices

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SUMMARY

Plasmodesmata provide symplastic continuity linking individual plant cells. However, specialized cells may be isolated, either by the absence of plasmodesmata or by down regulation of the cytoplasmic flux through these channels, resulting in the formation of symplastic domains. Maintenance of these domains may be essential for the co-ordination of growth and development. While cells in the center of the meristem divide slowly and remain undifferentiated, cells on the meristem periphery divide more frequently and respond to signals determining organ fate. Such symplastic domains were visualized within shoot apices of *Arabidopsis*, by monitoring fluorescent symplastic tracers (HPTS: 8-hydroxypyrene 1,3,6 trisulfonic acid and CF: carboxy fluorescein). Tracers were loaded through cut leaves and distributed throughout the whole plant.

Confocal laser scanning microscopy on living *Arabidopsis* plants indicates that HPTS moves via the vascular tissue from leaves to the apex where the tracer exits the phloem and moves symplastically into surrounding cells. The distribution of HPTS was monitored in vegetative apices, and just prior to, during, and after the switch to production of flowers. The apices of vegetative plants loaded with HPTS had detectable amounts of tracer in the tunica layer of the meristem and in very young primordia, whereas the corpus of the meristem excluded tracer uptake. Fluorescence signal intensity decreased prior to the onset of flowering. Moreover, at approximately the time the plants were committed to flowering, HPTS was undetectable in the inflorescence meristem or young primordia. Later in development, after several secondary inflorescences and mature siliques appeared, inflorescence apices again showed tracer loading at levels comparable to that of vegetative apices. Thus, analysis of fluorescent tracer movement via plasmodesmata reveals there is distinct temporal and spatial regulation of symplastic domains at the apex, dependent on the developmental stage of the plant.

Key words: *Arabidopsis thaliana*, Tracer loading, Shoot apex, Plasmodesmata, Symplastic fields, Transition to flowering

INTRODUCTION

Multicellular organisms rely on cell-to-cell communication to coordinate their development and growth. Extensive studies of animal cells show that this communication occurs by secretion of chemical signals, by the interaction of ligands with membrane-anchored proteins, and through symplastic cell-to-cell connections, via gap-junctions. Plants use similar modes of communication; however, instead of gap-junctions, plasmodesmata provide symplastic intercellular connections that act as gates for molecular trafficking from cell to cell. Plasmodesmata are elongated channels that span the cell wall providing cytoplasmic continuity between adjacent cells. They consist of a collar, a central core of endoplasmic reticulum and a cytoplasmic sleeve (Overall and Blackman, 1996; McLean et al., 1997; Kragler et al., 1998). Historically, plasmodesmata were considered to be non-selective pores, passively allowing movement of water, ions and nutrients. Now it is clear that plasmodesmata are dynamic and selective structures with the capacity to regulate cell-to-cell trafficking of varied molecules (reviewed in Lucas et al., 1993 Ghoshroy et al., 1997; McLean et al., 1997).

By connecting the cytoplasm of cells with their neighbors, plasmodesmata integrate cells symplastically. Early embryos of higher plants represent a single symplastic domain (Schulz and Jensen, 1968; Mansfield and Briarty, 1991). Later in development cells differentiate, and individual cells or groups of cells become more or less isolated forming new symplastic domains or fields, so that mature plants appear as mosaics of symplastic domains and fields (Erwee and Goodwin, 1985). Symplastic domain refers to cells or tissues that are permanently (and completely) symplastically isolated from the cells around them, such as root hair cells (Duckett et al., 1994; Meharg et al., 1994) and stomatal guard cells (Palevitz and Hepler, 1985). However, when regions are not isolated permanently (or completely), the term “field” may be used to imply a region of relative continuity – without implying strict symplastic isolation (after Rinne and van der Schoot, 1998). This isolation process, possibly by down regulation or loss of functional plasmodesmata, facilitates the development of distinct functions

within cells and tissues (Palevitz and Hepler, 1985; Bergmans et al., 1993; Lucas et al., 1993; Duckett et al., 1994).

Membrane impermeable fluorescent tracers can be used to track intercellular pathways and to elucidate symplastic domains and fields (Oparka, 1991 and references therein; Wright and Oparka, 1996). In *Arabidopsis*, symplastic fields in root tips have been studied with different tracer loading methods, and reveal that undifferentiated cells such as meristem cells and cells in the elongation zone are symplastically connected (Duckett et al., 1994). Later in development, however, specific root cells such as hair cells and maturing phloem cells, differentiate and gradually become symplastically isolated from the cells around them and define symplastic domains (Duckett et al., 1994; Oparka et al., 1994).

The shoot apex of a typical flowering plant is responsible for producing the bulk of the above-ground portion of the plant, and consists of the apical meristem and young organ primordia. The shoot apical meristem can roughly be divided into two divergent classes of cells: central zone cells which are a small array of slowly dividing and indeterminate cells, and the peripheral zone cells that divide rapidly and differentiate into organ primordia (Lyndon 1973). Shoot architecture results from the differential spacing of independent growth centers such as apices and buds, and the regulation of growth center activity by environmental conditions or by innate processes. Photoperiodic treatments serve as environmental cues, inducing a variety of plants, including *Arabidopsis*, to change their morphology by producing flowers (Bernier, 1981; Corbesier et al., 1996; Hempel et al., 1997). Flower-promoting factors are provided by different parts of the plant and the fate of the apical meristem – vegetative or reproductive – may be controlled by signals transported over long distances (Bernier et al., 1993). The proper coordination of these signals and their correct delivery to sites in the apex implies that cells must be able to receive, relay, and restrict signals (van der Schoot, 1996). Plasmodesmata appear to function as dynamic gates between cells, likely playing a role in regulating signal fluxes, generating symplastic fields, and opening or isolating regions which need to respond to or be protected from signaling factors (Rinne and van der Schoot, 1998).

In an effort to characterize potentially important changes in plasmodesmatal permeability within *Arabidopsis* shoot apices, we monitored symplastic fields in apices by following low-molecular-weight fluorescent tracers and examined the influence of environmental and innate signals on the patterns of cell-to-cell communication. Our results indicate that *Arabidopsis* apices contain symplastic fields that are temporally and spatially regulated during development.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana is a facultative long-day plant. When grown under our continuous SD conditions, the Landsberg *erecta* ecotype (and Columbia, Noessen, data not shown) used remains vegetative, producing only leaves, for up to 40 days. Thereafter, there is a slow conversion to the reproductive phase. An inductive LD treatment, however, rapidly switches the apex to reproductive mode and flower primordia are recognizable after 2 days.

To precisely monitor symplastic domains in *Arabidopsis* shoot apices and relate symplastic patterning to developmental events such as flowering, we used defined growth conditions. In all experiments, seeds were vernalized for 5 days at 4°C and subsequently sown in 3-inch pots on the surface of a 1:1 mix of Sunshine-mix (Fisons Horticultural Inc., Bellevue, Washington, USA) and vermiculite. Plants were grown at 22°C in Conviron growth chambers (Controlled Environments Ltd., Winnipeg, Manitoba, Canada). As soon as the soil surface became dry, the soil was soaked again from below. Seeds were initially grown in short-days (SDs; 8 hours light/16 hours dark; Fig. 1, yellow bar), thinned to a density of about 5 plants per pot to prevent overlapping and provide enough space for subsequent tracer loading. Irradiance during SD treatment was 120-130 μmol/m^{2}second. After 27 days of vegetative growth, plants were either transferred to long-day (LD; 16 hours light/8 hours dark; Fig. 1A, red bar) photoperiods to induce flowering where early and late inflorescence apices could be monitored or further grown under non-inductive SD conditions (Fig. 1B, yellow bar) to follow vegetative growth and a slower transition to flowering.

Experimental design for developmental studies

In SD experiments, the age of the plants was assessed by the number of visible leaves in addition to days after vernalization. Plants of the same age in days had different sizes and numbers of leaves, whereas plants with the same number of visible leaves were similar in size and developmental stage. Thus, to increase the uniformity of any particular experiment, plants were chosen which had the same age in

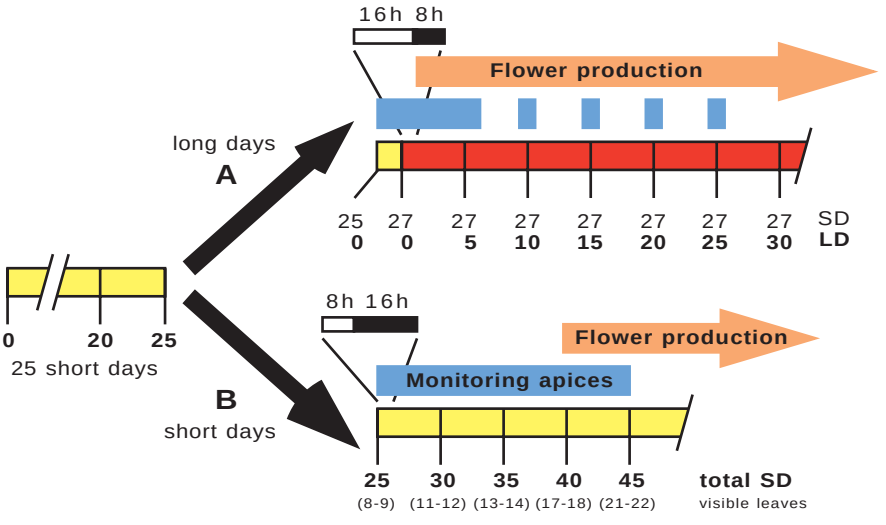

Fig. 1. Experimental design. To assay different developmental stages, subpopulations of 25-day-old SD-grown plants were exposed to two different light treatments. (A) After two additional SDs (yellow bar), the plants are transferred to LDs (red bar) to induce flowering. Flower primordia were visible after 2 LDs (orange arrow). Apices were monitored daily from time 25 SDs/0 LDs until 27/6 (blue bar) and again after 10, 15, 20, and 25 LDs. (B) Plants were kept under SD conditions (yellow bar) until flowering was initiated and flower primordia appeared (approximately 40 SDs, orange arrow). Apices were monitored daily between 25 and 45 SDs (blue bar).

days and the same number of visible leaves. To simplify the comparison of results we define plant age in number of SD and/or LD.

The smallest plants for which it was technically possible to routinely dissect and view apices, contain 9-10 visible leaves (24-25 SD). From this point on, meristem loading in plants induced for flowering was monitored daily for 6 LDs and then every 5 days for an additional 20 days (Fig. 1A). SD plants were monitored daily until the plants produced about 20-22 visible leaves (Fig. 1B) and first flower primordia were recognized around the inflorescence meristem (after 40-42 SD).

Scanning electron microscopy and morphological analysis

Methods for scanning electron microscopy were as described by Hempel and Feldman (1994, 1995). Flower and leaf primordia were distinguished as described by Hempel and Feldman (1994).

Tracers

Tracer-coupling in the meristem was monitored with HPTS (8-hydroxypyrene 1,3,6 trisulfonic acid; molecular mass, 520 Da), Lucifer yellow, Fluorescein diacetate, and FITC-dextran 3000 Da (Molecular Probes, Eugene OR). HPTS is water soluble, membrane impermeable, and a recommended symplastic tracer (Wright and Oparka, 1996). All tracers were dissolved in water and used at a concentration of 2.5 mg/ml.

Assay for monitoring symplastic continuity in *Arabidopsis* apices

Fluorescent tracers have been used to assess symplastic continuity in roots of *Arabidopsis* seedlings (Duckett et al., 1994; Oparka et al., 1994; Wright and Oparka, 1996) and unloading patterns in tobacco leaves (Roberts et al., 1997). In these studies, tracers were loaded into the cut edges of cotyledons or through abraded leaf surfaces, and their distribution was subsequently examined at the site of interest by confocal laser scanning microscopy (CLSM). The tracer is distributed symplastically through the mesophyll from the site of entry and ultimately reaches the vascular tissue. After entering the phloem, the tracer is transported long-distance throughout the plant and is unloaded into adjacent tissues (Duckett et al., 1994; Oparka et al., 1994, 1995; Roberts et al., 1997). For monitoring shoot apices, mature *Arabidopsis* plants are too large for loading through cotyledons, and too small and delicate for loading via abrading leaf surfaces. Additionally, we wished to view a region, the shoot apex, that is buried in a cluster of young rosette leaves. To accommodate these requirements, we developed a method to easily load and monitor *Arabidopsis* shoot apices.

A piece of silicon tubing approximately 1.5 cm long with an inner diameter of 1 mm was filled with 15 μl of a tracer. Three consecutive large leaves of the plant were cut at the base of their blades and free petioles were inserted into the tubing filled with tracer (Fig. 2A). The plants were returned to the growth chamber and incubated for 3 hours. As *Arabidopsis* leaves have a spiral phylotaxis and are clustered in a rosette, the 3 consecutive leaves are approximately equally spaced around the 360° circumference of the apex (Roebbelen, 1957). Tracer was evenly distributed over the plant and apex (data not shown). Loading was always performed at the beginning of the light period, although no clear differences were observed in plants loaded at different times during light or dark periods (data not shown). After incubation, loading tubes were removed and the plant cut just above the soil in the hypocotyl region. The whole plant was mounted on a stage designed to provide water for the plant during dissection and microscopy. Leaves covering the apical meristem were removed to allow viewing of the apex after which the meristem was immediately covered with water and a cover glass (Fig. 2C), and immediately moved to the CLSM for monitoring tracer distribution.

Microscopy

Apices were mounted and dissected on a stage consisting of a small box (15×30×15 mm, height:length:depth), which had silicon tubing inserted through a hole in the center of the lid. The cut plant was mounted with the stem in the tubing filled with water. The lowest leaves lay on the surface of the lid and together with the stem in the tubing provided stability for dissection (Fig. 2C).

Loaded and dissected apices were observed with a Nikon microscope (Nikon, Optiphot, Nikon, Tokyo, Japan) equipped for epifluorescence and a Molecular Dynamics (Sunnyvally, CA, USA) laser scanning device (confocal laser scanning microscope, CLSM). Silicon graphics (San Ramon, CA, USA) with SpaceLab software (Molecular Dynamics, Sunnyvally, CA, USA) was used to control the laser and to acquire and process images. Optical sections were made along the z-axis through the apex to facilitate determination of the 3-dimensional position of the tracer. The monitoring of loaded apices was performed with similar CLSM settings (all settings the same except detector gain 700-800) so that the results shown are comparable.

RESULTS

Developmental age dramatically affects symplastic movement into apices

Young *Arabidopsis* plants, 24-26 SD (9-11 visible small leaves), do not traffic fluorescent tracer to their vegetative apices after 3 hours incubation (Fig. 3A,B). This result was observed in 73% of apices in 9 independent experiments (Table 1). Further, both younger plants (4-8 visible leaves, albeit difficult to dissect), as well as shorter (2 hours) and longer (6 hours) incubation times gave similar results (data not shown). The lack of detection of tracer movement is significant since apices were monitored only when the remaining leaves on the dissected plant showed tracer in their veins (Fig. 2B); this requirement ensured that the tracer was distributed throughout the plant. Young plants containing tracer in their apices (27%, Table 1) showed only very weak fluorescence; such plants had 11 visible leaves and a 12th leaf primordium. The lack of HPTS

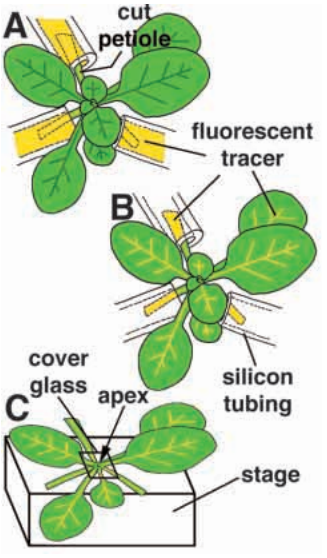

Fig. 2. Loading and mounting *Arabidopsis* plants for microscopy. (A) Three consecutive leaves of a soil grown plant were cut and inserted into tracer (yellow) filled tubing. (B) After 3 hours the tracer moved through the petioles and distributed over the whole plant and was visible in the vascular tissue. (C) The “loaded” plant was cut at the hypocotyl, mounted on a stage and the leaves covering the shoot apical meristem removed to expose the meristem for microscopy.

Table 1. Tracer loading into shoot apices of plants with different numbers of visible leaves

Number of visible leaves	*n**	% apices with no tracer loading	% apices with tracer loading
<12	11	73	27
12	14	21	79
12-17	67	18	82
>17	23	83	17

*Number of apices monitored in 9 independent experiments.

movement into very young apices was surprising since this area is developmentally active in the production of new organs, and is expected to be open for nutrient transport.

In contrast, 79% of older plants grown for another 2 SDs (total 27 SDs with about 12 visible leaves) showed significant HPTS import into the shoot apex (Table 1). Such apices reflect movement from vascular tissue below since fluorescent tracer was observed in leaf primordia radially around the meristem (Fig. 3C,D, arrowheads), and in the peripheral zone of the meristem (Fig. 3C, asterisk). The center of the vegetative meristem appeared to be tracer free, or loaded to a lesser degree, compared to the surrounding tissue (Fig. 3C,D, ap, and see also below). Subsequently, plants were induced for flowering under LD and further examined for trafficking into the apex.

Whereas the first LD did not affect the movement into apices (Table 2), after 2 LD, just before flower primordia were recognizable, transport between apices and vascular tissue was reduced to undetectable levels in 91 % of the tested plants (Fig. 4A; Table 2). Control experiments on plants of the same age but under continuous SD treatment continued to show tracer movement to the apex (Table 1; see also SD experiments 37 SD; Fig. 3E,F). This comparison between SD and LD plants indicates that the inductive LD treatment not only promotes flowering but also inhibits the movement of small molecules (520 Da) into the shoot apex. Plants kept further under LD conditions still restrict tracer movement (Fig. 4B; Table 2) but when producing secondary inflorescences and mature siliques (27 SD plus about 15 LD), tracer movement into the apex and small flower buds was again evident (Fig. 4C).

Note, confocal images of tracer loaded apices show HPTS in the cytoplasm and in the nuclei of all loaded cells exemplified by the leaf primordia and stipule cells shown in Fig. 5A,B. Since hydrophilic HPTS cannot readily pass through cell membranes and tracer could be detected in phloem cells in the stem (Fig. 5C) and veins of small leaves close to the apex (Fig. 5E), this cellular pattern suggests that HPTS moves symplastically into the cells of the apex, after symplastic unloading from the phloem (Wright and Oparka, 1996; Roberts et al., 1997). The observed changes in tracer loading within apices suggest symplastic transport is regulated at some step, or steps, between the phloem and the shoot apex.

In LD-induced plants, the rapid transition to flowering and restriction of tracer movement into the apex suggest these two events are related. To determine if this correlation also holds in a more gradual transition to flowering additional tracer

Table 2. Tracer loading into shoot apices after various numbers of LD

LD* after induction	*n*‡	% apices with no tracer loading	% apices with tracer loading
0	9	11	89
1	4	0	100
2	11	91	9
3	8	100	0
4	5	100	0
8	4	100	0

*Numbers of LD after growth for 27 days under SD conditions.
‡Numbers of apices monitored in 3 independent experiments.

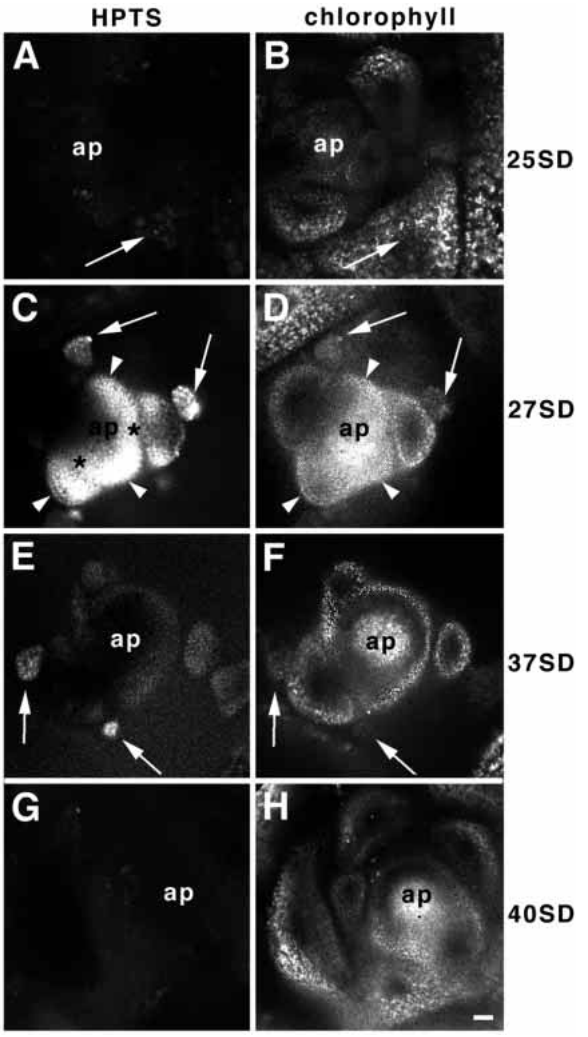

Fig. 3. Temporal tracer distribution in vegetative SD apices. Apices of tracer-loaded SD plants at different developmental stages were monitored by CLSM. (A,C,E,G) Fluorescent tracer distribution was monitored using a FITC-filter (green). (B,D,F,H) The chlorophyll autofluorescence, detected with a rhodamin-filter (red), outlines the morphological structure of the apex. (A,B) A 25 SD plant (10 visible leaves) which excludes the tracer from the apex (ap) although tracer is visible in the vascular tissue of the cut petiole of the 7th youngest leaf (arrow). (C,D) A 27 SD apex (12 visible leaves) brightly loaded with tracer. Tracer appears in meristem periphery (asterisks), primordia (arrowheads), and stipules (arrows). (E,F) A 37 SD apex (16 visible leaves) shows weaker fluorescence but in the same pattern as in C. Stipules (arrows) are more brightly loaded than the apex (ap) or primordia. (G,H) A 40 SD apex (18 visible leaves) where tracer movement is not detectable. Scale bar, 20 μm.

movement experiments were performed in older SD plants, which eventually commit to flowering without LD induction (see Fig. 6). Further loading experiments confirmed (82%; Table 1) the maintenance of trafficking between vascular tissue and apices after 27 SDs (see for example 37 SDs; Fig. 3E,F) and up to 39 SDs (17 visible leaves) (Fig. 7A,B). Note the amount of HPTS in plants older than 27 SDs is less than observed at 27 SDs, but sufficient to be readily detectable. However, 83% of plants older than 40 SDs (18 or more visible leaves) again restricted movement between the apex and vascular tissue (Figs 3G,H, 7D,E; Table 1). Flower production becomes obvious after 45 SDs (21 to 22 visible leaves); such plants do not have detectable levels of tracer in the apex (Fig. 7G,H), while tracer transport into leaves throughout the whole plant continues (data not shown).

The restriction of tracer movement into SD-grown apices just prior to flowering further supports the idea that the two events are related. Restricted movement continues until plants bolt at 55 SD (data not shown).That leaf number remains the same between 42 SDs and 55 SDs implies the plants were in the transition to flowering when they stopped trafficking to their apices. Further, scanning electron micrographs of these newly tracer-restricted apices confirm that these plants have altered their developmental program; at this time apices had initiated round flower type primordia low on the apical dome (Fig. 7F [42 SDs], I [45 SDs]) (Hempel and Feldman, 1994). These results together suggest that *Arabidopsis* maintains different symplastic fields in the apex before, during and after

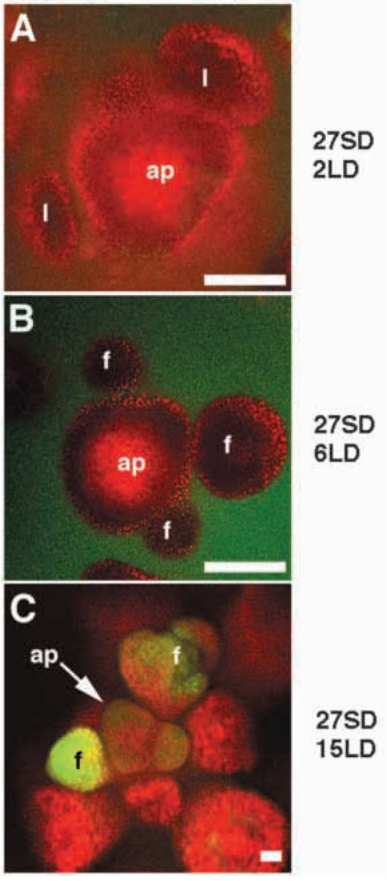

Fig. 4. Temporal tracer distribution in LD-induced inflorescence apices. CLSM images of apices of tracer-loaded plants induced under LD were monitored at different developmental times. Green represents the tracer distribution, the chlorophyll autofluorescence (red) outlines the morphological structure of the apices. (A) After 2 LDs tracer movement into the apex (ap) was restricted. Only leaf primordia (l) are morphologically recognizable. (B) After 6 LDs tracer movement into the apex is still restricted. Flowers (f) are macroscopically visible. (C) After 15-20 LDs, apices have produced secondary inflorescence and mature siliques, movement into the apex and surrounding flowers is again evident. Scale bars, 40 μm.

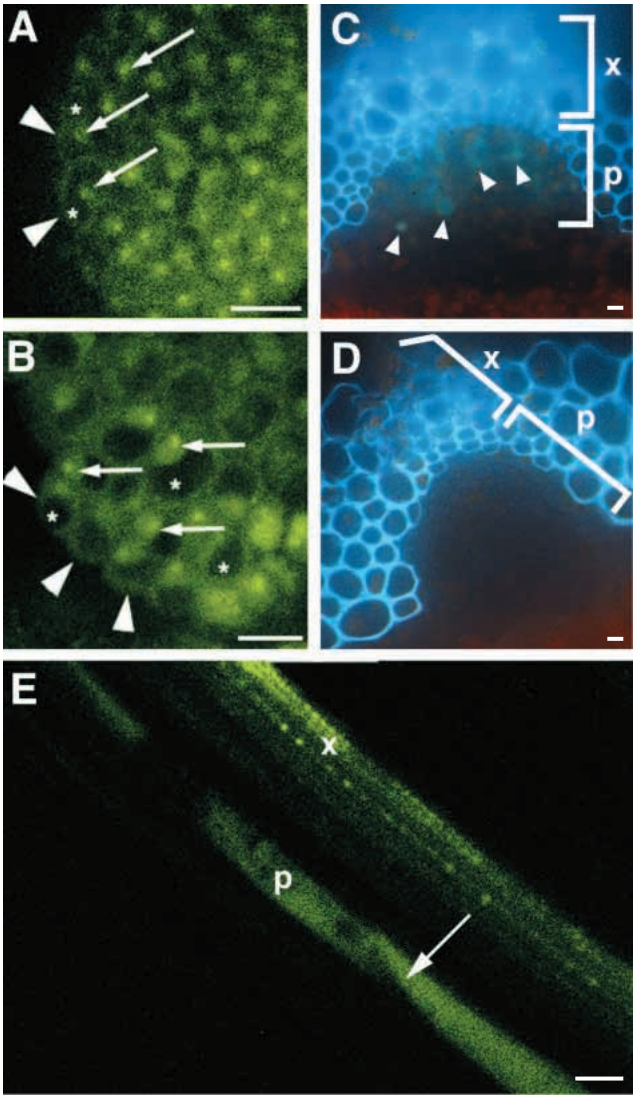

Fig. 5. Cellular localization of HPTS. (A) High magnification CLSM image from a leaf primordium shows tracer in the cytoplasm (arrowheads) and nuclei (arrows) but excluded from vacuoles (asterisks). (B) Detail from a stipule showing a similar HPTS distribution as in A. (C) Hand section of the stem below the apex. The section was illuminated with UV light, therefore the tissue such as the xylem (x) with lignified cell walls appear blue. Some single cells (arrowheads) in the phloem (p) contain greenish fluorescence due to the loaded tracer moving towards the apex. Note that the greenish fluorescence is clear upon direct viewing under the microscope, but is less clear upon photographic reproduction. (D) Hand section in a non-loaded control plant. Green fluorescence was never observed. (E) Small vascular bundle in a young leaf containing tracer in the xylem (x) and phloem (p). Scale bars, 10 μm.

the transition to flowering so that movement into shoot apices is altered.

Spatial regulation of symplastic movement in apices

Inflorescence apices of LD plants (27 SDs plus 15-20 LDs) were used to investigate the spatial distribution of symplastic domains in shoot apices. Longitudinal CLSM sections reveal an overall distribution of the tracer in cells of inflorescence apices. Only the three outermost cell layers of the shoot apical meristem contain tracer (Fig. 8). The central part of the corpus, hereafter referred to as the inner central zone (ICZ), did not contain tracer in detectable amounts, and appears to be symplastically isolated from the surrounding cells (Fig. 8, asterisk). This pattern is not due to lack of detection since bright red chlorophyll emission was detected in all areas of the meristem (Fig. 8).

Flower primordia and the meristem periphery, where new primordia are generated, contain cells with a higher tracer level than the central meristem (Fig. 8, p1, p2, and arrowheads). Incipient primordia (anlagen) as well as visible primordia appear to load both more uniformly and more deeply, i.e. at least 4 to 5 cell layers (Fig. 8, p1, p2, and arrowheads).

To determine whether tracer in the inner central zone of the meristem was not detected because it is the deepest area of the apex and the emitted light of the green fluorescent tracer was not detectable, we acquired confocal cross sections perpendicular to the plant axis (Fig. 9A). The first section is just above the epidermis of the meristem and faint tracer can be detected (Fig. 9B,C); on both sides of the meristem, flower primordia are sectioned deeper than the meristem itself and cells clearly are moving tracer symplastically (Fig. 9B, p1, p2). The next optical section (Fig. 9D,E) shows tracer within the cytoplasm of each cell in the first cell layer, and supports tracer moving symplastically in the epidermis of the apex. A deeper optical section (Fig. 9F,G) monitors tracer in the third cell layer of the meristem; since the optical plane cuts the dome-shaped meristem, outer layers are at the border of the apex (arrows). All three layers likely traffic tracer from the vascular tissue below (as in Fig. 8).

A fourth optical section cuts the fourth cell layer, the first cell layer of the ICZ (Fig. 9H,I). Here the three outermost cell layers contain tracer but tracer is excluded from the center of the apex (ICZ). Primordia surrounding the meristem, however, contain tracer through all cell layers (Fig. 9H, p1, p2). Since primordia protrude above the meristem (see Fig. 9A) tracer is detected in cells deeper than unloaded cells in the corpus of the meristem (Fig. 9H). Note again that very young, morphologically indistinct primordia (anlagen) reveal brighter tracer loading than outer cells in the meristem (Fig. 9F,H, arrowhead). The results in Fig. 9 were obtained after 3 hour incubation with fluorescent tracer; similar results were obtained following loading for 2 hours (data not shown) or 6 hours (Fig. 9J,K).

The distribution of HPTS suggests an inflorescence apex can be divided in two major symplastic fields: Field 1 is defined as ICZ and Field 2 as the three outermost cell layers plus the flanking primordia. Field 2 is symplastically linked with the vascular tissue and likely contains subfields recognizable by different staining intensities which may reflect developmental differences.

DISCUSSION

Co-ordination of development within plant apices requires intercellular signaling (van den Berg et al., 1997). The precise mechanisms by which signals pattern apices, however, are unknown. Although these signals are likely to move via either apoplastic or symplastic pathways, the degree to which each general pathway is used for developmental signaling is not known. Recent work, however, indicates that symplastic signaling pathways are altered during development (Bergmans et al., 1993; Duckett et al., 1994; Rinne and van der Schoot, 1998), and suggests that changes in symplastic signaling play a role in the differentiation of organs and tissues. The work described here indicates that symplastic signaling within the *Arabidopsis* shoot apex changes during development, and that the symplastic continuity between cells within the shoot apex varies according to location. The tracer movement patterns reported here also reveal a general direction for the flow of symplastic signals from the phloem to the shoot meristem within the *Arabidopsis* apex.

Using fluorescent tracers to identify symplastic fields within the shoot apex

Symplastic pathways are regulated within plant apices (Duckett et al., 1994; Rinne and van der Schoot, 1998), and plasmodesmata play a central role in the regulation of such cell-to-cell movement (Lucas et al., 1993; Oparka, 1993; McLean et al., 1997). Down regulation of plasmodesmata is assumed to be required for the establishment of symplastic fields, i.e., multicellular regions of symplastic continuity relatively distinct from the cells or tissues surrounding them (Erwee and Goodwin, 1985).

Symplastic fields have been visualized using two general techniques. In the first, fluorescent tracers are microinjected into single cells and subsequent movement is monitored

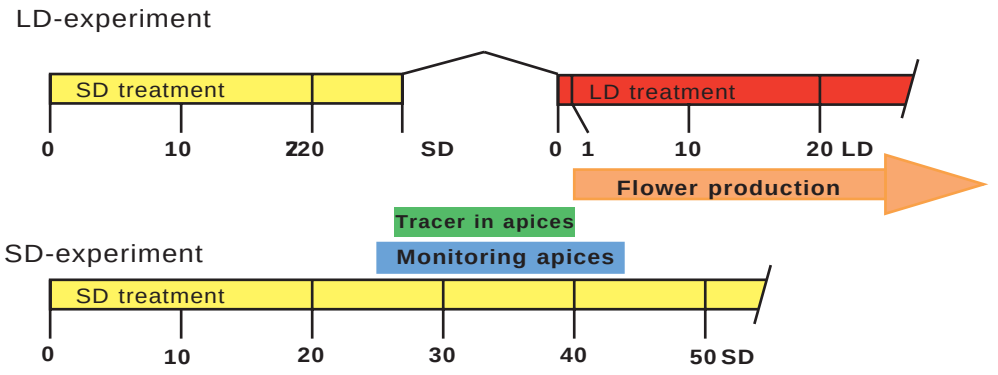

Fig. 6. Monitoring scheme. Overview of the temporal events described. The solid blue bar represents the times plants were examined for apex loading in SD- and LD-induction experiments. The solid green bar shows the time at which tracer was observed in shoot apices during the transition to flowering. Orange arrow marks when flowers are produced. SD conditions, yellow bar; LD conditions, red bar.

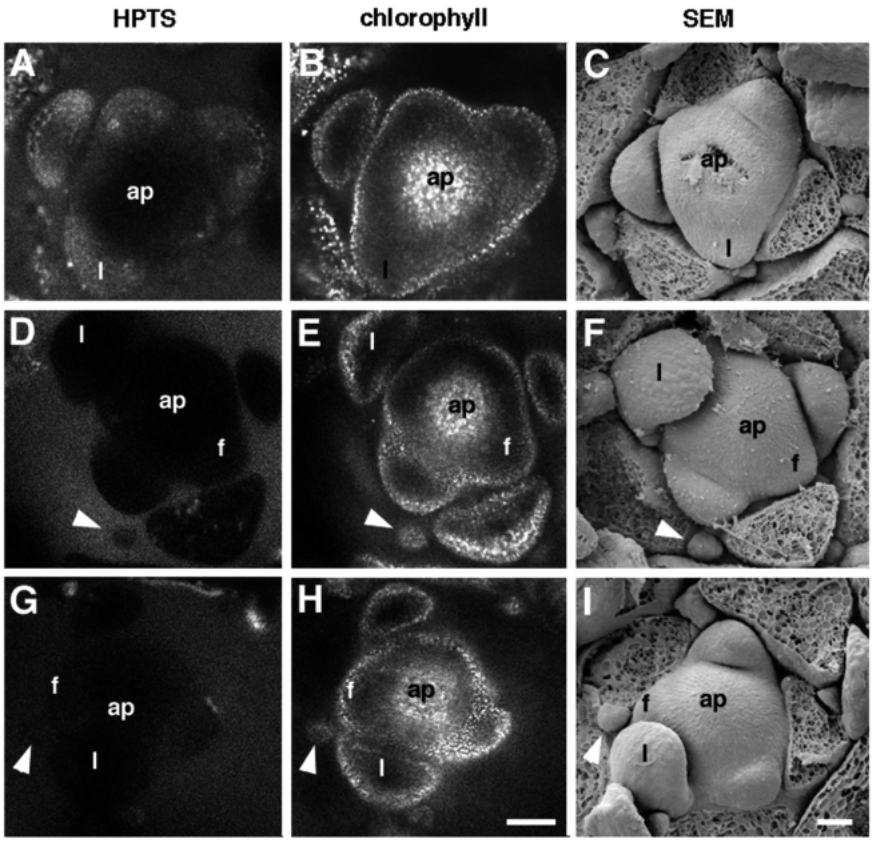

Fig. 7. CLSM images of apices of different ages and scanning electron micrographs (SEM) of the same apex to compare tracer movement and appearance of flower primordia. (A,D,G) Tracer distribution; (B,E,H) chlorophyll background; and (C,F,I) the corresponding SEM images. (A-C) A 40 SD apex (plant with 18 visible leaves); the plant allows modest movement of tracer into the apex (ap) and surrounding leaf primordia (l). (C) SEM image shows that the apex bears only leaf primordia (l). (D-F) A 42 SD apex (20 visible leaves); the plant restricts tracer movement into the apex (ap) and primordia (l and f). (F) SEM image reveals this apex is starting to initiate flowers (f). (G,H,I) A 45 SD apex (22 visible leaves); similar to that of the 42 SD apex. Scale bar, 40 μm in H (for A,B,D,G,E,H), in I (for C,F,I).

microscopically (Goodwin, 1983; Terry and Robards, 1987 van der Schoot and Lucas, 1995; Rinne and van der Schoot, 1998). The second technique monitors long-distance movement of fluorescent probes following the loading of wounded cells in one region of the plant, and then observation at distant sites. This technique has been utilized to monitor symplastic tracer movement following phloem unloading both in leaves and in root meristems (Oparka, 1991; Oparka et al., 1994; Roberts et al., 1997), and to identify symplastic fields within these regions. This method of probe loading has distinct advantages. Symplastic tracers can be loaded in amounts sufficient to label a whole plant (our results; Oparka et al., 1994; Roberts et al., 1997). Furthermore, probe movement is assessed in tissues that are undisturbed. One limitation of the phloem loading technique, however, is that only regions symplastically connected with the phloem are easily visualized.

We have used the second technique to monitor the movement of the symplastic tracer, HPTS, a cell-membrane-impermeant probe (Peterson et al., 1981; Wright and Oparka, 1996), within shoot apices. We found that the inner central zone (ICZ) of the *Arabidopsis* shoot apical meristem is symplastically isolated from the overlying three cell layers. We also found that the relative amount of symplastic coupling between the phloem and the shoot apex changes over time, correlating with the developmental stage of the plant. The localization of HPTS within the phloem in the stem below the apex and in the cytoplasm of apex cells and not their cell walls, indicates that tracer movement occurred symplastically, not apoplastically.

We have chosen to define regions within the shoot apex in which HPTS moves freely from cell-to-cell as symplastic fields (Rinne and van der Schoot, 1998), as opposed to symplastic domains, because the extent of these regions are dynamic (Fig. 10). The "symplastically isolated" cells or tissues that lie outside a field, empirically observed as cells or tissues that do not allow cell-to-cell movement of the symplastic tracer HPTS (520 Da), are not necessarily isolated to the movement of smaller molecules, as molecules below 500 Da may still move via plasmodesmata. Hence, the "fields" we observed may not be strictly isolated cytoplasmically. The term "domain" may better describe cells or tissues whose boundaries are delimited by plasmodesmata that are closed. For example, in the root epidermis of *Arabidopsis*, the cells in regions where tracer movement is restricted (Duckett et al., 1994) exhibit a high degree of electrical isolation (Meharg et al., 1994) suggesting that their plasmodesmata may be tightly closed.

The same general patterns of symplastic movement were observed within meristems when the similarly sized (approx. 460 Da) probes Lucifer yellow and carboxyfluorescein diacetate were used (data not shown). As expected from the size exclusion limits of plasmodesmata (Goodwin, 1983; Erwee and Goodwin, 1985; Terry and Robards, 1987), larger molecules, such as 3 kDa FITC-dextrans, do not move into shoot apices (data not shown). Thus, the restrictions in HPTS movement observed represent a general restriction of cell-to-cell continuity via the symplast, and differential regulation of plasmodesmatal size-exclusion limits.

Changes in symplastic tracer movement during the transition to flowering

Microscopic monitoring of HPTS movement in the shoot apex of Landsberg *erecta*, *Arabidopsis* plants (and Colombia, Noessen; data not shown) revealed that the symplastic

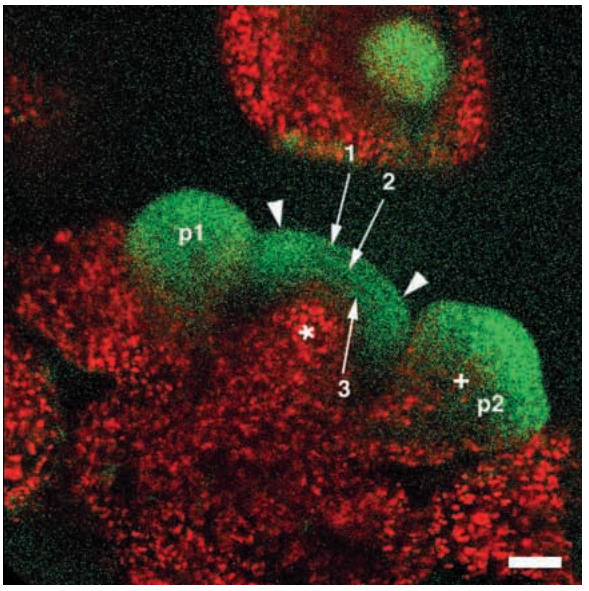

Fig. 8. Spatial tracer distribution in inflorescence meristem. Longitudinal CLSM section shows the spatial tracer distribution in the inflorescence meristem and surrounding flowers in different stages. Tracer appears in the three cell layers 1, 2 and 3 and slightly more brightly in flower anlagen (arrowheads). The center of the meristem (ICZ) excludes tracer (asterisk). Young flower primordia (p1) show a uniform distribution of tracer throughout, whereas older primordia (p2) start to exclude tracer from the inner central zone of the floral meristem (cross). Scale bar, 20 μm.

connections between the phloem and shoot apex change over developmental time. Young vegetative shoot apices (exposed to 25 SDs or containing less than 12 visible leaves) were symplastically isolated from the corresponding phloem. However, later in vegetative development, after approximately 12 visible leaves were formed (approximately 27 SDs), the shoot apex became symplastically connected to the phloem, as indicated by the movement of HPTS into the shoot apex. This symplastic coupling continued until the time when commitment to flowering occurred, but stopped before flower primordia were recognizable. Symplastic coupling between the phloem and the shoot apex, however, was restored later in inflorescence development after bolting (summarized in Fig. 6).

The significance of these changes in symplastic coupling between the phloem and the shoot apex observed during plant development is unknown. The restriction of dye movement into the shoot apex during the transition to flowering is surprising since it is commonly accepted that the transition is regulated by signals which are transported to the shoot apex during this period (Evans, 1969; Bernier, 1988). One possible explanation for this apparent contradiction is that induction signals may have reached the shoot apex prior to the visible formation of flower primordia. This hypothesis is consistent with recent work indicating that commitment to flowering in *Arabidopsis* can occur before visible changes are evident in the shoot apex (Hempel et al., 1997). The lack of tracer movement during the transition to flowering may also be an indication that the apex is restricting the symplastic movement of a floral repressor(s) (Martinez-Zapater et al., 1995). The time when the connection between phloem and apex is first evident (at 25 SDs) may then indicate a change in the relative competence for flowering of the shoot apical meristem (Shannon and Meeks-Wagner, 1991) during which a floral repressor(s) may be required to inhibit flowering until the appropriate time. The subsequent lack of tracer-movement during the transition to flowering may suggest a restriction of the movement of floral repressor(s) which would allow the transition to flowering.

The precise location at which the restriction of HPTS movement occurs is still unknown. Since the tracer moves symplastically, plasmodesmata are involved in this regulation. The phloem is likely flowing towards the apex when HPTS is excluded, because we observed HPTS in the phloem of the stem and in very small leaves close to the apex. In addition, a rapid increase in morphogenetic activity occurs during flowering (Hempel and Feldman, 1995; Corbesier et al., 1996), so that the shoot apex becomes an even stronger sink for nutrients and photoassimilates during this time. Increase in growth may be accompanied by an increase in apoplastic movement of photoassimilates, not monitored by tracer-coupling analyses. Or, there may also be increased symplastic movement of photoassimilates and other molecules smaller than the 500 Da probes used in this study. Since the apex is also a site of phloem differentiation and maturation, several different dynamic cell types (see Gilbertson and Lucas, 1996, Fig. 4) must be involved in unloading of the tracer from the mature sieve element. The restriction of tracer movement may be occurring in any or all or these cell types. A third site of restriction of symplastic movement could be between the protophloem and the apex, where a particular file of cells in the apex could regulate the size exclusion and direct movement of different compounds.

The restriction of symplastic movement of molecules into the shoot apex may be relevant to patterns of viral movement towards the shoot apex. It is known that some viruses are not able to infect apices (Matthews, 1991) and that bean dwarf mosaic gemini virus moves towards the shoot apex, but is restricted to the cells of the protophloem (Wang et al., 1996). This result highlights the potential importance of the protophloem and surrounding cells. The existence of an apical boundary for transport also is suggested by recent experiments on gene silencing in plants. Gene silencing occurs when DNA homologous to “endogenous” sequences is introduced ectopically, leading to the formation of a sequence-specific signal that spreads from initially transfected cells via a relay system employing plasmodesmata and phloem channels. It is especially relevant that this silencing is not observed in the apical meristem (Voinnet et al., 1998).

Further investigations obviously are needed to address the question of where symplastic movement of tracers and viruses are controlled at the shoot apex.

Variation in plasmodesmal permeability within the *Arabidopsis* shoot apex

To date there are few studies that asses symplastic tracer movement within apical meristems. Recently, Rinne and van der Schoot (1998) monitored such movement following microinjection of tracer into the corpus of birch seedling meristems; tracer was observed in the tunica and not the central or peripheral zones. Here, we observed a gradient in HPTS signal between the peripheral zone and the central zone of the

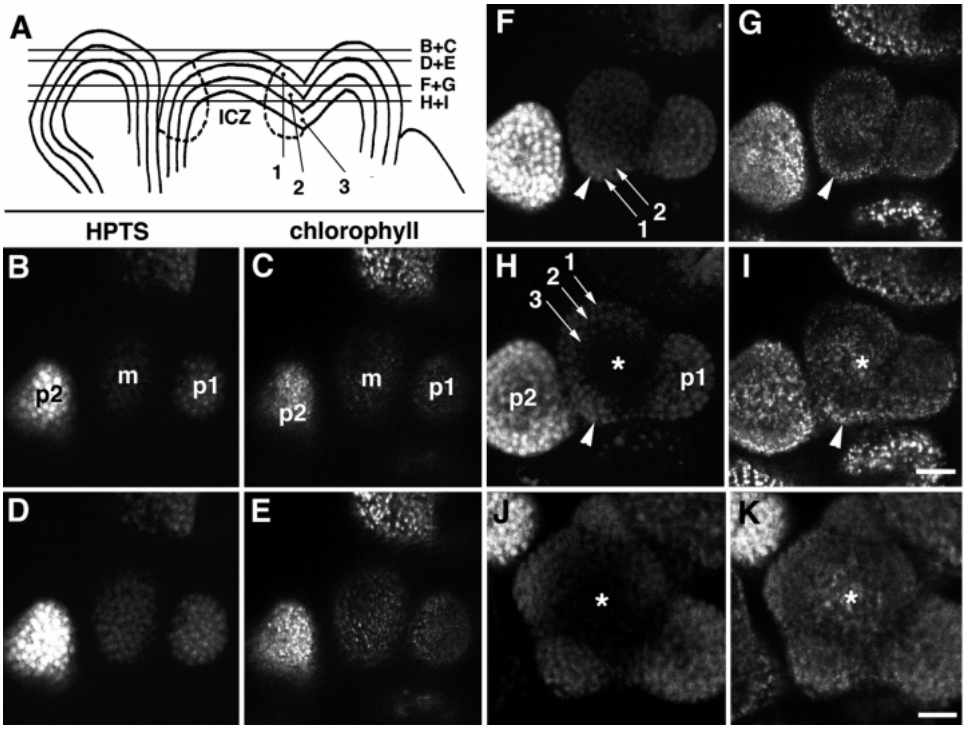

Fig. 9. Spatial tracer distribution in the inflorescence meristem. CLSM of cross sections at different depths in the same apex confirm the spatial distribution of the tracer in the longitudinal section in Fig. 8. (A) Drawing of an inflorescence meristem illustrates the location of four optical sections relating to the three cell layers 1, 2, and 3. B,D,F,H and J show the tracer distribution whereas C,E,G,I and K are the corresponding chlorophyll emission images. (B,C) Optical section just above the meristem shows faint tracer emission in the meristem (m) and clearly loaded cells in the primordia (p1 and p2) bulging from the meristem dome. (D,E) Section through the first cell layer shows tracer movement into all cells. (F,G) Section through the third cell layer shows tracer movement through layer 1, 2 and 3. The arrowhead indicates a brightly loaded flower anlage. (H,I) Section through the fourth cell layer shows tracer movement in cell layer 1, 2 and 3, whereas the ICZ (asterisk) is restricted for movement. Arrowhead indicates the same anlage as in F and G. Flower primordia (p1 and p2) still contain tracer in their centers as deep as 4-5 cell layers (see A). (J,K) Section through the fourth cell layer of another apex loaded for 6 hours confirming that the ICZ (asterisks) is restricting the flow of detectable amounts of the tracer. Scale bars, 20 μm.

apical meristem. The different connectivity between central and peripheral zone in birch and *Arabidopsis* apices may reflect a directional restriction; in birch, tracer moved outwards from the meristem, whereas in our experiments the tracer moves into the meristem from the periphery. In further contrast, Rinne and van der Schoot observed two cell layers in the tunica that trafficked tracer, while the present study shows tracer in three outer layers. The birch studies were performed on vegetative apices whereas inflorescence and vegetative (data not shown; A. Gisel, unpublished) *Arabidopsis* apices traffic tracer in the 3 outer layers of the meristem.

Classically the tunica is defined as the outer layers of the meristem that undergo anticlinal divisions resulting in expansion of the surface area (Schmidt, 1924); in contrast, the corpus undergoes growth in volume by division in all planes. While the *Arabidopsis* tunica is composed of only 2 cell layers when plants are grown in LD (Medford et al., 1992), plants that are grown in SD for extended periods (as in our experiments) seem to form an additional tunica cell-layer (present study, and see Fig. 4 in Hempel and Feldman, 1994); i.e., additional cell layers with exclusively anticlinal divisions. In fact, the tunica can consist of up to 5 cell layers, and in some species, the number of tunica layers increases during development (Chakravarti, 1953; Steeves and Sussex, 1989). As noted, birch and *Arabidopsis* apparently have different numbers of cell layers in their tunicas. What is notable in the present context, is that in both birch and *Arabidopsis* the layers composed of cells with anticlinal division patterns, i.e. the tunica, exhibit symplastic connectivity. However, it is not yet known how to relate these results to those on clonal analysis and cell fate determination. Classical clonal analyses suggest that dicotyledonous plants typically have a three layered meristem (Satina et al., 1940; reviewed by Tilney-Bassett, 1986); two outer cell layers, L1 and L2, that divide anticlinally, and an inner L3 comprising cells that divide both anticlinally and periclinally. Each of these 3 “layers” has been assigned to specify particular cell and tissue fates in the mature plant.

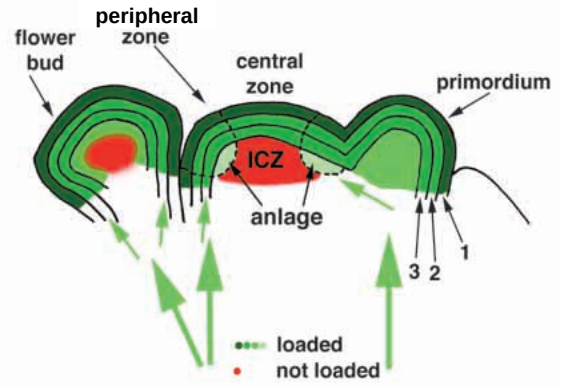

Fig. 10. Schematic of a shoot apical meristem summarizing the spatial tracer distribution. The zones defined in the text are depicted: tunica (cell layer 1, 2 and 3), central zone, inner central zone (ICZ), and peripheral zone. Tracer (green) moved into the tunica and into cells belonging to organ primordia or anlagen. The area which excludes tracer movement (red) is the ICZ below the three outer cell layers (tunica) and the central part of the flower bud. Large green arrows indicate the tracer movement in the phloem into the apex, the small green arrows the direction of cell-to-cell movement in the apex and flower bud.

However, clonal analyses has yet to be performed on plants that contain more than two cell layers in the tunica. Symplastic loading studies may serve as another parameter to assess the numbers of cell layers of the tunica in relation to parallel studies to assign cell fate by genetic approaches.

In young flower primordia, HPTS was distributed throughout all cell layers (Fig. 10, primordium, green). Then, shortly after the sepal primordia were visible, HPTS was excluded from the ICZ of the flower bud (Fig. 10, flower bud, green). The exclusion of HPTS from the ICZ of the shoot apical meristem and flower buds (Fig. 10, red) indicates that the tunica and ICZ become symplastically distinct, i.e., that they represent two developmental fields. If the center of the meristem does not traffic tracer, how does tracer move to the outer layers of the tunica? Tracer likely moves symplastically into the shoot apex following unloading from the phloem below (Fig. 10, large green arrows); and tracer must move through cells "around" the ICZ, through young anlagen before reaching the tunica in the central zone of the meristem (Fig. 10, small green arrows). Developmentally relevant signals that move symplastically may therefore act directly on developing primordia. This hypothesis is consistent with the observations of chimeric flower/paraclade shoots in *Arabidopsis* (Hempel, 1996) and recent analyses of the *Arabidopsis* root meristem. The specification of cell identity in the root meristem requires the direct action of signals from outside the apex on differentiating cell files (van den Berg et al., 1995). Additionally, very young primordia have a higher HPTS signal than the tunica of the meristem, suggesting that plasmodesmata may be important for the maintenance of a gradient of signal(s) between central and peripheral zone of the meristem (Wolpert 1996; Crick, 1970; Mueller, 1997). These gradient fields may provide positional information important in 'prepatterning' primordia.

These results show the potential of monitoring tracer movement in plants revealing symplastic compartmentalization and highlighting its importance in signaling during morphogenesis. Monitoring symplastic fields in developmental mutants will further define the role of symplastic fields in morphogenesis and provide a link between physiology and genetics.

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