Symplasmic fields in the tunica of the shoot apical meristem coordinate morphogenetic events

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SUMMARY

In plants, complex cellular interactions, which require the exchange of morphogenetic signals, underlie morphogenesis at the shoot apical meristem. Since all apical meristem cells are interconnected by plasmodesmata, we have investigated if symplasmic paths are available which may preferentially channel metabolites and potential morphogens in the apical meristem, and whether they could support both the formation of determinate appendages and the sustainment of an undifferentiated centre. Experiments in which the permeability of the symplasm was probed with fluorescent dye revealed that the tunica of the apical meristem of birch seedlings (Betula pubescence Ehrh.) is symplasmically compartmentalized into two concentric fields, which restrict the symplasmic diffusion of small potential morphogens to the cells inside their boundaries. A transient connection between the two fields was established early in a plastochron, potentiating the radial exchange of symplasmically diffusing signalling molecules. We suggest that the symplasmic subdivision of the tunica offers a means to unite cells into communication compartments, invoke boundary interactions between them, and shield the distal meristem cells from organogenesis. Electrophysiological measurements indicate that, in addition, the cells of these fields constitute metabolic working units. The relevance of these symplasmic fields for morphogenesis was established experimentally by treatment with short photoperiod, which induced breakdown of the fields into symplasmically isolated cells. Tannic acid staining and in situ immunolocalisation revealed that cell isolation was due to the activation of glucan synthase complexes intrinsic to sphincters. As a result callose plugs were formed on all plasmodesmata leading to morphogenetic deactivation.

Key words: Dormancy, Apical meristem, Vegetative meristem, Morphogenesis, Plasmodesmata, Plasmodesmal sphincter, Tunica

INTRODUCTION

In all higher plants the vegetative apical meristem (AM) gives rise to the shoot. The basic structure of the AM is formed as an integral part of the embryo (Jürgens, 1995), but the open growth habit of the shoot and its phyllotactic pattern emerge from the exclusive activity of the AM (Steeves and Sussex, 1989). The organizing principles are intrinsic to the AM proper since isolated AMs, with the primordia removed, sustain the ability for primary morphogenesis (e.g. Steeves and Sussex, 1989; Sachs, 1991). Even small groups of cells, surgically isolated at different AM loci, can rebuild an AM identical in size and function to the original (e.g. Sussex, 1989; Steeves and Sussex, 1989). How the AM is able to regenerate and sustain its dynamic organization is a key question in plant morphogenesis, but it has received surprisingly little attention.

In animal systems the ability of fragments to produce copies of the whole, demonstrated during early embryogenesis, is due to a morphogenetic field, i.e. ‘an area where the cells cooperatively bring about a specific set of structures’ (Müller, 1997). In the ‘positional information model’ (PI model) the patterning of morphogenetic fields, e.g. in limb formation, requires the graded presence of diffusing or non-diffusing morphogens (Wolpert, 1989, 1996). Such gradient fields (e.g. de Robertis et al., 1991) may provide positional information which is interpreted by the cells and recorded as positional values necessary for regeneration and development (Wolpert, 1989, 1996). In plants, morphogenesis at the AM has been explained in terms of the PI model by assuming the presence of morphogen gradients in the extracellular space (for review see Holder, 1979). In the AM two deviations from this situation may occur, however. Gradients may also be set up in the symplasmic space, and cells may be engaged in active forms of conversation. The morphogenetic field hosting such conversation networks must sustain infinite and repetitive development of the shoot. Regeneration as well as development at the AM would then involve self-organizing and self-maintaining signal networks in addition to gradients, a
situation which seems to go beyond the PI model. It is not clear how these gradients and hypothetical signal networks are positioned in the cellular matrix of the AM.

Importantly, the proliferating AM restricts pattern formation to its periphery and maintains an undifferentiated centre (e.g. Sawhney et al., 1981; Steeves and Sussex, 1989) which suggests the presence of physiological barriers (Sawhney et al., 1981), the position of which must be independent of cell proliferation. The mechanisms by which these barriers in the morphogenetic field are maintained and by which cell cycling is coordinated in space and time (Ferreira et al., 1994) operate at a level above that of the individual cell (Sachs, 1991; van der Schoot, 1996). The coordination of cellular events may involve biophysical processes (Green, 1985; Selker et al., 1992; Hernández and Green, 1993; Green et al., 1996; Fleming et al., 1997), morphogen diffusion and protein trafficking (Jackson et al., 1994; Hantke et al., 1995; Furner et al., 1996; Perbal et al., 1996; Szymkowiak and Sussex, 1996; van der Schoot, 1996).

The possibility that the organization of the symplasm underlies the functional subdivision of the AM into zones has not been previously considered as important in morphogenesis. Since an elaborate symplasmic network is present within the AM in the form of numerous cytoplasmic strands, plasmodesmata, this may provide a channel for distribution of signals. The way the symplasmic organization of the AM is built-up depends on the patterns of cell division. Cytoplasmic unity in cell lineages is created by incomplete cell divisions or ‘chambering’ (Kaplan and Hagemann, 1991) yielding cells which are exclusively connected by cyto-kinetic or primary plasmodesmata (e.g. Gunning, 1978). The multiple branched lineages in the AM are postcytokerinetically connected through existing walls by secondary plasmodesmata (van der Schoot, 1996; Bergmans et al., 1997) indicating that a three-dimensional symplasm is required in AM functioning (van der Schoot and Rinne, 1996). Primary and secondary plasmodesmata have been suggested to play partially different roles in the spatiotemporal control of signal flow (Lucas et al., 1993), but the specific distribution of plasmodesmata types within the AM (van der Schoot, 1996; Bergmans et al., 1997) is not necessarily sufficient to achieve adequate control over signal flow. In the floral meristem (FM) of Antirrhinum the difference in the transport of the transcription factor, GLOBOSA, between and within layers was suggested to be due to differential regulation of secondary and primary plasmodesmata, respectively (Perbal et al., 1996). Since secondary plasmodesmata are also formed within a layer, between and within branched clonal cell groups (van der Schoot, 1996; van der Schoot and Rinne, 1996, Bergmans et al., 1997), GLO would be able to reach all FM cells without restrictions. Additional mechanisms, supracellular in nature, must therefore be operational in the spatial control over morphogen distribution.

The existence of two separate growth zones, tunica and corpus, is also considered of importance in morphogenesis (Steeves and Sussex, 1989), but their individual roles and the way they integrate their activities are unclear. Bidirectional interactions between tunica and corpus occur at the AM periphery during the development of leaf primordia, but spatiotemporal positioning of a primordium and the development of dorsiventrality rely on mechanisms that act in a radial fashion (Steeves and Sussex, 1989). Mechanisms that shield a central population of tunica cells from organogenesis must also act radially (van der Schoot and Rinne, 1996). When the FM is formed, the entire tunica, not only its peripheral part, responds to signals sent out from the corpus resulting in the sequential production of floral organs (Szymkowiak and Sussex, 1992, 1996). Biophysical models depict primordium formation as an event initiated in the tunica (e.g. Green, 1985; Hernández and Green, 1993; Green et al., 1996), but input from morphogen sources (e.g. Fleming et al., 1997) may be necessary to sustain the local growth alterations at the future site of primordia.

Therefore, the organization of the symplasm may underlie not only the division of the AM into a central and peripheral area, but also the division into a tunica and corpus. Diffusion may thus be limited to compartments in the AM that correspond to this pattern of subdivision. Diffusion of morphogens in communication compartments is important in developing animal systems where gap junctions, the ‘equivalents of plasmodesmata’ (Pitts, 1990; Lucas et al., 1993), are involved in cell determination (de Laat et al., 1980; Canveney, 1990; Serras, 1989), but direct experimental investigations of diffusion paths in the AM are lacking. We here report on the presence of symplasmic fields in the tunica of proliferating AMs of birch seedlings, which potentially harbour gradient fields of diffusing morphogens. These symplasmic fields subdivide the tunica into two radially organized diffusion compartments, which keep their position in the proliferating cellular context and may support the peripheral formation of leaf primordia and the maintenance of an undifferentiated centre. AMs often switch to an alternative behaviour in response to environmental changes. Photoperiod length, for example, influences flower evocation, tuberization and dormancy (Bernier, 1988; Vince-Prue, 1994). In general, environmental stimuli may affect symplasmic communication (e.g. McLean et al., 1997), and in the case of photoperiodically induced switches this may lead to altered symplasmic patterns at the AM, serving its new state or function. We have demonstrated here for birch that exposure to short photoperiod induced breakdown of the symplasmic fields, stopped primary morphogenesis, and eventually resulted in dormancy. We therefore propose that the symplasmic network in the tunica is important in the coordination of morphogenetic events at the AM.

MATERIALS AND METHODS

Plant material

Field-grown Northern Scandinavian seedlings of birch (Betula pubescens Ehrh.), approximately 40 cm in height, were transplanted in pots at the start of the second growing season, and grown further in a growth chamber at 20°C and 16 hours photosynthetically active radiation (PAR) with photon flux density of 100 μmol·m⁻²·s⁻¹. After one month, about 10 plastochrons, half of the plants was subjected to a short photoperiod, 8 hours of PAR. Growth activity, elongation and production of new leaves were monitored throughout.

Iontophoretic microinjection and electrophysiology

Iontophoresis and electrophysiological measurements were performed as described previously (van der Schoot and Lucas, 1995). Apical shoot parts (with 3 mm of stem), with the AM exposed, were submerged and fixed upright in a bathing chamber. The AM surface
was inspected under low-irradiance white light. The extent of green autofluorescence of cut leaf tissues was assessed by short epillumination with blue-violet (BV) and blue light (B) in a fluorescence microscope (Nikon Optiphot II, Nikon, Tokyo, Japan). The (untouched) AM was free of green autofluorescence but could show red fluorescence. Excitation and barrier filters were standard BV and B filter-sets (BV: excitation 400-440/barrier 470/dichroic mirror 455; B: excitation 470-490/barrier 515/dichroic mirror 510). Entry of the microelectrode into a tunica cell was signalled by a sharp voltage drop. After the recording a stable membrane potential (Em) (a criterion for the ability of the impaled cell to make an adequate membrane seal around the microelectrode tip) Lucifer Yellow CH (LYCH; 457 Da) was iontophoresed (I = 1 to 5 nA) into the cell. In most experiments, however, the small tunica cells (diameter approx. 10 μm) did not survive the impalement with the microelectrode (diameter approx. 1 μm), or they had difficulties in sealing the membrane around the tip. In the latter case LYCH-input resulted in leakage through membranes, and in restricted dye-transfer to adjacent cells due to early collapse of the impaled cell. These experiments were discarded altogether. Vigorously growing plants all cells of the AM. Diffusing morphogens could be of significance in the AM, particularly if there were preferential symplasmic pathways to distribute them.

In search of such pathways we have microinjected the non-toxic and membrane-impermeable fluorescent probe LYCH, which visualizes diffusion pathways in the symplasm (Stewart, 1981). By direct observation we established that injection of LYCH, into cells at the peripheral zone of the AM (Fig. 2A), resulted in consistent dye-coupling patterns. In some cases (Table 1) LYCH injected into a single L1 cell, moved full circle in both directions along the base of the AM dome. In approximately 20 seconds LYCH occupied the entire peripheral zone of the tunica layer, excluding the leaf primordia, rising up along the outer edge of the peripheral zone (Fig. 2B). A single radial diffusion boundary inside the peripheral zone was typically present, where plasmodesmata were not able to pass on LYCH to neighbouring cells (Fig. 2C).

In agreement with the literature this is interpreted as being due to a decrease in the size exclusion limit of the plasmodesmata (references in Lucas et al., 1993), and which may even lead to complete plasmodesmata closure (Duckett et al., 1994). Hereafter we will refer to these permeability changes as ‘plasmodesmata narrowing or closure’, which probably occurs at the plasmodesmata neck region and/or at the level of subchannels (Lucas et al., 1993). In some cases the injection into the AM periphery resulted only in the formation of segments of the symplasmic ring (Table 1). In all cases, however, LYCH never entered subjacent tissue, nor the central AM area, which remained sharply delineated from the peripheral zone of the AM.

Microinjection of LYCH into a single cell of the central zone of AMs revealed the presence of a central zone symplasmic field in L1, complementary in shape to that at the peripheral zone (Fig. 3). The boundaries became visible within seconds and were usually irregular due to the protrusion of small rows of cells, probably part of a lineage, which still retained symplasmic continuity with the central zone field (Fig. 3).

When the AM was relatively small, i.e. at the beginning of a plastochron, microinjection of LYCH into a cell of the central zone revealed the presence of a central zone field which connected to a minor segment of the peripheral zone field (Fig. 4A-C). This fusion event was encountered a few times (Table 1) and probably existed only briefly.

Distinct Em-values in cells of the central zone and peripheral zone symplasmic fields

The symplasmic fields at the periphery and the centre of the AM (Figs 2, 3) matched the central and peripheral zones of the

<p>| Table 1. Observations on symplasmic permeability in actively proliferating (under long day) and deactivated (under short day) AMs |
|---------------------------------|------------------|------------------|</p>
<table>
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<tr>
<th>Plant type</th>
<th>Type of symplasmic field</th>
<th>Number of AM</th>
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<tbody>
<tr>
<td>LD</td>
<td>czf, distinct border</td>
<td>23</td>
</tr>
<tr>
<td>LD</td>
<td>pzf, distinct border to czf</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>closed ring</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>closed ring, 2-3 segments</td>
<td>7</td>
</tr>
<tr>
<td>LD</td>
<td>czf fused with pzf segment</td>
<td>3</td>
</tr>
<tr>
<td>SD</td>
<td>cell isolation, in pzf and czf</td>
<td>12</td>
</tr>
</tbody>
</table>

LD, long day grown plants; SD, short day grown plants; czf, central zone field; pzf, peripheral zone field.
zonation model (Fig. 1A; Steeves and Sussex, 1989; Sachs, 1991) both in position and activity level. Electrophysiological recording of the cell Em in the symplasmic field at the AM periphery showed strongly negative values (Table 2) due to the presence of an electrogenic component ($\Delta V$), which may locally drive active protonated uptake of substrates (van der Schoot and van Bel, 1990, and references therein).

In contrast, the Em values of tunica cells in the central zone fields were considerably smaller than those in peripheral zone fields of the AM (Table 1), indicating a low proton pumping activity and carrier-mediated uptake of substrates. The low variation of Em values between cells of the same field showed that the cells making up those fields may have a strong electric coupling and may also represent metabolic coupling groups.

### Short day induced breakdown of tunica fields

An important effect of short photoperiod, exploited here, is the deactivation of the AMs of birch seedlings (Rinne et al., 1994). To test the significance of the observed symplasmic fields for primary morphogenesis, we deactivated birch seedlings by treatment with short photoperiod. A 2-week exposure to short photoperiod induced growth cessation and disappearance of histological zonation (Fig. 1). In plants exposed to 4 subsequent short days, cells in the peripheral zone and central zone of the AM tunica, microinjected with LYCH, did not pass on the dye to other cells anymore (Fig. 4D) indicating a breakdown of both types of symplasmic field. Cell isolation was accompanied by a decrease of the Em values

<table>
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<th>Table 2. Membrane potentials of cells in symplasmic fields at the peripheral zone and central zone loci of actively proliferating and deactivated AMs</th>
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<tr>
<td>AM status</td>
</tr>
<tr>
<td>Active</td>
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<td></td>
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<tr>
<td>Deactivated</td>
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*The mean Em values of the cells at various loci were statistically different from each other ($P<0.001$, Student’s $t$-test, paired comparisons). cz, central zone; pz, peripheral zone; Em, membrane potential.
in both fields (Table 2).Apparently, some days after short photoperiod exposure the symplasmically isolated cells retained only the basic activity level characteristic for cells in a resting state.

Reversibility, up to a point, is typical for photoperiodic responses in general (e.g. Bernier, 1986). In birch, growth reversibility is already severely reduced after 10 days of exposure to short photoperiod (Welling et al., 1997). We have used this property to test in a separate experiment whether this was reflected in the symplasmic structure of the AM. The described breakdown of symplasmic fields by short photoperiod appeared to be reversible after 1 but not 3 weeks under short photoperiodic conditions. After 3 weeks, impalement of a cell with a microelectrode resulted in a strong osmotic withdrawal of LYCH solution from the electrode tip (Fig. 4E). The cells retained the LYCH and their swollen state at least until the next day, indicating firm plasmodesmata closure and water-proofing of cell walls.

Since all main temperate tree species have photoperiodic ecotypes differing in their response to day length (references in Olsen et al., 1997), we have confirmed in a separate experiment that a more southern ecotype (from Southern Scandinavia) showed the same response although the dissipation of the symplasmic fields was delayed in correspondence with their morphogenetic activity by approximately 1 week.

Different plasmodesmata mechanisms are involved in the formation and dissipation of fields

In active AMs versatile mechanisms must be operational for the

**Fig. 3.** Dye-coupling pattern emerging from a single injected L₁ cell at the AM centre. Diagrams indicate position of dye-coupled cells in surface views and longitudinal section; curved arrows show direction of spread. (A) LYCH moved symplasmically in approx. 5 seconds through the AM centre. Irregularity of the boundary is due to cell proliferation. (B) Final dye-coupling pattern reached after approx. 15 seconds. Photograph was taken after 30 minutes. Scale bar, 50 μm. AM, apical meristem; cz, central zone; L, leaf; me, microelectrode; pz, peripheral zone; s, section plane for cross section diagram.

**Fig. 4.** Dye-coupling pattern representing a ‘fusion field’ (A-C) and lack of dye-coupling under short photoperiodic conditions (D,E). Diagrams indicate the positions of dye-coupled L₁ cells and symplasmically isolated cells in surface views and longitudinal sections. The top four diagrams refer to A-C and the bottom two to E. (A) LYCH moves symplasmically in approx. 5 seconds from the injected L₁ cell at the AM centre towards the AM periphery (curved arrow in diagram). (B) After 15 seconds symplasmic boundaries are distinct. (C) Dye-coupling pattern in the L₁ after 30 minutes. (D) AM L₁ cell injected after 4 days exposure to short photoperiod shows absence of dye-coupling. (E) AM L₁ cells in former peripheral zone and central zone, injected after 4 weeks of short photoperiod. Absence of dye-coupling and swelling of dehydrated cells due to water absorption from the impaling microelectrode. Scale bar, 50 μm. AM, apical meristem; cz, central zone; L, leaf; me, microelectrode; pz, peripheral zone; s₁-3, section planes 1-3 for cross section diagrams.
transient narrowing or closure of the plasmodesmata at the boundaries of a symplasmic field. Local activation of 1,3-β-glucan synthase resulting in controlled callose deposition around the plasmodesmata neck has been considered as an important mechanism for the regulation of the plasmodesmal permeability (e.g. Lucas et al., 1993). The lack of labelling with anti-callose antibody shows, however, that this mechanism is not involved in the creation of symplasmic field boundaries (e.g. Fig. 5A,B).

In contrast, callose was evidently involved in the breakdown of symplasmic fields in the AM during morphogenetic deactivation by short photoperiod (Fig. 5C-E). This was shown, firstly, by fixation in the presence of tannic acid, which visualizes putative glucan synthase complexes at the plasmodesmata neck (Olesen, 1980; Olesen and Robards, 1990) in patterns typical for sphincters (Fig. 5C). These sphincters could not be detected in the morphogenetically active AM (Fig. 5A,B). Secondly, anti-callose antibodies clustered at the plasmodesmata neck (Fig. 5D,E) in patterns identical to those found with tannic acid (Fig. 5C), demonstrating that short photoperiod induced formation and subsequent activation of glucan synthase complexes at the plasmodesmata entrances. Physical closure of the plasmodesmata (Fig. 5D,E), categorically preventing symplasmic signalling, effectively blocked further formation of leaf primordia (not shown).

**DISCUSSION**

Control over AM function resides at the supracellular level (e.g. Poethig, 1990; Sachs, 1991; Steeves and Sussex, 1989; van der Schoot, 1996). We therefore addressed the question of whether the organization of the symplasm could provide a setting for such control. We investigated the tunica, which in birch is normally one layer thick (Fig. 1). Whether the degree of AM stratification is significant for primary morphogenesis has remained unclear (Szymkowski and Sussex, 1996). In sunflower, for example, the two layers show a similar zonation (Sawhney et al., 1981), and in Arabidopsis similar growth dynamics occur (Schnittger et al., 1996). It is feasible that when more tunica layers are present they form distinct physiological compartments with very similar growth dynamics. Below we discuss the functional structure of the symplasmic network in the tunica of birch and its relevance for the coordination of morphogenetic events at the AM.

**Symplasmic fields subdivide the tunica**

We have probed the symplasm of the AM by microinjecting LYCH into single tunica cells at different locations. Since LYCH does not pass through the cell membrane (Stewart, 1981), its spread from injected cells visualizes all symplasmic pathways available to small (up to 1 kDa) diffusing morphogens. The groups of symplasmically united tunica cells in which gradients of diffusing morphogens can be formed have been defined here as ‘symplasmic fields’. In mature tissues of plants, e.g. stem and leaves, groups of symplasmically coupled cells have been characterised as ’symplastic domains’ (Erwee and Goodwin, 1985), which stabilize the tissue and serve as storage and for internal redistribution of metabolites (van der Schoot and Van Bel, 1990; Lucas et al., 1993). Our earlier use of the term ‘symplasmic domain’ for coupling groups in the AM (van der Schoot and Rinne, 1996, and references therein) seems now inappropriate; since their cellular composition changes continuously due to cell proliferation and they collectively constitute a morphogenetic field, these cell-coupling groups should be conceptualized as ‘fields’ rather than ‘domains’.

In the AM of birch we found two different symplasmic fields. A concentric, ring-like field was present in the primordium forming part of the tunica (Fig. 2) which enclosed a second field, situated in the central undifferentiated part of the tunica (Fig. 3). The partial fields observed at the AM periphery may in some cases represent the complementary field of a fusion-field. Since the peripheral zone symplasmic field could be locally only a few cells wide, particularly where central cells were protruding into the periphery of the AM (Fig. 3C), the symplasmic movement may be slowed down or stopped at these locations. Possible local damage to the symplasmic connections in these corridors, due to primordia removal, cannot be ruled out. The presence of these fields demonstrates that plasmodesmata connections, although produced continuously between dividing cells, and within and between cell layers (van der Schoot, 1996; Bergmans et al., 1997), are narrowed or closed at certain boundaries within the AM. The resulting symplasmic fields, in analogy to those created by gap junctions in animal systems (de Laat et al., 1980; Serras, 1996; Bergmans et al., 1997), in patterns typical for sphincters (Fig. 5C). These sphincters could not be detected in the morphogenetically active AM (Fig. 5A,B). Secondly, anti-callose antibodies clustered at the plasmodesmata neck (Olesen, 1980; Olesen and Robards, 1990) in patterns identical to those found with tannic acid (Fig. 5C), demonstrating that short photoperiod induced formation and subsequent activation of glucan synthase complexes at the plasmodesmata entrances. Physical closure of the plasmodesmata (Fig. 5D,E), categorically preventing symplasmic signalling, effectively blocked further formation of leaf primordia (not shown).

**Fig. 5.** Ultrastructure of plasmodesmata in active (A,B) and deactivated (C-E) AMs. (A) Standard fixation with tannic acid. Absence of sphincters on plasmodesmata between L1 and L2 (as elsewhere). (B) Immuno-fixation and anti-callose labelling. Scant labelling of plasmodesmata between L1 and L2 (as elsewhere). (C) Standard fixation with tannic acid. Sphincters at the cytoplasmic ends of plasmodesmata between cell membrane and wall. (D) Immuno-fixation and anti-callose labelling. Sphincters, slightly visible due to naturally occurring tannins. Antibodies cluster at sphincters. (E) As in D, but without staining. c, plasmodesmata channel; cw, cell wall; s, section through sphincter ring. Scale bar (A-E), 100 nm.
plasmodesmata is lowered. inside the central zone field may determine the size of the histological central zone (Fig. 1A) and, as schematically cells. The central zone field in birch corresponds to the could, therefore, be based on a gradient set up by the initial plasmodesmata narrowing or closure at the field boundaries the central zone field. The mechanism which controls anchoring point for the mechanism which controls the size of the central zone field. The mechanism which controls plasmodesmata narrowing or closure at the field boundaries could, therefore, be based on a gradient set up by the initial cells. The central zone field in birch corresponds to the histological central zone (Fig. 1A) and, as schematically shown in Fig. 6B, the steepness of the hypothetical gradient inside the central zone field may determine the size of the field by influencing where the size exclusion limit of the plasmodesmata is lowered. The low E_m values in the central zone field (Table 2) match the low metabolism and cell cycling rate described for this distal area (Esau, 1977; Steeves and Sussex, 1989). This histological zone often extends beyond the tunica into the corpus (Steeves and Sussex, 1989) but the tunica, which lies within the central zone (Fig. 6), can be very distinctive and physiologically different from the subjacent central zone cells (Sawhney et al., 1981). This fact was, rightly in view of our findings, considered to be due to physiological isolation of these tunica cells, but the presence of numerous plasmodesmata connections did not seem to support such isolation (Sawhney et al., 1981). As it appears now it is not the absence of plasmodesmata which physiologically separates the distinct zones but their temporary narrowing or closure, as demonstrated by the present microinjection experiments. The large E_m values of cells in the peripheral zone field reflect the active proton pumping which drives competitive nutrient uptake, needed to maintain a high metabolic activity during cell proliferation. The large difference in cell E_m values between the two fields (Table 2) indicates that the symplastically united tunica cells are functioning as metabolic working units with distinct morphogenetic tasks. Subjacent cell layers may also be subdivided into metabolic working units since they also show a histological zonation (see e.g. Fig. 1A).

The internal diffusion boundaries in the AM of birch coincide with patterns of gene expression in other species. Certain genes, some of which have a ‘household’ function, are expressed in the entire vegetative AM, but not in the upper layer, or vice versa (Hake and Freeling, 1986; Pri-Hadash et al., 1992; Fleming et al., 1993). These expression patterns can change during the floral transition (Pri-Hadash et al., 1992) and may be influenced by spatial alterations in symplastic fields. In Iris, plasmodesmata frequencies between L_2 cells and surrounding cells diminish in early floral development (Bergmans et al., 1997), which implies a specific alteration in the density of connections in the overall symplastic network. This probably affects diffusion patterns in the AM quantitatively, but it is unknown if the pattern of symplastic fields have also changed during the transition to flowering. This is feasible, however, since the physiological zonation of the AM is also altered.

The diffusion boundaries described here may not present a barrier to diffusing molecules and ions that are smaller than LYCH (457 Da). In the case of root hair cells of Arabidopsis, which exhibit a high degree of electrical isolation (Meharg et al., 1994), the absence of dye-coupling has been argued to be due to occlusion or developmentally regulated closure (Duckett et al., 1994). The distinct difference in Em’s of the central and peripheral field in the birch tunica similarly suggests that the plasmodesmata size exclusion limit at the field boundaries is close to zero. Certain larger factors may pass the field boundaries by plasmodesmata gating, e.g. those involved in the regulation of non-autonomous gene expression or those involved in the coordination of cellular events across layers. For example, GLO in Antirrhinum is suggested to move between layers through plasmodesmata (Perbal et al., 1996), and may pass the diffusion boundary by transient opening and widening of plasmodesmata. Such trafficking would also provide a possibility for transient cotransport of diffusing morphogens. In addition, a lipid signalling pathway in the endoplasmic reticulum (Grabski et al., 1993) may be operational across symplastic field boundaries.

**Symplastic fields may temporarily fuse or dissipate**

A fusion between the two symplastic fields in the tunica was observed a few times when the AM was relatively small (Fig. 4A-C), probably at the beginning of a plastochron. This event, possibly coinciding with the initiation of the leaf primordium that will emerge at the end of the plastochron, may serve the symplastic exchange of certain morphogenetic signals between the two fields. Such signals may be chemical or electrical in nature (Lucas et al., 1993). Fusion events are also of interest for photoperiodic processes, such as flowering and dormancy, that typically take place in the leaf and are expressed at the AM (Bernier, 1988). In Anagallis (pimpernel), for example, the AM is only responsive (competent) to the inductive photoperiod during a short period in its plastochronic trajectory (Bernier, 1988). As the ‘florigenic signal’ probably travels via the symplasm from the leaf to the AM (Gunning and Robards, 1976; Bernier, 1988), it may reach the central meristem during a fusion event.

The uncoupling of AM cells in birch, induced by short photoperiod (Fig. 4D-E), was due to plasmodesmata being plugged with callose (Fig. 5C-E) and is obviously an effective means of preventing symplastic diffusion, macromolecular trafficking or lipid transfer. Symplastic uncoupling of cells in fern prothallia releases individual cells from global tissue constraints and results in redifferentiation and subsequent development of new prothallia from each individual cell (Tucker, 1990). In higher plants, some cellular constraints must remain, since redifferentiation is held in check and tissue
functioning can be restored by recoupling the cells (Erwee and Goodwin, 1984). In birch the symplasmic uncoupling of AM cells, and the resulting arrest of development, was reversible at the early stage by transfer to long photoperiod. This indicates that the inert state of the AM becomes fixed – serving the survival through winter – by biochemical processes which develop in symplasmically uncoupled cells. Biochemical adjustments involve changes in cell-autonomous gene expression (Welling et al., 1997), but it is unknown if they relate to the fixing of the AM.

Plasmodesmata closure due to short photoperiod is controlled by a different mechanism than plasmodesmata closure/narrowing at field boundaries, since sphincters were present only in the former (Fig. 5). Sphincters, found in a limited number of plants, have been suggested to regulate cell-cell transfer through plasmodesmata by the local deposition of callose (Olesen and Robards, 1990; Lucas et al., 1993). Sphincters are often absent, however, even in species for which they have been described. Explanations for this include the possibility that sphincters develop only late at certain stages of cell differentiation (Olesen and Robards, 1990), that they reflect a particular functional stage of plasmodesmata, or that they are stress-induced (Turner et al., 1994). Our observations show that in the birch AM sphincter formation occurs during developmental transitions induced by the environment. The sensing of day length and the subsequent formation of sphincters are likely part of the phylogenetically acquired mechanisms by which the plant anticipates the arrival of harsh conditions, and enters a dormant state.

In the above we have characterized regular and dynamically sustained symplasmic patterns of cell coupling in the AM. The spatiotemporal correlation of these symplasmic fields with metabolic and organogenetic activities, and field breakdown during AM deactivation, provide evidence for a role of the symplasm in coordinating cellular activities. Symplasmic fields control the symplasmic diffusion of potential morphogens and metabolites inside the AM and permit field interactions at their boundaries, thereby mediating various types of cellular collaborations. Given the generality of AM geometry and function, symplasmic fields may canalize morphogenesis in all higher plants.

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