The cytoskeleton facilitates a three-dimensional symplasmic continuum in the long-lived ray and axial parenchyma cells of angiosperm trees

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Abstract The microtubule (MT), microfilament (MF) and myosin components of the cytoskeleton were studied in the long-lived ray and axial parenchyma cells of the secondary xylem (wood) and secondary phloem of two angiosperm trees, Aesculus hippocastanum L. (horse-chestnut) and Populus tremula L. × P. tremuloides Michx. (hybrid aspen), using indirect immunofluorescence localisation and transmission electron microscopy. MTs and MFs were bundled and oriented axially (parallel to the cell’s long axis) within all parenchyma cell types after they had fully differentiated. Additionally, actin and myosin were immunolocalised at the thin-walled membranes of the pits, which linked cells in neighbouring files of both ray and axial parenchyma, and at the pits between axial and ray parenchyma cells themselves. Anti-callose antibody immunolocalised the plasmodesmata at the pit membranes, and in the same pattern as that of anti-myosin. Ray cells are important symplasmic pathways between the xylem and the phloem throughout the life of trees. We hypothesise that the MT and MF components of the cytoskeleton in the ray and axial parenchyma cells are involved in the transport of materials within those cells, and, in association with the acto-myosin of plasmodesmata at pit fields, are also important in intercellular transport. Thus, the symplasmic coupling between ray cells, between axial parenchyma cells, and between axial parenchyma and ray cells represents an extensive three-dimensional communication pathway permeating the tree from the phloem through the cambium into the wood. We suggest that this cytoskeletal pathway has an important role in delivery of photosynthate, and mobilised reserves, to the actively dividing cambium, and in the movement of materials to sites of reserve deposition, principally within the wood. This pathway could also have an important role in co-ordinating developmental processes throughout the tree.

Keywords Aesculus (cytoskeleton) · Cytoskeleton (immunofluorescence localisation) · Myosin (unconventional, VIII) · Parenchyma cells (long-lived) · Populus (cytoskeleton) · Secondary vascular tissues

Abbreviations MF: microfilament · MT: microtubule · SVS: secondary vascular system

Introduction

In plant cells, four principal arrays of the microtubule (MT) cytoskeleton are generally recognised (Goddard et al. 1994; Chan et al. 1996; Barlow and Baluška 2000): the pre-prophase band, the mitotic and meiotic spindles, the phragmoplast array, and the interphase array (both cortical and endoplasmic components). The first three are involved with various phases of cell division; the fourth has largely been implicated in cell morphogenesis and cell wall formation.

Previous immunofluorescence studies of MTs in plant cells have concentrated on relatively short-lived cells, or meristematic cells in the process of leaving the division zone and, with few exceptions (see below regarding secondary vascular tissues), deal almost exclusively with cells or tissues of the primary plant body (e.g. see chapters in Lloyd 1991). This bias fails to reveal the true diversity of MT arrays existing within plant cells, and, in the case of single-cell systems, provides little or no information about any role of the cytoskeleton in co-ordination of development between cells (see also Kost et al. 1999). For example, although the cortical MT array of interphase cells is generally described as transverse, oblique or longitudinal, the diversity of patterns it can adopt is dramatically displayed in cells of the secondary...
vascular system (SVS) of trees when its cells differentiate from cambium into woody derivatives (Chaffey et al. 1999; Funada et al. 2000). A similar dynamism has recently been reported for the microfibril (MF) cytoskeleton during wood formation in angiosperm (Chaffey et al. 2000a) and gymnosperm (Funada et al. 2000) trees.

The generally prevailing view of the role of the MT cytoskeleton in interphase cells is considered to be participation in deposition of cellulose microfibrils (‘wall formation’) (reviewed by Giddings and Staehelin 1991). This applies to cells as they differentiate to a ‘final’ state. In many situations that have been studied, this final state is short-lived. But in long-lived cells it is possible to see what type of cytoskeleton occupies the cell once its wall-building phase has been completed. Particularly good examples of such long-lived cell types are the ray and axial parenchyma cells of the secondary phloem and xylem of angiosperm trees. In contrast to the majority of the axial components of wood, which depend for their function (water-transport and support) upon their early death, the ray and axial parenchyma cells of this tissue remain alive and function for several years (e.g. Fahn and Arnon 1963). Furthermore, although the phloem as a whole remains a living tissue, its parenchyma cells also function for up to 1 or 2 years before being crushed by newly produced secondary phloem.

Does a cytoskeleton persist in these long-lived parenchyma cells? What form does it adopt, and can this give an indication of its function? We have attempted to answer these questions by indirect immunofluorescence examination of the microtubule, microfilament and myosin cytoskeleton of the ray and axial parenchyma cells of the secondary xylem and phloem of two angiosperm trees, *A. hippocastanum* L. (horse-chestnut) and *P. tremula* L. *X. tremuloides* Michx. (hybrid aspen).

Materials and methods

Plant material

Seedlings of *Aesculus hippocastanum* L. (Hippocastanaceae) were raised as described previously (Chaffey et al. 1996). Samples were taken from the base of the taproot and mid-epicotyl region during the first 3 years of seedling growth, and from stems of 5-year old saplings during periods when the vascular cambium was active in cell production. Stem and root samples were taken from saplings of *Populus tremula* L. *X. tremuloides* Michx, during their first 2 years of growth. The hybrid aspen was raised at the Swedish University of Agricultural Sciences (as described in Nilsson et al. 1997), and transferred to unheated greenhouses at IACR-Long Ashton Research Station. Saplings were watered daily but were fed once a week with a solution containing 31.25 mg l⁻¹ NH₄NO₃.

Indirect immunofluorescence microscopy

Chemical fixation

The procedure is described in detail in Chaffey (in press, a). In brief, material was pre-fixed with 100 μM m-maleimidobenzoyl N-hydroxysuccinimide ester (MBS) (Sigma Chemical Company, Poole, UK) in 25 mM Pipes buffer (pH 6.9), fixed with 3.7% formaldehyde, either with or without 0.2% (v/v) glutaraldehyde, in 25 mM Pipes buffer (pH 6.9), embedded in a butyl-methacrylate resin mixture, and sectioned at 6–10 μm. After resin removal, sections were incubated in a blocking solution (containing bovine serum albumen, fish-skin gelatin, normal goat serum and glycine in phosphate-buffered saline (PBS; 1 containing: 8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, 0.2 g KH₂PO₄, 0.2 g Na₂SO₄, pH 7.3–7.6)) for 30 min before application of the appropriate primary antibody (see below). Sections were incubated for ca. 2 h at room temperature before a PBS-wash and application of the relevant fluorescein isothiocyanate (FITC)- conjugated secondary antibody (Sigma Chemical Company, Poole, UK) (see below), diluted 1:30 in a solution containing bovine serum albumen, fish-skin gelatin, and NaN₃ in PBS (PBSA). Sections were incubated for ca. 1 h at room temperature, before washing in PBS, storing nuclei with propidium iodide, lightly staining with Toluidine Blue (to reduce autofluorescence), and mounting in Vectashield anti-fade mountant (Vector Laboratories, Peterborough, UK). Sections were examined in either a Zeiss Axiohot microscope under epifluorescence illumination of appropriate wavelength, and photographed on Kodak EPP100 colour slide film, or examined with a Leica TCS SP confocal laser scanning microscope (excitation/emission wavelengths used were: 488/503–553 nm for autofluorescence and propidium iodide, 568/582–704 nm) and recorded as two-colour micrographs. All images were subsequently converted to grey scale and processed further using PaintShop Pro version 5.01 or Corel Photo Paint version 8.0.

Antibodies

The following antibodies were used:

- For microtubules, anti-α-tubulin monoclonal antibody (Amer sham International), diluted 1:10 in PBS; and anti-mouse secondary antibody.
- For microfilaments, C4 anti-actin monoclonal antibody (ICN Biomedical, Thame, Oxfordshire, UK), diluted 1:50 in PBS; and anti-mouse secondary antibody.
- For myosin, anti-unconventional myosin VIII polyclonal antibody (a gift from Dr. F. Balsioka, University of Bonn, Germany), diluted 1: 70 in PBS; and anti-rabbit secondary antibody. This antibody has recently been characterised by Reichelt et al. (1999).
- For callose, anti-(1→3)-β-glucan (Biosupplies, Parkville, Victoria, Australia) diluted 1:20 in PBS; and anti-mouse secondary antibody. This antibody has been characterised by Meikle et al. (1991) and is specific for (1→3)-β-glucan (callose). The immunofluorescence staining pattern with this antibody in the SVS of *Aesculus* and hybrid aspen was the same as that of aniline blue (the standard fluorescent stain for callose – Eschrich and Currier 1964) (not shown).

Controls for immunolocalisation

Replacement of primary antibody or of secondary antibody with PBS or PBSA respectively, were used as controls for all antibodies. Control sections were devoid of FITC fluorescence illustrated for tubulin in Fig. 2A in Chaffey et al. (1999) and for actin in Fig. 1a in Chaffey et al. (2000a)]. Additionally, the distinct localisations of callose and myosin were used as controls for immunolocalisation of microfilaments and microtubules, and vice versa (e.g. Šamaj et al. 1998).

Freeze-fixation

Since concerns have been expressed that formation of callose may be induced by glutaraldehyde-fixation (Hughes and Gunning 1980), some material was fixed as above but without glutaraldehyde. It has also been suggested that callose synthesis can occur in response to injury (such as excision of material for sampling; Eschrich and