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Patterned secondary cell-wall assembly in tracheary elements occurs in a self-perpetuating cascade

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SUMMARY

The behaviours are presented of newly-synthesized xylan and putative glycine-rich protein during patterned secondary cell-wall biogenesis in drug-treated tracheary elements (TEs) differentiating in culture from isolated mesophyll cells of *Zinnia elegans*. The normal secondary wall thickenings contain cellulose, xylan, and lignin, and the results reported here suggest that they also contain glycine-rich protein (GRP). However, qualifications to this definitive interpretation are discussed. The specific cellulose synthesis inhibitors, 2,6-dichlorobenzonitrile (DCB) and isoxaben, were applied near the onset of differentiation. When they were fully effective in inhibiting deposition of detectable cellulose in the thickenings, no labelling of the thickenings was observed with probes for xylan (xylanase and an antibody to xylose) or GRP (an antibody). When the drugs were partially effective, a small amount of detectable cellulose was still deposited in the thickenings. In such TEs, patches of xylan and GRP were observed between thickenings, suggesting that these components were exocytosed but not able to localize at the altered thickenings. A model for cell-wall assembly is presented in which some molecules themselves are able to mediate the patterning of others, so that patterned secondary cell-wall assembly partly occurs by a self-perpetuating cascade.

Key-words: cellulose, cellulose synthesis inhibitors, glycine-rich protein, microtubules, xylan, *Zinnia elegans*.

INTRODUCTION

Plant cell walls have important roles in plant development, environmental adaptation and stress responses, and these varied functions are reflected in diverse molecular and spatial structure (Roberts 1990). To advance the incomplete understanding of how the complex, highly co-ordinated process of wall deposition is controlled, we have focused on the patterned secondary cell walls of tracheary elements (TEs). The secondary thickenings prevent the walls of TEs, which remain as the functional element after autolysis, from collapsing under the stress of water transport. The specificity of diverse thickening

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This paper is dedicated to Professor Dr M. M. A. Sassen on the occasion of his retirement.

patterns to different stages of plant development and particular species (O'Brien 1981; Preston 1988) indicates that secondary wall deposition must be under precise cellular control. Our experiments have been aided by use of isolated mesophyll cells of *Zinnia elegans* L. that can be induced to differentiate in suspension culture (Fukuda & Komamine 1980; Kohlenbach & Schmidt 1975). This cell system offers the advantages of: (1) semi-synchronous differentiation of up to 60% of the living cells; (2) deposition of one type of specialized cell wall near the end of a terminal differentiation process; and (3) optimum accessibility of differentiating cells to experimental drugs and microscopic analyses.

TE secondary wall thickenings contain cellulose, non-cellulosic polysaccharides and lignin (Roberts 1974). As in TEs of other dicots (Thornber & Northcote 1962), thickenings of suspension-cultured *Zinnia elegans* TEs contain a β-1,4-linked xylan as the major non-cellulosic polysaccharide as shown by biochemical and microscopic analyses (Ingold *et al.* 1988; Northcote *et al.* 1989; Suzuki *et al.* 1991; Taylor *et al.* 1992). Recent evidence shows that xylem cell walls also contain proteins, including proline-rich proteins, hydroxyproline-rich proteins and glycine-rich proteins (GRP) (Bao *et al.* 1992; Ye *et al.* 1991). Immunofluorescence demonstrates that some members of these protein families localize preferentially in dicot xylem and phloem walls that are lignified or destined to be lignified (Keller *et al.* 1989; Ye *et al.* 1991; Ye & Varner 1991).

Consideration of control mechanisms for wall patterning must take into account modes of biosynthesis of the components, cytoplasmic determinants of patterning and molecular interactions between cell-wall components. Cellulose is synthesized at the plasma membrane, with crystallized microfibrils directly integrating into the forming wall. In contrast, the other polysaccharide components, proteins, and (probably) monomers of lignin are exocytosed by cytoplasmic vesicles into the wall milieu (Dixon & Northcote 1985). Cortical cytoplasmic microtubules, which form bands that predict the pattern of secondary wall thickenings, have an as-yet unspecified role in control of TE secondary wall patterning (Hepler 1981; Hogetsu 1991; Seagull 1991). However, lignin may be deposited in a pattern after banded microtubules have disappeared from differentiating TEs (Roberts *et al.* 1985). The proteins of the secondary wall evoke particular interest because of speculation about possible roles as nucleation sites for lignin polymerization. Similarly, xylan is expected to associate closely with cellulose by hydrogen bonding and also shows evidence of cross-linking with lignin in some walls (see Taylor *et al.* 1992 for review). To seek additional evidence about molecular interactions that might mediate wall patterning, we applied cellulose synthesis inhibitors to differentiating TEs of *Zinnia elegans* and analysed the behaviour of xylan and GRP. The results provide additional information about mechanisms underlying the previously-reported dispersion of lignin in TEs treated with cellulose synthesis inhibitors (Taylor *et al.* 1992; Suzuki *et al.* 1992).

MATERIALS AND METHODS

Reagents

All chemicals and probes were purchased from Sigma Chemical Company (St Louis, Missouri) except as otherwise noted in the text.

Tinopal LPW® (a gift from Ciba Geigy, Greensboro, NC, USA) contains the same effective molecule as Calcofluor White®, which is an original trade name of American

Cyanamid, Bound Brook, NJ. It is a fluorescent brightener (FBA 28) that binds cellulose and sometimes other β-linked polysaccharides (Hughes & McCully 1975). It is interesting to note, however, that the patches of xylan described below do not fluoresce with Tinopal LPW, which is consistent with previous data showing lack of interaction with arabinoxylan (Wood *et al.* 1983). The intensity of Tinopal LPW staining of the thickenings of differentiating *Zinnia elegans* TEs increases co-ordinately with the intensity of birefringence under polarization optics and the intensity of labelling with a histologically-specific cellulase (Taylor *et al.* 1992), suggesting that the brightener mainly recognizes cellulose in these thickenings. Tinopal LPW is unequivocally identified by *Color Index* number 40622.

Culture methods

Single-cell suspension cultures were established using mesophyll cells isolated from the first true leaves of *Zinnia elegans* L. var. *envy* (G.S. Grimes Seeds, Smethport, PA) and induced to differentiate as described previously (Roberts *et al.* 1992) with the exception of the experimental variations noted below. In all experiments reported here, TE differentiation was initiated at around 48 h as determined by fluorescence of cell-wall thickenings with Tinopal LPW (Ingold *et al.* 1988) and up to 60% of the living cells were differentiated by 72 h. Cellulose synthesis inhibitors, 7·5 μM 2,6-dichlorobenzonitrile (DCB; Montezinos & Delmer 1980; Fluka Chemika-BioChemika, Buchs, Switzerland) or 0·3 μM isoxaben (Heim *et al.* 1990; a gift from Dr K. Burrow, Lilly Research Laboratories, Indianapolis, Indiana), were added to experimental cultures between 45 and 48 h. Stock solutions (1000 ×) of each inhibitor were made in DMSO and stored at −20°C for up to 3 months. DMSO at 0·1% does not affect these cultures (Taylor *et al.* 1992). Both inhibitors gave equivalent results.

Fluorescent labelling of TEs

Xylan in TEs was labelled using an antibody to β-1,4-xylose$_{3-8}$ (Northcote *et al.* 1989; Cambridge Research Biochemicals, Norwich, UK) or a cloned xylanase A and its antibody (Mondou *et al.* 1986; gifts from Prof. D. Kluepfel, University of Québec) and secondary fluorescent tags as previously described (Taylor *et al.* 1992) with minor changes. Briefly, TEs attached to polylysine-coated Teflon®-well slides (ICN Biomedicals, Inc. Costa Mesa, CA, USA) were incubated with xylanase A (10 μg/ml^{-1} for 20 min), washed once in Tris-buffered saline (TBS, pH 7·4), incubated with antibody to xylanase A (1:1000 in TBS, 0·01% azide, 1% BSA; 1 h at RT or over night at 4°C), washed 3 × 10 min in TBS, incubated with goat anti-rabbit FITC (FITC is fluorescein isothiocyanate; antibody diluted 1:200 or 1:400 as above; 1 h at RT), washed 3 times in TBS, stained 1 min in 0·005% Tinopal LPW (in 0·025 M Na-phosphate buffer, pH 7·2), washed 2 times with 0·025 M Na-phosphate buffer, pH 6·5, and mounted in a phenylene diamine mountant to suppress fading of fluorescence. The antibody to xylose (1:200) was applied in the same way. For the antibody to xylanase A, the pre-immune serum from the rabbit used to raise the antibody (provided by Prof. D. Kluepfel) was applied as a control (1:400). For the antibody to xylose, the primary antibody was replaced with TBS/BSA as a control. The secondary antibody was also replaced with TBS/BSA as a control.

Probes for xylan were also applied to fixed cells (3% freshly prepared formaldehyde, 0·05 M Na-phosphate buffer, pH 7·2; 1 h RT) and to unfixed cells that had been sonicated with a microtip. If cells were fixed, a 5 min rinse in 0·1% $NaBH_4$/TBS was added before

application of the primary antibody or xylanase A to block fluorescence of aldehyde groups.

A polyclonal antibody raised against a fusion protein containing amino acids 15–333 of a glycine-rich protein from French bean (Keller *et al.* 1988; a gift from Drs C. Lamb and B. Keller) was applied similarly with the following changes: (1) TEs were routinely sonicated with a microtip to create breaks in cell walls, but not to destroy the cells; (2) 1:200, 1:400 and 1:800 dilutions of primary antibody were tested; and (3) stringency of washing was increased by using 3 × 15 min washes in TBST (TBS + 0·2% Tween 20) with gentle rocking. Since the pre-immune serum from the rabbit used to generate this antibody is not available (Dr B. Keller, personal communication), 3 other pre-immune sera available in our laboratory were tested in dilutions from 1:100 to 1:1000 as controls. The primary and secondary antibodies were also replaced with TBS/BSA as controls.

The labelled samples were observed using an Olympus BH2 microscope equipped with epifluorescence, polarized light, and differential interference contrast (DIC) optics. Filter packages used for epifluorescence included: IB for green FITC fluorescence, 15 nm BP 495 and, for blue Tinopal LPW fluorescence, either a modified UG1, 20 nm BP 365, or V, 20 nm BP 405. The UG1 and V filter packages yielded equivalent information for Tinopal LPW, but the background was reduced with the V set. No crossover of Tinopal LPW fluorescence was observed with the IB filter pack for FITC. A Zeiss KP560 filter set was sometimes used to block autofluorescence of chlorophyll. Micrographs were recorded on Kodak TMax 400 film. TEs were examined at an early stage before autofluorescence of the thickenings became apparent.

Western blotting of GRP

Total cell protein from cultures at time of initiation and 45–70 h later (period of active differentiation) was extracted and probed with the antibody to GRP after the separation of 50 μg/lane of each protein extract on an SDS/polycrylamide gel (10%) followed by blotting onto Immobilon® nitrocellulose (Millipore, Bedford, Massachusetts, USA). Immunodetection was carried out by blocking the membrane (5% non-fat dry milk, TBS, 1 h, RT), washing in TBS, incubating with antibody to GRP (1:500 in TBS/5% dry milk, 1 h RT or overnight at 4°C), washing 4 × 5 min in TBST, incubating with donkey anti-rabbit Ig-horse-radish peroxidase (1:1000 diluted as above, 1 h RT), and washing 4 × 5 min in TBST and once in TBS. Detection was performed using enhanced chemiluminescence (ECL) and recorded on Hyperfilm ECL film (Amersham International PLC, Amersham, UK).

RESULTS

Staining for xylan

As previously reported (Taylor *et al.* 1992) and shown again here for direct comparison with other results, the patterned secondary wall thickenings of control TEs (Fig. 1a) contain xylan as indicated by binding of xylanase A (Fig. 1b) or antibody to xylose (data not shown; Northcote *et al.* 1989). Thickenings with xylan also stained positively with Tinopal LPW (Fig. 1c) and exhibited birefringence in polarized light (not shown), both of which support the presence of cellulose. The unique placement of xylan in the secondary wall is emphasized by the lack of staining of the primary wall of the adjacent non-differentiating cell (Fig 1b), which is visible by DIC (Fig. 1a). The previous report (Taylor

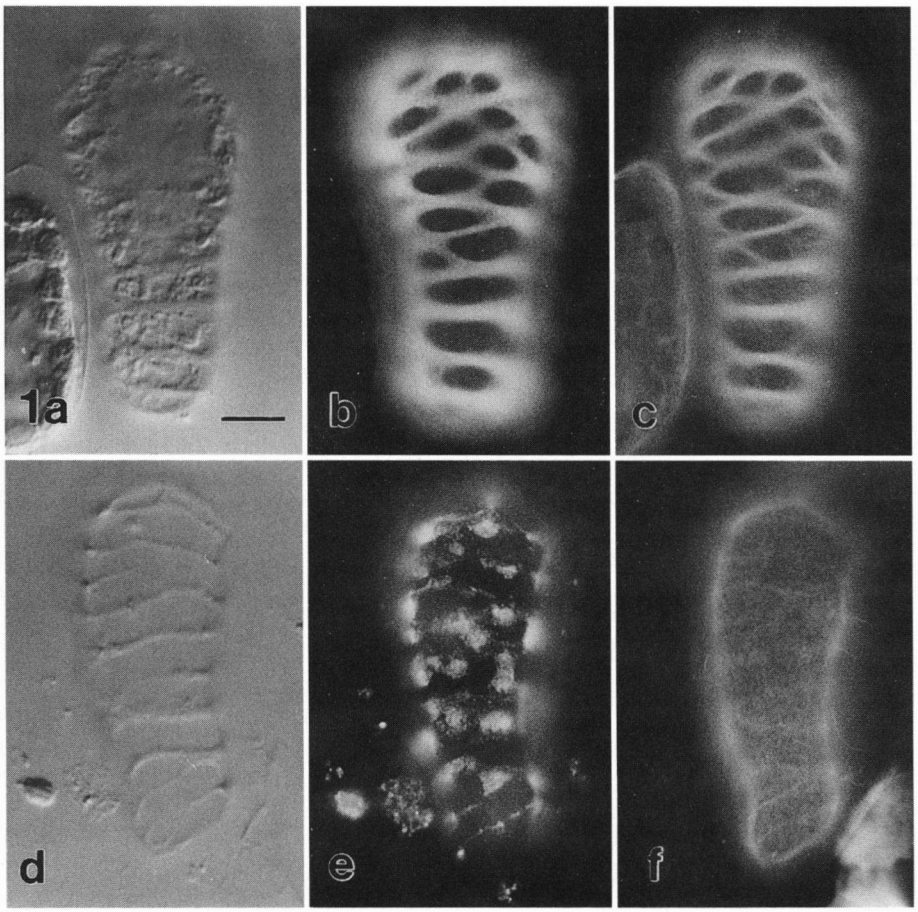

Fig. 1. Light microscopy by different optical methods of a control (a–c) and a DCB-treated TE (d–f) for xylan localization. The thickenings of control TEs visualized with DIC optics (Fig. 1a) contain xylan (Fig. 1b; localized with a cloned xylanase A and its antibody) and abundant cellulose (Fig. 1c; localized with Tinopal LPW). The abnormal thickenings of DCB-treated TEs can also be visualized with DIC (Fig. 1d). Some treated TEs show patches of xylan between the thickenings and little or no xylan over the thickenings (localized as above; Fig. 1e). TEs with patches commonly show evidence of partial 'escape' of the DCB inhibition, as indicated by faint fluoresence of thickenings with Tinopal LPW, which binds to cellulose (Fig. 1f). The general fluorescence all over the cell is due to Tinopal LPW binding to cellulose in the primary wall. These images suggest that in the absence of control amounts of cellulose, xylan is exocytosed but diffuses away from the abnormal thickening site.

et al. 1992) showed that thickenings of DCB- or isoxaben-treated TEs that contained no detectable cellulose remained unlabelled by probes for xylan. (DCB- or isoxaben-treated TEs still deposit an unidentified component of the secondary cell wall in a pattern; see Fig. 1d.)

Subsequently, we discovered that TEs in which cellulose synthesis was only partially inhibited by DCB or isoxaben ('escaped' TEs) could be labelled with probes for xylan, but in a different manner than controls. 'Escaped' TEs, as indicated by faint staining of the patterned wall material with Tinopal LPW and/or faint birefringence, occurred if old stock solutions of the inhibitors were used and if the inhibitors were added just after the onset of differentiation in some cells. (The 'old' stock solutions probably imply a reduced effective drug concentration, but proof through detailed threshold concentration studies

has not yet been obtained. Later times of addition imply that some cellulose was deposited before inhibition occurred). Patches of xylan (Fig. 1e) that often bridged the space between secondary wall thickenings (Fig. 1d) were commonly observed in treated TEs with thickenings that contained some cellulose (Fig. 1f, see approximately horizontal stripes near the bottom of the cell). The thickenings of such cells were either unstained or lightly stained with probes for xylan (see examples of both types in Fig. 1e). If the amount of cellulose in treated TEs was extensive as indicated by strong birefringence and strong Tinopal LPW staining, the probes for xylan corresponded to the pattern of the secondary wall as in control TEs (results indistinguishable from Fig. 1b).

Controls with the primary antibodies omitted, with substitution of the serum for the anti-xylanase A, and with the secondary antibody omitted showed no comparable images; cells were either black or showed dull green background fluorescence.

Staining for GRP

The patterned secondary wall thickenings of control TEs (Fig. 2a) could also be labelled with the antibody to GRP (Fig. 2b), and the staining had a fine granularity compared to the smooth staining of xylan in control thickenings (compare Figs 1b and 2b). Non-differentiating cells in the same preparations showed no fluorescence (not shown, but accurately represented by the non-differentiating cell in Fig. 1b). GRP staining also differed from that of xylan by being less frequent, more adversely affected by fixation in formaldehyde before sonication, and more enhanced by sonication of the TEs. Thickenings that stained positively for GRP also stained with Tinopal LPW (Fig. 2c), but Tinopal LPW-positive thickenings were observed that did not stain for GRP (not shown). Many DCB- and isoxaben-treated TEs did not stain with antibody to GRP, but some with patterned secondary walls (Fig. 2d) showed patches between the thickenings (Fig. 2e). Such cells were partially 'escaped' TEs as indicated by faint staining with Tinopal LPW (Fig. 2f, see horizontal stripes on cell) and often faint birefringence (not shown). As described for xylan, 'escaped' TEs with strong indications of cellulose in the wall showed GRP staining equivalent to control (results indistinguishable from Fig. 2b). Equivalent results were recorded photographically at 1:800 dilution of the GRP antiserum, although staining was brighter at 1:200 and 1:400.

When the primary or secondary antibodies were omitted, cells were either black or showed dull green background fluorescence. In all previous immunolocalization studies, the control was pre-immune serum from a different rabbit than that used to generate the anti-GRP. Three rabbit pre-immune sera available in our lab gave very different results when applied and followed by FITC-tagged secondary antibody: (1) pre-immune 1 was correlated with green fluorescence of some thickenings at 1:100, but not at 1:1000; (2) pre-immune 2 was correlated with strong cytoplasmic fluorescence at 1:100, which was very dim at 1:1000; and (3) pre-immune 3 was correlated with smooth green fluorescence of some thickenings at 1:100, 1:200 and 1:1000.

Western blotting of GRP

Using the sensitive method of chemiluminescent detection, no GRP was detected in extracts of *Zinnia* mesophyll cells just after isolation for culture at $t=0$. By 45 h, a band at about 48 kDa was detected, which persisted strongly at 54 and 70 h ($t=54$ and $t=0$ h shown in Fig. 3). Less intense higher molecular weight bands become visible later in culture that may represent cross-linked molecules of GRP in the cell wall, as has been previously proposed for later stages of xylem development in tissues (Keller *et al.* 1989).

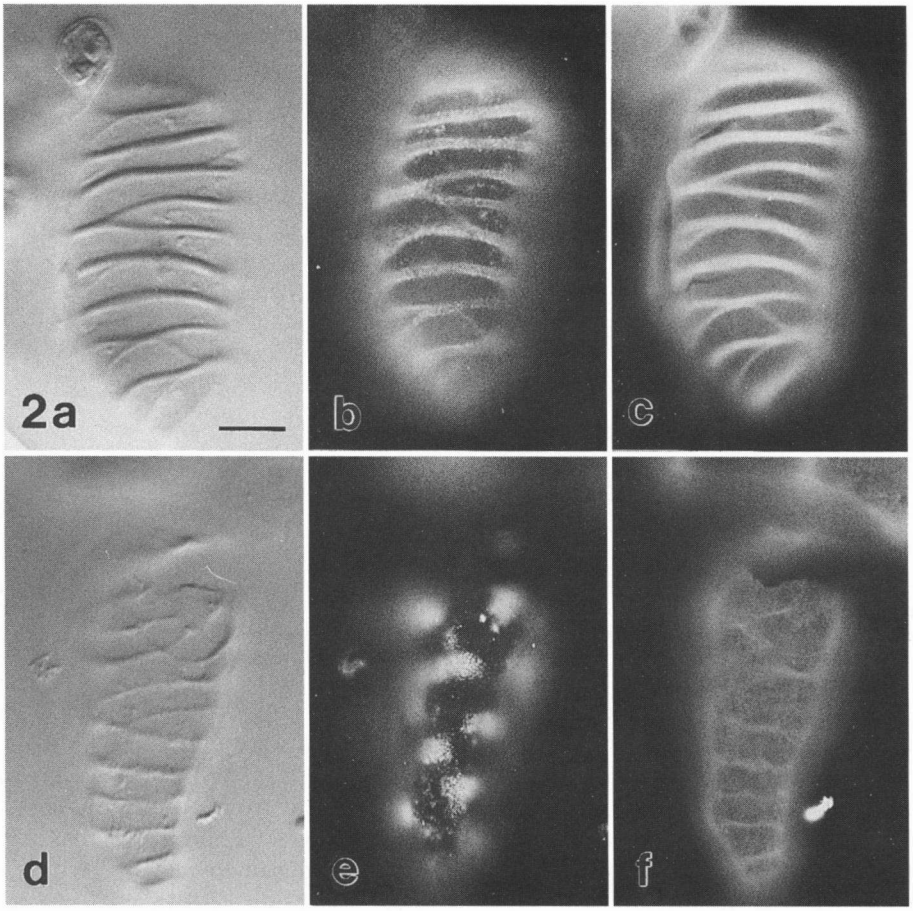

Fig. 2. Light microscopy by different optical methods of a control (a–c) and a DCB-treated TE (d–f) for glycine-rich protein (GRP) localization. The thickenings of control TEs visualized with DIC optics (Fig. 2a) probably contain GRP (Fig. 2b; localized with an antibody to GRP; see text for further discussion) and abundant cellulose (Fig. 2c; localized with Tinopal LPW). The abnormal thickenings of DCB-treated TEs can also be visualized with DIC (Fig. 2d). Some treated TEs show patches of GRP between thickenings, but no GRP over the thickenings (localized as above; Fig. 2e). TEs with patches commonly show evidence of partial 'escape' of the DCB inhibition, as indicated by faint fluorescence with Tinopal LPW, which binds to cellulose (Fig. 2f). These images suggest that in the absence of control amounts of cellulose, GRP is exocytosed but diffuses away from the abnormal thickening site.

DISCUSSION

These results support the hypothesis that patterning of TE secondary cell wall is partly controlled by a self-perpetuating cascade in which localized cellulose mediates the patterning of other molecules. This hypothesis was formulated based on the observation of dispersed lignin in DCB- and isoxaben-treated TEs that had undisturbed banded microtubules but lacked detectable cellulose and xylan in their patterned secondary walls (these walls are of still unknown composition) (Taylor *et al.* 1992). Because DCB and isoxaben are thought to be very specific inhibitors of cellulose biosynthesis (Heim *et al.* 1990; Montezinos & Delmer 1980), we suggested that xylan might be synthesized but diffuse into the culture medium in treated TEs. The patches of xylan reported here in TEs

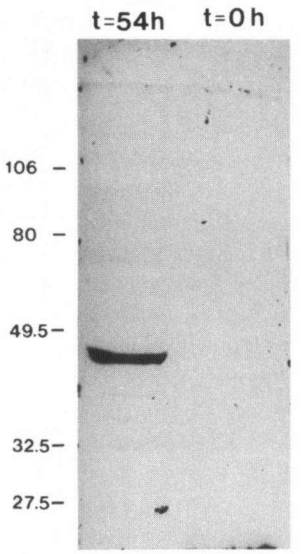

Fig. 3. Western blotting of whole cell extracts of *Zinnia elegans* mesophyll cells prior to ($t = 0$ h of culture) and during tracheary element differentiation ($t = 54$ h of culture). (Tracheary element differentiation started at 48 h and finished by 72 h after culture initiation.) A single band near 48 kDa is detected by the GRP antiserum.

that partially 'escape' the effects of DCB and isoxaben support the idea that xylan is synthesized and exocytosed. The certainty of the interpretation is enhanced by the staining of xylan with two different probes and the negative results with the pre-immune serum for the xylanase antibody. The diffusion of xylan away from the cellulose-depleted thickening site is reasonable based on its ability to hydrogen bond with cellulose (Reis *et al.* 1992) and the failure of hydrogen-bonding xyloglucan to remain fixed in the cellulose-depleted primary walls of DCB-adapted suspension cells (Shedletzky *et al.* 1990).

Results with the GRP antiserum support the model of synthesis, exocytosis and diffusion of wall components in the absence of control levels of cellulose. Images of putative GRP were similar to those of xylan, but were more granular, less frequent and more enhanced by sonication. In addition, the antiserum recognized one 48-kDa band at the time of TE differentiation and immunofluorescence was observed only in differentiating TEs, consistent with previous binding of the antiserum to protoxylem (Keller *et al.* 1989; Ye *et al.* 1991). These observations argue against non-specific binding of the GRP antiserum and suggest that the antigenic sites may be available only for short times, possibly due to interaction of GRP with other molecules of the wall. Our labelling of secondary thickenings with this antiserum suggests that the statistically significant colloidal gold labelling of French bean secondary walls that was *sometimes* observed in electron microscope preparations (Ryser & Keller 1992) may reflect reality. However, because the pre-immune serum from the rabbit injected with the GRP fusion protein is not available as a control, we cannot assert definitively that the only component localized by immunofluorescence is GRP. The GRP antiserum recognizes the *in vitro* translation product of mRNA synthesized *in vitro* from the cloned GRP gene (Keller *et al.* 1988), confirming its cross-reactivity with GRP epitopes. However, this powerful result cannot be extended to specificity in immunofluorescence with complete certainty; of three rabbit

pre-immune sera that we tested, one did stain thickenings at 1:1000. We eluted enough anti-GRP from gel slices to perform a Western blot by the sensitive chemiluminescent method, but low titre probably accounted for subsequent unsuccessful immunofluorescence. Until affinity purification of the antiserum is accomplished or other antibodies are available, we, along with others, can tentatively propose to have localized GRP.

Even when partially effective DCB and isoxaben allowed small amounts of cellulose to be deposited in the thickenings, the xylan and GRP did not become associated with the thickenings in a normal manner. Therefore, particular ratios between cellulose and other molecules are probably necessary for normal assembly of the wall. This is supported by the effects of DCB on helicoidal cell-wall assembly, which led to the proposal that a threshold ratio between cellulose and hemicellulose had a role in determining wall structure (Satiat-Jeunemaitre 1987). Patches of xylan and GRP may appear more frequently in incompletely inhibited cells because the small amount of cellulose provides a temporary anchoring site after exocytosis. Patches of xylan often appear as blocks that bridge the thickening sites, as if the molecules at the edge of the aggregate were bonded to the small amount of cellulose in each. Patches of xylan are more common and more coherent than those of GRP, which is perhaps related to the tendency of non-cellulosic polysaccharides like xylan to self-associate (Jarvis 1992).

Recently a microtubule antagonist was applied to developing TEs in pisum and commelina roots, and it was shown that cellulose (as indicated by Calcofluor staining) and a non-cellulosic polysaccharide (putatively xylan, labelled with FITC-wheat-germ agglutinin) were co-ordinately altered in distribution (Hogetsu 1991). From these results, it was concluded that microtubules determined the localization of a non-cellulosic polysaccharide as well as cellulose. However, application of the same lectin (FITC-labelled from *Triticum vulgaris*) to DCB-treated *Zinnia elegans* TEs revealed patches between thickenings reminiscent of the results with the xylan probes (data not shown). Therefore, the results of Hogetsu can be reinterpreted to suggest that the lectin-recognized polysaccharide merely follows the altered distribution of newly synthesized cellulose in TEs treated with microtubule antagonists. The cellulose itself may be the critical pattern-perpetuating molecule that is required to be localized by a microtubule-mediated mechanism under normal conditions. This hypothesis is consistent with other evidence, including timed addition of microtubule antagonists, that banded microtubules have accomplished their primary pattern-determining role by the time that patterned cellulose deposition begins (Haigler *et al.* 1992; Haigler & Koonce 1992).

In summary, normal quantities of cellulose are necessary for the fixation of xylan and putative GRP into the patterned thickenings. Depletion of these three molecules is associated with dispersion of lignin all over the wall (Taylor *et al.* 1992; Suzuki *et al.* 1992), in contrast to its normal restriction in the TE secondary wall pattern. We have not yet obtained immunofluorescence evidence for uniformly distributed xylan or GRP in cell walls of inhibitor-treated TEs that would correlate with the dispersed lignin. These molecules may diffuse into the medium, and ongoing analyses of the medium composition and the altered TEs at the electron microscope level should clarify the final fate of xylan and GRP. Other molecules not yet analysed may also be missing from the thickenings, and since cellulose and xylan associate intimately and extensively by hydrogen bonding, some of them might depend directly on xylan and indirectly on cellulose for their normal localization. Definitive determination of where various molecules fit into the self-perpetuating cascade of the secondary wall assembly must await more detailed determination of the molecular interactions within cell walls. The idea that the presence of localized cellulose establishes an

essential framework for the localization of other secondary cell-wall molecules is consistent with a 'hierarchical self-assembly process' for development of wall structure outlined previously for primary walls (Satiat-Jeunemaitre 1987).

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