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Comparative subcellular immunolocation of polypeptides associated with xylan and callose synthases in French bean (*Phaseolus vulgaris*) during secondary wall formation

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Abstract

The Golgi apparatus of plant cells is thought to be the main site of synthesis of cell wall matrix polysaccharides and the terminal glycosylation of glycoproteins. Much of this evidence still depends on earlier biochemical studies employing subcellular fractionation. However acquiring pure Golgi membranes is still difficult and the question of spatial organisation of glycosyl transferases can be addressed by immunolocation of the enzymes. An antibody to a xylan synthase-associated polypeptide from French bean, the enzyme which synthesises the core polysaccharide for secondary wall xylan, has been raised and shown to inhibit its activity. Xylan is deposited in secondary thickenings and the xylan synthase was only detected in appreciable amounts in developing xylem cells. The location within the Golgi stack was observed throughout the dictyosomes. Some enzyme subunits were also detected in post-Golgi vesicles. A second antibody to a non-catalytic M_r 65 000 subunit of β 1,3- glucan (callose) synthase was used for a comparative study. Although the bulk of this enzyme has been detected in previous studies at plasmamembrane-wall interfaces in sieve plates and stressed tissue, a Golgi-location can be observed in root tip meristematic cells during cell plate formation. The enzyme was present throughout the stacks. Callose was also immunolocated in a similar manner to xylan in secondary walls and thickenings and in pits in developing xylem. In these cells, the callose synthase was detected at the surface of the growing thickenings and the plasmamembrane within the pits.

Keywords: *Phaseolus vulgaris*; Leguminosae; Xylogenesis; Cell wall polysaccharides; Xylan synthase; Callose synthase; Immunolocation

1. Introduction

Despite the fact that plant cells synthesise and secrete mainly polysaccharides into the cell wall, it is only very recently that molecular data has become available for the biosynthetic enzymes involved, following the cloning of the first glycosyl transferases involved in cell wall synthesis (Pear et al., 1996; Arioli et al., 1998; Perrin et al., 1999; Edwards et al., 1999). Similarly, there is a lack of knowledge of the precise subcellular localisation of these enzymes, although it has been long realised that cellulose and callose biosynthesis takes place at the cell surface while the matrix polysaccharides, pectins, hemicelluloses and some glucans are synthesised and exported from the Golgi (Bolwell, 1993). These earlier biochemical studies involved subcellular fractionation. However, understanding regulation of polysaccharide biosynthesis subcellular localisation is important, since there is an indication that components of the pathway may be spatially and temporally separated. Increasingly, immunolocation (Bolwell, 1993, 2000; Dhugga et al., 1997) and heterologous expression (Wee et al., 1998; Palapac et al., 1999) have been used to elucidate targetting of proteins involved in cell wall biosynthesis. The present hypothesis for the regulation of matrix polysaccharide synthases is that the enzymes of UDP-sugar provision are soluble (Robertson et al., 1995) or loosely membrane associated (Amor et al., 1995) and, although subject to some regulation, sufficient levels are present in cells to maintain a substantial pool of intermediates. However the flux, measured using radioactive precursors, through the pathway can increase under certain

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developmental and environmental conditions (Robertson et al., 1999). In contrast, there is biochemical evidence that the expression of the Golgi-localised polysaccharide synthases, for example, xylan synthase (Bolwell and Northcote, 1983; Blee, et al., 2001), is tissue specific (Bolwell, 1993, 2000). Thus, the qualitative production of wall polysaccharides would be dependent on gene expression and turnover of the synthases and the process is tightly developmentally controlled. If this hypothesis is correct, then synthases should only be detectable in Golgi in cells at the relevant developmental stage and the full complement of enzymes involved in cell wall polysaccharide biosynthesis would not be expressed at any one time.

The present work concerns the location of two polysaccharide synthases in Golgi membranes in healthy French bean tissue. A xylan synthase has been previously characterised in Golgi membranes from hypocotyls of French bean (Bolwell and Northcote, 1983) from which it was subsequently purified to homogeneity (Rodgers and Bolwell, 1992). Besides its purification to a single band of M_r 40 000 observed by silver staining on a SDS acrylamide gel, the work also described the partial purification of a second xylosyl transferase and an arabinosyl transferase. Xylan itself has been immunolocated in cell walls of French bean suspension cultured cells stimulated to form secondary walls (Robertson et al., 1999) and in secondary thickenings of xylem vessels in bean stems (Northcote et al., 1989). Although a small amount of xylan is synthesised in the primary wall, the bulk of the synthesis occurs during differentiation of vascular cambium in the stem.

A second polysaccharide synthase involved in callose synthesis has been purified from the French bean (McCormack et al., 1997). This was purified as a dimer of M_r 55 000 and M_r 65 000 polypeptides. This biochemical approach to purification and identification of callose synthase has recently been qualified by the identification of plant homologues of the yeast β 1,3-glucan synthase FKS gene family in cotton, Arabidopsis and Nicotiana (Cui et al., 2001; Doblin et al., 2001; Hong et al., 2001). Since the cognate protein for these would have a potential M_r of around 200 kDa this is clearly at variance with the calcium-dependent pea and bean enzymes (Dhugga and Ray, 1994; McCormack et al., 1997) but not with the calcium-independent pollen tube enzyme (Turner et al., 1998). Peptides of around 200 kDa were not observed in abundance in preparations of the bean enzyme. However, the co-purifying 55 and 65kDa subunits could be chemically cross-linked and immunoprecipitated using an anti-(65 kDa callose synthase-associated polypeptide) IgG (Kerry, 2000) indicating their presence as two components of the callose synthase complex at least.

Immunolocation of callose synthase, using the antibody to the M_r 65 000 non-catalytic subunit as a marker has been reported in infected tissue in plasmodesmata and paramural deposits at infection sites in intimate association with its product, callose, using a double labelling technique (Brown et al., 1998). In healthy tissue, the M_r 65 000 subunit similarly used as a marker for callose synthase was also located in sieve plates of phloem and at the cell plates in dividing cells (McCormack et al., 1997). A monclonal antibody has also been used to localise an M_r 65 000 polypeptide that associated cationically with callose synthase (Delmer et al., 1993), but it is unknown whether the antigens are identical. In cells forming cell plates, the callose synthase enzyme subunits were also detected in the Golgi but at low resolution due to the fixation procedure, which is necessary to preserve antigenicity of the protein (McCormack et al., 1997). In contrast, a combination of rapid freezing and freeze substitution without a fixation stage, which is not required to preserve antigenicity of the polysaccharide, has shown high resolution localisation of the product in forming cell plates (Samuels et al., 1995). The presence of the enzyme in this compartment is intriguing since it may only be active at the cell surface (McCormack et al., 1997; Turner et al., 1998). It may mean that it is detected in transit but if it were functional in the Golgi, it may not necessarily indicate that callose would be the product.

There is evidence for differentiation of membranes of the Golgi stack in their component enzymes (Dupree and Sherrier, 1998). Some of this evidence derives from studies of the transit of the products of polysaccharide synthases (Moore et al., 1991). This is essentially indirect evidence and the direct location of xylan and callose synthase subunits in the Golgi stack is demonstrated in this study using the improved technique for tissue preparation of freeze substitution. This better structural resolution gives further insights into the compartmentation of these processes. In addition, callose could be detected in secondary walls of xylem, a rarely mentioned location since first described (Currier, 1957) and in pits and the callose synthase was detected at the surface in these locations.

2. Results

2.1. Co-immunolocation of xylan and callose in secondary walls and thickenings of developing xylem

Immunolocation of polysaccharides has been reinvestigated using an improvement on previous techniques used by us, which involves freeze substitution in tissue preparation to improve membrane integrity (Wojtaszek et al., 1999). An initial chemical fixation is still required to preserve antigenicity, however, and the preservation of structures is not as good as true freeze substitution (Samuels et al., 1995). Use of antiserum to

xylan located xylan to secondary wall and thickenings (Fig 1a,b). This has been reported previously (Northcote et al., 1989), but is repeated here to emphasise the exclusive association of xylan with secondary thickenings (Fig. 1a) and wall (Fig. 1b) with the absence of any gold particles over the primary wall. The other reason is to show co-localisation with callose (Fig. 1c,d) in these two structures. In Fig. 1c, the callose can be associated with the darker fully structured thickening, and also with a layer of electron translucent material which is an indication of the presence of callose (Brown et al., 1998). This indicates that callose may still be added at a late stage even when the thickening has had xylan (shown by immunolocation) and lignin (as suggested by electron dense deposits) deposited. This clear absence of callose from primary wall is reminiscent of its deposition in

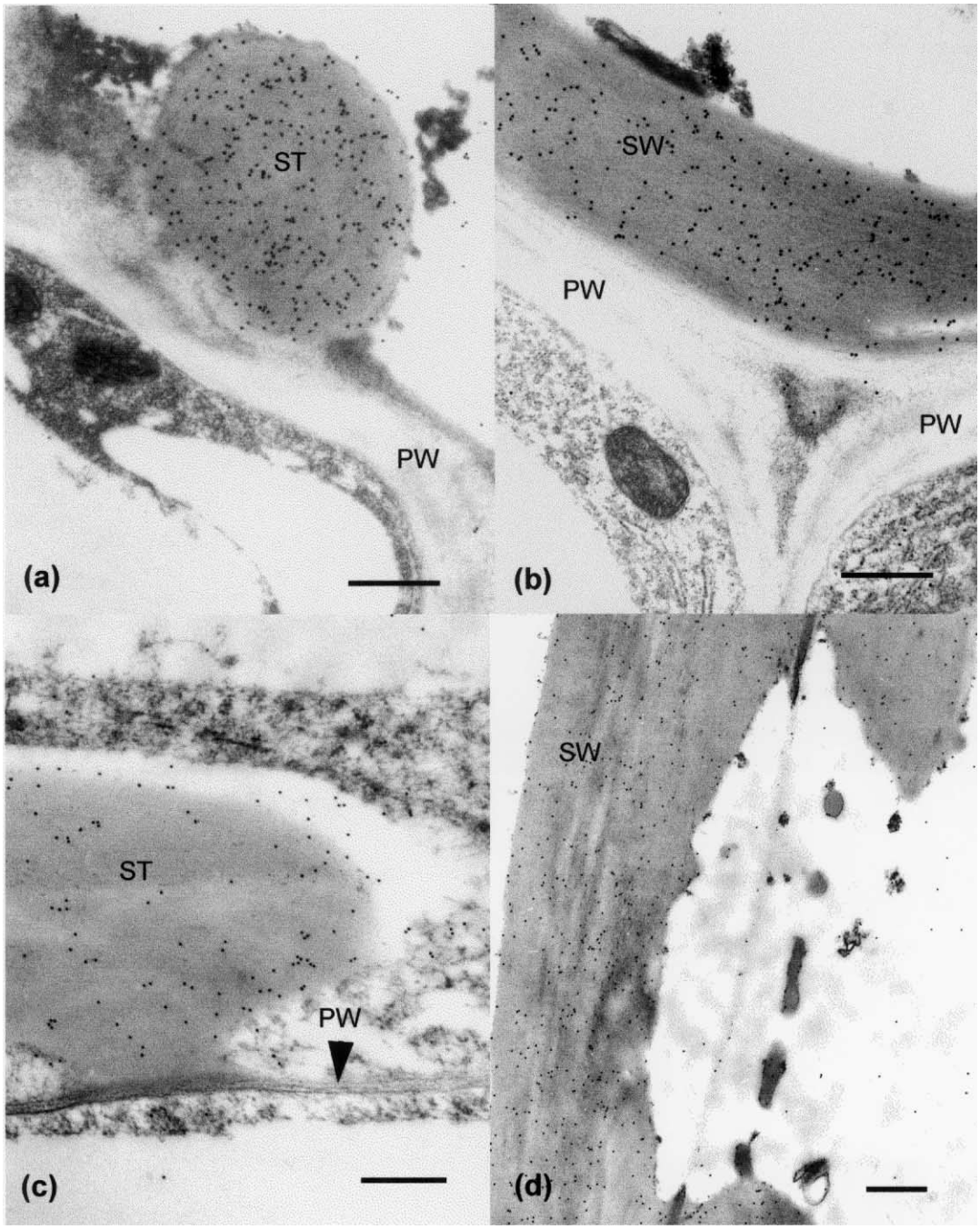

Fig. 1. Co-immunolocation of callose and xylan in secondary thickenings. (a) Detection of xylan in a single secondary thickening in a developing xylem vessel. (b) Detection of xylan in more extensive secondary wall. Note in (a) and (b) the virtual absence of xylan in primary wall. (c) Example of callose deposition in secondary thickenings. Note detection of callose in secondary thickening material which is probably lignified (more electron dense material) and on the surface of this in translucent material. (d) Even distribution of callose in a continuous stretch of secondary wall in a developing xylem vessel. PW, primary wall; ST, secondary thickening; SW, secondary wall; bars = 0.6 μm.

papillae on the inside of primary walls of the French bean challenged by pathogens (Brown et al., 1998). Callose is mainly reported to be confined to sieve plates and pits of phloem in vascular tissue. However, there have been occasional reports of callose being localised to "spiral thickenings" of some vessels in leaf and young stem of *Phaseolus vulgaris* and *Cucurbita pepo* (Currier, 1957). Aniline blue was used in the previous study and we now confirm the validity of this observation using highly specific antibodies to callose (Brown et al., 1998). The density of labelling was greater than might be expected, suggesting that the process of lignification may modify the intensity of staining seen with aniline blue and the amount of callose in secondary wall may have hitherto been underestimated. The enzymes responsible for the synthesis of xylan and callose were immunolocated in the light of these findings.

2.2. Characterisation of antisera

Xylan synthase was purified by the HPLC method described by Rodgers and Bolwell (1992) and a rabbit polyclonal anti-(French bean xylan synthase-associated polypeptide) serum raised. This proved to be inhibitory towards both the membrane-bound enzyme and following solubilisation (Fig. 2). Complete inhibition could be achieved for titres up to 1:100. Western blotting of intact microsomes and solubilised microsomes prepared from French bean hypocotyls undergoing differentiation showed only a background staining indicating a low abundance of the xylan synthase in these subcellular fractions. However, partial purification of the enzyme as far as the first chromatographic step in the protocol (Rodgers and Bolwell, 1992), allowed strong detection of the antigen. Fig. 3 shows fractionation of xylan synthase by HPLC on DEAE that had been solubilised from microsomal membranes prepared from French bean hypocotyls at a stage when they are expressing the form of xylan synthase, designated XS2 (Rodgers and Bolwell, 1992) which is associated with secondary wall formation. Fig. 3A shows the elution profile over the period 18-34 min indicating desorption of the xylan synthase activity at 24–26 min. Fig. 3B shows the different banding pattern of polypeptides in each of the fractions collected every 2 min. Fig. 3C shows a Western blot of each of the active fractions. There is clear cross reactivity with a series of bands in the region of M_r 50 000 only in the fractions exhibiting xylan synthase activity. However, there is some background in all fractions, which includes the well-characterised artifact of SDS/PAGE and western blots at M_r 65 000/60 000. This is due to keratin contamination and the existence of anti-(human keratin) in the rabbit serum. We have previously noted the existence of this in preparations of xylan synthase and have been unable to eliminate it (Rodgers and Bolwell, 1992). There are other apparent

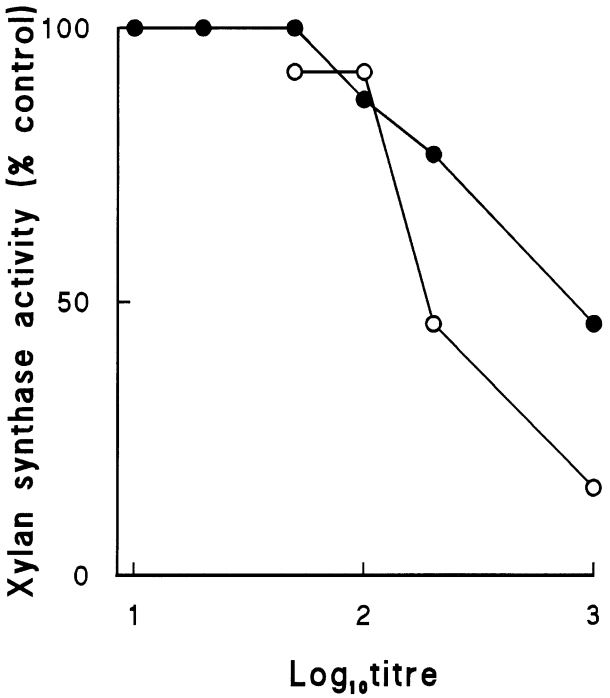

Fig. 2. Inhibition of xylan synthase activity by anti-(bean xylan synthase) serum. Microsomal (●) and soluble microsome (○) fractions were incubated with a titration series of serum for 15 min, before addition of substrate. Reactions were allowed to proceed for 1 h before determination of activity.

bands in the Western blot but which are present in all fractions with identical patterns. Such artifacts are common with preparations of membrane bound enzymes and are associated with use of detergents. However, we conclude that they are artifacts since, (1) they are present in all fractions even ones (34 min) after all the protein has apparently eluted, and (2) the banding patterns of individual fractions are very different and do not contain bands of the M_r of the artifacts that consistently appear in all fractions following Western blotting.

Specific cross reactivity is seen for bands that correspond in intensity to the distribution in xylan synthase activity eluting in fractions collected at 24, 26 and 28 min. The major band recognised by the antiserum and corresponding in intensity to the xylan synthase activity is M_r 50 000. This M_r is consistent with and within the range found for the type I and II membrane glycosyl transferases so far cloned (Edwards et al., 1999; Perrin et al., 1999). Lower M_r bands of M_r 46 000 and 40 000 that also correspond in intensity to the distribution of xylan synthase in the fractions are apparent in much lower abundance. In fact, the anti-(xylan synthase) serum was raised against active fractions following further purification beyond the DEAE purification stage, involving size exclusion and a reverse phase concentration step. The single band in these preparations revealed by silver staining has been previously reported to be M_r 40 000

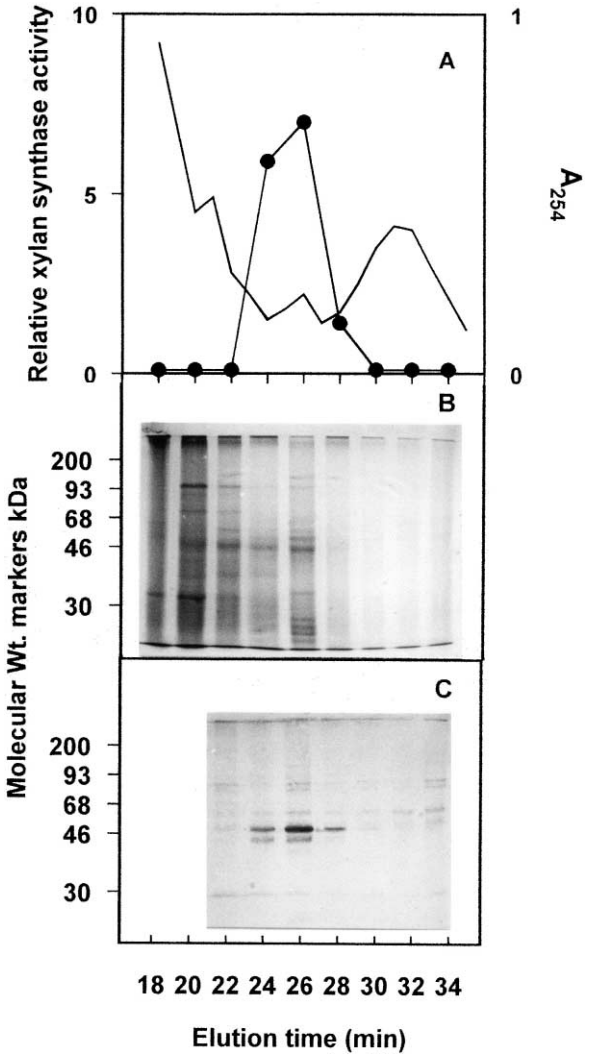

Fig. 3. Analysis and Western blotting of partially purified xylan synthase. Xylan synthase was purified from microsomes from 100 g of 13 day hypocotyls of French bean as far as the DEAE-stage (Rodgers and Bolwell, 1992). Fractions were taken and subjected to (A) determination of activity (●) and protein (—); (B) SDS/PAGE followed by staining with Coomassie Brilliant Blue and (C) Western blotting using anti-(French bean) xylan synthase with alkaline phosphatase detection. Note only the fractionation during the period of xylan synthase elution is depicted. The A254 nm peak at 32 min is mainly detergent.

(Rodgers and Bolwell, 1992). A likely explanation for this is proteolysis and that this is already occuring at the DEAE stage. Evidence from the French bean suggests that this proteolysis may be integral to regulation so that under certain circumstances xylan synthase may have a half-life as short as 2 h (Robertson et al., 1995). Furthermore we have observed this for other proteins from the French bean. Phenylalanine ammonia lyase (PAL) was initially purified as an enzymically active M_r 70 000 polypeptide product of proteolysis which was used to raise an antiserum. This recognised an M_r 77 000 native PAL enzyme subunit. Improved purification protocols enabled the purification of the M_r 77 000 form subsequently (Bolwell et al., 1985). We therefore conclude that it is likely that the antibody is specific and inhibitory towards a xylan synthase of M_r 50 000 in its intact and native state. However the possibility that that the detection of a 50 kDa polypeptide by the antiserum is a result of it being a minor component of the purified 40 kDa antigen and the enzyme itself is a multi-component complex is not dismissed completely. In this case, the 50 kDa polypeptide would constitute a xylan synthase-associated polypeptide but the antiserum would still be valid to immunolocate the enzyme. Furthermore, it should be noted that the presence of reactivity towards artifacts on Western blots in which the proteins are denatured does not negate the use of antisera in immunolocation, where the antigen is in its native state.

2.3. Immunolocation of xylan synthase associated polypeptide in Golgi bodies associated with secondary wall synthesis and secretion

Numerous pieces of work carried out by us on the French bean and potato (Shaw et al., 1990; Millar et al., 1992a,b; Smith et al., 1994; Robertson et al., 1996; Wojtaszek and Bolwell 1995; McCormack et al., 1997; Brown et al., 1998; Wojtaszek et al., 1999) support the contention that the methodologies used are not subject to artifactual locations when the antiserum is raised against native soluble proteins or membrane bound proteins that have been solubilised and demonstrated to be active. The antigen was in insufficient general location to allow low power images to be generated of vascular tissue location unlike other vascular-specific antigens in the French bean (Millar et al., 1992a,b; Smith et al., 1994; Robertson et al., 1996; Wojtaszek and Bolwell, 1995). At the subcellular level, the xylan synthase-associated polypeptide was detected in Golgi in cells forming secondary walls in vascular tissue of bean hypocotyls. Binding of immunogold particles was only detected in planes of section that included Golgi fields; there was no binding to any other part of the cell explaining the lack of detection above the subcellular levels. Figs. 4 and 5 show improved preservation of Golgi structure by including a freeze substitution stage. This methodology has previously aided localisation of plant prolyl hydroxylase to pre-Golgi vesicles (Wojtaszek et al., 1999). The xylan synthase-associated polypeptide is localised in vesicles on both the *cis*- and *trans*-Golgi side. There has been speculation that polysaccharide synthases show compartmentation wthin the Golgi stack (reviewed by Dupree and Sherrier, 1998). In the case of the xylan synthase from the images in Fig. 4a–d and many other images there is no evidence for such a compartmentation. The density of labelling on the stacks suggests that dwell time is short. Presence of the enzyme in vesicles close to the wall suggests that it traverses the stack and is present in secretory vesicles

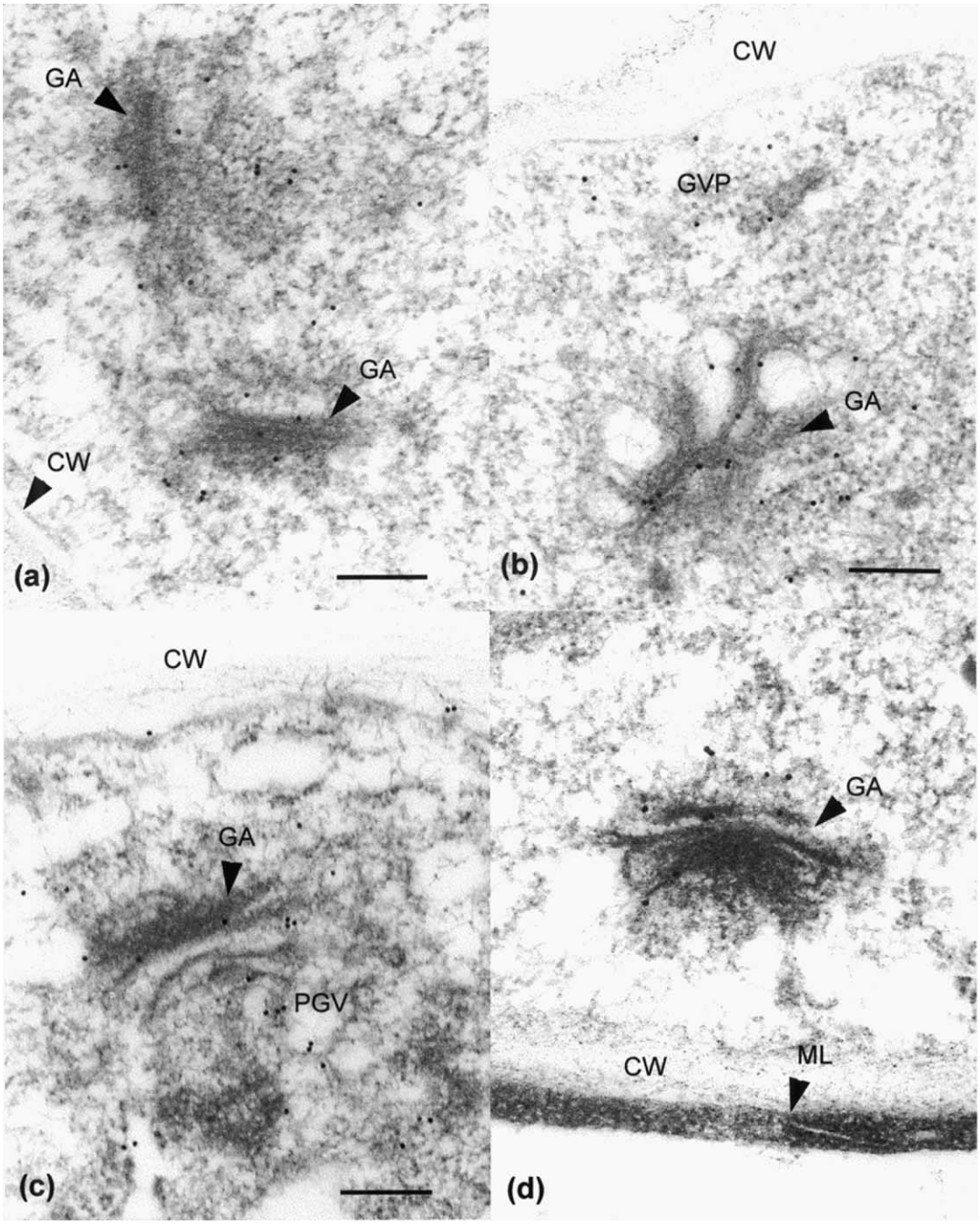

Fig. 4. Immunolocation of xylan synthase. (a–d) Examples of Golgi localisation in developing xylem cells in stem of French bean. Note the material was processed by freeze substitution, which improves the preservation of Golgi membranes and associated vesicles. CW, cell wall of indeterminate origin; GA, Golgi apparatus; GVP, post-Golgi vesicle; ML, middle lammella; PGV, pre-Golgi vesicles. Bars = 0.25 μm.

(Fig. 4b,c) so that synthesis of xylan could continue. However no immunogold particles were found associated with the wall per se. In order to investigate this hypothesis further we carried out a comparative study using an anti-(M_r 65 000 callose synthase-associated polypeptide) serum where this has been associated with the wall (McCormack et al., 1997; Brown et al., 1998).

2.4. Comparative immunolocation of an M_r 65 000 callose synthase-associated polypeptide during cell plate formation

The antiserum to the M_r 65 000 component of β 1,3-glucan synthase (callose synthase) has been previously characterised (McCormack et al., 1997; Brown et al., 1998) with respect to recognition of the antigen and its

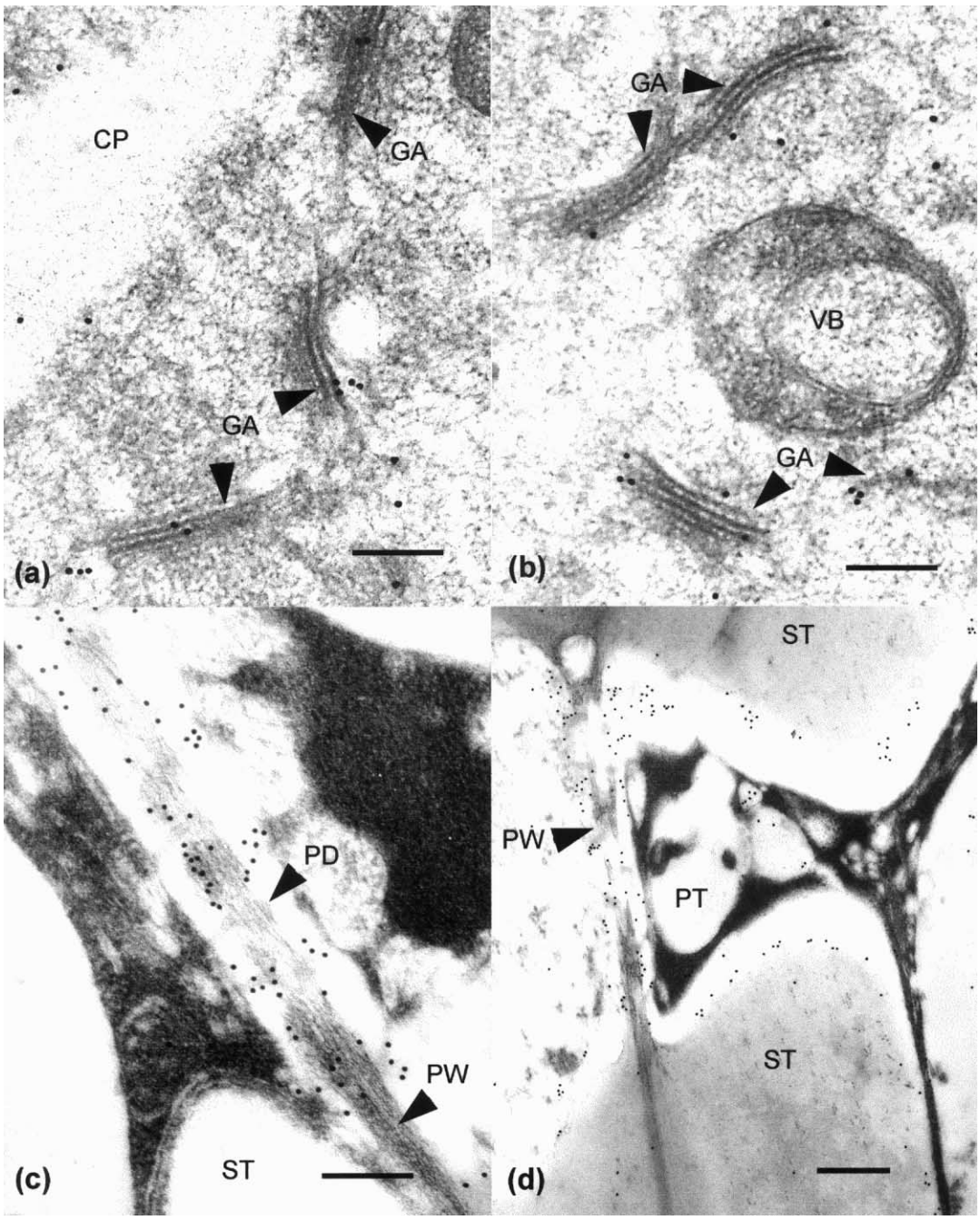

Fig. 5. Localisation of callose synthase in healthy plant cells. (a,b) Detection of callose synthase in Golgi in dividing root-tip cells. The use of freeze substitution gives improved Golgi preservation. (c) Detection of callose synthase associated with a plasmodesmatal connection in a pit in a developing xylem vessel. Gold labelling is associated with the plasmadesmata as is found in primary walls (Brown et al., 1998). A further example but showing that it is further localised to callose deposits on the associated secondary wall. CP, cell plate; GA, Golgi apparatus; PW, primary wall; PD plasmodesmata; PT, cell wall pit; ST, secondary thickening; VB, vesicular body. Bars = (a–c), 0.25 μm; (d), 0.5 μm.

validity as a marker for the complex and immunolocation in stressed tissue. This work has located this polysaccharide to plasmamembrane-wall interfaces in cells actively synthesising callose. We did note however that in unstressed root tissue there was binding to Golgi bodies and this has been further investigated using an improvement on our previous technology by including a freeze substitution step (Fig. 5a,b). This gave a much-improved resolution and allowed visualisation of the enzyme in the stacks. The density of labelling was greater but once again label was found throughout the stacks when many such images were examined. In this case the evidence from the immunlocation of the polypeptides used as markers for the respective enzymes is

greater than in the case of the xylan synthase that the callose synthase traverses the stacks and arrives at the plasmamembrane where the cell plate is forming in these cells.

2.5. Detection of callose and callose synthase-associated polypeptide in secondary thickenings in xylem

In connection with our studies in the location of callose and the callose synthase-associated polypeptide in infected bean plants (Brown et al., 1998), callose was detected in secondary thickenings of tracheids (Brown, I., Mansfield, J.W., Bolwell, G.P., unpublished data). The present work now reports their co-localisation during the assembly of these structures in healthy tissue. This shows the specific detection of callose in Fig. 1c, d and suggests the occurrence of callose may be more extensive than when detected using aniline blue (Currier, 1957). In the vascular tissue at this stage of development we were unable to detect the marker for callose synthase in the Golgi which exhibited the presence of that for xylan synthase. However, Fig. 5c,d shows the presence of callose synthase within the callose deposits of pit wall (Fig. 5c) and the adjacent secondary wall (Fig. 5d). Although double labelling was not performed, the co-localisation of enzyme within the callose deposits is similar to the situation found with papilla formation (Brown et al., 1998). This detection of callose in secondary thickenings has not been shown previously with this amount of certainty. In contrast, the occurrence of callose in sieve plates of phloem is well recognised. It is interesting that secondary thickenings and sieve plates, which form in cells that undergo programmed cell death as paramural deposits, contain callose in the same way as other paramural deposits such as papillae and tyloses do in infected tissue (Brown et al., 1998).

3. Discussion

Relatively few polysaccharide synthases involved in cell wall biosynthesis have been characterised at the biochemical and cellular level in plants despite their importance and diversity of action. However there is much speculation about their subcellular location, site of action and developmental regulation. Available evidence, much of it indirect and circumstantial, suggests that they are expressed in a tissue-specific manner and are localised to the Golgi only during the period of actual synthesis of the product (Bolwell, 1993). They are also subjected to turnover with a half-life which can be as short as 2 h under certain circumstances (Robertson et al., 1995). With respect to Golgi localisation, there is evidence for compartmentation in the Golgi stack from biochemical fractionation studies (Baydoun and Brett, 1997), location of products of glycosylation (Moore et al., 1991; Lerouge et al., 1998; Vicre et al., 1998) and location of heterologously expressed glycosyl transferases (Wee et al., 1998; Palapac et al., 1999). The present work investigates these hypotheses with respect to a xylan synthase involved in secondary wall formation in hypocotyls of the French bean and a callose synthase, as detected using antibodies to a non-catalytic M_r 65 000 subunit of the complex, similarly associated with secondary cell wall synthesis and also cell plate formation in root cells.

Limitation of detection to cells in tissues expressing the designated product supports the proposition that polysaccharide synthases are expressed in a cell specific manner. Thus in Figs. 4 and 5 with the use of freeze substitution, it is possible to discern the *cis* and *trans* cisternae on the basis of decreasing width of the stacks (Moore et al., 1991; Winicur et al., 1998). Despite ideas of compartmentalisation of polysaccharide synthesis in the stacks, both enzymes were found physically throughout the stacks. Both appeared to traverse since they were also found in pre- and post-Golgi vesicles. However, since there is some data showing immunogold labelling of differentially localised polysaccharide products in stacks as they traverse the Golgi (Moore et al., 1991), this probably indicates that there is subtle regulation going on that limits the activity of the enzymes in the stacks. This could include the role of nucleotide sugar transporters for which there is growing evidence for multiple forms (Wulff et al., 2000).

The detection of both the xylan synthase-associated polypeptide in Golgi bodies and, callose synthase-associated polypeptide at the surface of secondary walls in xylem-forming cells in healthy tissue adds to our understanding of secondary wall synthesis. In the case of the xylan synthase this was confined to these cells. Xylogenesis involves programmed cell death (PCD) as does the hypersensitive response. In the latter, paramural deposits, the papillae, contain abundant amounts of callose which co-localises with the M_r 65 000 subunit of the callose synthase complex (Brown et al., 1998). The synthesis was so rapid that the enzyme became embedded in the product. The occurrence of detectable callose in secondary thickenings, which like papillae are paramural deposits that arise by directed secretion, has not been reported previously at this level of precision. The distribution of the enzyme and product was similar to those found in papillae emphasising some similarities between xylogenesis and hypersensitive cells. The other major glucan in secondary walls is, of course, cellulose and this appears to be the product of a distinct group of cellulose synthases (Taylor et al., 1999). Although it is probably accepted now that callose synthase is a different enzyme certainly with the discovery of FKS1 homologues in plants (Cui et al., 2001; Doblin et al., 2001; Hong et al., 2001) the exact relationship between β 1,4- and β 1,3-synthesising capacity of glucan synthases requires further functional genomic studies. Bio-

chemically, the possibility and extent of dual specificity still exists (Kerry et al., 2001). It has long been recognised that secondary wall synthesis involves increased deposition of cellulose, however, the presence of callose in secondary wall and surrounding pits indicates a further complex regulation of synthesis of the two respective glucans.

4. Experimental

4.1. Materials

Seedlings of French bean (*Phaseolus vulgaris* L. cv. The Prince; Nickersons, Lincoln, UK) were grown in vermiculite under a 16 h light, 8 h dark regime at 15 °C. Uniformly labelled UDP-[^{14}C]-xylose (11 GBq.mmol^{-1}) was obtained from NEN, Stevenage, UK. Derivation and characterisation of anti-(bean callose synthase) IgG was described in McCormack et al. (1997).

4.2. Assay and purification of xylan synthase

Incubation conditions using radiolabelled substrate were as described previously (Bolwell and Northcote, 1981, 1983). Product determination was carried out by high voltage paper electrophoresis in 8% acetic acid 2% formic acid (w/w) also previously described (Bolwell and Northcote, 1981), or by precipitation and multiple washes of the polymer with cold 70% ethanol containing 1 mM EDTA before (Bolwell and Northcote, 1983) determination of radioactivity by liquid scintillation counting. Xylan synthase was purified by the HPLC method described by Rogers and Bolwell (1992). The enzyme was batch purified from approximately 200 g of 13-day-old bean hypocotyls. Batches of purified enzyme were combined for derivation of antiserum.

4.3. Derivation of antiserum

Purified xylan synthase in Freund's incomplete adjuvant was administered subcutaneously to a rabbit. For the initial immunisation, 100 μg was used followed by two booster immunisations of the same amount.

4.4. Analysis of anti-(bean xylan synthase) serum

Antiserum was characterised by Western blotting after SDS/PAGE. IgG binding was carried out using goat anti-(rabbit IgG)-serum conjugated to alkaline phosphatase. Cross-reactivity was determined for the partially purified enzyme since the xylan synthase was not readily detected in crude extracts or membrane prepapration. Briefly, solubilised microsomal membrane preparations (Rodgers and Bolwell, 1992) were subjected to HPLC on anion exchange using a 7.5×75 mm TSK DEAE-5PW column (Anachem, Luton, UK). Samples were loaded in 20 mM Tris pH 7.2, 1% reduced Triton X-100, and eluted using a linear gradient of zero to 0.75 M sodium chloride over 30 min at an overall flow rate of 0.5 ml/min. Fractions were collected and microdialysed against 20 mM Tris pH 7.2, 10 mM magnesium chloride, 1% reduced Triton X-100, 1.4 mM mercaptoethanol at 4 °C for 1 h before being assayed for xylan synthase activity. Fractions were concentrated and subjected to SDS/PAGE and western blotting.

4.5. Preparation of tissue using freeze substitution and immunostaining

French bean hypocotyl sections were excised from 13 day old plants of French bean and fixed in freshly prepared 1% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 0.05 M sodium phosphate buffer (pH 6.8) at 4 °C for 2 h. Young root tips were harvested and fixed similarly. Following an overnight incubation in 0.05 M sodium phosphate buffer (pH 6.8), fixed stem and root material was subjected to a series of incubations in 10, 20 (1 h each) and 30% (overnight) glycerol in 50 mM sodium phosphate buffer pH 6.8 at 4 °C. The tissue was then plunged into liquid N_2 under vacuum and transferred in liquid N_2 to an Ambient Freeze Substitution apparatus (Leica, UK). The tissue was incubated sequentially in: dry methanol, methanol containing 1% uranyl acetate and methanol at −90 °C for 12 h each which was followed by incubation in methanol at −90 °C for 72 h with changes of methanol every 24 h. After increasing the temperature to −50 °C, methanol was replaced with Lowacryl HM20 resin (Polysciences, Warrington, PA) and infiltrated for 72 h with several changes of resin. Infiltrated tissue was transferred to gelatin capsules, the fresh resin was added and allowed to polymerise at −50 °C. Following gradual increase of temperature to reach ambient over the period of 24–48 h, the tissue was left at this temperature for the next 24 h to complete polymerisation. Ultrathin sections were transferred to nickel grids. Immunogold labelling was carried out by first incubating sections at room temperature in 20 μl of 3% bovine serum albumin (BSA)/1% normal goat serum (v/v) in 20 mM Tris/HCl buffer (pH 7.6) containing 0.23 M NaCl (TBS) for 20 min. Excess solution was then drained from the section and replaced with 20 μl of either anti-(bean M_r 65 000 glucan synthase polypeptide) IgG at 1:300 dilution, or anti-(bean xylan synthase) serum at 1:1500, or anti-(callose) serum [Genosys Biotechnologies (Europe) Inc, Cambridge, UK] at 1:250, or anti-(xylan) serum (Northcote et al., 1989) at 1:600 in TBS-BSA-normal goat serum containing 0.05% Tween 20. They were then incubated for 18 h at room temperature. Sections were then washed thoroughly and repeatedly for 5 min in

TBS (pH 7.6) and the area around the sections carefully dried. Sections were immersed in 20 μl goat anti-rabbit IgG conjugated to colloidal gold (British Biocell International, Cardiff, UK; 5 nm particle size; 1:200 working solution in TBS-ovalbumin-normal goat serum) for 60 min at room temperature. The grids were then washed thoroughly with TBS followed by distilled water and dried. Silver enhancement of the colloidal gold was carried out using the Biocell SE kit (British Biocell International, Cardiff, UK) for 2 min at room temperature. Following a thorough washing with distilled water, the grids were counterstained with 2% aqueous uranyl acetate and lead citrate.

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