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Xylose-specific antibodies as markers of subcompartmentation of terminal glycosylation in the Golgi apparatus of sycamore cells

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Antibodies specific for xylose-containing plant complex *N*-linked glycans are used for indirect immunolocalization of xylosyltransferase in sycamore cells. The use of high pressure freezing and freeze substitution for sample preparation resulted in very good morphological preservation of the different Golgi cisternae. Xylosyltransferase shows a diffuse distribution all over the Golgi stacks and xylosylation appears to be an early processing event that is initiated in the *cis* Golgi compartment.

Xylosyltransferase immunolocalization; Plant glycoprotein biosynthesis; Plant glycosyltransferase; Plant Golgi apparatus

1. INTRODUCTION

Plant glycoprotein *N*-linked oligosaccharides are sequentially processed from oligomannose-type to complex-type during their intracellular transport through the secretory pathway giving rise to a large variety of mature oligosaccharide sidechains [1–3]. The sequence of events by which complex glycans are built is not as well known in plant cells as it is in animal cells. In animal cells, complex-type oligosaccharides are mainly processed in the Golgi apparatus by a sequence of events following from *cis* to medial to *trans* cisternae [4,5].

The substrate specificities of Golgi processing enzymes responsible for complex glycan biosynthesis have been described in several plant systems [6,7] and a number of overlapping sequences are possible for terminal glycosylation of plant complex glycans on the basis of their specificities. Consequently, a subcellular localization of these processing enzymes would be necessary for the definition of the sequential glycan processing events in plant cells. Unfortunately, fractionation of plant Golgi membranes by density gradient centrifugation has shown that the compartmentation of enzymes involved in glycoprotein oligosaccharide processing was not as clear as in animal cells [2,8].

Complex oligosaccharides of plant glycoproteins often differ from those of mammals in that they (i) lack sialic acid, (ii) have an L-fucose residue $\alpha 1 \rightarrow 3$ linked to the *N*-acetylglucosamine residue at the reducing end of the asparagine-linked chitobiose unit, and (iii) have a xylose attached $\beta 1 \rightarrow 2$ to the β-linked mannose. In the present paper, we have used immunocytochemical labeling to study the compartmentation in the Golgi apparatus of the plant-specific xylosyltransferase. Using antibodies specific for xylose-containing plant complex glycans [9,10], we have visualized the subcellular distribution of glycoproteins harboring these complex glycans in high-pressure frozen and freeze-substituted sycamore cells. Although this immunochemical approach only indirectly localizes a glycosyltransferase by mapping its oligosaccharide products, our results are entirely consistent with the conclusion that xylosyltransferase in sycamore cells has a diffuse distribution all over the Golgi stacks, including the *cis* Golgi cisternae.

Abbreviations: GlcNAc, *N*-acetylglucosamine; Man, mannose; Xyl, xylose; Fuc, L-fucose; Gal, galactose; RER, rough endoplasmic reticulum; TGN, *trans* Golgi network.

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2. MATERIALS AND METHODS

2.1. *Materials*

Sycamore suspension-cultured cells (*Acer pseudoplatanus*) were grown in a M6PT medium [11] or in the medium of Lamport as modified by Lescure [12]. An immune serum specific for xylose-containing plant complex glycans was raised in rabbits by injecting carrot cell wall β-fructosidase [13]. Before its use for immunolabeling, this immune serum was further purified on a phospholipase A2-Sepharose column to remove L-fucose-specific antibodies from a serum which was largely anti-xylose. Protoplasts were prepared from sycamore cells as described in [14]. Culture medium, cell wall and protoplast extracts were prepared from sycamore cell cultures according to [15].

2.2. *SDS-PAGE, affinodetection and immunodetection*

Polypeptides were separated by SDS-PAGE according to Laemmli [16] and transferred to a nitrocellulose membrane (Schleicher & Schuell, BAS 45) prior to detection. Immunodetection and affinode-

tection, using the ConA/peroxidase method, were carried out as previously described [17,18].

2.3. *High-pressure freezing, freeze substitution and immunolabeling*

Sycamore cells were frozen in a Balzers HPM 010 high-pressure freezing apparatus as described in [19]. The samples were freeze-substituted in acetone containing 2% (w/v) osmium tetroxide at −80°C for 2.5 days, then gradually warmed up to room temperature and embedded in Epon (Electron microscopy Sciences).

After quenching with 5% non-fat-dried milk in 10 mM sodium phosphate buffer, pH 7.2, containing 0.5 M NaCl, 0.1% Tween 20 (PBST1), thin sections were incubated overnight with the anti-xylose serum diluted 1:10 in PBST1. After several washes in 10 mM sodium phosphate buffer, pH 7.2, containing 0.5 M NaCl, 0.5% Tween 20 (PBST5), grids were incubated for 30 min in the presence of protein A-gold conjugate (PAG10, Bio Cell) diluted 1:50 in PBST1. After incubations, sections were stream washed with PBST5, rinsed in distilled water and dried. Eventually, sections were stained with uranyl acetate and lead citrate, before being observed with a Phillips CM10 or a Zeiss EM109 electron microscope.

3. RESULTS AND DISCUSSION

3.1. *Characterization of a xylose-specific antiserum*

A major limitation in the use of lectins or glycan-specific antibodies to study the distribution of glycoprotein oligosaccharides in the different Golgi cisternae of plant cells is that the Golgi apparatus in plants not only matures glycoprotein glycans, but in addition synthesizes complex polysaccharides found in cell walls, such as pectins and xyloglucans [20]. Consequently, lectins and carbohydrate-specific antibodies used as markers of Golgi subcompartments in plant cells may react not only with glycoprotein glycans, but also with precursors of cell wall polymers.

Xylose-specific antibodies used in the present study were purified from an immune serum obtained by immunizing a rabbit with native β-fructosidase from carrot cell wall [9,10]. It has been previously shown that the anti-glycan antibodies in this immune serum bind primarily to the xylose β1→2 linked to the β-linked mannose of the core in the complex-type oligosaccharide of carrot cell wall β-fructosidase. This glycoprotein has a complex glycan that is identical to an oligosaccharide structure commonly found in plant glycoproteins [1] and recently described for phytohemagglutinin A (PHA) ([21], Sturm, unpublished result). Antibodies specific for plant complex glycans have been purified on a PHA- Sepharose 4B column [9] whereas we used a phospholipase A2-Sepharose 4B column to isolate xylose-specific antibodies from this glycan-specific antibody fraction containing a minor amount of L-fucose-specific antibody. Phospholipase A2, a glycoprotein from honeybee venom, has a glycan with α1→3 and/or α1→6 linked fucose residue(s), but no xylose as shown on Fig. 1C [22]. Fucose-specific antibodies retained on the phospholipase A2-Sepharose 4B column are able to bind phospholipase A2 glycan or pineapple stem bromelain glycan on a blot (Fig. 2, right panel) whereas xylose- specific antibodies, unretained on this column, bind bromelain but do not detect phospholipase A2 on

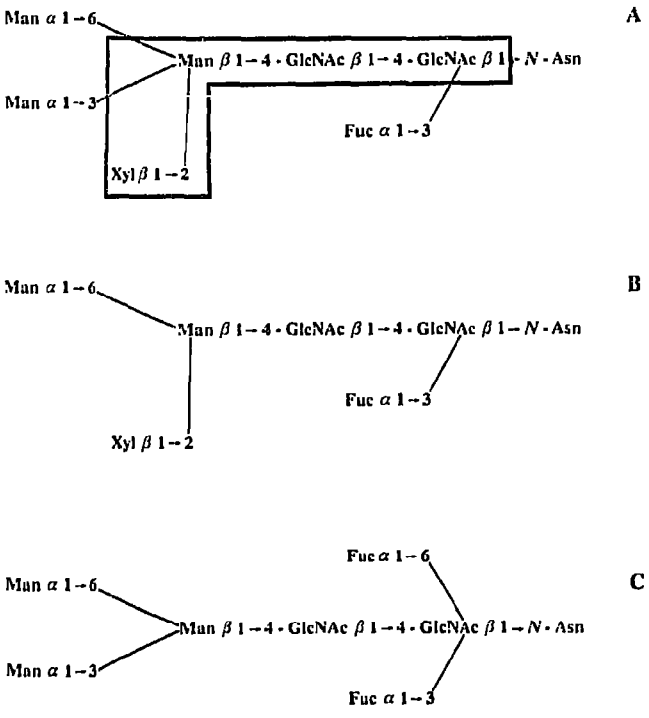

Fig. 1. Structures of the complex oligosaccharides *N*-linked to some glycoproteins used in the present study. (A) Typical *N*-linked complex oligosaccharide found in a number of plant secreted glycoproteins. Epitope of xylose-specific antibodies is surrounded with a solid line. (B, C) *N*-linked complex-type glycans from pineapple stem bromelain [20] and from phospholipase A2 [22], respectively.

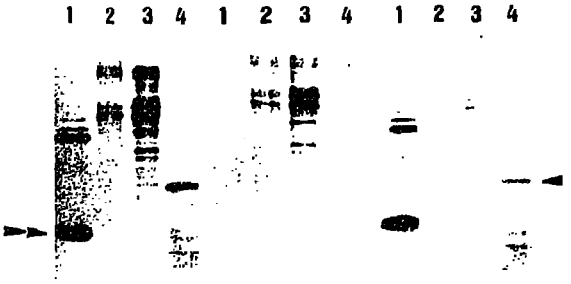

Fig. 2. Immunodetection with purified and unpurified xylose-specific antibodies. Phospholipase A2 (PA2) (double arrowhead on lane 1), proteins from suspension-cultured sycamore cells (lanes 2 and 3) and pineapple stem bromelain (arrowhead on lane 4) were separated with SDS-PAGE and analyzed on blots with antibodies specific for plant complex glycans. The antibodies used for immunodetection were purified from an immune serum specific for the complex glycan of carrot cell wall β-fructosidase. Antibodies retained from the immune serum on a PHA-Sepharose column were used directly for immunoblotting (CRUDE) or further purified on a PA2-Sepharose column. Antibodies that bound or did not bind immobilized PA2 were used for immunodetection on panels (RETAINED) and (UNRETAINED), respectively.

a blot (Fig. 2, middle panel). Mild periodate oxidation of these glycoprotein glycans on blots completely abolished subsequent immunodetection with these antibodies (result not shown) [18].

Hemicelluloses and pectic polysaccharides are synthesized in the Golgi apparatus. In dicots, the most abundant hemicellulose is xyloglucan, a polysaccharide consisting of a backbone of $\beta 1 \rightarrow 4$-linked glucose residues and short sidechains containing xylose, fucose and galactose. The dot blot experiment presented in Fig. 3 shows that our xylose-specific antiserum does not cross-react with xyloglucan, polygalacturonic acid or rhamnogalacturonan. These results indicate that the antibodies used in the present study are specific for xylose ($\beta 1 \rightarrow 2$) linked to the β-mannose in plant glycoprotein complex glycans but do not react with cell wall polysaccharides, particularly with xylose residues constitutive of xyloglucans.

3.2. *Glycoproteins with xylose in their glycans are abundant in sycamore cell cultures*

Successful labeling of plant dictyosomes with antibodies specific for proteins en route for other compartments was described almost exclusively in tissues specialized in the storage of proteins in their vacuoles (see [23] for illustration). The glycan-specific immunoprobe used in the present study has, potentially, a larger number of targets in the Golgi apparatus than antibodies specific for a single protein in transit in this organelle. However, before going further into the indirect localization of xylosyltransferase, we tried to evaluate the level of activity of this enzyme on its endogenous substrates in sycamore cells.

Vacuolar and extracellular proteins, divided into culture medium and cell wall proteins, were purified from suspension cultured sycamore cells [14,15]. These 2 abundant protein populations are transported via the Golgi apparatus from the RER to their final localization [24]. Proteins, obtained from extracellular compartments or solubilized from purified vacuoles, were fractionated by SDS-PAGE and electroblotted onto nitrocellulose paper. The affino- and immunodetection data presented on Fig. 4 indicate that most vacuolar or extracellular proteins in suspension cultured sycamore cells are glycoproteins. These glycoproteins have high-mannose- (affinodetection with ConA) and/or complex-type glycans containing xylose residues (immunodetection).

3.3. *Immunolocalization of xylose containing glycoproteins*

A preliminary condition for immunolocalization of glycoproteins with xylose-containing complex glycans was to obtain a good ultrastructural preservation of sycamore cells. The use of high-pressure freezing in conjunction with freeze substitution resulted in a very good

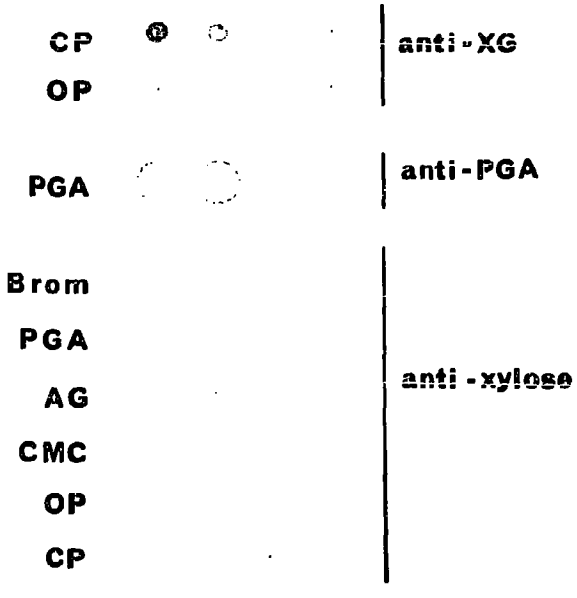

Fig. 3. Xylose-specific antibodies do not cross-react with cell wall constitutive polysaccharides. Dot blots were realized by applying onto nitrocellulose (from left to right) 5, 1, 0.2 and 0.04 μg of pineapple stem bromelain (Brom), polygalacturonic acid (PGA), arabinogalactans from larch wood (AG), carboxymethylcellulose (CMC) and pectins from orange (OP) and citrus (CP). Antisera used for immunodetection are specific for hemicellulose (anti-XG), pectins (anti-PGA) and Xyl-containing complex glycans (anti-xylose).

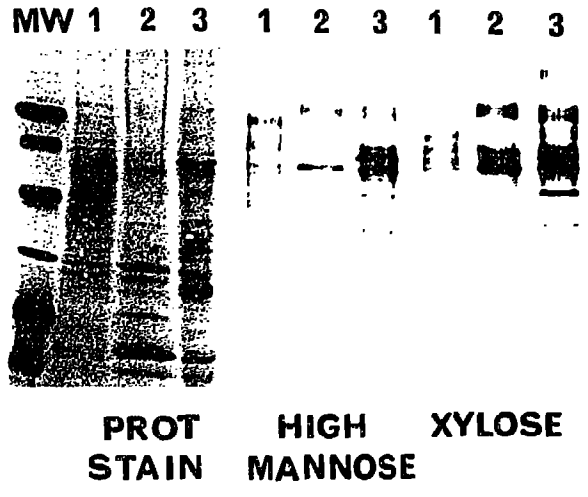

Fig. 4. Affino- and immunodetection and glycoproteins with oligomannose and/or xylose containing complex glycans in suspension cultured sycamore cells. Proteins from protoplasts (1), culture medium (2) and cell walls (3) were separated with SDS-PAGE and either stained on the gel with Coomassie blue (PROT STAIN) or transferred on blots stained for glycoproteins with high-Man- (HIGH MANNOSE) or complex- (XYLOSE) type glycans. The amounts of culture medium and cell wall proteins loaded on gels used for blotting have been divided by 3 and by 30, respectively, compared to protein amounts loaded on the gel stained for proteins. The same amount of protoplast proteins has been loaded for each detection. Molecular weight standards (MW) are, from top to bottom, 106 000, 80 000, 49 500, 32 500, 27 500 and 18 500.

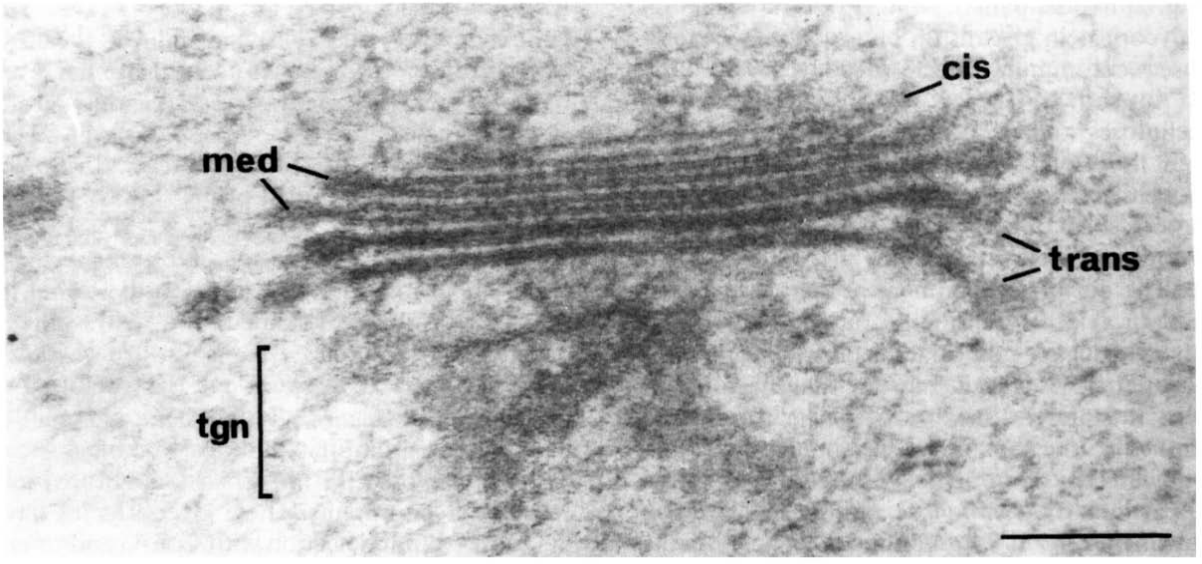

Fig. 5. Ultrastructure of a Golgi stack in high-pressure frozen/freeze-substituted sycamore cells. cis, *cis* Golgi; med, medial Golgi; trans, *trans* Golgi; TGN, trans Golgi network. Scale bar = 0.2 μm.

preservation of subcellular structures, particularly of the differences in the morphology between Golgi cisternae within individual Golgi stacks. They are designated as *cis*, medial and *trans* Golgi cisternae and the *trans* Golgi network (TGN) on Fig. 5.

It is generally admitted that secreted proteins of both plant and animal cells are synthesized in the RER and then are transported to the *cis* face of the Golgi apparatus. After vesicle-mediated transport through the different Golgi cisternae, secretory proteins are delivered to their final subcellular location. Many plant secreted proteins are co-translationally *N*-glycosylated and their glycans are modified during their intracellular transport under the action of at least a dozen glycosidases and glycosyltransferases. Higher plants appear to have many of the glycan processing enzymes that are found in animal cells in addition to at least one unique xylosyltransferase [1,6,24]. We have focused our interest on the later glycosyltransferase which has never been described in vertebrates [9].

As illustrated in Fig. 6 most of the anti-xylose gold labeling is concentrated in the cell wall while immunolabeling in the vacuole is very low. This result is highly consistent with the high concentration of glycoproteins in the extracellular compartment of sycamore cells as shown in Fig. 4. In contrast, blotting and immunogold localization results are consistent with the conclusion that glycoproteins are highly diluted in sycamore cell vacuolar sap.

Looking upstream in the secretory pathway, we have used the results obtained from immunolabeling detection of xylose-containing glycoproteins as an indirect approach to determine the subcellular localization of xylosyltransferase. Consistent with results obtained from subcellular fractionation [25], the immunolabeling of its products shows that xylosyltransferase in sycamore cells is present and/or biologically active in the Golgi apparatus but not in the RER (Fig. 6). The xylose- specific immunolabeling through the Golgi compartments appears early in the *cis* compartment, but gold particles are more concentrated over the medial and *trans* Golgi cisternae and the TGN. To confirm these qualitative observations, we have quantified the distribution of anti-xylose label by counting the gold particles in the different Golgi compartments. Of 50 Golgi stacks scored in this experiment, 9.5% of gold particles were observed in the *cis* cisternae, while as much as 41, 29.5 and 20% of the labeling was concentrated, respectively, in the medial and *trans* cisternae and TGN. A statistical analysis of our data has shown these results to be significantly different from a uniform distribution of the gold particles over the Golgi stacks.

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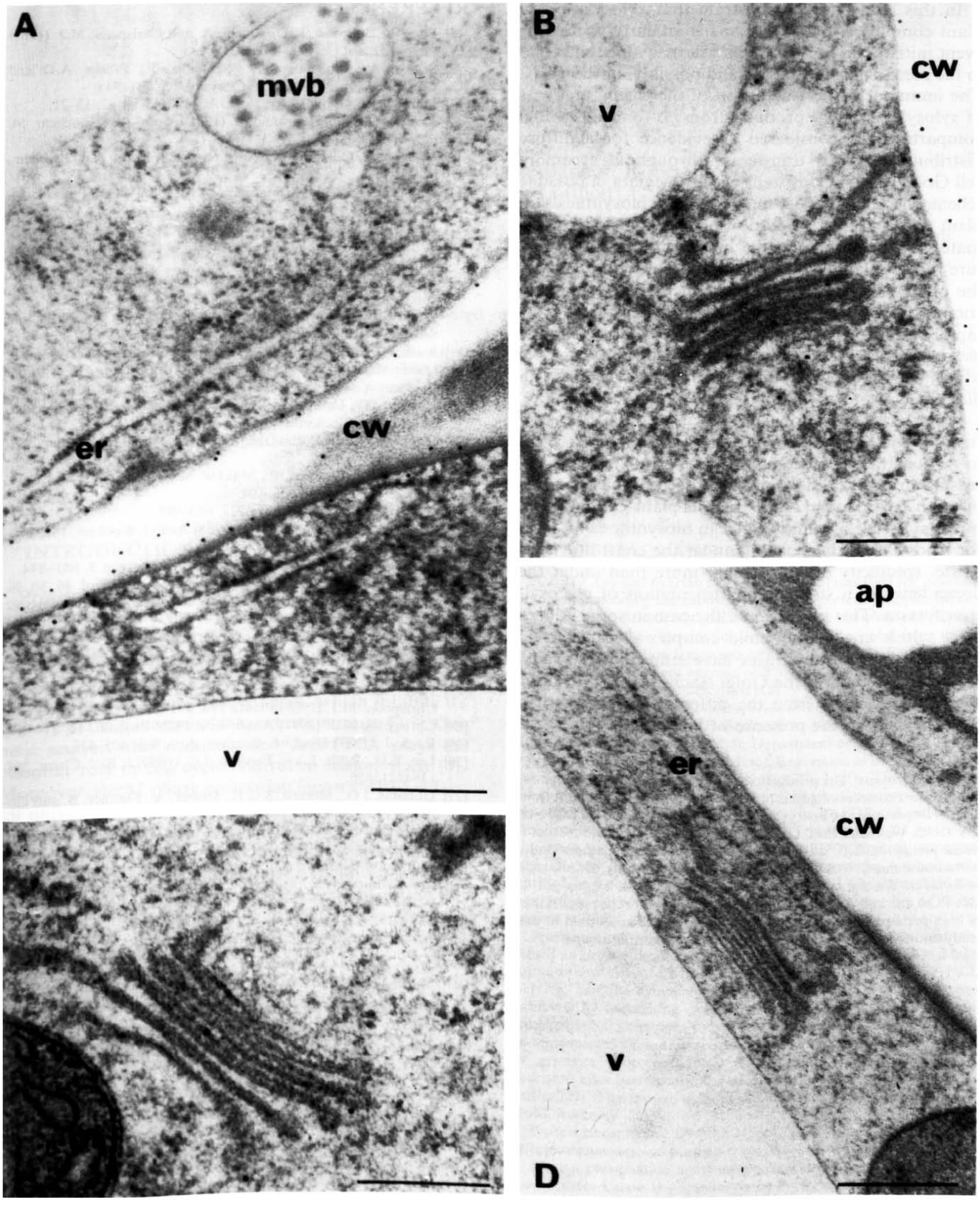
Fig. 6. Immunogold-localization of glycoproteins with xylose containing oligosaccharide sidechains in high-pressure frozen/freeze-substituted cultured sycamore cells (A, B and C) after treatment with xylose-specific antibodies followed by protein A-gold conjugate (10 nm). The specificity of the reaction is demonstrated by the lack of gold particles in the control section (D) incubated with pre-immune rabbit serum prior to treatment with protein A-gold. cw, cell wall; er, endoplasmic reticulum; mvb, multivesicular body; v, vacuole; m, mitochondria; ap, amyloplast. Scale bars are 0.3 μm for A, B and C and 0.4 μm for D.


A
mvb
er
cw
v
B
v
cw
ap
er
cw
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D

In this study, we demonstrate that xylosylation of plant complex *N*-linked glycans is an early processing event initiated in the *cis* Golgi cisternae and completed in the medial–*trans* cisternae and possibly, in the TGN. The immunochemical detection of increasing amounts of xylosyltransferase products from *cis* to medial Golgi compartment is considered as evidence for a diffuse distribution of this transferase throughout sycamore cell Golgi stacks. Consistent with these data, a possible scheme for *N*-linked complex glycan biosynthesis in plant cells is that the addition of xylose residues is initiated in *cis*-citernae on the high-mannose-type structure $Man_{4-3}GlcNAc_2$. This xylosylation is completed in the other Golgi compartments. Fucosylation of these complex glycans occurring in the same compartments rapidly after, or in parallel with xylosylation (Lainé et al., unpublished result) would explain the abundance of $Man_3Fuc_1Xyl_1GlcNAc_2$ oligosaccharide sidechains in plant glycoproteins [1].

Previous investigations, using density gradient centrifugation methods, were also unsuccessful in an attempt to demonstrate a subcompartmentation of glycan-processing enzymes in the Golgi cisternae of sycamore cells or bean cotyledons [2,8]. Thus, in plant cells, the sequential nature of complex glycan biosynthesis (at least for xylosylation) is probably under the control of substrate- specificity or accessibility more than under the dependence of a strict compartmentation of glycosyltransferases. This may also be the case in some animal cells, which are able to build complex glycans, when terminal glycosyltransferases have either a diffuse distribution throughout the Golgi stacks [25–27], or after their redistribution from the different Golgi cisternae into the RER in the presence of brefeldin A [28,29].

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