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Biosynthesis and Immunolocalization of Lewis a-Containing *N*-Glycans in the Plant Cell[1]

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We recently demonstrated the presence of a new asparagine-linked complex glycan on plant glycoproteins that harbors the Lewis a (Le^a), or Galβ(1-3)[Fucα(1-4)]GlcNAc, epitope, which in mammalian cells plays an important role in cell-to-cell recognition. Here we show that the monoclonal antibody JIM 84, which is widely used as a Golgi marker in light and electron microscopy of plant cells, is specific for the Le^a antigen. This antigen is present on glycoproteins of a number of flowering and non-flowering plants, but is less apparent in the Cruciferae, the family that includes Arabidopsis. Le^a-containing oligosaccharides are found in the Golgi apparatus, and our immunocytochemical experiments suggest that it is synthesized in the trans-most part of the Golgi apparatus. Le^a epitopes are abundantly present on extracellular glycoproteins, either soluble or membrane bound, but are never observed on vacuolar glycoproteins. Double-labeling experiments suggest that vacuolar glycoproteins do not bypass the late Golgi compartments where Le^a is built, and that the absence of the Le^a epitope from vacuolar glycoproteins is probably the result of its degradation by glycosidases en route to or after arrival in the vacuole.

Many secretory proteins are co-translationally *N*-glycosylated into the ER by receiving oligosaccharide side chains onto specific Asn residues constitutive of potential glycosylation sites (Abeijon and Hirschberg, 1992). These oligosaccharides are then matured successively into high-Man-type and eventually complex-type *N*-glycans, while the glycoprotein is transported through the different compartments of the secretory pathway. The initial events and the intermediary products of precursor oligosaccharide maturation are similar in plant and mammalian cells, but fully matured plant and mammalian *N*-glycans differ structurally. For instance, the most common mature glycan *N*-linked to plant glycoproteins has a paucimannosidic-type structure made of a core $Man_3GlcNAc_2$, which is common to all of the *N*-linked glycans in eukaryotic cells, but decorated with a β-(1,2)Xyl residue linked to the β-Man of the core and an α-(1,3)Fuc residue linked to the proximal GlcNAc of the core. This paucimannosidic-type *N*-glycan is a typical product of the plant *N*-glycosylation pathway and has been already described for a wide range of plant glycoproteins, including plant lectins and enzymes such as horseradish peroxidase (for a recent review, see Lerouge et al., 1998).

More complex bi-antennary plant *N*-glycans have recently been described. They have one or two terminal antennae containing an oligosaccharide sequence, Galβ(1-3)[Fucα(1-4)]GlcNAc, named Lewis a (Le^a) after their mammalian counterparts (Fitchette-Lainé et al., 1997; Melo et al., 1997). In humans, Lewis structures are responsible for histo-blood groups (Henry et al., 1995) and are involved in cell-to-cell recognition processes (Feizi, 1993). We have recently purified antibodies specific for the Le^a epitope from the serum of a rabbit immunized with sycamore laccase. These antibodies were named anti-plant Lewis antibodies (Fitchette-Lainé et al., 1997). In this paper, we show that Le^a-containing glycans are highly conserved and can be immunodetected on glycoproteins of most higher and lower plants analyzed so far. The high immunogenicity and wide distribution of Le^a in plants is also illustrated here through the demonstration that JIM 84, a monoclonal antibody widely used as a Golgi marker for both light and electron microscopy (Horsley et al., 1993), shares a similar specificity for Le^a as the polyclonal anti-plant Lewis antibodies that we previously obtained and characterized. Anti-plant Lewis antibodies were also used to further characterize the biosynthesis and location of Le^a-containing *N*-glycans within the plant cell.

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MATERIALS AND METHODS

Materials

Anti-β-(1,2)Xyl and anti-plant Lewis antibodies were prepared according to the method of Faye et al. (1993) and Fitchette-Lainé et al. (1997), respectively. Peptide *N*-glycosidase from almond (PNGase A) was from Boehringer Mannheim (Basel, Switzerland). The coding sequence of phytohemagglutinin (PHA) L (Sturm et al., 1988) was inserted in the PDE 1001 binary vector and then introduced in tobacco (*Nicotiana tabacum*) BY2 cells by co-culture with *Agrobacterium tumefaciens*, as described in Gomord et al. (1998). Suspension-cultured BY2 cells and transgenic cells were grown as previously described (Gomord et al., 1998). Maize (LG11) and onion seeds (White Lisbon) were grown on damp filter paper in the dark at room temperature for 4 d prior to harvesting. The Le^a trisaccharide was provided by Dr C. Auger (Laboratorie de Chimie Organique Multifonctionnelle, Orsay, France). BSA-Le^a,Le^x was obtained by coupling BSA with a 1:1 mixture of Galβ(1-3)[Fucα(1-4)]GlcNAcβ(1-3)Galβ(1-4)Glc (lacto-*N*-fucopentaose II) and Galβ(1-4)[Fucα(1-3)]GlcNAcβ(1-3)Galβ(1-4)Glc (lacto-*N*-fucopentaose III), and was a generous gift from J.-C. Michalski (Centre National de la Recherche Scientifique UMR 8576, Lille, France).

Protein Sample Preparation and Immunoblotting Experiments

Crude protein extracts were prepared from whole organisms: fungi (Ascomycetae: *Morchella esculenta*; Basidiomycetae: *Agaricus campestris*), algae (*Euglena subtilis*, *Ulva latuca*), lichens (*Evernia punastris*, *Hypogymnia physodes*), and bryophytes (*Mnium undulatum*, *Leucobrium glaucum*, *Polytrichum commune*), as well as from leaves of Pterydophytae (*Equisetum arvensis, Asplinium scolopendium, Adiantum* sp., *Dryopteris filix-mas*), gymnosperms (Cycadopsidae: *Cycas revoluta*; Ginkgopsidae: *Ginkgo biloba*; Pinopsidae: *Araucaria raucana, Abies* sp., *Larix decidua, Cedrus libanis, Pinus tabulasformis, Sequoia sempervirens, Juniperus horizontalis, Thuja* sp., *Taxus baccata*), monocots (Commelinidae: *Cyperus* sp., *Pleiobatus pumilus*; Arecedae: *Spathiphyllum* sp.; Liliidae: *Tulipa* sp., *Chrocus* sp., *Alium schanoprasum, Narcissus* sp., *Aloe* sp.), and dicots (Magnoliidae: *Laurus nobilis, Calycanthus praecox, Nuphar luteum*; Ranunculiidae: *Paeonia officinalis, Aquilegia vulgaris, Berberis vulgaris, Chelidonium majus*; Hammameliidae: *Cercidiphyllum japonicum, Hammamelis virginia, Liquidambar stiraciflua, Urtica dioica, Morus nigra, Maclura orantiaca, Quecus suber, Castanea sativa, Betula verrucosa, Coryllus avelana, Juglans regia;* Caryophyllidae: *Dianthus caryophyllus, Opuntia* sp., *Salicorna* sp., *Rumex acetosa, Althaea* sp., *Hibiscus* sp., *Viola* sp., *Cucurbita pepo, Hypericum perforatum, Raphanus sativus,* Arabidopsis, *Brassica oleracea, Brassica napus, Salix caprea, Primula* sp.; Rosidae: *Cerasum avium, Hydrangea macrophylla, Kalanchoe* sp; *Cytisus scoparus, Cercis siliquastrum, Cornus florida, Aucuba* sp., *Phellodendron japonicum, Ailanthus glandulosa, Rhus typhina, Acer pseudoplatanus, Pelargonium zonale, Carum petroselinum, Euonymus fortunei, Rhamnus imeritina*; Asteridae: *Synringa vulgaris, Solanum tuberosum, Nicotiana tabacum, Covolvulus sepium, Mentha* sp., *Digitalis purpurea, Catalpa bigonoïdes, Saintpaulia ionantha*).

Protein extracts were obtained by homogenizing plant material in a solution containing 0.7 M saccharose, 0.5 M Tris, 30 mM HCl, 0.1 M KCl, and 2% (v/v) β-mercapthoethanol. After incubation on ice for 30 min, the homogenate was centrifuged for 5 min at 5,000*g*. The supernatant was mixed vigorously with 1 volume of saturated phenol, left on ice for at least 30 min, and centrifuged at 10,000*g* for 30 min. The upper phenolic phase was precipitated overnight at 4°C by the addition of 5 volumes of methanol containing 0.1 M ammonium acetate. The preparation was then centrifuged for 30 min at 10,000*g*. The pellet was washed once with 0.1 M ammonium acetate in methanol before being resuspended in sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 10 mM DTT, 10% [v/v] glycerol, and 1% [w/v] SDS).

Laccase was purified from suspension-cultured sycamore cells according to the method of Sterjades et al. (1992). Protoplasts and vacuoles were prepared from tobacco BY2 cultured cells according to the method of Gomord et al. (1997). α-Mannosidase activity was determined in protoplasts and vacuoles following the method of Chrispeels and Boulder (1975) to load the same amount of vacuolar proteins on electrophoresis gels regardless of the protein content of the samples. Protein extracts from protoplasts and vacuoles were prepared by incubating the BY2 protoplasts and vacuoles at 100°C in the sample buffer. Protein extract from the culture medium of BY2 cultured cells was prepared by precipitating the medium with 12.5% (w/v) TCA at 4°C overnight, washing the precipitate three times in cold acetone, and resuspending it in the sample buffer.

For immunoblotting experiments, proteins were separated in SDS-PAGE gels according to the method of Laemmli (1970), transferred onto a nitrocellulose membrane, and detected with anti-plant Lewis antibodies (Fitchette-Lainé et al., 1997) or anti-Xyl antibodies (Faye et al., 1993).

Preparation of *N*-Glycans from Tobacco Plant Glycoproteins

A crude protein extract was obtained from tobacco plants by homogenizing 50 g of dried leaf material in 1 L of 50 mM HEPES, pH 7.5, containing 2 mM sodium bisulfite and 0.1% (w/v) SDS. Insoluble material was eliminated by centrifugation (4,400*g*, 15 min) at 4°C. Proteins were precipitated at 0°C overnight with 12.5% (w/v) TCA (final concentration). After washing the pellet twice with 90% (v/v) acetone, the proteins were digested with 10 mg of pepsin in 30 mL of 0.01 N HCl, pH 2.2, at 37°C for 48 h. After neutralization with 1 N ammonium hydroxide, the solution was heated for 5 min at 100°C, centrifuged, and lyophilized. The sample was desalted on a gel-filtration column (45 × 2.6 cm; Bio Gel P4, Bio-Rad Laboratories, Hercules, CA) equilibrated with 0.1 N acetic acid. The glycopeptide fractions were pooled and then deglycosylated overnight at 37°C with PNGase A (10 milliunits, Boehringer Mannheim) in a 100 mM sodium acetate buffer,

pH 5.0. *N*-Glycans were purified by successive elution through a AG 50W-X2 column (5 × 1 cm) and a C_{18} cartridge. The mixture of *N*-glycans was then separated into high-Man *N*-glycans and mature *N*-glycans by chromatography on a concanavalin A-Sepharose column as previously described (Rayon et al., 1996).

Analysis of Tobacco *N*-Glycans

Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra were measured on a mass spectrometer (Spec E, Micromass, Manchester, UK) using the reflector mode. This instrument was operated at an accelerating voltage of 20 kV with a reflector potential of 26 kV and a pressure of approximately 10^{-7} mbars in the source and 10^{-6} mbars in the analyzer. Samples were desorbed and ionized from the probe tip with a nitrogen laser (λ = 337 nm) with a pulsewidth of 4 ns. The laser shots were summed for each mass spectrum to achieve an acceptable signal-to-noise ratio. The solution containing the sample was prepared at a concentration of approximately 10 pmol μL^{-1} in water. Two or 5 μL of this solution was dissolved in the same volume of matrix solution prepared by dissolution of 2 mg of 2,5-dihydroxybenzoic acid in 200 μL of 70% (v/v) acetonitrile in 0.1% (v/v) TFA. The sample-matrix mixture obtained was homogenized, and 2 μL of this solution was deposited onto probe tips and allowed to dry for a few minutes under vacuum. The target was then applied in the MALDI-TOF mass spectrometer.

Immunofluorescence Microscopy

Immunostaining was essentially as described by Satiat-Jeunemaitre et al. (1996). Root apices were fixed for 1 h in 3% (w/v) paraformaldehyde in 0.1 M PIPES buffer, pH 6.9, and digested for 10 to 15 min in 1% (w/v) cellulase (Onozuka R10, Yakult Honshua, Tokyo) and 1% (w/v) pectinase (Sigma, St. Louis) before being squashed onto coated multiwell slides (Vectabond, Vector Laboratories, London) and air dried. Prior to immunostaining, cells were further permeabilized with 0.5% (v/v) Triton X-100 for 10 min. BY2 cells were fixed, lightly digested with enzymes for 10 min as described above, dried onto coated slides, and permeabilized with Triton X-100 prior to immunostaining.

For immunostaining, all cells were treated with 1% (w/v) BSA and 1% (v/v) fish gelatin (Sigma) prior to incubation in primary antibodies (anti-plant Lewis antibodies, 1:500 dilution in buffer, or JIM84 monoclonal antibody) for 1 h at room temperature. Following washing in buffer with 1% (v/v) fish gelatin and labeling for 1 h in the appropriate fluorescein isothiocyanate (FITC)-conjugated second antibody, some cells were stained in propidium iodide (3 μg mL^{-1}), washed, mounted in Citifluor (City University, London), and observed with a confocal microscope (model LSM 410, Zeiss, Jena, Germany). For double-immunofluorescence labeling, root tip slides were first stained with JIM 84 and anti-rat Cy3 (Jackson Immunoresearch, West Grove, PA) diluted 1:800, blocked, and then stained with anti-plant Lewis antibodies and second antibodies conjugated to FITC. Images from FITC-conjugated antibodies were collected using a 488-nm argon ion laser through a narrow barrier filter set (510–525 nm) and from Cy3-stained material with a 543-nm helium neon laser through a 570-nm long-pass filter set. The absence of bleed-through between channels was confirmed from specimens stained with single fluorochromes. Co-localization was assessed using co-localization software (Zeiss).

For methacrylate embedding, the technique of Baskin et al. (1992) was used. Root tips were fixed as above and dehydrated in a water/ethanol series containing 10 mM DTT, progressively reducing the temperature to −20°C, and infiltrated at low temperature with a (4:1, v/v) mixture of butyl methacrylate:methacrylate containing 0.5% (w/v) benzoin ethyl ether and 10 mM DTT. The resin mixture was degassed with gaseous nitrogen immediately before use. Samples were flat-dish-embedded in fresh resin and polymerized for 12 h at 0°C in a nitrogen atmosphere under an indirect UV light (a black light). One- to 2-μm-thick sections were cut dry on glass knives with an ultramicrotome (model MT 7000, Research and Manufacturing Company, Tucson, AZ) and allowed to dry from a water drop on poly-L-Lys coated multiwell slides. After removal of the resin in acetone for 10 min, sections were immunolabeled as above.

Immunogold Labeling

For immunogold labeling, root tips and cells were fixed in 1% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer and embedded in LR White resin using the progressive lowering of temperature technique described in Satiat-Jeunemaitre and Hawes (1992). Sections were cut on an ultramicrotome (Ultracut E, Reichert-Jung, Vienna), collected on nickel grids, and blocked sequentially with goat serum (1:30 [v/v] in PBS), 0.1% (v/v) Tween 20, 0.02 M Gly, and 1% (v/v) fish gelatin all in PBS plus 1% (w/v) BSA. Incubation in primary antibodies (anti-plant Lewis antibodies 1:500, anti-Xyl antibodies 1:500 dilution) was at 4°C overnight or 1 h at room temperature. After washing in PBS/BSA buffer, grids were incubated for 1 h at room temperature in rabbit secondary antibody conjugated to 10 nm of gold (British BioCell, Cardiff, UK), washed, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (model 1200 EXII, JEOL, Tokyo).

For double labeling of LR White-embedded BY2 cells expressing PHA, sections on grids were blocked sequentially with 0.1% (v/v) Tween 20, 0.02 M Gly, and 1% (v/v) fish gelatin in PBS prior to incubation in the first antibody (anti-PHA, 1:500 dilution), followed by washing in blocking buffer and labeling with protein A conjugated to 20 nm of colloidal gold. Grids were then further blocked in 1% (v/v) fish gelatin in PBS plus 0.2 mg mL^{-1} protein A for 1 h prior to incubation in the second primary antibodies (anti-plant Lewis antibodies, 1:500 dilution; anti-Xyl antibodies, 1:500 dilution), followed by washing and incubation in secondary antibodies conjugated to 10 nm of colloidal gold. For controls, wild-type BY2 cells were subjected to the double-labeling protocol or the second primary antibodies were omitted from the labeling protocol to confirm the

efficacy of the blocking step between the two different antibodies.

ELISA

The binding of JIM 84 to the BSA-Le^a,Le^x was measured as described in Fitchette-Lainé et al. (1997). Inhibition of recognition was measured in the presence of a range of oligosaccharides: Le^a, Galβ(1-3)[Fucα(1-4)]GlcNAc; Le^x, Galβ(1-4)[Fucα(1-3)]GlcNAc; Fucα(1-4)GlcNAc; Gal β(1-3)GlcNAc; Fuc and GlcNAc in 10^{-3} to 10^{-7} M concentrations.

RESULTS

The Addition of Le^a Is a Highly Conserved Modification of *N*-Glycans in Plants

To study the distribution of the Le^a epitope among plant *N*-glycans, total protein extracts prepared from leaves (Pterydophytae, gymnosperms, monocots, and dicots) or total organisms (algae, lichens, fungi, and bryophytes) were analyzed by immunoblotting after SDS-PAGE. Glycoproteins bearing Le^a were identified on blots using the anti-plant Lewis antibodies prepared as described in Fitchette-Lainé et al. (1997). As illustrated in Table I, results obtained from this immunoscreening show that Le^a-containing *N*-glycans are present on glycoproteins from mosses, ferns, gymnosperms, monocots, and dicots, but are absent on glycoproteins from lower organisms such as algae, lichens, and fungi. Arabidopsis was previously identified as being a major exception in the dicots, as it does not contain easily detectable amounts of the Le^a epitope (Fitchette-Lainé et al., 1997). Furthermore, no Le^a-containing *N*-glycans could be identified in the total population of *N*-linked glycans released from glycoproteins of Arabidopsis leaves (Rayon et al., 1999). As indicated in Table I, we have now found by immunoscreening that not just Arabidopsis but also other members of the Cruciferae family, including cauliflower, radish, and rape, do not present any detectable amounts of Le^a-bearing glycoproteins. We therefore conclude that the whole Cruciferae family lacks such glycoproteins. Notably, this family currently represents the only dicots whose *N*-linked glycans do not exhibit the high complexity otherwise found in this group.

Table I. *Occurence of Le^a epitope among plants*

Le^a was immunodetected on blot in plant extracts using anti-plant Lewis antibodies (Fitchette-Lainé et al., 1997).

Extract from	Le^a Detection[a]
Fungi	
Ascomyceteae	−
Basidiomycetae	−
Algae	
Chlorophytaea	−
Lichens	−
Bryophyta	+
Pterydophyta	
Equisetopsida	+
Gymnosperms	
Cycadopsidae	+
Ginkgopsidae	+
Pinopsidae	+
Angiosperms-Monocots	
Comelinidae	+
Arecedae	+
Liliidae	+
Angiosperms-Dicots	
Magnoliidae	+
Ranunculiidae	+
Hamamelidiidae	+
Caryophillidae	+
Cruciferae	−
Rosidae	+
Asteridae	+

[a] Similar amount of proteins was loaded on the gel for each sample analyzed.

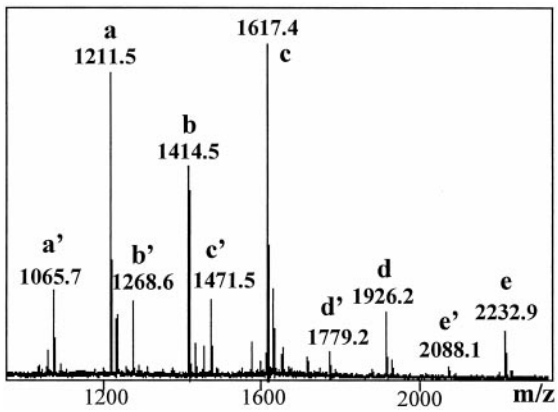

Figure 1. MALDI-TOF spectrum of paucimannosidic-type and complex-type *N*-glycans released from glycoproteins isolated from tobacco plants. a to e, Sodium adducts of the corresponding *N*-glycans represented in Figure 2. a′ to e′, Structures lacking one Fuc residue.

Le^a-Containing *N*-Glycans Are Abundant in Tobacco Plants

To investigate whether Le^a-containing *N*-glycans, detected on blots with specific antibodies, are only minor components or correspond to abundant oligosaccharides, the *N*-glycan pattern of a reporter plant was established. The material used in this study was the tobacco cv Xanthi, since this plant is widely used in many laboratories and could be obtained in an unlimited quantity. *N*-Glycans were released from tobacco leaf glycoproteins by successive digestions with pepsin and PNGase A, as previously reported (Rayon et al., 1996). PNGase A is able to release all plant *N*-linked glycans (including oligosaccharides having a Fuc residue α-linked to the O-3 of the proximal glucosamine). The mixture of *N*-glycans was separated into high-Man-type *N*-glycans and mature *N*-glycans by chromatography on a concanavalin A-Sepharose column, as previously described (Rayon et al., 1996). The non-retained fraction containing mature *N*-glycans was analyzed by MALDI-TOF MS. As illustrated in Figure 1, $(M + Na)^+$ molecular ions at *m/z* from 1,065 to 2,232 were detected and assigned to the plant *N*-linked oligosaccharides a to e rep-

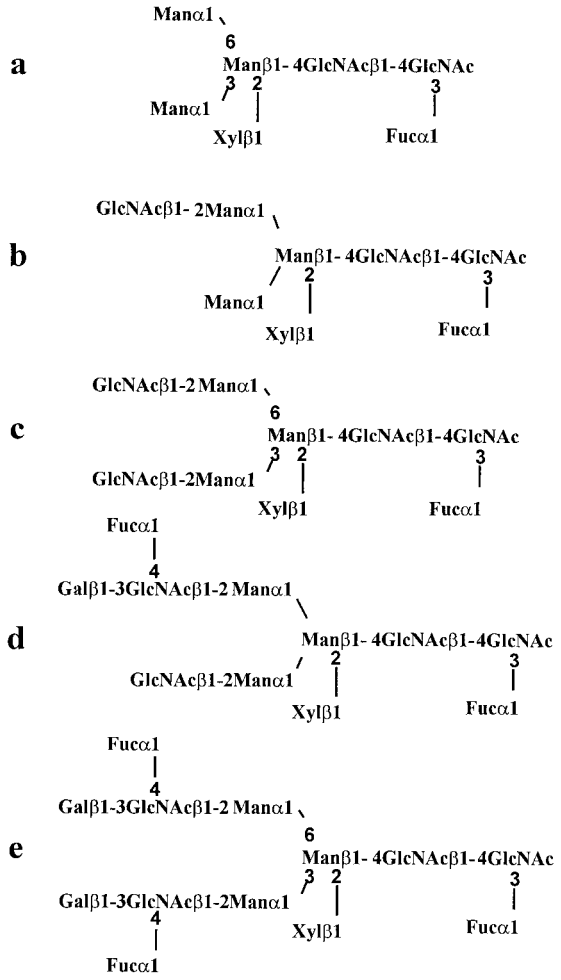

Figure 2. Structures of paucimannosidic-type (compound a) and complex-type *N*-glycans (compounds b–e) identified from tobacco plants. Compounds b and e correspond to two possible isomers according to the location of the GlcNAc residue or the Le^a epitope on the α-(1,3)- or the α-(1,6)-Man arm.

resented in Figure 2, on the basis of the mass values. $(M + Na)^+$ molecular ions at *m/z* 1,926 and 2,232 were assigned to mono- and bi-antennary *N*-glycans having one and two Le^a antennae linked to the core, respectively. These Le^a-containing *N*-glycans are abundant and were estimated to represent about 15% of the total *N*-glycan population in tobacco.

Glycoproteins Containing Le^a Are Detected in the Golgi Apparatus and Plasma Membrane in Plant Cells

Previous results have shown that Le^a-containing *N*-glycans are associated with proteins present at the plasma membrane of non-permeabilized plant cells (Fitchette-Lainé et al., 1997) and in soluble glycoproteins found in the culture medium of suspension-cultured plant cells (Fitchette-Lainé et al., 1997; Melo et al., 1997). To determine more precisely the distribution of glycoproteins bearing Le^a-containing *N*-glycans, immunofluorescence experiments with anti-plant Lewis antibodies were performed on BY2 tobacco cells and on tomato, onion, and maize root tip cells after Triton X-100 permeabilization. The results presented in Figure 3 (A–E and J) show punctate labeling within the cytoplasm, which can be doughnut-shaped in some single confocal sections (Fig. 3D). By electron microscopy, it can be seen that these organelles are the Golgi stacks (Fig. 4, A and B).

As illustrated in tomato root cells (Fig. 4A), labeling was much heavier toward the trans face of the dictyosome, suggesting that the addition of Le^a on plant complex *N*-glycans is a late Golgi event. In tomato, onion, and maize root cells, the plasma membrane is heavily labeled with the anti-plant Lewis antibodies (Fig. 3, B–E, and Fig. 4D), but in suspension-cultured tobacco BY2 cells, cell surface labeling was not detectable (Figs. 3A and 4B). In no preparations was there any labeling of the ER (Fig. 4C), the tonoplast, or the vacuolar contents (Fig. 4B). These observations show that in most plant cells, the anti-plant Lewis antibodies are good immunocytochemical markers of the Golgi apparatus and the plasma membrane, and indicate that Le^a-containing glycans are *N*-linked not only to soluble extracellular glycoproteins but also to glycoproteins found in the Golgi apparatus and the plasma membrane.

JIM84 Specificity Further Illustrates Abundance and Immunogenicity of Le^a Antennae

JIM 84, a monoclonal antibody raised against a carrot coated vesicle fraction, is generally accepted as one of the best Golgi markers for both immunofluorescence (Fig. 3F) and immunogold microscopy (Satiat-Jeunemaitre and Hawes, 1992; Horsley et al., 1993; Satiat-Jeunemaitre et al., 1994). However, JIM 84 specificity was unknown, although preliminary results were in favor of binding of this monoclonal antibody to the oligosaccharide side chains of plant glycoproteins (Horsley et al., 1993). Indeed, after oxidation of microsomal glycoproteins with sodium periodate, we found that the JIM84 binding was completely abolished (data not shown; Horsley et al., 1993). To further analyze which oligosaccharide epitope, *O*- or *N*-linked to microsomal proteins, is specifically recognized by this monoclonal antibody, we used an immunoblotting approach with purified glycoproteins bearing *N*-linked glycans of known structure, such as horseradish peroxidase (Kurosaka et al., 1991), bean PHA (Rayon et al., 1996), soybean agglutinin (Lis and Sharon, 1978), snail hemocyanin (van Kuik et al., 1985), human transferrin, honey bee venom phospholipase A_2 (Kubelka et al., 1993), and sycamore laccase (Fitchette-Lainé et al., 1997).

Among those glycoproteins, JIM 84 binds exclusively sycamore laccase, a secreted glycoprotein known to contain Le^a antennae on its *N*-linked glycans (Fitchette-Lainé et al., 1997) (Fig. 5A, lane 1). To further investigate whether JIM84 is specific for the Le^a epitope, the antibody was assayed for recognition of BSA-Le^a,Le^x, a neoglycoprotein bearing both Galβ(1-3)[Fuc α(1-4)]GlcNAc (Le^a) and Galβ(1-4)[Fuc α(1-3)]GlcNAc (Le^x) epitopes. As illustrated in Figure 5A, JIM84 clearly recognizes both sycamore laccase (lane 1) and BSA-Le^a,Le^x (lane 2). A similar result was obtained using the anti-plant Lewis antibodies that were

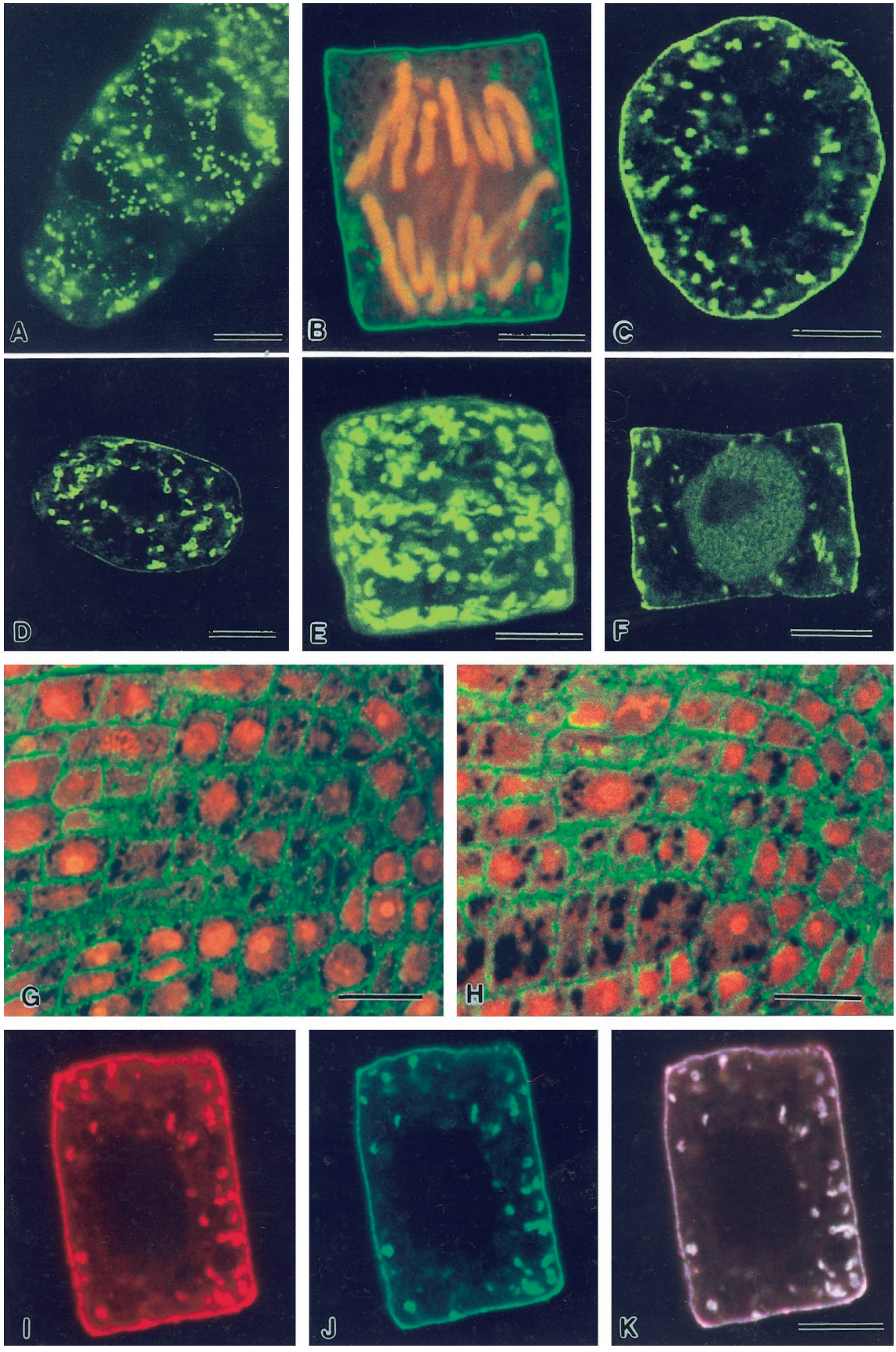

Figure 3. (Legend appears on facing page.)

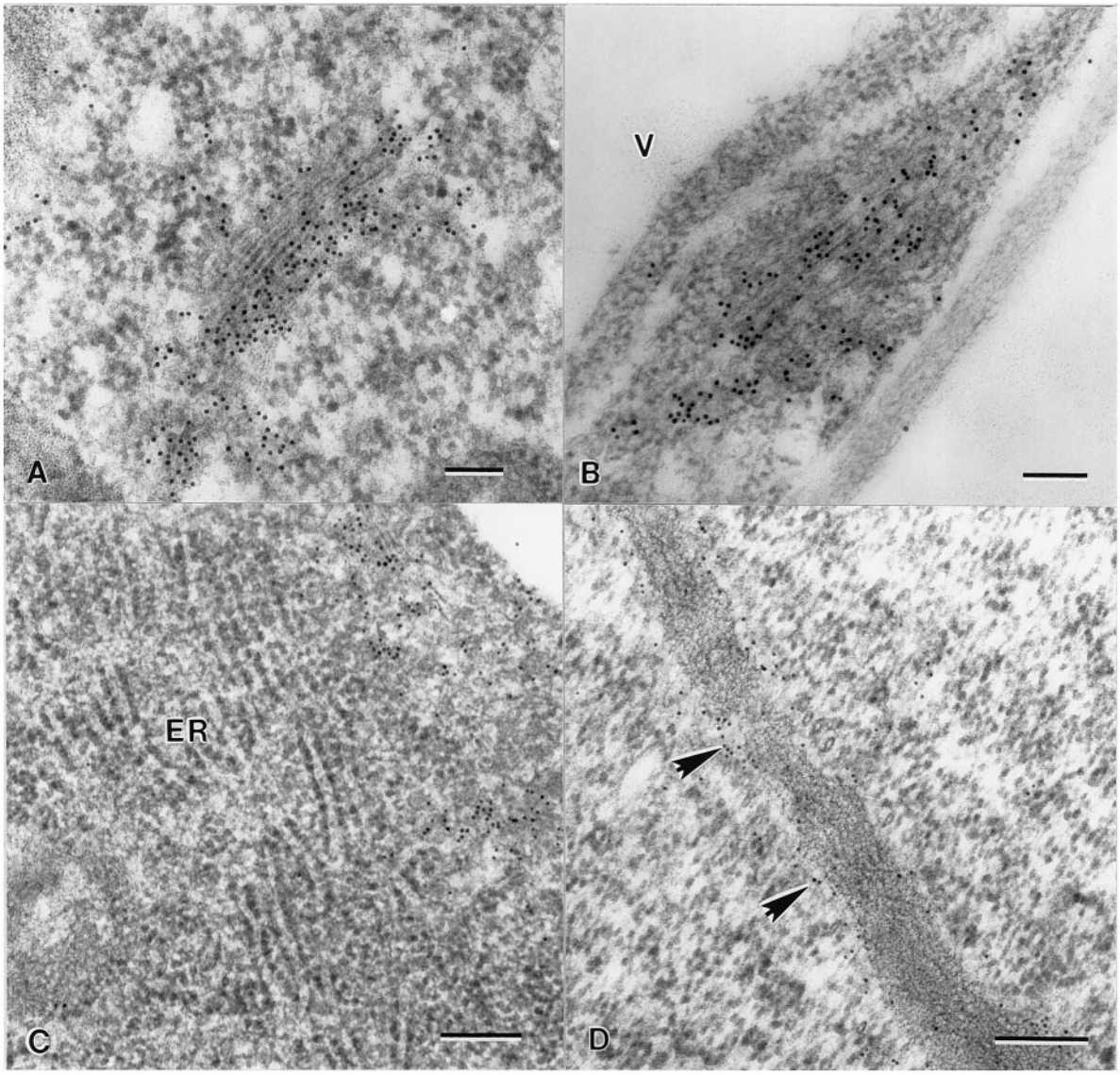

Figure 4. Immunogold labeling of Le^a-containing *N*-glycans in tomato and tobacco cells. A, Tomato root Golgi showing preferential distribution of gold particles over the trans-half of a Golgi stack. Bar = 100 nm. B, BY2 tobacco suspension-cultured cell showing Golgi labeling. Note lack of labeling in the vacuole (V) and tonoplast. Bar = 100 nm. C, ER in a tomato root cell not labeled with the anti-plant Lewis antibodies. Bar = 200 nm. D, Plasma membrane labeling of a tomato root tip cell. Compare with the suspension-cultured tobacco cells, which show little cell surface labeling. Bar = 200 nm.

Figure 3. (Legend continued from facing page.)
Confocal immunofluorescence micrographs of the Golgi apparatus and cell surface stained with anti-plant Lewis and JIM 84 antibodies. A, BY2 tobacco cell with Le^a staining. Note lack of cell surface labeling. Bar = 10 μm. B, Onion root cell in anaphase with Le^a staining. The cell was counterstained with propidium iodide to reveal the chromosomes. Bar = 10 μm. C, Maize root tip cell with Le^a staining. Bar = 10 μm. D, Tomato root tip meristem cell with Le^a staining. Note the presence of several doughnut-shaped Golgi bodies. Bar = 10 μm. E, Reconstruction of 15 0.5-μm-thick optical sections of a newly divided tomato root tip cell to demonstrate the large number of individual Golgi stacks in a cell with Le^a staining. Bar = 10 μm. F, Onion root meristem cell with JIM 84 staining. Bar = 10 μm. G and H, Longitudinal sections of methacrylate embedded maize root tips counter stained with propidium iodide, showing cell surface labeling across all cell types. G, Le^a staining; H, JIM 84 staining. Bar = 20 μm. I to K, Double labeling of a maize root tip cell showing co-localization of the Lewis and JIM 84 epitopes. I, JIM 84 staining; J, Le^a staining; K, merged image of I and J where the lilac color indicates areas of co-localization. Bar = 10 μm.

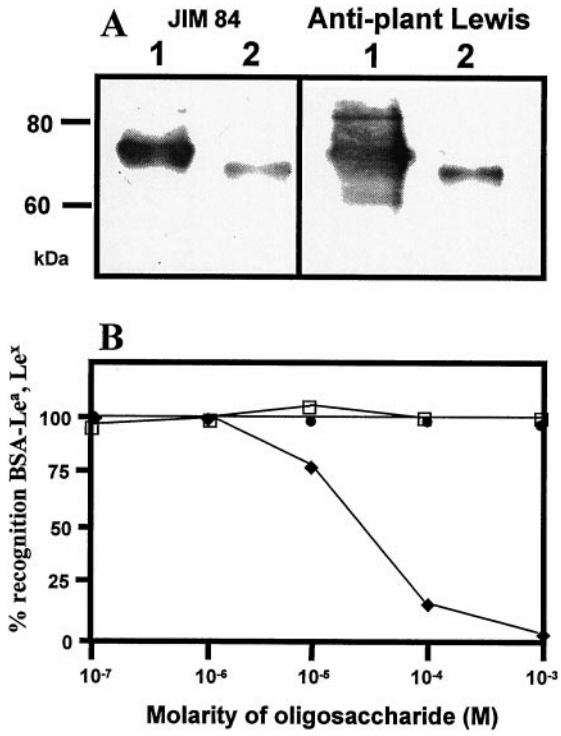

Figure 5. A, Immunodetection of Le^a epitope with JIM 84 and with the anti plant-Lewis antibodies. Lanes 1, Purified sycamore laccase; lanes 2, BSA-Le^a, Le^x. B, Inhibition of the binding capacity of JIM 84 monoclonal antibody on BSA-Le^a, Le^x by Le^a (◆); Galβ(1-3)GlcNAc (□); and Fucα(1-4)GlcNAc (●).

previously reported to be specific to Le^a (Fitchette-Lainé et al., 1997). To confirm the specificity of JIM84 for the Le^a epitope, ELISA assays were carried out on BSA-Le^a,Le^x (Fig. 5B). Binding of JIM84 to this neoglycoprotein was found to be inhibited by 50% in competition with 2.3×10^{-5} M Le^a trisaccharide. In contrast, no inhibition was observed with Le^x trisaccharide (data not shown) or with a range of oligosaccharides such as the Galβ(1-3)GlcNAc and Fucα(1-4)GlcNAc (Fig. 5B). From these data, it appears that the monoclonal antibody JIM84 recognizes the Le^a epitope and shows a specificity similar to the anti-plant Lewis antibodies.

Since both JIM84 and the anti-plant Lewis antibodies appear to be specific for Le^a, root squashes of onions (Fig. 3F) and methacrylate sections of maize root tips were labeled with JIM 84 (Fig. 3H). Labeling patterns obtained using this monoclonal antibody as a probe were found to be identical to those obtained using the anti-plant Lewis antibodies (Fig. 3, B and G). Furthermore, double immunofluorescence labeling experiments showed that both antibodies bind to the same Golgi stacks in a cell and may compete for the same binding sites (Fig. 3, I–K). In particular, the avidity of polyclonal anti-plant Lewis antibodies is so high that if staining with anti-plant Lewis occurs first, then there is no JIM 84 recognition (data not shown). Taken together, these observations confirm that these antibodies share the same specificity.

Le^a-Containing *N*-Glycans Are Associated with Extracellular Glycoproteins While These Oligosaccharides Are Never Found on Vacuolar Glycoproteins

Immunocytochemical experiments performed with either JIM 84 or polyclonal anti-plant Lewis antibodies have shown that Le^a-containing *N*-glycans are present in the Golgi apparatus and at the plasma membrane. However, they cannot be found in the vacuole. To obtain further information on the distribution of complex *N*-glycans in plant cells, we have analyzed glycoproteins from the culture medium, protoplasts, and vacuoles prepared from suspension-cultured tobacco cells. These different protein extracts were analyzed by immunoblotting after SDS-PAGE. Glycoproteins were probed on the blots with antibodies directed against the β-(1,2)Xyl residue linked to the β-Man of the glycan core (Faye et al., 1993) and with the anti-plant Lewis antibodies.

Results presented in Figure 6 show that many extracellular glycoproteins are immunodetected with both antibodies (Fig. 6, lanes 1 and 2), indicating the high frequency of extracellular glycoproteins having modified *N*-glycans and, particularly, glycans with terminal Le^a antennae. Vacuolar glycoproteins are immunodetected exclusively with the anti-β-(1,2) Xyl antibodies, but never react with the anti-plant Lewis antibodies (Fig. 6, lanes 3). The latter observation is consistent with results obtained from immunocytochemical experiments in which no labeling of the vacuole with anti-plant Lewis antibodies was observed (Figs. 3 and 4). Both approaches strongly suggest that extracellular glycoproteins, either soluble or membrane bound, are decorated with elaborated Le^a-containing

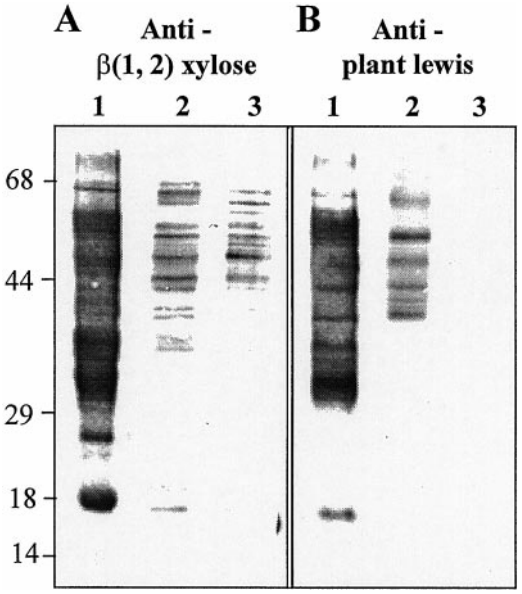

Figure 6. Immunodetection of Le^a-containing *N*-glycans and β-(1,2)Xyl-containing *N*-glycans on glycoproteins from culture medium, protoplast, and vacuole of cultured tobacco cells. Protein extracts from culture medium (lanes 1), protoplasts (lanes 2), and vacuoles (lanes 3) of suspension-cultured tobacco cells were separated in SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoprobed with anti-Xyl (A) and anti-plant Lewis antibodies (B).

N-glycan structures, while vacuolar glycoproteins never contain Le^a epitopes. This conclusion is also consistent with our previous results showing that, when expressed in tobacco BY2 cells or in tobacco cv Xanthi plants, the bean PHA, a vacuolar protein, is *N*-glycosylated by high-Man-type and by $Man_3XylFucGlcNAc_2$ (Fig. 2, glycan a) paucimannosidic-type *N*-glycans, but does not harbor Le^a-containing *N*-glycans (Rayon et al., 1996, 1998).

Vacuolar Proteins Cross the Golgi Stacks Where Le^a Antennae Are Assembled

The fact that extracellular glycoproteins have *N*-glycan structures that are not found on vacuolar proteins could beexplained by differences in the intracellular transport of both protein populations. Indeed, one could hypothesize that, during their transport to the plasma membrane, extracellular glycoproteins travel through Golgi compartments containing both β-(1,3)galactosyltransferase and α-(1,4)fucosyltransferase, while vacuolar proteins do not. Consequently, vacuolar proteins never have their *N*-glycans exposed to these transferases. To test this hypothesis, we performed double-immunolabeling experiments on BY2 cells expressing the vacuolar protein PHA from bean. First, the double immunolabeling on these cells was performed with anti-PHA antibodies and anti-β-(1,2)Xyl antibodies. Results indicated that both antibodies recognized proteinaceous deposits in the vacuolar lumen (Fig. 7A). The level of PHA labeling in Golgi stacks was low but, as expected, occurred in the same stacks as anti-β-(1,2)Xyl antibody labeling (Fig. 7B). The transformed BY2 cells were immunodetected with anti-PHA and anti-plant Lewis antibodies. Both types of antibodies were able to label the same Golgi stacks (Fig. 7, C and D), indicating that both vacuolar proteins and extracellular/plasma membrane proteins cross the same Golgi compartments, particularly the cisternae where Le^a antennae are built, before being sent to their respective target compartments.

DISCUSSION

Biantennary complex-type *N*-glycans, called in the past "laccase-type *N*-glycans" and now renamed "Le^a-containing *N*-glycans" after examination of their structure, have been so far described in only a few secreted glycoproteins (Takahashi et al., 1986, 1990; Ogawa et al., 1996). We showed in a previous study that a large number of higher plant glycoproteins are glycosylated with Le^a-containing *N*-glycans (Fitchette-Lainé et al., 1997). In this paper, the search for glycoproteins bearing Le^a-containing *N*-glycans has been extended across the whole plant kingdom. We found that, except for members of the Cruciferae family (including Arabidopsis), *N*-glycans bearing the Le^a epitope are abundant in the higher and in some lower

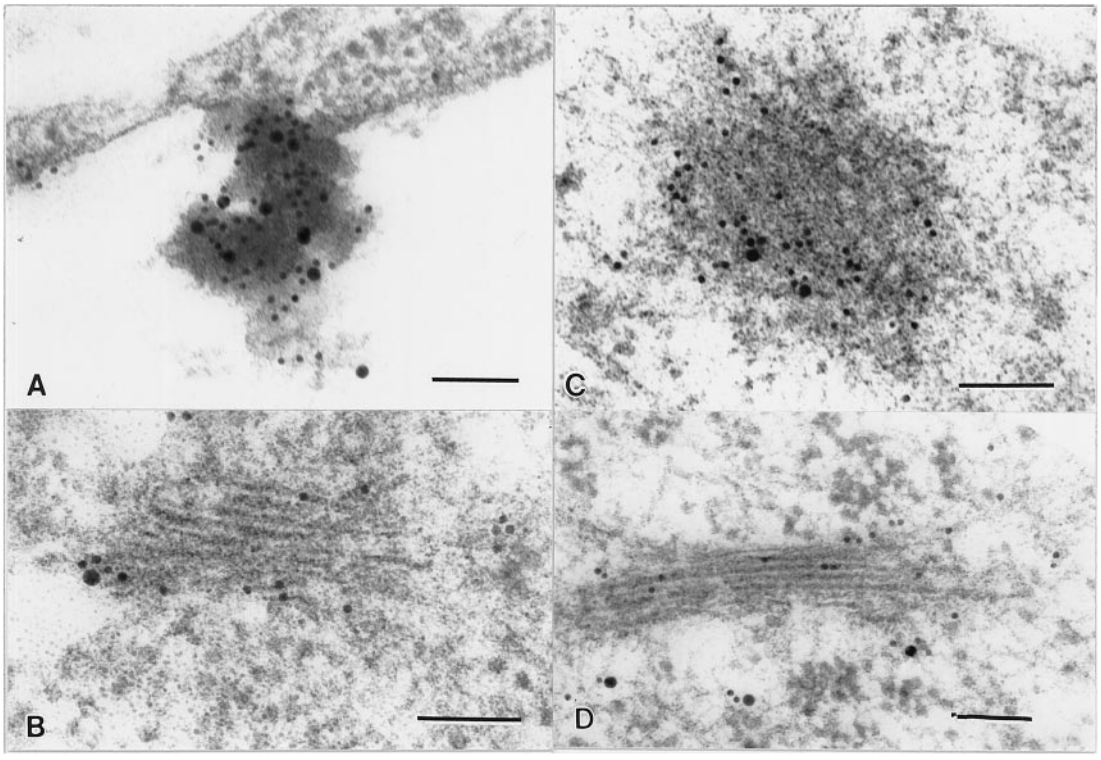

Figure 7. Double labeling of PHA and *N*-glycans in transformed BY2 suspension-cultured cells. A, Vacuolar inclusion in a transformed BY2 cell expressing PHA stained with anti-PHA antibodies (20 nm of gold particles) followed by anti-β-(1,2) Xyl antibodies (10 nm of gold particles). Bar = 100 nm. B, Golgi stack stained with anti-PHA antibodies (20 nm of gold particles) and anti-β-(1,2) Xyl antibodies (10 nm of gold particles). Bar = 100 nm. C, Golgi stack double labeled with anti-PHA antibodies (20 nm of gold particles) and anti-plant Lewis antibodies (10 nm). Bar =100 nm. D, Golgi stack double labeled with anti-PHA (20 nm gold) and anti-plant Lewis antibodies (10 nm gold). Note that PHA labeling is at the trans face. Bar = 100 nm.

representatives of the plant kingdom, and therefore seem to have appeared rather early in evolution. The absence of Le^a epitopes in protein extracts of members of the Cruciferae does not definitively demonstrate that this antigen is not synthesized in these plants. However, it does suggests that the expression of the related glycosyltransferase activities is likely to be organ or time dependent.

Le^a-containing *N*-glycans are not only abundant, they are also highly immunogenic in mammals. This is illus-trated here in plants through the characterization of awell-known Golgi marker, JIM 84. The monoclonal antibody JIM 84, which was raised against a carrot coated vesicle fraction, has been shown by immunofluorescence and immunogold microscopy to recognize the Golgi apparatus in a range of plant cells, as well as the plasma membrane in many cell types. Immunodetection of various glycoproteins on blots, as well as analysis of the binding of JIM 84 on BSA-Le^a, Le^x clearly demonstrates that this monoclonal antibody is specific for the glycan Le^a epitope, as previously demonstrated for the anti-plant Lewis antibodies.

Both JIM 84 and the purified polyclonal anti-plant Lewis antibodies can be used as Golgi markers. The strong labeling obtained by immunogold microscopy with the latter has permitted an analysis of gold particle distribution on a Golgi stack. Labeling of the plant Golgi apparatus with anti-plant Lewis antibodies is mostly observed over the trans-most part of the stack. This suggests that Le^a is synthesized in the trans-Golgi by transfer of Gal and Fuc residues by the Golgi β-(1,3)galactosyltransferase and the α-(1,4)fucosyltransferase onto terminal glucosamine residues of mature plant *N*-glycans. This suggests that the formation of the Lewis antigen is primarily a late event occurring after the transfer of β-(1,2)Xyl and α-(1,2)Fuc residues on the core *N*-glycans that were previously reported to occur in the medial and trans Golgi apparatus (Lainé et al., 1991; Zhang and Staehelin, 1992; Fitchette-Lainé et al., 1994).

Extracellular glycoproteins, either soluble or membrane bound (i.e. integral plasma membrane glycoproteins), present complex, elaborated *N*-linked glycans bearing Le^a antennae. This was illustrated through both immunoblotting and immunocytochemical experiments. In contrast, vacuolar glycoproteins do not present Le^a-containing complex glycans and could not be probed with the anti-plant Lewis antibodies. This obvious discrepancy in the complex *N*-glycan distribution within plant cells raises one question: Are there two different types of Golgi stacks or two different Golgi pathways involved in protein secretion in plant cells, one dedicated to the vacuolar proteins, devoid of the enzymatic machinery able to assemble the Lewis antennae, and one specific for the extracellular proteins in which the Lewis antennae are synthesized? When double-immunolabeling experiments using both anti-plant Lewis antibodies and antibodies directed at PHA were performed on transgenic tobacco BY2 cells expressing PHA, a reporter vacuolar protein (Rayon et al., 1996), glycoproteins bearing Le^a co-localized with vacuolar proteins (i.e. PHA) within the same cisternae of a Golgi stack. These results rule out the hypothesis that vacuolar and extracellular (i.e. Le^a-containing) glycoproteins do not travel through the same Golgi stack, or that vacuolar proteins leave the Golgi stacks before the compartments containing β-(1,3)galactosyltransferase and α-(1,4)fucosyltransferase, which are responsible for Le^a biosynthesis.

In bean seeds the lectin PHA, which is stored in the protein storage vacuole, has a paucimannosidic-type *N*-glycan *N*-linked to Asn-60 (glycan a, Fig. 2). However, it has been shown that during its transport, immature PHA bears a complex-type *N*-glycan presenting terminal GlcNAc residues. These GlcNAc residues are eliminated just before or rapidly after the arrival of the lectin in the protein bodies of bean cotyledons (Vitale and Chrispeels, 1984). We have recently shown that, when expressed in suspension-cultured tobacco cells or in tobacco plants, PHA is *N*-glycosylated by the paucimannosidic $Man_3XylFucGlcNAc_2$, as is also observed in bean (Rayon et al., 1996, 1998). As a consequence, a rapid trimming of terminal GlcNAc from intermediate complex *N*-glycans also occurs in tobacco, and this mechanism is probably highly conserved in plants. Indeed, the vacuole is a highly hydrolytic compartment, containing all of the glycosidase activities necessary not only to cleave terminal GlcNAc residues but also to degrade the terminal Le^a epitope.

We investigated whether vacuolar glycoproteins acquire Le^a structures before further trimming during their transport to or within the vacuole. Although the degradation of Le^a antennae should be fast enough to be undetectable using pulse-chase experiments (result not shown), degradation by glycosidases is likely to be the best explanation for the absence of Le^a on glycoproteins stored in the vacuoles. Furthermore, since Le^a-containing *N*-glycans are only found on extracellular glycoproteins in plants, this antigen can serve as a useful marker of the glycoprotein transport through the plant secretory system and as a marker of the final location of glycoproteins within the plant cell.

As demonstrated by immunolabeling experiments, the Le^a epitope is highly expressed on the plasma membrane of a wide range of plant cells. By analogy with data from animal cells, this location may suggest some involvement of Lewis antigens in cell-to-cell recognition or interaction with plant pathogens. Efforts must now be made to examine the implication of cell-surface glycans, such as Lewis antigens, in plant communication processes.

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LITERATURE CITED

Abeijon C, Hirschberg CB (1992) Topography of glycosylation reactions in the endoplasmic reticulum. Trends Biochem Sci **17:** 32–36

Baskin TI, Busby CH, Fowke LC, Sammut M, Gubler F (1992) Improvements in immunostaining samples embedded in methacrylate: localisation of microtubules and other antigens

throughout developing organs in plants of diverse taxa. Planta **187:** 405–413

Chrispeels MJ, Boulder D (1975) Control of storage protein metabolism in the cotyledon of germinating mung bean: role of the endopeptidase. Plant Physiol **55:** 1031–1037

Faye L, Gomord V, Fitchette-Lainé A-C, Chrispeels MJ (1993) Affinity purification of antibodies specific for Asn-linked glycans containing α1,3 fucose or β1,2 xylose. Anal Biochem **109:** 104–108

Feizi T (1993) Oligosaccharides that mediate cell-cell adhesion. Curr Opin Struct Biol **3:** 701–710

Fitchette-Lainé A-C, Gomord V, Cabanes M, Michalski J-C, Saint Macary M, Foucher B, Cavelier B, Hawes C, Lerouge P, Faye L (1997) *N*-glycans harboring the Lewis a epitope are expressed at the surface of plant cells. Plant J **12:** 1411–1417

Fitchette-Lainé A-C, Gomord V, Chekkafi A, Faye L (1994) Distribution of xylosylation and fucosylation in the plant Golgi apparatus. Plant J **5:** 673–682

Gomord V, Denmat L-A, Fitchette-Lainé A-C, Satiat-Jeunemaitre B, Hawes C, Faye L (1997) The C-terminal HDEL sequence is sufficient for retention of the secretory proteins in the endoplasmic reticulum (ER) but promotes vacuolar targeting of proteins that escape the ER. Plant J **11:** 313–325

Gomord V, Fitchette-Lainé A-C, Denmat L-A, Michaud D, Faye L (1998) Production of foreign proteins in tobacco cell suspension culture. *In* C Cunningham, AJR Porter, eds, Methods in Biotechnology, Vol 3. Humana Press, Totowa, NJ, pp 155–164

Henry S, Oriol R, Samuelson B (1995) Lewis histo-blood group system and associated secretory phenotypes. Vox Sang **6:** 166–182

Horsley D, Coleman J, Evans D, Crooks K, Peart J, Satiat-Jeunemaitre B, Hawes C (1993) A monoclonal antibody, JIM 84, recognizes the Golgi apparatus and plasma membrane in plant cells. J Exp Bot **44:** 223–229

Kubelka V, Altmann F, Staudacher E, Tretter V, Marz L, Hard K, Kamerling JP, Vliegenthart JFG (1993) Primary structures of the *N*-linked carbohydrate chains from honeybee venom phospholipase A2. Eur J Biochem **213:** 1193–1204

Kurosaka A, Yano A, Itoh N, Kuroda Y, Nakagawa T, Kawasaki T (1991) The structure of a neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antiserum. J Biol Chem **266:** 4168–4172

Laemmli U (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **227:** 680–685

Lainé A-C, Gomord V, Faye L (1991) Xylose-specific antibodies as markers of subcompartmentation of terminal glycosylation in the Golgi apparatus of sycamore cells. FEBS Lett **295:** 179–184

Lerouge P, Cabanes-Macheteau M, Rayon C, Fitchette-Lainé A-C, Gomord V, Faye L (1998) N-glycoprotein biosynthesis: recent development and future trends. Plant Mol Biol **38:** 31–48

Lis H, Sharon N (1978) Soybean agglutinin: a plant glycoprotein. J Biol Chem **253:** 3468–3476

Melo NS, Nimtz M, Conradt HS, Feveiro PS, Costa J (1997) Identification of the human Lewis^a carbohydrate motif in a secretory peroxidase from a plant cell suspension culture (*Vaccinium myrtillus* L.). FEBS Lett **415:** 186–191

Ogawa H, Hijikata A, Amano M, Kojima K, Fukushima H, Ishizuka I, Kuruhara Y, Matsumoto L (1996) Structures and contribution to the antigenicity of oligosacharides of Japanese cedar (*Cryptomeria japonica*) pollen allergen *Cry j* I: relationship between the structures and antigenic epitopes of plant *N*-linked complex-type glycans. Glycoconj J **13:** 555–566

Rayon C, Cabanes-Macheteau M, Loutelier-Bourhis C, Saliot-Maire I, Lemoine J, Reiter WD, Lerouge P, Faye L (1999) Characterization of *N*-glycans from Arabidopsis: application to a fucose-deficient mutant. Plant Physiol **119:** 725–734

Rayon C, Gomord V, Faye L, Lerouge P (1996) *N*-Glycosylation of phytohemagglutinin expressed in bean cotyledons or in transgenic tobacco cells. Plant Biochem Biophys **34:** 273–281

Rayon C, Lerouge P, Faye L (1998) The protein *N*-glycosylation in plants. J Exp Bot **49:** 1463–1472

Satiat-Jeunemaitre B, Fitchette-Lainé A-C, Alabouvette J, Marty-Mazars D, Hawes C, Faye L, Marty F (1994) Differential effects of monensin on the plant secretory pathway. J Exp Bot **45:** 685–698

Satiat-Jeunemaitre B, Hawes C (1992) Redistribution of a Golgi glycoprotein in plant cells treated with brefeldin A. J Cell Sci **103:** 1153–1166

Satiat-Jeunemaitre B, Steele C, Hawes C (1996) Golgi-membrane dynamics are cytoskeletonN dependent: a study on Golgi stack movement induced by brefeldin A. Protoplasma **191:** 21–33

Sterjades R, Dean JF, Eriksson K-E (1992) Laccase from sycamore maple (*Acer pseudoplatanus*) polymerizes monolignols. Plant Physiol **99:** 1162–1168

Sturm A, Voelker TA, Hermann EM, Chrispeels MJ (1988) Correct glycosylation, Golgi-processing, and targeting to protein bodies of the vacuolar protein phytohemagglutinin in transgenic tobacco. Planta **175:** 170–183

Takahashi N, Hitotsuya H, Hanzawa H, Arata Y, Kurihara Y (1990) Structural study of asparagine-linked oligosaccharide moiety of taste-modifying protein, miraculin. J Biol Chem **265:** 7793–7798

Takahashi N, Hotta T, Ishihara H, Mori M, Tejima S, Bligny R, Akazawa T, Endo S, Arata Y (1986) Xylose-containing common structural unit in *N*-linked oligosaccharides of laccase from sycamore cells. Biochemistry **25:** 388–395

van Kuik JA, van Halbeek H, Kamerling JP, Vliegenthart JFG (1985) Primary structure of the low-molecular-weight carbohydrate chains of *Helix pomatia* α-hemocyanin. J Biol Chem **260:** 13984–13988

Vitale A, Chrispeels MJ (1984) Transient *N*-acetylglucosamine in the biosynthesis of phytohemagglutinin: attachment in the Golgi apparatus and removal in the protein bodies. J Cell Biol **99:** 133–140

Zhang GF, Staehelin LA (1992) Functional compartmentation of the Golgi apparatus of plant cells. Plant Physiol **99:** 1070–1083