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Biochemical characterization of *Silene alba* α4-fucosyltransferase and Lewis a products

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α1,4-Fucosylation has been recently detected in *Arabidopsis thaliana* [Léonard *et al.* (2002), *Glycobiology* 12: 299–306], and corresponding enzymes have also been characterized in *Beta vulgaris* [Bakker *et al.* (2001), *FEBS Lett*, 507: 307–312], and *Lycopersicum aesculentum* [Wilson (2001), *Glycoconjugate J.*, 18: 439–447]. Here we demonstrated fucosyltransferase activity (FucT) in *Silene alba* cells and tissues. The Fuc linkage to GlcNAc residues of the *lactosamine* moiety of the Type I acceptor was confirmed by mass spectrometry experiments. Le^a-glycoconjugates are found in the Golgi apparatus and plasma membrane of plant cells. *In planta*, the highest levels of activity were detected in seedlings, young roots and male flowers. The enzyme was stable up to 45°C and the optimum pH of reaction was 8.0. The enzyme required Mg^{2+} or Mn^{2+} for activity and was inhibited by Zn^{2+} and ethylenediaminetetraacetic acid. Chemical modification of the enzyme with group-selective reagents revealed that selective modifications of arginine and lysine residues had no effect on enzyme activity. However the enzyme contains histidine and tryptophan residues that are essential for its activity. In contrast to human FUT3, the *S. alba* α4-FucT was insensitive to *N*-ethylmaleimide (NEM) treatment. Measurement of enzyme activity in *S. alba* cell fractions indicated that the enzyme is bound to microsomal membranes, furthermore a soluble isoform of the protein may be present.
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Abbreviations: **Con A, concanavaline A; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; GlcNAc, *N*-acetylglucosamine; NEM, *N*-Ethylmaleimide; DEPC, Diethylpyrocarbonate; Mes, 4-Morpholinoethanesulfonic acid; PBE94, Polybuffer-exchange 94; FucT, fucosyltransferase; Le^a, Lewis a.**

Introduction

Glycoproteins are widely distributed in plants, but the role of complex glycosylation in plant development is still unknown. In fact mutants defective in complex N-linked glycans (cgl) have a normal growth, without any strong phenotypic modifications [1]. Plant oligosaccharide structures can be divided in 3 groups: oligomannosidic, paucimannosidic and complex-type N-glycans. The core structure of complex-type oligosaccharides may contain a Xyl residue linked to the β-mannosyl residue by β1,2 linkage, and Fuc attached by a α (1-3) bound to the asparagine-linked GlcNAc. Complex-type oligosaccharides are well described, *e.g.*, in rice α-amylase [2], in Sycamore cell laccase [3,4] and in soybean peroxidase [5].

In laccase-type N-glycan*s*, an additional Fuc residue is linked to the *O*-4 GlcNAc [4] at the non-reducing ends of the glycan thus forming the Le^a determinant (Galβ1-3(Fucα1-4)GlcNAc-R). Plant Le^a-products were localized by immuno-staining in Golgi apparatus and in the outer face of the plasma membrane [4,6,7]. The requisite α4-FucT activity has been first measured in mungbean [8] and more recently in sycamore cells [4], in *Vaccinus myrtillus* [9], in *Silene alba* [10] and in *Arabidopsis thaliana* [11]. Corresponding cDNA have been cloned from *Beta vulgaris* [6], *Lycopersicum aesculentum* [12] and

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Arabidopsis thaliana [11]. However purification of a plant α4-FucT to homogeneity has failed yet except a partial purification of α4- FucT from *Vaccinus myrtillus* [13]. In that study, the authors demonstrated that α4-FucT is found in the microsomal fractions. Numerous glycosyltranferases of eukaryotic cells are described as endogenous Golgi proteins with a type II membrane topology. They are characterized by a short N-terminal cytoplasmic tail, typically 6 to 12 amino acids, a trans-membrane domain of about 17 to 20 amino acids, a relatively short luminal stem region and a large luminal catalytic domain. In addition to Golgi anchored enzymes [14], soluble forms of them (galactosyltranferase, fucosyltransferase, sialyltransferase) have been detected in mammalian serum [15], milk [16], colostrum [17], saliva [18] and in the culture media of normal and tumoral cells [19]. The mechanisms for processing and secretion of soluble glycosyltransferases are not well understood. N-terminal sequence analysis of soluble forms of α(2,6)-sialyltransferases and β(1,4)-galactosyltranferases revealed that they result from proteolysis within their luminal stem regions, which release the catalytic domains [20]. The proteases responsible for the enzymatic cleavage have not been formally identified. Nevertheless a cathepsin D like protease is involved in the cleavage of the rat liver α(2,6)-sialyltransferase during the acute phase of inflammation [21]. Such proteases have been described in plants [22] but not their role in generation of soluble plant glycosyltranferases.

In this work, we demonstrated that Le^a-determinant and the corresponding α4-FucT activity are well expressed in *Silene alba* cells suspension culture and plant tissues through *in vitro* tests using a Type I acceptor substrate. A biochemical characterization and a subcellular localization of this enzyme were then undertaken. Our results confirmed its localization into the plasma membrane and the Golgi apparatus, but seem also to suggest the existence of a soluble form.

Materials and methods

Plant culture

White campion (*Silene alba* [Miller] E.H.L. Krause) cells were maintened according to the procedure of Morvan [23] and were grown in climate chamber under continuous light (80 μE m^{-2} s^{-1}) and rotation (120 rpm) in a vertical flask agitator. *Silene alba* plants were grown in greenhouse and harvested at different stages corresponding to vegetative formation or flower development.

Enzyme assays

Fucosyltransferase assays were conducted in 60 μL containing 25 mM sodium cacodylate (pH 7), 5 mM ATP, 20 mM $MnCl_2$, 10 mM α-L-fucose, 0–9 μM GDP-[^{14}C]-fucose (310 mCi/mmol ; Amersham Pharmacia Biotech), 10 to 200 μM unlabelled GDP-Fuc, and 25 μg of crude protein extracts from *S. alba* cells or plants. The mixture was incubated between 5 min and 1 h at 37°C in presence of 0.1 mM of acceptor substrate (Type I, Type II, H-Type I and H-Type II). The reaction was stopped by addition of 3 mL of cold water. The reaction mixture was then applied to a conditioned Sep-Pack C_{18} reverse chromatography cartridge (Waters Millipore, Bedford MA) attached to a 10 ml syringe. Unreacted GDP-[^{14}C]-fucose was washed off with 15 mL of water.The radio-labelled reaction product was eluted with 2 times 5 mL of ethanol, collected directly into scintillation vials and counted with 2 volumes of Biodegradable Counting Scintillant (Amersham Pharmacia Biotech.) in a liquid scintillation beta counter (Liquid scintillation analyser, Tri-Carb-2100TR, Packard).

For mass spectrometry experiments, fucosyltransferase assays are carried out at 37°C for 7 h in a similar reactional medium as described above modified by 1 mM unlabelled GDP-Fuc and 3 mg mL^{-1} acceptor. Three independent reactions were gathered for one measurement of mass spectrometry.

Triton-stimulated UDPase activity was examined in 1 mL of 10 mM UDP, 20 mM Mes-KOH pH 7 in presence or absence of 0.3% detergent. G6Pase (Glucose 6 phosphatase) activity was examined in 1 mL of 10 mM Glc6P, 20 mM Mes KOH, pH 7. The amount of inorganic phosphate released was used to measure the activity.

Immunocytochemical procedure

The immuno-localization of Le^a containing glycans was performed with rabbit antibodies raised against laccase Le^a epitope [4].

S. alba cells were fixed for 15 to 30 min in a mixture of 1.5% (w/v) paraformaldehyde and 0.5% glutaraldehyde in 0.05 mM phosphate buffer pH 7.2 [24]. Rigorous washing in the same buffer was followed by 4 min post-fixation in 1% (w/v) Na_2SO_4, dehydration in an ethanol series, and overnight embedding in London Resin White. Polymerization occurred in gelatine capsules at 60°C for 24 h.

The imunogold reaction on thin sections, carefully spread with toluene vapour on parlodion-coated gold grids, was performed at 20°C, as previously described [25]. The procedure accommodated a compromise between the preservation of both structure and antigenicity. The sections, hydrated in deionised water, were then etched in the dark by 0.56 M $NalO_4$ and 0.1 M HCl, and washed for 15 min in PBS, 0.1% (v/v) Triton X-100, and 0.2% (v/v) glycine at pH 7.2. Unspecific sites were saturated for 45 min by goat serum in PBS, 0.2% (v/v) Triton X-100, 0.2% (v/v). Tween and 0.1% (w/v) BSA, and sections were incubated overnight with the Le^a antibody (diluted at 1/50). After washing in PBS, sections were placed for 40 min in TBS pH 8.2, 0.2% (v/v) Triton X-100, 1% (w/v) BSA, and goat serum before the 3 h application of a 15 nm gold particle-labelled goat anti-mouse IgG at a 1/40 dilution. The sections, washed in TBS and deionised water, were contrasted using a saturated aqueous solution of uranyl acetate in lead citrate. Controls were done as for the semi-thin sections. Samples were observed with an electronic microscope (100C, JEOL) operated at 80 kV.

Protein extraction and western blot experiments

Cells were harvested by filtration on Whatman 41 paper with a Büchner funnel, and disrupted with liquid nitrogen. The protein fraction used to measure α4-FucT activity was extracted in cacodylate buffer (200 mM sodium cacodylate pH 7.0 containing 1% (w/v) Triton X-100). The homogenate was centrifuged at 14,000 g for 30 min and the crude protein fraction collected form the supernatant. For microsomes preparation, disrupted cells were homogenised on ice in Hepes-KOH buffer pH 7.4 containing 1 mM DTT and 0.4 M sucrose [26]. The homogenate was filtered through cloth nylon and centrifuged at 5,000 g at 4°C for 10 min. The supernatant was again centrifuged (at 100,000 g, 4°C for 1 h) and the pellet was resuspended in cacodylate buffer.

Protein extracts for immunochemical analysis were obtained by homogenising plant material in a solution containing 0.7 M sucrose, 0.5 M Tris, 30 mM HCl and 2% (v/v) β-mercaptoethanol. After incubation on ice for 30 min, the homogenate was centrifuged at 5.000 g for 5 min. 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 addition of 5 volumes of methanol containing 0.1 M ammonium acetate. The preparation was then centrifuged at 10,000 g for 30 min. The pellet was washed once with 0.1 M ammonium acetate in methanol and twice with acetone before being resuspended in sample buffer (62.5 mM Tris-HCl pH 6.8, 10 mM DTT, 10% (v/v) glycerol).

Protein content was determined by the Bradford method using BSA as a standard [27].

The crude extracts were analyzed by SDS-PAGE using a 12% acrylamide Tris-Glycine gel [28]. Proteins were visualised after Coomassie blue staining and transferred onto nitrocellulose membrane (Schleicher & Schuell, Germany). Membranes were blocked overnight at 4°C using Tris-buffered saline pH 7.5 containing 0.1% (v/v) Tween 20 and 5% (w/v) bovine serum albumin (TBS-T-BSA). After three washings in TBS-T, the membrane was incubated for 1 h at 20°C with a mouse anti-Le^a antibody in TBS-T-BSA buffer at 1:100 dilution rabbit anti-mouse immunoglobulin coupled to horseradish peroxidase (Amersham,England) was used at 1:1000 dilution for detection.

Chromatography experiments

Affinity (ConA and GDP-agarose) and electrofocusing chromatography were performed on samples previously desalted on Sephacryl S200. The Sephacryl S200 colum (Vol = 350 mL) was equilibrated with 20 mM Mes-KOH, pH 7.0, containing 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% Triton X 100 (Buffer A) at a flow rate of 10 ml·h^{-1} at 4°C.

Before loading onto the ConA Sepharose 4B column (Vol = 100 mL), NaCl was added to desalted samples at a final concentration of 0.5 M NaCl. Bound glycoproteins were eluted with a gradient of methyl α-D-glucopyranoside (0 to 2 M) in Buffer A at a flow rate of 25 mL h^{-1} at 4°C.

For GDP-agarose chromatography, desalted samples were dialysed against buffer A before loading onto GDP-agarose column equilibrated with buffer A at a flow rate of 60 mL h^{-1}. The column was washed with buffer A then with A completed by 100 mM NaCl. The enzyme was finally eluted with a gradient of NaCl (100 mM to 2 M) in the same buffer.

For electro-focusing chromatography, desalted samples were first dialysed against 25 mM ethanolamine buffer, pH 9.5. A column containing 25 mM of PBE94 gel was prepared. The sample was loaded onto the column and elution was performed using a 2% Pharmalytes pH 8-10.5 solution containing 0.1% Triton X100 at a flow rate of 1 mL·min^{-1}.

Carbohydrate composition analysis

Monosaccharide composition was determined after methanolysis by gas-liquid chromatography of pertrimethyl-silylated methylglycosides according to Kamerling *et al.* [29], as modified by Montreuil *et al.* [30]. The fucosyltransferase products were also methylated according to Finne *et al.* [31], and analyzed as their partially methylated partially acetylated methyl glycosides by GC-MS according to Fournet *et al.* [32]. The molecular weight of the fucosylated product was characterised by Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) on a reflectron-type Vision 2000 time-of-flight mass spectrometer (Finnigan MAT, Bremen, Germany).

Results

α4-fucosyltransferase activity in cells and plants

L-Fuc transfer to the Galβ1,3GlcNAcβ-O-$(CH_2)_7CH_3$ acceptor (Type I) was monitored by the quantification of radio-labelled enzyme reaction products. As shown in Figure 1, a steady state of α4-FucT activity (22 pmol $min^{-1}mg^{-1}$ protein) was obtained

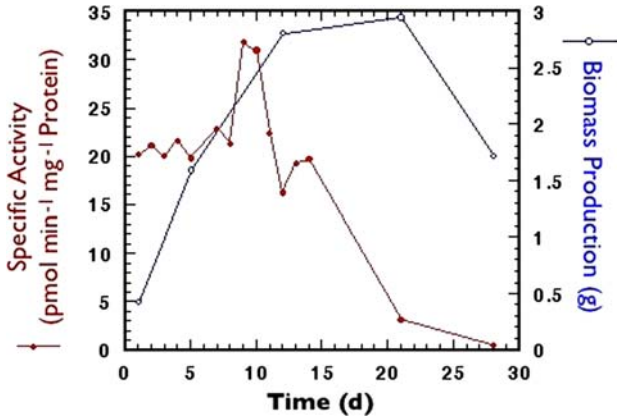

Figure 1. Variation of the α4-FucT activity and the biomass of the cells according to the age of a cell suspension culture of *Silene alba.*

Table 1. *In vitro* α4-FucT activity measured for different plant tissues extracts. Activity is expressed in pmol $min^{-1}mg^{-1}$ protein and each value is the mean of 3 replicates

	Protein level (pmol min^{-1} mg^{-1} protein)	*α4-FucT specific activity (pmol min^{-1} (mg^{-1} protein))*
Vegetative stage		
Seedling	0.24	50
Shoot	0.42	2
Root	0.34	15
Floral stage		
Petal	0.1	2
Sepal	0.08	1
Pistil	0.24	15
Stamen	0.42	67
Leaves		
Old	0.18	1
Young	0.39	26
Roots		
Old	0.2	2
Young	0.41	39

for young cells (from 0 to 8 days). A transitory increase in the activity was measured for 9–10 day old cells (up to 32 pmol min^{-1} mg^{-1} protein), followed by a reduction after the 10th day. The kinetics of α4-FucT activity follow a similar curve with the biomass, except for the first phase of growth.

The presence of α4-fucosyltransferase activity in *Silene alba* was then performed in whole plant. The enzyme activity was particularly associated with flower organs (Table 1). A strong activity was found in male flowers (67 pmol min^{-1} mg^{-1} protein) compared to female ones (15 pmol min^{-1} mg^{-1} protein). At the vegetative stage, seedlings (50 pmol min^{-1} mg^{-1} protein) and roots (15 pmol min^{-1} mg^{-1} protein) exhibited the highest activities whereas a low activity was measured in shoots (2 pmol min^{-1} mg^{-1} protein). Anti-Le^a antibodies were used to identify the products of the α4-FucT activity. The Le^a-epitopes are found at a high level in flowers (Figure 3A, line 2) as in seedlings, roots, and shoots without any significant difference in their distribution (data not shown).

Mass analysis α4-fucosyltransferase products

The attachment of Fuc at the substrate acceptor Galβ1-3GlcNAcβ-O-$(CH_2)_7CH_3$ (Type I) was confirmed by mass spectrometry. Composition and MALDI-TOF MS clearly confirmed the presence of Fuc residue which could be identified by its characteristic retention time on the GC chromatogram, and by the presence on the MALDI-TOF mass spectrum of the pseudo-molecular ion ($M + Na^+$) at m/z 664.7. The linkage of L-Fuc to the type I acceptor substrate was determined from the diagnostic ions of the EI mass spectrum of the partially methylated, partially acetylated methyl glycosides obtained from the transfer product. The mass spectra analysis (Figure 2), confirmed the formation of 6-*O*-methyl-3,4-*O*-diacetyl-GlcNAcMe (which has been assessed by the presence of the main ion fragments m/z 115, 170 and 256), per-*O*-methyl-Fuc and per-*O*-methyl-Gal indicating that Fuc is linked to the GlcNAc residue at the *O*-4 position.

Subcellular localization of Le^a-glycoproteins and α4-fucosyltransferase

In plant tissues, Le^a-determinants were detected by western blot and revealed 5 polypeptides of molecular mass ranging from 66 to 100 kDa (Figure 3A, lane 1). Furthermore, subcellular

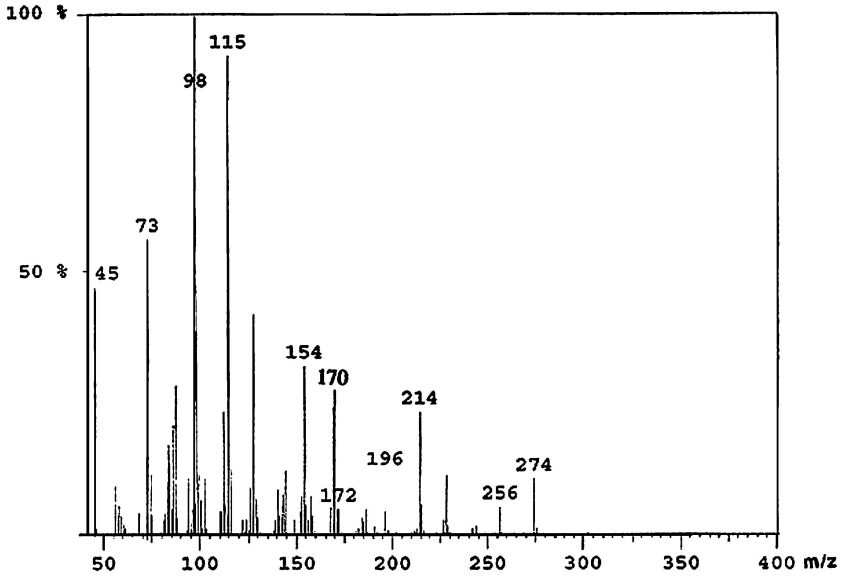

Figure 2. EI-MS spectrum of the 6-*O*-methyl-3,4-*O*-di-acetyl-GlcNAcMe obtained after L-Fuc transfer from GDP-Fuc to Type I acceptor by a α4-FucT from *S. alba*.

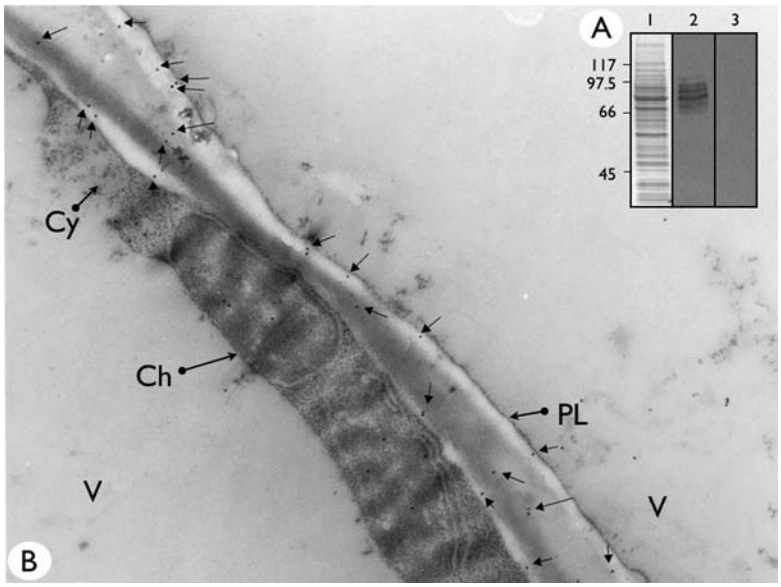

Figure 3. (A) Coomassie Blue staining of *S. alba* proteins in 10-day-old cells separated by SDS-PAGE as described in Materials and Methods (lane 1). Western blotting of *S. alba* Le^a-glycoproteins of 10-day-old cells (lane 2) with anti Le^a-antibody. Absorption of anti-plant Le^a-antibodies with human saliva prior to immunodetection (lane 3) demonstrated the reaction specificity. (B) Detection of Le^a-epitope in *S. alba* cell thin sections. Note the strong labeling (arrow) observed on the outer face of the plasma membrane. PL: plasma membrane, V: large vacuole, Ch: chloroplast, Cy: cytoplasm.

localization of *S. alba* Le^a-glycoproteins was determined by electron microscopy (Figure 3B). Le^a-determinants were found on the plasma membrane and in the Golgi of *S. alba* cells (Figure 3B) as previously demonstrated for *A. thaliana* [11]. Anti-Le^a pre-incubated with boiled human saliva abolished the protein labelling as shown western blot (Figure 3 lane 2) as microscopy experiments (data not shown).

Subcellular localization of the corresponding enzyme was then undertaken. Microsomal fractions were obtained by sucrose density gradient ultracentrifugation. Enzymatic markers of Golgi, plasma membrane and endoplasmic reticulum (Triton-stimulated UDPase for Golgi, ATPases for plasma membrane, Glc-6P-phosphatase for endoplasmic reticulum) were then assayed simultaneously with the α4-FucT activity of various no-microsomal (soluble fraction) and microsomal fractions (Table 2).

Both Golgi-enriched and soluble fractions contained a significant α4-FucT activity. The specific activity of the Golgi-enriched fraction (312 pmol min^{-1} mg^{-1} protein) was 6 fold higher than in the soluble one (52 pmol min^{-1} mg^{-1} protein). To be sure that the soluble fraction was membrane free, we made several successive ultra-centrifugations with or without Triton X-100. After checking of the absence of membrane contaminants in this soluble fraction, we could still measure a α4-FucT activity. Moreover Triton X-100 treatments of soluble fraction had no effect on α4- FucT activity, whereas similar experiments with Golgi-enriched one induced variable levels of α4-FucT activities (For Triton X-100 0.5% ∼300 pmol min^{-1} mg^{-1} protein and for 0.1% or 1% is reduced up to 85% of the optimal activity). All these data seem to suggest the presence of 2 forms of this enzyme: one soluble and one anchored to the membrane of the Golgi apparatus and sensitive to Triton X-100.

Substrate specificity and biochemical properties of α4-fucosyltransferase

The *in vitro* substrate specificity was determined using disaccharide Type I and Type II acceptor substrates and the trisaccharide acceptor H-type I, (Table 2). Both the soluble and membrane bound enzymes exhibited a α4-fucosyltransferase activity with Type I and H-type I but not with Type II acceptor sustrate. Moreover, the Type I/H-type I activity ratio was identical for the two putative forms.

The two potential forms of α4-FucT were then compared on the basis of their biochemical properties (pH, ion dependency, MW, pI and glycosylation state). For both, the enzymatic reaction proceeds linearly up to 2 h. The activity increased proportionally when the amount of protein varied from 10 to 100 μg. The optimal experimental conditions (pH, temperature and ions concentrations) were similar for the soluble and membrane anchored enzymes. A pH-dependent activity curve obtained using cacodylate (pH 5 to 7) and Tris-HCl (pH6 to 8) gave the strongest activity at pH 8. The activity slightly increased with temperature between 25 and 45°C (the optimal temperature), and strongly decreased above 50°C. Mg^{2+} and Mn^{2+} at 5 mM were optimal divalent cations for FucT activity whereas addition of Zn^{2+} or EDTA resulted to a complete inhibition. By selective ultra-filtration, we showed that the molecular mass was over 100 kDa for the membrane-anchored enzyme and

Table 2. Percentage of Golgi, endoplasmic reticulum and plasmalemma membrane found in the enriched and the soluble fraction (see Materials and Methods) and α4-FucT activity measured in the Golgi-enriched and the soluble fraction. Kinetic enzymatic analysis for sub-cellular compartment markers as performed during 2 h. The value correspond to the last point of the kinetic analysis. For α4-FucT, the reaction was performed during 15 min in a mixture containing 25 μg of proteins from Golgi-enriched or soluble fraction. Activity is expressed in $p$mol $min^{-1}mg^{-1}$ protein and each value is the mean of 3 replicates

Enzymatic markers	*Golgi-enriched fraction (%)*	*Soluble fraction (%)*
Golgi marker		
Triton stimulated UDPase	83	0
RE marker		
Glc-6-Phosphatase	11	0
Plasmalemma marker		
H^+-ATPase	6	2
Acceptor	***Golgi-enriched fraction*** *(pmol $min^{-1}mg^{-1}$protein)*	***Soluble fraction*** *(pmol $min^{-1}mg^{-1}$protein)*
Type I		
Galβ1-3GlcNAcβ- O-R [a]	312 $\pm$ 10	52 $\pm$ 2
H-Type I		
Fucα1-2Galβ1-3GlcNAc-O-R	265 $\pm$ 21	45$\pm$ 3
Type II		
Galβ1-4GlcNAcβ- O-R [a]	$<$0.1	$<$0.1
H-Type II		
Fucα1-2Galβ1-4GlcNAc- O-R	$<$0.1	$<$0.1

[a] R, $(CH_2)_7CH_3$
[b] R, C_6H_5

between 50 and 100 kDa for the soluble one. Isoelectric points were estimated by isoelectric gel electrophoresis and by chromatofocusing. Two different pIs have been determined for the membrane one, 8.9 ($\pm$0.1) and $>$10.0 and only one pI 8.8 ($\pm$0.1) for the soluble form. From the ability of the enzyme to bind to Sepharose Con A-4B, it was concluded that both are glycosylated. The soluble and membrane anchored enzymes were eluted at 250 mM α-methylglucoside suggesting a strong interaction between mannosyl residues and Con A.

To investigate possible differences in the catalytic mechanism of soluble and membrane bound enzymes, amino acid-specific inhibitors at variable concentrations were tested. The effect of five of these inhibitors (phenylglyoxal for Arg, *N*-ethylmaleimide for Cys, *N*-bromosuccinimide for Trp, 2,4,6-trinitrobenzenesulfonate for Lys and DEPC for His) on both the soluble and membrane bound *S. alba* α4-FucT were comparable to that obtained with a human α4-FucT, FUT3 (Table 3). As expected, 5 mM NEM reduced FUT3 activity by 50% [33], whereas no effect on the plant α4-FucT activity was measured. Phenylglyoxal had no effect on FUT3 activity whereas it slightly enhanced the soluble and membrane anchored α4-FucT activities. 2,4,6-trinitrobenzenesulfonate slightly reduced

Table 3. Effect of inhibitors on *S. alba* α4-FucT activities. The amino acids Cys, His, Arg, Lys and Trp of human FUT3 and *S. alba* α4-FucT were modified by NEM, DEPC, phenylglyoxal, 2,4,6-trinitrobenzenesulfonate and *N*-bromosuccinimide respectively. The recombinant FUT3 used for there experiments was kind gift of Dupuy *et al.* [35]. Enzymes were pre-treated for 30 min with inhibitors prior to measurement of α4-FucT activity. The reaction mixture contained Type I acceptor substrate and 50 μg of proteins, the incubation time was 30 min). The percentage of inhibition was calculated in comparison to untreated sample. Activity is expressed in pmol $min^{-1}mg^{-1}$ protein and each value is the mean of triplicate assays

			S. alba α4-FucT (%)	
	Concentration (mM)	*FUT3 (%)*	*Soluble isoform*	*Membrane isoform*
NEM	5	51	100	104
Phenylglyoxal	5	104	114	118
2,4,6-trinitrobenzenesulfonate	5	97	82	86
DEPC	0.5	10	11	13
N-bromosuccinimide	0.5	6	8	9

the plant α4-FucT activity but not FUT3 activity. In contrast, DEPC and *N*-bromosuccinimide strongly reduced the activities of FUT3, soluble and Golgi-enriched *Silene alba* α4-fucosyltransferase to 10% of the control.

Discussion

In this work we studied the α4-FucT activity from *Silene alba*. Le^a-epitopes were identified by immuno-detection (immunolocalization and western blotting) and the α1,4 linkage was demonstrated by structural analyses of the *in vitro* enzyme products. At a cellular level, Le^a-containing glycoconjugates were localized on the outer face of the plasmalemma and in Golgi apparatus, as in tomato, tobacco, onion, maize [7] and *Arabidopsis thaliana* cells [11]. In the whole plant, the Le^a-epitope was detected in all the tissues tested (flowers, leaves, roots and seedlings) without significant differences in the western blotting pattern (data not show). However α4-FucT activity measurements showed up predominance in young tissues (young leaves and roots) as well as in male flowers and seeds and it seems that growing tissues require *de novo* synthesis of Le^a-glycoconjugates. These data suggest that *de novo* Le^a-glycoconjugates could be involved in cell elongation and/or differentiation. The strong α4-FucT activity in seeds and male flowers suggests that *de novo* synthesis of Le^a-determinants could be associated with androgenesis, and later with seed maturation, as suggested by Joly *et al.* [34].

Use of specific inhibitors showed that Trp and His seem to be key amino acids for *S. alba* α4-FucT activity as for mammalian FUT3 [35,36] suggesting a possible similar implication of these amino acids in the conserved domain associated with α4-FucT activity. The only slight difference between plant (soluble or membrane anchored enzyme) and FUT3 was found with Cys inhibitor (NEM). Cys modification by NEM completely inhibited a α3-FucT (FUT6) [35], partially reduced FUT3 [33] activity but had no effect on *S. alba* α4-FucT. The catalytic domain of FUT3 contains five Cys residues, Cys^{143} being responsible for NEM-sensitivity of FUT [33]. In comparison, all plant sequences examined to date have eleven Cys residues [6,11,12]. However, the counterpart of human Cys^{143} seems not to be conserved which could explain the resistance of *S. alba* α4-FucT to NEM.

Biochemical analysis of the *S. alba* FucT suggests the occurrence of a soluble and a membrane anchored form of α4-fucosyltransferase. Microsomal contamination in soluble fraction could be discounted due to complementary experiments such as Triton X-100 extraction with or without protease inhibitors, and supernatant ultra-centrifugation with or without sucrose. Nevertheless, the biochemical properties, sensitivity to amino-acid-modifying agents and substrate specificity of the two forms are very similar. The single obvious difference concerns their isoelectric point and molecular masses. The soluble form of α4-FucT has a pI of 8.7–8.9 and a molecular mass between 50 and 100 kDa, whereas the membrane anchored form has a pI $>$ 10 and a MW $>$ 100 kDa. As demonstrated for some mammalian glycosyltransferases [15–18], the soluble form of α4-FucT of *S. alba* may result from proteolytic cleavage of the membrane-anchored enzyme. Richardson *et al.* [21] suggested a possible role for an aspartic proteinase in glycosyltransferase cleavage. Such proteinases, including cathepsin like proteinase, are well distributed in the plant kingdom [37]. If such proteolytic cleavage occurred in *S. alba*, it should explain the different isoelectric points of the two enzyme forms. Our experiments performed in presence of protease inhibitor (PMSF, Protease Inhibitor Cocktail: 4(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), bestanin, pepstatinA, E-64, leupeptin, and 1, 10-phenanthroline) seem to suggest that proteolytic cleavage during purification is not the origin of the soluble form of this enzyme. New experiments, based on knowledge of the corresponding genes could enable us to identify the mechanism leading to the expression of two putative forms of *S. alba* α4-FucT. If we assume that *S. alba* contains a soluble and a membrane-anchored α4-FucT, their specific functions within the cell should be determined.

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