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Biosynthesis of arabinogalactan-protein in *Lolium multiflorum* (Italian ryegrass) endosperm cells*

Subcellular distribution of galactosyltransferases

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Intracellular membranes from protoplasts of Italian-ryegrass (*Lolium multiflorum*) endosperm cells have been fractionated on sucrose density gradients and identified on the basis of putative-marker-enzyme assays. Galactosyltransferases capable of incorporating galactose from UDP galactose into 66% ethanol-soluble products are associated with all membrane fractions. Affinity chromatography of the ethanol-insoluble products on (murine myeloma protein J539)–Sepharose reveals that the enzymes responsible for the synthesis of polymers containing (1→6)-β-D-galactose residues are associated exclusively with subcellular fractions enriched in Golgi-derived membranes. This suggests that the Golgi apparatus plays an important part in the synthesis of the carbohydrate component of the ryegrass arabinogalactan-protein.

The arabinogalactan-protein secreted by suspension-cultured endosperm cells of Italian ryegrass (*Lolium multiflorum*) consists of a peptide core to which arabinogalactan chains are appended. The polysaccharide, comprising more than 90% by weight of the molecule, contains a backbone of (1→3)-β-D-galactose residues heavily substituted with oligomeric side chains of (1→6)-β-D-galactose and L-arabinose residues (Anderson *et al.*, 1977). In addition to the secreted arabinogalactan-protein, a polymer with analogous structural features is bound to the plasma membrane of the cells (Schibeci *et al.*, 1982; Fincher *et al.*, 1983).

Mascara & Fincher (1982) examined the biosynthesis of the carbohydrate moiety of the ryegrass arabinogalactan-protein by providing a total cellular membrane preparation with UDP-[^{14}C]galactose in the presence of ADP-ribose, which is an inhibitor of UDP-galactose 4-epimerase. Radioactive galactose residues were incorporated into products that were insoluble in aq. 66% ethanol. A significant proportion of these products was bound to affinity columns of (murine myeloma protein J539)–Sepharose (Mascara & Fincher, 1982) indicating that oligo- or poly-saccharides analogous in structure to the arabinogalactan-protein were present. Murine myeloma protein J539 is a monoclonal antibody highly specific for (1→6)-β-D-galactose residues (Glaudemans *et al.*, 1974).

A more detailed study of individual polysaccharide synthases responsible for arabinogalactan-protein synthesis was precluded by the presence of other membrane-bound enzymes competing for the UDP-galactose substrate. In the present study we have used the assay system of Mascara & Fincher (1982) in conjunction with recently developed membrane-fractionation procedures (Schibeci *et al.*, 1982; Cohen *et al.*, 1983; Henry *et al.*, 1983). These procedures permitted the isolation of membrane fractions enriched in RER, SER, Golgi membranes and 'secretory vesicles', enabling us to purify partially enzymes capable of transferring galactose from UDP-galactose into 66% ethanol-insoluble polymers containing (1→6)-β-galactose residues, and to identify the subcellular location of the enzymes.

Abbreviations used: Mops, 4-morpholine-ethanesulphonic acid; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

* The present paper is part IV of a series of papers of this topic. Part I is Pollard & Fincher (1981); part II is Mascara & Fincher (1982); and part III is Cohen *et al.* (1983).

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Materials and methods

Ryegrass endosperm cell culture

Italian-ryegrass (*Lolium multiflorum* Lam.) endosperm cells were grown in suspension culture at

28°C in the dark on a modified White's medium containing 4% (w/v) sucrose (Smith & Stone, 1973). Mid-exponential-phase (7-day-old) cells were used in all experiments.

Preparation of plasma membrane and intracellular membranes

Ryegrass endosperm protoplasts, prepared by incubation of suspension-cultured cells with 1% Driselase (Kyowa Hakka Kogyo Co., Tokyo, Japan) (Keller & Stone, 1978) were coated with murine myeloma protein J539, lysed in 50mM-potassium phosphate buffer, pH8.0 (containing 5mM-$MgCl_2$), and the plasma-membrane fraction isolated by low-speed centrifugation of the lysate on a discontinuous sucrose/sorbitol gradient as described by Schibeci *et al.* (1982). The fraction containing the bulk of the intracellular membranes and cytoplasmic components (fraction F1) was centrifuged at 100000*g* for 1h, the pellet resuspended in 20mM-Mops/NaOH buffer, pH7.5 (containing 5mM-dithiothreitol) with or without 5mM $MgCl_2$, and the suspension centrifuged at 180000*g* for 2h on linear 5–42% (w/w)-sucrose density gradients in a Beckman SW41 rotor (Cohen *et al.*, 1983). Gradient fractions were collected by using a J-shaped Pasteur pipette and the membranes collected by centrifugation at 200000*g* for 1h and finally resuspended in 20mM-Mops/NaOH buffer, pH7.5 (containing 5mM-dithiothreitol). Refractive indices of sucrose-gradient fractions were measured with an Abbé refractometer.

Marker-enzyme assays

NADH- and NADPH-cytochrome *c* reductases were measured spectrophotometrically at 550nm (Hodges & Leonard, 1974). Latent IDPase was determined by the method of M'Voula-Tsieri *et al.* (1981) and the phosphate released by the method of Peterson (1978).

Assay of galactosyltransferases and isolation of ethanol-insoluble products

The assay mixture contained, in 250μl, 0.1M-Mops/NaOH buffer, pH7.5, 10mM-$MgCl_2$, 5mM-dithiothreitol, 3.2μM-UDP-[^{14}C]galactose (302mCi/mmol; New England Nuclear Corp.) and 2.5mM-ADP-ribose (Sigma) (Mascara & Fincher, 1982). The membrane suspensions (50μl, 0.5mg of protein) were preincubated in the assay mixture for 30min at 4°C before initiation of the reaction by addition of UDP-[^{14}C]galactose. After shaking the mixture for 30min at 20°C, the reaction was stopped by the addition of 2ml of chloroform/methanol (1:1, v/v). Assays were routinely performed in triplicate, and zero-time and boiled-enzyme controls were included.

Non-incorporated UDP-[^{14}C]galactose, lipid and low-M_r compounds were removed by successively washing with cold (4°C) chloroform/methanol/water (10:10:3, by vol.; 2×5ml), 66% (v/v) ethanol containing 0.05mM-EDTA (2×5ml) and 66% (v/v) ethanol (2×5ml) (Mascara & Fincher, 1982). The dried pellet was suspended in 100μl of water, dissolved in 1ml of NCS scintillation fluid (Amersham Australia) and counted for radioactivity in PCS scintillation fluid (Amersham) at an efficiency of 80–90% (Mascara & Fincher, 1982).

Affinity chromatography on (myeloma protein J539)–Sepharose

Ethanol-insoluble products were dissolved in 1M-NaOH containing 0.2% (w/v) $NaBH_4$ (Mascara & Fincher, 1982) and, after exhaustive dialysis against water, were concentrated to approx. 9500c.p.m./ml of water and applied to the (myeloma protein J539)–Sepharose 4B column. The column was prepared and operated as described by Mascara & Fincher (1982). Myeloma J539 protein was purified from mouse ascites fluid by affinity chromatography on acid-treated Sepharose 2B (Eichmann *et al.*, 1976).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. When dithiothreitol was present in samples, protein was determined after pretreatment with 0.2M-*N*-ethylmaleimide (Hughes *et al.*, 1981).

Results and discussion

Fractionation of intracellular membranes

The identity of the ryegrass subcellular-membrane fractions was based on putative-marker-enzyme distributions and on the density shifts caused by dissociation of ribosomes from RER to form SER at low Mg^{2+} concentrations (Lord *et al.*, 1973; Quail, 1979). The results shown in Table 1 are in agreement with previous results (Cohen *et al.*, 1983), although it was observed here that careful preparation and fractionation of the sucrose gradient led to a clear and reproducible resolution of two broad membrane bands in fraction L3. The lower-density component (fraction L3a) accounted for only a few per cent of total protein at a high Mg^{2+} concentration, but at a lower Mg^{2+} concentration it contained a much higher proportion of the protein (Table 1).

Latent IDPase has been correlated with the Golgi apparatus (Ray *et al.*, 1969) and antimycin A-insensitive NAD(P)H-cytochrome *c* reductases as markers for the endoplasmic reticulum (Lord *et al.*, 1973; Quail, 1979). On the basis of the marker-

Table 1. *Marker-enzyme distribution in subcellular membrane fractions prepared either with low or high Mg^{2+}*
Recoveries of enzyme activities were 60–85%. The relative specific activity (RSA) is the ratio of the specific activity of the subcellular-membrane fraction to that of the total intracellular membrane suspension loaded on the sucrose gradient. The percentage of the total recovered activity in each fraction (%TA) is also shown. Total enzyme activities in the low- and high-Mg^{2+} gradients respectively were: NADH-cytochrome *c* reductase, 1487.5 and 2849.9 nmol·min^{-1}; NADPH-cytochrome *c* reductase, 445.5 and 807.2 nmol·min^{-1}; IDPase, 792.6 and 759.8 nmol·min^{-1}.

				NADH-cytochrome *c* reductase				NADPH-cytochrome *c* reductase				IDPase			
		Protein (%)		Low Mg^{2+}		High Mg^{2+}		Low Mg^{2+}		High Mg^{2+}		Low Mg^{2+}		High Mg^{2+}	
Fraction	Density (g·cm^{-3})	Low Mg^{2+}	High Mg^{2+}	RSA	%TA	RSA	%TA	RSA	%TA	RSA	%TA	RSA	%TA	RSA	%TA
L1	<1.01	24.8	29.4	0.03	0.8	0.25	6.1	0.54	13.6	0.37	11.0	2.0	49.6	1.41	41.1
L2a	1.02	5.9	1.6*	0.30	1.7	0.57	0.8	0.53	3.1	1.00	1.6	0	0	0.56	0.9
L2b	1.03	7.4		0.34	2.5			0.83	6.2			0	0		
L2c	1.04	19.7	2.1	0.83	16.4	0.67	1.1	1.28	25.1	1.29	2.7	0.87	17.2	0.69	1.4
L3a	1.04–1.08	20.4	2.8	1.15	23.5	1.22	2.8	1.24	25.5	1.54	4.3	0.56	11.4	1.03	2.9
L3b	1.08–1.12	11.2	19.3	2.66	29.8	1.30	20.5	1.47	16.4	1.59	30.7	1.84	20.6	2.45	47.1
L4	1.13–1.15	5.8	30.2	3.73	21.7	2.53	62.4	1.35	7.9	1.32	39.7	0.18	1.1	0.17	5.2
L5 (pellet)	>1.17	4.7	14.6	0.77	3.6	0.53	6.3	0.46	2.2	0.68	9.9	0.02	0.1	0.06	0.9

* Values so positioned are from pooled fractions L2a + L2b.

enzyme assays it appears that fraction L3b is enriched in Golgi-derived membranes, whereas SER is concentrated in fraction L3a, and RER is located mainly in fraction L4 (Table 1). The successful resolution of fractions L3a and L3b therefore represents a significant improvement to the membrane-fractionation procedure of Cohen *et al.* (1983). The identities of fractions L2a, L2b and L2c are uncertain, but they may contain transport vesicles. The pellet (fraction L5) probably contains mitochondria, protein bodies, peroxisomes, tonoplasts and membrane aggregates (Cohen *et al.*, 1983). The recovery of 40–50% of latent IDPase in the floating layer (fraction L1) may be due to non-particulate forms of the enzyme (Quail, 1979) or to non-specific soluble phosphatases (Hodges & Leonard, 1974).

Fraction L2c isolated with low Mg^{2+} is also enriched in IDPase activity. This suggests that these membranes are Golgi-derived, and presumably include 'secretory vesicles'. Subfractionation of rat liver Golgi apparatus has been demonstrated (Ehrenreich *et al.*, 1973; Bergeron *et al.*, 1982; Deutscher *et al.*, 1983). Ray *et al.* (1976) fractionated Golgi membranes isolated from pea cells into vesicles and cisternae, using rate-zonal centrifugation in a continuous sucrose gradient, and Owens & Northcote (1981) observed two peaks of IDPase activity on centrifugation of potato tissue-culture-cell homogenates on a discontinuous sucrose density gradient.

Many plant cell gradient-fractionation schemes employ density gradients starting from 20% (w/w) sucrose. When centrifuged in these gradients, the light 'secretory vesicles' remain in the 'soluble' or top fraction. Hence a Mg^{2+} density shift in IDPase activity associated with these Golgi secretory vesicles is not observed. The presence of NADH- and NADPH-cytochrome *c* reductases in fraction L3b could be due to contamination with endoplasmic-reticulum vesicles. Alternatively, these reductases may be authentic components of Golgi membranes, as demonstrated for rat liver (Ito & Palade, 1978).

Distribution of galactosyltransferases

Because of the better resolution observed in the presence of high Mg^{2+} concentrations, in particular with respect to the separation of membranes derived from RER, SER and Golgi, the distribution of galactosyltransferases was examined in these gradients. Galactosyltransferases capable of catalysing the transfer of galactose from UDP-galactose into 66%-ethanol-insoluble products were detected in all sucrose gradient fractions at high Mg^{2+} concentrations (Table 2). This is consistent with earlier suggestions that a multiplicity of enzymes can participate in such reactions (Mascara & Fincher, 1982). Most of the activity appears to be loosely bound to the membranes, since it is recovered in the floating 'soluble' fraction L1 after membrane fractionation.

Of prime importance in the localization of galactosyltransferases which might be involved specifically in arabinogalactan-protein biosynthesis is the origin of membranes synthesizing polymeric products containing (1→6)-β-galactose residues (Mascara & Fincher, 1982). When the

Table 2. *Distribution of galactosyltransferases in sucrose density gradients prepared at high Mg^{2+} concentrations*

Enzyme activities are given as the ratio of the specific activity in the subcellular fraction to that in the total intracellular membrane fraction loaded on the gradient (relative specific activity, RSA) and as the percentage of the total activity recovered in each gradient fraction (%TA). Total enzyme activity recovered in the gradient fractions was 10.6 pmol·min^{-1}.

Fraction	RSA	%TA
L1	2.85	74.4
L2a+L2b	2.38	3.5
L2c	0.92	1.7
L3a	0.42	1.1
L3b	0.40	6.7
L4	0.22	6.0
L5	0.51	6.7

products formed from each of the sucrose-gradient fractions (Table 2) were applied to (myeloma protein J539)–Sepharose columns, only those derived from membranes in fraction L3b contained polymers which were specifically bound to the affinity columns. Of the radioactive products formed by galactosyltransferases in this fraction, 53% were bound. Pooled L2 fractions also produced significant amounts of (1→6)-β-galactose-containing polymer (37% of the 66% ethanol-insoluble product), lending support to the suggestion that Golgi-derived 'secretory vesicles' are present in fraction L2c. Thus the enzyme responsible for the biosynthesis of products containing (1→6)-β-galactose residues appears to be exclusively associated with subcellular fractions enriched in Golgi-derived membranes.

It has also been shown that the prolyl hydroxylase involved in arabinogalactan-protein biosynthesis is located, in part at least, on dictyosomes (Cohen *et al.*, 1983). Furthermore, arabinosyltransferases implicated in the glycosylation of the hydroxyproline-rich cell-wall glycoproteins, which share some similarities in compositions with the arabinogalactan-proteins (Fincher *et al.*, 1983), are Golgi-associated (Kawasaki, 1981; Owens & Northcote, 1981).

The availability of the membrane-fractionation scheme described here will greatly facilitate the isolation of sufficient material for detailed structural analysis of polymers containing (1→6)-β-galactose residues and enable the molecular mechanisms for the assembly of the polysaccharide moiety of the ryegrass arabinogalactan-protein to be examined.

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