Biochem. J. (1983) **209**, 627–633
Printed in Great Britain

Localization of β-glucan synthases on the membranes of cultured *Lolium multiflorum* (ryegrass) endosperm cells

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(*Received 20 July 1982/Accepted 1 October 1982*)

The distribution of β-glucan synthases between plasma membranes and intracellular membranes of suspension-cultured Italian-ryegrass (*Lolium multiflorum* Lam.) endosperm cells was examined. Highly purified plasma membranes prepared from protoplasts were only slightly enriched in β-glucan synthases assayed at 10μM- and 1mM-UDP-glucose. Most β-glucan synthase was associated with intracellular membranes. These membranes were fractionated on a linear sucrose density gradient and were resolved into different membrane fractions containing β-glucan synthases. β-Glucan synthases assayed at 10μM-UDP-glucose were found in a fraction banding at a density of $1.11\,g\cdot cm^{-3}$, but most of the β-glucan synthase assayed at 1mM-UDP-glucose was at a density of $1.04\,g\cdot cm^{-3}$.

Mixed-membrane preparations from ryegrass (*Lolium multiflorum*) endosperm cells grown in suspension culture produce 1,3-β-glucans, 1,4-β-glucans and 1,3;1,4-glucans from UDP-glucose (Smith & Stone, 1973*a*; Henry & Stone, 1982), but the subcellular location of the enzymes involved in their synthesis has not been established.

Golgi membranes (Ray *et al.*, 1969; Van der Woude *et al.*, 1974; Helsper *et al.*, 1977) and plasma membranes (Van der Woude *et al.*, 1974; Anderson & Ray, 1978; Ray, 1979) have been reported as the major subcellular locations of β-glucan synthases in cells from higher plants. These subcellular fractions were isolated by sucrose-density-gradient centrifugation of whole-cell homogenates and characterized by their density, by the presence of marker enzymes and by electron microscopy. However, the variability in density reported for membranes from different sources and the lack of unequivocal markers make the identification of membrane fractions from plants difficult (Quail, 1979). A recently developed method for the isolation of highly purified plasma membranes from protoplasts from ryegrass permits circumvention of some of the difficulties associated with the fractionation of whole-cell homogenates (Schibeci *et al.*, 1982).

The approach taken in the present study has been to fractionate protoplast lysates by first separating the plasma membrane from the intracellular membranes by centrifugation on a discontinuous sucrose/sorbitol gradient (Schibeci *et al.*, 1982) and then to fractionate the intracellular membranes (free of plasma membranes) on a linear sucrose gradient. This approach allowed a determination of the distribution of β-glucan synthases between the plasma membrane and intracellular membranes and an investigation of the location of the synthases on intracellular membrane fractions.

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Materials and methods

Cell culture

Italian-ryegrass (*Lolium multiflorum* Lam.) endosperm cells were maintained in liquid suspension culture on modified White's medium with 4% (w/v) sucrose as the carbon source (Smith & Stone, 1973*b*),

Isolation of protoplasts

Ryegrass endosperm cells in mid-exponential phase of growth were incubated with 1% Driselase (Kyowa Hakko Kogyo Co., Tokyo, Japan) as described by Keller & Stone (1978). Removal of the cell wall was monitored by staining with Calcofluor White ST (Nagata & Takebe, 1970). Viability was determined by fluorescein diacetate staining (Heslop-Harrison & Heslop-Harrison, 1970). To avoid possible contamination of plasma membranes with intact protoplasts of similar density in a subsequent discontinuous sucrose/sorbitol gradient, the protoplasts, suspended in 0.5M-sucrose and overlayered with 10% (w/v) sorbitol containing 0.5mM-$CaCl_2$, were centrifuged at 400**g** for 10min. The protoplasts at the interface were collected and

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used for membrane fractionation (Schibeci *et al.*, 1982). β-Glucan synthase activities in the membrane fractions from these protoplasts and those found in the pellet were not significantly different when assayed at either low or high UDP-glucose concentrations.

Isolation of membrane fractions

Membrane fractions were isolated as previously described (Schibeci *et al.*, 1982). Protoplasts were coated with myeloma protein J539, allowed to swell in 50mM-potassium phosphate buffer, pH 8.0, and blended gently in a Sorvall Omnimixer (Wagner & Siegelman, 1975). The presence of the myeloma protein J539 did not influence β-glucan synthase activities of the membrane fractions. The lysate was layered on a discontinuous sucrose/sorbitol gradient and centrifuged at 140**g** for 30min. Fractions (F1–F4) from the gradient were collected as described by Schibeci *et al.* (1982) and centrifuged at 100000**g** for 1h. This procedure removed soluble components and floating 'lipid bodies'. Membranes were resuspended in 20mM-Mops (4-morpholinepropanesulphonic acid)/NaOH buffer, pH 7.5, before assay of β-glucan synthases. Fraction F1 from the discontinuous sorbitol/sucrose gradient contains most of the intracellular membranes, and the plasma-membrane sheets are found in fraction F3 (Schibeci *et al.*, 1982).

Density-gradient centrifugation of intracellular membranes

The F1 fraction was fractionated by resuspending the 100000**g** pellet in 10mM-Tris/HCl buffer, pH 7.5, and centrifuging on a linear density gradient ranging from 5% (w/w) to 42% (w/w) sucrose at 180000**g** for 2h.

Fractions were collected by upward-flow displacement with an ISCO gradient fractionator equipped with a model UA-2 ultraviolet analyser (Instrument Specialities Co., Lincoln, NE, U.S.A.). The refractive index was measured with an Abbé refractometer. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Enzyme assays

β-Glucan synthase assays were performed as described by Henry & Stone (1982) with either 10μM- or 1mM-UDP-glucose. Membrane fractions were incubated in 50mM-Mops buffer, pH 7.5, containing 20mM-$MgCl_2$ and 2mM-dithiothreitol with UDP-[^{14}C]glucose at 25°C for 15min. Portions of the reaction mixture were transferred to glass-fibre discs (Whatman GF/A), which were washed with ethanol solutions to remove unincorporated UDP/[^{14}C]glucose (Henry & Stone, 1982), and the radioactivity incorporated into the 66%-(v/v)-ethanol-insoluble fraction was determined.

Latent inosine diphosphatase activity was measured as described by M'Voula-Tsieri *et al.* (1981). The phosphate released was determined by using the method of Taussky & Shorr (1953). NADH– and NADPH–cytochrome *c* reductase activities were measured spectrophotometrically at 550nm, and phosphodiesterase activity was determined with 0.4mM-bis-*p*-nitrophenyl phosphate as substrate (Hodges & Leonard, 1974).

Characterization of products of β-glucan synthases

The products formed by β-glucan synthases were characterized by hydrolysis with specific β-glucan hydrolases as described by Henry & Stone (1982). The oligosaccharides released by β-glucan hydrolases were characterized by gel filtration on Bio-Gel P-2 (Henry & Stone, 1982).

Results

Distribution of β-glucan synthases between plasma membranes and intracellular membranes

The specific activities of the β-glucan synthases in the lysed protoplast membrane fractions were similar to those in mixed-membrane preparations from cultured ryegrass endosperm cells (Henry & Stone, 1982). Lysed ryegrass protoplasts were fractionated on a discontinuous sorbitol/sucrose gradient, and four fractions were obtained as described by Schibeci *et al.* (1982). The membrane components of these fractions were assayed for total β-glucan synthase activity. Most of the activity (82% and 86%) assayed at both 10μM- and 1mM-UDP-glucose concentrations respectively was found in fraction F1, which contained most of the intracellular membranes (Table 1). Fraction F3, which was highly enriched in plasma membranes (Schibeci *et al.*, 1982), contained only 6% of the activity assayed at 10μM-UDP-glucose and 4% of the activity assayed at 1mM-UDP-glucose. The specific activity of the β-glucan synthases was highest in fraction F3 but was only slightly lower in fractions F2 and F1.

With GDP-glucose as substrate very low activities were found in all fractions (Table 2). This is consistent with the lower activity of mixed-membrane preparations assayed with GDP-glucose compared with UDP-glucose (Henry & Stone, 1982), and indicated that none of the fractions is enriched in a GDP-glucose-dependent β-glucan synthase.

Distribution of β-glucan synthases among intracellular membrane fractions

Since most of the β-glucan synthase activity was associated with the intracellular membranes (fraction F1), with only a slight enrichment in the plasma

Table 1. *β-Glucan synthases in membrane fractions*

Membrane fractions obtained by discontinuous-sucrose-density-gradient centrifugation were assayed with either 10 μM- or 1 mM-UDP-glucose as substrate. For full experimental details see the text.

	With 10 μM-UDP-glucose			With 1 mM-UDP-glucose		
	Activity		Specific activity (pmol of glucose incorporated/s per mg of protein)	Activity		Specific activity (pmol of glucose incorporated/s per mg of protein)
Membrane fraction	(pmol of glucose incorporated/s)	(%)		(pmol of glucose incorporated/s)	(%)	
F1	225	82.4	1.32	61300	86.0	360
F2	29.8	10.9	2.06	6260	8.8	432
F3	16.4	6.0	2.35	3116	4.4	445
F4	1.42	0.5	0.47	518	0.7	173
Total (F1 + F2 + F3 + F4)	273	100	—	71200	100	—
Protoplast lysate	435	—	1.65	113000	—	428

Table 2. *Comparison of UDP-glucose and GDP-glucose as substrates for β-glucan synthases from different membrane fractions*

Membrane fractions obtained by discontinuous-sucrose-density-gradient centrifugation were assayed with either 10 μM-UDP-glucose or 10 μM-GDP-glucose as substrate. For full experimental details see the text.

Membrane fraction	Activity with 10 μM-UDP-glucose (pmol of glucose incorporated/s)	Activity with 10 μM-GDP-glucose (pmol of glucose incorporated/s)
F1	82.7	3.3
F2	12.2	1.8
F3	2.1	0.3
F4	3.4	1.2
Total (F1 + F2 + F3 + F4)	100.4	6.6

membrane, the intracellular membranes were further fractionated on linear sucrose density gradients. Initially a linear sucrose density gradient ranging from 20% to 50% (w/w) was used, but most of the β-glucan synthase and protein floated on the top of the gradient. A gradient ranging from 5% to 42% (w/w) sucrose improved the resolution.

The absence of any contaminating plasma membrane in the linear sucrose gradient was confirmed by adding ^{125}I-labelled plasma-membrane sheets, prepared as described by Schibeci *et al.* (1982), to the intracellular membrane suspension before fractionation. Almost all the labelled plasma membrane was found in the pellet.

Three major membrane components were resolved in this gradient (Fig. 1, peaks I, II and III). A peak of β-glucan synthase assayed with 1 mM-UDP-glucose was located at a density of 1.04 $g \cdot cm^{-3}$ (Fig. 1, peak I), with a smaller peak at a density of 1.11 $g \cdot cm^{-3}$ (Fig. 1, peak III). With the assay at 10 μM-UDP-glucose most activity was in peak III. Thus it appears that the various particulate synthases may originate from different intracellular compartments.

Distribution of marker enzymes

In an attempt to identify the subcellular origin of the membrane-bound synthases from fraction F1, we determined the distribution of marker enzymes in the linear sucrose gradient fractions. Inosine diphosphatase, NADH-cytochrome *c* reductase and phosphodiesterase activities (Fig. 2) were coincident with the major protein peak I ($\rho = 1.04\,g \cdot cm^{-3}$). Peak III ($\rho = 1.11\,g \cdot cm^{-3}$) contains inosine diphosphatase and NADH–cytochrome *c* reductase with trace amounts of phosphodiesterase activity. The activity of NADPH–cytochrome *c* reductase was very low in all fractions and was much lower than that of NADH–cytochrome *c* reductase. Phosphodiesterase activity was maximal in the pellet.

Glucosylation of lipids

Incorporation of radioactivity from 10 μM-UDP-glucose into the fraction soluble in chloroform/methanol/water (10:10:3, by vol.) showed a distribution of activity similar to that of the β-glucan synthases (Table 3). Most of the activity was found in fraction F1. The lipids labelled in these assays do not bind to DEAE-cellulose and are neutral lipids, probably sterol glucosides (Henry & Stone, 1982).

Glucosyl transfer from 10 μM-UDP-glucose to products soluble in chloroform/methanol/water (10:10:3) was maximal in the intracellular fraction banding at a density of 1.04 $g \cdot cm^{-3}$ (peak I), coinciding with the β-glucan synthase assayed at 1 mM-UDP-glucose and most of the protein (Fig. 1).

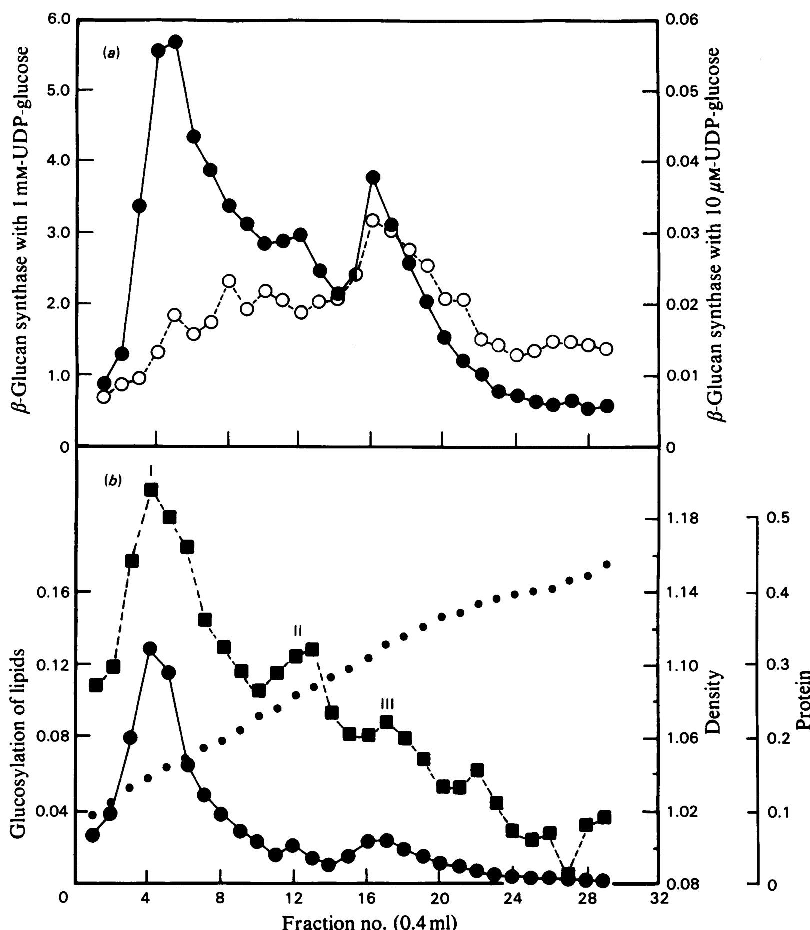

Fig. 1. *Distribution of β-glucan synthase activities from intracellular membranes within the linear sucrose density gradient*

All values for enzyme activity represent the total activity in the gradient fraction. (*a*) ○, β-Glucan synthase activity assayed with 10 μM-UDP-glucose (pmol of glucose incorporated/s); ●, β-glucan synthase activity assayed with 1 mM-UDP-glucose (pmol of glucose incorporated/s). (*b*) ■, Protein (mg/ml); ······, density ($g \cdot cm^{-3}$); ●, glucosylation of lipids (pmol/s) (transfer from 10 μM-UDP-glucose to products soluble in chloroform/methanol). For full experimental details see the text.

Table 3. *Incorporation of radioactivity from 10 μM-UDP-[^{14}C]glucose into products soluble in chloroform/methanol/water (10:10:3, by vol.)*

Membrane fractions obtained by discontinuous-sucrose-density-gradient centrifugation were assayed for the ability to transfer glucosyl residues from 10 μM-UDP-glucose to products soluble in chloroform/methanol/water (10:10:3). The fractions were washed with 0.5% KCl to remove UDP-[^{14}C]glucose. For full experimental details see the text.

Membrane fraction	Activity (pmol of glucose incorporated/s)	Activity (%)	Specific activity (pmol of glucose incorporated/s per mg of protein)
F1	497	88.0	2.93
F2	50	8.8	3.46
F3	15	2.6	2.15
F4	2.8	0.5	0.93
Total (F1 + F2 + F3 + F4)	565	100	—
Protoplast lysate	961		3.64

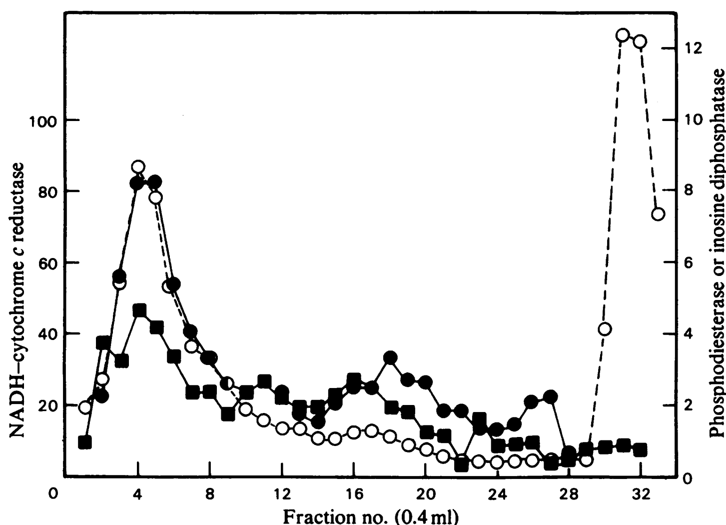

Fig. 2. *Distribution of marker enzymes within the linear sucrose density gradient (same gradient as in Fig. 1)*
All values for enzyme activity represent the total activity in the gradient fraction. ●, NADH–cytochrome *c* reductase (nmol/min); ○, phosphodiesterase (nmol/min); ■, inosine diphosphatase (nmol/min). For all experimental details see the text.

Characterization of the products of β-glucan synthases

Mixed membranes from both protoplasts and whole cells produce about 50% 1,3-β-glucan when assayed with 1 mM-UDP-glucose (Table 4). However, at 10 μM-UDP-glucose 1,3-β-glucans represent only 6% of the product of membranes from whole cells but about 70% of the products of protoplast membranes. Fractions F1 (intracellular membranes) and F3 (plasma membrane) produced β-glucans in similar proportions to those formed by the total protoplast membranes (Table 4). The relative amounts of β-glucans formed by intracellular membranes at the two UDP-glucose concentrations indicate that peak I ($\rho = 1.04\,g \cdot cm^{-3}$) is enriched in 1,3-β-glucan synthase relative to the peak of lower density (peak III, $\rho = 1.11\,g \cdot cm^{-3}$). Enzymic analysis of the β-glucans produced by these fractions, with the use of specific β-glucan hydrolases, is consistent with this conclusion.

Table 4. *Products of β-glucan synthases*
The β-glucans produced from UDP-glucose by the membrane fractions were characterized by the use of specific β-glucan hydrolases (Cook *et al.*, 1980). Total β-glucan synthase activities from the whole-cell homogenate (see Henry & Stone, 1982) at 10 μM- and 1 mM-UDP-glucose were 1.32 and 283 pmol/s per mg of protein, and for the protoplast lysate (see Table 1) the corresponding values were 1.65 and 428 pmol/s per mg of protein respectively.

	1,3-β-Glucans* (%)	1,4-β-Glucans + 1,3;1,4-β-glucans† (%)
With 10 μM-UDP-glucose as substrate		
Whole-cell homogenate‡	6	94
Protoplast lysate	72	28
Fraction F1	69	31
Fraction F3	71	29
With 1 mM-UDP-glucose as substrate		
Whole-cell homogenate‡	50	50
Protoplast lysate	57	43
Fraction F1	54	46
Fraction F3	47	53

* Determined by hydrolysis with 1,3-β-glucan exohydrolase.
† Determined by hydrolysis with 1,4-β-glucan endohydrolase (includes 1,4-β-glucans and 1,3;1,4-β-glucans).
‡ Henry & Stone (1982).

Discussion

The preliminary fractionation of protoplast membranes into plasma membrane and intracellular membranes (Schibeci *et al.*, 1982) eliminated the complication of the presence of plasma membrane in the subsequent fractionation of the intracellular membranes. The separation of the intracellular membrane fraction (F1) from the protoplast lysate on the discontinuous gradient and its collection by ultracentrifugation produced a membrane fraction that was free of soluble components and 'lipid bodies'. Jones (1980) showed that gel filtration of a whole-cell homogenate of barley aleurone layers on Sepharose 4B separates the particulate from soluble

components and yields an organelle fraction that can be more readily and quantitatively fractionated into its components on continuous sucrose density gradients. The procedure used to isolate intracellular membranes in the present study offers similar advantages.

Membranes from lysed ryegrass protoplasts show greater 1,3-β-glucan synthesis at both UDP-glucose concentrations than do those from whole cells. Incorporation into 1,4- and 1,3;1.4-β-glucans by whole cells and protoplast homogenates is similar at 1 mM-UDP-glucose. Thus at this UDP-glucose concentration the increase in the proportion of glucose incorporated into 1,3-β-glucan by the protoplast homogenates is due to increased 1,3-β-glucan synthesis. At 10 μM-glucose the increased proportion of 1,3-β-glucan appears to be due to both increased 1,3-β-glucan synthesis and decreased 1,4-β-glucan synthesis. These observations are consistent with the detection of Aniline Blue-staining deposits (callose) and the chemical determinations of 1,3-β-glucans on some regenerating plant protoplasts (Eschrich, 1957; Pearce *et al.*, 1974; Blaschek *et al.*, 1981; Takeuchi & Komamine, 1981). 1,3-β-Glucans are not normally found as components of the ryegrass cell walls (Smith & Stone, 1973*a*; Anderson & Stone, 1978). However, mixed membranes from whole cells synthesize appreciable amounts of 1,3-β-glucan from UDP-glucose (Smith & Stone, 1973*a*; Cook *et al.*, 1980; Henry & Stone, 1982). This observation has been made for cell-free preparations from many higher plants (for a review see Fincher & Stone, 1981). Enhanced 1,3-β-glucan synthesis has also been observed in cells that have been physiologically stressed or injured (see Fincher & Stone, 1981). Increased 1,3-β-glucan synthesis may also result from exposure of isolated membrane fractions to sucrose during density-gradient centrifugation (Ephritikhine *et al.*, 1980). The mechanism of enhancement of 1,3-β-glucan synthesis in these situations is not understood, but presumably relates to a change in the environment of the membrane-bound synthases (e.g. substrate availability, transmembrane potential etc.).

The plasma membranes from peas (*Pisum sativum*) (Anderson & Ray, 1978), onion (*Allium cepa*) (Van der Woude *et al.*, 1974) and maize (*Zea mays*) (Ray, 1979) have been shown to contain β-glucan synthases. In the present study ryegrass protoplast plasma membranes showed some enrichment in β-glucan synthase, but the bulk of the enzymes were associated with intracellular membranes. In onion (Van der Woude *et al.*, 1974), maize (Ray, 1979) and peas (Ray *et al.*, 1969) β-glucan synthases have been reported on intracellular membranes identified as Golgi membranes. There has been little attention paid to the possible association of β-glucan synthases with the endoplasmic reticulum, although indirect evidence suggests an association (Northcote, 1974; Fincher & Stone, 1981).

Our studies with enzyme markers do not permit the unambiguous identification of the two major intracellular membrane components. Latent inosine diphosphatase is often used as a marker for Golgi membranes (Quail, 1979). Membrane fractions from both peaks of β-glucan synthase activity in the gradient also contained inosine diphosphatase activity, suggesting that both of these fractions might contain Golgi membranes (Rothman, 1981). However, recent evidence has indicated that inosine diphosphatase may be located not only in Golgi membranes but also in plasma membranes and possibly endoplasmic reticulum (M'Voula-Tsieri *et al.*, 1981). The activity of NADH–cytochrome *c* reductase, a commonly used marker for endoplasmic reticulum, was coincident with both peaks of β-glucan synthase in the gradient. Phosphodiesterase is probably associated with vacuoles (Boller & Kende, 1979), and most of this enzyme was found in the pellet although some remained in peak I.

The location of enzymes involved in the glucosylation of sterols is also uncertain. In *Allium cepa* and *Calendula officinalis*, UDP-glucose: sterol glucosyltransferase has been suggested as a Golgi-membrane marker (Lercher & Wojclechowski, 1976). Chadwick & Northcote (1980) found the enzyme was distributed on different intracellular membranes from soya-bean (*Glycine max*) protoplasts and was also present on plasma membranes. In ryegrass, enzymes transferring glucosyl residues from UDP-glucose to products soluble in chloroform/methanol were mostly in the intracellular membrane fraction with the lowest density, but some enzyme was also associated with plasma membranes (fraction F3).

Since neither of these intracellular membrane fractions contains plasma membranes, they may be derived from different intracellular organelles (e.g. Golgi apparatus and endoplasmic reticulum), or alternatively from different parts of the endoplasmic reticulum (e.g. rough or smooth) or different regions within the Golgi complex (Rothman, 1981). Thus the β-glucan synthases in ryegrass endosperm are distributed throughout the endomembrane system.

We thank Miss Julie Friedrichsen for maintaining the ryegrass cultures and Dr. G. B. Fincher, Dr. P. J. Harris and Dr. G. M. Polya for critically reading the manuscript. This work was supported by the Australian Research Grants Scheme.

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