# The Maize Mixed-Linkage (1→3),(1→4)-β-D-Glucan Polysaccharide Is Synthesized at the Golgi Membrane[1][OA]

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With the exception of cellulose and callose, the cell wall polysaccharides are synthesized in Golgi membranes, packaged into vesicles, and exported to the plasma membrane where they are integrated into the microfibrillar structure. Consistent with this paradigm, several published reports have shown that the maize (*Zea mays*) mixed-linkage (1→3),(1→4)-β-D-glucan, a polysaccharide that among angiosperms is unique to the grasses and related Poales species, is synthesized in vitro with isolated maize coleoptile Golgi membranes and the nucleotide-sugar substrate, UDP-glucose. However, a recent study reported the inability to detect the β-glucan immunocytochemically at the Golgi, resulting in a hypothesis that the mixed-linkage β-glucan oligomers may be initiated at the Golgi but are polymerized at the plasma membrane surface. Here, we demonstrate that (1→3),(1→4)-β-D-glucans are detected immunocytochemically at the Golgi of the developing maize coleoptiles. Further, when maize seedlings at the third-leaf stage were pulse labeled with [$^{14}$C]$O_2$ and Golgi membranes were isolated from elongating cells at the base of the developing leaves, (1→3),(1→4)-β-D-glucans of an average molecular mass of 250 kD and higher were detected in isolated Golgi membranes. When the pulse was followed by a chase period, the labeled polysaccharides of the Golgi membrane diminished with subsequent transfer to the cell wall. (1→3),(1→4)-β-D-Glucans of at least 250 kD were isolated from cell walls, but much larger aggregates were also detected, indicating a potential for intermolecular interactions with glucuronoarabinoxylans or intermolecular grafting in muro.

An overwhelming body of evidence accumulated has established that the (1→4)-β-D-glucan chains of cellulose microfibrils are synthesized and assembled at the plasma membrane surface (Delmer, 1999; Saxena and Brown, 2005), whereas, with the lone exception of the (1→3)-β-D-glucan, callose, all noncellulosic pectin and cross-linking glycan polysaccharides are synthesized in Golgi membranes (Northcote and Pickett-Heaps, 1966; Ray et al., 1969, 1976; Harris and Northcote, 1971; Zhang and Staehelin, 1992). Using several plant systems, including grass species, autoradiography and membrane fractionation showed that monosaccharides from $^{14}$C-labeled substrates accumulated in cell wall polysaccharides in Golgi vesicles during a pulse were subsequently transferred to the cell wall when chased with unlabeled substrates (Northcote and Pickett-Heaps, 1966; Pickett-Heaps, 1967; Jilka et al., 1972). Early studies showed that labeled sugars from nucleotide-sugar substrates could be incorporated into alcohol-insoluble polysaccharides using microsomal membranes, and later refined by isolation of Golgi membranes and the synthesis of defined polysaccharides with combinations of nucleotide sugars (Bailey and Hassid, 1966; Ray et al., 1969, 1976; Smith and Stone, 1973; Ray, 1980; Hayashi and Matsuda, 1981a; Gordon and Maclachlan, 1989; Gibeaut and Carpita, 1993).

When micromolar concentrations of substrates were used, only small chains of the glycan products were typically made in vitro. For example, xyloglucan oligomers with the characteristic α-D-Xyl-(1→6)-D-glucosyl unit, isoprimeverose, were synthesized with isolated microsomal membranes and low concentrations of UDP-Glc and UDP-Xyl (Ray et al., 1976; Hayashi and Matsuda, 1981b). When concentrations of each nucleotide sugar were increased to millimolar concentrations, then polysaccharides of about 250 kD were synthesized containing the characteristic XXXG heptasaccharide unit structure (Gordon and Maclachlan, 1989). Immunocytochemical evidence with antibodies directed against the terminal nonreducing xylosyl and fucosyl residues confirm that synthesis of the xyloglucan backbone begins in the cis-Golgi membrane and culminates with fucosylation in the trans-Golgi membrane and trans-Golgi network (Moore et al., 1991; Lynch and Staehelin, 1992; Zhang and Staehelin, 1992). The fucosyl transferase responsible for xyloglucan side chain decoration was also shown to be a Golgi-resident protein by in vitro synthesis of xyloglucan polymers (Camirand and Maclachlan, 1986).

In Poales species, including all grasses, the mixed-linkage (1→3),(1→4)-β-D-glucan is a major cross-linking glycan that appears transiently during cell elongation in growing tissues and accumulates to

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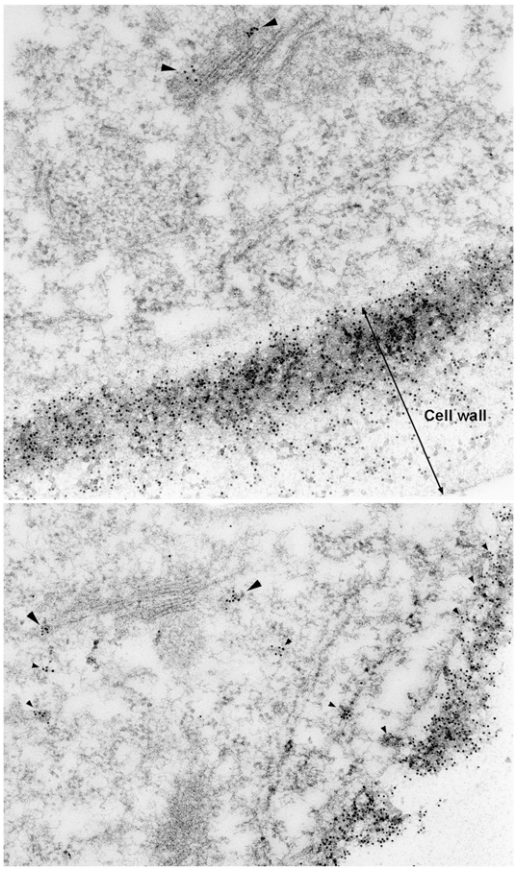


**Figure 1.** Identification of (1→3),(1→4)-β-D-glucan by immunocytochemistry. Immunogold staining is detected at the periphery of Golgi stacks (large arrowheads) and vesicles (small arrowheads). In some instances, the vesicles with (1→3),(1→4)-β-D-glucan are found just fusing to the plasma membrane (small arrowheads). Labeling in the cell wall is more concentrated near the plasma membrane.

large amounts in the cell walls of the endosperm of certain grains (Stone and Clarke, 1992; Trethewey et al., 2005). Bailey and Hassid (1966) demonstrated the synthesis in vitro of noncellulosic glucans with microsomal membranes from grasses. Henry and Stone (1982) used the *Bacillus subtilis* endoglucanase, an enzyme that generates diagnostic cellodextrin-(1→3)-β-Glc units from (1→3),(1→4)-β-D-glucan to show that the mixed-linkage β-glucan was made specifically with UDP-Glc and microsomal membranes. We used flotation centrifugation to obtain highly enriched Golgi membranes from which (1→3),(1→4)-β-D-glucans of an average of about 250 kD were synthesized (Gibeaut and Carpita, 1993).

The BG1 monoclonal antibody recognizes the (1→3),(1→4)-β-D-glucan with high specificity (Meikle et al., 1994). This monoclonal antibody has been used to show dramatic changes in epitope abundance of (1→3),(1→4)-β-D-glucan in the cell walls of developing tissues (Meikle et al., 1994; Trethewey et al., 2005; McCann et al., 2007) and its appearance in the cell walls of Arabidopsis (*Arabidopsis thaliana*) following heterologous expression of genes thought to encode its synthases (Burton et al., 2006; Doblin et al., 2009). The failure to detect (1→3),(1→4)-β-D-glucan in Golgi membranes and only in the cell wall prompted Fincher (2009) to conclude that cellodextrin oligomers of the (1→3),(1→4)-β-D-glucan may be initiated in the Golgi membrane, but the actual polymerization of the polysaccharide occurs at the plasma membrane.

While there is little question that synthesis of full-length polymers is possible in vitro with isolated Golgi membranes and UDP-Glc (Gibeaut and Carpita, 1993; Buckeridge et al., 1999, 2001; Urbanowicz et al., 2004), Fincher (2009) asserts correctly that there exists no experimental evidence that the polymer is made in vivo within the Golgi membrane in intact tissues. In fact, earlier work showing the paucity of immunolabeling of (1→3),(1→4)-β-D-glucan in Golgi membranes of developing wheat (*Triticum aestivum*) endosperm at a time of active deposition called to question the site of synthesis in vivo (Philippe et al., 2006). There is precedence for the synthesis of chitin in vitro with precociously activated chitisomes (Bracker

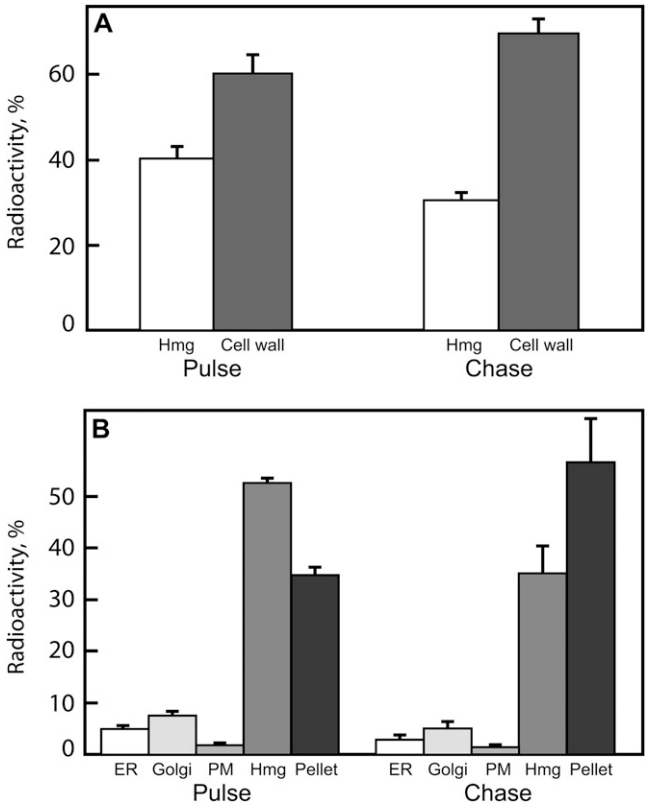


**Figure 2.** Kinetics of labeling of homogenate and cell wall fractions. A, Percentage of radioactivity incorporated into the total homogenate (Hmg) and cell wall after a 6-h pulse with $[^{14}C]O_2$ and after a subsequent 18-h chase with ambient $CO_2$. B, Proportion of radioactivity recovered in ER, Golgi, plasma membrane-mitochondria (PM), cleared homogenate (Hmg), and pellet fractions after extensive dialysis to remove soluble sugars. Values are ±variance of two independent extractions from plants taken from the same labeling periods.

et al., 1976), a vesicular package of chitin synthase that in vivo is quiescent until reaching the plasma membrane. No activity of chitin synthase from isolated plasma membranes could be demonstrated. In a similar way, the Golgi synthase activity of (1→3),(1→4)-β-D-glucan could be a precocious activation in vitro of a plasma membrane activity.

As in vitro synthesis studies clearly show synthesis of full-length (1→3),(1→4)-β-D-glucan only at the Golgi, we reexamined the puzzling finding of its absence from Golgi bodies to determine the true site of synthesis in vivo. In contrast to Fincher (2009), our own immunocytochemistry shows (1→3),(1→4)-β-D-glucan is indeed in the Golgi membrane in 2-d-old coleoptiles, when rapid growth is just beginning. However, we are unable to detect the β-glucan in Golgi after the peak rate of elongation. We pulse labeled maize (*Zea mays*) seedlings with radiolabeled $CO_2$ and followed the fate of label captured by photosynthesis and translocated to elongating cells at the base of the seedling. We found by flotation centrifugation that Golgi membranes contain (1→3),(1→4)-β-D-glucan of at least 250 kD, similar to that of the product of in vitro synthesis at optimal UDP-Glc concentrations and commercial preparations of barley (*Hordeum vulgare*) endosperm (1→3),(1→4)-β-D-glucan (Gibeaut and Carpita, 1993; Buckeridge et al., 1999, 2001; Urbanowicz et al., 2004). When polysaccharides are extracted sequentially from the cell walls by hot ammonium oxalate, and increasing concentrations of NaOH to 4 M, the (1→3),(1→4)-β-D-glucans are found mostly in the higher concentrations of alkali fractions. While 250 kD polymers are observed, most of the (1→3),(1→4)-β-D-glucans eluted in fractions containing glucuronoarabinoxylans (GAXs), which are much larger, indicating either that an aggregation with GAXs increase the apparent size or that transglucosylation events increase the degree of polymerization of the (1→3),(1→4)-β-D-glucans.

## RESULTS

### Localization of (1→3),(1→4)-β-D-Glucan at the Golgi by Immunocytochemistry

We have demonstrated previously the deposition of (1→3),(1→4)-β-D-glucan epitopes into the cell wall, during elongation of the maize coleoptile, using the BG1 antibody (Carpita et al., 2001; McCann et al., 2007). The amount of (1→3),(1→4)-β-D-glucan is tightly correlated with epitope abundance in cell walls using transmission electron microscopy. Here, we show that vesicles associated with Golgi stacks are

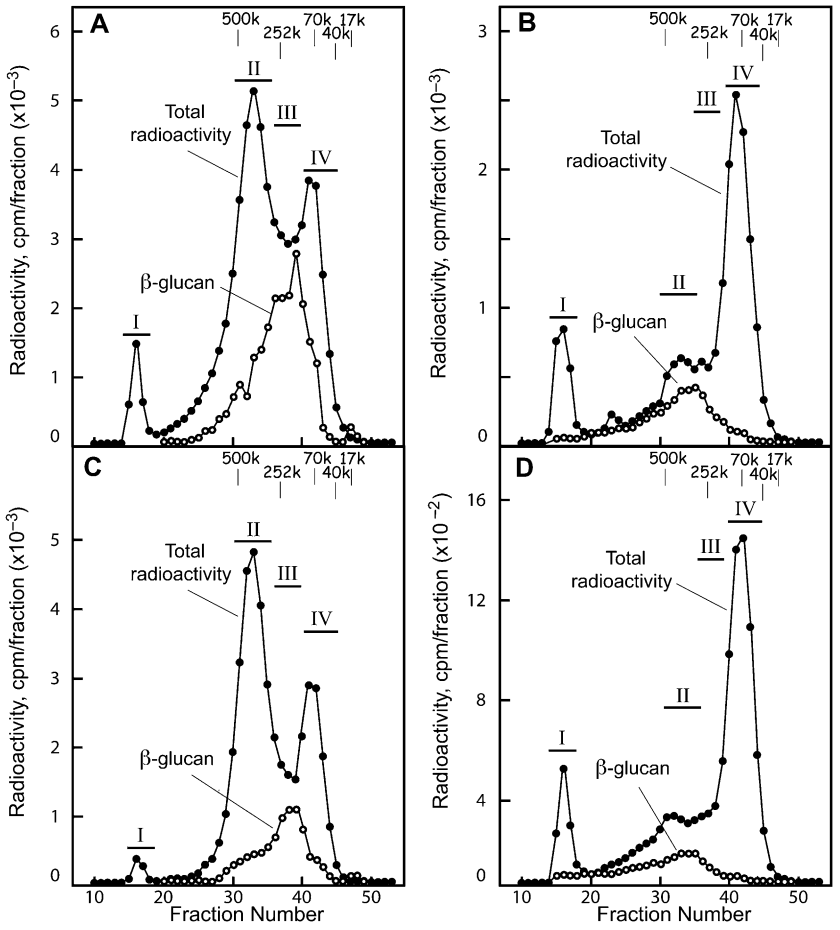


**Figure 3.** Gel-permeation chromatography of radiolabeled polysaccharides from Golgi and cleared homogenates in pulse and chase experiments. The (1→3),(1→4)-β-D-glucan (β-glucan) is estimated as the solubilized radioactivity in each fraction after digestion with the *B. subtilis* endo-β-glucanase after subtraction of enzyme minus controls. A, Cleared homogenate after 6-h pulse labeling. B, Golgi membranes isolated after 6-h labeling. C, Cleared homogenate after subsequent 18-h chase. D, Golgi membranes after 18-h chase. Indications of molecular mass were established with dextran standards (Sigma); Roman numerals indicate fractions pooled for further analysis.

labeled with the BG1 antibody when epidermal and cortical cells of 2-d-old maize coleoptiles are actively synthesizing (1→3),(1→4)-β-D-glucan (Fig. 1). Because low-temperature embedding without fixation and staining by osmium tetroxide is required to preserve antigenicity in materials processed for immunocytochemistry, the membranes have little contrast in these transmission electron micrographs. However, the Golgi stacks are easily recognizable and the small amount of cytoplasmic labeling is consistently associated with them. In other images, labeled material in vesicles, perhaps en route to the plasma membrane, can be seen (Fig. 1). Golgi stacks from older coleoptiles taken after maximal growth rates and peak levels of (1→3),(1→4)-β-D-glucan in the cell wall contained much less, or even lacked, labeling with the BG1 antibody (data not shown).

### Kinetics of Labeling

Because the incorporation of labeled Glc into (1→3),(1→4)-β-D-glucan in the cell walls of the expansion zone of leaf cells at the base of the sheath depends on fixation of $[^{14}C]O_2$ in cells of the leaf blade, conversion to Suc, and translocation to the cells of sheath, pulse labeling is a slow process that achieves a steady state of flux only after several hours. Likewise, a chase with unlabeled $CO_2$ for 18 h still results in accumulation of the label in the cell wall mass of the developing leaf (Fig. 2A). Because of differences in the amounts of labeled $CO_2$ that was taken up during the pulse and chase periods, the total incorporation per plant is variable but the proportions of total incorporation in the isolated fractions show initial high incorporation of label into the Golgi membrane and cleared homogenate fractions during the pulse period, and then loss of the proportion of radioactivity from these fractions and the increasing accumulation in the cell wall (Fig. 2A). Most of the radioactivity in the homogenate was soluble (dialyzable) material (data not shown), but after dialysis the polysaccharides in membrane and cleared homogenate also showed high proportions of labeling during pulse and marked decreases during subsequent chase periods with ambient unlabeled $CO_2$ (Fig. 2B). The exception is the pellet of the flotation centrifugation, which contains starch grains and wall fragments, and this fraction has higher proportions of incorporation of radioactivity in the chase samples (Fig. 2B).

### Identification of (1→3),(1→4)-β-D-Glucan and Other Noncellulosic Polysaccharides at the Golgi Membrane

When the polysaccharides solubilized from the homogenate cleared of membrane and Golgi fractions

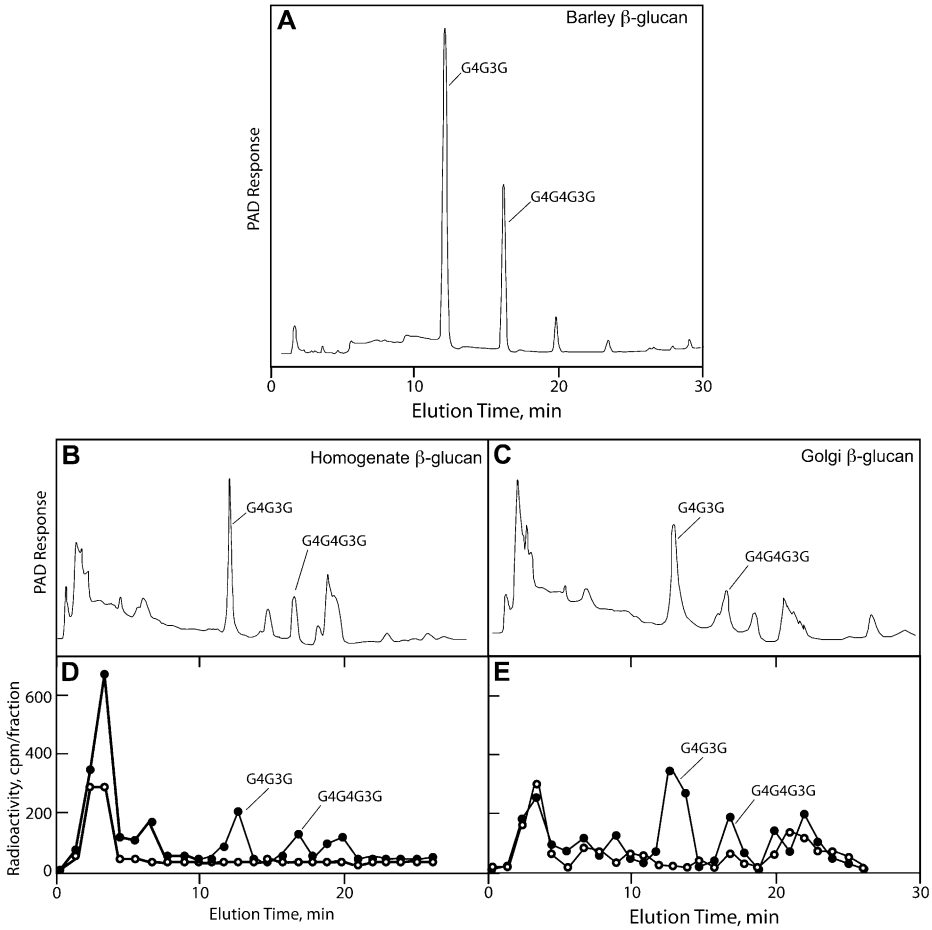


**Figure 4.** Separation of (1→3),(1→4)-β-D-glucan oligomers by HPAEC after digestion with the *B. subtilis* endo-β-glucanase. A, Digestion of purified barley (1→3),(1→4)-β-D-glucan (Sigma) yields predominantly cellobiosyl-(1→3)-D-Glc (G4G3G) and cellotriosyl-(1→3)-D-Glc (G4G4G3G) in a ratio of about 2.5:1. Smaller amounts of cellotetraosyl-(1→3)-D-Glc, cellopentaosyl-(1→3)-D-Glc, and higher cellodextrin-(1→3)-D-Glc oligomers are seen. B and C, Detection of the digestion products of fraction III cleared homogenate and fraction II Golgi membranes, respectively, by pulsed amperometric detection (see fraction designations in Fig. 3). D and E, Radioactivity in 0.35-mL fractions (1 min). The identity of the other radioactive fractions is unknown. Black symbols are radioactivity soluble in 80% (v/v) ethanol after endo-β-glucanase digestion; white symbols are digestion controls without enzyme.

were separated by gel-permeation chromatography and the endo-β-D-glucanase used to estimate the amounts of authentic (1→3),(1→4)-β-D-glucan in each fraction based on solubilization of oligomers in 80% (v/v) ethanol; these oligomers of the β-glucan are soluble in fractions corresponding to an average of about 250 kD or higher (Fig. 3). No oligomers above radioactivity in the endo-β-D-glucanase minus controls are observed in other fractions, such as endoplasmic reticulum (ER) or plasma membrane-mitochondria (data not shown). When the void fraction and three included fractions of major pools of radioactivity are recovered and concentrated, digestion with the endo-β-D-glucanase results in the cellobiosyl- and cellotriosyl-(1→3)-D-glucose in fractions II and III, corresponding to about 250 kD and higher (Fig. 4). No cellobiosyl- and cellotriosyl-(1→3)-D-glucose oligomers are detected in the absence of the endo-β-D-glucanase, indicating that no oligomers of this size are present nascently in fraction IV (data not shown). The size of the (1→3),(1→4)-β-D-glucan peak is not reduced by treatment with protease K (data not shown), but significant amounts of label shift to the total included volume by treatment with the endo-β-D-glucanase (Fig. 5). In all instances, the chase results in a reduction of radioactivity recovered in (1→3),(1→4)-β-D-glucans (Figs. 3 and 4).

The polysaccharide distribution of the Golgi membranes of the coleoptile and leaves were used as measures of the contents at steady-state wall deposition in this organ. The monosaccharide distribution was not reflective of the distribution found in the cell walls in either of these organs (Table I). Several studies show that, because of the predominance of GAXs and (1→3),(1→4)-β-D-glucans in the cell wall, Xyl, Ara, Glc, and Gal are the relative distribution in order of abundance in the cell wall. In contrast, the cleared homogenate and Golgi fractions contain Ara, Man, and Gal in much greater abundance than Glc and Gal in the Golgi membranes and the soluble fraction of the homogenate (Table I). Based on the monosaccharide distribution, arabinogalactan proteins (AGPs) and GAXs are more abundant in these two fractions (Table I). Although IDPase specific activity was highest in the Golgi fraction, only 31% of the total IDPase activity was associated with these membranes, whereas 59% of the IDPase activity was associated with the membrane-cleared homogenate. The remaining 10% of the activity was found, in order of decreasing abundance, in ER, plasma membrane-mitochondrial, and pellet fractions.

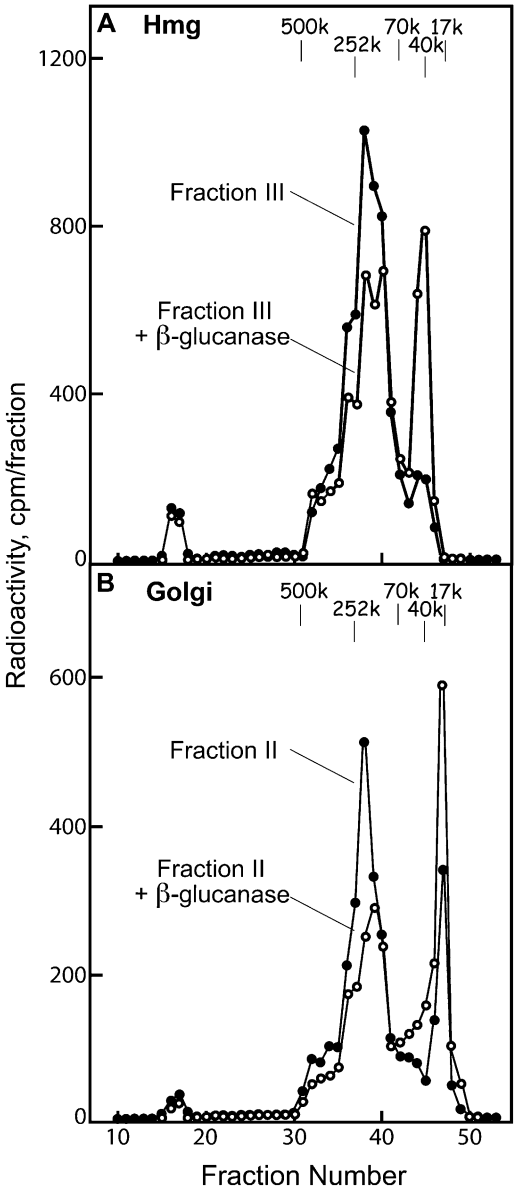


**Figure 5.** Digestion of the (1→3),(1→4)-β-D-glucan-rich fractions with the endo-β-glucanase results in shift of radioactivity to total included fraction. Fractions of polysaccharide (see Fig. 3) were dialyzed and freeze dried; this material was digested with the endo-β-glucanase and rerun on the gel-permeation column. The shift in radioactivity is consistent with the estimation of (1→3),(1→4)-β-D-glucan in fractions III and II from the cleared homogenate (Hmg; A) and Golgi membranes (B), respectively.

### Transit of Noncellulosic Polysaccharides and Incorporation into the Cell Wall

The majority of the radioactivity from fixed [$^{14}$C]$O_2$ is recovered from the cell wall, and higher proportions are found after chase with unlabeled ambient $CO_2$ (Fig. 2A). Sequential fractionation of the isolated walls with hot ammonium oxalate, followed by increasing concentrations of NaOH, shows that the (1→3),(1→4)-β-D-glucan is mostly accumulated in the 4 M NaOH-soluble fraction (Fig. 6). When the soluble polysaccharides from the 4 M NaOH-soluble fraction were separated by gel-permeation chromatography, some (1→3),(1→4)-β-D-glucan of 250 kD was found, but most of the oligomers from (1→3),(1→4)-β-D-glucan generated by action of the endo-β-glucanase came from much larger material (Fig. 6). However, the smaller amounts of (1→3),(1→4)-β-D-glucan oligo-

**Table I.** *Neutral monosaccharide distribution in Golgi membranes and the membrane-free homogenate fractions*

Polysaccharides in four fractions identical to those designated in Figure 3, A (homogenate) and B (Golgi), were dialyzed against deionized water and freeze dried before analysis of monosaccharide as alditol acetate derivatives by gas chromatography-mass spectrometry.

| Fraction | Rha | Fuc | Ara | Xyl | Man | Gal | Glc |
|---|---|---|---|---|---|---|---|
| | | | | *mol%* | | | |
| Golgi | | | | | | | |
| Fraction I (void) | 22 | tr | 1 | 3 | 21 | 19 | 34 |
| Fraction II | 17 | tr | 17 | 10 | 6 | 18 | 32 |
| Fraction III | 15 | tr | 13 | 11 | 8 | 16 | 37 |
| Fraction IV | 25 | tr | 3 | 9 | 13 | 11 | 39 |
| Homogenate | | | | | | | |
| Fraction I (void) | 30 | tr | 6 | 7 | 18 | 12 | 27 |
| Fraction II | 3 | tr | 39 | 3 | 2 | 48 | 4 |
| Fraction III | 5 | tr | 34 | 6 | 3 | 37 | 16 |
| Fraction IV | 8 | tr | 9 | 11 | 16 | 15 | 41 |

mers released by hydrolysis from the 0.1 M NaOH-extracted material was in the size range of that found in the Golgi membranes and cleared homogenate, indicating that the integration with GAX into the cell wall fractions more recalcitrant to NaOH extraction occurred in muro.

## DISCUSSION

Full-length polymers of (1→3),(1→4)-β-D-glucan are synthesized in the Golgi membrane. In contrast to the results of Fincher and his colleagues (Wilson et al., 2006; Fincher, 2009), we observe labeling of the trans-Golgi peripheral membranes, vesicles derived from them, and vesicles fusing with the plasma membrane (Fig. 1). Further, in vivo labeling studies show β-glucan polymers of 250 kD and higher are enriched in the Golgi membrane and a soluble homogenate fraction most likely derived from lysis of secretory vesicles (Figs. 3 and 4). Thus, the hypothesis that the (1→3),(1→4)-β-D-glucan begins with short (1→4)-β-linked cellodextrin oligomers made in the Golgi, which are then stitched together in (1→4)-β-linkages at the plasma membrane by trans-glucosylation (Fincher, 2009), is not supported by two lines of evidence.

These findings are completely consistent with a rather large body of work. Golgi membranes have been enriched by flotation centrifugation, a technique that begins with a homogenate adjusted to 42% Suc, and floats the Golgi membranes to a 35%/29% interface, trapping the much denser plasma membranes mostly to the 42%/35% interface (Gibeaut and Carpita, 1990, 1993). Subsequent evaluation of downward versus flotation centrifugation showed 7-fold improvements in the enrichment of Golgi from plasma membrane (Gibeaut and Carpita, 1994). The *B. subtilis* endo-β-glucanase has strict activity against (1→4)-β-D-glucosyl linkages but also requires a penultimate (1→3)-β-D-linked glucosyl residue at the nonreducing end of the site of cleavage (Anderson and Stone, 1975). By action of the *B. subtilis* endo-β-glucanase, (1→3),(1→4)-β-D-glucan is hydrolyzed mostly into cellobiosyl- and cellotriosyl-(1→3)-β-D-Glc oligomers in a ratio of about 2.5:1 (Fig. 4). Small amounts of longer cellodextrin-(1→3)-β-D-Glc oligomers to about degree of polymerization 9 are also made. These diagnostic oligomers indicate that the (1→3),(1→4)-β-D-glucan is made primarily of cellotriosyl and cellotetraosyl units connected by single (1→3)-β-D-glucosyl linkages. All of these are separated from laminaridextrin and cellodextrin oligomers by high-performance anion-exchange HPLC and used to assay specifically for radiolabel incorporated into the (1→3),(1→4)-β-D-glucan synthesized in vitro (Gibeaut and Carpita, 1993; Buckeridge et al., 1999, 2001; Urbanowicz et al., 2004). In all of these studies, the products of in vitro reactions with isolated Golgi membranes were separated by gel-permeation chromatography. Under conditions with adequate substrate concentration, the (1→3),(1→4)-β-D-glucan synthesized in vitro averaged about 250 kD, essentially equivalent in size to commercial preparations of barley endosperm β-glucan and those isolated from senescent maize coleoptile walls by 4 M NaOH (Gibeaut and Carpita, 1993; Buckeridge et al., 1999, 2001; Urbanowicz et al., 2004).

The activities demonstrated in vitro do not result from a few residues that might be added to preexisting polymers, as several lines of evidence indicate that the synthesis of full-length polymers is made ab initio at the Golgi membrane with UDP-[$^{14}$C]Glc as substrate. First, suboptimal concentrations of UDP-[$^{14}$C]Glc result in much shorter oligomers and polymers enriched in cellotetraosyl rather than cellotriosyl units (Buckeridge et al., 1999). When concentrations of UDP-Glc in the millimolar range are added, then radioactivity is no longer used and polymers of high molecular mass are synthesized, and these are detected by electrochemical detection alone (Buckeridge et al., 1999, 2001). In fact, as higher and higher amounts of UDP-Glc are used, the higher the ratio of cellotriose:cellotetraose, from 2.5:1 to 11:1, and the cellopentosyl unit, whose ratio with the cellohexaosyl unit is always

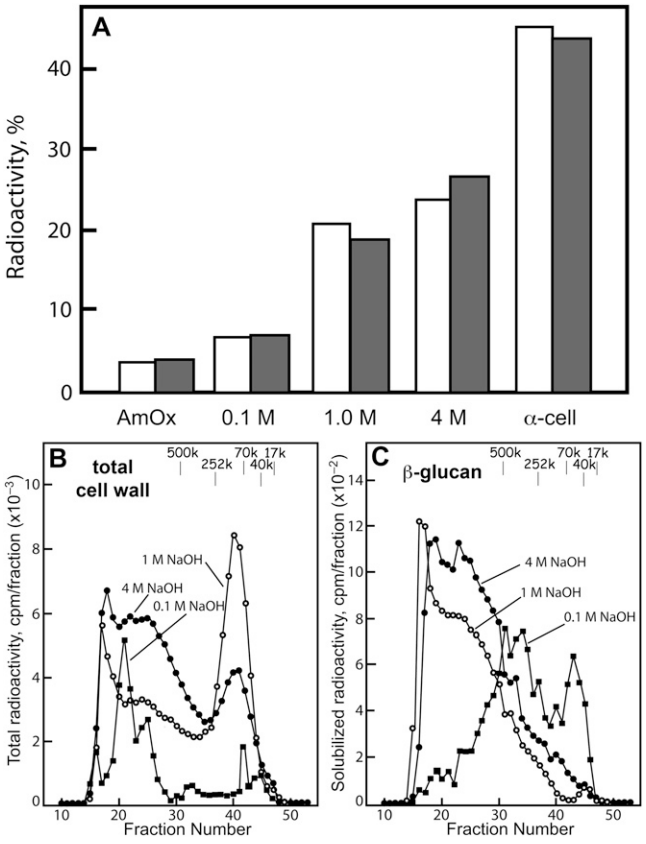


**Figure 6.** Separation of cell wall fractions. A, Proportion of radioactivity recovered after sequential extraction with hot ammonium oxalate, and 0.1, 1.0, and 4 M NaOH. The insoluble material remaining after exhaustive 4 M NaOH extraction is α-cellulose. The white bars are cell walls isolated after the 6-h pulse, and the shaded bars are after the 18-h chase. Variance of two samples was less than ±5% in all samples. Polysaccharides recovered after dialysis of neutralized 0.1, 1.0, and 4 M NaOH extracts were suspended in 0.1 M MES [NaOH], pH 5.5, and separated by gel-permeation chromatography. The (1→3),(1→4)-β-D-glucan in each fraction was estimated by radioactivity recovered in the oligomers soluble in 80% (v/v) ethanol in column buffer in each fraction after digestion with the *B. subtilis* endo-β-glucanase after subtraction of enzyme minus controls. B, Total radioactivity recovered in each fraction. C, Radioactivity from (1→3),(1→4)-β-D-glucan oligomers soluble in 80% (v/v) ethanol.

similar to the cellotriosyl:cellotetraosyl ratio, becomes the second most abundant oligomer, mimicking the unit structure of lichenin (Buckeridge et al., 1999). Even at lower concentrations of substrate using radioactivity, the specific activity of label in the products matches the specific activity of the substrate, which is only possible in ab initio synthesis (Buckeridge et al., 1999). In a study of topology of the synthesis, intact Golgi membranes treated with proteinase K lost their ability to make the cellotriosyl units specifically, whereas the synthesis of the cellotetraosyl and higher order even-numbered units was unaffected, with loss of cellotriosyl unit synthesis correlated with significant loss in size of the (1→3),(1→4)-β-D-glucan product (Urbanowicz et al., 2004).

The mixed-linkage (1→3),(1→4)-β-D-glucan is also present in the homogenate fraction, and in higher amounts than recovered from the Golgi membranes. Although this material could come from the plasma membrane-cell wall interface before incorporation into the cell wall, it is more likely from lysis of the fragile secretory vesicles delivering the β-glucan to the plasma membrane (Fig. 1). In fact, the fragility of these vesicles has been documented, and various means have been employed with limited success in preventing their lysis during isolation (Morré et al., 1965). No (1→3),(1→4)-β-D-glucan was detected in a fraction enriched in plasma membrane, but a small amount of the β-glucan is recovered from a pellet that contains some cell wall fragments (data not shown). The homogenate cleared of membrane shares a monosaccharide profile that is similar to that of the Golgi (Table I), and contains nearly twice the total IDPase activity as does the Golgi membrane. As described previously (Carpita and Gibeaut, 1988), cell wall polymers are a surprisingly small amount of the Golgi-associated polymers, and instead, AGPs are the predominant material. The gel-permeation chromatography indicates two fractions enriched in AGP, fraction IV containing polymers 70 kD and less, and fraction II containing polymers averaging about 500 kD or higher. Although a lengthy chase period still results in small increases in label in total membrane and homogenate fractions, the label in water-soluble material and specifically in the (1→3),(1→4)-β-D-glucan decreases in both Golgi and homogenate fractions, indicating turnover of the β-glucan from these fractions. The AGP-rich fractions maintained higher proportions of label, indicating a rapid turnover of these molecules returned label to the pool through nucleotide-sugar salvage pathways (Gibeaut and Carpita, 1991).

In contrast to the endosperm β-glucan that is soluble in hot water, in growing tissues, such as developing leaves, the (1→3),(1→4)-β-D-glucan is tightly bound to the cellulose microfibrils and requires in excess of 1 M NaOH to remove a majority of it (Fig. 6). Architecturally, most of the β-glucan does not form interstitial material but tightly coats the microfibrils during maximal rates of cell growth and is then largely degraded when growth has ceased (Carpita et al., 2001; McCann et al., 2007). While the (1→3),(1→4)-β-D-glucan chases from the Golgi and homogenate fractions, there is a net accumulation of the β-glucan in the cell wall. Very little β-glucan is detected in the hot ammonium oxalate fraction or 0.1 M NaOH fraction in either the pulse or chase, indicating that the (1→3),(1→4)-β-D-glucan is very quickly integrated with the newly synthesized microfibrils upon fusion of the β-glucan-laden vesicles to the plasma membrane surface (Fig. 1).

Xyloglucan endo-β-transglucosylases (XETs) are implicated in the remodeling of xyloglucans in dicotyledonous species, where a disproportional cleavage and transfer is implicated in lengthening the hybrid chains (Thompson and Fry, 2001). Evidence for such

a function in vivo comes from the finding that the Golgi-associated xyloglucans, like (1→3),(1→4)-β-D-glucans, are initially about 250 to 300 kD, even in maize cells in culture, but quickly enlarge to over 2 million during secretion to the wall (Kerr and Fry, 2003). Microscopical and chromatographic evidence shows those extracted from the cell wall are even bigger (McCann et al., 1992; Kerr and Fry, 2003). Mutants with severely modified xyloglucans altered in their ability to be recognized by XETs resulted in loss of tensile strength of the hypocotyl, indicating that the enzyme is necessary to retether the long interstitial xyloglucans during microfibril rearrangment and formation of new partners (Peña et al., 2004). Because the xyloglucan endo-β-transglucosylase/hydrolase gene family is quite large in what are considered xyloglucan-poor grass species (Yokoyama et al., 2004; Penning et al., 2009), other substrates were considered for its disproportioning activity that could increase polymer length or make covalently linked heteropolymers (Hrmova et al., 2007). In light of the inability to detect β-glucans in the Golgi membrane, Fincher (2009) proposed that an XET might be responsible for polymerizing (1→4)-β-D-glucan oligomers into the (1→3),(1→4)-β-D-glucan extracellularly. While Fincher (2009) suggests that the grafting of xyloglucans and cellulose oligomers reported by Hrmova et al. (2007) was catalyzed at quite reasonable rates, it should be noted that those activities were about 1/500 the rate of xyloglucan transglucosylation. Fry and colleagues (Fry et al., 2008a, 2008b) reported the presence of a (1→3),(1→4)-β-D-glucan and its trans-glucosylase in the primitive vascular plant, *Equisetum*. Little is yet known about the synthase for this mixed-linkage β-glucan but it appears to have arisen by convergent evolution given the predominate cellotetraosyl oligomeric structure (Fry et al., 2008a). The *Equisetum* trans-glucosylase grafts (1→3),(1→4)-β-D-glucan to xyloglucan with specificity—an activity they were unable to detect with the transglucosylases from grass species (Fry et al., 2008b). While interactions with GAX or molecular grafting may have resulted in increases in the molecular size of the (1→3),(1→4)-β-D-glucans (Fig. 6), it is doubtful that XET activity is responsible for it.

In conclusion, we show that the synthesis of the maize (1→3),(1→4)-β-D-glucan, consistent with all other noncellulosic cell wall polysaccharides, occurs at the Golgi. This finding is fully consistent with the finding of *CslF* and *CslH* gene products, the synthases of (1→3),(1→4)-β-D-glucan (Burton et al., 2006; Doblin et al., 2009), at the Golgi membrane (Fincher, 2009). Members of the *CesA/Csl* gene superfamily are among a large group of type 2 membrane-associated synthases with processive activity, meaning they continually add residues to the nonreducing end of a growing polymer chain without letting go of the acceptor chain (Saxena and Brown, 1997; Coutinho et al., 2003). Our findings underscore the cautionary adage that absence of evidence is not evidence of absence.

## MATERIALS AND METHODS

### Plant Material, Growth, and Pulse Labeling

For studies of (1→3),(1→4)-β-D-glucan synthesized in vivo, maize (*Zea mays*) caryopses were soaked overnight in deionized water bubbled with air at 28°C, sown in 20 × 20 cm (8-cm deep) trays of moist, medium-grade potting mixture, and incubated under greenhouse conditions at 25°C to 28°C for 7 d until the initial emergence of the third leaf. For pulse labeling, the trays of seedlings were placed on a shelf of clear Plexiglas fumigation chambers above a fan constructed by taping paper wings on a magnetic stir bar, stirred magnetically in a growth chamber with 500 μmol of light at 25°C. Pulse labeling was initiated by addition of $H_2SO_4$ via serum stopper into a dish containing 1.0 mCi of Na [$^{14}$C]bicarbonate (MP Biomedical; 55 mCi/mmol). Upon completion of the pulse labeling at 6 h, ambient air was introduced into the chamber and effluent passed through a NaOH trap. The plants were either harvested directly or incubated up to 18 h in ambient $CO_2$ with this forced air-effluent trapping system. Radioactivity recovered trapped in the NaOH was negligible.

For estimation of Golgi polysaccharide distribution, unlabeled maize coleoptiles were soaked overnight as described above, sown in moist vermiculite, and incubated for 2.5 to 3 d in darkness at 28°C when coleoptiles were 1.5 cm long, before the most rapid stage of elongation.

### Transmission Electron Microscopy and Immunocytochemistry

Tissue blocks of about 2 mm$^3$ were taken from the tip region and midsection of 2- to 4-d-old etiolated maize coleoptiles and fixed overnight in 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, then low-temperature embedded as described previously (Wells, 1985; Hills et al., 1987). Thin sections (100 nm) were cut on an ultramicrotome (Leica) and picked up on carbon-coated and plastic-filmed gold grids. The (1→3),(1→4)-β-D-glucan epitopes were detected with the BG1 monoclonal antibody that specifically recognizes this mixed-linkage β-glucan (Meikle et al., 1994; available from Biosupplies Australia), and visualized by a 10-nm colloidal gold-conjugated rabbit anti-mouse secondary antibody (Sigma). The blocking solution was 1% (w/v) acetylated bovine serum albumen (Aurion) in phosphate-buffered saline plus 0.1% (w/v) Tween 20, and incubation solution for washes was 0.1% acetylated bovine serum albumen in phosphate-buffered saline plus 0.01% (w/v) Tween 20.

### Isolation of Membrane Fractions

After labeling and relevant chase periods, blades and first two elongated leaves were excised, and the remaining developing leaves at the base of the sheath were collected in a chilled beaker and overlaid with an equal volume of ice-cold homogenization buffer consisting of 84% (w/v) Suc in 20 mM HEPES [KOH], pH 7.2, containing 20 mM KCl, 10 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. The material was gently stirred in homogenization buffer for about 5 min before gentle mashing in a chilled mortar and pestle (Gibeaut and Carpita, 1994). The homogenates were squeezed through a nylon mesh (45-μm$^2$ pores) and adjusted to about 42% (w/v) Suc. About 20 mL of the homogenate was pipetted into each 38.5-mL centrifuge tube (Ultraclear, Beckman) and overlaid with 6 mL each of 35%, 29%, and 18% (w/v) Suc in a gradient buffer containing 20 mM HEPES[Bis Tris-propane], pH 7.6, and the remaining volume was made up with 9.5% (w/v) Suc in the same buffer. After Suc gradient centrifugation at 140,000*g* in a rotor (model SW28, Beckman) for 90 min, the interfaces containing the enriched ER and Golgi apparatus were collected with a plastic Pasteur pipette. The homogenate was diluted 1:1 (v/v) with gradient buffer and centrifuged at 140,000*g* for 30 min to pellet plasma membrane and mitochondria, and the clear homogenate fraction was filtered through a circle of GF/F glass fiber filter mat (Whatman) to recover soluble sugars and polysaccharides from the homogenate. Golgi membranes and membrane-free homogenates were also prepared from the etiolated coleoptiles by this same protocol. All fractions were collected in 15- or 50-mL Corning plastic tubes, boiled 5 min, dialyzed against several changes of deionized water until radioactivity reached background, and freeze dried.

Samples of unlabeled maize plants treated equally were taken for assay of (1→3),(1→4)-β-D-glucan in membrane fractions and for protein determination and enzyme marker assays to establish membrane identity. Protein was determined by bicinchoninic acid assay (Pierce), and assays for IDPase

(Golgi), NADH oxidase (mitochondria), vanadate-sensitive ATPase (plasma membrane), and NADP reductase (ER) were performed as optimized for maize from published methods described in Gibeaut and Carpita (1994).

## Gel-Permeation Chromatography

One milliliter of each of the cleared supernatants from the homogenate and membrane fractions was mixed with 4 mL of 0.1 M MES[NaOH], pH 5.5, in water and applied to a 2.5- × 40-cm column of Sepharose 4B (Sigma) equilibrated in the same buffer. Fractions (4 mL) were collected, 0.5 mL was assayed for total radioactivity by liquid scintillation spectroscopy, and to 400 $\mu$L each in paired 2-mL Eppendorf tubes was added 10 $\mu$L of the preparation of lichenase in 1 mM sodium acetate, pH 5.5, or acetate buffer alone. Reactions were incubated as before, terminated by boiling for 2 min, and then 1.6 mL of ethanol containing 5 mg/mL cellulose powder were added to precipitate undigested polysaccharides at −80°C at least overnight. After centrifugation for 5 min at 14,000 rpm in a microfuge, 1 mL of the soluble fractions was assayed for radioactivity by liquid scintillation spectroscopy. In separate column runs, either dextran standards ranging from 17 to 500 kD (Sigma) or 5 mL of a 0.5% (w/v) solution of barley (*Hordeum vulgare*) $\beta$-glucan (Sigma) were run under conditions identical to those for the labeled products.

The remainders of the column fractions were pooled into five fractions representing the major peaks of radioactivity and (1→3),(1→4)-$\beta$-D-glucan dialyzed against running deionized water for 36 h, followed by two changes of nanopure water for 4 h each, and then freeze dried. The freeze-dried material was dissolved in 1 mL of water as before, and samples taken for assay of total monosaccharide and lichenase digestion. For monosaccharides, the samples were brought to 2 N trifluoroacetic acid (TFA) in 4-mL borosilicate glass vials sealed with Teflon-lined screw caps and heated to 120°C for 90 min with occasional vortex mixing. After cooling, 1 mL of *t*-butyl alcohol was added, and the TFA mixture was evaporated in a water bath at 45°C under a stream of nitrogen gas. Monosaccharides were separated isocratically in 15 mN NaOH at 0.35 mL $min^{-1}$ by high-performance anion-exchange chromatography (HPAEC) and detected by pulsed amperometry; 0.3-mL fractions were collected and assayed for radioactivity by liquid scintillation spectroscopy. Products of lichenase digestion were separated and detected by HPAEC as described below.

## Assay of (1→3),(1→4)-$\beta$-D-Glucan

Polysaccharides in freeze-dried homogenate and membrane fractions were dissolved in 2 mL of water by a combination of sonication in a water bath and boiling. The small amount of precipitate (mostly denatured protein) was pelleted by centrifugation and the soluble portions transferred to 2-mL Eppendorf centrifuge tubes. A small sample (100 $\mu$L) was taken from each for measurement of total radioactivity by liquid scintillation spectroscopy. Ten microliters of a 1:100 (v/v) dilution preparation of lichenase (*Bacillus subtilis* endo-$\beta$-D-glucanase; Megazyme) in 10 mM sodium acetate, pH 5.5, was added to 0.25 to 0.5 mL of the soluble fractions, and the samples were incubated for 3 h at 37°C. To controls without enzyme were added 10 mM sodium acetate only. The products released from digestion of purified $\beta$-glucan were mostly cellobiosyl- and cellotriosyl-(1→3)-D-Glc, with decreasingly smaller amounts of cellotetraosyl- and cellopentaosyl-(1→3)-D-Glc. The reactions were terminated by heating for 2 min in a boiling water bath, cooled to ambient temperature, and centrifuged at 10,000*g* for 5 min. Radioactivity in $\beta$-glucan was estimated as the difference in dpm in the supernatants between digested and undigested samples.

The oligomers produced by the lichenase digestions were separated on an anion-exchange column (CarboPac PA-20, Dionex) equilibrated in 0.2 N NaOH and eluted in a multistep gradient of sodium acetate to 0.2 M in 0.2 N NaOH. Radioactivity in 0.35-mL fractions collected in 1.0 mL of 0.5 N acetic acid was determined by liquid scintillation spectroscopy.

## Monosaccharide Analysis

Heat-inactivated membrane and cell wall fractions from labeled and unlabeled seedlings and unlabeled etiolated coleoptiles were used for analysis of total monosaccharide composition and linkage analysis. Dialyzed and freeze-dried materials were hydrolyzed with 1 mL of 2 M TFA at 120°C for 90 min, as described previously (Gibeaut and Carpita, 1991). After cooling, 1 mL of *t*-butyl alcohol was added, and the mixture evaporated in a stream of nitrogen gas at 45°C. The dry residue was dissolved in water, and the monosaccharides separated by HPAEC isocratically in 15 mM NaOH and detected by pulsed amperometry. Radioactivity in 0.35-mL fractions was sufficient to resolve the major monosaccharides.

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