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# Endotransglycosylation of xyloglucans in plant cell suspension cultures

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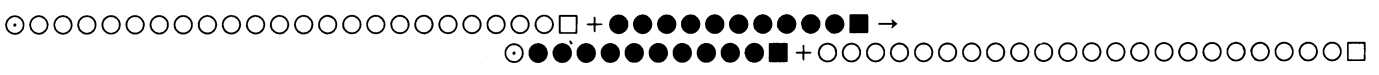
1. A xyloglucan-derived nonasaccharide ([$^3$H]XG9; $Glc_4$,$Xyl_3$,Gal,Fuc) was neither taken up by cultured plant cells nor appreciably hydrolysed by them, but a proportion of it became incorporated into extracellular polymers in all cultures tested (*Spinacia*, *Daucus*, *Rosa*, *Acer*, *Capsicum*, *Zea* and *Festuca*). 2. In *Spinacia* these polymers were soluble in 20% (w/v) trichloroacetic acid, had apparent $M_r$ 20000–30000, were able to bind reversibly to cellulose powder and were susceptible to hydrolysis by endo-$\beta$-(1→4)-D-glucanase, indicating that they were xyloglucans. 3. The linkage formed between [$^3$H]XG9 and the xyloglucan was alkali-stable and glucanase-labile, indicating that the reaction responsible for the incorporation was a transglycosylation. 4. The reducing terminus of the XG9 moiety remained reducing (convertible into [$^3$H]glucitol by $NaBH_4$) after incorporation into the polymer, showing that the XG9 was the glycosyl acceptor and the polysaccharide the donor. 5. The results provide the first evidence that polymeric xyloglucans are subject *in vivo* to cleavage followed by transfer of the cut end on to other xyloglucan-related molecules. 6. Similar endotransglycosylation reactions could occur within the primary cell wall, between pairs of high-$M_r$ structural xyloglucan molecules. Such a reaction would provide a mechanism for reversible wall loosening and may thus be relevant to our understanding of plant cell growth.

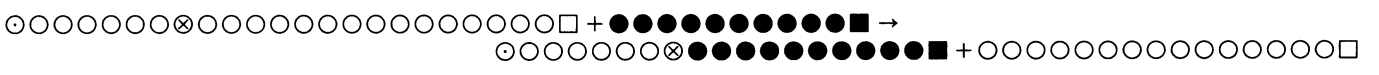
## INTRODUCTION

Xyloglucan is a structural polysaccharide that hydrogen-bonds to cellulosic microfibrils in the primary cell walls of higher plants (Hayashi & Maclachlan, 1984; Hayashi *et al.*, 1987; Fry, 1989*a*). It has been proposed that xyloglucan molecules, which are extended rods of total contour length approx. 150–1500 nm, may hydrogen-bond simultaneously to two or more microfibrils (which have a spacing within a given wall layer of approx. 20–40 nm) and act to tether adjacent microfibrils (Fry, 1989*b*; McCann *et al.*, 1990). If this picture is correct, wall loosening and consequently cell expansion would be faciliated by the cleavage of intermicrofibrillar xyloglucan chains, permitting microfibrils to move apart and/or past one another. However, repeated cleavage would weaken the cell wall; therefore the re-formation of bonds is equally important (Albersheim, 1976; Cleland, 1981). We are investigating the hypothesis that endotransglycosylation of structural xyloglucans is the mechanism for concurrent bond breakage and resynthesis during cell expansion.

Transglycosylation is the transfer of a sugar residue from a donor to an acceptor. When the donor is a polysaccharide (...○○○○○...), the transferred sugar residue may be a non-reducing terminus (⊙), in which case the reaction is exo-transglycosylation [in the following schemes, the acceptor (●●●●●●●●●■) is also a polysaccharide]:



or it may be a mid-chain residue (⊗), in which case the reaction is endotransglycosylation:



Both the original reducing termini (□ and ■) remain as reducing groups.

Many glycosidases exhibit exotransglycosylase activity *in vitro*, especially if high concentrations of a suitable acceptor are provided (Cooper & Greenshields, 1964; Dey, 1979; Tanaka *et al.*, 1982; Kaushal *et al.*, 1989). Plant cell walls contain a $\beta$-D-glucosidase with a tendency to favour transglucosylation (e.g. of cellobiose) over hydrolysis; it could possibly catalyse exotransglucosylation in a wall polysaccharide possessing a non-reducing terminal $\beta$-D-glucose residue (Nari *et al.*, 1983). The biological significance of this is unclear.

An example of an endotransglycosylase is the '*D*-enzyme' (EC 2.4.1.25) of *Bacillus*, which can break a mid-chain $\alpha$-(1→4)-glucosyl bond in one starch molecule and then re-form a similar $\alpha$-(1→4)-glucosyl bond with a different acceptor molecule (Pazur & Okada, 1968). Similarly, an endo-(1→3)-$\beta$-D-glucanase from a mollusc (Bezukladnikov & Elyakova, 1990) and a fungal chitinase (Usui *et al.*, 1990) can catalyse the endotransglycosylation of their respective substrates. No evidence has yet been presented for a plant endotransglycosylase acting on cell-wall polysaccharides *in vivo*, although the potential role of such an enzyme in cell expansion has long been recognized (Albersheim, 1976).

In endotransglycosylation, a substantial segment of the

Abbreviations used: BAB, butanone/acetic acid/$H_3BO_3$-saturated water (9:1:1, by vol.); BPW, butan-1-ol/pyridine/water (4:3:4, by vol.); EAW, ethyl acetate/acetic acid/water (10:5:6, by vol.); EPW, ethyl acetate/pyridine/water (8:2:1, by vol.); g.p.c., gel-permeation chromatography; [*r.t.*-1-$^3$H]XG9, xyloglucan-derived nonasaccharide $^3$H-labelled at position 1 of its reducing terminal D-glucose unit.

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attacked polysaccharide may be transferred from donor to acceptor. In contrast with exotransglycosylation, endotransglycosylation might be expected to exert a profound influence on wall extensibility by cutting load-bearing polysaccharides in mid-chain. McDougall & Fry (1990) have presented evidence that an enzyme from young *Phaseolus* leaves can catalyse endotransglycosylation *in vitro* with xyloglucan as both donor and acceptor. The enzyme caused xyloglucan molecules in an initially bimodal population to converge towards their average $M_r$ (Figs. 7*a* and 7*b* of McDougall & Fry, 1990).

Does endotransglycosylation of xyloglucans occur *in vivo*? Since the products may be identical with the substrates, and the reaction causes no change in their mean $M_r$, endotransglycosylation is very difficult to study *in vivo*. One approach is to monitor the fate of $^3$H-labelled xyloglucan–oligosaccharides fed to cultured cells (Baydoun & Fry, 1989). Although oligosaccharides are not structural components of the cell wall, they can nevertheless permeate it and may thus participate in transglycosylation reactions catalysed by wall enzymes. The [$^3$H]oligosaccharides were not taken up by the cells, and underwent little degradation, but the $^3$H became associated with high-$M_r$ extracellular material via alkali-stable bonds. This was interpreted as being due to transglycosylation, and two models were proposed to account for the reaction: (*a*) the [$^3$H]oligosaccharide (●●●■) is cut (e.g. ↑ = bond cleaved) and a portion of it transferred to a non-radioactive polysaccharide (⊙○○○○○○○○○○○○○○○○○○○□) (hypothesis *a*):

●●●■ + ⊙○○○○○○○○○○○○○○○○○○○□ → ●●●⊙○○○○○○○○○○○○○○○○○○○□ + ■
↑

or (*b*) a non-radioactive polysaccharide is cut and a portion of it transferred on to the [$^3$H]oligosaccharide (hypothesis *b*):

●●●■ + ⊙○○○○○○○○○○○○○○○○○○○□ → ⊙○○○○○○○○○○○○●●●■ + ○○○○○○□
↑

Of these two models, only (*b*) involves the mid-chain cleavage of high-$M_r$ endogenous polysaccharides, the step proposed to be responsible for loosening the cell wall. Therefore it was important to distinguish between the two models. The major difference between them is that in (*b*) the reducing group of the oligosaccharide is incorporated into the polymer, whereas in (*a*) it is not. In the present paper we show that hypothesis (*b*) is correct, and thus provide the first evidence for the endotransglycosylation of a plant cell-wall polysaccharide *in vivo*.

## EXPERIMENTAL

### Preparation of [*r.t.* -1-$^3$H]XG9

Non-radioactive xyloglucan nonasaccharide (XG9) was prepared by cellulase-catalysed digestion of *Rosa* hemicellulose, and purified by gel-permeation chromatography (g.p.c.) on Bio-Gel P-2 followed by preparative paper chromatography in EAW and BPW, all as described before (McDougall & Fry, 1989). Acid hydrolysis of the non-radioactive XG9 yielded glucose, xylose, galactose and fucose in the molar proportions ~4:3:1:1, as expected for the major nonasaccharide of primary cell-wall xyloglucan, α-D-Xyl*p*-(1→6)-β-D-Glc*p*-(1→4)-[α-D-Xyl*p*-(1→6)]-β-D-Glc*p*-(1→4)-[α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→2)-α-D-Xyl*p*-(1→6)]-β-D-Glc*p*-(1→4)-D-Glc (Valent *et al.*, 1980). A portion of this XG9 was custom tritiated by Amersham International (Amersham, Bucks., U.K.) by the 'TL·7' method, which involves incubation of the sample in the presence of $^3H_2$ and a catalyst. Under these conditions the hydrogen atom at position 1 of the reducing terminus is expected to exchange with the $^3H_2$. The radioactive oligosaccharide (specific radioactivity approx. 12 TBq/mol) was re-purified by paper chromatography in EAW and BPW, eluted with water and stored in 25% (v/v) ethanol at 4 °C.

### Incubation of spinach cells with [*r.t.*-1-$^3$H]XG9

Suspension cultures of spinach (*Spinacia oleracea* L., cv. Monstrous Viroflay) were maintained as described previously (Fry, 1982). Portions (3 ml) of day-4 culture were transferred into 60 ml Sterilin pots containing 0.5 MBq of [*r.t.*-1-$^3$H]XG9 [sterilized by drying from 75% (v/v) ethanol in a stream of sterile air] and incubated aseptically under standard cultural conditions. The added [$^3$H]XG9, diluted to 3 ml, would contribute approx 14 μM-XG9 (calculated from specific radioactivity); in addition, stale culture medium already contains approx. 0.1–1.0 μM-XG9 (Fry, 1986). Controls were performed with fresh culture medium in place of suspension culture. At intervals (see Fig. 2), cultures were harvested and centrifuged (2500 *g* for 5 min). The pelleted cells were re-suspended in 3 ml of water and re-centrifuged, and the two supernatants were pooled (solution *S*). The cells were washed three more times in water and finally assayed for $^3$H radioactivity. Solution *S* was loaded on to a column of Bio-Gel P-2 (bed volume 160 ml) with internal markers glucose and dextran ($M_r$ 9400) (each 0.17%, w/v) and eluted with 0.05% chlorbutol (1,1,1-trichloro-2-methylpropan-2-ol) in water. Fractions were assayed for hexose by the anthrone method (Dische, 1962) and for $^3$H radioactivity. Fractions containing [$^3$H]polymer (co-eluted with dextran) were pooled and divided into portions (1.32 or 0.33 kBq), which were dried *in vacuo* and analysed as follows. The analysis was performed on [$^3$H]polymer from each sampling time shown in Fig. 2, with similar results; data for the 48 h sample are shown. (In all the following tests, a sample of pure [*r.t.*-1-$^3$H]XG9 was processed in parallel.)

**Acid hydrolysis.** One portion (0.33 kBq) was incubated in 1 ml of 2 M-trifluoroacetic acid at 120 °C for 1 h. Products were chromatographed in EPW.

**Driselase digestion.** A second portion (0.33 kBq) was incubated with 125 μl of 0.25% (w/v) Driselase [Sigma Chemical Co., Poole, Dorset, U.K.; partially purified as described previously (Fry, 1982)] in pyridine/acetic acid/water (1:1:98, by vol., pH 4.7) at 25 °C for 20 h. Products were chromatrographed in EPW. Driselase is a mixture of enzymes from the fungus *Irpex lacteus*; it contains enzymes that hydrolyse all the glycosidic linkages of xyloglucan except α-D-xylosyl (Fry, 1988), and it therefore releases the xylose residues of xyloglucan and XG9 in the form of a diagnostic disaccharide, isoprimeverose [α-D-Xyl*p*-(1→6)-D-Glc].

**Cellulase digestion.** A third portion (1.32 kBq) was incubated with 200 μl of 0.1% (w/v) *Trichoderma viride* cellulase (Sigma Chemical Co.; not further purified) in pyridine/acetic acid/water (1:1:98, by vol., pH 4.7) at 25 °C for 3 h. Products were chromatographed in EAW. Under these conditions, this enzyme preparation hyrolyses xyloglucan to a high yield of hepta-, octa- and nona-saccharides, but has little effect on these oligosaccharides other than to hydrolyse a small proportion (< 10%) of the L-fucose residues of XG9.

**$NaBH_4$ reduction.** A fourth portion (0.33 kBq) was treated with 200 μl of 0.5 M-$NaBH_4$ in 1 M-$NH_3$ at 25 °C for 4 h to

reduce the reducing terminus to an alditol, freed of borate and cations (Fry, 1988), dried and hydrolysed with trifluoracetic acid as above. Products were chromatographed in BAB.

**G.p.c.** A fifth portion (1.32 kBq) was re-dissolved in 10 mM-NaOH and chromatographed on Sepharose CL-6B equilibrated and eluted with 10 mM-NaOH.

**Trichloroacetic acid precipitation.** A sixth portion (1.32 kBq) was redissolved in 1% (w/v) BSA, and trichloroacetic acid was added to 20% (w/v). The mixture was incubated at 0 °C for 18 h, then centrifuged at 1500 *g* for 5 min. The pellet was re-suspended in 2 ml of 20% trichloroacetic acid and centrifuged as before. The supernatants were pooled and portions thereof were assayed for $^3H$ radioactivity; the pellet was re-suspended in water and assayed for $^3H$ radioactivity.

**Binding to cellulose.** A seventh portion (0.33 kBq) was re-dissolved in 0.5 ml of 20 mM-sodium acetate buffer, pH 5.0, containing 0.12% (w/v) *Tropaeolum* xyloglucan (prepared by the method of Edwards *et al.*, 1985) as non-radioactive carrier. The solution was applied to a 3 ml-bed-volume column of cellulose powder (pre-washed in 1 M-NaOH followed by water), which was eluted (flow rate 0.1 ml/min) sequentially with the same buffer (13 ml), buffer containing 8 M-urea (12.5 ml) and 1 M-NaOH (12.5 ml). The elution of radioactivity was monitored.

**$Cu^{2+}$ precipitation (Rao, 1959; Edwards *et al.*, 1985).** An eighth portion (1.32 kBq) was re-dissolved in 475 $\mu$l of 0.5% (w/v) *Tropaeolum* xyloglucan. To this was added 70 $\mu$l of Fehling's solution A [35% (w/v) potassium sodium tartrate tetrahydrate in 25% (w/v) KOH] followed by 70 $\mu$l of Fehling's solution B [7.5% (w/v) $CuSO_4,5H_2O$]. The precipitated xyloglucan was collected by centrifugation, washed in 70% (v/v) ethanol and assayed for $^3H$ radioactivity.

**$I_2$ precipitation.** A ninth portion (1.32 kBq) was dissolved in 3.4 ml of 0.038% *Tropaeolum* xyloglucan, to which 3 ml of 3% (w/v) $I_2$ containing 4% (w/v) KI was added. The precipitate of polysaccharide was allowed to develop for 2 h at 0 °C, collected by centrifugation at 20000 *g* for 10 min and assayed for $^3H$ radioactivity.

### Chromatography

Paper chromatography was on Whatman 3MM paper, by the descending method, in the following solvents: EPW (ethyl acetate/pyridine/water, 8:2:1, by vol.) for 16 h; EAW (ethyl acetate/acetic acid/water, 10:5:6, by vol.) for 36 h; BAB (butanone/acetic acid/$H_3BO_3$-saturated water, 9:1:1, by vol.) for 24 h (Lewis & Smith, 1967); BPW (butan-1-ol/pyridine/water, 4:3:4 by vol.) for 16 h. External markers were stained with $AgNO_3$/NaOH (Fry, 1988). Sample lanes were cut into 1 cm-long strips and assayed for $^3H$ radioactivity.

H.p.l.c. of oligosaccharides was performed on a Brownlee Amino-Spheri-5 column eluted isocratically with 60% (v/v) acetonitrile as described previously (McDougall & Fry, 1990). Total carbohydrate was monitored by refractive index.

### Assay of radioactivity

Aqueous solutions and suspensions were mixed with 10 vol. of toluene/Triton X-100 (2:1, v/v) containing 0.33% 2,5-diphenyloxazole and 0.033% 1,4-bis-(5-phenyloxazol-2-yl)benzene. Dried strips of chromatography paper were added to 2 ml of toluene containing 0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene. Radioactivities of samples were scintillation-counted at efficiencies of approx. 35% for aqueous solutions and of approx. 6% for dried samples. Radioactive material eluted from the h.p.l.c. column was recorded by use of a RAMONA flow-through monitor (Raytest Instruments, Sheffield, U.K.).

### Survey of different plant species

Cell-suspension cultures of four other dicotyledonous plants [carrot (*Daucus carota*), rose (*Rosa* sp., cv. Paul's Scarlet), sycamore (*Acer pseudoplatanus*) and chilli pepper (*Capsicum frutescens*)] and of two monocotyledonous plants [maize (*Zea mays*) and tall fescue grass (*Festuca arundinacea*)] (all used 3 days after sub-culturing) were incubated with 14 $\mu$M-[*r.t.*-1-$^3H$]XG9 for up to 72 h. Samples (150 $\mu$l) of culture supernatant were then loaded on to Whatman 3MM paper and developed in BPW for 16 h; material of $R_F$ 0.00 was assayed for $^3H$ radioactivity.

## RESULTS

### Characterization of [*r.t.*-1-$^3H$]XG9

The $^3H$-labelled oligosaccharide was indistinguishable from authentic XG9 on paper chromatography in EAW and BPW (McDougall & Fry, 1989), on Bio-Gel P-2 (Fry, 1986; see also Fig. 3) and by h.p.l.c. (Fig. 1). T.l.c. of an acid hydrolysate of the [$^3H$]oligosaccharide followed by staining with aniline hydrogen phthalate revealed a sugar composition of glucose, galactose, xylose and fucose in the molar proportions ~4:1:3:1, consistent with XG9. Acid hydrolysis of the oligosacchides before and after reduction with $NaBH_4$ yielded [$^3H$]glucose and [$^3H$]glucitol respectively as sole radioactive product (results not shown), confirming that the $^3H$ was restricted to the reducing terminus. Driselase digestion of the $^3H$-labelled oligosaccharide followed by paper chromatography yielded [$^3H$]glucose but no [$^3H$]isoprimeverose, again showing that the XG9 was labelled specifically in its reducing terminal glucose unit, the only one of the four glucose units that is not xylosylated. These observations, together with its method of preparation (Evans *et al.*, 1974), indicate that the material was [*r.t.*-1-$^3H$]XG9.

### [$^3H$]XG9 is incorporated intact into extracellular spinach polymers

To determine whether the XG9 was the glycosyl donor or acceptor during transglycosylation *in vivo*, spinach cell cultures were incubated with [*r.t.*-1-$^3H$]XG9 for up to 72 h. If the XG9 were cleaved during the transglycosylation, the $^3H$ would be released as a smaller [$^3H$]oligosaccharide or [$^3H$]glucose, and not efficiently incorporated into polymer.

Uptake of $^3H$ and/or binding to the cells was negligible (Fig. 2). Nevertheless, g.p.c. of the culture filtrate showed that a substantial proportion of $^3H$ became incorporated into soluble polymeric material. Negligible amounts of $^3H$ were associated

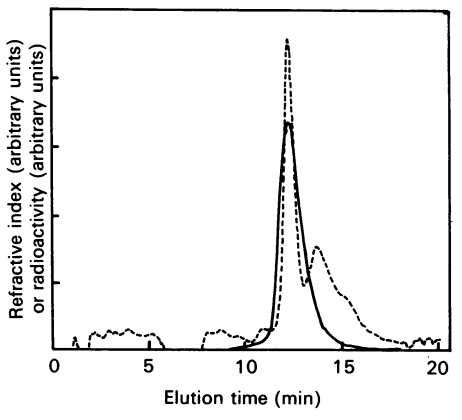



**Fig. 1. H.p.l.c. analysis of [*r.t.*-1-$^3H$]XG9 on amino-substituted silica**

The main peak of refractive index (-----) co-chromatographs with the sole peak of $^3H$ (——).

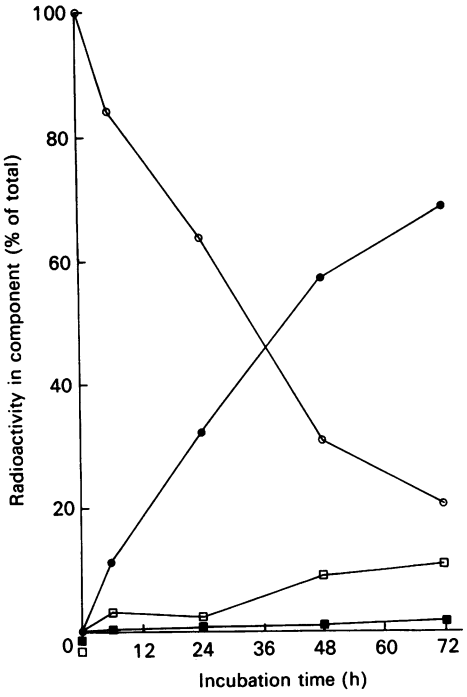



**Fig. 2. Incorporation of $^3H$ from [*r.t.*-1-$^3H$]XG9 into fractions of spinach cell cultures**

Components assayed were as follows: spinach cells and insoluble cell-wall material (■); low-$M_r$ XG9-breakdown products (□); soluble apoplastic polymer (●); unchanged [$^3H$]XG9 (○). The data for □, ○ and ● were obtained from Bio-Gel P-2 chromatography.

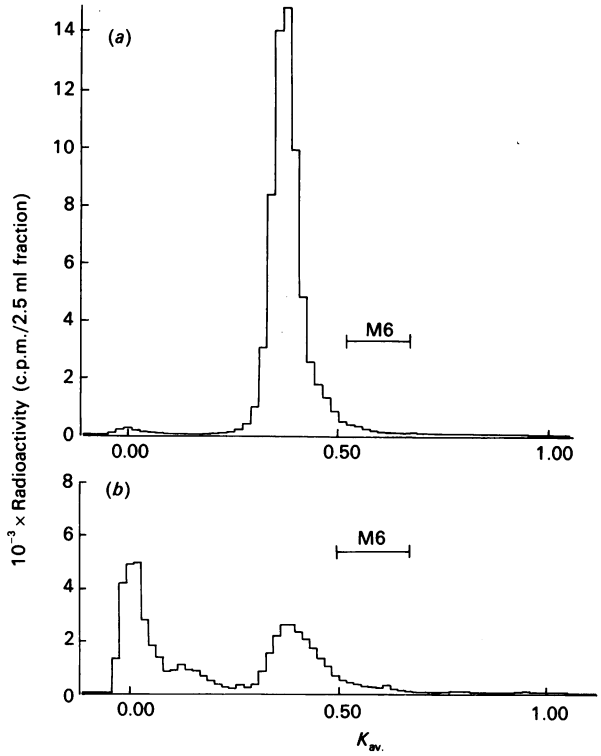



**Fig. 3. G.p.c. of the products of incubating [*r.t.*-1-$^3H$]XG9 in spinach culture medium**

(*a*) [$^3H$]XG9 incubated with fresh culture medium in the absence of cells for 48 h; (*b*) [$^3H$]XG9 incubated with whole spinach culture for 48 h. Internal markers were dextran ($K_{av.}$ 0.00), glucose ($K_{av.}$ 1.00) and maltohexaose (M6). Recovery of applied $^3H$ from the columns was 89.4% (*a*) and 98.5% (*b*).

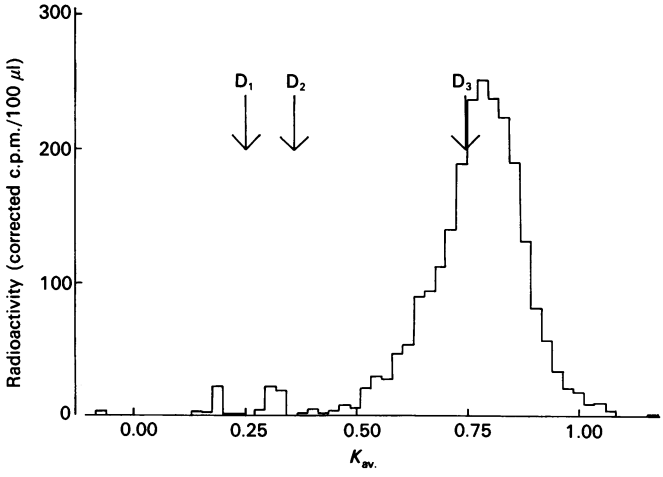



**Fig. 4. G.p.c. of high-$M_r$ transglycosylation products**

The Figure shows Sepharose CL-6B chromatography of material recovered from the void volume of the g.p.c. shown in Fig. 3(*b*). Dextrans of $M_r$ 488000 ($D_1$), 249000 ($D_2$) and 40200 ($D_3$) were the external markers. Internal markers were industrial-grade dextran ($M_r$ $5 \times 10^6$–$40 \times 10^6$; $K_{av.}$ 0.00) and glucose ($K_{av.}$ 1.00). The eluent was 10 mM-NaOH. Apparent recovery of applied $^3H$ from the column was 104.5%.

with breakdown products, which would have included any $^3H_2O$ formed by respiration of [$^3H$]glucose (Figs. 2 and 3). Non-radioactive glucose, which was the carbon source in the culture medium, was less than 50% utilized during the 72 h incubation (results not shown); therefore most of any [$^3H$]glucose that was liberated by an apoplastic reaction would have been recovered in the spent medium. These observations support model (*b*) (see the Introduction), i.e. that the XG9 acts as the glycosyl acceptor without itself being cleaved.

The $M_r$ of the $^3H$-labelled polymer was estimated to be 20000–30000 by g.p.c. on a Sepharose CL-6B column calibrated with linear dextran $M_r$ markers (Fig. 4).

Confirmation that the $^3H$ was incorporated into the extracellular polymer without appreciable degradation of the XG9 moiety was provided by the demonstration that the radioactive polymer yielded [$^3H$]glucose as sole radioactive product upon hydrolysis with acid (Fig. 5*a*) or Driselase (Fig. 5*b*). If the XG9 had been degraded to [$^3H$]glucose and the $^3H$ then re-incorporated into new polymers, the radioactivity would have been distributed among many different sugar residues.

The $^3H$-labelled unit remained as a reducing terminus after incorporation into the polymer. This was shown by reduction by $NaBH_4$ of the labelled polymer followed by acid hydrolysis to yield [$^3H$]glucitol rather than [$^3H$]glucose (Fig. 5*c*). This again strongly supports model (*b*), confirming that the XG9 is the glycosyl acceptor.

### Nature of the polymer that binds to [$^3H$]XG9

The polymer–XG9 bond was stable to paper-chromatography solvents (therefore probably covalent) and resistant to alkali (therefore not an ester) (Baydoun & Fry, 1989; R. C. Smith & S. C. Fry, unpublished work). Treatment of the radioactive polymer with cellulase released a product that was indistinguishable by paper chromatography from [$^3H$]XG9 (Fig. 5*d*), indicating that the linkage between the polymer and the XG9 moiety was probably formed through a β-(1→4)-D-glucosyl bond. Since XG9 does not contain any cellulase-labile bonds, this indicates

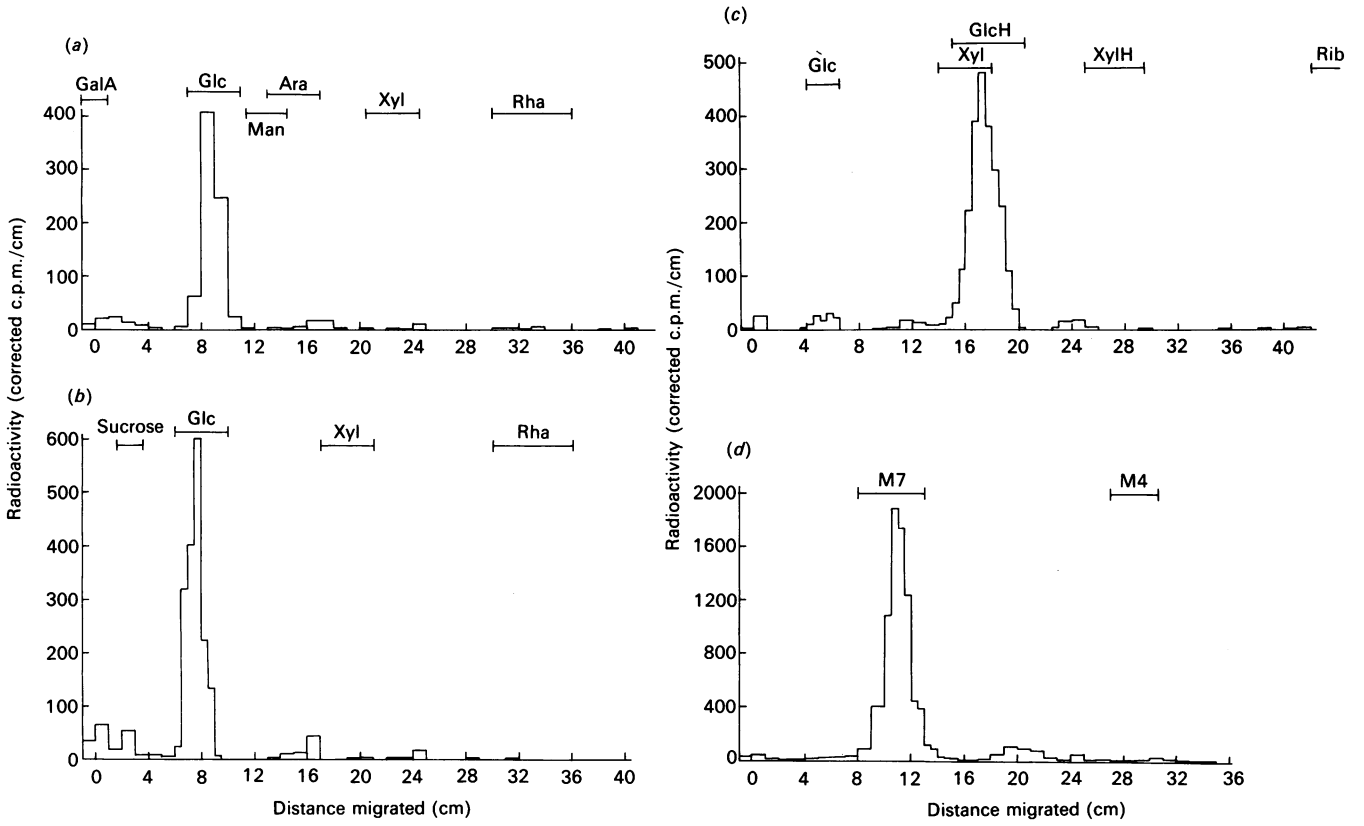



**Fig. 5. Paper chromatography of high-$M_r$ transglycosylation products after hydrolysis**

Material recovered from the void volume of the g.p.c. shown in Fig. 3(*b*) was subjected to: (*a*) trifluoroacetic acid hydrolysis (products separated in EPW); (*b*) Driselase digestion [products separated in EPW; isoprimeverose and cellobiose have $R_F$ values near or slightly below that of the marker sucrose (Fry, 1988)]; (*c*) trifluoroacetic acid hydrolysis of borohyride-reduced material (products separated in BAB); (*d*) *Trichoderma*-cellulase digestion (products separated in EAW). When a chromatogram similar to that shown in (*d*) was developed for a shorter time such that marker glucose remained on the paper, no $^3H$ migrated faster than M4. Positions of external markers are shown (GlcH, D-glucitol; XylH, xylitol; M7, maltoheptaose; M4, maltotetraose). Recovery of applied $^3H$ was 69.0% (*a*), 86.7% (*b*), 91.0% (*c*) and 101.9% (*d*).

**Table 1. Precipitability of water-soluble [$^3$H]polymer produced by spinach cells from exogenous [$^3$H]XG9**

The extracellular water-soluble [$^3$H]polymer produced upon incubation of spinach cell cultures in the presence of [$^3$H]XG9 was mixed with an appropriate non-radioactive carrier and treated with the precipitant. As a control, a sample of pure [$^3$H]XG9 was treated in the same way. The non-precipitated $^3H$ radioactivity was confirmed to be present in the supernatant.

| Precipitant used | Non-radioactive carrier used | Proportion of $^3H$ precipitated (%) | |
|---|---|---|---|
| | | [$^3$H]Polymer | [$^3$H]XG9 |
| 20% trichloroacetic acid | BSA | 0.28 | 0.14 |
| $Cu^{2+}$ (Fehling's solution) | Xyloglucan | 2.65 | 0.15 |
| $I_2$/KI | Xyloglucan | 3.53 | 2.99 |

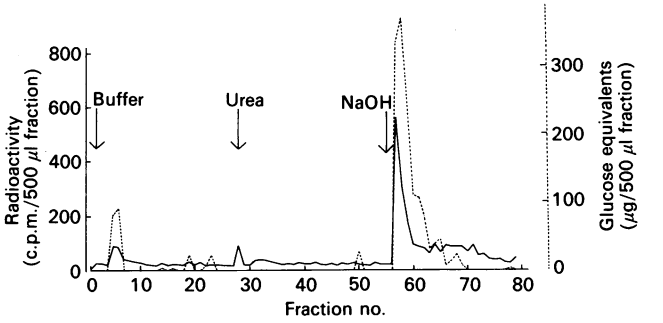



**Fig. 6. Cellulose affinity chromatography of high-$M_r$ transglycosylation products**

Material recovered from the void volume of the g.p.c. shown in Fig. 3(*b*) (——) and an internal marker of nasturtium xyloglucan (-----) were passed through the cellulose column. The eluents were 20 mM-sodium acetate buffer, pH 5.0, followed by 8 M-urea, followed by 1 M-NaOH. Recovery of applied $^3H$ from the column was 92.7% (70% of the applied $^3H$ eluted with NaOH).

that the polymer that acts as glycosyl donor probably contributes a $\beta$-(1→4)-D-glucosyl bond.

The radioactive polymer was not precipitated by 20% trichloroacetic acid (Table 1), indicating that any proteinaceous component was quantitatively minor.

The $^3H$-labelled polymer was able to bind to cellulose and could subsequently be eluted with 1 M-NaOH (Fig. 6). This indicates that the polymer had the properties of a hemicellulose.

The only known plant polymer that fits the above description is xyloglucan. However, the radioactive polymer was not precipitated by $I_2$ or $Cu^{2+}$ (Table 1), despite the presence of non-

radioactive carrier xyloglucan (from *Tropaeolum* seeds), which was precipitated from the same solution. The $M_r$ of the $^3$H-labelled polymer (20000–30000) was relatively low, and this may explain its failure to be precipitated by $I_2$ or $Cu^{2+}$; cell-wall-bound xyloglucans usually have $M_r$ approx. $10^5$–$10^6$ (for review see Fry, 1989*a*). Ring & Selvendran (1981) have reported the existence of both $Cu^{2+}$-precipitable and $Cu^{2+}$-non-precipitable xyloglucans in extracts of potato-tuber cell walls.

The data therefore suggest that endogenous polymeric xyloglucans are cleaved by an endotransglycosylase present in the apoplast, and then the cut end is transferred on to a non-reducing residue of the exogenous [*r.t.*-1-$^3$H]XG9.

### Xyloglucan endotransglycosylation is not unique to spinach cultures

If the endotransglycosylation observed in spinach cultures reflects an important process in cell-wall expansion, we would expect to find it in other plant species.

When [*r.t.*-1-$^3$H]XG9 was fed to young rapidly growing cultures of four other dicotyledonous plants and two members of the Gramineae, a proportion of the $^3$H was in each case incorporated into extracellular polymers (Fig. 7). The $^3$H-labelled polymeric material ($R_F$ 0.00) was clearly resolved from XG9 ($R_F$ 0.16) and is taken to be the product of apoplastic transglycosylation. None of the six cultures showed appreciable uptake or binding of $^3$H or breakdown of [$^3$H]XG9 to products with $R_F$ greater than 0.16.

Of the six species studied, the two members of the Gramineae (*Zea* and *Festuca*) showed relatively low transglycosylation activity. This could reflect the lower xyloglucan content of graminaceous cell walls, and thus a lower rate of xyloglucan endotransglycosylation. The *Capsicum* cultures, which also showed low apparent rates of transglycosylation, were composed of large clumps of the tightly aggregated cells, only the outermost of which would have had ready access to the [$^3$H]XG9.

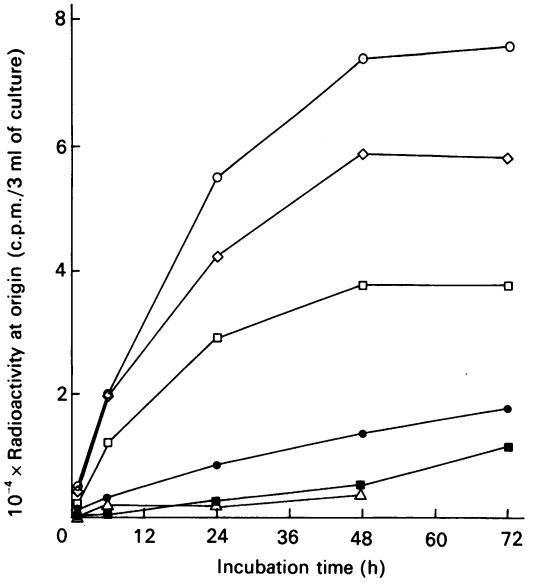



**Fig. 7. Incorporation of $^3$H from [*r.t.*-1-$^3$H]XG9 into soluble apoplastic polymer in cultures of six other species**

Cell suspension cultures of the following species were tested: ○, rose (*Rosa* sp.); ◇, sycamore (*Acer pseudoplatanus*); □, carrot (*Daucus carota*); △, chilli pepper (*Capsicum frutescens*); ●, maize (*Zea mays*); ■, fescue (*Festuca arundinacea*).

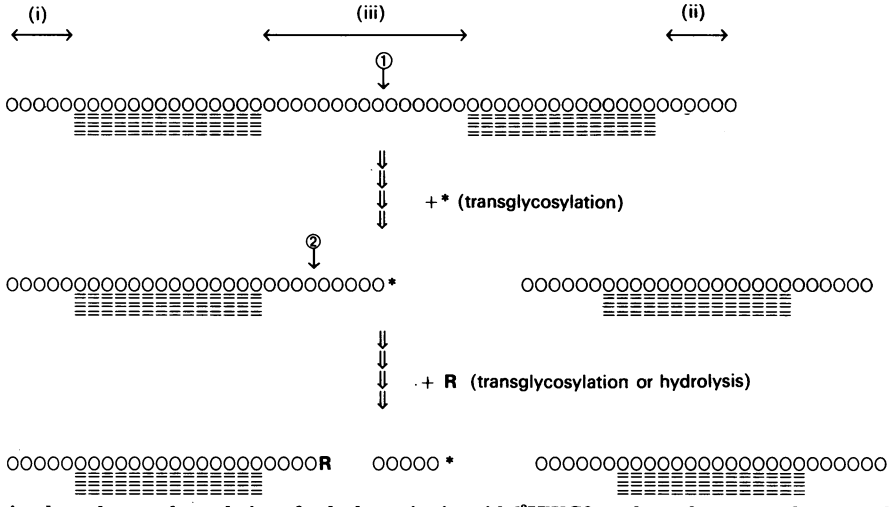
## DISCUSSION

The data demonstrate the activity of an apoplastic transglycosylase that appears to cleave a glycosidic bond in the backbone of polymeric xyloglucan and transfer the new (potentially reducing) terminus on to a non-reducing residue of XG9, re-forming a glycosidic linkage. The reaction must occur external to the plasma membrane because the substrate, [*r.t.*-1-$^3$H]XG9, though not being taken up by the cells, is incorporated intact into an extracellular polymer. Evidence that the XG9 is not cleaved during the reaction is the fact that $^3$H from it is incorporated into the polymer whether the XG9 is labelled in its fucose or xylose residues (Baydoun & Fry, 1989) or in its reducing terminal glucose unit (present work). The incorporation of [*fucosyl*-$^3$H]XG9 and [*xylosyl*-$^3$H]XG9 also shows that we were not simply observing an exchange of the aldehydic hydrogen atom at the reducing terminus of the oligosaccharide. If the reaction primarily involved cleavage of the side chains of the polymeric xyloglucan, only short (at most trisaccharide) portions would be added to the [$^3$H]XG9, and this would not give the observed large increase in $M_r$; therefore the backbone of the xyloglucan must be cleaved. The bond cleaved and re-synthesized appears to be cellulase-labile, as shown by the release of [$^3$H]XG9 from the labelled polymer by fungal cellulase. This suggests that the acceptor hydroxy group in XG9 is O-4 of the glucose residue furthest from the reducing terminus, and that the transglycosylase may be a cellulase (McDougall & Fry, 1990) or have a similar substrate-specificity.

The fact that the acceptor ([$^3$H]XG9) has a low $M_r$ would partly explain why all the observed (i.e. radioactive) products of transglycosylation had a lower $M_r$ than is typical for xyloglucans. Transglycosylation leads the reactants to converge towards their average $M_r$, which in the case of XG9 + xyloglucan will be about half the $M_r$ of the xyloglucan.

However, the radioactive transglycosylation products had $M_r$ approx. 20000–30000, which is less than one-tenth of the $M_r$ of typical wall-bound xyloglucan. Furthermore, almost all the products were found in solution in the culture medium rather than bound to the cell wall. In explanation of this, it may be relevant that xyloglucan appears to occur in the cell wall in two states differing in their molecular mobility (MacKay *et al.*, 1988) and accessibility to enzymes (Hayashi *et al.*, 1984): approx. 30% of wall-bound xyloglucan is readily released as water-soluble products by a few hours' treatment with cellulase, whereas the other approx. 70% is much less susceptible (Table VI of Hayashi *et al.*, 1984). It seems likely that enzymes preferentially attack segments of xyloglucan that are not directly hydrogen-bonded to cellulose and that therefore have a high molecular mobility.

Non-cellulose-bound segments of xyloglucan would be of three types: (i) loose non-reducing terminal segments, (ii) loose reducing terminal segments and (iii) intermicrofibrillar segments (Scheme 1 and Fry, 1989*b*). Endotransglycosylation of xyloglucan in these three situations (with [$^3$H]XG9 as acceptor) would have the following consequences respectively: (i) the immediate $^3$H-labelled product would be a short free polysaccharide: (ii) a non-radioactive fragment would be released and the [$^3$H]XG9 unit would become the new reducing terminus of the remaining loose segment, so that, at the next attack (transglycosylation or hydrolysis) on this segment, the $^3$H would be released on a short free polysaccharide; (iii) no fragment would be released at the first attack, but two loose segments would be created, one of which ($^3$H-labelled at its reducing end) would generate a $^3$H-labelled short free polysaccharide at the




**Scheme 1. Hypothesis to explain why endotransglycosylation of xyloglucan *in vivo* with [$^3$H]XG9 as glycosyl acceptor always results in low-$M_r$ non-wall-bound $^3$H-labelled products**

It is assumed that the endotransglycosylase can most readily attack segments of xyloglucan that are not hydrogen-bonded to cellulose, and that the enzyme tends to carry out multiple attacks on a particular segment of xyloglucan. (i), (ii), (iii) are the three types of non-cellulose-bound xyloglucan segment considered in the text; the consequences of attack at segment (iii) are shown. Key: OOOOOOOO..., xyloglucan chain (reducing terminus to right); ≡≡≡, cellulosic microfibril; *, [$^3$H]XG9; **R**, $H_2O$ or xyloglucan; ① and ②, first and second sites of enzymic attack.

next attack on this segment (Scheme 1). Concerning (ii) and (iii), where two enzymic attacks have to be proposed to account for the observed product, it is possible that the transglycosylase tends to carry out multiple attacks on a single polysaccharide segment, as reported for an endo-$\beta$-(1→3)-glucanase (Bezukladnikov & Elyakova, 1990). In the plant cell wall this tendency could be due to the substrate-binding properties of the enzyme [as with the endo-$\beta$-(1→3)-glucanase] and/or to the limited mobility of the enzyme in the cell-wall matrix (confining the enzyme within the vicinity of one particular segment of a xyloglucan molecule).

The measured enzyme activity, with XG9 as acceptor, may be atypical of what occurs *in vivo* in that the acceptor is of very low $M_r$. We suggest that the major transglycosylation reaction *in vivo*, in the absence of exogenous XG9, occurs between pairs of essentially identical high-$M_r$ xyloglucan molecules [especially the type (iii) segments shown in Scheme 1]. Such endotransglycosylation could cause a temporary breakage of load-bearing intermicrofibrillar xyloglucan chains, sufficient to allow limited cell-wall extension and thereby relieving some of the wall's mechanical stress. Following incremental wall expansion, similar bonds would be re-formed between new partners. Since no net bond cleavage would be involved, the cell wall would regain much of its mechanical strength after expansion, as appears to occur (Cleland, 1981).

The proposed tendency of the endotransglycosylase to catalyse repeated attacks on non-cellulose-bound xyloglucan segments [type (ii), or attack ② on type (iii); Scheme 1] would, when the acceptor is the non-reducing end of another high-$M_r$ xyloglucan chain, act as a repair mechanism creating a new intermicrofibrillar bridge. This could explain the increase in mean $M_r$ of wall-bound xyloglucan (and decrease in growth rate) observed when $H^+$-treated plant tissue is returned to a neutral buffer (Nishitani & Masuda, 1983).

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