XYLOGLUCAN ENDOTRANSGLYCOSYLATION IN THE APOPLAST OF PLANT CELL SUSPENSION CULTURES

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Abbreviations

ADP	Adenosine diphosphate
Ara	Arabinose
BAB	Paper chromatography solvent; butanone/acetic acid/boric acid-saturated water (9:1:1. v/v/v)
BAP	6-Benzylaminopurine
BPW	Paper chromatography solvent; butan-1-ol/pyridine/water (4:3:4, v/v/v)
Chlorobutol	1,1,1-tricholo-2-methylpropan-2-ol
CMC	Carboxymethylcellulose
CPA	*para*-Chlorophenoxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
DCB	2,6-dichlorobenzonitrile
EAW	Paper chromatography solvent system; ethyl acetate/acetic acid/water (10:5:6, v/v/v)
EPW	Paper chromatography solvent system; ethyl actate/pyridine/water (8:2:1, v/v/v)
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
GDP	Guanidine diphosphate
Glc	Glucose
g.p.c.	Gel permeation chromatography
h.p.l.c.	High performance liquid chromatography
IAA	Indole acetic acid
Isoprimeverose	α-D-xylopyranose-(1-6)-D-glucose
K_{av}	The volume in which a substrance will elute from a g.p.c. column, as a proportionof the total included volume
M2	Maltose
M3	Maltotriose
M4	Maltotetraose
M6	Maltohexaose
M7	Maltoheptaose

P	Tugor pressure
p.c.	Paper chromatography/paper chromatogram
PCV	Packed cell volume
	[= (volume cells/3 ml following centrifugation)x3.33]
PGA	Polygalacturonic acid
POPOP	1,4-bis-(5-phenyloxazol-2-yl)benzene
PPO	2,5-diphenyloxazole
r	Relative increase in cell volume
RAMONA	Radio-isotope flow detector (Raytest Instruments)
R_f	The distance migrated by a substance during paper chromatograph or thin layer chromatography, relative to the solvent front
Rha	Rhamnose
SCV	Settled cell volume
	[= (volume of cells/3 ml) x 33.33]
TCA	Trichloroacetic acid
TFA	trifluoroacetic acid
t.l.c.	Thin layer chromatography
UA	Uronic acids
UDP	Uridine diphosphate
XET	Xyloglucan endotransglycosylase
XG5	Xyloglucan pentasaccharide (Fig. 1.4.1)
XG7	Xyloglucan heptasaccharide (Fig. 1.3.1)
XG7f	Fucosylated xyloglucan heptasaccharide (Fig. 4.3.1)
XG8	Xyloglucan octasaccharide (Fig. 1.3.1)
XG9	Xyloglucan nonasaccharide (Fig. 1.3.1)
XG9n	non-fucosylated xyloglucan nonasaccaride (Fig. 1.4.2)
XG10	Xyloglucan decasaccharide (Fig. 1.3.1)
Xyl	Xylose
Y	Yield threshold
ϕ	Wall extensibility
π	Osmotic potential
ψ	Water potential

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Abstract

Xyloglucan is thought to form cross-links between cellulose microfibrils within the plant cell wall. The hydrolysis of these 'tethers' may be involved in wall loosening, and a control of cell expansion. Transglycosylation reactions between xyloglucan 'tethers' has for some years been suggested as an alternative mechanism to hydrolysis by which the plant cell wall may be loosened. The work described in this thesis is an investigation of this hypothesis.

Spinach (*Spinacia oleracea* L.) cell suspension cultures were incubated with [*xylosyl*-^{3}H]xyloglucan nonasaccharide (XG9; $Glc_4.Xyl_3$.Gal.Fuc) and [*reducing terminus*-^{3}H]XG9. The majority of ^{3}H-labelled material remained soluble and extracellular (96-98%), and 53-57% of it underwent an apparent increase in molecular weight as shown by gel permeation chromatography. This result could suggest the occurrence of a transglycosylation reaction in the apoplast. A proportion of XG9 was hydrolysed to low molecular weight ^{3}H-labelled products.

[^{3}H]XG9 was found to have undergone no appreciable re-arrangement on incorporation into the high molecular weight product as complete acid hydrolysis and Driselase digestion released similar ^{3}H-labelled hydrolysis products from the putative transglycosylation product, as from [^{3}H]XG9. The reducing terminus of the oligosaccharide remained as a reducing terminus of the high molecular weight product. This was shown by sodium borohydride reduction of the product formed during incubation of cells with [*reducing terminus*-^{3}H]XG9. This would suggest that XG9 acts as the acceptor substrate, an apoplastic donor being cleaved and the newly formed potentially reducing terminus of this polymer being transferred onto XG9.

Cellulase digestion of the transglycosylation products apparently released [^{3}H]XG9, suggesting the oligosaccharide-polymer association was via a cellulase-labile bond. The formation of a cellulase-labile bond indicates that the soluble polymeric donor substrate may be xyloglucan. Evidence for the involvement of xyloglucan in this reaction is that the product was found to bind to cellulose (as does xyloglucan) and was removed by 1 M-sodium hydroxide. However, the product was not precipitable with either iodine or copper unlike some xyloglucans. Possibly this was due to the low molecular weight of the product, which was estimated by gel permeation chromatography to be 20-30 kDa. XG9 can be considered to be a low molecular weight xyloglucan. The endogenous acceptor substrate to this reaction may

therefore be cell wall bound xyloglucan.

Xyloglucan endo-transglycosylation was detected in suspension cultures derived from other dicotyledonous plants (*Rosa*, *Acer*, *Daucus* and *Capsicum*) and some Graminaceous plants (*Festuca* and *Zea*). It would therefore appear that this activity may be ubiquitous in plants.

The proposed role of apoplastic transglycosylation in cell expansion was also investigated by monitoring xyloglucan endo-transglycosylation in the apoplast of suspension-cultured spinach cells throughout the growth cycle. During the initial rapid growth of the culture, elevated transglycosylase activities were found to be associated with the cells (removed from their spent medium and washed prior to incubation with XG9). This finding suggests that xyloglucan endo-transglycosylation activity may be associated with cell expansion. However, from the data collected it is not possible to determine whether the role of this activity is in wall loosening or the synthesis of new xyloglucan tethers. Activity was also detected in the spent medium, particularly of older, slower growing cultures. I propose that the physiologically important activity is that catalysed by enzyme molecules in transit through the cell wall, following secretion from the cell.

1. INTRODUCTION

1.1 The expansion of plant cells

The growth and development of higher plants involves the processes of cell division, cell expansion and differentiation. The meristems of higher plants (apical, lateral or intercalary) produce new cells by the process of mitosis (Esau, 1965). Cell division alone does not lead to an irreversible increase in volume of the tissue (i.e. growth), although this process is usually accompanied by some cell expansion (Brown, 1976). The greatest increase in cell volume occurs once the cell has left the meristematic region of the plant (or once division has ceased, in the case of determinate meristems) (Esau, 1965). For example, the diameter of the isodiametric cells in the root apical meristem is typically 10 μm. Once these cells have left the meristematic region of the root, they may expand to an average length of 150 μm and an average diameter of 20 μm (Salisbury and Ross, 1978), which is a 90-fold increase in cell volume. This expansion involves the uptake of water into vacuoles which coalesce as the cell matures.

Plant cells are surrounded by a wall which plays a structural role, determining cell shape. An increase in cell volume is associated with an increase in cell wall area. For example, a 90-fold increase in volume of a root cell is associated with an approximately 32-fold increase in cell wall area. It is thought that the plant cell wall controls the extent, direction and cessation of plant cell expansion.

1.2 The physical properties of the plant cell wall control cell expansion

Cell expansion occurs by the action of turgor pressure on the plant cell wall (Cleland, 1971; Taiz, 1984; Cosgrove, 1986). Turgor pressure in a plant cell is generated as water passes through the plasma membrane (a semipermeable membrane), down the gradient in water potential formed by the accumulation of solutes within the protoplast. Since the plant cell is surrounded by a cell wall, the entry of water into the protoplast will generate a hydrostatic pressure or turgor pressure (Husken *et al.*, 1978; Zimmermann *et al*, 1978). The magnitude of the cell's turgor pressure (P) is determined by the difference between osmotic potential inside the symplast and that in the apoplast, assuming that the hydraulic conductivity is not limiting, which it may be in some plant tissues (Boyer, 1985; Tomos, 1985, 1989).

At steady-state (i.e. no net water movement into or out of the cell), the water potential (ψ) outside the protoplast (ψ_o = osmotic potential ouside the protoplast, π_o) is equal to that inside the protoplast ($\psi_i = P-\pi_i$) i.e. $\psi_o = \psi_i$.

In a turgid cell, the load-bearing parts of the wall are under tension (due to the extension of elastic elements within the wall). Relaxation of wall tension by

biochemical wall loosening, results in a decrease in turgor pressure. Water will enter the cell down the water potential gradient and the cell will expand (Tomos, 1985, 1989; Cosgrove, 1986, 1987). This increase in cell volume results in the dilution of cell solutes, which can be balanced by solute assimilation.

The process of cell expansion can be thought of within the framework of the Lochart equation (Lochart, 1965):

$r = \phi(P-Y)$ where r = increase in cell volume relative to initial volume, ϕ = wall extensibility, Y = yield threshold of the wall

Wall loosening may involve a decrease in yield threshold and/or an increase in wall extensibility (Taylor and Davies, 1986; Smit *et al.*, 1989; Pritchard *et al.*, 1990, 1991). The biochemical bases of these parameters have not yet been demonstrated. Consideration should be given to the molecular architecture of the primary cell wall and the load-bearing bonds within this structure.

1.3 The molecular structure of the primary cell wall

The primary plant cell wall is a biphasic structure composed of a relatively inextensible network of insoluble cellulose microfibrils embedded in a gel-like matrix of polysaccharides and glycoproteins (Northcote, 1972; Preston, 1979; McNeil *et al.*, 1984; Fry, 1986a). Cellulose molecules (straight chained β-(1-4)-D-glucan) are present throughout the primary cell wall as crystalline aggregates, or microfibrils (Atalla and van der Hart, 1984). Microfibrils are made up of 60-70 glucan chains, have a diameter of 4.5-8.5 nm and are separated from one another by approximately 20-40 nm (Monro *et al.*, 1976; McCann *et al.*, 1990). During cell expansion, cellulose microfibrils do not appear to undergo appreciable hydrolysis (Maclachlan, 1977), but move apart or past one another (Preston, 1982). The orientation of cellulose microfibrils within the primary cell wall is thought to determine the direction in which the cell will expand (Green, 1980; Preston, 1982). The processes contributing to wall loosening are believed to involve the cleavage of bonds restricting the movement of microfibrils relative to one another. Therefore, a consideration of the molecular structure of the primary cell wall is necessary.

Many models of the molecular structure of the plant primary cell wall have been proposed. In 1973, Albersheim and co-workers published a model of the walls of suspension cultured sycamore cells (Keegstra *et al.*, 1973; Talmadge *et al.*, 1973). In this model xyloglucan (section 1.3.1) molecules are hydrogen bonded to cellulose microfibrils. Pectins (i.e. polysaccharides with an α-(1-4)-linked galacturonic acid

backbone containing α-linked rhamnose residues; for review, Jarvis, 1984) are covalently linked to the hydrogen bonded xyloglucan so forming cross-links between microfibrils. There is some evidence that xyloglucan-pectin linkages do exist in the primary cell wall (Bauer *et al.*, 1973; Stevens and Selvendran, 1984a,b; Chambat *et al.*, 1984; O'Neill and Selvendran, 1985a; Redgwell and Selvendran, 1986; du Pont and Selvendran, 1987); however an insufficient number of such bonds appear to be present to support Albersheim's model (Monro *et al.*, 1976; Darvill *et al.*, 1980; McNeil *et al.*, 1984), which demands that pectin is covalently linked to xyloglucan.

A second model is that proposed by Lamport and Epstein (1983), in which cellulose and extensin (a hydroxyproline-rich glycoprotein; Darvill *et al.*, 1980) make up a concatenated network suspended in a gel of pectins and hemicellulose. This 'warp-weft' structure is stabilised by isodityrosine linkages between extensin molecules producing loops through which cellulose microfibrils run. Isodityrosine (a specific dimer of tyrosine) has been found in the plant cell wall (Fry, 1982a), but it is still not clear if such linkages form intermolecular cross-links.

Perhaps the most widely accepted model of the primary cell wall of dicotyledonous plants is that in which xyloglucan molecules form the cross-links between cellulose microfibrils (Hayashi and Maclachlan, 1984a; Hayashi *et al.*, 1987; Fry, 1989a,b; Hayashi, 1989; McCann *et al.*, 1990). Xyloglucan is known to hydrogen bond to cellulose *in vitro* (Bauer *et al.*, 1973; Chambat *et al.*, 1984; Hayashi *et al.*, 1987) and it is believed to bind to the cellulose of the cell wall in a similar manner (Monro *et al.*, 1976; Chambat *et al.*, 1984; Hayashi and Maclachlan, 1984a; Hayashi *et al.*, 1987; Levy *et al.*, 1991). Hydrogen bonds are thought to be formed between the β-(1-4)-linked glucan backbone of xyloglucan and cellulose (Hayashi *et al.*, 1987; Levy *et al.*, 1991). Xyloglucan is unable to bind to other xyloglucan molecules in this manner and so forms a monolayer covering the microfibrils. The cell wall of dicotyledonous plants contains too much xyloglucan for all of it to be bound directly to cellulose (Hayashi *et al.*, 1987). It has been proposed that one xyloglucan molecule may bind to one, two or more microfibrils along its length, and so form 'tethers' between neighbouring microfibrils (Hayashi and Maclachlan, 1984a; Hayashi *et al.*, 1987; Fry, 1989a,b; Hayashi, 1989; McCann *et al.*, 1990). A typical xyloglucan molecule has a length of between 150-1500 nm (for review Fry, 1989b; McCann *et al.*, 1990) which is long enough to span the distance between several cellulose microfibrils (estimated to be 20-40 nm; McCann *et al.*, 1990). It is proposed that xyloglucan forms load-bearing 'tethers' between microfibrils, with other wall polysaccharides and glycoproteins forming a matrix around this structure, stabilising the 'tethers' by preventing slippage of hydrogen bonds (Fry, 1986a, 1989a).

Xyloglucan can be extracted from the cell wall with concentrated alkali (e.g. 6 M-sodium hydroxide: Hayashi *et al.*, 1980; Jarvis *et al.*, 1981; Carpita, 1984) but is not much solubilised by mild chaotropic agents (e.g. 8 M-urea or 4.5-6.0 M-guanidinium thiocyanate: Monro *et al.*, 1976; Edelmann and Fry, 1992). Alkali treatment has been shown to cause swelling of cellulose (Ring and Selvendran, 1981; Edelmann and Fry, 1992) which is believed to increase the accessibility of xyloglucan to the extractant. Alkali treatment (i.e. 6 M-sodium hydroxide extraction carried out for 1 hour at room temperature) has been shown to cause an incomplete extraction of xyloglucan (Chambat *et al.*, 1981; Shedletzky *et al.*, 1990; Edelmann and Fry, 1992). De-pectination of the walls prior to alkali treatment has been reported to enhance the efficiency of extraction of xyloglucan (Edelmann and Fry, 1992). Evidence for the existence of glycosidic linkages between xyloglucan and cell wall pectin has been presented. The putative xyloglucan-pectin association has been proposed to be formed over several hours following xyloglucan secretion into the wall (Edelmann and Fry, 1992). Other candidates for xyloglucan cross-linking to the cell wall are ester bonds (Brown and Fry, 1992) and diferulic acid bridges between polysaccharide bound ferulic acid (Markwalder and Neukom, 1976; Hartley and Jones, 1976; Shibuya, 1984). However, such ester linkages could not contribute to alkali resistance of wall bound xyloglucan.

1.3.1 The structure of xyloglucan

Xyloglucan is a major component of the primary cell walls of dicotyledonous plants (20-25% of the dry weight of the wall; McNeil *et al.*, 1984; Fry, 1989b) and is also present to a lesser extent in the wall graminaceous plants (Kato and Matsuda, 1985). Xyloglucan has been found to be present throughout the primary cell wall (Hayashi and Maclachlan, 1984a; Moore *et al.*, 1986). In this section the chemical structure of this polysaccharide will be outlined.

Xyloglucan is a rod-shaped molecule (O'Neill and Selvendran, 1983) with a length of typically between 0.15 and 1.5 μm (for review, Fry, 1989b). The xyloglucan molecule consists of a backbone of β-(1-4)-linked D-glucopyranose (Aspinall *et al.*, 1969), with a chain length of between 200 and 3000 residues (Hayashi *et al.*, 1980; Nishitani and Matsuda, 1982, 1983; for review Fry, 1989b). This backbone is substituted with α-D-xylopyranose (60-75% of the glucose residues of xyloglucan in the dicotyledonous cell wall are xylosylated) linked through *O*-6 (Bauer *et al.*, 1973; Kato and Matsuda, 1976; Hayashi *et al.*, 1980; Joseleau and Chambat, 1984). The distribution of xylose residues is such that the backbone is usually made up of sequences of three consecutive substituted glucose residues followed by an

unsubstituted glucose residue (Bauer *et al.*, 1973; Kato and Matsuda, 1980a; Hayashi and Maclachlan, 1984a). Further substitution of the α-xylosyl side groups (about 20-25%) is found in xyloglucan derived from dicotyledonous plant cell walls (Bauer *et al.*, 1973; Kato and Matsuda, 1980ab; Valent *et al.*, 1980; O'Neill and Selvendran, 1983, 1985b; Bacic *et al.*, 1988). The β-glucan backbone may carry the following *O*-6 linked side chains;

(1). L-Fuc*p*-α-(1-2)-D-Gal*p*-β-(1-2)-D-Xyl*p*-α-(1-

(2). D-Gal*p*-β-(1-2)-D-Xyl*p*-α-(1-

(3). L-Ara*f*-?-(1-2)-D-Xyl*p*-β-(1- (e.g. Akiyama and Kato, 1982)

(4). D-Xyl*p*-α-(1-

Xyloglucan is susceptible to cellulase (endo-β-(1-4)-D-glucanase) hydrolysis at unsubstituted glucosyl units along the backbone (Wong *et al.*, 1977; Hayashi and Maclachlan, 1984a; Hayashi *et al.*, 1984). Complete cellulase digestion of cell wall xyloglucan derived from dicotyledonous plants therefore releases oligosaccharides by cleavage of approximately each fourth glucose residue. The major oligosaccharides released are XG7, XG8, XG9 and XG10 (i.e. hepta-, octa-, nona-, and decasaccharides of xyloglucan: Fig. 1.3.1: Bauer *et al.*, 1973; Hayashi *et al.*, 1980; Kato and Matsuda, 1980a; Valent *et al.*, 1980; Hayashi and Maclachlan, 1984a; Kato *et al.*, 1985; Matsushita *et al.*, 1985). These findings suggest that xyloglucan is composed of a series of linked repeating sequences.

Xyloglucan derived from Graminaceous plants has a similar structure to that described above, but contains fewer α-xylosyl side groups (Shibuya and Misaki, 1978; Kato *et al.*, 1981), and is very much less fucosylated (Kato and Matsuda, 1985). Cellulase treatment of xyloglucan derived from the cell walls of Graminaceous plants yields mostly hepta- and pentasaccharides, cellobiose and monomeric glucose (Labavitch and Ray, 1978; Kato *et al.*, 1981, 1982) suggesting that the repeating sequences of this xyloglucan differ from those found in dicotyledonous xyloglucan.

A third family of xyloglucans are the seed storage amyloids, for example those found in tamarind (Kooiman, 1959) and nasturtium (le Dizet, 1972). These non-cell wall xyloglucans (Reis *et al.*, 1987) are non-fucosylated and in some cases contain a ratio of Glc:Xyl-Glc:Gal-Xyl-Glc of 1:1:1 (Kooiman, 1961; le Dizet, 1972).

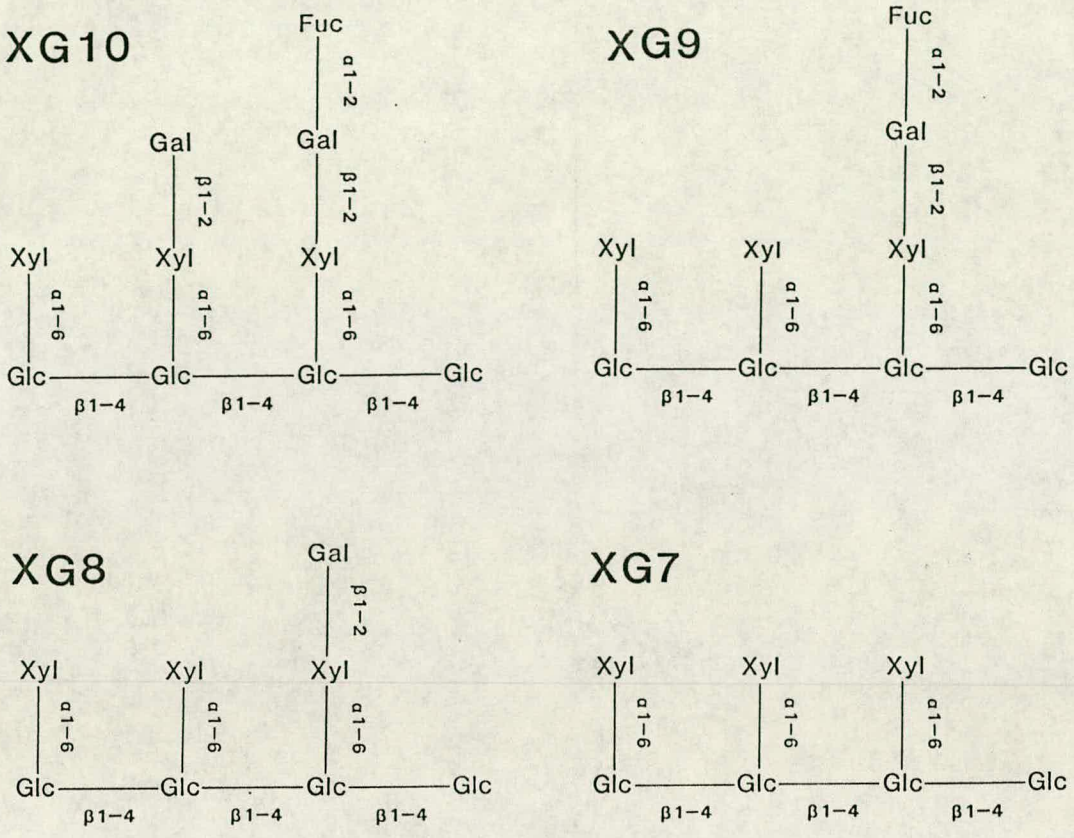


Fig. 1.3.1 The oligosaccharides released by cellulase digestion of xyloglucan derived from the cell walls of dicotyledonous plants.

1.4 Xyloglucan and cell expansion

If, as it has been proposed in section 1.3, xyloglucan molecules form 'tethers' between cellulose microfibrils, xyloglucan may contribute to the mechanical properties of the cell wall and so be involved in the biochemical processes of wall loosening and cell expansion (Cleland, 1981; Taiz, 1984; Fry, 1989a; Hayashi, 1989). Much evidence has been published suggesting that cell wall xyloglucan is subject to hydrolysis during auxin-stimulated (and acid-induced) growth of plant tissues (for reviews Hayashi, 1989; Fry, 1989a,b). A proportion of cell wall xyloglucan has been

shown to be solubilised from the wall in the presence of indole acetic acid (IAA; an auxin) (Labavitch and Ray, 1974; Terry *et al.*, 1981) and at low pH (Jacobs and Ray, 1975). It has been suggested that this 'solubilisation' of previously wall-bound, alkali extractable xyloglucan is due to cleavage of insoluble xyloglucan molecules so releasing non-hydrogen-bonded fragments into solution in the apoplast. The molecular weight of alkali-extractable xyloglucan has been found to decrease during auxin or acid treatment of tissues (Nishitani and Masuda, 1982, 1983; Inohue *et al*,. 1984; Lorences and Zarra, 1987; Arribas *et al.*, 1991; Wakabayashi *et al.*, 1991). These findings provide further evidence that hydrolysis of xyloglucan molecules occurs during cell expansion. It has also been reported that a small proportion of wall-bound xyloglucan undergoes hydrolysis to dialysable products during auxin-induced cell expansion (Labavitch and Ray, 1974; Gilkes and Hall, 1977), although the role of this more complete hydrolysis of xyloglucan in wall loosening is not clear. Further evidence for the role of xyloglucan hydrolysis in cell expansion is the finding that antibodies raised against cell wall xyloglucan and fucose-binding lectins inhibit auxin-stimulated cell expansion when applied to plant tissue (Hoson and Masuda, 1991; Hoson *et al.*, 1991).

Cellulase has been found to be present in the apoplast of plant cells (Byrne *et al.*, 1975; Bal *et al.*, 1976; Wong *et al.*, 1977; Hatfield and Nevins, 1987). Some plant cellulases cleave the backbone of xyloglucan molecules (Wong *et al.*, 1977; Hayashi and Maclachlan, 1984; Hayashi *et al.*, 1984a) and could therefore be responsible for the changes in cell wall xyloglucans which have been detected in the expanding plant cell (Fan and Maclachlan, 1966; Hayashi *et al.*, 1984; Fry, 1989). Cellulase has been found to be synthesised *de novo* during auxin treatment of plant tissues (Verma *et al.*, 1975). The activity of cellulase has also been found to be enhanced at low pH (Hayashi *et al.*, 1984), the pH optimum for cellulase being 5.5-6.0 (Byrne *et al.*, 1975).

Other enzymes which exhibit hydrolytic activity towards xyloglucan are also present in the apoplast of plant cells, e.g. α-D-xylosidase, β-D-galactosidase and α-L-fucosidase (Koyama *et al.*, 1981, 1983; O'Neill *et al.*, 1988). As none of these activities results in the cleavage of the backbone of xyloglucan, their role in cell wall loosening is not clear. However, increases in the activities of α-xylosidase and β-galactosidase have been detected in response to auxin (O'Neill *et al.*, 1988).

1.4.1 The effects of xyloglucan oligosaccharides on plant cell expansion

Specific xyloglucan oligosaccharides, produced by cellulase digestion of xyloglucan (section 1.3.1), have been found to act as 'anti-auxins' and 'auxin-mimics',

depending on the concentration at which they are applied to plant tissue and their structure (for review Fry *et al.*, 1990). Oligosaccharides exhibiting a biological activity, other than the supply of a metabolisable carbon source, are known as oligosaccharins (McNeil *et al.*, 1984; Thran Thanh Van *et al.*, 1985).

1.4.1.1 Xyloglucan oligosaccharides as 'anti-auxins'

The 2,4-dichlorophenoxyacetic acid- (2,4-D-) stimulated elongation observed in pea stem segments has been reported to be inhibited in the presence of XG9 applied at a concentration of approximately 1-10 nM (York *et al.*, 1984; McDougall and Fry, 1988). Acid-induced cell expansion has also been found to be inhibited at similar concentrations of this oligosaccharide (Lorences and Fry, 1989). This inhibition of auxin-stimulated cell expansion by XG9 has been shown not to be due to cytotoxicity of the oligosaccharide (York *et al.*, 1984; McDougall and Fry, 1989a). The α-linked fucosyl unit of XG9 is believed to be a structural requirement for the oligosaccharide to act as an 'anti-auxin' (McDougall and Fry, 1989b). For example; XG8 does not have an inhibitory effect on 2,4-D-stimulated growth (McDougall and Fry, 1989a), whereas XG5 (Fig. 1.4.1) and the trisaccharide L-fucopyranosyl-α-(1-2)-D-galactopyranosyl-β-(1-4)-D-glucose (purified from human milk) both exhibit this inhibitory activity (McDougall and Fry, 1989b).

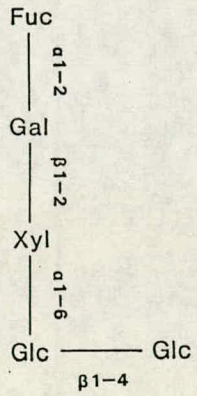


Fig. 1.4.1 The structure of xyloglucan pentosaccharide (XG5), an 'anti-auxin'.

It has been suggested that XG9 may be a natural 'anti-auxin', and indeed this putative oligosaccharin has been found to be present in the apoplast of cultured plant cells (Fry, 1986b). However, the concentration of apoplastic XG9 has been estimated to be approximately 400 nM (Fry, 1986b), which is considerably higher than the 'anti-auxin' concentration determined in pea stem straight-growth bioassays (York *et al.*, 1984; McDougall and Fry, 1989a).

1.4.1.2 Xyloglucan oligosaccharides as 'auxin-mimics'

Certain xyloglucan oligosaccharides, at concentrations of 1-100 μM, have been shown to stimulate 'cellulase' activity in a viscometric assay using xyloglucan as the viscous substrate (Farkas and Maclachlan, 1988; McDougall and Fry, 1990). However, this stimulation in 'cellulase' activity is not detected if carboxymethylcellulose (CMC) is used as the substrate (Farkas and Maclachlan, 1986; McDougall and Fry, 1990). The same oligosaccharides, at a concentration of 1 μM, have also been reported to promote cell expansion in pea stem straight-growth bioassays (McDougall and Fry, 1990).

The oligosaccharides found to stimulate 'cellulase' activity and to promote cell elongation are XG9n (non-fucosylated nonasaccharide derived from nasturtium seed xyloglucan by cellulase hydrolysis Fig. 1.4.2), XG8, XG7, and XG9 (these oligosaccharides are listed in order of effectiveness as promoters of cellulase and of cell expansion, XG9n being the most effective: McDougall and Fry, 1990). As the specificity of oligosaccharide action is similar in both 'cellulase' promotion and cell expansion stimulation, it seems likely that both effects share a common mechanism. The effects of these oligosaccharides are thought to be stoichiometric rather than regulatory, and hence they are not strictly auxin-mimics.

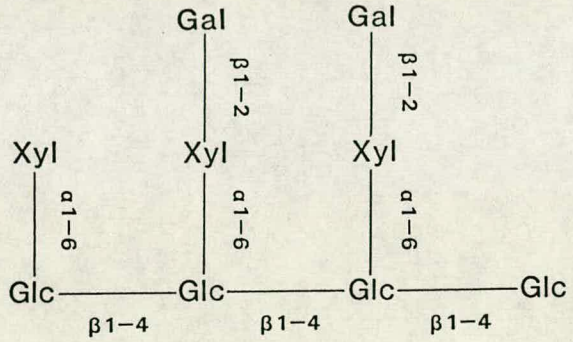


Fig. 1.4.2 The structure of non-fucosylated xyloglucan nonasaccharide (derived from nasturtium xyloglucan), an 'auxin-mimic'

Presumably the mechanisms by which xyloglucan oligosaccharides promote cell expansion or inhibit 2,4-D-stimulated cell expansion are different. The bases of these oligosaccharin activities of xyloglucan oligosaccharides are unknown.

1.5 Transglycosylation

In an enzyme catalysed transglycosylation reaction a glycosyl moiety is

transferred from a donor molecule onto an acceptor molecule, typically a hydroxylic organic compound (e.g. a sugar) by the enzyme (Nomenclature Commitee of I.U.B., 1984). The probable mechanism of such a reaction, catalysed by a transglycosylase, is given below.

1). Glycosyl-O-R + Enzyme-H ⟷ Glycosyl-Enzyme + ROH
(DONOR) *(INTERMEDIATE)* *(PRODUCT)*

2). Glycosyl-Enzyme + R'OH ⟷ Glycosyl-O-R' + Enzyme-H
(INTERMEDIATE) *(ACCEPTOR)* *(PRODUCT)*

(Doudoroff *et al.*, 1947; Edelman, 1956)

In enzyme-catalysed transglycosylation the donor substrate is cleaved, either mid-chain (endo-transglycosylation) or at a non-reducing terminus (exo-transglycosylation) by the transglycosylase, and the newly formed, potentially reducing terminus is transferred probably via a glycosyl-enzyme intermediate (Voet and Abeles, 1970), onto a glycosyl acceptor. The size of the glycosyl moiety which is transferred is dependent on which bond is cleaved in the donor substrate; exo-transglycosylation results in the transfer of a single glycosyl unit, whereas a larger moiety is transferred during endo-transglycosylation.

Synthetic processes within the protoplasm of plant cells often involve transglycosylation reactions. The glycosyl donors to these reactions are often sugar nucleotides (for reviews; Nikaido and Hassid, 1971; Feingold and Avigad, 1980; Watkins, 1986). The free energy of hydolysis of a sugar nucleotide (e.g. uridine-diphosphate- (UDP-) glucose) is typically 30.5 $kJ.mol^{-1}$, whereas the free energy of hydrolysis of a typical glycosyl-glycosyl linkage is approximately 8.4-21.0 $kJ.mol^{-1}$ (Feingold and Avigad, 1980). Transglycosylation reactions involving the transfer of a glycosyl moiety from a sugar nucleotide donor to a sugar acceptor therefore typically favour synthesis of a glycosidic linkage. Examples of such protoplasmic reactions occurring in plants include: sucrose-phosphate synthesis catalysed by sucrose-phosphate synthase (Mendicino, 1960); synthesis of xyloglucan backbone is thought to involve the co-operative action of UDP-glucose: β-(1-4)-D-glucan 4-β-D-glucosyl transferase and a UDP-xylose: β-(1-4)-D-glucan 6-α-D-xylosyltransferase (Hayashi and Matsuda, 1981abc; Cambell *et al.*, 1988); the fucosylation of xyloglucan by GDP-fucose: xyloglucan 2-α-L-fucosyltransferase (Hanna *et al.*, 1991); the synthesis of the α-(1-4)linked glucan backbone of starch by ADP-glucose: α-(1-4)-D-glucan 4-α-D-glucosyltransferase (for reveiw; Beck and Zeigler, 1989).

Alternatively, transglycosylation may involve the cleavage of a glycosidic bond within an oligosaccharide, polysaccharide or glycoprotein, transfer of a glycosyl moiety and the synthesis of a new glycosidic bond with a sugar acceptor. Examples of such reactions are given in sections 1.5.1 and 1.5.2.

In many transglycosylation reactions the number of reducing groups in the substrates is identical to that in the products, and the mean molecular weight of the products is equal to that of the substrates. As a transglycosylation reaction may result in no net chemical change, the reaction can be difficult to detect *in vivo*. However, many hydrolases have been shown to exhibit transglycosylase activity *in vitro*.

1.5.1 Many hydrolases exhibit transglycosylase activity *in vitro*

The mechanism of transglycosylation is closely related to that of hydrolysis, the difference being the nature of the glycosyl acceptor (Sharon and Flowers, 1979). In a hydrolysis reaction the acceptor substrate is water.

1).Glycosyl-O-R + Enzyme-H ⟷ Glycosyl-Enzyme + ROH
(*DONOR*) (*INTERMEDIATE*)(*PRODUCT*)

2).Glycosyl-Enzyme + HOH ⟷ Glycosyl-O-H + Enzyme-H
(*INTERMEDIATE*) (*ACCEPTOR*) (*PRODUCT*)

(Evidence for a glycosyl-enzyme intermediate in a glycosyl transfer reaction was reported by Lai *et al.*, 1974.)

Hydrolysis therefore results in the cleavage of a glycosidic bond, and an increase in the number of reducing ends in the products relative to the substrate. Given the similarity in mechanism, it is perhaps not surprising that many plant hydrolases (mostly glycosidases) have been shown to exhibit transglycosylase activity, *in vitro* (first shown by Bacon and Edelman, 1950; Blachard and Albon, 1950). For example, glucosidases (Barker and Bourne, 1952; Crook and Stone, 1953; Barker *et al.*, 1955; Yamamoto and Nevins, 1979; Tanaka *et al.*, 1982; Kaushal *et al.*, 1989), galactosidases (Pazur, 1954; Dey and Pridham, 1972; Dey, 1979), and fructosidases (Carnie and Porteous, 1963; Cooper and Greenshields, 1964) will catalyse exotransglycosylation reactions provided sufficient concentrations of an appropriate acceptor substrate are available.

It has been claimed that an extracellular dextranase will catalyse an endo-transglycosylation reaction *in vitro* (Heyn, 1981). However, the role of such an enzyme in the plant cell wall is unclear, as no dextran is present in the plant cell wall.

1.5.2 Intracellular transglycosylation reactions associated with starch metabolism in plants

Starch metabolism in plants, bacteria and fungi is believed to involve a number of transglycosylation reactions (for reveiw, Amylase Research Society of Japan, 1988). The α-(1-4)-glucan backbone is thought to be synthesised by the action of ADP-glucose: α-(1-4)-D-glucan 4-α-D-glucosyltransferase (for reveiw; Beck and Ziegler, 1989). Other transglycosylase enzymes involved in starch metabolism do not have sugar nucleotide donor substrates. For example, the starch branching enzyme or Q-enzyme (1,4-α-D-glucan: α-(1-4,1-6)-D-glucan 6-α-D-glucanotransferase, E.C. 2.4.1.18) catalyses an endo-transglycosylation reaction in which an α-(1-4)-glucosidic linkage (e.g. in amylose) is split and the 'released' segment is transferred onto an α-(1-4)-linked glucan via an α-(1-6) bond (Barker *et al.*, 1950). The activity of a plant Q-enzyme was first demonstrated in potato (Haworth *et al.*, 1944), but has since been reported in many starch synthesising plant tissues (Manners *et al.*, 1968; Hawker *et al.*, 1974). The usual donor substrate to this reaction is amylose, the reaction resulting in the formation of a highly branched amylopectin molecule (Kitahata and Okada, 1988). The free energy of hydrolysis of an α-(1-4)-glucosyl bond is greater than that for the hydrolysis of an α-(1-6)-glucosyl bond, and therefore branching activity is favoured rather than the reverse reaction.

The starch disproportionating enzyme or D-enzyme (α-(1-4)-D-glucan: α-(1-4)-D-glucan 4-α-glucanosyltransferase, E.C. 2.4.1.25) is another endo-transglycosylase involved in starch metabolism in plants (Peat *et al.*, 1956; Manners and Rowe, 1969; Lin and Preiss, 1988). This enzyme catalyses the cleavage of an α-(1-4) bond, splitting out a maltosyl or larger segment from an α-(1-4)-glucan chain (the donor substrate) and transfers this moiety onto an acceptor via an α-(1-4) linkage (Walker and Whelan, 1957). The enzyme will cleave an α-(1-4) bond within the substrate molecule apart from the linkage at the non-reducing end of the donor and the penultimate bond to the reducing end (Jones and Whelan, 1969). In this reaction, catalysed by the D-enzyme, neither the forward nor reverse reactions are energetically favoured, as the free energy of hydrolysis of both the bond formed and broken is equal. The role of this activity in starch metabolism is unknown, although it has been suggested that such activity may synthesise primers and chains to be transferred by the Q-enzyme (Maeda, 1988).

1.5.3 A putative transglycosylation activity has been detected in the apoplast of plant cell suspension cultures

In 1989, Baydoun and Fry reported the occurrence of a putative apoplastic transglycosylation reaction in spinach (*Spinacia oleracea* L.) cell suspension cultures. This may be the first report of an apoplastic transglycosylation activity detected *in vivo*. A transglycosylase activity, apparently present in the apoplast, has been reported in wounded soybean suspension cultures (Brett, 1978). Exogenous UDP-glucose was incorporated into a β-(1-3)-glucan (i.e. callose); synthesis of this polysaccharide being a common wound response in plants (Currier, 1957). It has been suggested that this synthetase activity may not be truly apoplastic, but rather rupture of the plasma membrane may be necessary for the substrate (UDP-glucose) to become available to the enzyme involved (the plasma membrane usually being impermeable to sugar nucleotides).

When spinach cell suspension cultures were incubated with [*xylosyl*-^{3}H]xyloglucan nonasaccharide (XG9; Fig. 1.3.1) or [*fucosyl*-^{3}H]XG9, a high molecular weight ^{3}H-labelled product was formed in the apoplast of the culture. This reaction did not appear to involve any significant breakdown or re-arrangement of the [^{3}H]oligosaccharide, but rather may have been the incorporation of [^{3}H]XG9 into a soluble extracellular polymer, possibly by a transglycosylation reaction. Two possible mechanisms for this detected reaction have been proposed (Fig. 1.5.1). These mechanisms differ in the nature of the glycosyl donor and acceptor substrates.

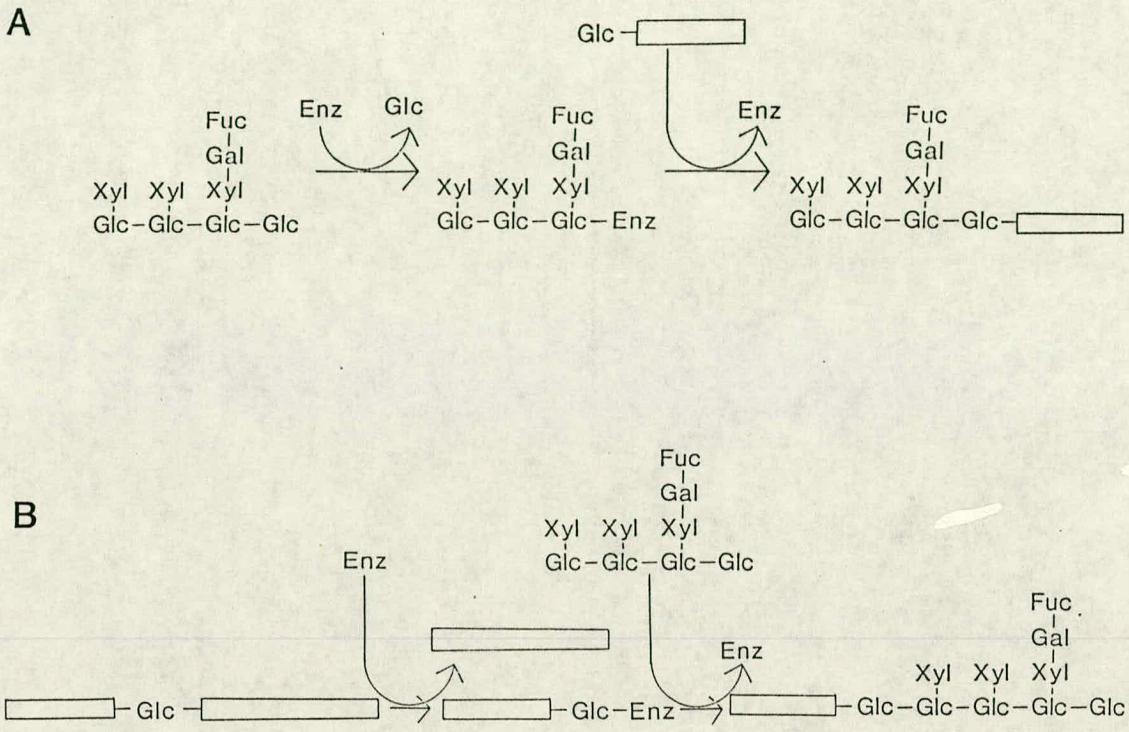


Fig. 1.5.1 Two mechanisms by which [^{3}H]XG9 may be incorporated into an apoplastic polymer (Baydoun and Fry, 1989).

A. XG9 (the donor substrate) is cleaved adjacent to its reducing terminus, releasing monomeric glucose. The newly formed, potentially reducing terminus of the donor is transferred onto a soluble, extracellular polymer, possibly xyloglucan (the acceptor substrate).

B. A soluble extracellular polymer, possibly xyloglucan (the donor substrate), is cleaved mid-chain, releasing a fragment of the polymer. The newly formed, potentially reducing terminus of the polymer is transferred onto the non-reducing terminus of XG9 (the acceptor substrate). This reaction involves the transfer of a large glycosyl moiety.

1.6 A possible role for apoplast xyloglucan transglycosylation in cell expansion

Cell wall loosening is believed to involve the hydrolysis of load-bearing bonds within the wall (section 1.4). However, continued bond cleavage would result in the loss of the physical integrity of the wall (Albersheim, 1976; Cleland, 1981). Therefore, during prolonged periods of cell expansion, cleaved bonds must be replaced by the synthesis of new load-bearing bonds. *De novo* synthesis of polysaccharides (particularly xyloglucan) and their deposition into the wall have been shown to increase during periods of cell expansion (Baker and Ray, 1965ab; Abdul-Baki and Ray, 1971; Shore and Maclachlan, 1973; Takeuchi and Komamine, 1980; Hayashi and Maclachlan, 1984b; Brummell and Hall, 1983, 1985; Kutschera and Briggs, 1987). However, transglycosylation of cell wall polysaccharides could provide a mechanism whereby load bearing bonds within the cell wall would be broken for a limited period, allowing for incremental turgor-driven expansion of the cell, prior to re-synthesis of the bonds (Albersheim, 1976; Cleland, 1981; Taiz, 1984). Such a mechanism of wall loosening has been given the name 'chemical creep' (Cleland and Rayle, 1972).

The evidence presented in section 1.3 suggests that xyloglucan may form load-bearing cross-links between microfibrils within the wall. Transglycosylation reactions between cell wall bound xyloglucan molecules may therefore be involved in the processes of cell wall loosening. Evidence of a transglycosylation reaction possibly involving xyloglucan as one (or maybe both) of the substrates has been discussed in section 1.5.3. In order for a transglycosylation reaction between xyloglucan molecules within the wall to cause any appreciable change in the mechanical properties of the wall, and so contribute to cell wall loosening, the enzyme involved should be endo-acting, catalysing mid-chain cleavage of xyloglucan tethers. The transfer of terminal sugar residues from one molecule to another (e.g. an exo-transglycosylation reaction) is unlikely to produce any appreciable effect on the wall's mechanical properties. It is therefore important that the mechanism of the putative transglycosylation reaction, detected in the apoplast of spinach suspension cultures, be elucidated.

1.7 Aims and objectives

The aim of this work is to investigate the biochemical mechanisms involved in the process of cell wall loosening during plant cell expansion.

It has been suggested that apoplastic transglycosylation reactions may be involved

in cell wall loosening during cell expansion. Evidence for a transglycosylation activity in the apoplast of plant cells has been reported. However, the mechanism of this putative apoplastic transglycosylation reaction has yet to be elucidated. The main objectives of the work described in this thesis are as follows.

(1). To determine the mechanism by which exogenous XG9 apparently becomes incorporated into a soluble, extracellular polymer.

(2). To determine the nature of the endogenous extracellular polymeric substrate proposed to be involved in the *in vivo* reaction.

(3). To determine the nature of the endogenous substrates, both acceptor and donor.

(4). To investigate the possible physiological role of this putative transglycosylation reaction in the control of cell wall loosening, during cell expansion.

(5). To investigate whether the oligosaccharin activities of XG9 (and other xyloglucan oligosaccharides), either as an 'anti-auxin' or as an 'auxin-mimic', are due to XG9's involvement as a substrate in the putative apoplastic transglycosylation reaction.

2. MATERIALS AND METHODS

2.1 Plant material

2.1.1 Cell suspension cultures

2.1.1.1 Maintenance of cell suspension cultures

Cell suspension cultures from the following plants were used as experimental material: carrot (*Daucus carota*), chilli pepper (*Capsicum frutescens*), maize (*Zea mays*), rose (*Rosa* sp., cv Paul's Scarlet), spinach (*Spinacia oleracea* L., cv Monstrous Viroflay), sycamore (*Acer pseudoplatanus*), tall fescue grass (*Festuca arundinacea*).

Spinach and fescue cultures were incubated at 25°C with: photon flux density; 20 $\mu mol.m^{-2}.s^{-1}$; rotation in a horizontal plane, 110 rpm with an amplitude of 3.5 cm. Remaining cultures were incubated at 25°C with: photon flux density; 2.5 $\mu mol.m^{-2}.s^{-1}$; rotation in a horizontal plane, 100 rpm with an amplitude of 1.5 cm (Fry, 1980; Fry and Street, 1980).

All cultures were sub-cultured every two weeks, into fresh medium (appendix 1).

2.1.1.2 Sterilization techniques

All media and glassware used in the maintenance and manipulation of cell suspension cultures were sterilised by autoclave (temperature, 121°C, pressure, 15 psi). Aseptic manipulations of cultures were carried out in a laminar flow cabinet.

Xyloglucan nonasaccharides were transferred to culture pots (60 ml 'Sterilin' culture pots) in 75% ethanol. This was dried in a stream of sterile air (in a laminar flow cabinet), prior to the addition of cell culture or medium.

2.1.1.3 Measurement of cell growth in suspension cultures

Percent settled and packed cell volumes were the parameters measured to determine growth of cell suspension cultures. Changes in percent cell volume of a culture reflect changes in cell wall area, irrespective of the occurrence or otherwise of cell division.

Settled cell volume (SCV) measurement

A portion of a well shaken cell suspension culture (3 ml) was transferred (with a wide ended pipette) to a 15 ml conical, graduated centrifuge tube, and allowed to stand for 15 min, at room temperature. After this time the volume occupied by cells was read from the tube graduations:

% SCV = (volume of cells, ml/3 ml) x 33.333

Packed cell volume (PCV) measurement

The same sample of culture used to determine SCV was then centrifuged (300xg for 5 min). The volume occupied by the cells following centrifugation was read from the tube graduations;

% PCV = (volume of cells, ml/3 ml) x 33.333

2.2 Extraction of xyloglucan from *Tropaeolum* (nasturtium) seeds (Rao, 1959; Edwards *et al.*, 1985).

Nasturtium (*Tropaeolum majus*) seeds (approx. 200) were imbibed for 16 h and then ground in water (25 ml), to which sodium borohydride (final concentration, 0.05%) and 2 M-sodium hydroxide (475 ml) were later added. Following a 60 min incubation at 100°C, the suspension was centrifuged (4000xg for 20 min). Polymeric material was precipitated from the supernatant by the addition of 1 l of acetic acid/ethanol (1:10, v/v). The precipitate was collected by filtration and washed in 50% ethanol. This crude xyloglucan fraction was re-dissolved at 100°C (for 1 h) in 360 ml of 4% (w/v) potassium, sodium tartrate in 2.8% (w/v) potassium hydroxide. Any undissolved material was removed by filtration. Xyloglucan was precipitated from the solution by the addition of 50 ml Fehling's solution A (7.5% w/v copper sulphate). The copper-xyloglucan complex was collected by centrifugation (4000xg for 10 min) and re-dissolved in acetic acid/water (1:4, v/v). Partially de-complexed xyloglucan was precipitated in 67% ethanol and collected by filtration. Remaining copper was removed by incubation with 5% (w/v) EDTA (McDougall and Fry, 1989a). Purified xyloglucan was collected by preciptiation with ethanol (final concentration, 50%) and washed in 50% ethanol. This preparation was freeze dried, and stored dry.

2.3 Preparation of [*xylosyl*-^{3}H]xyloglucan nonasaccharide

2.3.1 Incubation of cells with [^{3}H]arabinose

Rose cells (*Rosa* sp., cv. Paul's Scarlet) from a day-7 culture were transferred into 100 ml fresh rose medium (appendix 1) containing 250 MBq (molarity, 0.26 mM) of L-[1-^{3}H]arabinose (97 TBq.mol^{-1}). Arabinose was tritiated by the 'TR7' method by Amersham International, and purified by preparative paper chromatography (Baydoun and Fry, 1989). The culture was incubated at 25°C, under standard rose culture conditions, for 8 days. The tritium from [1-^{3}H]arabinose is incorporated into L-

arabinosyl and D-xylosyl residues of glycoproteins and polysaccharides (Fry and Northcote, 1983).

2.3.2 Extraction of [*pentosyl*-^{3}H]hemicellulose

Cells were harvested from the culture medium by filtration, and suspended in 50 ml with water. Cell contents and non-covalently bound proteins were removed by a 16 h phenol/acetic acid/water extraction (50 ml acetic acid and 200 g phenol) at 25°C (Selvendran *et al.*, 1985). The crude cell wall preparation was harvested by centrifugation (800xg for 10 min). The pellet was washed in phenol/acetic acid/water (2:1:1, w/v/v), followed by 70% ethanol, to remove traces of phenol. Hemicelluloses were extracted at 25°C (16 h) in 20% (w/v) potassium hydroxide (Carpita, 1984; Hayashi *et al.*, 1980) containing 1% (w/v) sodium borohydride (to protect reducing termini from alkali degradation). The alkali insoluble residue was removed by centrifugation (800xg for 10 min). The supernatant containing [^{3}H]hemicelluloses, was brought to pH 5.0 by acetic acid (oxidising borohydride to borate with the release of hydrogen). The salts were removed from the hemicellulose preparation by dialysis, against 0.05% (w/v) chlorbutol (1,1,1-trichloro-2-methylpropan-2-ol) in water (2 d) and the dialysate was freeze dried.

2.3.3 *Trichoderma* cellulase digestion of [*pentosyl*-^{3}H]hemicellulose

Trichoderma cellulase (Sigma Chemical Co.) is a cellulase preparation from *Trichoderma viride*. Under the conditions used cellulase digestion of xyloglucan will yield hepta-, octa-, and nona-saccharides (Bauer *et al.*, 1973; Hayashi *et al.*, 1980; Valent *et al.*, 1980), with little further effect on these oligosaccharides (apart from hydrolysis of less than 10% of the fucose residues) (Fry,1988).

Dried [*xylosyl*-^{3}H]hemicellulose (250 mg) was dissolved in 20 ml sodium acetate buffer (20 mM, pH 4.7) to which 100 mg *Trichoderma* cellulase was added. The reaction mixture was incubated at 25°C, with shaking for 1 h.

2.3.4 Purification of [*xylosyl*-^{3}H]xyloglucan nonasaccharide (XG9) by Bio-Gel P-2 gel permeation chromatography (McDougall and Fry, 1988).

The *Trichoderma* cellulase digest was centrifuged (800xg for 10 min), and the pellet discarded. The supernatant (19 ml) was applied to a Bio-Gel P-2 column (bed volume, 500 ml) together with internal markers of blue dextran and glucose (0.053%, w/v). The sample was eluted with 0.05% chlorbutol in water (flow rate, 0.287 ml.min^{-1}). Fractions (3 ml) were collected and assayed for hexose (section 2.8.1) and tritium (section 2.8.2.1). Fractions eluting with a K_{av} of 0.39 (that of XG9)

were pooled and freeze dried. [*xylosyl*-^{3}H]XG9 (specific activity, 0.5 TBq.mol^{-1}) was stored in 25% ethanol at 4°C.

2.4 Preparation of [*reducing terminus*-^{3}H]xyloglucan nonasaccharide

2.4.1 Purification of xyloglucan nonasaccharide

Xyloglucan nonasaccharide (XG9) was prepared from hemicellulose, extracted from rose cells with alkali. Oliosaccharides were prepared by *Trichoderma* cellulase digestion, and the nonasaccharide was purified by gel permeation chromatography (on Bio-Gel P-2), followed by preparative paper chromatography in EAW and BPW (McDougall and Fry, 1989b).

2.4.2 Tritiation of xyloglucan nonasaccharide

Purified XG9 was custom-tritiated by Amersham Intenational. The hydrogen at position 1 of the reducing terminal glucose was exchanged for tritium, by the 'TL-7' method. This involves the incubation of XG9 with $^3H_2$ in the presence of a catalyst. The [*reducing terminus*-^{3}H]XG was then re-purified by preparative paper chromatography in EAW and BPW (McDougall and Fry, 1989b). The oligosaccharide (specific activity, 12 TBq.mol^{-1}) was stored in 25% ethanol at 4°C.

2.5 Assays for the putative transglycosylase, using xyloglucan nonasaccharide as a substrate

2.5.1 Incubation of cell suspension cultures with [^{3}H]xyloglucan nonasaccharide

(Baydoun and Fry, 1989)

Xyloglucan nonasaccharide (0.5 MBq of [*reducing terminus*-^{3}H]XG9 or 0.046 MBq of [*xylosyl*-^{3}H]XG9) was transferred, in 75% (v/v) ethanol, into 60 ml 'Sterilin' culture pots. This was dried in a stream of sterile air (section 2.1.1.2). Aliquots (3 ml) of cell suspension culture were aseptically transferred into the 'Sterilin' pots, together with fresh medium (3 ml). The final concentration of added xyloglucan nonasaccharide was approximately 14 μM.

Cells were incubated in XG9-containing medium under standard conditions (section 2.1.1.1). At intervals, cells were harvested from the culture medium by centrifugation (500xg for 5 min). The pelleted cells were resuspended in water (3 ml) and centrifuged, to wash remaining non-incorporated ^{3}H from the cells. The two

supernatants were pooled and stored frozen (-20°C). Cells were washed a further three times in water (3 ml) and the supernatants discarded. The volume of washed cells was made up to 2 ml with water, and a portion was assayed for tritium (section 2.8.2.1).

2.5.2 Incubation of spinach suspension culture spent medium with [*reducing terminus*-^{3}H]xyloglucan nonasaccharide

Aliquots (7 ml) of spinach cell suspension culture (*Spinacia oleracea* L., cv Monstrous Viroflay) were removed aseptically from 400 ml cultures, at intervals. These aliquots were filtered through two layers of nylon mesh (pore size, 64 μm; Lockertex), under sterile conditions. The spent medium was collected and 3 ml aliquots were transferred to 60 ml 'Sterilin' culture pots, together with 3 ml of fresh medium. Each culture pot contained 0.5 MBq [*reducing terminus*-^{3}H]XG9, transferred in 75% ethanol, and dried in a stream of sterile air. These samples were incubated for 9 h under standard spinach culture conditions (section 2.1.1.1). A small portion (100 μl) of each sample was then streaked onto Whatman 3MM chromatography paper, and the ^{3}H-products separated in BPW (section 2.6.3).

2.5.3 Incubation of filtered spinach cells with fresh medium containing [*reducing terminus*-^{3}H]xyloglucan nonasaccharide

The cells filtered from the spinach suspension culture in section 2.5.2 were washed on the nylon mesh with 20 ml of fresh spinach culture medium. Cells (fresh weight, 400 mg) were aseptically transferred into 60 ml 'Sterilin' culture pots, together with 6 ml of fresh spinach culture medium. Each culture pot contained 0.5 MBq [*reducing terminus*-^{3}H]XG9 (section 2.1.1.2). Samples were incubated under standard spinach culture conditions (section 2.1.1.1) for 9 h. Cells were then harvested by centrifugation, as in section 2.5.1. A portion (150 μl) of the pooled supernatants were streaked onto Whatman 3MM chromatography paper. The ^{3}H-labelled products were separated in BPW (section 2.6.3)

2.6 Fractionation Methods

2.6.1 Gel permeation chromatography (g.p.c.); Bio-Gel P-2 (McDougall and Fry, 1988)

Samples of culture medium (7.5 ml) were applied to a Bio-Gel P-2 column (bed volume, 160 ml) together with internal molecular weight markers (0.17%, w/v) of

glucose and dextran (molecular weight, 9,400). The samples were eluted with 0.05% (w/v) chlorbutol in water, at a flow rate of 0.2-0.25 ml.min^{-1}. Fractions (2.5 ml) were collected and assayed for hexose (section 2.8.1) and radioactivity (section 2.8.2.1). Fractions containing the dextran marker (i.e. ^{3}H-labelled polymers) were pooled, and stored frozen.

The position of a particular fraction within the eluent from the column is given as a K_{AV} value, where;

$$K_{av} = \frac{\text{(volume in which fraction eluted)-(void volume)}}{\text{(included volume)-(void volume)}}$$

2.6.2 Gel permeation chromatography (g.p.c.); Sepharose CL-6B

Portions (1ml) of high molecular weight material, prepared by Bio-Gel P-2 chromatography (section 2.6.1), were dried *in vacuo* (in a SpeedVac). Dried material was taken up in 10 mM-sodium hydroxide (3 ml), together with internal markers (0.17%, w/v) of glucose and industrial grade dextran (molecular weight 5,000-40,000kD). Samples were applied to a Sepharose CL-6B column (bed volume, 80 ml), and eluted with 10 mM-sodium hydroxide (flow rate, 0.3 ml.min^{-1}). Fractions (1 ml) were assayed for hexose (section 2.8.1) and radioactivity (section 2.8.2.1).

The column was calibrated with dextrans of molecular weights 488, 249, 74.3, and 40 kD.

2.6.3 Paper chromatography (p.c.)

Samples were applied to Whatman 3MM chromatography paper (Whatman International Ltd.), as spots or streaks. Sugar (0.05 mg) and alditol (0.5 mg) standards were used as external markers. Chromatograms were developed by descending chromatography in one of the following solvent systems:

EAW; ethyl acetate/acetic acid/water (10:5:6, v/v/v), run for 24-48 h;

EPW; ethyl acetate/pyridine/water (8:2:1, v/v/v), run for 16 h;

BPW; butan-1-ol/pyridine/water (4:3:4, v/v/v), run for 16 h;

BAB; butanone/acetic acid/boric acid-saturated water (9:1:1, v/v/v), run for 24 h.

The position of material on a paper chromatogram is given as an R_F value. That is, the position of a particular material, relative to the solvent front.

$$R_f = \frac{\text{(distance of material from origin)}}{\text{(distance of solvent front from origin)}}$$

2.6.4 Thin layer chromatography (t.l.c.)

Samples were applied to cellulose thin layer chromatography (t.l.c.) plates (E. Merck) as spots. Sugar standards (up to 100 μg) were used as external markers. Chromatograms were developed by ascending chromatography in EPW [ethyl acetate/pyridine/water (8:2:1, v/v/v)] for approximately 5 h.

2.6.5 High performance liquid chromatography (h.p.l.c.)

Analytical separation of the components of the [*reducing terminus*-^{3}H]xyloglucan nonasaccharide preparation was performed by h.p.l.c., using a Gilson liquid chromatograph, fitted with an Amino-Spheri-5 (Brownlee) 220x4.2 mm column (containing a resin of amino-substituted silica). The mobile phase was an aqueous 60% (v/v) acetonitrile solution, which had been filtered through a Nylon-66 membrane filter (pore size, 0.45 μm; Rainin Instrument Co.). All solvents used were HyperSolv grade (BDH).

A sample of [*reducing terminus*-^{3}H]XG9 was dried *in vacuo*, resuspended in HyperSolv water (volume, 100 μl), and subjected to centrifugation (2000xg.) to remove any insoluble particles. A fraction (20 μl) of the sample supernatant was injected onto the Amino-Spheri-5 column via a Rheodyne sample injection valve. Material was eluted from the column with 60% acetonitrile, at a flow rate of 0.4 ml.min^{-1}. Under these conditions, oligosaccharides are separated on the basis of the number of hydoxyl groups present per oligosaccharide molecule (McDougall and Fry, 1991).

Sugars eluted from the column were detected by refractive index measurements. The elution of ^{3}H-labelled material was monitered by a RAMONA, a flow radio-isotope monitor (section 2.8.2.3).

2.6.6 Cellulose affinity chromatography (Aspinall *et al.*, 1969; Hayashi *et al.*, 1987)

A portion of high molecular weight material, prepared by Bio-Gel P-2 chromatography (section 2.6.1), was dried *in vacuo* (in a SpeedVac). Material was re-dissolved in 20 mM-sodium acetate (0.5 ml) at pH 5.0, containing 0.12% (w/v) *Tropaeolum* xyloglucan (section 2.2). The sample was applied to a column of microgranular cellulose powder (Sigma Chemical Co.: bed volume, 3 ml), which had been pre-washed in 1 M-sodium hydroxide and equilibrated with 20 mM-sodium acetate. Material was eluted (flow rate, 0.1 ml.min^{-1}) with 13 ml of 20 mM-sodium acetate (pH 5.0); 12.5 ml sodium acetate buffer containing 8 M-urea; followed by 12.5 ml of 1 M-sodium hydroxide. Fractions (0.5 ml) were collected and assayed for hexose (section 2.8.1) and radioactivity (section 2.8.2.1).

2.7 Methods of analysis of oligo- and polysaccharides

2.7.1 Trifluoroacetic acid (TFA) hydrolysis (Fry, 1988)

Dried samples were dissolved in 2 M-trifluoroacetic acid (TFA), and incubated at 120°C for 1 h. The acid was removed by drying *in vacuo*. Hydrolysed material was dissolved in 50-100 µl of water and applied to chromatography paper. Chromatograms were developed in EPW (section 2.6.3 and 2.6.4).

2.7.2 Alkali hydrolysis

Ester-linkages are susceptible to alkali hydrolysis, unlike glycosidic bonds (Fry, 1989c). Alkali hydrolysis was used as an analytical test for the presence of ester bonds, within polysaccharides.

Dried samples were dissolved in 40 µl of 0.5 M-potassium hydroxide (containing 0.3% (w/v) sodium borohydride, to minimise alkali degradation of reducing termini of sugars), and incubated at room temperature for 1 h. The samples were neutralised with acetic acid (2 µl), and spotted onto chromatography paper. Products of hydrolysis were separated in EAW (section 2.6.3).

2.7.3 Sodium borohydride reduction followed by acid hydrolysis (Fry, 1988)

Dried samples were dissolved in 200 µl of 0.5 M-sodium borohydride (in 1 M-ammonia solution), and incubated for 4 h at 25°C. Sodium borohydride will only reduce the reducing terminal sugar of an oligosaccharide or polysaccharide. Acetic acid (30 µl) was added (to remove excess borohydride by oxidation) and the sample was applied to a column of Dowex 50 (bed volume, 1.5 ml) previously washed in 1 M-hydrochloric acid and equilibrated with water. Dowex removes ammonium and sodium ions from the mixture. The sample was eluted from the column with water (3 ml), and then dried *in vacuo*. The reduced material was repeatedly dried from methanol/acetic acid (10:1, v/v) to remove boric acid as the methyl ester, and subjected to TFA hydrolysis (section 2.7.1). The products of hydrolysis were separated by paper chromatography in BAB (section 2.6.3).

2.7.4 Driselase digestion

Driselase (Sigma Chemical Co.) is an enzyme preparation from *Irpex lacteus*. It contains enzymes that will hydrolyse all glycosidic linkages in xyloglucan, except α-D-xylosyl. Driselase hydrolysis of xyloglucan will yield monomeric galactose, glucose and fucose, and the disaccharide isoprimeverose (α-D-xylopyranose-(1-6)-D-glucose) (Fry, 1988).

Driselase was partially purified by precipitation in 52% (w/v) ammonium sulphate. The precipitate was collected by centrifugation, dissolved in water, and de-salted on Sephadex G-25. The 'purified' Driselase was stored dry below 0°C (Fry, 1982b).

Dried samples were dissovled in 125 µl of 0.25% (w/v) partially purified Driselase in pyridine/acetic acid/water (1:1:98, v/v/v) at pH 4.7, containing 0.05% chlorbutol. Enzyme mixtures were incubated for 20 h at 25°C. The products of Driselase digestion were separated by paper chromatography in EPW (section 2.6.3).

2.7.5 *Trichoderma* cellulase digestion

Dried samples were disolved in 200 µl of 0.1% (w/v) *Trichoderma* cellulase in pyridine/acetic acid/water (1:1:98, v/v/v) at pH 4.7, containing 0.05% chorbutol. Enzyme mixtures were incubated for 30 min and 3 h at 25°C. The products of cellulase digestion were separated in EAW, by paper chromatography (section 2.6.3).

2.7.6 Copper precipitation (Rao, 1959; Edwards *et al.*, 1985)

Dried sample were re-dissolved in 475 µl of 0.5% (w/v) *Tropaeolum* xyloglucan (section 2.2). Alkali Fehling's reagent (70 µl of 35% (w/v) potassium, sodium tartrate (tetrahydrate) in 25% potassium hydroxide) was added, followed by 70 µl of Fehling's solution B (7.5% (w/v) copper sulphate). The precipitate was collected by centrifugation (800xg for 5 min) and washed in 70% ethanol. The two supernatants were pooled, and both the pellet and supernatants were assayed for tritium (section 2.8.2.1).

2.7.7 Iodine precipitation (Kato and Matsuda, 1976; Kooiman, 1960)

Dried samples were re-dissolved in 3.4 ml of 0.038% (w/v) *Tropaeolum* xylogucan (section 2.2). To this, 3 ml of 3% iodine (in 4% potassium iodide) solution was added. This mixture was kept on ice for 2 h to allow the precititate to from. The precipitate was collected by centrifugation (20,000xg for 10 min), and both supernatant and pellet were assayed for tritium (section 2.8.2.1)

2.7.8 Trichloroacetic acid (TCA) precipitation.

Samples (1.5 ml) were mixed with 1 ml of 5% (w/v) bovine serum albumin (BSA), used as a non-radioactive carrier. To these 40% trichloroacetic acid (2.5 ml) was added. The mixture was kept on ice (16 h) to allow the precipitate to form. Xyloglucan was shown not to precipitate at this concentration of TCA. The precipitate was collected by centrifugation (800xg for 5 min), and washed in ice cold

20% TCA. The two supernatants were pooled, and both the pellet and supernatants were assayed for tritium (section 2.8.2.1).

2.8 General Methods

2.8.1 Assay for hexose (Dische, 1962)

The hexose content of an aqueous sample (0.5 ml) was determined by addition of 0.2% anthrone in concentrated sulphuric acid (1 ml). The solution was mixed and incubated in a boiling water bath for 5 min. Once cool, the absorbance of the solution was measured at 600 nm. Glucose was used as the standard.

2.8.2 Assay for tritium

2.8.2.1 Aqueous samples

Aqueous radioactive samples were mixed with ten volumes of toluene/Triton X-100 (2:1, v/v) scintillation cocktail (containing 0.33% (w/v) PPO (2,5-diphenyloxazole) and 0.033% (w/v) POPOP (1,4-bis-(5-phenyloxazol-2-yl)benzene)). Quench curves were constructed, using [^{3}H]toluene as a radioactive standard, successively quenched by addition of acetone.

2.8.2.2 Non-aqueous samples

Non-aqueous radioactive samples (e.g. strips of chromatography paper) were soaked in a toluene scintillation cocktail (containing 0.5% (w/v) PPO, 0.05% (w/v) POPOP)

2.8.2.3 H.p.l.c. eluent

A RAMONA monitor (Raytest Instruments) was used to detect radioactivity present in the eluent from analytical h.p.l.c. (section 2.6.5). The eluent was mixed with scintillation cocktail and subjected to scintillation counting in a flow cell.

2.8.3 Chromatography stains

Sugar markers on paper chromatograms were stained with an alkaline silver nitrate reagent (Harborne, 1976). Chromatograms were dipped into a solution of silver nitrate (2 ml of saturated aqueous silver nitrate solution in 400 ml acetone), followed by an ethanolic sodium hydroxide solution (5 ml of 10 M-sodium hydroxide in 100 ml ethanol). Chromatograms were 'de-stained' in 10% sodium thiosulphate solution.

Alditol markers were stained with potassium permanganate. Chromatograms were sprayed with a 1% potassium permanganate solution (made up in 2% sodium carbonate) (Fry, 1988)

Aniline hydrogen-phthalate stain was used as a stain for monosaccharides (Partridge, 1949: modified by Fry, 1988). Chromatograms were dipped into 1.6% phthalic acid in acetone/diethyl ether/water (49:49:2, v/v/v), to which aniline (0.1%) had been added immediately before use. Colour was developed by heating the dried chromatograms to 105°C for 5 min.

2.8.3.1 Densitometry of aniline hydrogen-phthalate stained t.l.c. plates

The density of staining of t.l.c. plates with aniline hydrogen-phthalate (section 2.8.3), was determined by the measurement of reflection factor (i.e. Luminous flux reflected by body/luminous flux incident upon the body), by a scanning densitometer (Helena Laboratories, Quick Scan R & D). Quantitative assessment of the amount of aniline hydrogen-phthalate staining material present was acheived by comparing the density of staining of known amounts of external sugar markers with that of samples. Density of staining was determined by calculating the area under each curve.

3. RESULTS

3.1 Preparation of [*xylosyl*-^{3}H]xyloglucan oligosaccharides

3.1.1 Introduction

A putative transglycosylation reaction has been detected in the apoplast of spinach suspension cultures (Baydoun and Fry, 1989). An apparent increase in molecular weight of ^{3}H-label was detected during incubation of cells with [*fucosyl*-^{3}H]XG9, acetylated [*fucosyl*-^{3}H]XG9 and acetylated [*xylosyl*-^{3}H]XG9 (Baydoun and Fry, 1989). This increase in molecular weight occurred in the apoplast of the cultures and did not involve appreciable breakdown of the oligosaccharides.

The [*xylosyl*-^{3}H]xyloglucan oligosaccharides prepared in this section were subsequently used as ^{3}H-labelled exogenous substrates for the putative extracellular transglycosylase (section 3.2).

3.1.2 Preparation and purification of [*xylosyl*-^{3}H]xyloglucan oligosaccharides

[*xylosyl*-^{3}H]Xyloglucan oligosaccharides were prepared by *Trichoderma* cellulase digestion of [*pentosyl*-^{3}H]hemicellulose extracted from the cell walls of [^{3}H]arabinose fed rose cells (section 2.3). The cellulase digest was subjected to g.p.c. on Bio-gel P-2 (section 2.3.4) together with internal molecular weight markers, dextran and glucose. Fractions (2.5 ml) were collected, and assayed for hexose (section 2.8.1) and tritium (section 2.8.2.1).

The *Trichoderma* cellulase digest contained high molecular weight ^{3}H-labelled material co-eluting with dextran, low molecular weight ^{3}H-labelled material co-eluting with glucose and three major ^{3}H-labelled components of intermediate molecular weight (Fig. 3.1.1).

XG9 is known to elute from Bio-gel P-2 with a K_{AV} of 0.4 (McDougall and Fry, 1988). Fractions exhibiting approximately this K_{AV} were pooled and dried *in vacuo*, in a SpeedVac. These pooled fractions will be referred to as the [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation.

3.1.3 Analysis of the [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation

The [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation was analysed to determine both its purity (i.e. the number and nature of ^{3}H-labelled components present) and the specificity of the ^{3}H-label (i.e. which residues are ^{3}H-labelled).

3.1.3.1 Paper chromatographic behaviour of [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation

Samples (1.3 kBq) of the [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation

were separated by p.c. in EAW and BPW (section 2.6.3). The positions of external markers were determined by alkali-silver nitrate stain (section 2.8.3).

Five ^{3}H-labelled components were resolved by BPW. A proportion of the material remained at the origin. Small amounts of ^{3}H were associated with material with R_Fs similar to maltotetraose and the monosaccharides (maybe xylose or arabinose). The majority of ^{3}H-labelled material had an R_F similar to maltoheptaose (approximately, 69.1%). This material may be made up of two components. One component (the major part) had an R_F just below that of maltoheptaose, and the second was a slightly less mobile material (Fig. 3.1.2a).

P.c. of the [^{3}H]xyloglucan oligosaccharide preparation in EAW separated four or five ^{3}H-labelled components. A proportion of the material remained at the origin. Small amounts of ^{3}H were associated with materials of (1) R_F between that of maltoheptaose and maltotetraose, and (2) with an R_F similar to maltotetraose. The major ^{3}H-labelled material separated by p.c. had a similar mobility to maltoheptaose (approximately, 63.5%). The profile of this peak suggests the presence of two partially resolved compounds (Fig. 3.1.2b).

3.1.3.2 TFA hydrolysis of [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation

A sample of the preparation (4.4 kBq) was acid hydrolysed with 2 M-TFA (section 2.7.1). The products were separated by p.c. in EPW (section 2.6.3). External markers were visualised with alkali-silver nitrate stain (section 2.8.3).

The major ^{3}H-labelled product co-chromatographed with the xylose external marker (approximately, 72.1%). A smaller amount of ^{3}H was associated with material of similar R_F to the arabinose external marker (Fig. 3.1.3a). A third component of the hydrolysate remained at the origin of the p.c..

3.1.3.3 Driselase digestion of [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation

A sample (4.4 kBq) of [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation was subjected to Driselase digestion (section 2.7.4). The products of enzymatic digestion were separated by p.c. in EPW (section 2.6.3). External markers were stained with alkali-silver nitrate (section 2.8.3).

The major ^{3}H-labelled product co-chromatographed with the sucrose external marker (approximately, 74.3%). Isoprimeverose is known to exhibit a similar mobility to sucrose in EPW (Fry, 1988). Other ^{3}H-labelled material present in the digest remained at the origin of the p.c. (Fig. 3.1.3b).

3.1.4 Specific activity of [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation

The specific activity of the preparation was estimated by determination of the

amount of hexose present in a 40 kBq sample (section 2.8.1).

The specific activity of the preparation was calculated to be approximately 0.2 $TBq.mol^{-1}$ of hexose.

3.1.5 Discussion of results

The major ^3H-labelled component of the [*xylosyl*-^3H]xyloglucan oligosaccharide preparation co-chromatographed with maltoheptaose in both BPW and EAW. Maltoheptaose and XG9 are known to have similar mobilities in these solvents (Fry, 1986b). This is compatible with the major ^3H-labelled component of this preparation being XG9. However, a slightly less mobile oligosaccharide may also have been present. *Trichoderma* cellulase digestion of xyloglucan releases hepta-, octa- , nona- and decasaccharides (Valent *et al.* 1980; Kato and Matsuda, 1980a). XG7 (the heptasaccharide) is slightly less mobile than XG9 in BPW and EAW (Fry, 1986b). The Bio-gel P-2 chromatography did not wholly resolve XG9 from XG7 (Fig. 3.1.1). It is probable therefore that the preparation recovered from g.p.c. was enriched in XG9 but did not contain solely XG9.

Other minor components of the [^3H]xyloglucan oligosaccharide preparation included the following.

(1) BPW and EAW immobile material. This was presumably polymeric material which was not fully hydrolysed by the *Trichoderma* cellulase treatment. All data collected in transglycosylation experiments concerning the molecular weight of the ^3H-labelled material must be corrected for the amount of high molecular weight ^3H-labelled material present in the substrate preparation.

(2) ^3H-Labelled oligosaccharides with a higher mobility in EAW and BPW than maltoheptaose. *Trichoderma* cellulase treatment of extracted xyloglucan released low molecular weight fragments other than XG9 and XG7 (Fig. 3.1.2ab). The preparation recovered from Bio-gel P-2 chromatography appears to have been contaminated with small amounts of these fragments.

(3) ^3H-Labelled monomeric sugars, were also present in the preparation. *Trichoderma* cellulase is not a pure cellulase, but does contain low levels of contaminant enzyme activities. Although the incubation conditions used here minimise the activities of such contaminants, small amounts of ^3H-labelled monomeric sugars could still have been released during incubation of the hemicellulose with *Trichoderma* cellulase

In summary, Bio-gel P-2 chromatography of the *Trichoderma* cellulase digest did not isolate [^3H]XG9, but rather produced a preparation enriched in [^3H]XG9.

Acid hydrolysis of the [^3H]xyloglucan oligosaccharide preparation yielded

[^{3}H]xylose as the major ^{3}H-labelled product. A small amount of [^{3}H]arabinose may also have been released. The majority of ^{3}H-label was therefore associated with the xylosyl residues present in the [^{3}H]oligosaccharide preparation.

Driselase digestion of [^{3}H]xyloglucan oligosaccharide preparation released a disaccharide as the major ^{3}H-labelled product. Driselase is an enzyme preparation containing many endo- and exo-hydrolases which act on the glycosidic bonds present in cell wall polysaccharides. However, Driselase lacks α-xylosidase activity (Fry, 1988). As a result, Driselase will hydrolyse all the glycosidic linkages within xyloglucan apart from the α(1-6) linkage between xylosyl and glucosyl residues. The release of [^{3}H]isoprimeverose (i.e. D-xylosyl-α(1-6)-D-glucose) from the [^{3}H]oligosaccharide preparation during incubation with Driselase, and the release of [^{3}H]xylose during TFA hydrolysis, indicate that the ^{3}H-label was associated with the α-xylosyl residues of xyloglucan oligosaccharides.

The [^{3}H]xyloglucan oligosaccharide preparation contained [*xylosyl*-^{3}H]XG9 and [*xylosyl*-^{3}H]XG7, and small amounts of [^{3}H]polymer, ^{3}H-labelled monomeric sugars, and some [^{3}H]oligosacchrides smaller than XG7. The specific activity of the preparation was found to be approximately 0.2 Bq.mol^{-1} of hexose. This preparation was used a substrate for the putative apoplastic transglycosylation reaction detected in the apoplast of spinach suspension cultures (Baydoun and Fry, 1989)

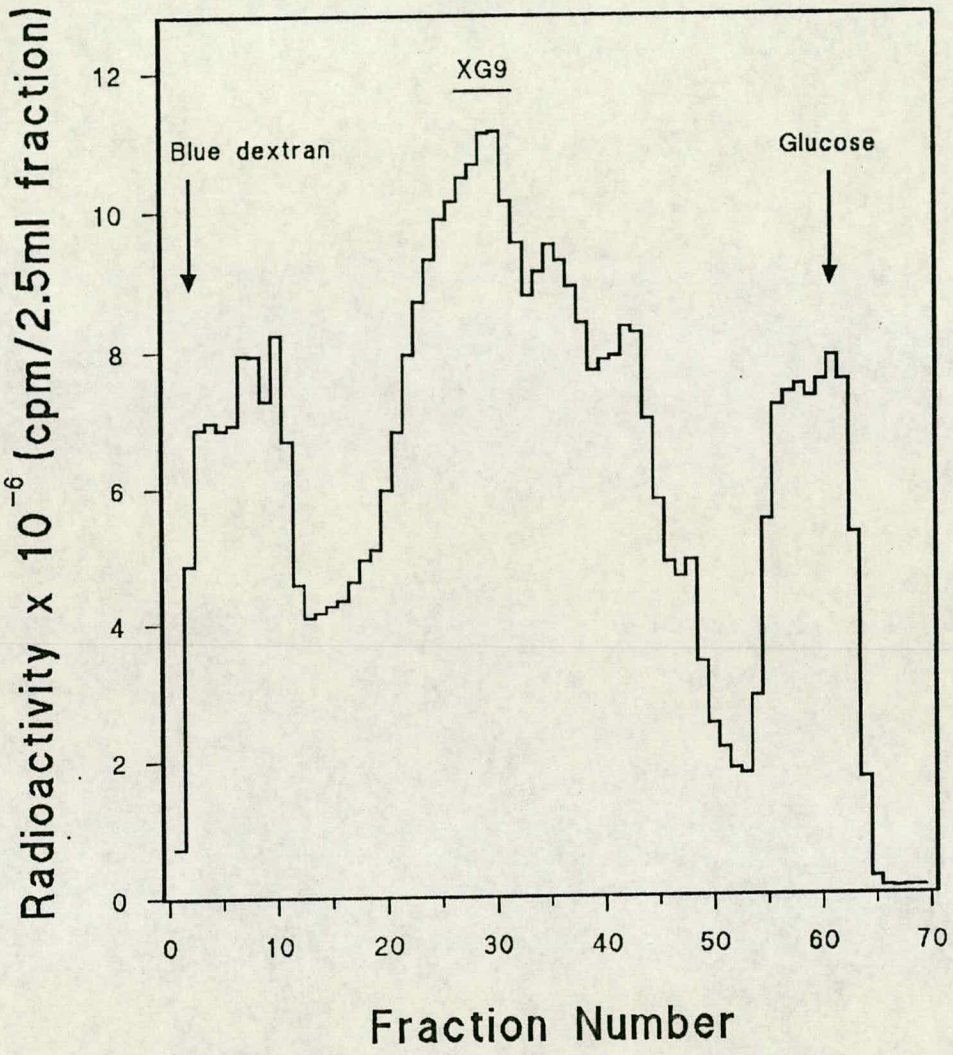


Fig 3.1.1 Purification of [*xylosyl*-^{3}H]xyloglucan oligosaccharides by Bio-Gel P-2 chromatography

Rose suspension cultures were incubated with [^{3}H]arabinose. [*pentosyl*-^{3}H]Hemicelluloses were extracted and subjected to *Trichoderma* cellulase digestion. The products released by enzymatic digestion were separated by Bio-Gel P-2 chromatography. The position of internal markers dextran (K_{AV}, 0.00) and glucose (K_{AV}, 1.00) are shown. Fractions containing ^{3}H-labelled material with a K_{AV} of approximately 0.4 (that of the xyloglucan nonsaccharide, XG9) were pooled. This ^{3}H-labelled material is referred to as the [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation.

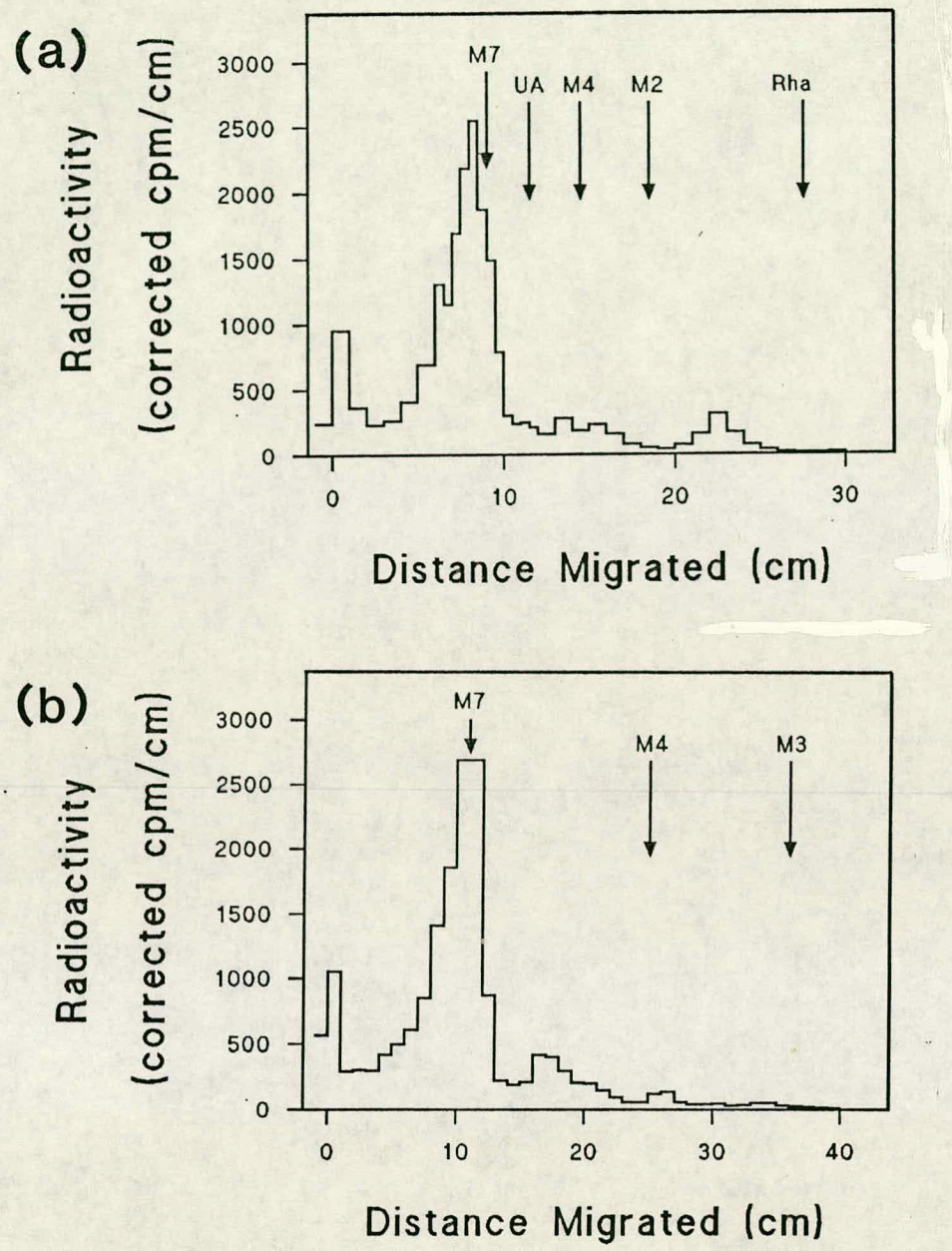


Fig 3.1.2 Paper chromatography of [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation

[*xylosyl*-^{3}H]Xyloglucan oligosaccharide preparation (see Fig 3.1.1) was applied to chromatography paper. Chromatograms were developed in (a) BPW and (b) EAW. The positions of the external markers are shown (maltoheptaose (M7), maltotetraose (M4), maltotriose (M3), maltose (M2), galacturonic and glucuronic acids (UA), and rhamnose (Rha).

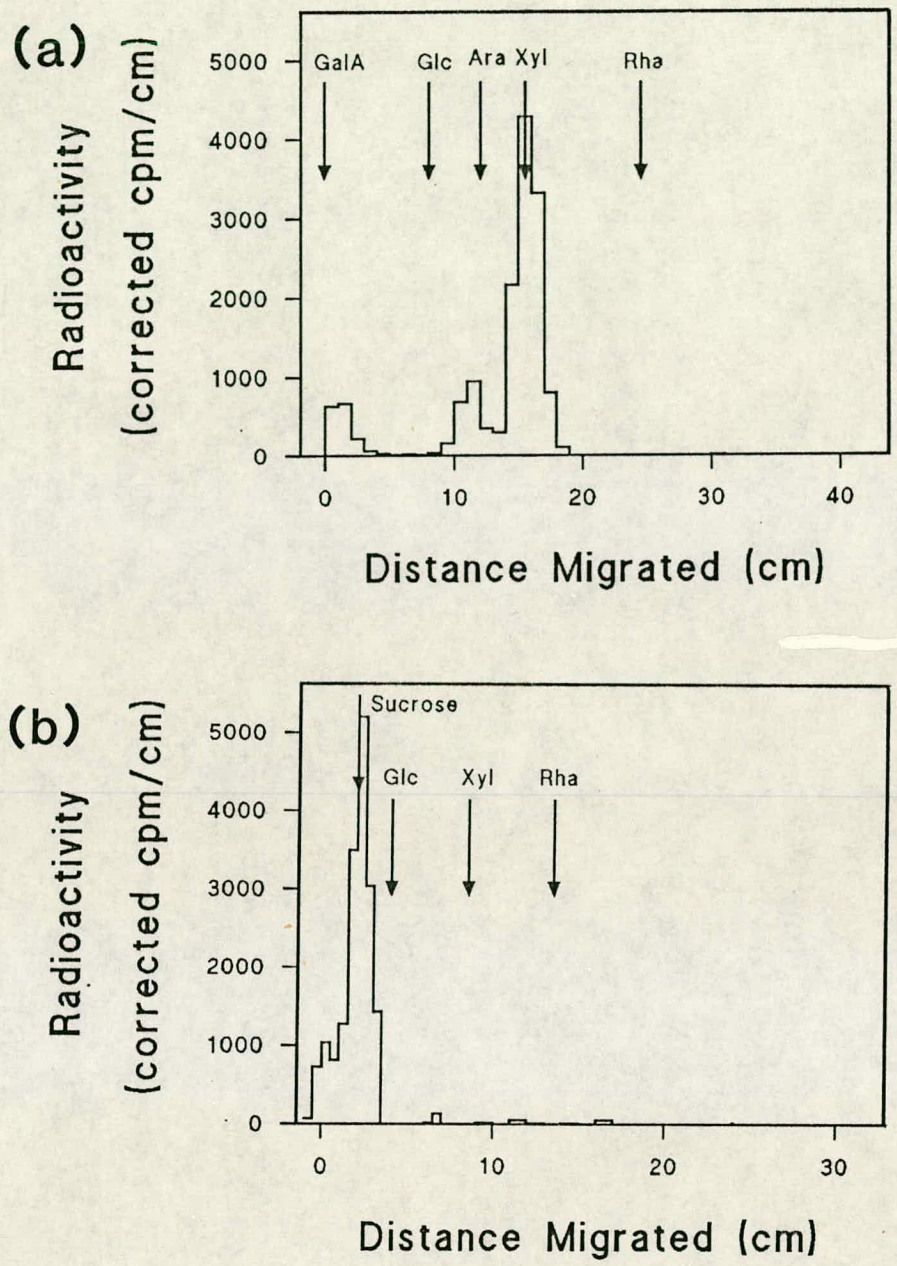


Fig 3.1.3 Paper chromatography of ^{3}H-labelled material released from [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation by TFA hydrolysis and Driselase digestion

[*xylosyl*-^{3}H]Xyloglucan oligosaccharide preparation was subjected to (a) TFA hydrolysis (products separated in EPW) and (b) Driselase digestion (products separated in EPW). The positions of external markers galacturonic acid (GalA), glucose (Glc), arabinose (Ara), xylose (Xyl), rhamnose (Rha) and sucrose are shown.

3.2 Investigation into the occurrence of a putative extracellular transglycosylation, using [*xylosyl*-^{3}H]oligosaccharides as a substrate

3.2.1 Introduction

A putative extracellular transglycosylation reaction was detected in spinach cell suspension cultures by Baydoun and Fry (1989). The aim of this experiment was to demonstrate extracellular transglycosylation, using xyloglucan oligosaccharides as an exogenous substrate (Baydoun and Fry, 1989). The oligosaccharides used were tritium labelled in their α-xylose residues. The β-(1-4)-glucosyl backbone of the oligosaccharide is substituted with α-xylosyl resides, apart from the reducing terminal glucose unit. With a ^{3}H-label in the xylosyl side groups, the metabolic fate of both the side groups and the substituted glucosyl backbone to which they are linked can be determined. It was neccessary to determine the fate of exogenous XG9 in spinach cultures before the putative transglycosylation reaction could be investigated further.

3.2.2 Incubation of spinach cell suspension cultures with [*xylosyl*-^{3}H]oligosaccharides

Spinach cell suspension cultures were incubated with [*xylosyl*-^{3}H]oligosaccharides (enriched in the nonasaccharide, section 3.1) as in section 2.5.1, for 6, 24, 48 and 72 h. Cell free controls, containing fresh medium in place of suspension culture, were also incubated with [*xylosyl*-^{3}H]oligosaccharides. Cells were separated from the incubation medium by centrifugation (section 2.5.1), and samples of both medium and cells were assayed for tritium (section 2.8.2.1).

During the 72 h incubation, 95.9% of total added ^{3}H remained soluble and extracellular. Small amounts of ^{3}H (maximum, 4.1% of the total added ^{3}H) were taken up into the cells or became associated with insoluble material (Table 3.2.1, Fig. 3.2.2).

Table 3.2.1 Proportion of ^3H taken up into cells and/or associated with insoluble (cell wall) material

Incubation time	Proportion of ^3H taken up into and/or associated with cells (%)
6 h	0.52
24 h	1.77
48 h	3.45
72 h	4.09

3.2.3 Bio-Gel P-2 chromatography of spinach cell suspension culture medium

Medium, removed from the cells (section 2.5.1) following incubation of the suspension culture with [*xylosyl*-^3H]oligosaccharides, was subjected to g.p.c. on Bio-Gel P-2 (section 2.6.1). Tritium labelled components were thus separated from one another on the basis of their apparent molecular weight. Molecules with an apparent molecular weight of between 100 and 1800 (determined by globular proteins) can be resolved on Bio-Gel P-2. Each fraction (2.5 ml) was assayed for hexose (section 2.8.1) to determine the position of the internal markers (dextran, maltohexaose and glucose), and for tritium (section 2.8.2.1).

Elution profiles of ^3H-labelled components of the incubation medium are given in Fig.3.2.1ab. Tritium from [*xylosyl*-^3H]xyloglucan oligosaccharides had become associated with both apparently larger and smaller molecules compared to the exogenous oligosaccharides. Over the 72 h incubation, a maximum of 53.4% of the total ^3H had become associated with high molecular weight material (eluting from the column with a K_{AV} of 0.0). Over the same time period, a maximum of 19.3% of the total ^3H had undergone an apparent decrease in molecular weight (eluting from the column with K_{AV}s of 0.63, 0.76 and 0.89). All data on the percent of ^3H from the exogenous xyloglucan oligosaccharides to become associated with larger or smaller molecules have been corrected, using results from cell free controls (Fig.3.2.2).

Some oligosaccharide substrate remained in the incubation medium following 72 h incubation with spinach suspension culture (approximately, 28-36% of the total ^3H added) (Fig.3.2.2).

3.2.4 Analysis of high molecular weight, ^{3}H-labelled components of incubation medium

Fractions co-eluting with dextran from the Bio-Gel P-2 were pooled, and portions were hydrolysed with TFA (section 2.7.1), Driselase (section 2.7.4) and *Trichoderma* cellulase (section 2.7.5). Analytical hydrolyses were carried out to determine the extent of breakdown and re-incorporation of ^{3}H from exogenous xyloglucan oligosacharides during incubation with spinach cell suspension cultures.

3.2.4.1 TFA hydrolysis

Samples of ^{3}H-labelled high molecular weight products (dried from 2.0 ml) were hydrolysed in 2 M-TFA (section 2.7.1). This procedure results in the complete hydrolysis of glycosidic linkages liberating all glycosyl residues as free monomers. Hydrolytic products were separated by p.c. (section 2.6.3) in EPW. External markers were visualised by alkaline-silver nitrate stain (section 2.8.3).

The major ^{3}H-labelled hydrolysis product released in all samples co-chromatographed with the xylose external marker. A typical chromatogram is shown in Fig. 3.2.3.

3.2.4.2 Driselase digestion

Samples of ^{3}H-labelled high molecular weight products (2.0 ml, dried *in vacuo*) were hydrolysed with Driselase (section 2.7.4). The products of digestion were separated by p.c. in EPW (section 2.6.3). External markers were visualised by alkaline-silver nitrate stain (section 2.8.3).

The major ^{3}H-labelled product released in all cases co-chromatographed with the sucrose external marker (Fig.3.2.4a shows a typical chromatogram). Isoprimeverose (D-xylopyranosyl-α-(1-6)-D-glucose) is known to exhibit a similar mobility to sucrose in EPW (Fry,1988).

3.2.4.3 *Trichoderma* cellulase digestion

Samples of ^{3}H-labelled high molecular weight products (4 ml) were subjected to *Trichoderma* cellulase digestion (section 2.7.5). Hydrolysis products were separated by p.c. in EAW (section 2.6.3).

The major ^{3}H-labelled product released on cellulase digestion co-chromatographed with the maltoheptaose external marker. A typical chromatogram is shown in Fig. 3.2.4b. Maltoheptaose is known to have a similar mobility to XG9 in EAW (Fry, 1986b). However, resolution of XG9 from other similar sized xyloglucan oligosaccharides (e.g. XG7) is poor, in this solvent system.

3.2.5 Discussion of results

During prolonged incubation of spinach suspension cultures with [*xylosyl*-^{3}H]xyloglucan oligosaccharides (enriched in XG9), negligible amounts of ^{3}H were taken up by the cells or became associated with insoluble (cell wall) material. This suggests that cells take up little oligosaccharide and that negligible exogenous oligosaccharide was broken down to release [^{3}H]xylose, or any other ^{3}H-labelled sugar which can be transported across the plasma membrane.

G.p.c. of the incubation medium revealed that although the apoplast of spinach suspension culture did contain hydrolytic activity towards the exogenous oligosaccharides, very little ^{3}H-labelled monomeric material was released. The major ^{3}H-labelled breakdown products of the [*xylosyl*-^{3}H]xyloglucan oligosaccharides eluted from the Bio-Gel P-2 column with K_{AV}s of: 0.63, 0.76, and 0.89. That is, they have molecular weights between that of XG9 (K_{AV}, 0.41) and monomeric sugars (K_{AV}, 1.00). Plant material has been shown to contain α-xylosidase, β-glucosidase, α-fucosidase and β-galactosidase activities which will cleave the glycosidic linkages of xyloglucan oligosaccharides (O'Neill *et al.*, 1988). An enzyme has been purified from *Aspergillus oryzae,* which hydrolyses the substituted β-(1-4)-glucosyl backbone of xyloglucan oligosaccharides (Kato *et al.*, 1985). It has been suggested that in plants the α-xylosyl-substituted β-(1-4)-glucosyl backbone of xyloglucan oligosaccharides, is cleaved by the sequential splitting of the α-xylosidic linkage, followed by the β-glucosidic linkage (Koyama *et al.*, 1983).

Transglycosylation involves both the cleavage and synthesis of glycosidic bonds. Therefore the process of incorporation of ^{3}H-labelled material from xyloglucan oligosaccharides into the high molecular weight product may involve the mid-chain cleavage of the oligosaccharide. This cleavage could result in the liberation of small, ^{3}H-labelled fragments from the oligosaccharides.

The amount of ^{3}H associated with products of oligosaccharide hydrolysis is high compared to that found by Baydoun and Fry (1989). This could be due to:

(1). a higher specificity of the hydrolytic enzymes for the contaminants of the [*xylosyl*-^{3}H]XG9 preparation than for XG9 itself,

(2). an enhancement of enzyme activity by these contaminants, or

(3). the cells used in this experiment were behaving differently towards [*xylosyl*-^{3}H]oligosaccharides (e.g. due to age).

The major ^{3}H-labelled metabolic product formed in the apoplast of spinach suspension cultures during incubation with [*xylosyl*-^{3}H]xyloglucan oligosaccharides was a high molecular weight material. That is, rather than eluting from Bio-Gel P-2 with a K_{AV} of 0.41 (that of XG9), some ^{3}H-labelled material eluted from the column

with a K_{AV} of 0.00. High molecular weight ^{3}H-labelled material could be produced either by extracellular transglycosylation, or by intracellular polymer synthesis, using [^{3}H]oligosaccharide breakdown products. ^{3}H-Labelled monomeric sugars can pass across the plasma membrane. Once intracellular, sugars can be phosphorylated and subsequently attached to nucleotides by a series of scavenger reactions. The activated sugars can then be interconverted (for review, Gander, 1976). However, plants do not have a xylose kinase which would phosphorylate xylose directly. Once intracellular, xylose is converted to xylulose by an isomerase. Xylulose kinase phosophorlylates the xylulose, producing xylulose-5-phosphate (Zahnley and Axelrod, 1965), an intermediate in the pentose phosphate pathway (for review, Gander, 1976) The ^{3}H-label associated with intracellular xylose will therefore become associated with numerous sugar phosphates. As a result, all polyasccharide fractions of the cell wall may become ^{3}H-labelled (Carpita *et al.*, 1982). Acid hydrolysis and Driselase digestion of a ^{3}H-labelled polysaccharide, synthesised intracellularly from 'scavenged' [^{3}H]xylose, would release many ^{3}H-labelled hydrolysis products. However, the major ^{3}H-labelled hydrolysis products released from the high molecular weight ^{3}H-labelled product by acid hydrolysis and Driselase digestion, were xylose and isoprimeverose, respectively. The original oligosaccharide preparation yielded the same ^{3}H-labelled hydrolysis products. This suggests that during incubation of [*xylosyl*-^{3}H]oligosaccharides with spinach culture, the ^{3}H-labelled material underwent an increase in apparent molecular weight without significant breakdown or re-arrangement. This supports the supposition that the high molecular weight ^{3}H-labelled material is a product of extracellular transglycosylation. *Trichoderma* cellulase treatment of the ^{3}H-labelled high molecular weight product released [*xylosyl*-^{3}H]XG9 or a ^{3}H-labelled oligosaccharide with a similar R_F to XG9 in EAW. There are no cellulase-labile bonds within the exogenous [*xylosyl*-^{3}H]xyloglucan oligosaccharides. The release of an XG9-like oligosaccharide from the high molecular weight product by cellulase therefore suggests that the [^{3}H]oligosaccharide was incorporated into an apoplastic polymer with little re-arrangement, directly through a cellulase-labile bond, or near to a cellulase-labile bond within the polymer.

These results confirm the findings of Baydoun and Fry (1989) and suggest that the exogenous oligosaccharide is a substrate for an extracellular transglycosylase. The mechanism of this putative extracellular transglycosylation was investigated further to determine if the exogenous oligosaccharide is the glycosyl donor or acceptor in the proposed reaction.

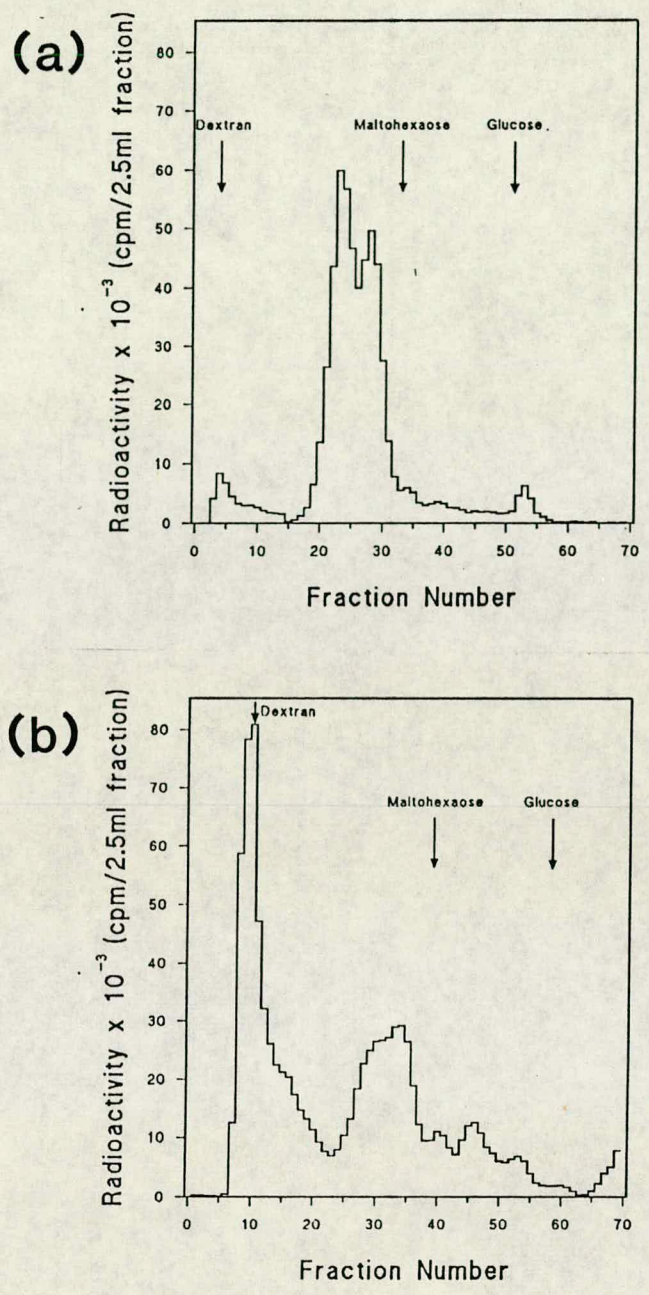


Fig 3.2.1 Bio-Gel P-2 chromatography of soluble extracellular products formed during incubation of [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation with spinach suspension cultures

[*xylosyl*-^{3}H]Xyloglucan oligosaccharides were incubated in (a) cell-free fresh medium for 72 h, and (b) day-4 spinach culture for 72 h. ^{3}H-Labelled material in the incubation media were separated by Bio-Gel P-2 chromatography. The positions of the internal markers dextran (K_{AV}, 0.00), maltohexaose (K_{AV} approximately, 0.57), and glucose (K_{AV}, 1.00) are shown.

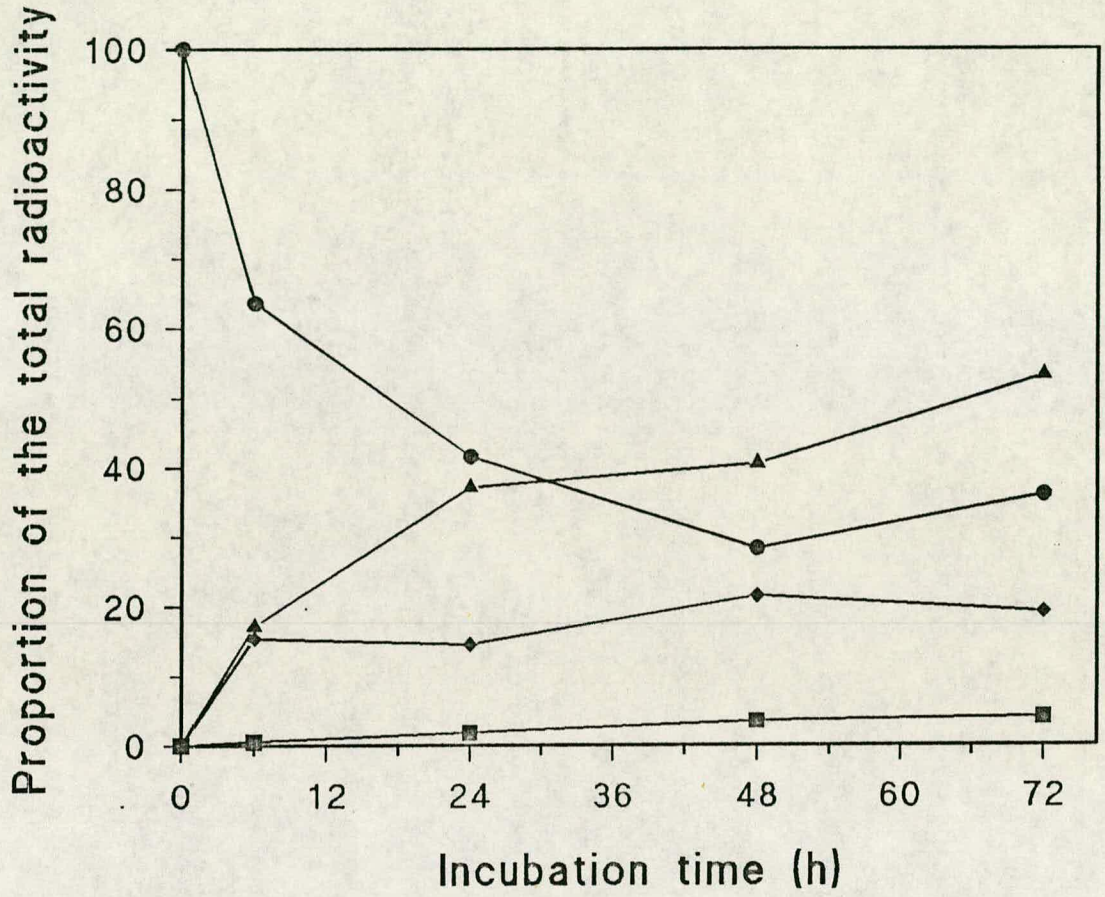


Fig 3.2.2 Fate of the xylosyl residues of xyloglucan oligosaccharides during incubation of spinach suspension cultures with [*xylosyl*-^{3}H]xyloglucan oligosaccharides

Spinach suspension cultures were incubated with [*xylosyl*-^{3}H]xyloglucan oligosaccharides for up to 72 h. The following components of suspension culture were assayed for tritium after 6, 24, 48, and 72 h incubation: spinach cells (■); low molecular weight breakdown products (◆); soluble apoplastic polymer (▲); and unchanged [^{3}H]XG9 and [^{3}H]XG7 (●). Data for ●, ▲, and ◆ were obtained from Bio-Gel P-2 chromatography (e.g. Fig. 3.2.1). All data are corrected relative to the cell-free controls.

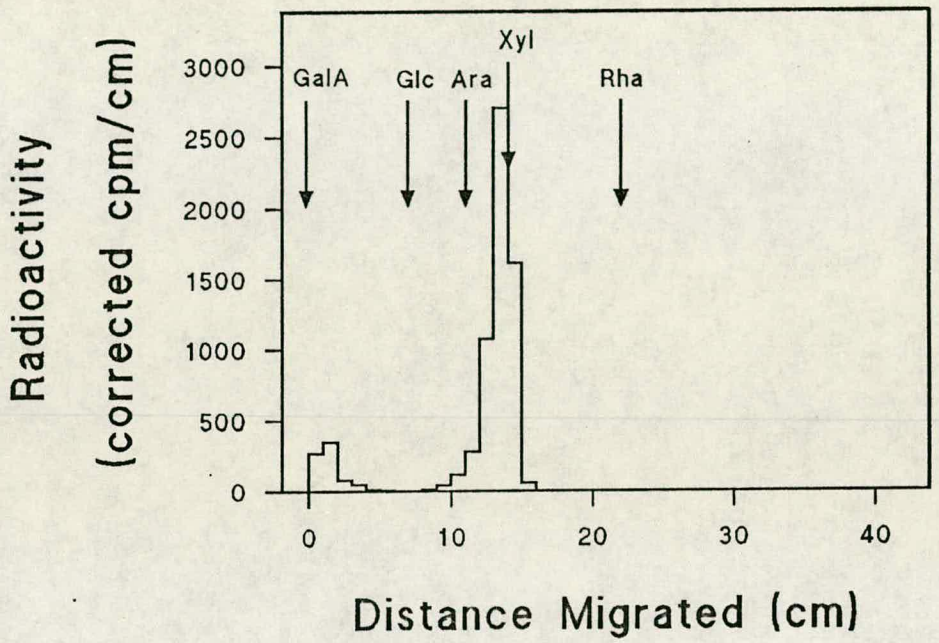


Fig 3.2.3 Paper chromatography of ^{3}H-labelled material released from high molecular weight incubation products, by acid hydrolysis

High molecular weight material was recovered from the void volume of the g.p.c. shown in Fig. 3.2.1, and subjected to TFA hydrolysis (products separated in EPW). The positions of the external markers galacturonic acid (GalA), glucose (Glc), arabinose (Ara), xylose (Xyl) and rhamnose (Rha) are shown

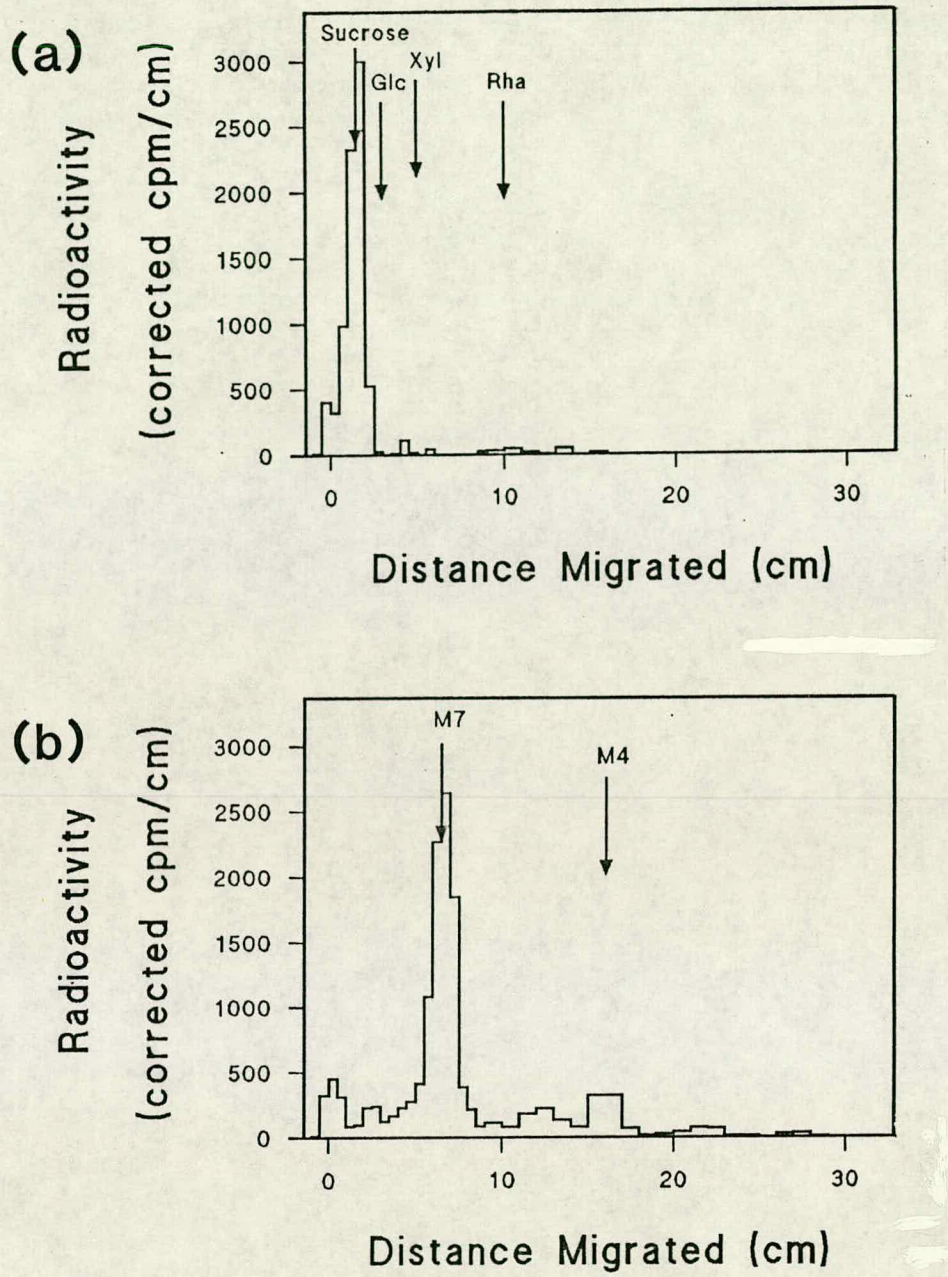


Fig 3.2.4 Paper chromatography of ^{3}H-labelled material released from high molecular weight incubation products, by Driselase digestion and *Trichoderma* cellulase digestion

High molecular weight material was recovered from the void volume of the g.p.c. shown in Fig. 3.2.1, and subjected to (a) Driselase digestion (products separated in EPW); (b) *Trichoderma* cellulase digestion (products separated in EAW). The positions of the external markers sucrose, glucose (Glc), xylose (Xyl), rhamnose (Rha), maltoheptaose (M7) and maltotetraose (M4) are shown.

3.3 Preparation of [*reducing terminus*-^{3}H]XG9

3.3.1 Introduction

In order of determine the nature of the glycosyl donor and acceptor in the putative extracellular endo-transglycosylation described in section 3.2, the fate of the XG9 reducing terminus in the reaction must be determined. In a transglycosylation reaction the glycosyl donor is cleaved, and a glycosyl-enzyme intermediate may be formed. The newly formed potentially reducing terminus of the glycosyl moiety of this intermediate is transferred onto an acceptor and a new glycosidic linkage is formed. Therefore the original reducing terminus of the glycosyl donor will not become incorporated into the high molecular weight transglycosylation product. The reducing terminus of the acceptor molecule in such a reaction remains unaltered as the reducing terminus of the high molecular weight product.

In order to follow the fate of the reducing terminus of XG9 in spinach suspension cultures, an oligosaccharide specifically ^{3}H-labelled in the reducing terminal glucose unit was prepared. This oligosaccharide was subsequently used as a substrate for the putative extracellular transglycosylation reaction detected in spinach cultures.

3.3.2 Analysis of [*reducing terminus*-^{3}H]XG9 preparation

The [*reducing terminus*-^{3}H]XG9 preparation was analysed to determine both its purity and the position of the ^{3}H-label in the oligosaccharide.

3.3.2.1 Chromatographic behaviour of [*reducing terminus*-^{3}H]XG9 preparation

3.3.2.1.1 Paper chromatography

Samples of [^{3}H]XG9 preparation (3.2 kBq) were applied to chromatography paper. One chromatogram was developed in BPW and another in EAW (section 2.6.3). External markers were visualised by alkali-silver nitrate stain (section 2.8.3).

All of the ^{3}H-labelled material co-chromatographed with maltoheptaose or authentic XG9 (Fig. 3.3.1ab). Maltoheptaose has a similar mobility to XG9 in BPW (Fry, 1986b).

3.3.2.1.2 H.p.l.c.

A sample of [*reducing terminus*-^{3}H]XG9 containing an internal XG9 marker was subjected to h.p.l.c. on a Brownlee Amino-Spheri-5 column (section 2.6.5). Total carbohydrate in the eluent was monitored by refractive index. Radioactive material

present in the eluent was detected by a RAMONA flow-through monitor (section 2.8.2.3).

A single peak of ^{3}H-labelled material was eluted from the column which co-chromatographed with the main peak in refractive index (Fig. 3.3.2).

3.3.2.2 TFA hydrolysis of [*reducing terminus*-^{3}H]XG9 preparation

Samples of [^{3}H]XG9 (0.8 kBq and 0.7 MBq) were subjected to complete acid hydrolysis in 2 M-TFA (section 2.7.1). The products of hydrolysis of the 0.8 kBq sample were separated by p.c. in EPW (section 2.6.3). External markers were visualised by alkali-silver nitrate stain (section 2.8.3). This preparation (0.8 kBq) yielded [^{3}H]glucose as the sole ^{3}H-labelled hydrolysis product. (Fig. 3.3.3a).

The larger sample of hydrolysed [^{3}H]XG9 (0.7 MBq) was subjected to t.l.c. in EPW (section 2.6.4). A mixture of the monomeric sugars which are known to make up XG9, in the appropriate molar ratio (i.e. glucose:xylose:galactose:fucose, 4:3:1:1), was also subjected to t.l.c. as the external marker. A range in amount of this marker mixture was applied to the t.l.c. plate. The external markers and hydrolysed sample were visualised by aniline hydrogen-phthalate stain (section 2.8.3). Measurements of the intensity of staining (a measurement of the amount of reducing sugar present on the t.l.c. plate) were made by densitometry (section 2.8.3.1)

Two spots of aniline hydrogen-phthalate staining material were separated by t.l.c. of the hydrolysate of [^{3}H]XG9 preparation (0.7 MBq sample; Fig. 3.3.3b). The less mobile spot appeared to be composed of two partially resolved components, galactose and glucose (which were found to co-chromatograph in the marker mixture). A second, more mobile spot, exhibited a similar R_F value to both fucose and xylose external markers.

The ratio of the densities of staining of the two resolved spots separated by t.l.c. was used to calculate the ratio of glucose-galactose to xylose-fucose present in the TFA hydrolysate of [^{3}H]XG9. This ratio was found to be approximately 5:4 (mol/mol).

3.3.2.2.1 Specific activity of [reducing terminus-^{3}H]XG9 preparation

The total amount of carbohydrate present in the acid hydrolyses 0.7 MBq sample of [^{3}H]XG9 was determined by the density of aniline hydrogen-phthalate staining of the glucose-galactose peak separated by t.l.c. (Fig. 3.3.3b). External markers of glucose-galactose (molar ratio, 4:1), containing a range in the amount of total sugar, were stained. A calibration curve relating total sugar to density of staining was constructed. Hence, the amount of glucose-galactose present in the acid hydrolysate could be calculated. The total amount of the nonasaccharide was determined, using

the expected ratios of the sugar residues known to be present. The specific activity of the [^{3}H]XG9 preparation was thus calculated (12 TBq.mol^{-1}).

3.3.2.3 Sodium borohydride reduction followed by TFA hydrolysis of [*reducing terminus*-^{3}H]XG9 preparation

A sample of [^{3}H]XG9 preparation (0.8 kBq) was subjected to sodium borohydride reduction (section 2.7.3). All reducing termini in the [^{3}H]XG9 preparation were thus assumed to be reduced to alditols. The reduced material was subsequently hydrolysed in 2 M-TFA (section 2.7.1). The hydrolytic products were separated by p.c. in BAB (section 2.6.3). External markers were visualised by potassium permanganate stain (section 2.8.3).

The sole ^{3}H-labelled product released from the reduced [^{3}H]XG9 preparation by acid hydrolysis co-chromatographed with glucitol (the alditol of glucose) external marker (Fig. 3.3.4).

3.3.2.4 Driselase digestion of [*reducing terminus*-^{3}H]XG9 preparation

A sample (0.8 kBq) of [^{3}H]XG9 preparation was incubated with 1.25% Driselase solution (section 2.7.4) and the products of digestion were separated by p.c. in EPW (section 2.6.3). External markers were visualised by alkali-silver nitrate stain (section 2.8.3).

The major ^{3}H-labelled product released from [^{3}H]XG9 preparation by Driselase digestion showed a similar mobility to the glucose external marker (Fig. 3.3.5a).

3.3.2.5 *Trichoderma* cellulase digestion of [*reducing terminus*-^{3}H]XG9 preparation

[^{3}H]XG9 (3.1 kBq) was incubated with 0.1% *Trichoderma* cellulase solution (section 2.7.5). An enzyme-free control ([^{3}H]XG9 in buffer alone) was incubated alongside. Products released from the oligosaccharide preparation were separated by p.c. in EAW (section 2.6.3). External markers were visualised with alkali-silver nitrate (section 2.8.3).

The only ^{3}H-labelled material found in the cellulase digests showed a similar mobility to the maltoheptaose external marker, and the ^{3}H-labelled substance in the enzyme-free control (Fig. 3.3.5b).

3.3.3 Discussion of results

Only one [^{3}H]oligosaccharide was found to be present in the [*reducing terminus*-^{3}H]XG9 preparation by p.c. in both EAW and BPW. This oligosaccharide co-chromatographed with maltoheptaose in BPW, and with authentic XG9 in EAW. However, XG9 is not clearly resolved from XG7 in either solvent. The [*reducing*

terminus-^{3}H]XG9 preparation was found to be h.p.l.c. pure. Thus XG9 was the sole ^{3}H-labelled material in a system that resolves XG9 from XG7 (McDougall and Fry, 1991).

TFA hydrolysis of the [^{3}H]XG9 preparation yielded [^{3}H]glucose as the sole ^{3}H-labelled product. Therefore all of the ^{3}H-label was associated with glucose units within the oligosaccharide. This ^{3}H-labelled unit was reduced by sodium borohydride, and released as [^{3}H]glucitol on acid hydrolysis. As sodium borohydride will only reduce the reducing terminus of an oligosaccharide, the ^{3}H-label was associated only with the reducing terminal glucose unit of XG9. Further evidence for this position of the ^{3}H-label is the release of [^{3}H]glucose by Driselase digestion of [*reducing terminus*-^{3}H]XG9 preparation. Driselase is a commercial enzyme preparation which catalyses the hydrolysis of all the glycosidic linkages within XG9, apart from the α-xylosyl branches (Fry, 1988). The only unsubstituted glucose unit in XG9 is at the reducing terminus of the oligosaccharide (Fig. 3.3.6). This glucose unit is the only glucose unit which will be released as monomeric glucose by the action of Driselase (all other glucose residues will be released as isoprimeverose).

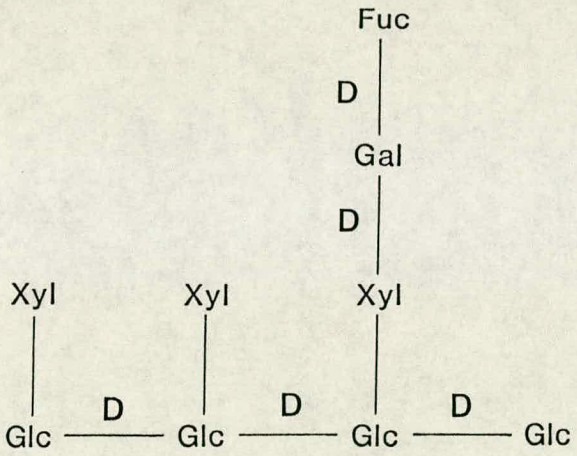


Fig. 3.3.6 The structure of xyloglucan nonasaccharide.
D, denotes Driselase labile bonds within the oligosaccharide

The four monomeric units known to be present in XG9 (i.e. glucose, xylose, galactose and fucose, Valent *et al.*, 1980) were not wholly resolved by t.l.c. in EPW. Galactose and glucose were partially resolved from one another, but xylose and fucose co-chromatographed. Complete acid hydrolysis of a 0.7 MBq sample of [*reducing terminus*-^{3}H]XG9 yielded glucose-galactose and xylose-fucose in a similar manner to the external markers. The ratio of glucose-galactose to xylose-fucose in the [^{3}H]XG9

preparation was found to be similar to that of XG9 (i.e. molar ratio, 5:4). This suggests that the [^{3}H]oligosaccharide in the [*reducing terminus*-^{3}H]XG9 preparation is indeed similar if not identical to XG9.

The [^{3}H]XG9 preparation was subjected to *Trichoderma* cellulase digestion. No ^{3}H-labelled digestion products were released. The [^{3}H]XG9 preparation therefore does not contain any cellulase labile bonds (i.e. unsubstituted β(1-4) linked glucosyl residues). This is consistent with the [^{3}H]XG9 preparation containing only XG9.

The [*reducing terminus*-^{3}H]XG9 prepared as described above was used as a substrate for the proposed extracellular transglycosylation reaction. The role of the exogenous oligosaccharide in the putative transglycosylation reaction could therefore be determined by demonstrating the fate of the ^{3}H-label during incubation of spinach suspension cultures with [*reducing terminus*-^{3}H]XG9. The [*reducing terminus*-^{3}H]XG9 preparation had been shown to contain XG9 as the sole ^{3}H-labelled component. The specific activity of the preparation was found to be 12 TBq.mol^{-1} with all of the ^{3}H-label in the prepation being present in the reducing terminal glucose unit of the oligosaccharide.

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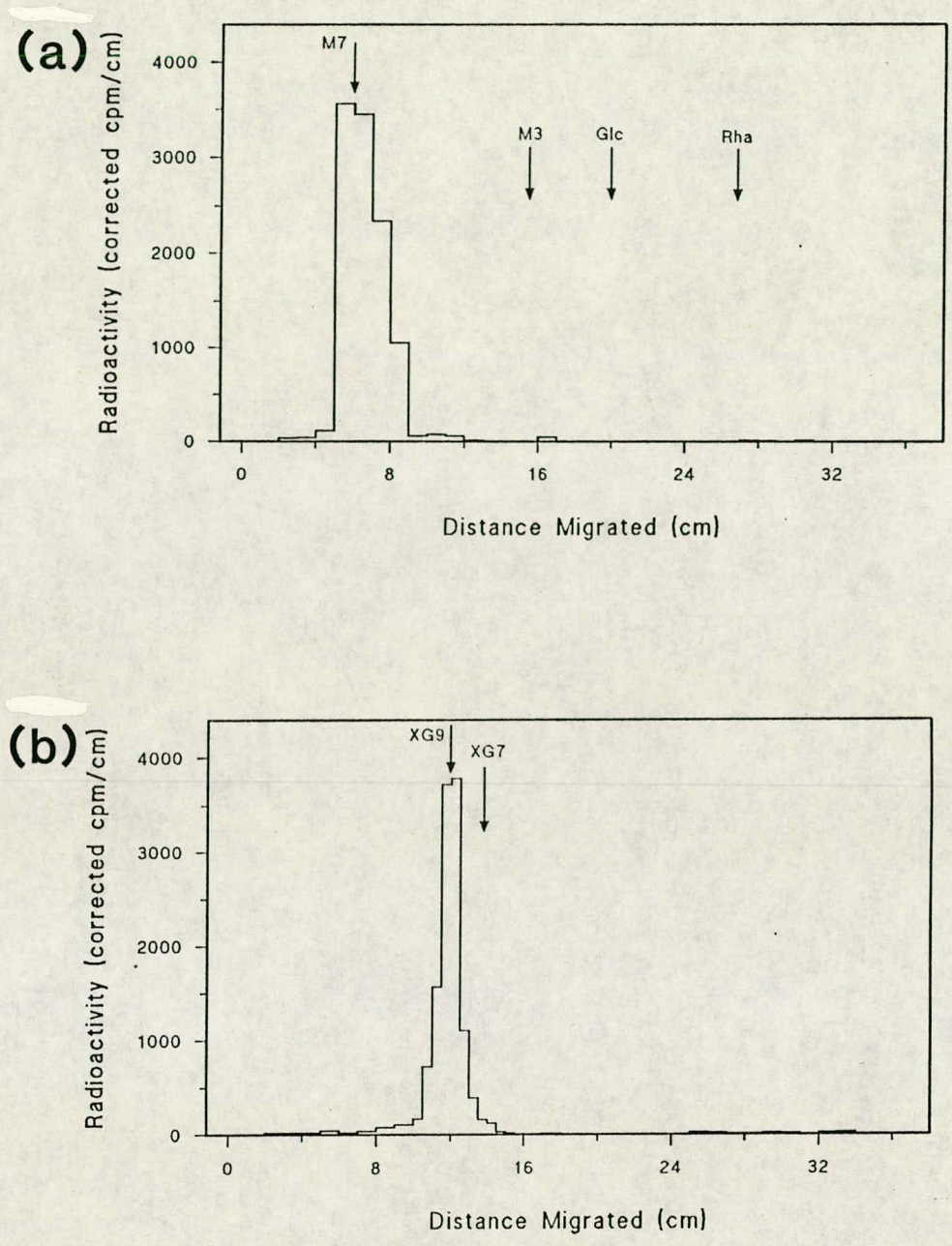


Fig 3.3.1 Paper chromatography of [*reducing terminus*-^{3}H]XG9 preparation

Samples of [*reducing terminus*-^{3}H]XG9 preparation were subjected to paper chromatography in (a) BPW and (b) EAW. The positions of external markers are shown.

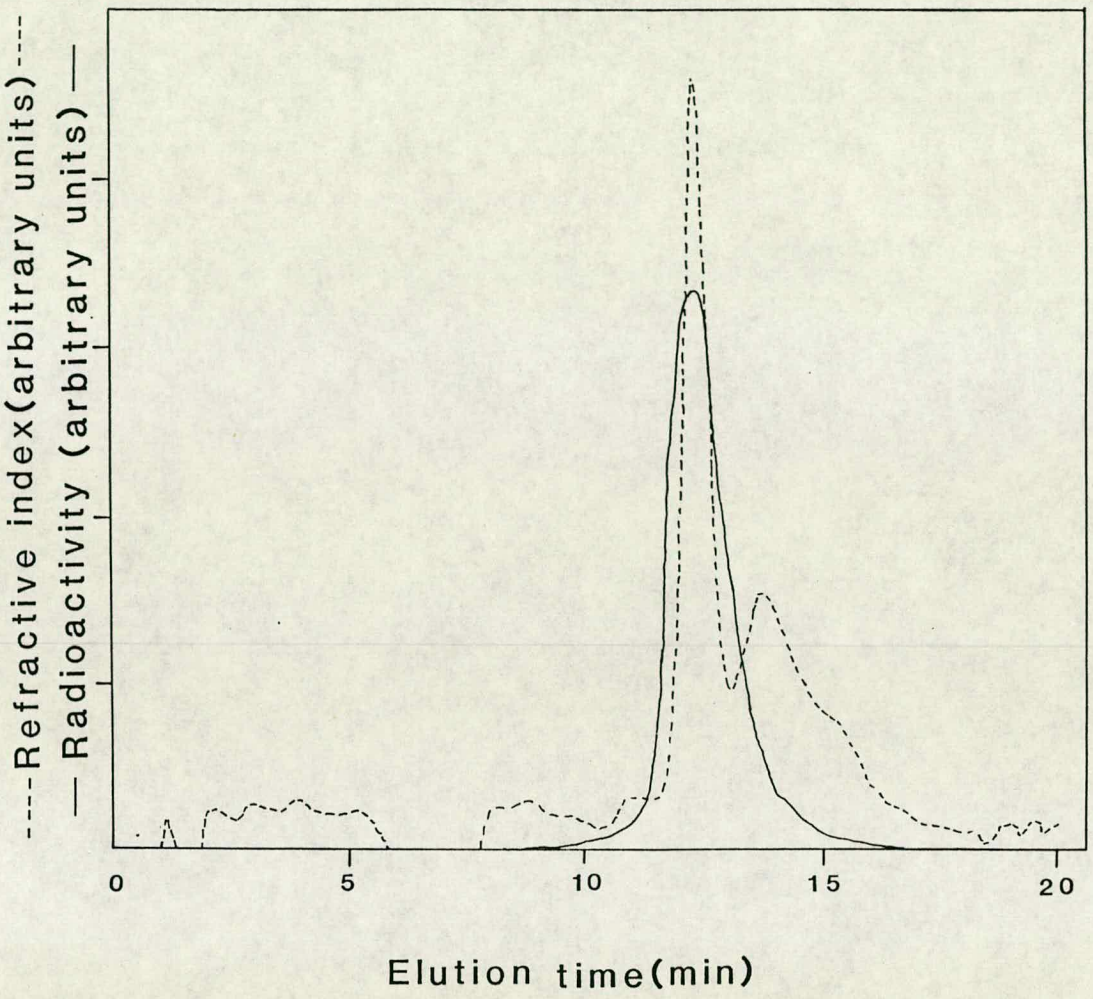


Fig 3.3.2 H.p.l.c. of [*reducing terminus*-^{3}H]XG9

A sample of [*reducing terminus*-^{3}H]XG9 was subjected to h.p.l.c. on an Amino-5-Spheri column (—). Material was eluted with 60% (v/v) aqueous acetonitrile. The elution of the internal marker of XG9 (---)is shown.

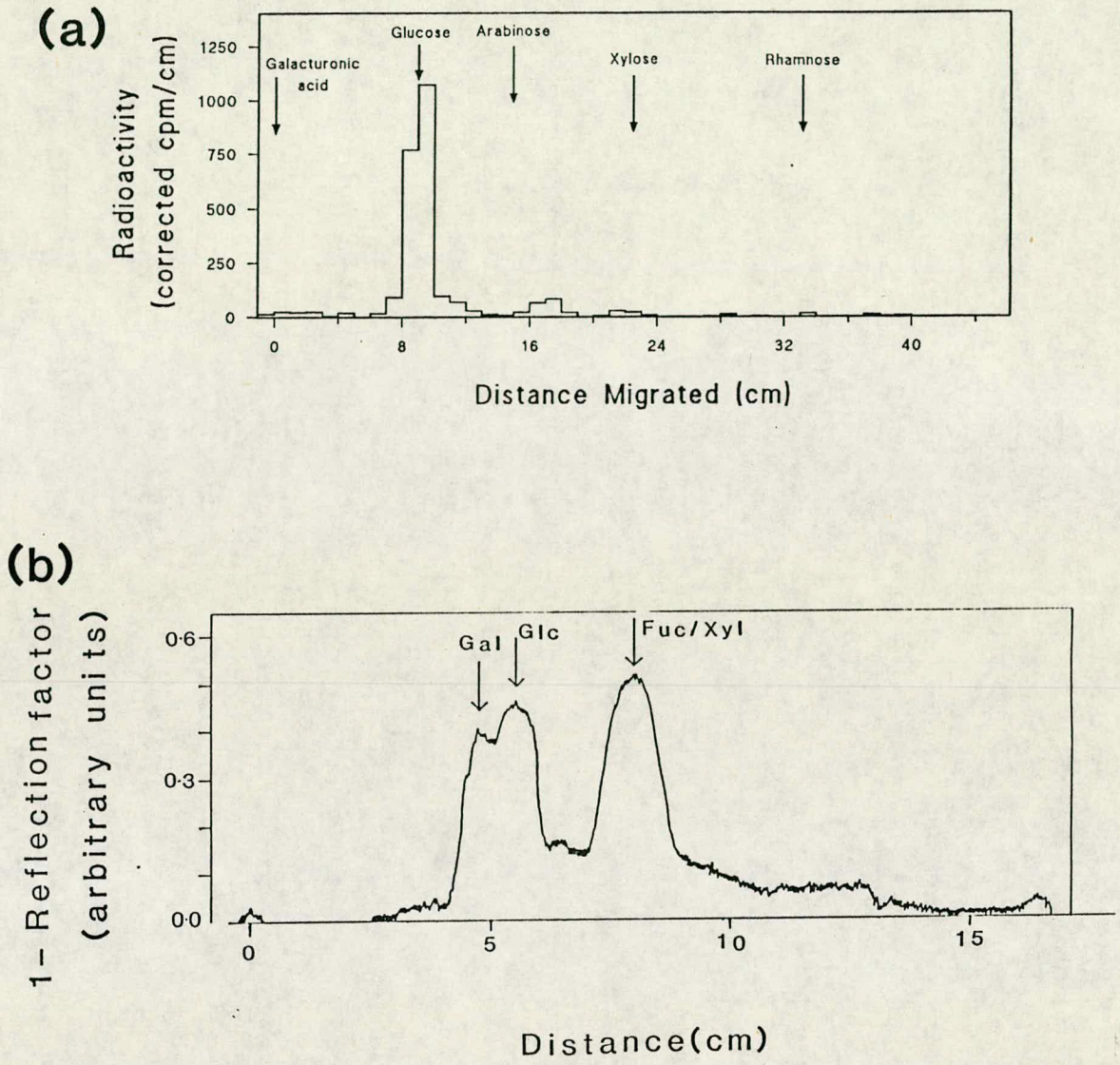


Fig 3.3.3 Chromatography of material released from [*reducing terminus*-^{3}H]XG9 preparation by acid hydrolysis

(a) A 0.8 kBq sample of [*reducing terminus*-^{3}H]XG9 preparation was subjected to TFA hydrolysis. The ^{3}H-labelled products were separated by p.c. in EPW. The positions of the external markers are shown.

(b) A 0.7 MBq sample of [*reducing terminus*-^{3}H]XG9 preparation was subjected to TFA hydrolysis. The products were separated by t.l.c. in EPW, stained with aniline hydrogen-phthalate, and the reflection factor measured across the plate by a scanning densitometer. The positions of the external markers are shown.

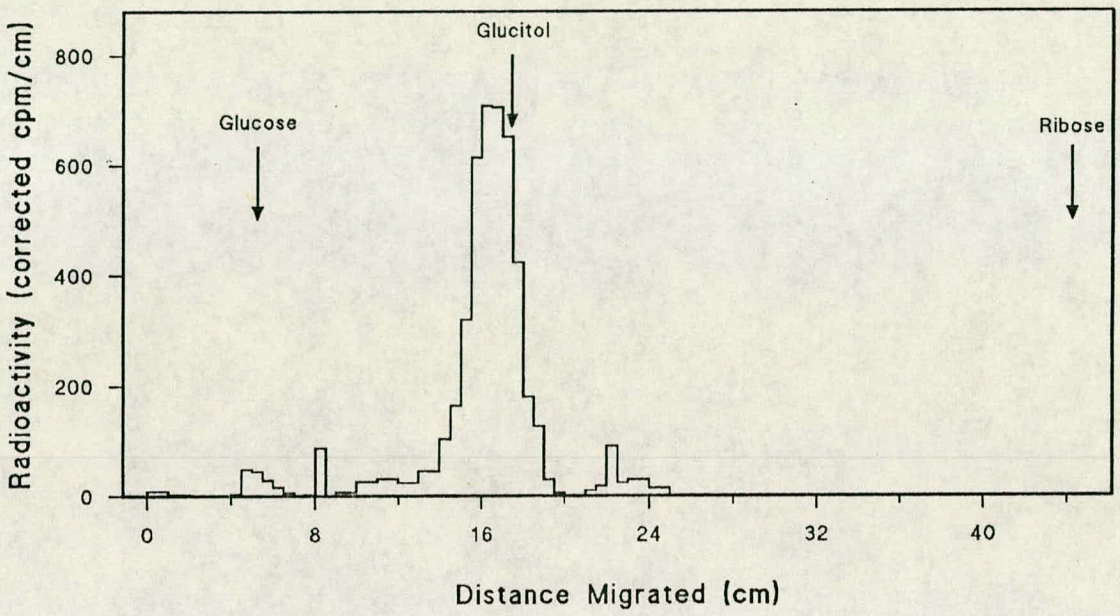


Fig 3.3.4 Paper chromatography of ^{3}H-labelled material released from [*reducing terminus*-^{3}H]XG9 preparation by acid hydrolysis after reduction with sodium borohydride.

A sample of [*reducing terminus*-^{3}H]XG9 preparation was subjected to sodium borohydride reduction followed by TFA hydrolysis. The products were separated in BAB. The positions of external markers are shown.

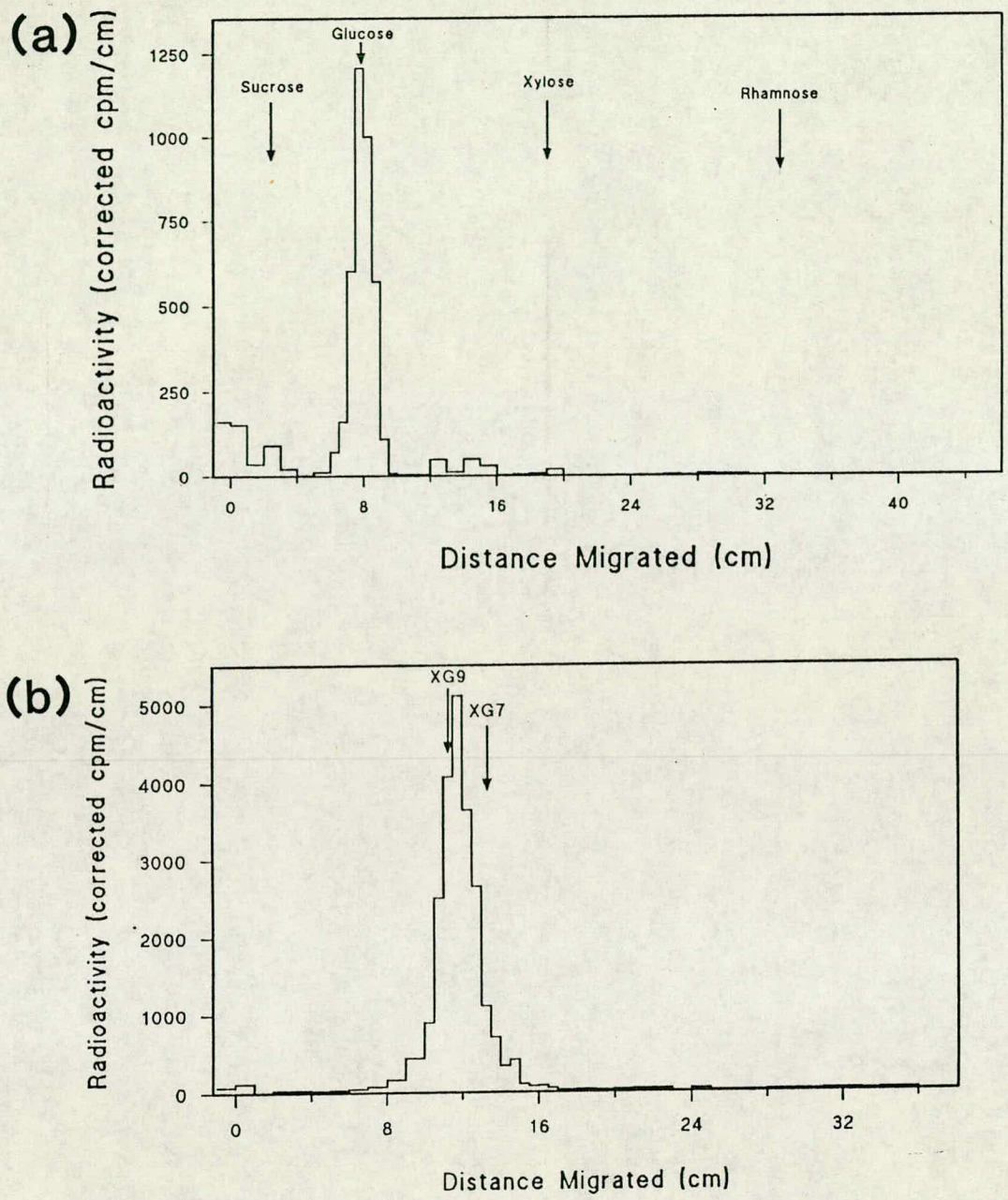


Fig 3.3.5 Paper chromatography of ^{3}H-labelled material released from [*reducing terminus*-^{3}H]XG9 preparation by Driselase and *Trichoderma* cellulase digestion

[*reducing terminus*-^{3}H]XG9 preparation was subjected to (a) Driselase digestion (products separated in EPW); and (b)*Trichoderma* cellulase digestion (products separated in EAW). The positions of external markers are shown.

3.4 Investigation into the nature of the glycosyl donor and acceptor in the putative extracellular transglycosylation reaction detected in spinach suspension cultures

3.4.1 Introduction

Exogenous [*xylosyl*-^{3}H]xyloglucan oligosaccharides were found to undergo an apparent increase in molecular weight in the apoplast of spinach cell suspension cultures (section 3.2). This increase in molecular weight does not involve significant breakdown or re-arrangement of the oligosaccharides. It has been postulated that this is a demonstration of extracellular transglycosylation. Transglycosylation is the transfer of a glycosyl moiety from a donor molecule onto an acceptor. The aim of this experiment was to determine if exogenous xyloglucan nonasaccharide is the glycosyl donor or acceptor in the putative transglycosylation reaction. The oligosaccharide used as a substrate was [*reducing terminus*-^{3}H]XG9. The tritium label was on carbon 1 of the reducing terminal glucose unit (section 3.3). If XG9 is the glycosyl donor in the proposed reaction (Fig. 3.4.1a), the reducing terminus of the oligosaccharide will be lost and the tritium label will not be transferred onto the polymeric acceptor.

Baydoun and Fry (1989) reported that few of the oligosaccharide α-xylosyl side groups were lost by breakdown of the XG9 substrate in the apoplast of spinach cultures. Consequently, the proposed transglycosylation mechanisms (Baydoun and Fry, 1989) did not involve any cleavage of the oligosaccharide which would result in the liberation of low molecular weight xylose-containing or fucose-containing material (Fig. 3.4.1). It was suggested that the reducing terminal glucose unit may have been cleaved from XG9 by a transglycosylase, and the newly formed reducing terminus of the oligosaccharide transferred onto a large, glycosyl acceptor. No plant cell wall enzyme is known to break the glycosidic bond between a substituted glucosyl residue and the reducing terminal glucose unit of XG9. However, evidence for such an enzyme activity is the detection of a xyloglucan oligosaccharide similar to XG9 but lacking the unsubstituted reducing terminal glucose unit in the apoplast of plant suspension cultures (Fry, 1986b). In the experiment described in section 3.2 significant amounts of low molecular weight ^{3}H-labelled material were lost from exogenous [*xylosyl*-^{3}H]XG9 in the apoplast of spinach suspension cultures. Incorporation of ^{3}H-labelled material into a polymer may therefore involve the mid-chain cleavage of XG9 (if the exogenous oligosaccharide is the glycosyl donor in the reaction). If XG9 is the glycosyl donor in the reaction the reducing terminal glucose

unit of the oligosaccharide will be lost, and not incorporated into the polymeric product, regardless of where in the oligosaccharide cleavage takes place.

However, if the oligosaccharide is the glycosyl acceptor in the reaction (Fig. 3.4.1b), the tritium label will become associated with the high molecular weight product. The initial bond cleavage in the polymeric glycosyl donor must occur mid-chain, by an endo-acting enzyme (Fig. 3.4.1b). Endo-transglycosylation of cell wall polysaccharides is more likely to alter significantly the physical properties of the wall than the simple transfer of terminal sugar residues from one molecule to another.

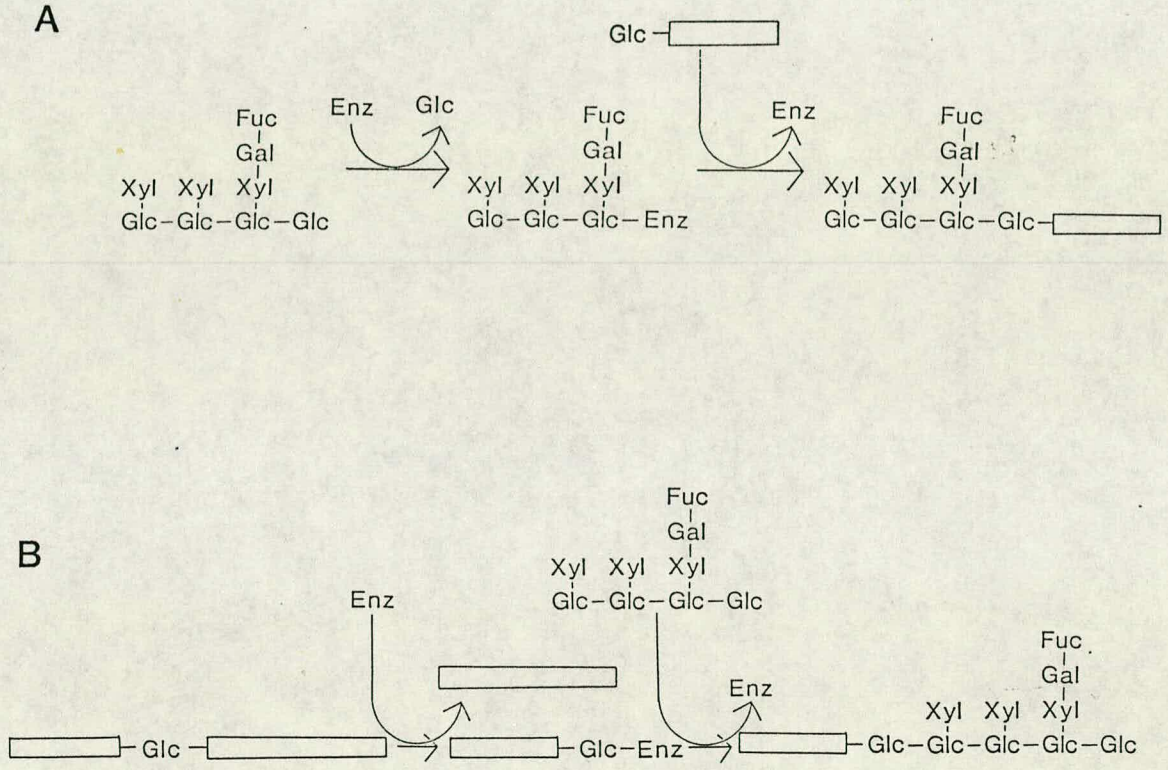


Fig. 3.4.1 Two possible mechanisms by which [*xylosyl*-^{3}H]XG9 may be incorporated into an apoplastic polymer during incubation of plant cell suspension cultures with this oligosaccharide.

A XG9 is the glycosyl donor to an apoplastic transglycosylation reaction.

B XG9 is the glycosyl acceptor the apoplastic transglycosylation reaction.

3.4.2 Incubation of spinach cell suspension cultures with [*reducing terminus*-^{3}H]XG9

Aliquots of spinach cell suspension culture (3 ml, of day-4 culture) were incubated with [*reducing terminus*-^{3}H]XG9 for 6, 24, 48, and 72 h (section 2.5.1). Cell-free controls, containing fresh medium in place of cell suspension culture, were also incubated with [^{3}H]XG9. Cells were then separated from the incubation medium by centrifugation (section 2.5.1). The cells were washed to remove non-incorporated ^{3}H. Samples of incubation medium and cells were assayed for radioactivity (section 2.8.2.1).

Table 3.4.1 Proportion of ^{3}H taken up into cells and/or associated with insoluble (cell wall) material

Incubation time	Proportion of ^{3}H taken up into and/or associated with cells (%)
6 h	0.41
24 h	0.63
48 h	0.19
72 h	1.90

Negligible amounts of ^{3}H were taken up into the cells or incorporated into insoluble (cell wall) material. During 72 h incubation of spinach cells with [*reducing terminus*-^{3}H]XG9, 98.1% of the total ^{3}H-labelled material added remained extracellular and soluble.

3.4.3 Bio-Gel P-2 chromatography of incubation medium

Incubation medium (section 2.5.1) was subjected to g.p.c. on a Bio-Gel P-2 column (section 2.6.1), together with internal molecular weight markers (dextran K_{AV}, 0.00; glucose K_{AV}, 1.00; and maltohexaose). Fractions (2.5 ml) were collected and a portion was assayed for tritium (section 2.8.2.1). The position of the internal markers was determined by the measurement of hexose in each fraction (section 2.8.1).

The results of Bio-Gel P-2 chromatography are shown in Fig. 3.4.2ab. Very little ^{3}H was associated with material of lower molecular weight than the exogenous XG9. During 72 h incubation with [*reducing terminus*-^{3}H]XG9, a maximum of 6.4% of the total added ^{3}H-labelled material eluted from Bio-Gel P-2 with a K_{AV} greater than that of XG9 (Fig. 3.4.2b). However, very low levels of ^{3}H-labelled monomers (K_{AV}, 1.00)

were released into the incubation medium. The major ^{3}H-labelled product in the incubation medium was a high molecular weight compound (K_{AV}, 0.00). During 72 h incubation of spinach cells with [*reducing terminus*-^{3}H]XG9 56.5% of the total ^{3}H underwent an apparent increase in molecular weight (Fig. 3.4.3). Some unchanged XG9 remained in the medium following 72 h incubation (Fig. 3.4.3).

3.4.4 Analysis of ^{3}H-labelled, high molecular weight product

Fractions from Bio-Gel P-2 chromatography (section 2.6.1) which contained [^{3}H]polymer (K_{AV}, 0.00) were pooled and divided into portions (250 µl and 1 ml) which were dried *in vacuo* and the ^{3}H-labelled high molecular weight material was analysed.

3.4.4.1 Chromatographic behaviour of [^{3}H]polymer

Samples of [^{3}H]polymer were applied to chromatography paper. The chromatogram was developed in BAW (section 2.6.3).

All ^{3}H-labelled material remained at the origin of the chromatogram (data not shown)

3.4.4.2 TFA hydrolysis

[^{3}H]Polymer (dried from 250 µl) was hydrolysed in 2 M-TFA (section 2.7.1). The products of hydrolysis were separated by p.c. in EPW (section 2.6.3).

The sole ^{3}H-labelled hydrolysis product co-chromatographed with the glucose external marker (Fig. 3.4.4a).

3.4.4.3 Alkali hydrolysis

^{3}H-Labelled high molecular weight material (dried from 250 µl) was incubated in a 0.5 M-potassium hydroxide solution (section 2.7.2). This alkali treatment will hydrolyse alkali-labile (e.g. ester) bonds within the [^{3}H]polymer. Glycosidic linkages are relatively alkali stable. The neutralised hydrolysate was applied to chromatography paper, and the chromatogram was developed in EAW (section 2.6.3).

All ^{3}H-labelled material remained at the origin of the chromatogram (data not shown).

3.4.4.4 Sodium borohydride reduction, followed by TFA hydrolysis

A portion of [^{3}H]polymer (dried from 250 µl) was subjected to sodium borohydride reduction (section 2.7.3). Any reducing terminal sugar of the polymer was therefore reduced to an alditol. The reduced [^{3}H]polymer was acid hydrolysed (section 2.7.1) and the products of hydrolysis were separated by p.c. in BAB (section 2.6.3).

The major ^{3}H-labelled product of reduction followed by acid hydrolysis co-chromatographed with the glucitol external marker (Fig. 3.4.4b). Negligible ^{3}H-labelled material co-chromatographed with the glucose external marker.

3.4.4.5 Driselase digestion

A portion of [^{3}H]polymer (dried from 250 µl) was incubated with a 0.25% Driselase solution (section 2.7.4). The products of digestion were separated by p.c. in EPW (section 2.6.3).

The major ^{3}H-labelled product released from the polymeric material by Driselase showed a similar mobility to the glucose external marker in EPW (Fig.3.4.5a).

3.4.4.6 *Trichoderma* cellulase digestion

A portion of [^{3}H]polymer (dried from 1 ml) was incubated with 0.1% *Trichoderma* cellulase for 3 h (section 2.7.5). A second portion was incubated, under the same conditions, in pyridine/acetic acid/water (1:1:98, pH 4.7) without cellulase, as a control. The products of digestion (and the controls) were separated by p.c. in EAW (section 2.6.3).

The major ^{3}H-labelled product released by *Trichoderma* cellulase digestion co-chromatographed with the XG9 external marker (Fig. 3.4.5b). The external markers of XG9 and XG7 were, however, poorly resolved.

3.4.4.7 Copper precipitation

Portions of [^{3}H]polymer (dried from 1 ml) were dissolved in 0.5% *Tropaeolum* xyloglucan solution (section 2.2). The non-radioactive XG carrier was precipitated with Fehling's solution (section 2.7.6).

Copper precipitated a negligible amount of ^{3}H-labelled material from samples of [^{3}H]polymer (2.7% of total [^{3}H]polymer), although the non-radioactive carrier XG did form a precipitate. [^{3}H]XG9 did not precipitate with copper (0.2% of total [^{3}H]XG9).

3.4.4.8 Iodine precipitation

Samples of [^{3}H]polymer (dried from 1 ml) were dissolved in 0.038% *Tropaeolum* xyloglucan solution (section 2.2). Xyloglucan present in this solution was precipitated with iodine/potassium iodide (section 2.7.7).

A negligible amount of ^{3}H-labelled material was precipitated from [^{3}H]polymer with iodine (approximatley, 3.5% of the total ^{3}H), even though *Tropaeolum* xyloglucan did produce a dark pellet. A similar amount of ^{3}H was found in the precipitate formed with [^{3}H]XG9 and *Tropaeolum* xyloglucan.

3.4.4.9 TCA precipitation

Samples of [^{3}H]polymer (dried from 1 ml) were disolved in 5% BSA. Protein in this solution was precipitated in 20% TCA (section 2.7.8).

BSA formed a thick precipitate with TCA but negligible amounts of either [^{3}H]polymer or [^{3}H]XG9 were found to precipitate.

3.4.4.10 Cellulose affinity chromatography

A sample of [^{3}H]polymer (dried from 250 µl) and an internal marker of *Tropaeolum* xyloglucan were applied to a cellulose column. Material was eluted from the column sequentially by 20 mM-sodium acetate buffer, 8 M-urea, and 1M-sodium hydroxide (section 2.6.6). The position of the internal xyloglucan marker was determined by the anthrone method (section 2.8.1)

Tropaeolum xyloglucan and 70% of [^{3}H]polymer bound to cellulose (i.e. was not eluted from the column with sodium acetate buffer or 8 M-urea), but was subsequently eluted with 1 M-sodium hydroxide (Fig. 3.4.6). [^{3}H]XG9 did not bind to cellulose (86% was eluted from the column in sodium acetate buffer).

3.4.4.11 Sepharose CL-6B chromatography

The molecular weight of [^{3}H]polymer was estimated by g.p.c. on a Sepharose CL-6B column (section 2.6.2). Sepharose CL-6B will resolve material of molecular weights between 10^4 and 10^6 (determined by polysaccharides). External molecular weight markers (dextrans) were applied to the column to calibrate the elution pattern from the column according to molecular weight (Fig. 3.4.7b).

[^{3}H]Polymer eluted from the column with a K_{AV} of approximately 0.81. The average apparent molecular weight of [^{3}H]polymer was therefore calculated to be 20,000-30,000 (Fig. 3.4.7a). The molecular weight of XG9 is 1370.

3.4.5 Discussion of results

Negligible amounts of ^{3}H-labelled material were taken up by spinach cells, or became associated with insoluble (cell wall) material, during incubation of cells with [*reducing terminus*-^{3}H]XG9. This suggests:

(1) negligible transport of XG9 across the plasma membrane;

(2) negligible breakdown of XG9 releasing ^{3}H-labelled material which is transported across the plasma membrane; and

(3) any product of XG9 metabolism in the apoplast of spinach cell suspension culture is soluble and not associated with the cell wall.

Bio-Gel P-2 chromatography of incubation medium revealed that [*reducing terminus*-^{3}H]XG9 is very stable in the apoplast of spinach suspension cultures. Very

little extracellular breakdown of XG9 was detected. Substantially higher amounts of ^{3}H-labelled breakdown products were released from [*xylosyl*-^{3}H]xyloglucan oligosaccharides than from [*reducing terminus*-^{3}H]XG9 during incubation with spinach suspension cultures. The apparent ambiguity between these two sets of data could be due to an effect of the contaminants of the [*xylosyl*-^{3}H]xyloglucan oligosaccharide preparation on the metabolism of XG9; or due to differing physiological states of the cells used in the two experiments. These explanations are discussed in section 3.2.5.

The major ^{3}H-labelled product formed in the incubation medium was a high molecular weight material. A substantial proportion of ^{3}H-labelled material from the incubation medium eluted from the Bio-Gel P-2 column with a K_{AV} of 0.00 (compared with the K_{AV} of XG9, 0.41). This suggests that XG9 was acting as the glycosyl acceptor, in this putative extracellular transglycosylation. However, it may be argued that the [^{3}H]polymer could be a product of XG9 breakdown followed by uptake of [^{3}H]glucose, conversion of [^{3}H]glucose into many ^{3}H-labelled sugar nucleotides and susbsequent intracellular polymer synthesis. To determine the metabolic route by which the [^{3}H]polymer was produced, the ^{3}H-labelled components of the polymer were analysed by TFA hydrolysis (both before and after sodium borohydride reduction) and Driselase digestion.

[*reducing terminus*-^{3}H]XG9 released [^{3}H]glucose on TFA hydrolysis, and [^{3}H]glucitol on borohydride reduction followed by TFA hydrolysis. When subjected to the same treatments, the [^{3}H]polymer also produced [^{3}H]glucose and [^{3}H]glucitol, respectively, as the major ^{3}H-labelled hydrolysis products. The only ^{3}H-labelled unit in [^{3}H]XG9 and [^{3}H]polymer is therefore the reducing terminal glucose. Driselase digestion of both [^{3}H]XG9 and [^{3}H]polymer released [^{3}H]glucose as the only ^{3}H-labelled product. Again, this is evidence that the only ^{3}H-labelled residue in both [^{3}H]XG9 and [^{3}H]polymer is the reducing terminal glucose. An α-xylosyl-substituted glucosyl residue would be released as isoprimeverose on Driselase digestion (Fry, 1988). These data provide evidence that exogenous XG9 remains unaltered on incorporation into a polymer. The ^{3}H-label remained associated with a glucose unit during incorporation into a soluble apoplastic polymer. The reducing terminus of XG9 was incorporated into the high molecular weight product, and formed the reducing terminus of this polymer. This suggests that the enzyme activity detected is an endo-acting enzyme, which causes the cleavage of a polymeric glycosyl donor, and the transfer of this glycosyl moiety onto XG9, the acceptor.

The nature of the linkage between XG9 and a polymer was investigated. In a transglycosylation reaction a glycosidic bond is cleaved and a new glycosidic linkage

is synthesised. [^{3}H]Polymer was alkali stable under conditions which would have caused the hydrolysis of an ester-linkage (Fry, 1989c). The attachment formed was strong, as [^{3}H]polymer remained intact during p.c. in BAW. The bond formed is therefore presumably covalent, and likely to be glycosidic, as expected for the product of a transglycosylation reaction.

Trichoderma cellulase treatment of [^{3}H]polymer released [^{3}H]XG9 or a ^{3}H-labelled oligosaccharide with very similar mobility to XG9 in EAW (e.g. XG7). The nonasaccharide substrate does not contain a cellulase-labile bond. This suggests that the linkage formed between polymer (donor) and XG9 (acceptor) is cellulase-labile, or at least is formed close to a cellulase-labile bond within the glycosyl donor. In either case the polymeric donor may be expected to be xyloglucan since this is a major cell wall polysaccharide which is soluble in water and contains unsubstituted β-(1-4)-glucosyl residues, the bonds between which are cellulase labile. The transglycosylation reaction detected may therefore be catalysed by a cellulase, acting as a transglycosylase. This possibility was investigated by further analyses of the [^{3}H]polymer product.

The molecular weight of the product was found to be 20,000-30,000. This is very low for a xyloglucan molecule (10^5-10^6; Fry, 1989b), but is consistent with the proposed mechanism, which involves cleavage of a glycosidic linkage in the backbone of the polysaccharide donor. The cleavage of side groups from the glycosyl donor, and their transfer onto XG9 would result in the formation of a product of significantly lower molecular weight than 20,000-30,000.

Xyloglucan is known to complex with both iodine (Kato and Matsuda; 1976, Kooiman, 1960) and copper (Rao, 1959; Edwards *et al.*, 1985). However, [^{3}H]polymer did not form a precipitate with either. This could be because:

(1) [^{3}H]polymer is not a xyloglucan;

(2) [^{3}H]polymer is a xyloglucan but is not large enough to form a precipitate on complexing with copper or iodine (e.g. Bauer *et al.* (1973) found that xyloglucan (molecular weight, 7600) purified from the soluble extracellular polysaccharides from sycamore suspension culture, gave a brown/violet colour with iodine, but did not precipitate) or;

(3) the [^{3}H]polymer is non-precipitable for other reasons. For example, Ring and Selvendran (1981) have reported copper-precipitable and non-precipitable xyloglucans in potato-tuber cell walls. The efficiency of precipitation with iodine is thought to depend on the extent of branching within the polysaccharide, and also the concentrations of both polysaccharide and iodine (Gaillard *et al.*, 1969). The addition of calcium ions aids in the precipitation reaction of iodine and polysaccharide

(Gaillard *et al.*, 1969). The exclusion of calcium ions may explain the lack of precipitation of [^{3}H]polymer on addition of iodine.

Evidence for xyloglucan being the glycosyl donor is the binding of [^{3}H]polymer to cellulose. Neither *Tropaeolum* xyloglucan nor [^{3}H]polymer could be eluted from the cellulose column with sodium acetate buffer or 8 M-urea; however, 1 M-sodium hydroxide removed both. Xyloglucan will bind to cellulose via hydrogen bonds (Bauer *et al.*, 1973; Aspinall *et al.*, 1969) as will xylans (Hayashi *et al.*, 1987). It has been suggested that this non-covalent binding to celluose is specific to the hemicellulose β-(1-4)-glycosyl backbone. Fractionation of cell wall xyloglucans by cellulose affinity has been reported. A proportion of xyloglucan is eluted from cellulose powder with 8 M-urea and a second proportion with sodium hydroxide (Joseleau and Chambat, 1984). The glucose/xylose ratios and the extent of fucosylation are reported to differ between these two xyloglucan fractions. In the work presented here, [^{3}H]polymer was eluted from the cellulose column with 1 M-sodium hydroxide only. [^{3}H]Polymer was found not to contain significant protein, as it was not precipitated in 20% TCA. Although proteins and glycoproteins usually do precipitate in TCA, extensins (cell wall glycoproteins) do not (Lamport, 1970).

In summary, the reaction detected in the apoplast of spinach cell suspension cultures appears to be an endo-transglycosylation reaction (possibly involving a cellulase). An endogenous, extracellular polymer (the glycosyl donor), probably xyloglucan, was cleaved by the extracellular enzyme which transferred the newly formed reducing terminus of the polymer onto exogenous XG9 (the glycosyl acceptor).

This activity had been detected only in suspension cultures of *Spinacia oleracea*. A further experiment was carried out to establish whether endo-transglycosylation of xyloglucan occurs in other plant species.

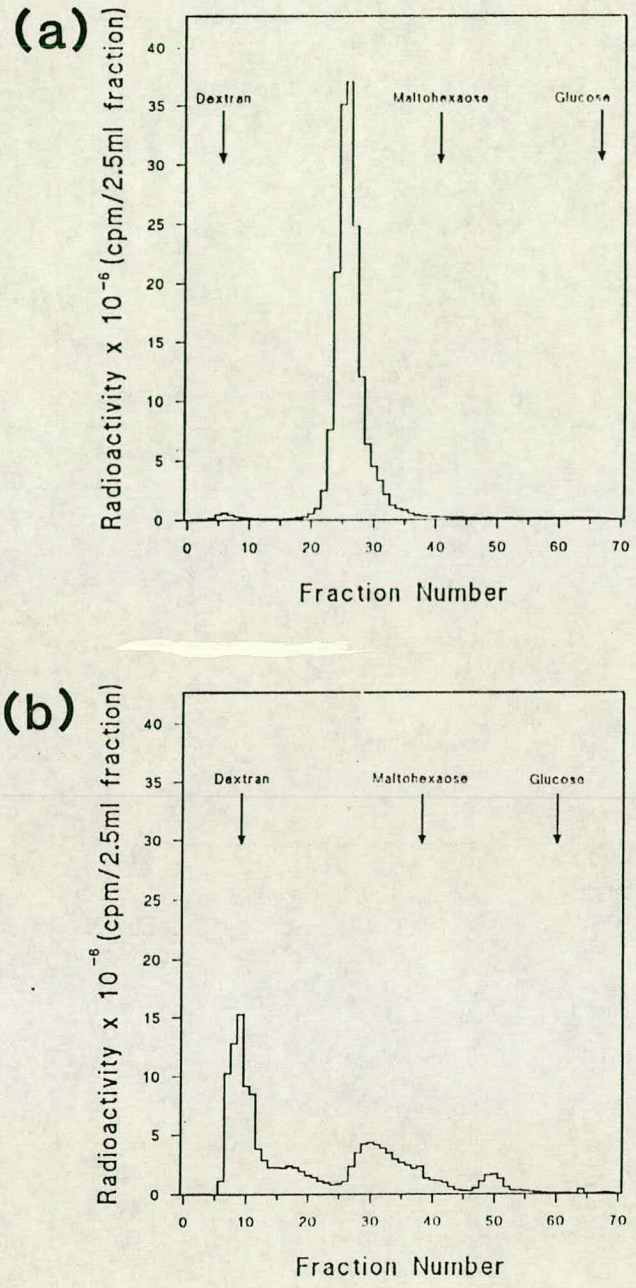


Fig 3.4.2 Bio-Gel P-2 chromatography of soluble extracellular products formed during incubation of [*reducing terminus*-^{3}H]XG9 with spinach suspension cultures

[*reducing terminus*-^{3}H]XG9 was incubated in (a) cell-free fresh medium for 72 h, and (b) day-4 spinach culture for 72 h. ^{3}H-Labelled material in the incubation media were separated by Bio-Gel P-2 chromatography. The positions of internal markers dextran (K_{AV}, 0.00), maltohexaose (K_{AV} approximately, 0.57) and glucose (K_{AV}, 1.00) are shown.

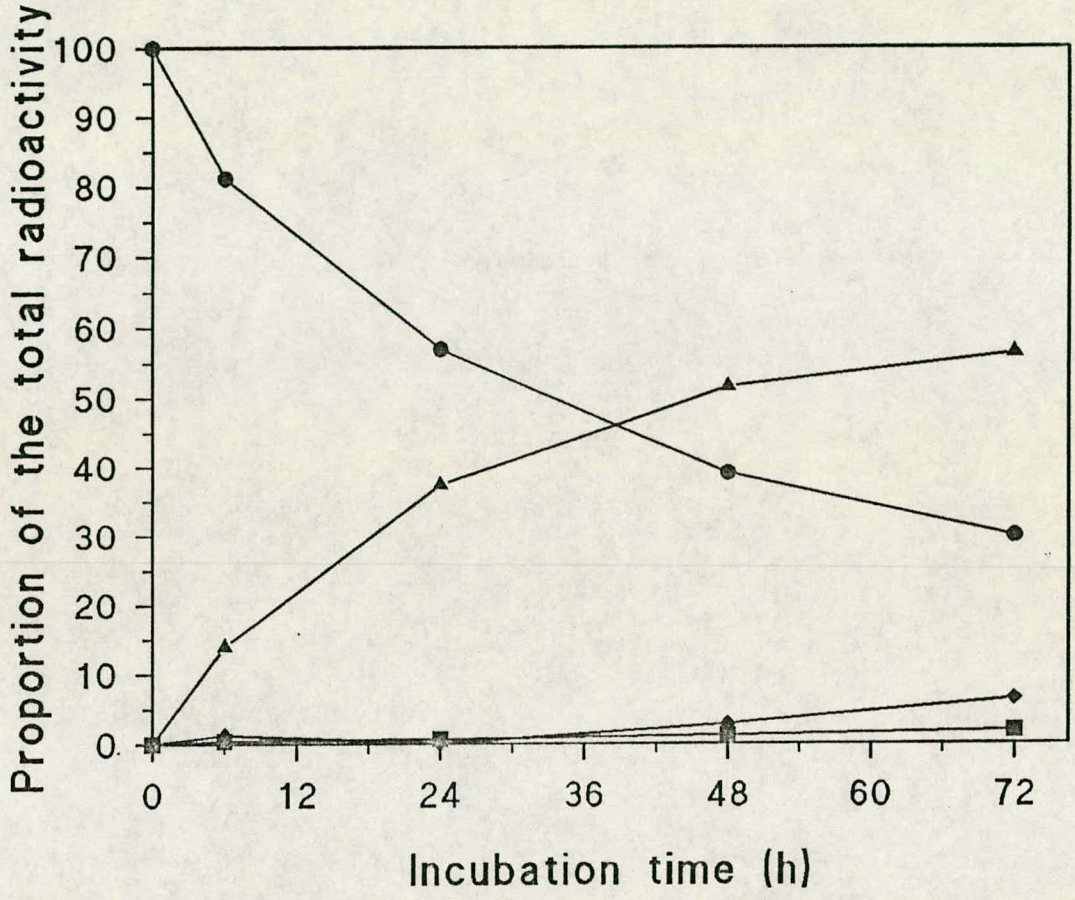


Fig 3.4.3 Fate of the reducing terminus of XG9 during incubation of spinach suspension cultures with [*reducing terminus*-^{3}H]XG9

Spinach suspension cultures were incubated with [*reducing terminus*-^{3}H]XG9 for up to 72 h. The following components of suspension culture were assayed for tritium after 6, 24, 48, and 72 h incubation: spinach cells (■); low molecular weight XG9 breakdown products (◆); soluble apoplastic polymer (▲); and unchanged [^{3}H]XG9 (●). Data for ●, ▲, and ◆ were obtained from Bio-Gel P-2 chromatography (e.g. Figs. 3.4.2). All data are corrected relative to the cell-free controls.

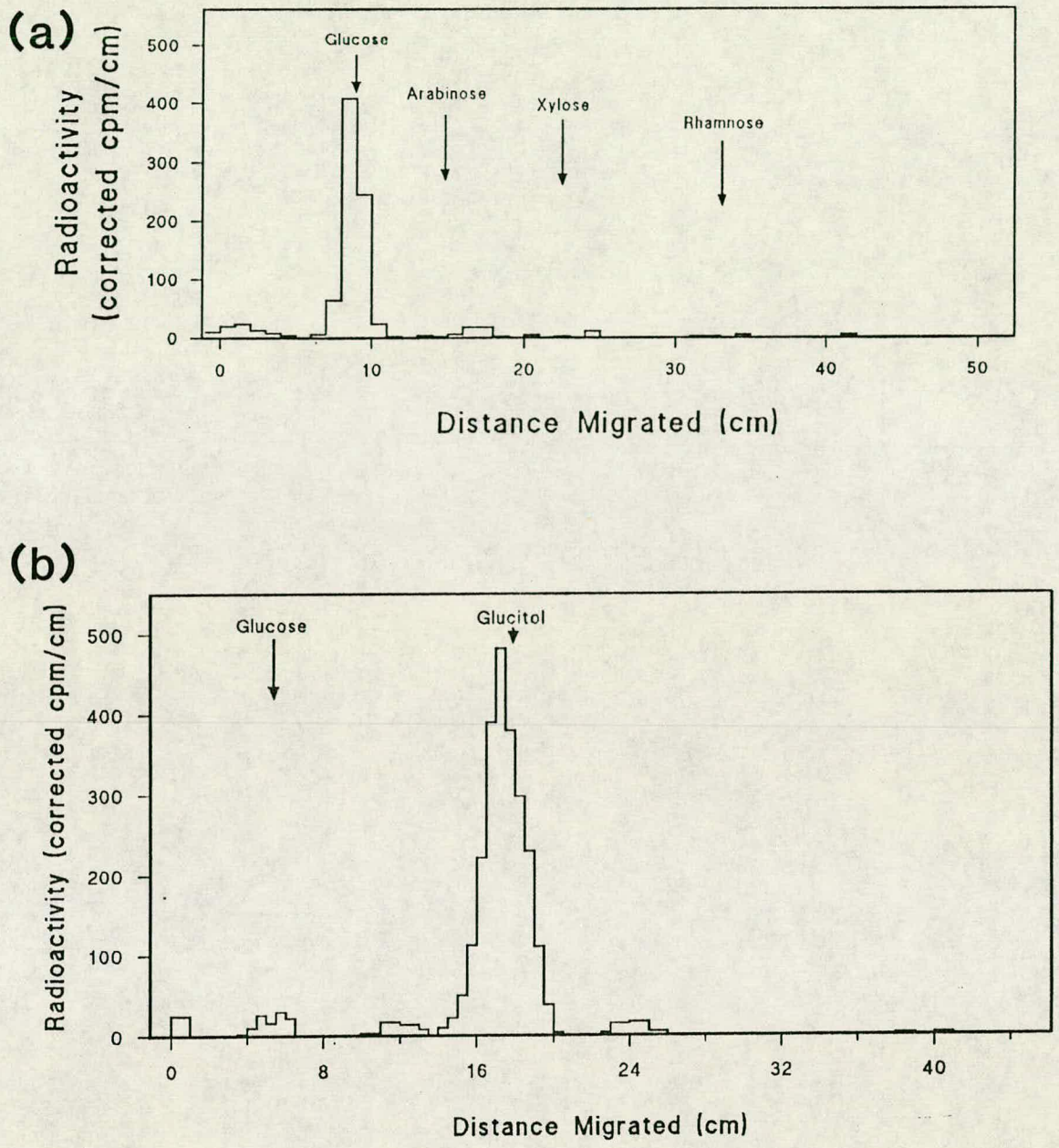


Fig 3.4.4 Paper chromatography of ^{3}H-labelled material released from high molecular weight incubation products by acid hydrolysis, before and after reduction with sodium borohydride.

High molecular weight material was recovered from the void volume of the g.p.c. shown in Fig 3.4.2, and subjected to (a) TFA hydrolysis (products separated in EPW); and (b) sodium borohydride reduction followed by TFA hydrolysis (products separated in BAB). The positions of external markers are shown.

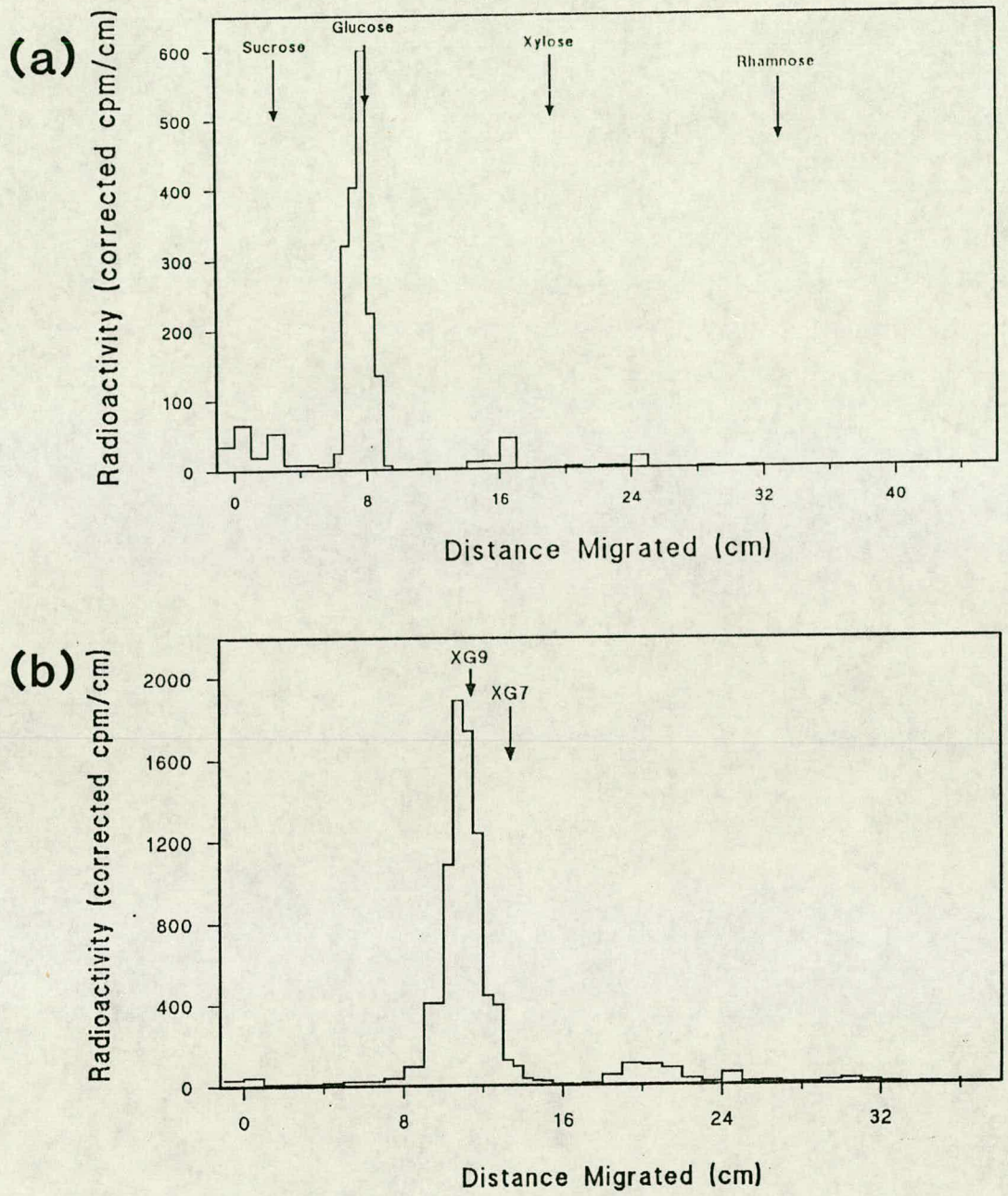


Fig 3.4.5 Paper chromatography of ^{3}H-labelled material released from high molecular weight incubation products by Driselase and *Trichoderma* cellulase digestion.

High molecular weight material was recovered from the void volume of the g.p.c. shown in Fig 3.4.2, and subjected to (a) Driselase digestion (products separated in EPW); and (b) *Trichoderma* cellulase digestion (products separated in EAW). The positions of external markers are shown.

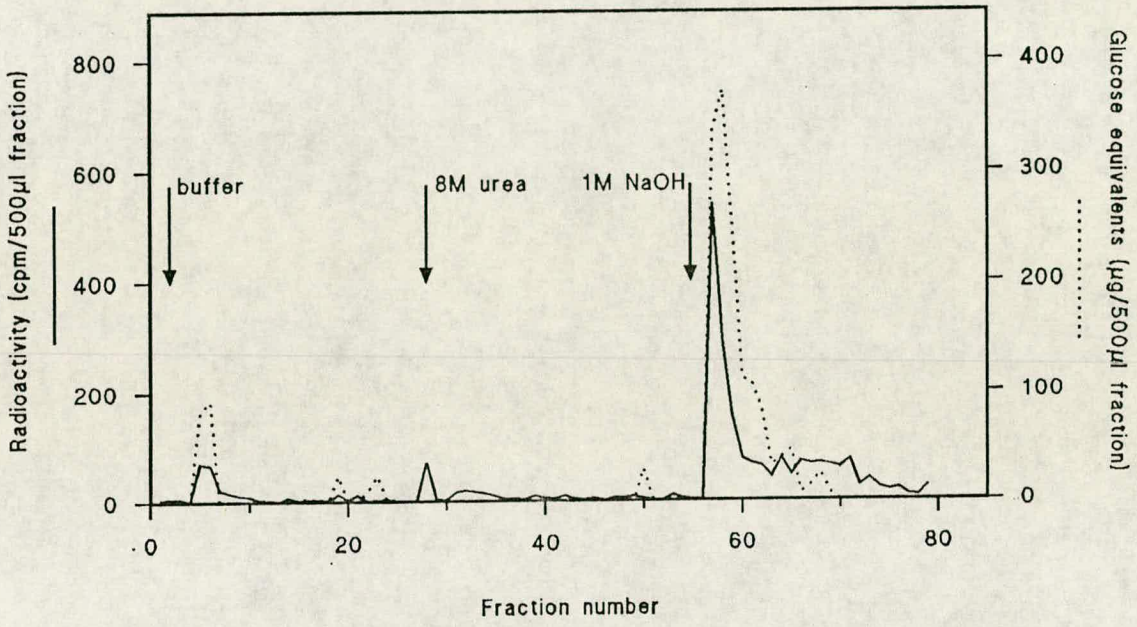


Fig 3.4.6 Cellulose affinity chromatography of high molecular weight incubation product

High molecular weight material, recovered from the void volume of the g.p.c. shown in Fig 3.4.2 (——), and an internal marker of nasturtium xyloglucan (····) were applied to a cellulose column. Material was sequentialy eluted with: 20 mM-sodium acetate buffer (pH 5.0); 8 M- urea; 1 Msodium hydroxide.

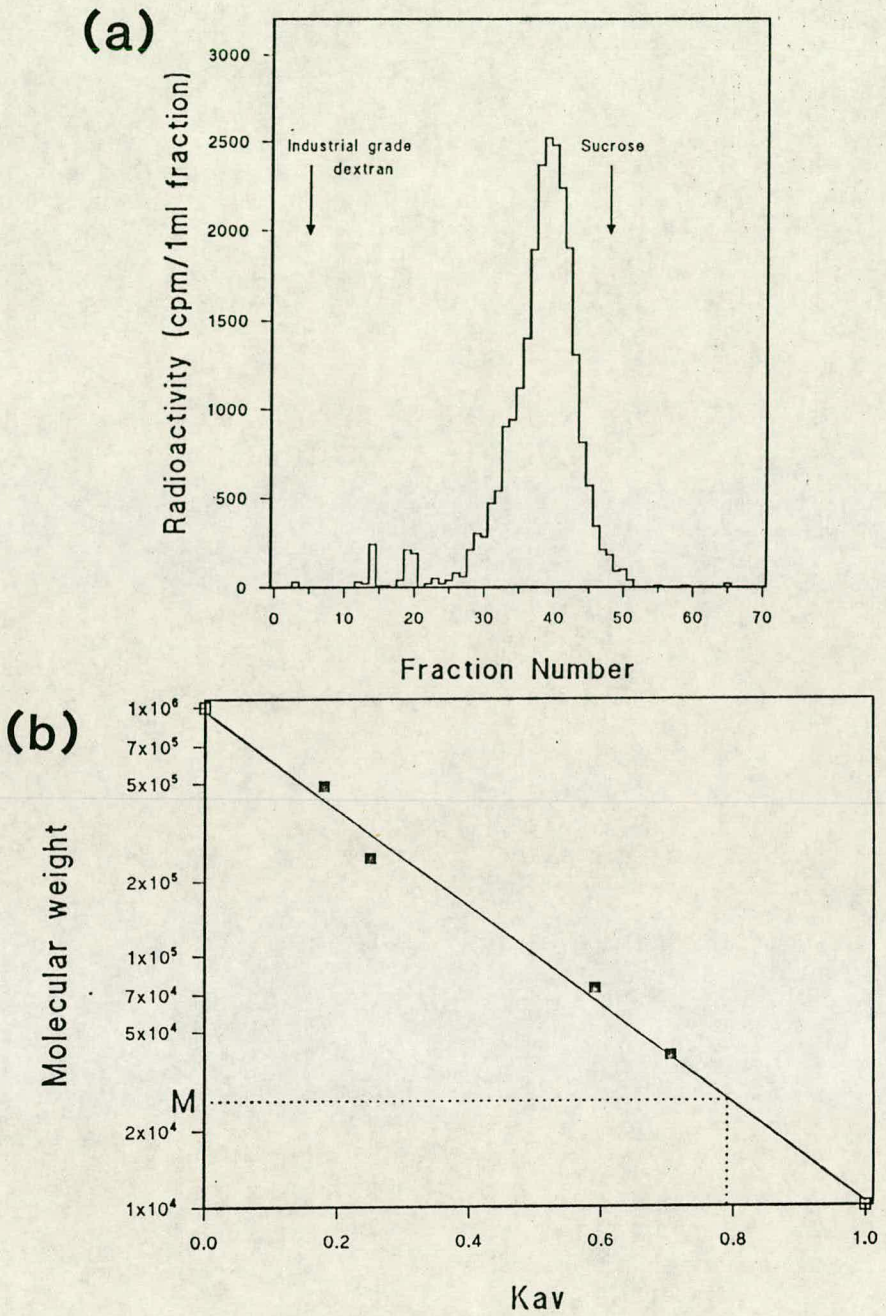


Fig 3.4.7 Sepharose CL-6B chromatography of high molecular weight incubation product; estimation of molecular weight

(a) High molecular weight material recovered from the void volume of the g.p.c. shown in Fig 3.4.2, was subjected to Sepharose CL-6B chromatography. The position of internal markers are shown. (b) The Sepharose CL-6B column was calibrated by external markers (dextrans of molecular weights; 488, 249, 74.3, and 40 kD). The molecular weight of the 'transglycosylation' product was estimated to be 20-30 kD (M).

3.5 Investigation into the occurrence of xyloglucan endo-transglycosylation activity in the apoplast of cell suspension cultures derived from a number of plant species

3.5.1 Introduction

It has been postulated (section 1.6) that extracellular transglycosylation has an important role in the enzymatic control of cell wall loosening during cell expansion. If this activity has a primary role in plant development, its presence should be distributed widely throughout the plant kingdom. This experiment was carried out to investigate the occurrence of extracellular xyloglucan endo-transglycosylation activity in a number of suspension cultures, derived from both monocotyledonous and dicotyledonous plants.

3.5.2 Incubation of plant cell suspension cultures with *[reducing terminus-*^{3}H]XG9

Samples of day-3 cell suspension culture (3 ml) of four 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*)] were incubated with [*reducing terminus*-^{3}H]XG9 for 1, 6, 24, 48, and 72 h (section 2.5.1). Cell-free controls were also incubated over similar time periods.

Table 3.5.1 The settled cell volumes and packed cell volumes of the original cultures which were incubated with [*reducing terminus*-^{3}H]XG9

Cell suspension culture	%SCV	%PCV
Carrot	26.7	11.7
Rose	13.3	10.0
Sycamore	11.7	10.0
Chilli pepper	6.7	5.0
Maize*	8.3	6.7
Tall fescue grass*	16.7	11.7

(* denotes cultures derived from monocotyledonous plants).

Cells were separated from the incubation medium by centrifugation (section 2.5.1). Samples of cells and medium were assayed for tritium (section 2.8.2.1)

Table 3.5.2 The proportion of ^{3}H-labelled material taken up into cells and/or associated with insoluble cell wall material.

Cell suspension culture	Proportion of ^{3}H-labelled material taken up into cells and/or associated with insoluble cell wall material, after incubation time = t			
	t=6h	t=24h	t=48h	t=72h
Carrot	0.46	1.00	1.11	1.51
Rose	0.51	0.90	1.05	0.75
Chilli pepper	0.56	0.44	0.62	NA
Sycamore	0.24	0.23	0.30	0.31
Tall fescue	0.35	0.40	0.58	0.69
Maize	0.35	0.31	0.34	0.29

The majority of ^{3}H-labelled material remained extracellular and soluble in all cultures during a 72 h incubation (Table 3.5.2).

3.5.3 Separation of ^{3}H-labelled products in the incubation medium

Samples of incubation medium (150 µl) from cultures (at each time point) were applied to chromatography paper (section 2.6.3). Samples from the cell-free controls (75 µl) were also applied to chromatography paper. Chromatograms were developed in BPW (section 2.6.3). External markers were visualised by alkali-silver nitrate stain (section 2.8.3). The position of all ^{3}H-labelled material on the chromatograms was determined by liquid scintillation counting (section 2.8.2.2).

P.c. separated two distinct ^{3}H-labelled compounds in the incubation media of all cultures. ^{3}H-Labelled material with a similar mobility to the maltoheptaose external marker was found on all chromatograms (R_F, 0.19). Maltoheptaose has a similar mobility in BPW to XG9 (Fry, 1986b). The amount of this ^{3}H-labelled compound decreased with time in all cultures. Each culture also accumulated ^{3}H-labelled material which remained at the origin of the chromatogram (R_F, 0.0), following 16 h irrigation in BPW. The rate of accumulation of BPW-immobile ^{3}H-labelled material varied from culture to culture. *Rosa* cultures showed the fastest rate of accumulation

of BPW-immobile ^{3}H-labelled product, followed by *Acer*, *Daucus*, *Zea*, *Festuca*, and lastly *Capsicum* (Fig. 3.5.1). The final amount of BPW-immobile ^{3}H-labelled material in the medium following 72 h incubation with [*reducing terminus*-^{3}H]XG9 also varied according to culture type. *Rosa* culture medium contained the most of this material, followed by *Acer*, *Daucus*, *Zea*, *Festuca*, and *Capsicum* (Fig. 3.5.1).

No appreciable ^{3}H-labelled material with a R_F of greater than that of maltoheptaose was found in medium of any culture, even after 72 h incubation with [^{3}H]XG9 (Figs. 3.5.2-3.5.7).

3.5.4 Discussion of results

Negligible ^{3}H-labelled material was taken up by cells and/or became associated with cells, during 72 h incubation of either monocotyledonous or dicotyledonous plant cells with [*reducing terminus*-^{3}H]XG9. This is equivalent to the data obtained for *Spinacia* cells (Table 3.4.1). Therefore, none of the plant cells studied here transport either XG9 or any ^{3}H-labelled XG9-breakdown products across their plasma membrane. The products of XG9 metabolism remained extracellular and soluble, as was found in *Spinacia* suspension cultures.

Negligible amounts of ^{3}H-labelled material had an R_F value of above that of XG9. This suggests that the apoplast of all the cultures investigated contained negligible hydrolytic activity towards the exogenous nonasaccharide. No ^{3}H-labelled monomeric material was released from the oligosaccharide. Therefore, any metabolic change in the ^{3}H-labelled material did not involve the loss of the reducing terminal glucose unit of XG9.

The major ^{3}H-labelled product which was found to accumulate in the incubation medium of all the cells under investigation, was soluble in water, and immobile in BPW. The mobility of a compound in a particular solvent system is predominantly dependent on the partitioning of the solute between the stationary and mobile phases. The ^{3}H-labelled material (XG9) fed to the cells could become less mobile in BPW by undergoing an increase in molecular weight, or by developing a charge. By considering the work carried out previously, it seems likely that ^{3}H-labelled material has become BPW-immobile as a result of an increase in molecular weight. I suggest that BPW-immobile ^{3}H-labelled material is the product of an extracellular transglycosylation reaction as demonstrated in spinach cell suspension culture.

Cultures derived from dicotyledonous plants (*Rosa*, *Daucus*, *Acer*), with the exception of *Capsicum*, exhibited a faster rate of accumulation of ^{3}H-labelled transglycosylation product over the initial 24 h of the incubation with [*reducing terminus*-^{3}H]XG9, than did cultures derived from monocotyledonous plants (i.e. *Zea*

and *Festuca*). Xyloglucan is present in the primary cell walls of both monocotyledonous and dicotyledonous plants. However, whereas dicotyledonous plant cell walls contain approximately 20% xyloglucan by dry weight, Graminaceous plant cell walls contain only 2 to 5% xyloglucan by dry weight (for review, Fry, 1989b). The chemistry of Graminaceous xyloglucan is different from that of xyloglucan from dicotyledonous plant cell walls. Xyloglucan in the Graminaceous cell wall is less branched (i.e. fewer xylosyl side groups) and contains few or no galactosyl or fucosyl residues (Kato and Matsuda, 1985; Kato *et al.*, 1981; Shibuya and Misaki, 1978). Cellulase treatment of xyloglucan from rice and barley cell walls does not yield an XG9 fragment as with dicotyledonous xyloglucan. Rather, hepta- and penta-saccharides are released, together with cellobiose and monomeric glucose (Kato *et al.*, 1981; Kato and Matsuda, 1985). The difference in rate of the extracellular transglycosylation reactions detected in cultures from dicotyledonous plants and cultures of Graminaceous plant cells could therefore be due to:

(1) lower enzyme activity, or amount of enzyme protein in the apoplast of Graminaceous cultures;

(2) lower concentration of glycosyl donor (xyloglucan) in the cell walls and medium of Graminaceous cell cultures;

(3) low specificity of the Graminaceous enzyme for the dicotyledonous plant-derived XG9.

The rate of accumulation of ^{3}H-labelled transglycosylation product is dependent on culture type; so too is the final amount of high molecular weight ^{3}H-labelled product in the medium following 72 h incubation with [^{3}H]XG9. The rate of the reactions in all cultures began to decrease after approximately 24 h. The net incorporation of ^{3}H-labelled material into the polymeric product had almost stopped by the end of the 72 h incubation period, despite a proportion of the [^{3}H]XG9 substrate being still present in the medium. Negligible ^{3}H-labelled material was released from XG9 by extracellular breakdown. The decrease in rate of net incorporation of ^{3}H-labelled material into polymer can not have been achieved by degradation or removal of [^{3}H]XG9. However, possible explanations are as follows:

(1) the enzyme responsible has a low affinity for the exogenous XG9 substrate. As the concentration of XG9 decreases, the enzyme becomes gradually less able to catalyse the conversion of XG9 substrate to high molecular weight product and so the reaction gradually slows.

(2) Exogenous XG9 is chemically similar to its parent polysaccharide, xyloglucan. The oligosaccharide may therefore be susceptible to similar enzymatic activity as xyloglucan. As XG9 is a small molecule, it can permeate the cell wall, and

so come into contact with wall enzymes which usually catalyse reactions with polymeric xyloglucan as a substrate. In other words, XG9 can act as a 'probe' for extracellular enzymes which act on cell wall xyloglucan. Polymeric xyloglucan may compete with [^{3}H]XG9 for the active site of the enzyme involved in the reaction investigated here. As the concentration of [^{3}H]XG9 falls (due to transglycosylation involving the oligosaccharide) the probability that the glycosyl acceptor in the reaction will be [^{3}H]XG9, also falls. The only ^{3}H-labelled material in the system is the exogenous oligosaccharide, therefore any products formed by transglycosylation between two endogenous xyloglucan molecules can not be detected.

Competition between non-radioactive oligosaccharides and [^{3}H]XG9 may also occur. Non-radioactive fragments will be released from their parent polysaccharide by the action of cellulase (Fry, 1986b), an enzyme which is proposed to play a role in cell wall loosening during cell growth (Verma *et al.*, 1975). As the concentration of [^{3}H]XG9 falls, and the amount of non-radioactive, native oligosaccharides increases, the rate of production of ^{3}H-labelled product would decrease.

Competition between non-radioactive material and the exogenous [^{3}H]XG9 is very likely to occur. There will be high concentrations of non-radioactive, available substrates present both in the cell wall and in the surrounding medium. Only a limited amount of ^{3}H-labelled material was added to the cultures, and the concentration of this substrate will fall as the incubation progresses.

(3) No ^{3}H-labelled breakdown products of [^{3}H]XG9 were detected in the incubation medium of any of the cell suspension cultures. However, the ^{3}H-labelled polymeric product may still be subject to breakdown in the apoplast of suspension cultures, if [^{3}H]XG9 were released from the [^{3}H]polymer. A balance between [^{3}H]polymer synthesis and [^{3}H]polymer degradation would result in the cessation of net incorporation of ^{3}H-labelled material into a transglycosylation product. This degradation activity cannot be detected by the method used here. However, if such activity were present in suspension culture medium, [^{3}H]XG9 would be released from [^{3}H]polymer during incubation of suspension cultures with the ^{3}H-labelled transglycosylation product.

The degradation of the ^{3}H-labelled polymeric product in the apoplast of suspension cultures is quite likely to occur. Cellulase is present in the apoplast of plant cells (Bal *et al.*, 1976; Hatfield and Nevins, 1986; Hayashi *et al.*, 1984). [^{3}H]Polymer yields [^{3}H]XG9 on *Trichoderma* cellulase digestion (section 3.4.4.6).

(4) The exogenous XG9 substrate or the endogenous xyloglucan substrate become unavailable to the enzyme during prolonged incubation with cultured plant cells.

(5) The amount and/or the activity of the enzyme involved in producing ^{3}H-

labelled BPW-immobile material, decreases over the 72 h incubation period.

Capsicum sp. cultures exhibited the lowest rate of transglycosylation and the lowest total amount of ^{3}H-labelled transglycosylation product, compared to all the other cultures investigated (including Graminaceous plant cell cultures). This could be explained by the following:

(1) *Capsicum frutescens* cultured cells have low extracellular transglycosylation activity.

(2) the enzyme which catalyses the apparent increase in molecular weight of XG9 in the apoplast of *Capsicum* cultures, has a low affinity for spinach derived XG9.

(3) the cells in the *Capsicum* cultures used were very clumped. The exogenous [*reducing terminus*-^{3}H]XG9 substrate was most probably not able to permeate the cell walls fully. This would restrict the access of XG9 to cell wall associated xyloglucan and enzyme. Of the explanations given, this is perhaps the most likely.

Xyloglucan endo-transglycosylation appears to occur in the apoplast of both dicotyledonous and Graminaceous plant cell suspension cultures. The apparent ubiquity of this activity in the plant kingdom may suggest a central role in plant metabolism. The proposed role of transglycosylation in plant cell expansion was investigated by measuring the activity in cultures of different ages, and therefore exhibiting different growth rates. The position of the detected activity in the apoplast (i.e. is it cell associated or present in the medium of suspension cultures?) was also investigated.

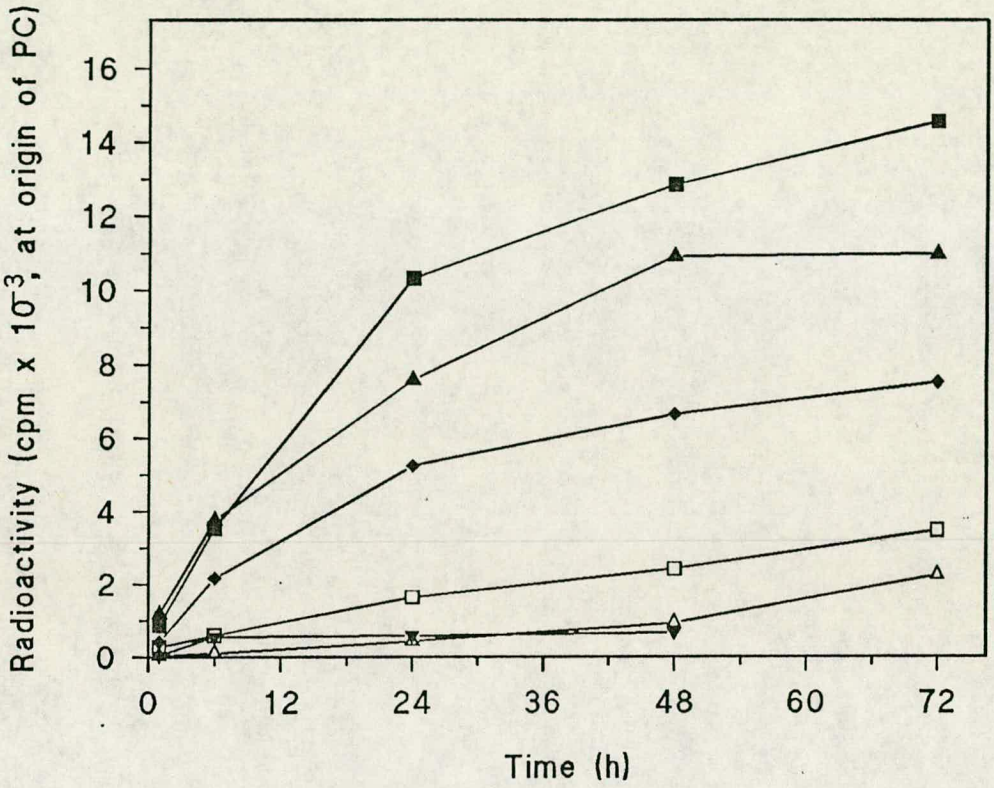


Fig 3.5.1 The synthesis of ^{3}H-labelled BPW-immobile material in the apoplast of plant cell suspension cultures during incubation with [*reducing terminus*-^{3}H]XG9.

Plant cell suspension cultures (◆ *Daucus*, ■ *Rosa*, ▲ *Acer*, ▼ *Capsicum*, □ *Zea* and Δ *Festuca*) were incubated with [^{3}H]XG9 for up to 72 h. ^{3}H-Labelled materials in 150 µl of incubation medium were separated by paper chromatography. The total amount of ^{3}H-labelled BPW-immobile material produced in 6 ml of incubation medium was calculated from the amount of tritium that remained at the origin of the p.c.'s (see Figs 3.5.2-3.5.7). These data were corrected relative to the cell-free controls.

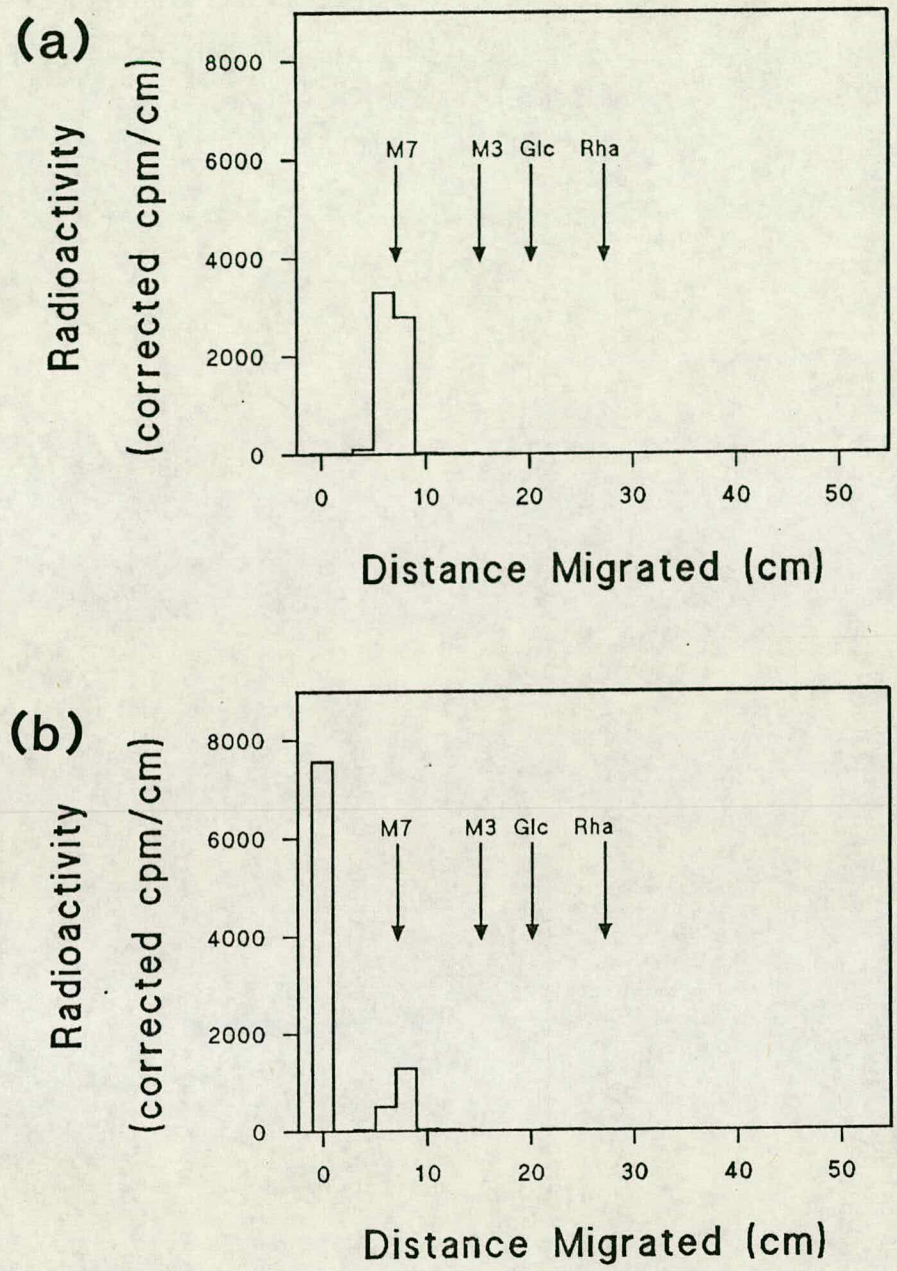


Fig 3.5.2 Paper chromatography of ^{3}H-labelled soluble extracellular products formed during incubation of [*reducing terminus*-^{3}H]XG9 with *Daucus* suspension cultures

[*reducing terminus*-^{3}H]XG9 (in 3 ml of fresh medium) was incubated (a) in fresh medium (in the absence of cells) for 72 h; and (b) with day-4 *Daucus* (carrot) suspension culture (3 ml) for 72 h. ^{3}H-Labelled material in 150 µl of incubation medium was separated by paper chromatography in BPW. The positions of the external markers maltoheptaose (M7), maltotriose (M3), glucose (Glc) and rhamnose (Rha) are shown.

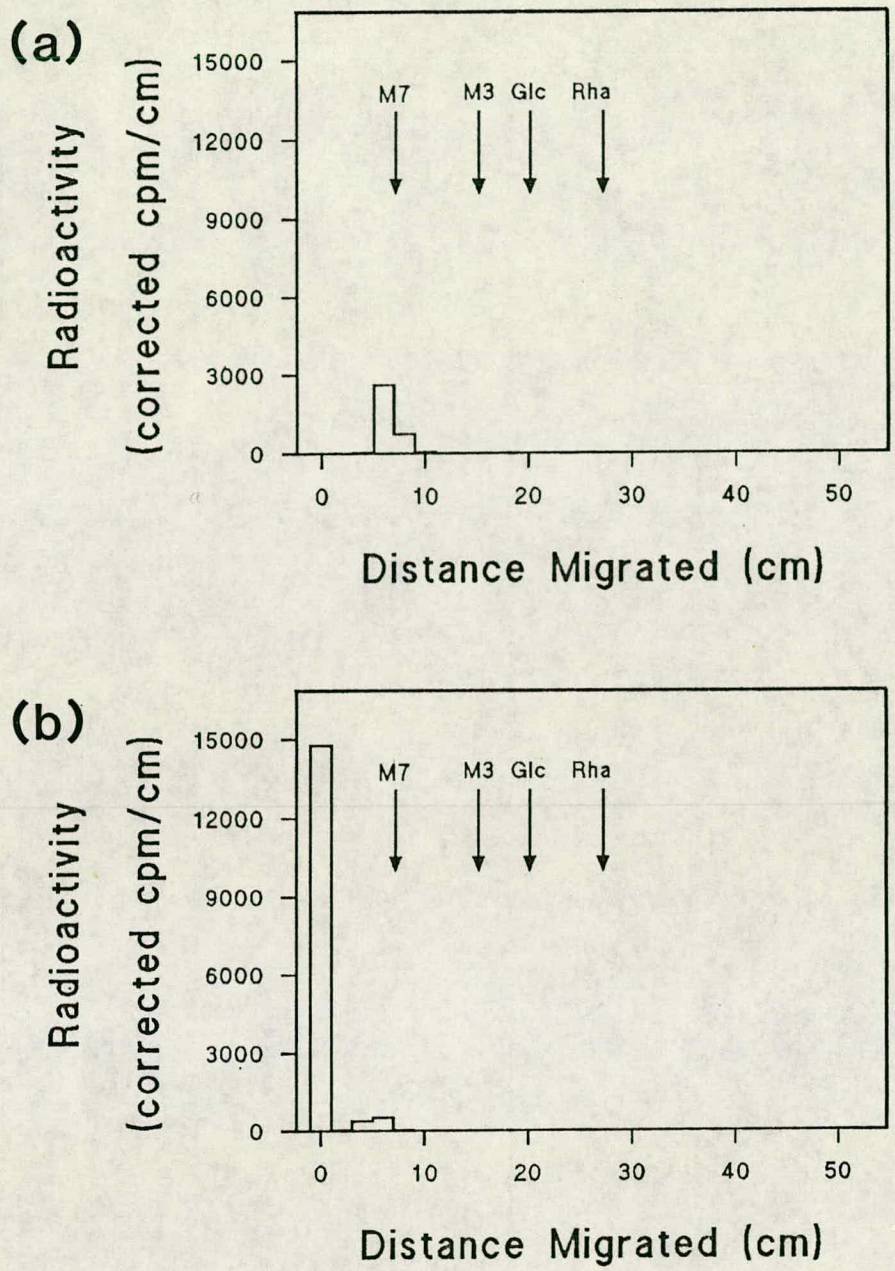


Fig 3.5.3 Paper chromatography of ^{3}H-labelled soluble extracellular products formed during incubation of [*reducing terminus*-^{3}H]XG9 with *Rosa* suspension cultures

As for Fig. 3.5.2, but with *Rosa* (rose) cell suspension cultures.

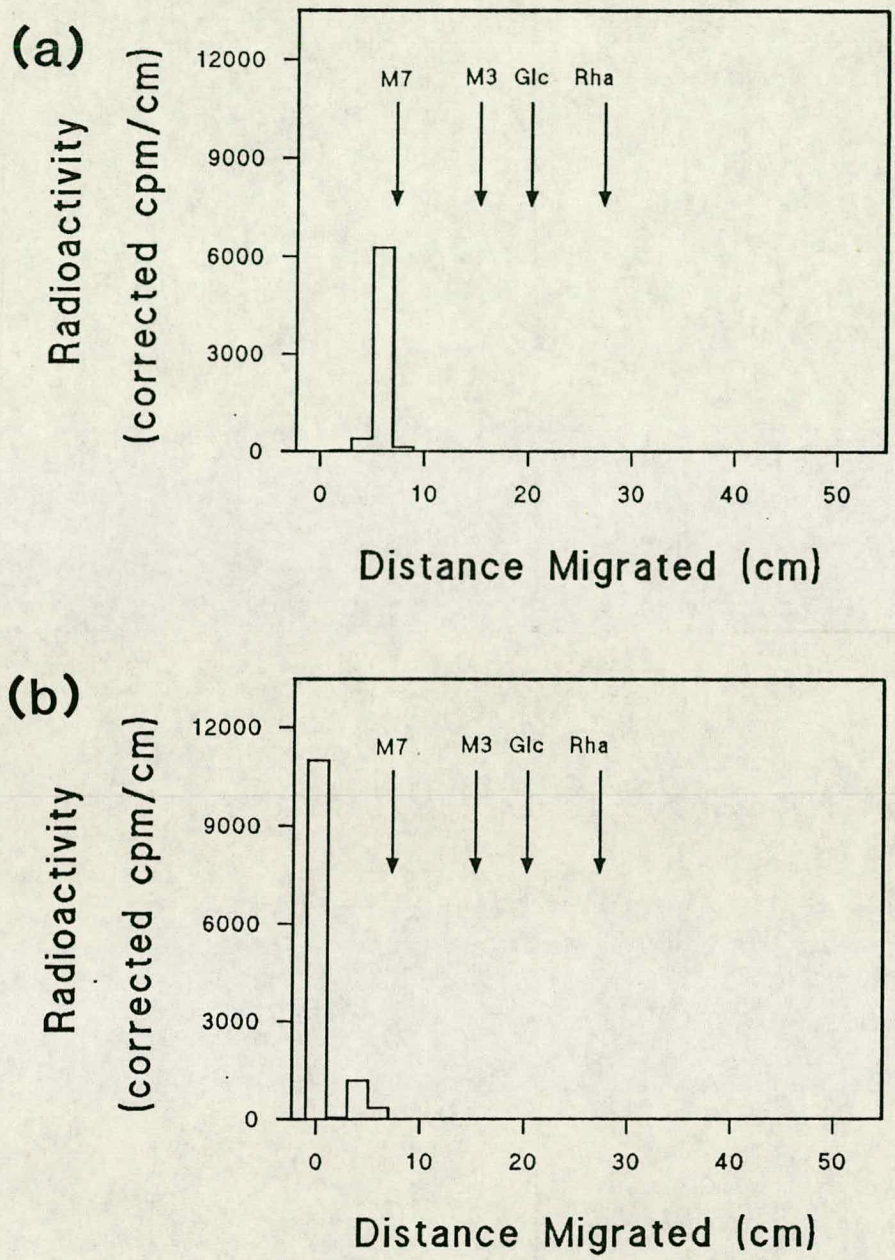


Fig 3.5.4 Paper chromatography of ^{3}H-labelled soluble extracellular products formed during incubation of [*reducing terminus*-^{3}H]XG9 with *Acer* suspension cultures

As for Fig. 3.5.2, but with *Acer* (sycamore) cell suspension cultures.

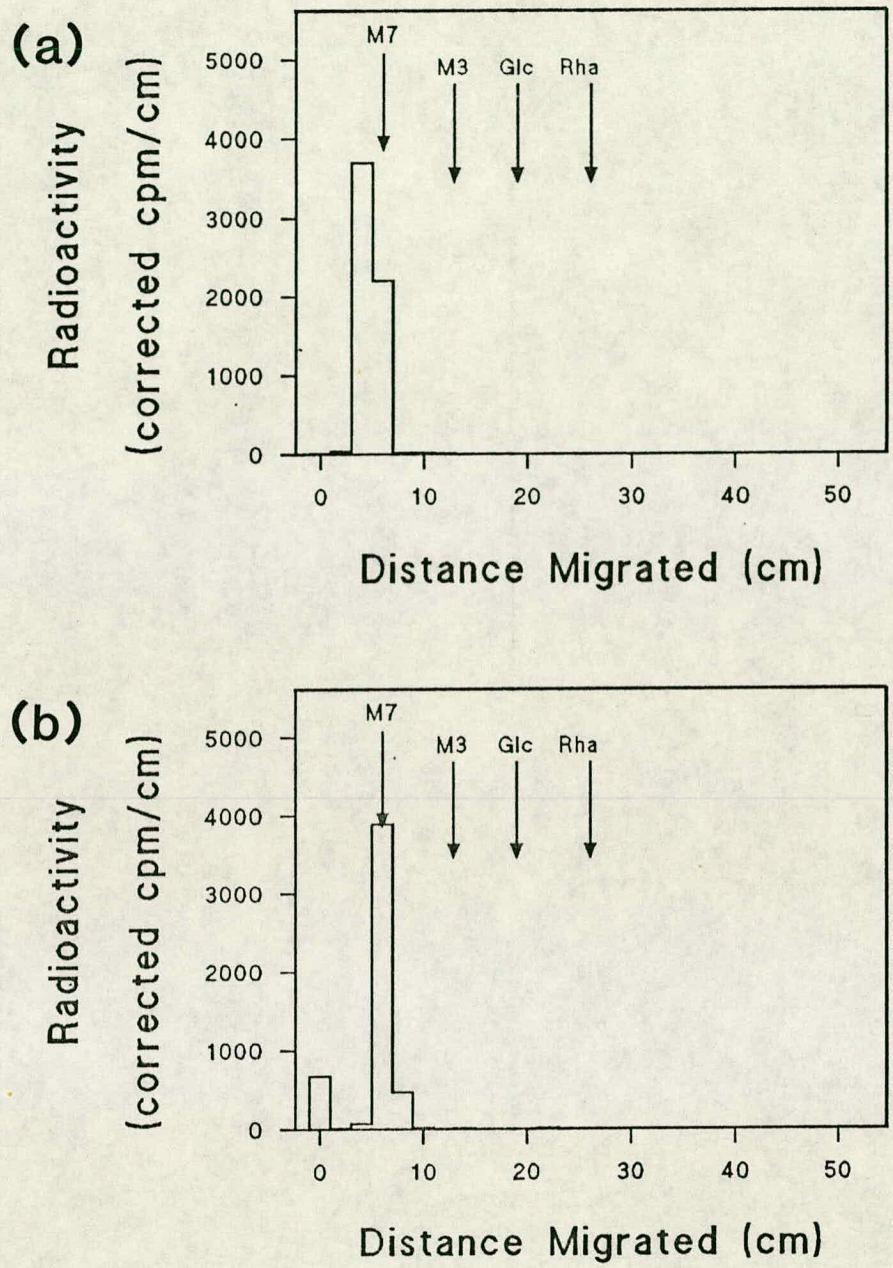


Fig 3.5.5 Paper chromatography of ^{3}H-labelled soluble extracellular products formed during incubation of [*reducing terminus*-^{3}H]XG9 with *Capsicum* suspension cultures

As for Fig. 3.5.2, but with *Capsicum* (chilli pepper) cell suspension cultures.

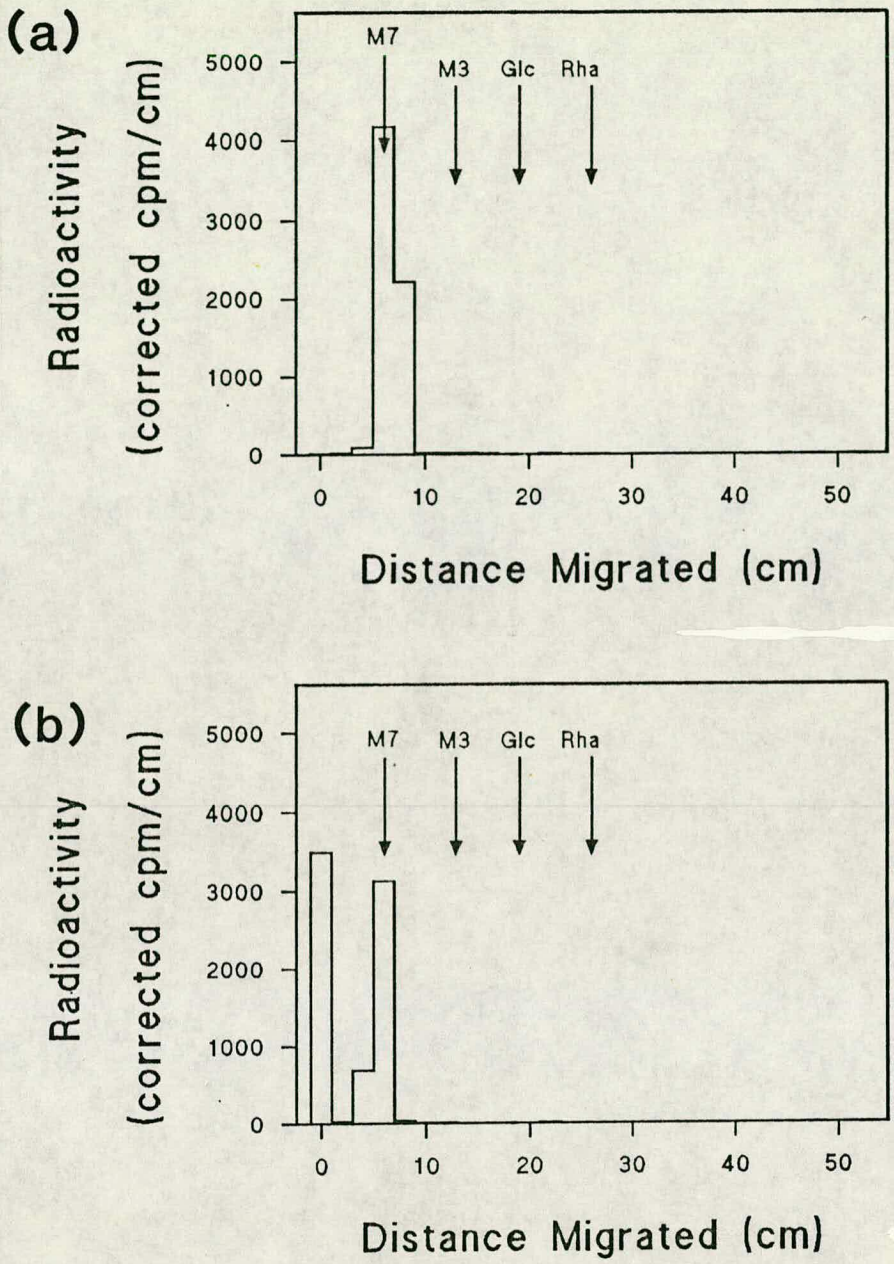


Fig 3.5.6 Paper chromatography of ^{3}H-labelled soluble extracellular products formed during incubation of [*reducing terminus*-^{3}H]XG9 with *Zea* suspension cultures

As for Fig. 3.5.2 but with *Zea* (maize) cell suspension cultures .

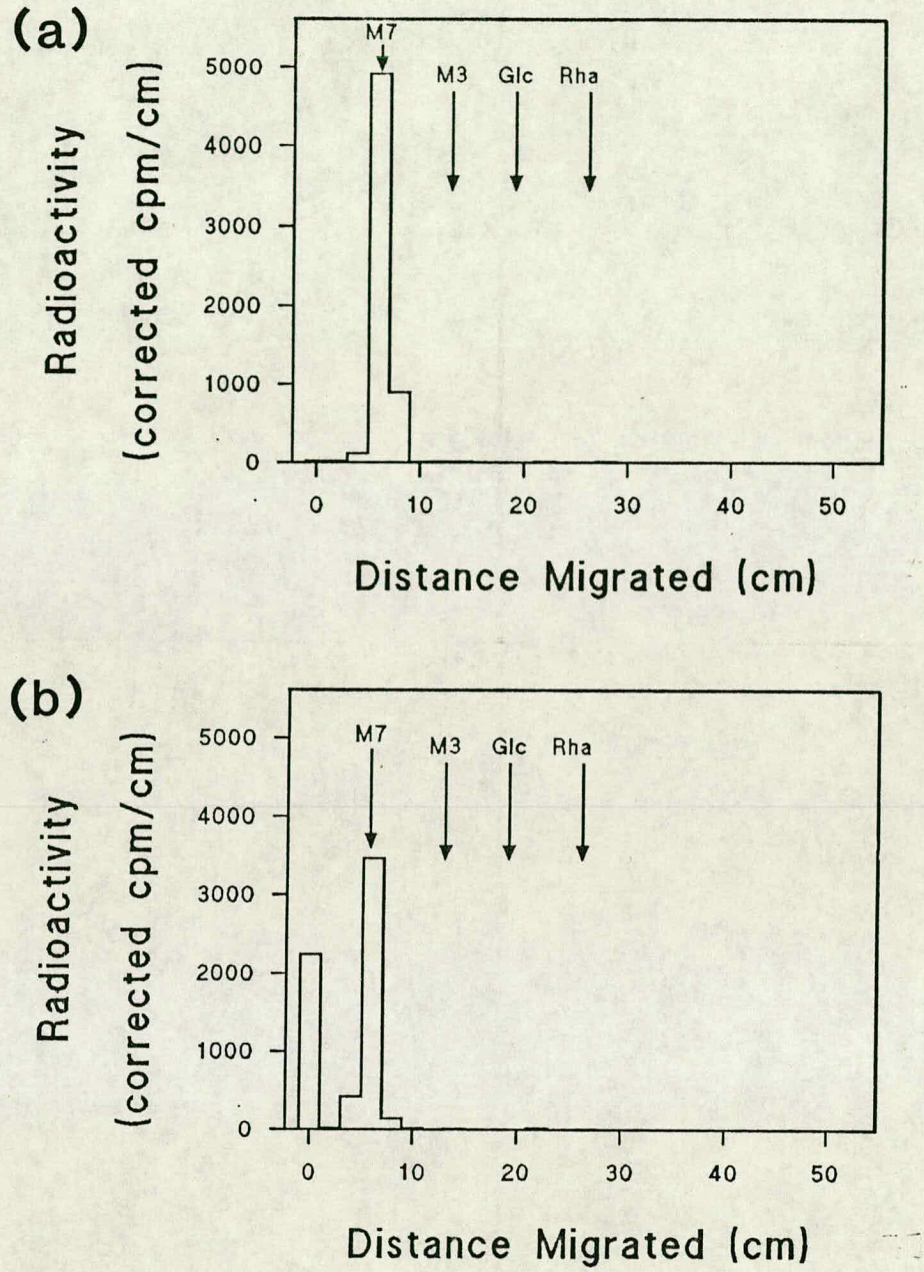


Fig 3.5.7 Paper chromatography of ^{3}H-labelled soluble extracellular products formed during incubation of [*reducing terminus*-^{3}H]XG9 with *Festuca* suspension cultures

As for Fig. 3.5.2, but with *Festuca* (tall fescue grass) cell suspension cultures.

3.6 Investigation into the localisation of xyloglucan transglycosylation activity within the apoplast of spinach suspension cultures. A time course for this activity throughout the growth cycle of the culture.

3.6.1 Introduction

Xyloglucan endo-transglycosylation activity has been detected in the apoplast of a number of dicotyledonous and Graminaceous plant cell suspension cultures (section 3.5). The aim of this experiment was firstly to determine the distribution of transglycosylation activity in the soluble fraction (spent medium) and insoluble fraction (bound to the cell wall) of the apoplast of a suspension culture.

The second aim of this experiment was to investigate the possible role of xyloglucan endo-transglycosylation in cell wall loosening during cell expansion. It has been proposed (section 1.6) that an enzyme activity which involves both bond cleavage and re-formation could allow a temporary loosening of the wall, so enabling turgor-driven expansion, prior to re-establishing the mechanical properties of the wall. This type of activity would allow prolonged cell expansion, without the loss of the physical integrity of the cell wall.

Suspension cultured cells exhibit a growth cycle involving an initial lag phase prior to a period of rapid cell growth. Growth rate subsequently slows until no net growth of the culture occurs. It was hoped that by measuring the transglycosylation activities of cultures in these three phases of their growth cycle (lag phase, rapid growth phase and finally stationary phase) conclusions as to the physiological role played by this activity could be made.

3.6.2 Growth of *Spinacia* suspension cultures

Growth of suspension cultures was estimated by measurement of the settled cell volume and packed cell volume (section 2.1.1.3) over 24 days. Samples (3 ml) were taken aseptically from two 250 ml cultures on days 0, 2, 4, 6, 8, 12, 16, 20 and 24 from subculture.

The most rapid increase in cell volume occurred between day 2 and day 6 from subculture (Fig. 3.6.1). Six days after subculture growth cell expansion was beginning to slow.

3.6.3 Incubation of *Spinacia* suspension cultures with [*reducing terminus*-^{3}H]XG9

Samples of suspension culture (10 ml) were removed aseptically from 250 ml

Spinacia suspension cultures. Samples were taken on days 0, 2, 4, 6, 8, 12, 16, 20, and 24 from subculture, from two independent cultures. Of each of these 10 ml samples, 3 ml of culture was transferred directly into 'Sterilin' culture pots and incubated with [^{3}H]XG9 for 9 h (section 2.5.1). These samples will be referred to as whole culture samples. The remaining 7 ml of culture was filtered to separate the cells from the spent medium (section 2.5.1). The washed cells were resuspended in fresh spinach culture medium. Both spent medium and cells were incubated with [^{3}H]XG9 for 9 h (sections 2.5.2 and 2.5.3). These samples will be referred to as spent medium and washed cell samples. On day 14 from subculture, a second 10 ml portion was removed from the 250 ml cultures. From this portion, whole culture, washed cells and spent medium samples were prepared (section 2.5.2) and then boiled for 20 min in order to de-nature any proteins present. These boiled samples were incubated with [^{3}H]XG9 and will be referred to as the boiled controls.

Following the 9 h incubation, cells were separated from incubation medium by centrifugation (section 2.5.1). Portions of both incubation medium and cells were assayed for tritium (section 2.8.2.1).

Table 3.6.1 The proportion of ^{3}H taken up into cells and/or associated with insoluble cell wall material

Age of cultures (days from subculture)	Proportion of ^{3}H taken up into cells and/or associated with cells (%) after 9 h incubation of cells with [^{3}H]XG9			
	Cells in spent medium		Cells in fresh medium	
0	0.19	0.21	0.21	0.30
2	0.61	0.51	0.55	0.46
6	0.99	1.15	0.91	0.38
8	1.32	0.75	0.95	0.42
12	1.06	0.86	0.51	0.56
16	1.34	1.06	0.59	0.27
20	1.40	1.28	0.55	0.39
24	1.79	1.45	0.59	0.46

The results given are the data obtained from samples taken from two independent

250 ml cultures. All data have been corrected relative to the boiled controls.

Negligible ^{3}H-labelled material was taken up into cells, or became associated with insoluble (cell wall) material (Table 3.6.1). Cells washed in fresh medium and then incubated with [^{3}H]XG9 in fresh medium took up less ^{3}H-labelled material than did cells incubated with [^{3}H]XG9 in spent medium (Table 3.6.1).

3.6.4 Separation of ^{3}H-labelled components of incubation media

Samples of incubation medium (150 µl of medium removed from whole culture incubations; 150 µl of medium removed from washed cell incubations; and 100 µl of spent medium incubations) were applied to chromatography paper. Chromatography was carried out in BPW (section 2.6.3). External markers were visualised by alkali-silver nitrate stain (section 2.8.3). The amount of ^{3}H-labelled BPW-immobile material in each sample was determined by assaying the origins of the p.c. for tritium (section 2.8.2.2)

P.c. of the incubation media of day 2 and day 24 whole culture, washed cells and spent medium separated two ^{3}H-labelled components (data not shown). The first of these remained at the origin (^{3}H-labelled BPW-immobile material) and the second component co-chromatographed with the maltoheptaose external marker. This marker migrated clear of the origin on all p.c.'s (data not shown). Maltoheptaose has a similar mobility to XG9 in BPW.

3.6.4.1 Activity detected in whole suspension culture

The amount of ^{3}H-labelled BPW-immobile material accumulated in the medium during a 9 h incubation increased over the first six days following subculture. Between day 6 and day 8 the activity decreased. However, the activity detected in samples of whole culture increased gradually from day 8 onwards (Fig. 3.6.2).

3.6.4.2 Activity detected in washed cells

Cells separated from their spent medium produced substantially lower amounts of ^{3}H-labelled BPW-immobile material during 9 h incubation with [^{3}H]XG9 (Fig. 3.6.3), compared to whole culture (Fig. 3.6.2). The activity was maximal in cells taken from cultures on days 0 to 6 (Fig. 3.6.3).

3.6.4.3 Activity detected in spent medium

^{3}H-Labelled BPW-immobile material was also formed during the incubation of cell-free spent medium with [^{3}H]XG9 (Fig. 3.6.4). The activity detected in spent medium increased slightly between day 2 and day 6, but decreased between day 6 and 8. The activity detected in samples taken from day 8 onwards gradually increased

with age of culture (Fig. 3.6.4).

3.6.5 Discussion of results

Growth rates of the cultures investigated were estimated by measurement of the changes in settled cell volume and packed cell volume. These parameters are an approximate measure of cell expansion, irrespective of cell division. Cell division without cell expansion could not result in any appreciable change in SCV or PCV. A measure of cell expansion reflects the extent that the cell wall area increases during growth.

Growth, as defined by changes in SCV and PCV, was most rapid between day 2 and day 4. By day 6 from subculture the rate of change of SCV and PCV appeared to be much lower. The precise behaviour of the cultures over this later period is however difficult to define. The growth curves could be interpreted in one of the following two ways.

(1) From day 8 onwards, SCV and PCV continued to increase gradually (deviations from the curves being due to sampling error).

(2) The cultures investigated demonstrated a biphasic growth pattern. Rapid cell expansion occurred initially between days 2 and 4, the growth rate then slowed until day 12 when a second phase of cell expansion occurred. More usually the net growth rate of cultures tends towards zero with increasing age of older cultures.

The method employed to measure SCV and PCV of cultures involved the removal of a small sample of culture. The remaining culture was grown on: as its volume decreased, the ratio of surface area to volume of the culture increased. By altering this ratio, many processes within the culture could have been altered, e.g. the extent of aeration would have increased. It is possible that this enhanced aeration may result in the continuing growth seen in old cultures.

Whatever the exact behaviour of the cultures was from day 12 onwards, the most rapid increase in total cell volume of the cultures occurred between day 2 to day 6.

The production of ^{3}H-labelled BPW-immobile material in the medium of cultures during incubation with [^{3}H]XG9 is thought to be as a result of an extracellular transglycosylation reaction (section 3.5). As discussed in section 3.5.4, the final amount of ^{3}H-labelled BPW-immobile material may be the net product of two reactions: the incorporation of [^{3}H]XG9 into an apoplastic polymer (a transglycosylation reaction) and possibly its subsequent removal by the reverse reaction. Cultures exhibiting a large accumulation of ^{3}H-labelled polymeric product in their spent medium may:

(1) contain high levels of endo-transglycosylation activity; or

(2) contain relatively low levels of activity removing the incorporated [^{3}H]XG9 from the apoplastic polymeric product.

Accumulation of ^{3}H-labelled BPW-immobile material should never be considered as a measure of total transglycosylation activity, as exogenous [^{3}H]XG9 may not be the only glycosyl acceptor available in the apoplast of the culture. For example, XG9's parent polysaccharide, xyloglucan, is most probably an endogenous glycosyl acceptor in the reaction (section 3.4). However, the accumulation of ^{3}H-labelled BPW-immobile material during the incubation of plant material with [*reducing terminus*-^{3}H]XG9, may give an indication of the xyloglucan endo-transglycosylation activity present.

The net transglycosylation activity (with [^{3}H]XG9 as the acceptor) detected in whole culture samples increased from day 0 to day 6, coincident with the period of rapid growth (i.e. increase in PCV and SCV). Net transglycosylation activity declined between days 6 and 8, when the rate of growth was declining.

Net extracellular transglycosylation activity detected in washed cell incubation medium was elevated during days 0 to 6, as with whole culture. Transglycosylation activity measured in spent medium also showed a small peak in activity on day 6.

Samples of whole culture contained less spent medium and slightly fewer cells than were incubated individually with [^{3}H]XG9. Despite this, the levels of transglycosylation activities detected in day 6 washed cell incubation medium and in day 6 spent medium do not account for the total activity measured in day 6 whole culture. Samples of cells were prepared by filtering whole culture and collecting the spent medium which was incubated with [^{3}H]XG9. The cells were then washed in fresh medium to remove any remaining spent medium. Appreciable levels of transglycosylation activity were found to be removed from cells during this washing procedure (i.e. the cell washing was incubated with [^{3}H]XG9, and was found to have transglycosylation activity). This would indicate that a proportion of the enzyme (or enzymes) responsible for the production of ^{3}H-labelled BPW-immobile material are freely solubilised from the wall in fresh medium and not bound to the wall. Cells, however, still exhibit transglycosylation activity after washing. This may suggest that:

(1) a proportion of the activity is not freely soluble but is bound, either ionically or covalently to the cell wall; or

(2) all extracellular activity is removed by washing the cell in fresh medium and that activity detected in the apoplast of washed cells is due to proteins secreted into the apoplast during incubation.

Washed cells only exhibit elevated net transglycosylation activities during the period of rapid growth. The activities detected whole culture and spent medium were

found to increase steadily from day 8 onwards. During this period, SCV and PCV of the cultures were no longer increasing rapidly.

The net transglycosylation activity detected in the apoplast of washed cells may be the physiologically important portion of the activity within a suspension culture. Data presented here suggest that the apoplastic source of this potentially important activity could be one of the following.

(1) The enzyme is bound to the cell wall or cell surface, and is therefore not removed by washing. The plant cell wall is known to contain many enzymes involved in apoplastic metabolism (Bligny and Douce, 1983; Grison and Pilet, 1985; Hatfield and Nevins, 1987; Hayashi *et al.*, 1984; Wallner and Nevins, 1974). The strength of binding of proteins to the wall varies.

(2) The physiologically important activity is the newly secreted activity. The enzyme responsible for the transglycosylation reaction may have been completely solubilised from the cells during filtration and subsequent washing. The activity detected with washed cells could be that secreted during the period of incubation. Secretion of the enzyme rather than its synthesis could be the factor controlling the extent of apoplastic transglycosylation. Intraprotoplasmic enzyme activity was not detected, as the substrate ([^{3}H]XG9) did not pass through the plasma membrane.

Wall-bound activity and/or activity in transit through the wall may play a role in wall loosening whereas activity apparently accumulating in the spent medium of cultures may not. The solubilised activity free in the spent medium, and activity either bound to the wall and/or in transit through the wall, both produced high molecular weight ^{3}H-labelled material during incubation with [^{3}H]XG9. Possible bases of the difference in physiological role of the two activities could be one of the following.

(1) The enzyme itself may differ in terms of the activity it exhibits towards glycosidic bonds within the cell wall. For example, binding of the enzyme to the wall may alter the activity of this protein towards the endogenous glycosyl acceptor (proposed to be polymeric xyloglucan).

(2) However, it is more probable that the substrates available to solubilised enzyme and wall bound activity and/or enzyme in transit throught the wall, may differ. Although activity appears to be completely solubilised by washing cells thoroughly in fresh medium, the protein involved may only be able to permeate the cell wall to a limited extent during normal culture conditions. The permeation of macromolecules through the cell wall is limited by the porosity of the wall. The maximum sizes of globular proteins which can permeate the wall have been estimated to have molecular weights of 17,000 (Carpita *et al.*, 1979; using cytorrhysis of cells) and 60,000 (Tepler and Taylor, 1981; using the gel permeation properties of cell wall prepartions). If

permeation of the enzyme involved is limited, the substrates most readily available to the solubilised enzyme (in the spent medium) may be soluble xyloglucans (known to accumulate in the medium of suspension cultures; Hayashi *et al.*, 1980; Bauer *et al.*, 1973). However, newly secreted enzyme and/or wall bound activity is more likely to come into contact with polymeric xyloglucan within the inner cell wall. It has been postulated (Richmond *et al.*, 1980; Eisinger *et al.*, 1983) that the inner portion of the cell wall is the load bearing part of the wall. Enzymatic loosening of the inner cell wall may therefore be the important enzymatic process involved in cell expansion.

The activity accumulating in spent medium of suspension culture may be partly derived from cell sloughing (i.e. erosion of the outer cell wall) and cell death. The time period over which samples were taken from spinach suspension cultures in this experiment exceeds the normal culture cycle of 14 days. The spent medium might therefore normally contain slightly lower levels of transglycosylation activity than was detected here in older cultures.

In conclusion, xyloglucan endo-transglycosylation activity was detected in both the soluble fraction and associated with the insoluble fraction of the apoplast of spinach suspension cultures. The activity detected in the apoplast of washed cells (i.e. either bound to the wall and/or newly secreted from the cell) appears to be associated with the phase of rapid cell expansion of the culture.

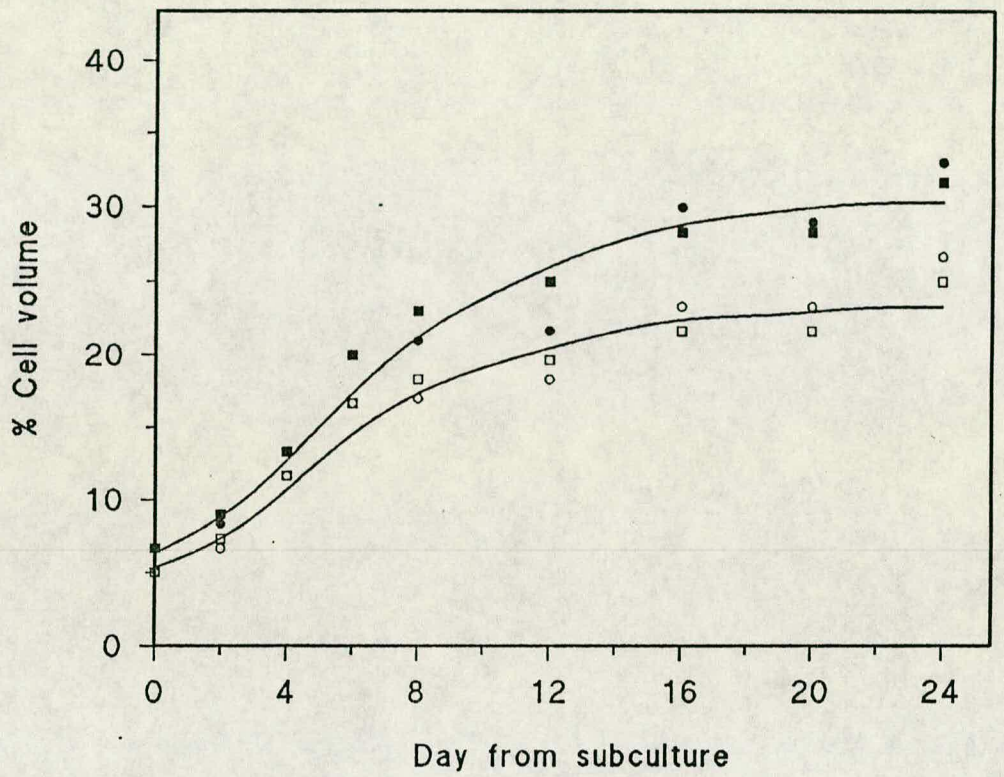


Fig 3.6.1 Estimation of cell expansion in *Spinacia* suspension cultures over a 24 day period

SCVs (● and ■) and PCVs (○ and □) were estimated throughout a 24 day growth cycle of two *Spinacia* cell suspension cultures. SCV and PCV values were calculated from the following; the volume occupied by cells (sett_led or packed) in a 3 ml sample of suspension culture).

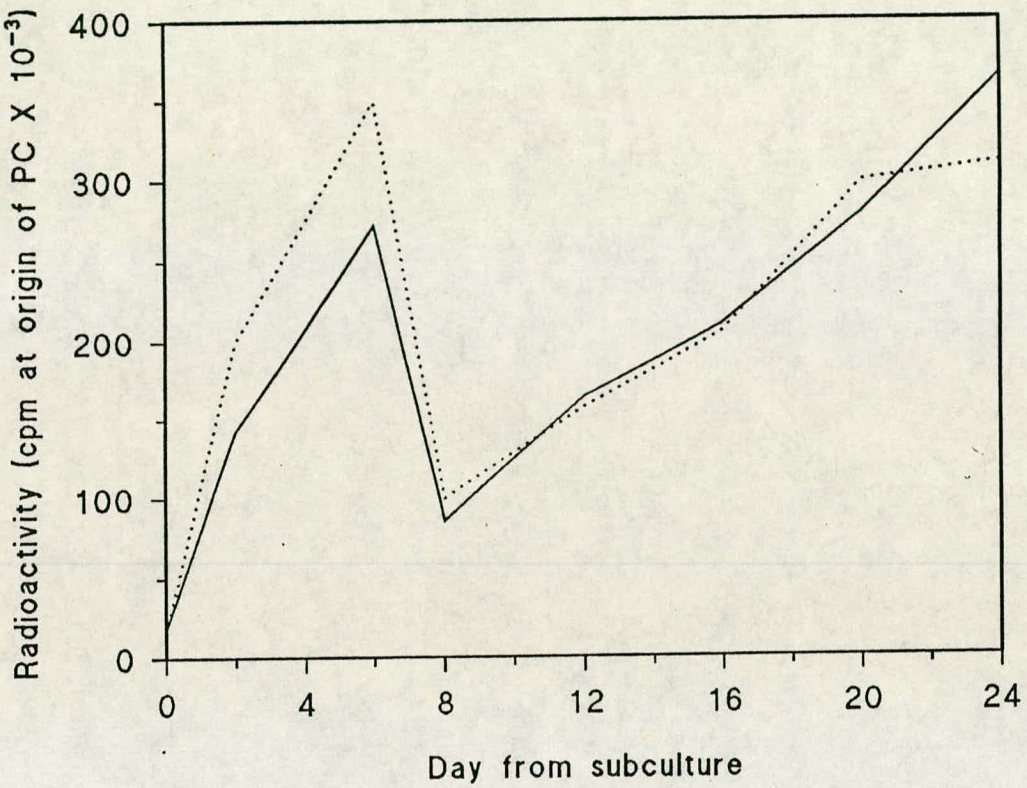


Fig 3.6.2 The synthesis of ^{3}H-labelled BPW-immobile material in the apoplast of whole *Spinacia* suspension culture during incubation with [*reducing terminus*-^{3}H]XG9

Samples of whole culture (3 ml) were taken from *Spinacia* suspension cultures at times throughout a 24 day growth cycle. These samples were incubated with [*reducing terminus*-^{3}H]XG9 (in 3 ml of fresh medium) for 9 h. ^{3}H-Labelled material in 150 µl of incubation medium was separated by p.c. in BPW. The total amount of ^{3}H-labelled BPW-immobile material (i.e. the amount of ^{3}H-labelled material remaining at the origin) produced in 6 ml of incubation medium was calculated (and corrected relative to boiled controls).

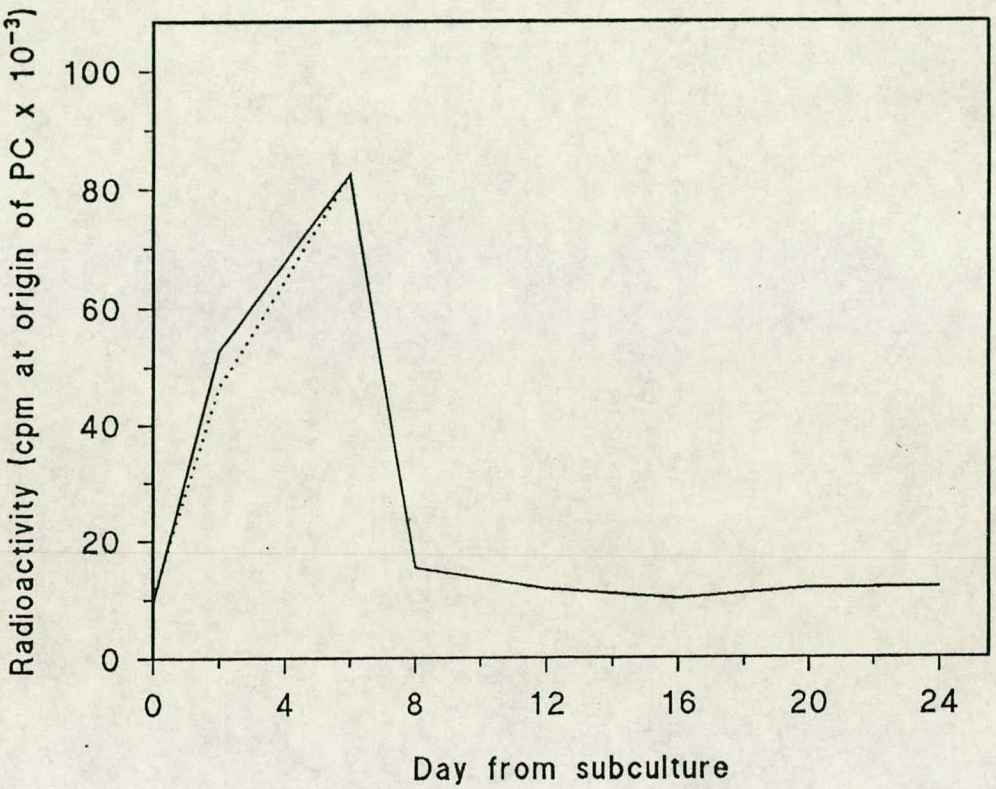


Fig 3.6.3 The synthesis of ^{3}H-labelled BPW-immobile material in the apoplast of washed *Spinacia* cells during incubation with [*reducing terminus*-^{3}H]XG9

Samples of washed cells (350 mg) were prepared from *Spinacia* suspension cultures at times throughout a 24 day growth cycle. These samples were incubated with [*reducing terminus*-^{3}H]XG9 (in 6 ml of fresh medium) for 9 h. ^{3}H-Labelled material in 150 µl of incubation medium was separated by p.c. in BPW. The total amount of ^{3}H-labelled BPW-immobile material produced in 6 ml of incubation medium was calculated

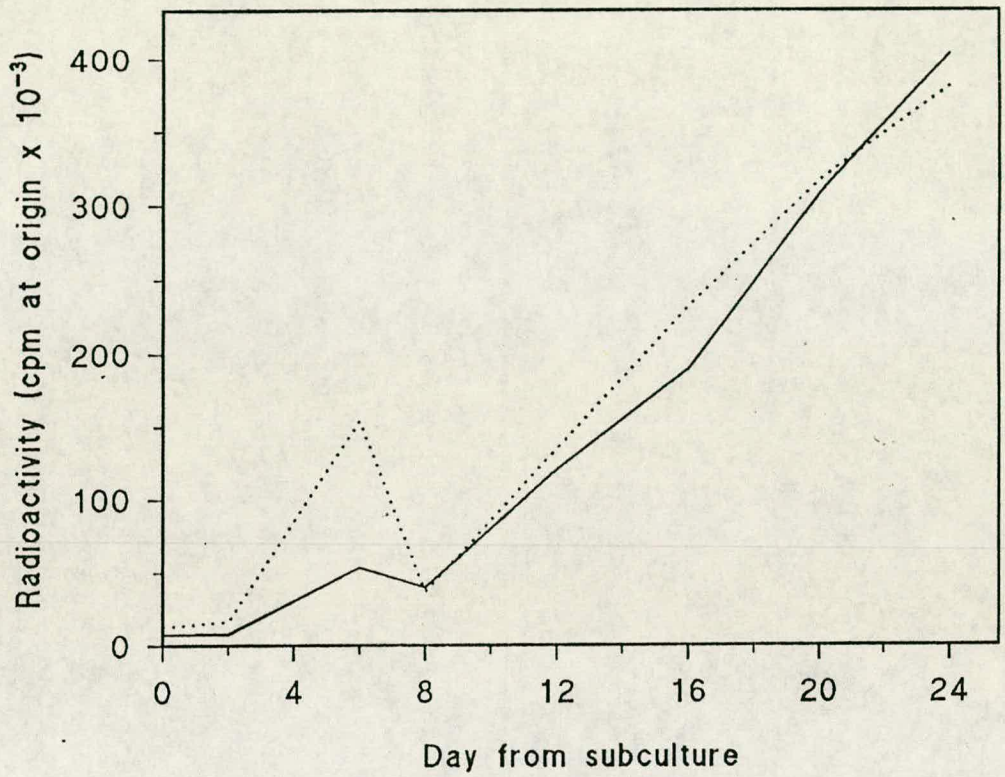


Fig 3.6.4 The synthesis of ^{3}H-labelled BPW-immobile material in *Spinacia* suspension culture spent medium during incubation with [*reducing terminus*-^{3}H]XG9

Samples of spent medium (3 ml) were prepared from *Spinacia* suspension cultures at times throughout a 24 day growth cycle. These samples were incubated with [*reducing terminus*-^{3}H]XG9 (in 3 ml of fresh medium) for 9 h. ^{3}H-Labelled material in 100 µl of medium was separated by p.c. in BPW. The total amount of ^{3}H-labelled BPW-immobile material produced in 6 ml of incubation medium was calculated.

4. DISCUSSION

4.1 Transglycosylation activity was detected in the apoplast of plant cell suspension cultures

When plant cell suspension cultures (derived from both dicotyledonous and graminaceous plants) were incubated in the presence of either [*xylosyl*-^{3}H]xyloglucan oligosaccharides or [*reducing terminus*-^{3}H]XG9 a soluble, apoplastic, high molecular weight, ^{3}H-labelled product was formed. The [^{3}H]oligosaccharides were shown to undergo little extracellular hydrolysis and were found not to be taken up by the cells to any great extent, and the incorporation of [^{3}H]XG9 into the product did not involve significant re-arrangement of the oligosaccharide. The findings presented in this thesis (chapter 3) provide evidence that transglycosylation activity is present in the apoplast of plant cells. The glycosyl donor in this reaction has been proposed to be polymeric xyloglucan (section 3.4). A glycosidic linkage in the backbone of xyloglucan is thought to be cleaved and the newly formed potential reducing terminus of the donor transferred onto the non-reducing terminus of XG9, the glycosyl acceptor.

Xyloglucan transglycosylation reactions have been recently demonstrated *in vitro*.

(1) A cellulase preparation from *Phaseolus* has been found to catalyse an increase in the molecular weight of rose [^{3}H]xyloglucan when incubated in the presence of an excess of larger, non-radioactive, xyloglucan (McDougall and Fry, 1990).

(2) The incubation of *Vigna* xyloglucan (molecular weight, 420,000) with apoplastic fluid derived from *Vigna* epicotyls resulted in the formation of lower molecular weight soluble xyloglucan and high molecular weight, water insoluble, xyloglucan (Nishitani and Tominago, 1991). This has been presented as evidence for the presence of a xyloglucan transglycosylase in the apoplast of plant cells.

(3) A xyloglucan specific endo-β-D-glucanase purified from germinated *Tropaeolum* seeds (Edwards *et al.*, 1986) has been reported to catalyse the transglycosylation of xyloglucan oligosaccharides derived from *Tamarindus* xyloglucan (C. Fanutti, Stirling University, personal communication).

(4) Endo-(1-4)-β-D-glucanase from *Tropaeolum* seeds has also been found to exhibit transglycosylation activity towards *Tropaeolum* xyloglucan and XG9n (V. Farkas *et al.*, Institute for Chemical Research, Bratislava, personal communication).

In the apoplastic reaction described here, the product formed is a soluble extracellular polymer with a molecular weight between 20,000 and 30,000, which is approximately one tenth the size of cell wall bound xyloglucan (molecular weight, 10^5 to 10^6; for review, Fry, 1989b). In a transglycosylation reaction the average molecular weight of the substrates is equal to that of the products. As the acceptor substrate is small, the molecular weight of the product would be expected to be lower than that of

the glycosyl donor, as the donor molecule is cleaved and only a portion is transferred onto the acceptor ([^{3}H]XG9).

The products formed by the incorporation of [^{3}H]XG9 into polymeric cell wall bound xyloglucan might have been expected to be wall bound. However, most of the ^{3}H-labelled transglycosylation product was found to be present in the culture medium.

4.2 A hypothesis for the formation of a low molecular weight, ^{3}H-labelled, soluble product by a transglycosylation reaction between wall-bound xyloglucan and XG9

Cell wall xyloglucan can exhibit two states of molecular mobility (Mackay *et al.*, 1988). The cell wall consists of a rigid component (64% of the primary cell wall mass) probably composed of xyloglucan bound to cellulose microfibrils and a highly mobile component made of molecules (pectic and hemicellulosic) perhaps indirectly attached to one or more microfibrils but loosely suspended in water (Mackay *et al.*, 1988). The accessibility of xyloglucan to enzymatic attack could be dependent on the molecular mobility of the substrate. For example, investigations into the susceptibility of cell wall bound xyloglucan to cellulase action has shown that approximately 30% of cell wall xyloglucan is hydrolysed releasing water-soluble products by a 6 h cellulase treatment. The other 70% of the bound xyloglucan is less susceptible to cellulase hydrolysis (Hayashi *et al.*, 1984). Segments of xyloglucan molecules not directly hydrogen bonded to cellulose microfibrils (i.e. xyloglucan with high molecular mobility) therefore may be more susceptible to enzyme action than non-mobile segments of xyloglucan.

There are three possible types of xyloglucan segment which are not directly hydrogen bonded to cellulose (Fig 4.2.1). If we assume that the mobile xyloglucan segments are those most susceptible to transglycosylation, there are three preferred products which we would expect to be formed by a transglycosylation reaction between xyloglucan (glycosyl donor) and [^{3}H]XG9 (glycosyl acceptor).

(1) The cleavage of a mobile, non-reducing terminal segment of xyloglucan, and the transfer of the 'released' glycosyl moiety onto [^{3}H]XG9, would result in the liberation of a soluble, medium molecular weight, ^{3}H-labelled xyloglucan fragment.

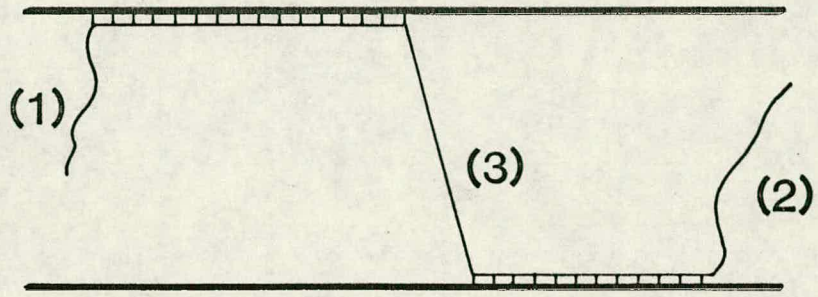


Fig. 4.2.1 A schematic representation of the mobile and non-mobile segments of cell wall xyloglucan

Non-mobile xyloglucan (⊥⊥) is thought to be hydrogen bonded to cellulose microfibrils (▬). Possible xyloglucan segments not directly hydrogen bonded to cellulose (—) present in the cell wall include; (1) non-reducing terminal segments of xyloglucan, (2) reducing terminal segments of xyloglucan, and (3) intermicrofibrillar segments of xyloglucan.

(2) A transglycosylation reaction between [^{3}H]XG9 and a mobile, reducing terminal segment of xyloglucan would result in the release of a soluble, medium molecular weight, non-radioactive xyloglucan fragment, with the glycosyl acceptor ([^{3}H]XG9) becoming the new reducing terminus of a wall-bound xyloglucan molecule.

(3) Transglycosylation between [^{3}H]XG9 and an intermicrofibrillar xyloglucan segment would also result in the incorporation of ^{3}H-labelled material into the insoluble wall, but no soluble xyloglucan fragments would be released.

However, almost all of the ^{3}H-labelled product formed was observed to be soluble and of medium molecular weight. This may suggest that the detected transglycosylation reaction occurs between [^{3}H]XG9 and mobile non-reducing terminal xyloglucan segments, or between [^{3}H]XG9 and the soluble xyloglucan present in the spent medium. However, medium molecular weight, soluble, ^{3}H-labelled products would be released from the cell wall if transglycosylation reactions between [^{3}H]XG9 and mobile reducing terminal or intermicrofibrillar xyloglucan segments were followed by a second enzyme attack (either hydrolysis or transglycosylation).

The possibility of multiple attack by an endo-β(1-3)-glucanase on a single polysaccharide has been reported (Bezukladnikov and Elyakova, 1990). This endo-glucanase exhibits transglycosylation and hydrolytic activities. The extent of

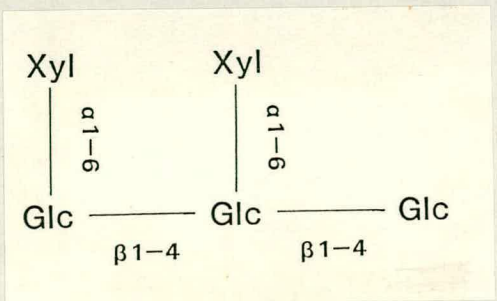
transglycosylation versus hydrolysis is dependent on the presence of a glycosyl acceptor. The substrate binding properties of this enzyme are such that following one attack on the substrate the enzyme moves along the polysaccharide (a few residues) prior to a second attack, and so on. A second example of multiple attack has been reported for a purified endopolygalacturonase secreted by cultures of *Colletotrichum lindemuthianum* (English *et al.*, 1972). The initial hydrolysis of polygalacturonic acid (PGA) chains appears to be random; however the enzyme then releases tri-, di- and monogalacturonic acid from PGA by multiple attack along the same substrate chain.

Multiple attack may also come about as a result of limited mobility of an enzyme through the cell wall. The enzyme may be restricted to one particular substrate molecule. The insoluble, wall-bound, ^{3}H-labelled products formed by a single transglycosylation reaction between [^{3}H]XG9 and cell wall xyloglucan may thus be highly susceptible to a second enzyme attack (hydrolysis or transglycosylation). As a result, a medium molecular weight, soluble, ^{3}H-labelled fragment would probably be released from the wall.

4.3 Substrate specificity of the transglycosylase

The donor and acceptor substrate specificities of the transglycosylase present in pea stem homogenate have been investigated *in vitro* (Fry *et al.*, 1992). The only polysaccharides found to act as glycosyl donors to the reaction were xyloglucans. Xyloglucans derived from rose cell walls (highly fucosylated) and from *Tropaeolum* seeds (non-fucosylated) act as donor substrates to the reaction. Fucosylation of the donor xyloglucan is therefore not essential; indeed the transglycosylase exhibited higher activity towards non-fucosylated xyloglucan. No other β-linked glucan nor any xylans, arabinoxylans or α-linked glucans acted as donor substrates to the detected *in vitro* reaction.

Competitive inhibition studies and incubation of the enzyme extract with specific ^{3}H-labelled substrates has been used to determine the acceptor substrate specificity of the transglycosylase present in pea stem homogenate (Fry *et al.*, 1992). The simplest structural unit for the glycosyl acceptor has been proposed to be:



Galactosylation and fucosylation of the xylosyl side group attached to the central glucose residue does not hinder the oligosaccharides' role as a glycosyl acceptor. Therefore, XG9, XG9n, XG7 and XG7f (Fig. 4.3.1) can all act as glycosyl acceptors to the apoplastic transglycosylation reaction (Fry *et al.*, 1992). In section 3.2 both XG9 and XG7 were shown to act as glycosyl acceptors in the *in vivo* reaction.

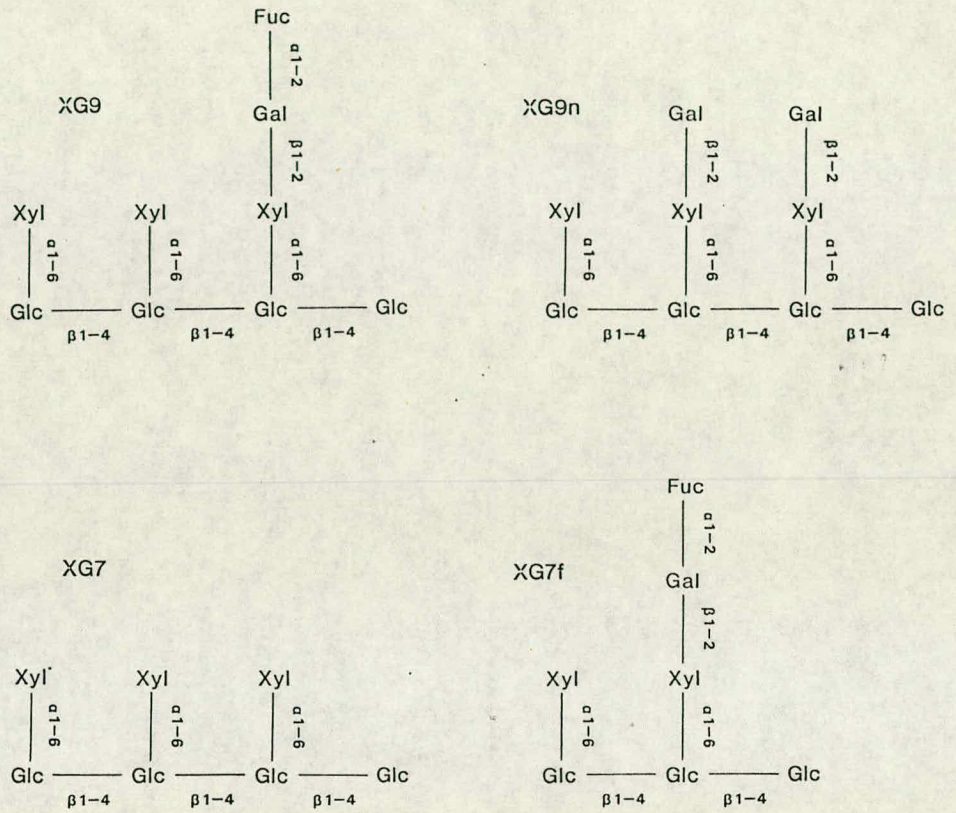


Fig. 4.3.1 The structure of xyloglucan oligosaccharides which can act as glycosyl acceptor substrates to the xyloglucan endotransglycosylase.

4.4 The endogenous glycosyl acceptor in the transglycosylation reaction may be polymeric xyloglucan

XG9 and XG7 act as the glycosyl acceptor in the detected apoplastic transglycosylation reaction. These oligosaccharides are derived from polymeric xyloglucan and are chemically similar to their parent polysaccharide and may therefore be susceptible to attack by xyloglucan acting enzymes (apart from cellulase).

Polymeric cell wall bound xyloglucan may be the endogenous glycosyl acceptor in the wall transglycosylation reaction as the non-reducing terminal group of this polymer may fulfil the structural requirements of the acceptor substrate. However, the only transglycosylation reactions detected were those involving the exogenous acceptor, [^{3}H]XG9. The products of such a reaction between two cell wall bound xyloglucan molecules would not be detected *in vivo* as the products and substrates would be indentical.

An enzyme present in the apoplast of *Vigna* epicotyls has been shown to catalyse an *in vitro* transglycosylation reaction between molecules of polymeric xyloglucan (Nishitani and Tominaga, 1991). Similarily, a *Phaseolus* cellulase preparation has been reported to catalyse a transglycosylation reaction between polymeric xyloglucan molecules (McDougall and Fry, 1990). This activity may be similar to that detected in the apoplast of plant cell suspension cultures (i.e. the reaction between [^{3}H]XG9 and polymeric xyloglucan).

4.5 Future work to detect the endogenous transglycosylation reaction

The products of a transglycosylation reaction between two cell wall-bound xyloglucan polymers (i.e. the proposed endogenous xyloglucan transglycosylation reaction) would be identical to the substrates. With no net chemical change to the wall the reaction would be difficult to detect. Therefore a technique is required in which any products of cell wall transglycosylation could be distinguished from the substrates.

4.5.1 The proposed technique to detect any transglycosylation of polymeric xyloglucan within the cell wall of growing cells (Fig. 4.5.1)

Transglycosylation between two cell wall-bound xyloglucan molecules would be detected if the two substrate molecules could be physically separated from one another once they had been extracted from the wall. For example, if a growing plant cell wall were initially to contain ^{12}C-xyloglucan and ^{13}C-xyloglucan, any transglycosylation occurring between these polymers could produce xyloglucan molecules containing both ^{12}C- and ^{13}C-residues. ^{12}C and ^{13}C are both stable isotopes of carbon differing only in their atomic weight. A polymeric molecule containing ^{13}C in place of ^{12}C will have a higher density than the same type of molecule containing solely ^{12}C. Some of the products formed by transglycosylation between ^{13}C-xyloglucan ('dense' xyloglucan) and ^{12}C-xyloglucan ('light' xyloglucan) may exhibit an intermediate density. Macromolecules of different bouyant densities can be separated from one

another by isopycnic centrifugation. By using isopycnic centrifugation ^{12}C-xyloglucan may be separable from ^{13}C-xyloglucan. If the resolution of these two xyloglucans is adequate, any intermediate density products formed by apoplastic transglycosylation could be separated from the xyloglucan substrates.

In order to produce plant cell walls containing both ^{12}C-xyloglucan and ^{13}C-xyloglucan it is proposed to subculture cells growing in glucose-$^{12}C_6$ medium, into medium containing glucose-$^{13}C_6$ so that they begin to synthesise ^{13}C-polysaccharides. Specific *in vivo* radio-labelleling of xyloglucan is not possible; however [^3H]fucose is only incorporated into fucose residues (Roberts, 1968), which occur in xyloglucan, some glycoproteins and a trace in rhamnogalacturonan-I/II (RG-I/II). The fate of ^{13}C-xyloglucan synthesised during a defined time interval could be determined by feeding [^3H]fucose in a pulse. Intermediate density polysaccharides may be produced transiently immediately following subculture. In order to minimise the detection of these, the cells should be incubated in glucose-$^{13}C_6$ medium for long enough to ensure that the intracellular pools of glucose are saturated with glucose-$^{13}C_6$ before the radio-labelled sugar is added to the culture medium. In this case the only ^3H-labelled polysaccharides synthesised by the cell are those maximally labelled with ^{13}C. The cells could then be transferred into glucose-$^{13}C_6$ medium containing no ^3H-labelled sugar or an excess of non-radioactive fucose and incubated in this medium for varying lengths of time to allow any apoplastic transglycosylation to occur.

The hemicellulose fraction would be extracted from the cell wall. Components of the extract would be separated by isopycnic centrifugation and so the bouyant density of [^3H]xyloglucan during prolonged incubation of cells in glucose-$^{13}C_6$ medium can be monitored. Any transglycosylation occurring between [^3H]xyloglucan and ^{12}C-xyloglucan would result in a decrease in the bouyant density of the ^3H-labelled material.

Variations in the bouyant density of cell wall bound xyloglucan may occur as a result of activities other than transglycosylation (e.g. hydrolysis). An appropriate control must therefore be used in which cells are fed a pulse of [^3H]fucose in the presence of glucose-$^{12}C_6$, and are not grown in glucose-$^{13}C_6$.

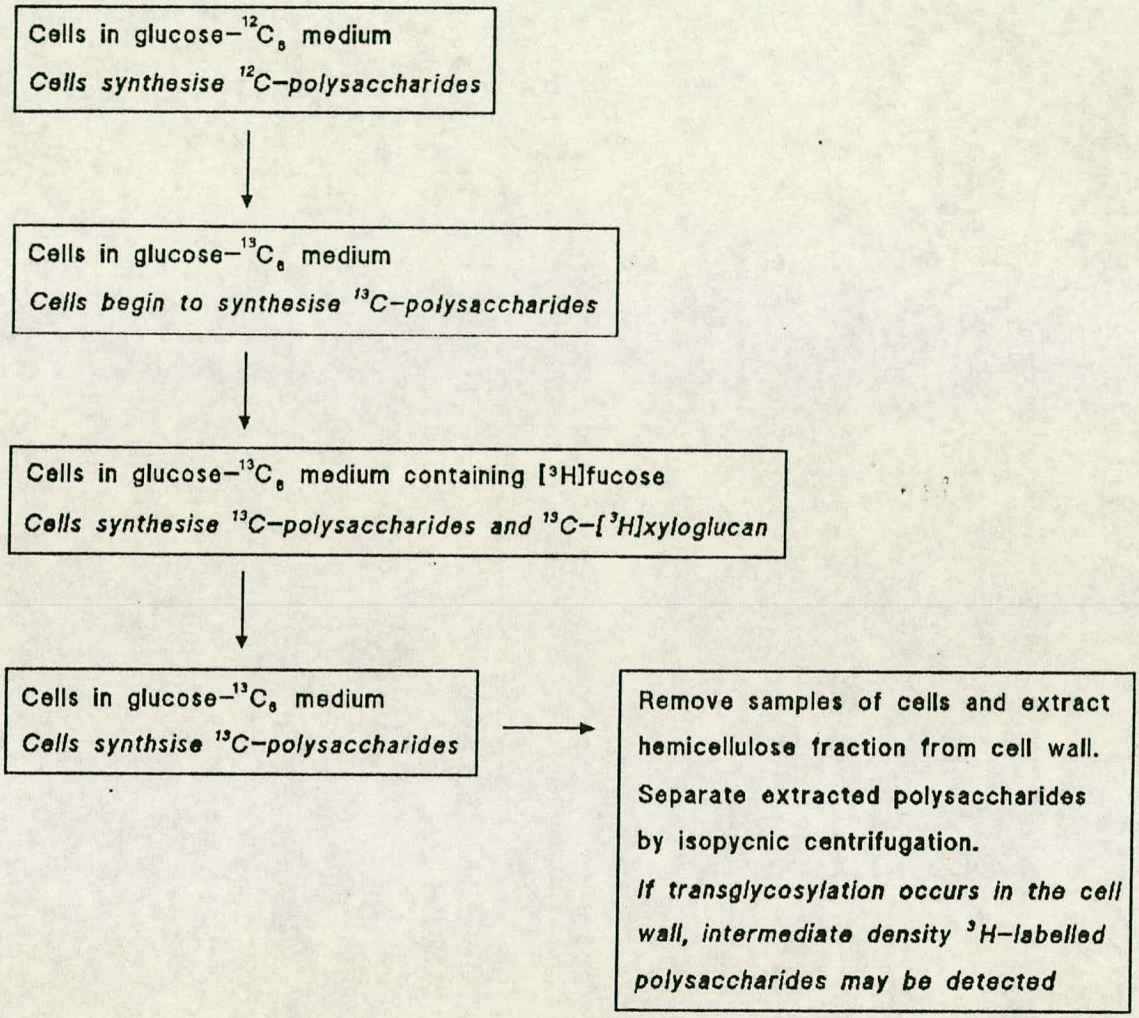


Fig. 4.5.1 Scheme of the proposed experimental regime to detect transglycosylation of polymeric xyloglucan within the cell walls of growing plant cells.

4.6 Is the transglycosylase a cellulase?

The bonds cleaved and synthesised during the detected transglycosylation reaction appear to be cellulase-labile (i.e. probably D-glucose-β(1-4)-D-glucose; section 3.4). The enzyme involved in the reaction may therefore be a cellulase acting as a transglycosylase rather than as a hydrolyase. A preparation of cellulase from *Phaseolus* has been reported to catalyse an apparent increase in molecular weight of [^{3}H]xyloglucan in the presence of non-radioactive higher molecular weight xyloglucan (McDougall and Fry, 1990). This *Phaseolus* preparation has also been shown to catalyse the incorporation of [^{3}H]XG9 into a copper-precipitable polymer (G.J. McDougall, SCRI, Invergowrie, personal communication). These observations suggest that the cellulase or a contaminant of the preparation may exhibit transglycosylation activity. However, by comparing the properties of cellulase (derived from pea stems) with the properties of the apoplastic xyloglucan transglycosylase, it appears that the detected transglycosylation reaction is mediated by a novel enzyme and not a cellulase (Fry *et al.*, 1992). The following observations support this conclusion:

(1) Cellulase derived from pea stems, efficiently hydrolyses carboxymethylcellulose (CMC) and β-(1-3),(1-4)-D-glucan (Wong *et al.*, 1977). Neither of these cellulase substrates can act as a donor substrate to the transglycosylase (Fry *et al.*, 1992).

(2) The hydrolysis of CMC by pea cellulase is inhibited by cello-oligosaccharides (Wong *et al.*, 1977). However, cellopentaose does not affect the transglycosylation activity present in pea homogenate (Fry *et al.*, 1992).

(3) 2,4-D (2,4-dichlorophenoxyacetic acid) induces an increase in both buffer-soluble and buffer-insoluble cellulase activity (Verma *et al.*, 1975; Hayashi *et al.*, 1984). The same auxin treatment results in a decrease in extractable transglycosylation activity in pea stems (Fry *et al.*, 1992).

(4) High salt extracts of 2,4-D-treated pea stems contained high cellulase activity but low transglycosylation activity (Fry *et al.*, 1992).

(5) Mercaptoethanol, which is claimed to inhibit pea cellulase activity (Wong *et al.*, 1977), promotes transglycosylase activity (Fry *et al.*, 1992).

The xyloglucan transglycosylation activity detected in the apoplast of suspension cultured cells may therefore be due to a novel enzyme, for example a xyloglucan-specific endo-(1-4)-β-glucanase with transglycosylation activity (Edwards *et al.*, 1986).

4.7 Possible roles for xyloglucan endo-transglycosylation in the plant cell wall

Transglycosylation of cell wall polysaccharides has been postulated as a mechanism which could contribute towards cell wall loosening without the loss of the wall's physical strength (section 1.6). In this section evidence for such a role in cell elongation is discussed, together with the possibility that xyloglucan transglycoslylation could also be involved in a process of xyloglucan inter-microfibrillar 'tether' repair.

4.7.1 Transglycosylation and cell wall loosening

Transglycosylation involving inter-microfibrillar xyloglucan chains could cause incremental cell wall expansion by the temporary cleavage of load-bearing bonds. Cleavage of a xyloglucan 'tether' could result in limited turgor driven extension prior to the synthesis of another glycosidic linkage between the glycosyl donor and a second xyloglucan molecule, the acceptor substrate (section 1.6). The enzyme may preferentially catalyse reactions involving those segments of xyloglucan molecules which are under tension, rather than between mobile segments of xyloglucan chains which are non-load-bearing. Transglycosylation of inter-microfibrillar xyloglucan could allow cell wall expansion without the loss of the wall's mechanical strength (Fig 4.7.1).

Evidence supporting the possible role of xyloglucan endotransglycosylation in cell wall loosening has been presented in this thesis (section 3.6). Xyloglucan endotransglycosylation activity was found to be associated with suspension cultured spinach cells only during rapid cell expansion. It has been postulated that this growth related activity is either bound to the cell wall or is newly secreted into the wall (section 3.6). From the work on transglycosylase extraction from homogenised pea stem tissue it appears that only negligible activity is bound to the cell wall (Fry *et al.*, 1992). It is therefore probable that during cell expansion the transglycosylase is secreted into the apoplast and catalyses the transglycosylation of load bearing xyloglucan 'tethers' during its transit through the wall (section 3.6).

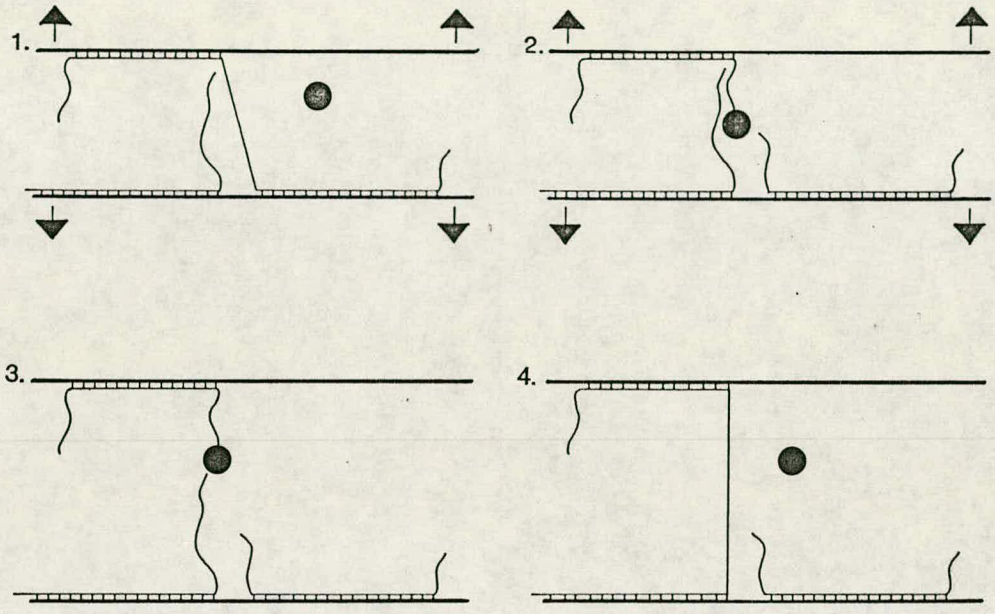


Fig. 4.7.1 Schematic representation of a transglycosylation reaction which could result in incremental cell wall expansion.

(1) A xyloglucan 'tether' is under tension due to turgor (↑). (2) Xyloglucan transglycosylase (●) cleaves the backbone of the xyloglucan 'tether', (3) allowing limited turgor-driven expansion. (4) The transglycosylase transfers the newly formed, potentially reducing terminus of the broken 'tether' onto the non-reducing terminus of a mobile segment of xyloglucan, so forming a new xyloglucan 'tether' replacing the original load-bearing bond.

Cellulose microfibrils (—): Hydrogen bonded xyloglucan chains (⊔): xyloglucan segments not directly hydrogen bonded to microfibrils (—).

Further evidence of the possible physiological role of xyloglucan endotransglycosylation is the distribution of this activity along the stem of a growing pea seedling. Homogenates of the third, rapidly elongating, internode of pea seedlings (a short distance from the apical bud) contained substantially greater transglycosylation activity than homogenates of the slower growing first internode (Fry *et al.*, 1992). A similar distribution of transglycosylation activity has been shown in the apoplast of *Vigna* epicotyls (Nishitani and Tominaga, 1991).

The appearance of transglycosylation activity in the apoplast of cells during cell expansion may result from synthesis of the enzyme rather than simply its secretion. Homogenisation of pea stem tissue would release both intercellular and intracellular activities. The protein synthesis inhibitor cycloheximide has been found to inhibit the appearance of xyloglucan transglycosylation activity in the apoplast of pre-washed 3 d carrot cultures by approximately 90% (P.R. Hetherington, Edinburgh University, personal communication). This result suggests that the levels of xyloglucan tranglycosylation activity are controlled at the point of protein synthesis.

4.7.2 Transglycosylation and the synthesis of xyloglucan 'tethers'

Inter-microfibrillar xyloglucan is not necessarily the only xyloglucan which is not directly hydrogen bonded to cellulose microfibrils and therefore accessible to apoplastic enzymes (section 4.2). Transglycosylation between mobile reducing and non-reducing terminal segments of xyloglucan would not contribute to cell wall loosening, as described for reactions involving inter-microfibrillar segments of xyloglucan. Rather such a reaction could result in the formation of new xyloglucan 'tethers', possibly contributing to a cell wall tightening process restricting wall expansion (Fig. 4.7.2). For example, when H^+-treated *Vigna* epicotyl tissue is transferred into a neutral buffer, a decrease in growth rate is observed along with an increase in the mean molecular weight of cell wall bound xyloglucan (Nishitani and Masuda, 1982). This increase in the average size of xyloglucan molecules could be brought about by xyloglucan transglycosylation.

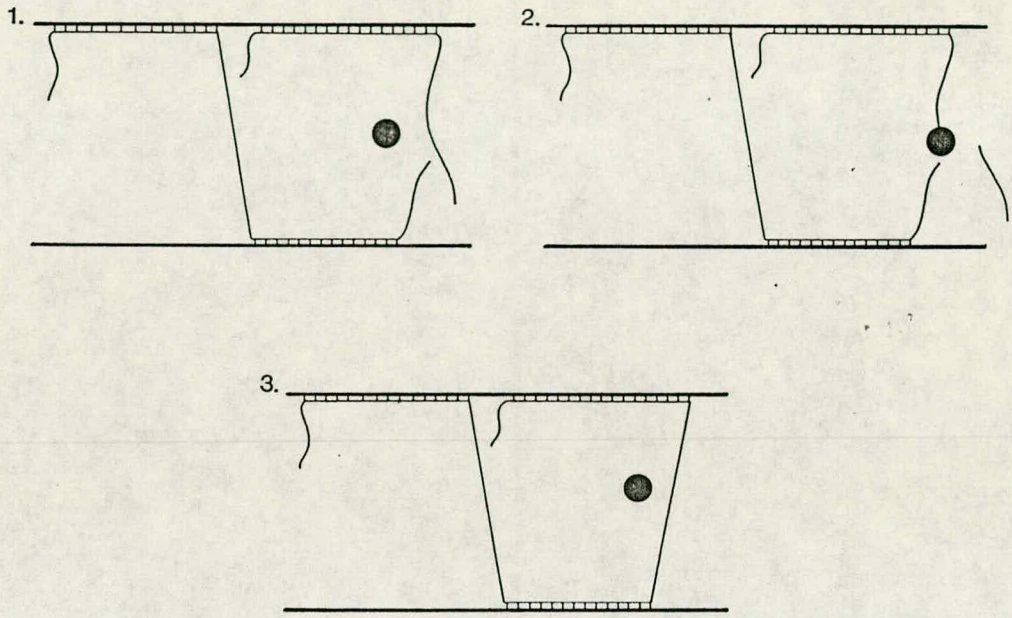


Fig. 4.7.2 Schematic representation of a transglycosylation reaction which could result in the synthesis of new xyloglucan 'tethers' between microfibrils.

(1) A mobile xyloglucan reducing terminal segment is cleaved by the xyloglucan transglycosylase (●) (2), and the newly formed, potentially reducing terminus is transfered onto a mobile, non-reducing teriminal xyloglucan segment (3), so forming a new xyloglucan 'tether' between two cellulose microfibrils (i.e. a new load-bearing bond).

Cellulose microfibrills (—): hydrogen-bonded xyloglucan chains (⫟⫟): mobile xyloglucan segments (—).

Such a process of cell wall tightening may contribute towards a cessation of cell elongation as a result of a loss in wall extensibility. It is therefore possible that the metabolism of cell wall bound xyloglucan is involved in both wall loosening and tightening. Cellulase- or xyloglucan endotransglycosylase-catalysed cleavage (i.e. hydrolysis) of xyloglucan 'tethers' could cause an increase in wall extensibility so allowing for turgor driven expansion. The subsequent repair of these 'tethers' by xyloglucan transglycosylation could bring about cell wall tightening so limiting cell expansion.

Evidence suggesting that xyloglucan endo-transglycosylation may be involved in wall tightening is that the dilution of 'Oxford cell line' carrot cultures (Cole *et al.*, 1990; Knox *et al.*, 1991) causes a rapid decrease in soluble, symplastic xyloglucan endotransglycosylation activity along with an onset of cell expansion (P.R. Hetherington, Edinburgh University, personal communication). In high cell density cultures of the 'Oxford cell line', grown in the presence of 2,4-D, 80 to 90% of the cells are small and spherical. Dilution of this culture (100 fold) and the removal of 2,4-D results in the rapid elongation (10 fold in 4 days) of between 80 to 90% of the cells. High levels of xyloglucan transglycosylation activity are present in the apoplast of the small non-expanding cells. The levels of this activity decrease (3 to 4 fold) with the onset of rapid cell expansion brought about by dilution of the culture. The decline in transglycosylation activity may remove a constraint of cell elongation.

Xyloglucan transglycosylation may also be involved in the process of incorporation of soluble, newly secreted xyloglucan into the insoluble cell wall. Newly secreted xyloglucan molecules are thought to become bound to the cellulose microfibrils via hydrogen bonds (section 1.3). However, treatment of pea internode segments with the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB) has been found not to affect the binding of newly secreted xyloglucan molecules to the cell wall over a 24 h period (H. Edelmann, Bonn University, personal communication). Newly synthesised xyloglucan may bind to existing xyloglucan in the cell wall via glycosidic linkages formed by the action of a transglycosylase.

From the data presented in this thesis it is not possible to infer in which, if either, of these processes (cell wall loosening or tightening) xyloglucan transglycosylation plays a role. Elevated activities detected in the apoplast of young spinach cells (section 3.6.4.2) may have been associated with rapid cell expansion, or with the onset of the deceleration of cell growth. Difficulty in the determination of the precise events in the culture cycle have made it impossible to show a correlation of high xyloglucan transglycosylation activity with either rapid cell expansion or with cell wall tightening. There is an apparent correlation between cell expansion in the stems of

etiolated pea seedlings and transglycosylation activity in stem segment homogenates (Fry *et al.*, 1992). The peak in detected activity occurs over the top 5 cm of the growing pea stem, whereas the most rapid cell elongation is thought to occur towards the top of this segment. This elevated transglycosylation activity could therefore be associated with cells leaving the zone of rapid expansion, and therefore with the deceleration of wall expansion rather than with cell wall loosening.

4.7.3 Future work to determine the physiological role of xyloglucan transglycosylation

Determination of the physiological role played by xyloglucan endotransglycosylase within the cell wall could involve biochemical, physiological and molecular biological techniques.

4.7.3.1 Xyloglucan transglycosylase activity in a plant tissue whose growth has been well characterised

The difficulty in studying the possible relationship between xyloglucan transglycosylation activity and plant cell expansion in plant cell suspension cultures is that the transglycosylase appears to be highly soluble and therefore accumulates in the medium of the culture. Suspension cultures also differ from whole plants in that they have an extended apoplast, and that in cultured cells a particular growth pattern is selected for by regular subculturing. Therefore, to investigate the role of xyloglucan transglycosylation in cell expansion it may be useful to use a whole plant as the experimental material. If the growth of a particular plant tissue is well characterised and the zones of rapid cell elongation, deceleration of cell expansion and cessation of cell expansion can be resolved, any correlation between cell elongation or the cessation of growth and xyloglucan endotransglycosyation activity could be investigated. Using such a system, the effect of plant growth regulators on both cell expansion and transglycosylation activity could be determined. For example, auxin or gibberellin treatment of plant tissue may result in an increase in xyloglucan endotransglycosylation activity together with an increase in cell elongation, which would indicate that apoplastic transglycosylation of xyloglucan may be involved in wall loosening.

4.7.3.2 The activity of the transglycosylase towards mobile and non-mobile, stressed and non-stressed xyloglucan segments

In the discussion thus far I have assumed that mobile xyloglucan chains are the most likely substrates of the transglycosylase *in vivo*, as opposed to xyloglucan segments which are hydrogen-bonded to cellulose microfibrils (section 4.2). If

xyloglucan endotransglycosylation is involved in either wall loosening or in the synthesis of new xyloglucan 'tethers' (Figs. 4.7.1 and 4.7.2), transglycosylation of mobile xyloglucan segments is likely. It may be possible to determine the affinity of purified xyloglucan endotransglycosylase to soluble xyloglucan and xyloglucan which is hydrogen-bonded to cellulose by the following method. Non-radioactive, large molecular weight xyloglucan (purified by g.p.c.) could be incubated with different quantities of cellulose granules ranging from an excess of cellulose to no cellulose. Presumably when xyloglucan is incubated with an excess of cellulose the majority of xyloglucan will hydrogen bond to the granules. By decreasing the amount of cellulose present in the pre-incubation mixture, the proportion of directly hydrogen bonded xyloglucan chains will decrease, until, when no cellulose is present, all the xyloglucan substrate will be mobile and soluble. This material could then be incubated with [^{3}H]XG9 in the presence of purified xyloglucan transglycosylase. The molecular weight of [^{3}H]xyloglucan (both soluble and insoluble) could be monitered over a period of time by g.p.c.. From the results of such an experiment it may be possible to infer whether the affinity of the transglycosylase for xyloglucan substrates is affected by the hydrogen bonding of xyloglucan to cellulose and so whether the *in vivo* reaction is more likely to involve mobile or soluble xyloglucans rather than hydrogen-bonded xyloglucan.

If the xyloglucan endotransglycosylase is involved in the processes of wall loosening during cell elongation, the transglycosylation reaction would involve intermicrofibrilar xyloglucan segments, i.e. xyloglucan chains which are accessible and under stress. The process of synthesising new xyloglucan 'tethers' would involve transglycosylation between mobile, non-stressed xyloglucan chains. The behaviour of xyloglucan endotransglycosylase towards stressed and non-stressed substrate molecules may indicate what the role of this activity is within the cell wall. Xyloglucan molecules could be subjected to stress *in vitro* by pumping (under pressure) through an inert matrix. For example, a reaction mixture of xyloglucan transglycosylase, high molecular weight non-radioactive xyloglucan and [^{3}H]XG9, could be slowly pumped through an hplc column, filled with an inert packing. By comparing the rate of such a reaction between stressed xyloglucan molecules, with that between non-stressed xyloglucan (i.e. not subjected to high pressure) it may be possible to ascertain whether the more probable *in vivo* reaction involves stressed xyloglucan segments or non-stressed chains.

4.7.3.3 The fate of newly secreted xyloglucan molecules in the apoplast

Xyloglucan endotransglycosylase may be involved in the synthesis of new xyloglucan 'tethers' between cellulose microfibrils (Fig. 4.7.2). If this were the case,

newly secreted xyloglucan molecules may be subject to transglycosylation with pre-exsisting wall bound xyloglucan. Some evidence for this type of reaction has been discussed in section 4.7.2. The result of a transglycosylation reaction between newly secreted xyloglucan and wall bound polysaccharides would be an increase in molecular weight of the newly secreted xyloglucan once it was in the apoplast. This apoplastic increase in molecular weight of newly secreted polysaccharides could be detected by feeding cells a radio-labelled precursor of xyloglucan (e.g. [^{3}H]fucose). [^{3}H]Fucose should be applied to cells in a pulse sufficiently long to ensure that a proportion of intraprotoplasmic [^{3}H]xyloglucan had attained a maximum intracellular molecular weight, but should not exceed the time taken for the ^{3}H-labelled polysaccharide to be secreted into the apoplast. The cells could then be transferred into non-radioactive medium. During incubation in this medium the cells will secrete [^{3}H]xyloglucan into the apoplast. The molecular weight of [^{3}H]xyloglucan bound to the cell wall could be determined by extraction and gpc. Comparisons of the maximum molecular weight of intracellular [^{3}H]xyloglucan with the maximum molecular weight of wall bound [^{3}H]xyloglucan would indicate if nascent xyloglucan molecules are subject to apoplastic transglycosylation, possibly forming new xyloglucan 'tethers' within the wall.

4.7.3.4 The control of transglycosylase synthesis and secretion into the apoplast

In order to investigate the control of synthesis and secretion of xyloglucan endotransglycosylase (XET) in growing plant cells, the enzyme should be purified. Once the protein has been isolated, it would be possible to raise antibodies against it and to identify the transglycosylase gene and its transcription products. These in turn could make it possible to localise the transglycosylase within the apoplast (by immuno-cytochemistry) and to investigate the controls of synthesis and secretion of the activity by growing plant cells; for example, the effect of various plant growth regulators (e.g. auxins, gibberellins, abscisic acid etc.) on the translation of XET-mRNA and the levels of enzyme secreted from the cells (by Western blotting). The effect of plant growth regulators on the steady state levels of XET-mRNA could also be determined (by Northern blotting). Before these techniques can be used, not only must the XET protein be isolated and antisera raised, but also cDNA encoding for the protein must be cloned (perhaps using an expression library).

Specific mutants could be used to help determine the physiological role of xyloglucan endotransglycosylase, for example, the production of heat-shock mutants (REF) producing increased or decreased levels of xyloglucan endotransglycosylase following an incubation at an elevated temperature. The effect of altering xyloglucan endotransglycosylation activity on the growth rate and cell wall properties of the

mutant cells could indicate what the role of this enzyme is in the plant cell wall.

4.8 A possible basis for the stimulation of plant cellulase activity and cell expansion by xyloglucan oligosaccharides

4.8.1 A possible basis of the stimulation of plant cellulase by xyloglucan oligosaccharides

Certain xyloglucan oligosaccharides (concentration, 1-100 μM) apparently cause a stimulation in 'cellulase' activity as determined by a viscometric assay using xyloglucan as the 'cellulase' substrate. In such an assay, the xyloglucan is hydrolysed by cellulase and as a result the viscosity of the substrate decreases (Farkas and Maclachlan, 1988). The addition of specific xyloglucan oligosaccharides to the assay mixture results in an increase in the rate of change in substrate viscosity.

The incubation of xyloglucan with a transglycosylase in the absence of oligosaccharides would probably result in little if any alteration of the viscosity. All transglycosylation reactions would involve the cleavage of a xyloglucan chain and the transfer of the newly formed potentially reducing terminus onto the non-reducing terminus of another xyloglucan molecule. However, the addition of xyloglucan oligosaccharides to the incubation mixture would provide the transglycosylase with low molecular weight glycosyl acceptors which would compete with xyloglucan. Transglycosylation reactions between polymeric xyloglucan and xyloglucan oligosaccharides could result in the loss of viscosity of the xyloglucan substrate. This mechanism (i.e. that xyloglucan transglycosylase was the activity causing xyloglucan breakdown, rather than cellulase) therefore may explain the apparent stimulation of 'cellulase' activity by xyloglucan oligosaccharides.

Evidence to support this mechanism is the donor and acceptor substrate specificity of xyloglucan endotransglycosylase. Firstly, the promotion of 'cellulase' activity by xyloglucan oligosaccharides is only observed if the viscous substrate used in the assay is xyloglucan. No oligosaccharide-stimulated activity is observed towards carboxymethylcellulose (CMC), a commonly used artificial substrate of cellulase. Similarily, CMC is not a donor substrate of xyloglucan endotransglycosylase (Fry *et al.*, 1992) and is therefore not susceptible to transglycosylation. Secondly, the structural requirements of an oligosaccharide to act as a glycosyl acceptor for xyloglucan endotransglycosylase are similar to those required to promote 'cellulase' activity in the viscometric assay (E.P. Lorences and S.C. Fry, University of Valencia/Edinburgh, personal communication). Further evidence for the role of

xyloglucan endotransglycosylase in the promotion of xyloglucan-depolymerisation activity by xyloglucan oligosaccharides is that the effect of oligosaccharides has been demonstrated to be stoichiometric rather than regulatory (McDougall and Fry, 1990), which suggests that the oligosaccharides are substrates in the reaction causing a drop in xyloglucan viscosity.

The activity of a cellulase preparation from *Phaseolus* towards xyloglucan in a viscometric assay has been shown to be promoted by the xyloglucan oligosaccharides and has also been shown to exhibit transglycosylation activity. Incubation of high molecular weight non-radioactive xyloglucan with lower molecular weight [^{3}H]xyloglucan in the presence of the cellulase preparation resulted in an increase in the molecular weight of the [^{3}H]xyloglucan (McDougall and Fry, 1990). This enzyme preparation has also been shown to catalyse the incorporation of [^{3}H]XG9 into copper-precipitable polymer, presumably xyloglucan (G.J. McDougall, SCRI, Invergowrie, personal communication). Although the xyloglucan endotransglycosylase is not likely to be a cellulase (section 4.6), the cellulase preparation may contain transglycosylase activity as a contaminant.

Some plant cellulase preparations catalyse a gradual decrease in the viscosity of polymeric xyloglucan in the absence of xyloglucan oligosaccharides. Hydrolysis of the xyloglucan substrate rather than xyloglucan transglycosylation may account for this observation. This hydrolytic activity may reside with cellulase present in the preparation, or with the transglycosylase which may exhibit hydrolytic activity towards xyloglucan at low substrate concentrations (section 1.5.1).

4.8.2 A possible basis for the promotion of cell expansion by xyloglucan oligosaccharides

Specific xyloglucan oligosaccharides (e.g. 1 μM-XG9) stimulate elongation of pea stem segments (McDougall and Fry, 1990). This 'auxin mimicking' activity of oligosaccharides may be due to their effect on the endogenous xyloglucan transglycosylation reactions in the apoplast of plant cells. These reactions may involve non-hydrogen bonded segments of polymeric cell wall xyloglucan (section 4.2) and may contribute to either cell wall loosening or wall tightening (section 4.7). If low molecular weight acceptor substrate molecules (i.e. xyloglucan oligosaccharides) were present in the apoplast of cells, they would compete with the proposed polymeric acceptor substrate (i.e. xyloglucan). As a result, the cleaved portions of the donor substrate (i.e. xyloglucan) may be transferred onto the oligosaccharide, rather than onto wall-bound xyloglucan. This in turn would result in a decrease in the number of load-bearing xyloglucan chains synthesised by

transglycosylation, whether the transglycosylase cleaves the xyloglucan donor molecules in intermicrofibrillar segments or in the mobile, terminal segments of the molecule. Transglycosylation of wall bound xyloglucan donor onto low molecular weight acceptors could therefore cause a long-term loosening of the wall, similar to that proposed for polysaccharide hydrolysis.

Evidence for such a mechanism can be found by comparing the structural requirements for 'auxin-mimicking' oligosaccharides with the acceptor substrate requirements for the transglycosylase. The oligosaccharides which have been shown to promote cell elongation are (in order of effectiveness) XG9n > XG8 > XG7 > XG9 (McDougall and Fry, 1990). XG9n, XG7 and XG9 are also acceptor substrates for xyloglucan transglycosylase; the order of affinity of the enzyme for the acceptor substrates being XG9n > XG7 > XG9 (Fry *et al.*, 1992). XG5 does not appear to act as an 'auxin mimic' (McDougall and Fry, 1990) and is not an acceptor substrate for the transglycosylase (Fry *et al.*, 1992).

4.8.3 Xyloglucan transglycosylation and the control of apoplastic XG9 concentration

Transglycosylation of xyloglucan onto XG9, a biologically active oligosaccharide, may also be an important process in controlling the concentration of apoplastic XG9. XG9 is present in the apoplast of plant cells (Fry, 1986b) and is believed to be produced by the cleavage of cell wall bound xyloglucan (McDougall and Fry, 1989a; 1990). Presumably, the concentration of this oligosaccharin is strictly controlled although XG9 does not appear to be subject to extensive hydrolysis in the cell apoplast (sections 3.2, 3.4, 3.5; Baydoun and Fry, 1989).

4.9 Conclusions

The apoplast of suspension cultured plant cells contains transglycosylation activity which catalyses the incorporation of low molecular weight acceptor substrate ([^{3}H]XG9) into soluble polymeric xyloglucan. This activity was detected in the apoplast of both dicotyledonous and graminaceous plant cell cultures, and was found to be highly soluble and present in the spent culture medium. A peak in xyloglucan transglycosylation activity was found to be associated with the period of most rapid cell expansion. This activity is probably that in transit through the cell wall following secretion from the cells rather than bound to the wall. The possible roles of this activity include wall loosening (during cell expansion) and wall tightening (during the deceleration of cell expansion).

I have also suggested that the stimulation of xyloglucan depolymerisation by certain xyloglucan oligosaccharides, as detected in viscometric assays, may have its basis in the action of the apoplastic xyloglucan endotransglycosylase.

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6. APPENDIX

A. Composition of cell suspension culture media

A1. *Acer pseudoplatanus* (sycamore) culture medium

KCl	37.5 $g.l^{-1}$
$MgSO_4.7H_2O$	12.5 $g.l^{-1}$
$NaNO_3$	30 $g.l^{-1}$
NaH_2PO_4	6.5 $g.l^{-1}$
$CaCl_2.2H_2O$	3.7 $g.l^{-1}$
$ZnSO_4.7H_2O$	1.0 $mg.l^{-1}$
H_3BO_3	1.0 $mg.l^{-1}$
$MnSO_4.4H_2O$	0.1 $mg.l^{-1}$
$CuSO_4.5H_2O$	0.03m $g.l^{-1}$
KI	0.01m $g.l^{-1}$
$NaFe(EDTA)_2$	1.5 $mg.l^{-1}$
Thiamine-HCl	1.0 $mg.l^{-1}$
Calcium pantothenate	2.5 $mg.l^{-1}$
Choline chloride	0.5 $mg.l^{-1}$
L-cysteine-HCl	10 $mg.l^{-1}$
2,4-D[1]	0.1 $g.l^{-1}$
Glucose	20 $g.l^{-1}$
pH	6.4

A2. *Capsicum frutescens* (chilli pepper) culture medium

Schenk and Hildebrandt, basal salts*	4.46 $g.l^{-1}$
CPA[2]	2 $mg.l^{-1}$
2,4-D[1]	0.5 $mg.l^{-1}$
Kinetin	0.1 $mg.l^{-1}$
Sucrose	30 $g.l^{-1}$
pH	5.8

A3. *Daucus carota* (carrot) culture medium

KNO_3	2.5 $g.l^{-1}$
$(NH_4)_2SO_4$	134 $mg.l^{-1}$
NaH_2PO_4	150 $mg.l^{-1}$
$MgSO_4.7H_2O$	250 $mg.l^{-1}$
$CaCl_2.2H_2O$	150 $mg.l^{-1}$
$FeSO_4.7H_2O$	15 $mg.l^{-1}$

$Na_2EDTA.2H_2O$	20 $mg.l^{-1}$
$MnSO_4.4H_2O$	10 $mg.l^{-1}$
H_3BO_3	2 $mg.l^{-1}$
$ZnSO_4.7H_2O$	0.25 $mg.l^{-1}$
$CuSO_4.5H_2O$	25 $g.l^{-1}$
$CoCl_2.6H_2O$	25 $g.l^{-1}$
KI	0.75 $mg.l^{-1}$
Nicotinic acid	1 $mg.l^{-1}$
Thiamine-HCl	10 $mg.l^{-1}$
Pyridoxine-HCl	1 $mg.l^{-1}$
myo-Inositol	100 $mg.l^{-1}$
2,4-D[1]	0.5 $mg.l^{-1}$
BAP[3]	0.25 mg.l-1
Sucrose	20 $g.l^{-1}$
pH	4.5

A4. *Festuca arundinacea* (tall fescue grass) culture medium

Murashige and Skoog, basal salts**	4.4 $g.l^{-1}$
Glucose	20 $g.l^{-1}$
pH	5.7

A5. *Rosa* sp., cv. Paul's Scarlet (rose) culture medium

$CaCl_2.2H_2O$	74 $mg.l^{-1}$
KH_2PO_4	140 $mg.l^{-1}$
KCl	750 $mg.l^{-1}$
$NaNO_3$	850 $mg.l^{-1}$
$MgSO_4.7H_2O$	250 $mg.l^{-1}$
$MnSO_4.4H_2O$	1 $mg.l^{-1}$
H_3BO_3	0.2 $mg.l^{-1}$
$ZnSO_4.7H_2O$	0.5 $mg.l^{-1}$
KI	0.1 $mg.l^{-1}$
$CuSO_4.5H_2O$	0.02 $mg.l^{-1}$
$CoCl_2.6H_2O$	0.01 $mg.l^{-1}$
$Na_2MoO_4.2H_2O$	0.02 $mg.l^{-1}$
$FeCl_3.6H_2O$	5.4 $mg.l^{-1}$
$Na_2EDTA.2H_2O$	7.4 $mg.l^{-1}$
2,4-D[1]	1.0 $mg.l^{-1}$

Kinetin	0.5 $mg.l^{-1}$
Glucose	20 $g.l^{-1}$
pH	6.0

A6. *Spinacia oleracea* (spinach) culture medium

Murashige and Skoog, basal salts**	4.4 $g.l^{-1}$
Glucose	10 $g.l^{-1}$
pH	4.4

A7. *Zea mays* (maize) culture medium

Murashige and Skoog, basal salts**	4.4 $g.l^{-1}$
Thiamine-HCl	0.1 $mg.l^{-1}$
Pyridoxine-HCl	0.5 $mg.l^{-1}$
Nicotinic acid	0.5 $mg.l^{-1}$
L-asparagine	300 $mg.l^{-1}$
2,4-D[1]	5 $mg.l^{-1}$
Glucose	20 $g.l^{-1}$
pH	5.9

* Shenk and Hildebrandt, basal salts (no phytohormones or sucrose/glucose); Imperial Laboratories. (Shenk and Hildebrandt, 1972)

** Murashige and Skoog, basal salts (no phytohormones or sucrose/glucose); Sigma Chemical Company. (Murashige and Skoog, 1962)

[1]2,4-D, 2,4-dichlorophenoxyacetic acid

[2]CPA, *para*-Chlorophenoxy-acetic acid

[3]BAP, 6-Benzylaminopurine

7. PUBLICATIONS

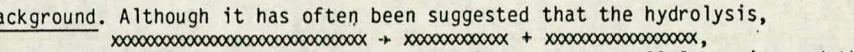
Poster presented at 5th Cell Wall Meeting, Edinburgh (1989)

Abstract Number: 139

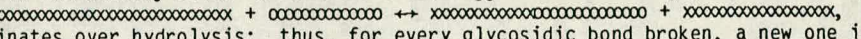
EXTRACELLULAR TRANSGLYCOSYLATION INVOLVING XYLOGLUCAN OLIGOSACCHARIDES IN VIVO

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Background. Although it has often been suggested that the hydrolysis,



of structural wall polysaccharides such as xyloglucan causes wall-loosening and thus enables cell growth (1), this process alone seems likely to weaken the wall more than occurs in the healthy cell. A refinement (2) suggests that transglycosylation, e.g.



predominates over hydrolysis: thus, for every glycosidic bond broken, a new one is formed. This would allow molecular creep, and thus growth, but would tend to maintain the strength of the wall. However, no extracellular transglycosylation of any wall-related carbohydrate has yet been shown to occur in vivo. Transglycosylation of wall-bound polysaccharides is difficult to look for because substrates are not distinguishable from products [mean M_r unchanged]. We obtained tentative evidence for in-vivo transglycosylation by showing that exogenous [^{3}H]oligosaccharides become incorporated into high-M_r apoplastic materials (3). Oligosaccharides, although not a structural part of the wall, will nevertheless permeate it and may thus participate in reactions catalysed by wall enzymes. We are re-investigating the incorporation reaction to try to test whether transglycosylation is responsible.

Methods. Radioactive xyloglucan nonasaccharide (XG9) was isolated by cellulase-digestion of [^{3}H]xyloglucan. [xylosyl-^{3}H]XG9 and [reducing terminus glucosyl-^{3}H]XG9 were fed to spinach cell cultures for 6, 24, 48 and 72 h. High-M_r (Bio-Gel P-2), soluble, extracellular, ^{3}H-labelled products were analysed by acid-, Driselase- and cellulase-hydrolysis, $NaBH_4$-reduction, and bromine-water oxidation.

Results. After 24 h, 21-36% of the [^{3}H]XG9 had been incorporated into high-M_r material. Only small amounts of ^{3}H were found in break-down products or taken up into the cells. The ^{3}H could be released from the high-M_r material in the form of XG9-like oligosaccharides by treatment with cellulase, indicating no major rearrangement of the oligosaccharide. The reducing terminus of XG9 remained a reducing terminus after incorporation; thus the oligosaccharide appears to act as the acceptor of glycosyl transfer, possibly from a non-radioactive polysaccharide.

Conclusions. Extracellular oligosaccharides undergo a reaction in vivo which has the characteristics of transglycosylation. We speculate that similar reactions occur in the structural polysaccharides of the growing cell wall to permit creep.

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Poster presented at 7th Congress of the Federation of European Societies of Plant Physiology, Umea, Sweden. (1990)

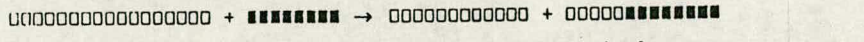
Abstract Number: P2-774

P2 - 774

EVIDENCE FOR EXTRACELLULAR TRANSGLYCOSYLATION OF XYLOGLUCAN *IN VIVO* IN CULTURED *SPINACIA* CELLS

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It has been suggested that endo-transglycosylation reactions involving structural polysaccharides of the primary cell wall contribute to the mechanism which allows cell expansion. The reactions would be (a) the enzymic cleavage of a polysaccharide chain followed by (b) the transfer of the cut end on to a second chain; the net reaction would thus be:



This process might permit wall-loosening and chemical creep, whilst not causing undue weakening of the cell wall. However, there is no definitive evidence for endo-transglycosylation of polysaccharides *in vivo*, and its occurrence is difficult to study because the reactants are chemically similar to the products [no change in mean molecular weight]. We have infiltrated short [^{3}H]xyloglucan chains into the apoplast *in vivo*, and thereby generated much longer [^{3}H]polysaccharide chains. We interpret this as evidence for transglycosylation. Characteristics of the reaction will be described.

Paper presented at 1st Scottish Cell Wall Group Meeting, Edinburgh. (1991)

Abstract Number: 4

Transglycosylation of xyloglucan in the apoplast: a possible role in cell wall expansion.

R. C. Smith, Centre for Plant Science, Division of Biological Sciences, University of Edinburgh

BACKGROUND The hydrolysis of structural polysaccharides, (e.g. xyloglucan), has been postulated as a possible mechanism of cell wall loosening during cell growth (1). However, wall loosening must be accompanied by bond synthesis if the structural integrity of the wall is to be maintained. It has been postulated that transglycosylation of structural polysaccharides may occur in preference to hydrolysis (2). Transglycosylation is the cleavage of a glycosidic bond and the subsequent transfer of the newly formed reducing terminus onto a different acceptor. New evidence for extracellular transglycosylation *in vivo* is given here (*cf.* 3).

METHODS Spinach cell suspension cultures were fed [*xylosyl*-^{3}H]xyloglucan nonasaccharide (XG9) and [*reducing terminus*-^{3}H]XG9. Culture medium was found to contain the majority of the added ^{3}H, and was subjected to gel permeation chromatography. High molecular weight ^{3}H-labelled material was subsequently analysed by the following hydrolyses: (1) trifluoroacetic acid, (2) Driselase, (3) *Trichoderma* cellulase, (4) alkali, (5) trifluoroacetic acid following borohydride-reduction. The chemical nature of the high molecular product was invesigated by: (1) copper precipitation, (2) iodine precipitation, (3) cellulose affinity, (4) trichloroacetic acid precipitation.

RESULTS A substantial proportion of [^{3}H]XG9 was found to have been incorporated into a polymer of molecular weight *ca.* 25000, without significant breakdown or uptake into cells. This product is soluble, apoplastic, and is made up of the unaltered oligosaccharide, linked to a high molecular weight compound via or near to a cellulase-labile bond. The ^{3}H-labelled polymeric product is soluble in 20% trichloroacetic acid and was found to bind to cellulose powder (eluting with 1M sodium hydroxide). However, both copper and iodine precipitation were ineffective.

CONCLUSIONS It is postulated here that this high molecular weight ^{3}H-labelled compound is the product of an apoplastic transglycosylase. As the reducing terminus is transferred with the body of the oligosaccharide, it is suggested that xyloglucan is cleaved and the newly formed reducing terminus either transferred onto another xyloglucan molecule or onto exogenous XG9, by an extracellular enzyme. The activity was found to correlate with cell growth rate in spinach suspension cultures and was shown to occur in a number of other cell suspension cultures, both dicotyledonous and monocotyledonous.

Refereces: (1). Labavitch J. M. and Ray P. M. (1974) *Plant Physiol 53*: 669-73 (2). Albersheim P. (1977) In: Tissue Culture and Plant Science, Ed. H. E. Street, pp379-404 (3). Baydoun E. A-H. Fry S. C. (1989) *J Plant Physiol 134*: 453-9.

Paper presented at Society of Experimental Biology Meeting, Birmingham. (1991)

Abstract Number: P6-12

Transglycosylation of xyloglucan in the apoplast: a possible role in cell wall expansion. [P6.12]

R.C. Smith and S.C. Fry; Centre for Plant Science, University of Edinburgh.

The hydrolysis of structural polysaccharides, (e.g. xyloglucan), has been proposed as a mechanism of cell wall loosening during plant cell growth (1). However, wall loosening must be accompanied by bond synthesis if the structural integrity of the wall is to be maintained. It has been proposed that transglycosylation of structural polysaccharides may occur in preference to hydrolysis (2). Transglycosylation is the cleavage of a glycosidic bond and the subsequent transfer of the newly formed reducing terminus onto a different acceptor. New evidence for extracellular transglycosylation *in vivo* is given here (*cf.* 3).

Spinach cell suspension cultures were fed [*xylosyl*-^{3}H]xyloglucan nonasaccharide (XG9) and [*reducing terminus*-^{3}H]XG9. A substantial proportion of [^{3}H]XG9 was found to have been incorporated into a xyloglucan-like polymer (molecular weight *ca.*20000), without significant rearrangement, breakdown or uptake into cells. As the reducing terminus of XG9 is transferred with the body of the oligosaccharide, it is postulated here that the high molecular weight ^{3}H-labelled compound is the product of an apoplastic transglycosylase. Xyloglucan was cleaved and the newly formed reducing terminus either transferred onto another xyloglucan molecule or onto exogenous XG9. The activity was found to correlate with cell growth rate in spinach suspension cultures and was shown in a number of other cell suspension cultures, both dicotyledonous and monocotyledonous.

1. J.M. Labavitch and P.M. Ray (1974) *Plant Physiol* 53:669-73.
2. P. Albersheim (1977) in: Tissue Culture and Plant Science, Ed. H.E. Street, pp. 379-404.
3. Baydoun E.A-H. and S.C. Fry (1989) *J. Plant Physiol* 134:453-9.

1974

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OLIGOSACCHARINS FROM XYLOGLUCAN AND CELLULOSE: MODULATORS OF THE ACTION OF AUXIN AND H^+ ON PLANT GROWTH

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Summary

The dose/response curve for auxin's action as a plant growth promoter is extremely wide. How does the cell vary its response to such a wide range of signal intensity? We suggest that cell wall-bound cellulase, and the products of its action on the wall polysaccharides xyloglucan and cellulose, provide a complex regulatory system, acting at three or possibly four different levels. Our recent observations will be presented in terms of the following speculations.

(1) *Speculation*: Low levels of auxin activate cellulase (by inducing H^+-secretion?), which cuts inter-microfibrillar xyloglucan chains, loosening the cell wall and tending to promote growth.

Observation: auxins induce xyloglucan turnover and decrease the mean M_r of wall xyloglucan. Re-incorporation of xyloglucan fragments suggests that transglycosylation predominates over hydrolysis.

(2) *Speculation*: Moderate levels of auxin activate cellulase further; the enzyme generates small amounts (nM) of oligosaccharides, some of which are growth-inhibitory. This process tends to moderate growth.

Observation: Xyloglucan nonasaccharide (XG9 – see Fig. 2) at 1–10 nM antagonises auxin-induced growth. The effect is seen with fucose-containing oligosaccharides (XG9, XG5, fucosyl-lactose), but not with XG7 or XG8. XG9 (1 nM) and XG10 (0.1 nM) and cellohexaose (10 nM) also block H^+-induced growth.

(3) *Speculation*: Higher levels of auxin induce cellulase synthesis leading to the generation of higher concentrations of oligosaccharides that can mimic auxin. This process thus tends to restore growth.

Observation: all xyloglucan-oligosaccharides tested (HPLC-pure XG7, XG8, XG9, XG9n – Fig. 2) promote growth at 1 μM, the order of effectiveness being

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Key words: cellulase; cellulose; xyloglucan; oligosaccharin; auxin; acid-growth; growth (plant).

XG9n>XG8>XG7≃XG9. The same oligosaccharides (at 1–100 μM) promote the *mid-chain* hydrolysis of xyloglucan by plant cellulase, while slightly inhibiting total hydrolysis. The order of effectiveness is XG9n≃XG8>XG7≃XG9. The promotion of mid-chain hydrolysis (and/or transglycosylation) might be expected to be particularly effective at loosening the cell wall: most of the wall's xyloglucan molecules might suffer a single cleavage rather than a few of them undergoing complete degradation.

(4) *Speculation*: Very high levels of auxin induce excessive levels of cellulase, and therefore of oligosaccharides. This inhibits growth.

Observation: XG7, XG9 and XG9n lose their auxin-like activity at 10 μM.

Introduction

The dose/response curves for the action of auxins as growth-promoting plant hormones are extremely wide, but supra-optimal concentrations are herbicidal and inhibit cell growth (Pilet, 1961; Thimann, 1969). It is not known how the growing plant cell is able to vary its response to such a wide range of concentrations of the same signal. Here we summarise evidence which suggests that a cell wall enzyme, cellulase, modulates the signalling actions of auxins at three or possibly four distinct levels. We suggest that the various consequences of cellulase action serve to broaden the dose/response curve of auxin.

Cellulase [β-(1→4)-glucan 4-glucanohydrolase; EC 3.2.1.4] hydrolyses the glycosidic linkage ... -D-glucopyranosyl-β-(1→4)-D-glucopyranosyl-... occurring mid-chain in a polysaccharide (Wong, Fincher and Maclachlan, 1976):

... D-Glc*p*-β-(1→4)-D-Glc*p*-β-(1→4)-D-**Glc*p***-β-(1→4)-D-Glc*p*-β-(1→4)-...
↑
hydrolysis

The enzyme seems to require that the glucose residue (shown in **bold** type) whose glycosidic bond is to be hydrolysed should be unsubstituted except at position *O*-4. Other, neighbouring glucose residues can, however, be substituted, e.g. with α-D-xylose at position *O*-6,

D-Xyl*p* D-Xyl*p* D-Xyl*p*
α |1→6 α |1→6 α |1→6
... D-Glc*p*-β-(1→4)-D-Glc*p*-β-(1→4)-D-**Glc*p***-β-(1→4)-D-Glc*p*-β-(1→4)-...
↑ ↑
no hydrolysis hydrolysis

or with glucose at position *O*-3, without preventing hydrolysis. Although the best known substrate of cellulase is cellulose, the enzyme is actually better able to hydrolyse the glucan-based hemicelluloses, xyloglucan and β-(1→3),(1→4)-D-glucan, than cellulose (Hayashi and Maclachlan, 1984). Native cellulose, unlike

the hemicelluloses, is highly crystalline and not readily accessible to enzymic hydrolysis. The present account will focus on Dicots, which do not possess β-(1→3),(1→4)-D-glucan, and thus the emphasis will be on xyloglucan metabolism (for a review of the structure and functions of xyloglucan, see Fry, 1989*a*; for a review of methodology, see Fry, 1988).

A structural rôle: Xyloglucan as an intermicrofibrillar cross link

Xyloglucan chains can hydrogen-bond on to the surface of the cellulosic microfibrils (Aspinall, Molloy and Craig, 1969). However, the cell wall has been reported to contain somewhat more than enough xyloglucan to coat all the microfibrils with a molecular monolayer (Hayash, Marsden and Delmer, 1987). It therefore seems likely that newly secreted xyloglucan molecules will compete with each other for hydrogen-bonding sites on the nascent, naked, cellulosic microfibrils (Fry, 1989*b*). Xyloglucan chains have a backbone of total contour length≃150–1500 nm. This is extremely long compared with the diameter of a microfibril (approximately 4 nm in the primary cell wall) and compared with the average spacing between adjacent microfibrils within any given layer of the cell wall (about 20–40 nm – McCann, Wells and Roberts, 1989). The two ends of each xyloglucan molecule will therefore have a high probability of finding *different* microfibrils to which to hydrogen-bond; indeed a single xyloglucan molecule could hydrogen-bond to more than two different microfibrils at different regions along its length. Xyloglucan molecules may thus act as 'strings', tethering adjacent microfibrils and thereby contributing to the coherency of the cell wall (Fry, 1989*b*). There is some analytical evidence in favour of such an arrangement (Hayashi and Maclachlan, 1984) and structures have been visualised by electron microscopy of depectinated cell walls that can be interpreted as indicating xyloglucan cross-links (McCann *et al.* 1989).

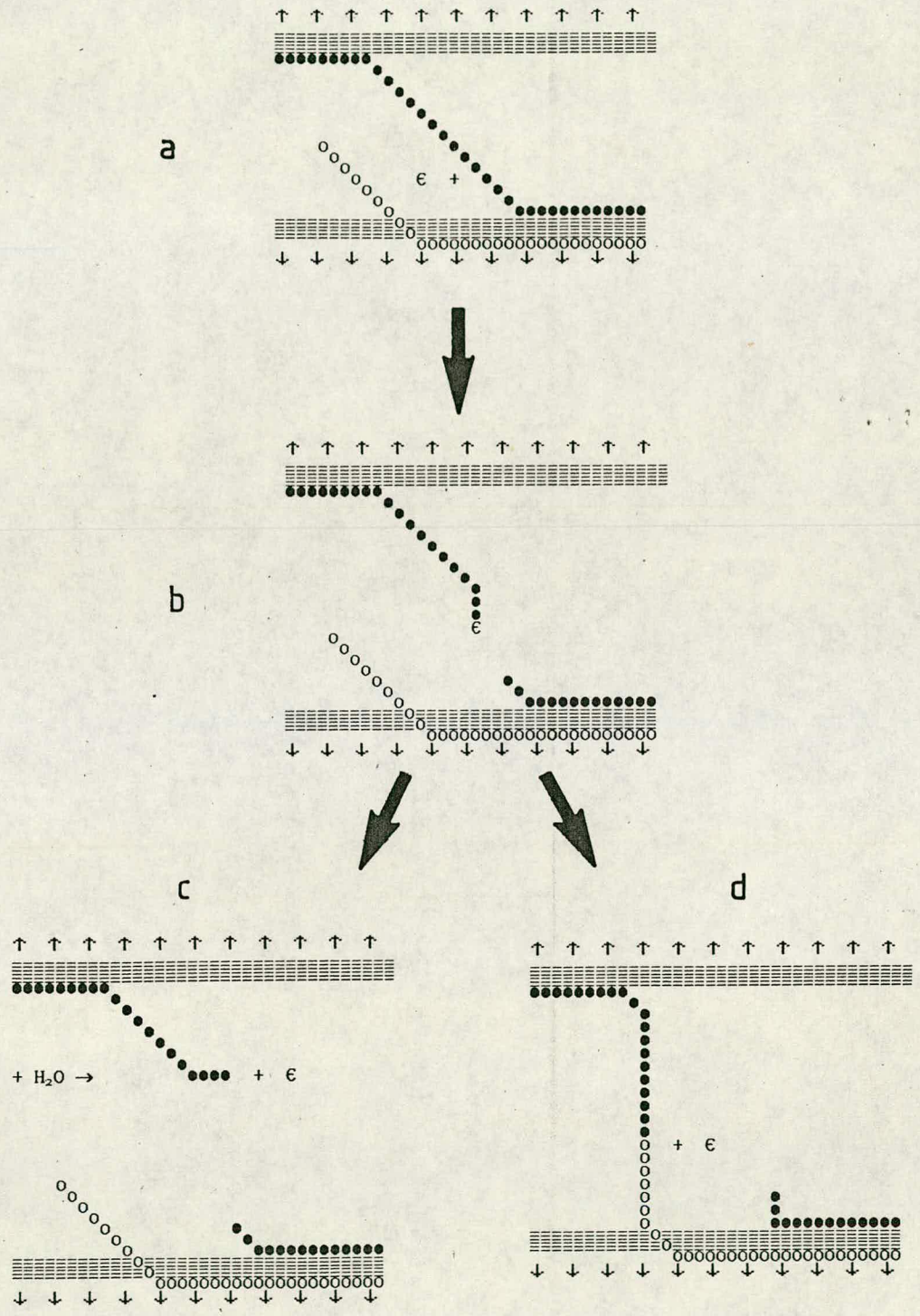
If xyloglucan serves such a key 'tethering' rôle in the cell wall, it is easy to imagine that enzymic cleavage of the xyloglucan backbone could loosen the cell wall and facilitate turgor-driven cell expansion (Davies and Özbay, 1980; Fry, 1989*b*). It is therefore of great interest to find that tissues whose growth has been promoted by the application of small amounts of auxin (or of H^+, auxin's probable mediator of short-term wall loosening) show evidence of degradation of wall-bound xyloglucan. This degradation can be detected as the complete loss (presumably by hydrolysis to dialysable mono- and oligosaccharides) of a small proportion of the total wall xyloglucan (Labavitch & Ray, 1974; Gilkes and Hall, 1977), as the possible solubilisation of some of the formerly tightly wall-bound xyloglucan (Terry, Jones and Bonner, 1981), and as a small decrease in the mean M_r of the bulk of the remaining wall-bound xyloglucan (Nishitani and Masuda, 1982, 1983; Inohue, Yamamoto and Masuda, 1984; Reville and Zarra, 1987; Lorences and Zarra, 1987).

Although it has often been suggested that the hydrolysis of xyloglucan causes wall-loosening (Labavitch and Ray, 1974), such hydrolysis alone seems likely to

weaken the cell wall more than actually occurs in the healthy cell. A refinement (Albersheim, 1974) suggests that transglycosylation (Fig. 1) predominates over hydrolysis: for every bond broken a new one is formed. This would allow molecular creep, and thus growth, but would tend to maintain the strength of the cell wall. There is, as yet, no evidence for the transglycosylation of polysaccharides in the walls of living cells, and such evidence would be extremely difficult to obtain because the substrates are indistinguishable from the products [mean M_r unchanged]. However, the occurrence of transglycosylation can be probed by infiltration of [^{3}H]oligosaccharides into the apoplast of living plant tissue (Baydoun and Fry, 1989; Smith and Fry, 1989): although oligosaccharides are not structural components of the cell wall, they will nevertheless permeate it and may participate in reactions catalysed by wall-bound enzymes whose normal substrates are the structural polysaccharides.

To obtain evidence for transglycosylation, we fed [^{3}H]oligosaccharides (most of the work was done with XG9 – see Fig. 2) aseptically to living suspension cultured cells of *Spinacia oleracea* (Baydoun and Fry, 1989; Smith and Fry, 1989). Little breakdown to free monosaccharides occurred and less than 2 % of the ^{3}H entered the cells during a 72 h incubation. However, within 24 h, 21–36 % of the ^{3}H was incorporated into 'polymer' (soluble, extracellular material that voids a Bio-Gel P-2 gel-permeation chromatography column). The association between the [^{3}H]oligosaccharide and the polymer was covalent and was shown not to be an ester as it was alkali-stable. The ^{3}H could be released from the polymer in the form of XG9-like [^{3}H]oligosaccharide(s) by treatment with fungal cellulase, indicating that no gross re-arrangement of the oligosaccharide had occurred during incorporation and that the bond between the XG9 and the polymer was formed at or near a cellulase-labile linkage. The reducing terminus of the [^{3}H]XG9 remained a reducing terminus (convertible to [^{3}H]sorbitol by $NaBH_4$) after incorporation into the polymer (Smith and Fry, 1989). The most plausible explanation for these observations is that extracellular polysaccharides underwent transglycosylation reactions resulting in the transfer of part of the polysaccharide on to [^{3}H]XG9, acting as the glycosyl acceptor. We speculate that similar transglycosylation reactions occur between intermicrofibrillar xyloglucan molecules within the cell wall, permitting creep and thus growth, and enhanced by auxin. The observed hydrolysis of xyloglucan in response to auxin may represent only a small

Fig. 1. Hydrolysis and transglycosylation as means of permitting wall growth. ≡≡≡≡≡≡≡≡≡≡ microfibril; ○○○, ●●● xyloglucan molecules; ϵ 'cellulase'; ↑ ↑, ↓ ↓ turgor-generated force tending to separate microfibrils. (a) depicts two neighbouring microfibrils, within a load-bearing layer of the cell wall, associated with xyloglucan chains, some of which (●●●●●●) are intermicrofibrillar bridges. The surface of the wall is in the plane of the paper. (b) Cellulase cuts the intermicrofibrillar xyloglucan chain and forms an enzyme·polysaccharide complex. This has two possible fates: (c) In hydrolysis, the enzyme·polysaccharide complex reacts with H_2O. (d) In transglycosylation, the enzyme·polysaccharide complex reacts with a neighbouring xyloglucan molecule (○○○○○○), to re-form an intermicrofibrillar polysaccharide chain. We argue that this would maintain the strength of the cell wall while allowing creep.


a
b
c
d
+ H_2O →

proportion of the total xyloglucan cleavage events if transglycosylation predominates over hydrolysis. It seems plausible that cellulase, in the environment of the cell wall (where there is a relatively low concentration of free water) acts mainly as a transglycosylase rather than as a hydrolase.

Xyloglucan oligosaccharides at 10^{-10}–10^{-8} M: Antagonists of the growth-promoting effects of auxins and H^+

Moderate levels of auxin may activate cellulase further; the enzyme might then not only tend to split xyloglucan chains, loosening the cell wall, but also have an appreciable chance of splitting a given xyloglucan chain twice in close proximity. This would generate small quantities of oligosaccharides (possibly amounting to nM concentrations in the apoplast?), some of which have been shown to inhibit auxin-induced elongation. This process would tend to moderate cell expansion and could participate in a feedback control loop precluding excessive growth (Fry, 1985).

Certain oligosaccharides enzymically fragmented from xyloglucan *in vitro* are potent plant growth regulators at nM concentrations, i.e. they are 'oligosaccharins' (York, Darvill and Albersheim, 1984; McDougall and Fry, 1988). These oligosaccharides are formed from xyloglucan by the action of cellulase, which cleaves the polysaccharide's backbone at unsubstituted glucose residues. The major penta- to decasaccharides produced are composed of glucose+xylose±galactose±fucose. These and related oligosaccharides that can readily be obtained are shown in Fig. 2 (Bauer, Talmadge, Keegstra and Albersheim, 1973; Kato and Matsuda, 1980; Valent, Darvill, McNeil, Robertsen and Albersheim, 1980; Hayashi and Maclachlan, 1984; Matsushita, Kato and Matsuda, 1985; Fry, 1989*a*).

The first xyloglucan oligosaccharide to be shown to possess biological activity was XG9: at 1–10 nM it antagonised the elongation-promoting effect of 1 μM 2,4-D (2,4-dichlorophenoxyacetic acid – an artificial auxin) on stem segments cut from etiolated pea seedlings (York *et al.* 1984; McDougall and Fry, 1988). Segments treated with XG9+2,4-D grow less in length than do those treated with 2,4-D alone. The effect of nM XG9 is mimicked by XG5 and 2′-fucosyl-lactose (Fuc→Gal→Glc) at similar concentrations but not by methyl-α-L-fucopyranoside (McDougall and Fry, 1989*a*). This suggests that the α-L-fucose residue is necessary, but not sufficient, for the anti-auxin action of XG9.

At the higher concentration of 0.1–1 μM, XG9 loses this anti-auxin effect (York *et al.* 1984; McDougall and Fry, 1989*b*). This is surprising because growth inhibitors usually increase in effectiveness with increasing concentration. XG5 and Fuc→Gal→Glc do not lose their anti-auxin effect at 0.1–1.0 μM (McDougall and Fry, 1989*a*).

The anti-auxin effect of XG9 and related oligosaccharides is absolutely dependent on the terminal α-L-fucose residue. Thus, XG8, which differs from XG9 only in lacking the terminal α-L-fucose residue, totally lacks anti-auxin activity (McDougall and Fry, 1989*b*). Similarly, XG7 is inactive. XG10, which

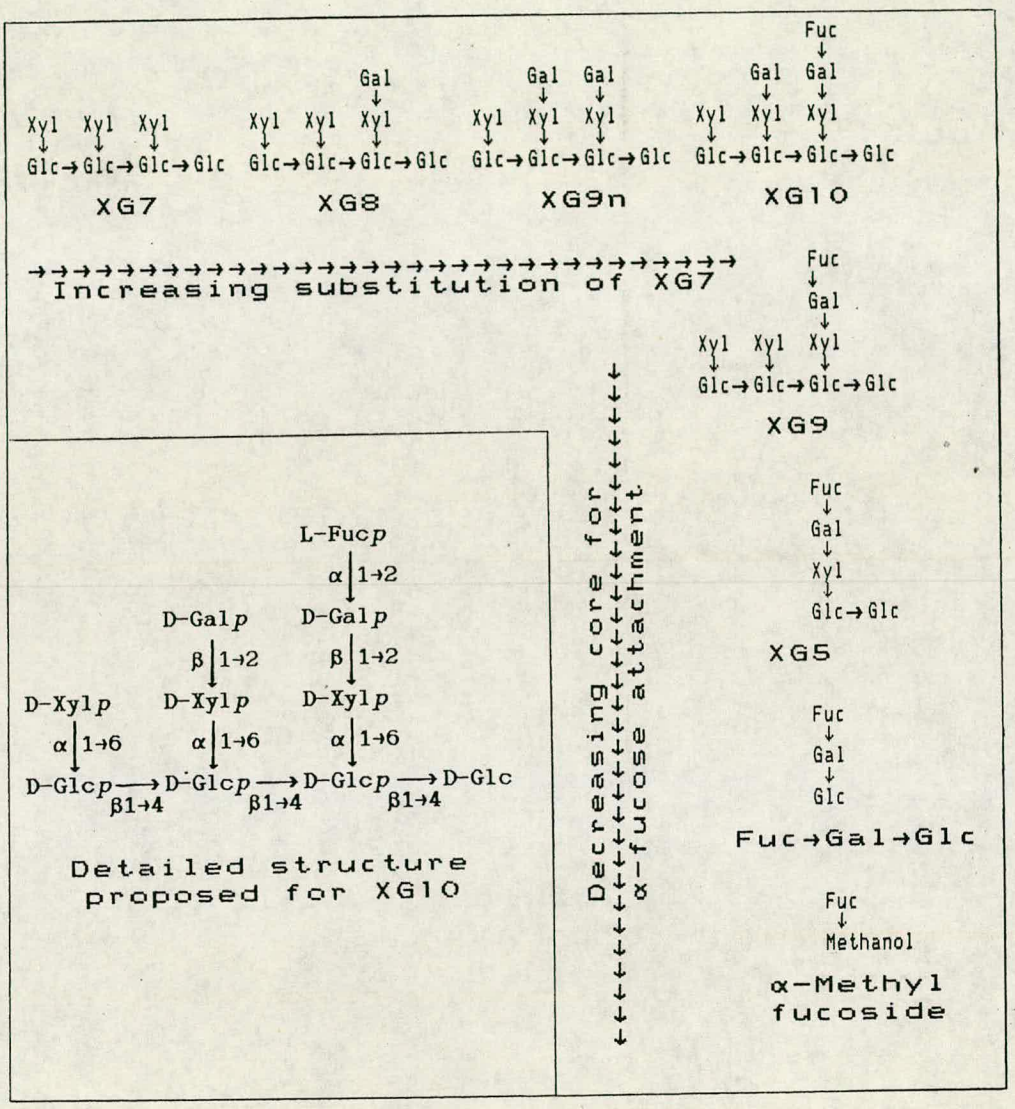


Fig. 2. Probable structure of the xyloglucan oligosaccharides mentioned in the text.

differs from XG9 in having an additional galactose residue on the second xylose residue, also lacks anti-auxin activity at 1 nM (McDougall and Fry, 1989*b*) [but it has not yet been tested at 0.1 nM – cf. below].

Oligogalacturonides have also recently been shown to exhibit anti-auxin activity in the pea stem segment straight-growth bioassay (Branca, de Lorenzo and Cervone, 1988). However, the concentrations required to demonstrate this effect are approximately 0.1–1.0 mM and it seems likely that a rather different mechanism, more akin to a general reduction in cell vitality, is responsible for the inhibition of growth here.

The mechanism of action of XG9, XG5 and Fuc→Gal→Glc as anti-auxins is

unknown. Preliminary evidence suggests that, although these oligosaccharides antagonise the effect of 2,4-D on stem segment elongation (straight-growth), they do not influence 2,4-D's effect on total cell expansion, measured as fresh weight (McDougall and Fry, unpublished). If this is so, XG9-treated stem segments will have an increased girth and thus, at least to this extent, will resemble ethylene-treated specimens. The possibility that XG9 acts as an anti-auxin by evoking the synthesis of ethylene deserves attention.

It has been suggested that the early stages of auxin-induced growth are mediated by increased H^+ extrusion into the cell wall and that the decreased extracellular pH causes wall loosening, facilitating turgor-driven cell expansion (Cleland, 1981). It is thus of interest to ask whether XG9 blocks the action of H^+ on elongation. Our recent studies have shown that XG9 at 1 nM does inhibit H^+-induced elongation in pea stem segments just as effectively as it inhibits auxin-induced elongation (Lorences and Fry, 1989). This shows that the anti-auxin effect of XG9 is not solely mediated by a suppression of H^+-secretion, but rather that XG9 blocks some step that is common to the action of both auxin and H^+ on growth. Possibilities include a tightening of the cell wall (Fry, 1986*b*), a re-orientation of the microfibrils (Green, 1980), or a reduction in turgor pressure (Katsumi and Kazama, 1978).

Of the other oligosaccharides tested for their ability to antagonise H^+-induced elongation, XG7 and XG8 were ineffective – as expected owing to their lack of an α-L-fucose residue. However, XG10 did block H^+-induced elongation at 0.1 nM (but not at 1 nM); it will be of interest to re-test XG10 as an anti-auxin at such low concentrations (cf. McDougall and Fry, 1989*b*). In addition, cellohexaose was found to antagonise H^+-induced elongation; the optimum inhibitory concentration was about 10 nM. This would suggest that certain cello-oligosaccharides (which, like XG9 and XG5, are possible products of the action of cellulase on a cell wall polysaccharide) are growth-inhibitory oligosaccharins. Cellohexaose at 10 nM has no detectable effect on the action of *Phaseolus vulgaris* cellulase *in vitro* (cf. next section), although at much higher concentrations (1–10 mM) it is known to act as a competitive inhibitor of the hydrolysis of carboxymethylcellulose by a plant cellulase (Wong *et al.* 1975). At 10 nM, cellohexaose appears more likely to act as a 'conventional' oligosaccharin, binding to a specific receptor. It will be of great interest to investigate the possible natural occurrence of cellohexaose and related cello-oligosaccharides at *ca.* 10-nM concentrations in the apoplast *in vivo*.

Although all the bioassays described above have all been performed with factitious oligosaccharides, generated by the action of acids and enzymes *in vitro*, XG9 does accumulate *in vivo* – in the spent medium of suspension cultured cells of *Spinacia oleracea* – to levels of about 430 nM (Fry, 1986*a*). This supports the idea that xyloglucan oligosaccharides can act as natural growth-regulators. The kinetics of pulse-labelling experiments have suggested that XG9 is generated in cultured *Spinacia* cells by the breakdown of pre-formed polysaccharide molecules rather than by the *de novo* synthesis and secretion of ready-made nonasaccharides (McDougall and Fry, unpublished). The XG9 can therefore be classed as an

oligosaccharin in the strict sense, i.e. as a fragment liberated by partial degradation of the plant cell wall.

Xyloglucan oligosaccharides at 10^{-7}–10^{-6} M: Growth-promoters

Higher levels of auxin can induce the synthesis of cellulase (Verma, Maclachlan, Byrne and Ewings, 1975), and the increased action of increased amounts of the enzyme is then even more likely to cleave a single xyloglucan molecule at neighbouring vulnerable sites, leading to the generation of higher concentrations of oligosaccharides than the nM levels discussed in the previous section. Some of these oligosaccharides have recently been shown to promote the straight-growth of etiolated pea stem segments and thus, at least by that criterion, to mimic auxin. This process would thus tend to prevent an excessive reduction in growth at relatively high auxin concentrations.

We purified four xyloglucan oligosaccharides (XG7, XG8, XG9 and XG9n – see Fig. 2) by gel-permeation chromatography on Bio-Gel P-2, paper chromatography in ethyl acetate/acetic acid/water (10:5:6), and HPLC on a Brownlee Amino Spheri-5 semi-preparative column in 55 % acetonitrile. Each oligosaccharide was virtually homogeneous upon analytical HPLC on Dionex CarboPac PA1 in 0.1 M NaOH with a 50→100 mM linear gradient of sodium acetate (McDougall and Fry, 1990). Each pure oligosaccharide promoted the elongation of pea stem segments, the order of effectiveness at 1 μM being XG9n>XG8>XG7≃XG9 (McDougall and Fry, 1989*c*, 1990).

This is the first report of elongation in an auxin bioassay being promoted by oligosaccharides. The effect clearly had structural requirements different from those of the anti-auxin effect of XG9. An α-L-fucose residue was not necessary for the effect; addition of an α-L-fucose residue to XG8 *decreased* its effectiveness. The optimum concentration for the promotion of straight-growth was generally about 1 μM, and (in all cases except XG8) 10 μM was less effective (McDougall and Fry, 1990). For comparison, the optimal growth-promoting concentration of 2,4-D in this tissue was also about 1 μM (McDougall and Fry, 1988).

The same four oligosaccharides promoted, in viscometric assays, the hydrolysis of xyloglucan by an acidic isozyme of cellulase extracted from young bean (*Phaseolus vulgaris*) leaves. The promotion was detectable at 1 μM oligosaccharide, and appeared to saturate at about 200 μM. This effect was not seen with fungal (*Trichoderma*) cellulase acting on xyloglucan nor on plant cellulase acting on carboxymethylcellulose, in agreement with the report of Farkas and Maclachlan (1988). Curiously, no promotion of digestion was seen when the *Phaseolus* cellulase was assayed with [^{3}H]xyloglucan as substrate, in experiments that measured the conversion of [^{3}H]xyloglucan to radioactive fragments small enough to be soluble in 50 % ethanol. XG9 even gave a significant (35 %) decrease in enzyme activity in the radiochemical assay (McDougall and Fry, 1990).

Viscometric assays favour the detection of mid-chain cleavage of polysaccharides, whereas the radiochemical assay favours the detection of cleavage events

occurring relatively near the termini of the polysaccharide (although negligible cleavage occurred *very* near the termini, as demonstrated by the absence of [^{3}H]XG9 and similarly small-sized fragments in the digestion products). The data thus indicate that the presence of xyloglucan oligosaccharides at 1–100 μM shifts the action pattern of *Phaseolus* cellulase to more frequent cleavage in mid-chain positions relative to positions near the chain termini. We suggest that the oligosaccharides can form a transient enzyme·oligosaccharide complex with the cellulase; the enzyme must therefore frequently relinquish its hold on any particular polysaccharide molecule. In the presence of xyloglucan oligosaccharides, any tendency of the cellulase to cleave a single polysaccharide molecule repeatedly will be minimised, and the result will be that a large proportion of the polysaccharide molecules in the population will undergo a single cleavage rather than a small proportion of them undergoing repeated cleavages.

In the context of the growing cell wall, a relative promotion of mid-chain cleavage such as this (albeit possibly by transglycosylation rather than hydrolysis) might be expected to be particularly effective at loosening the cell wall by cutting the intermicrofibrillar xyloglucan chains.

The order of effectiveness of individual pure xyloglucan oligosaccharides in promoting the action of cellulase in the viscometric assay was XG9n≃XG8>XG7≃XG9 (McDougall and Fry, 1990). This is similar to the order of effectiveness for promotion of pea stem segment elongation, and strengthens the idea that the ability of the oligosaccharides to promote mid-chain cleavage of xyloglucan *causes* their ability to promote straight-growth.

As mentioned in the previous section, XG9 loses its anti-auxin activity at 0.1–1.0 μM. We have suggested (McDougall and Fry, 1989*a*) that this is related to XG9's gaining the ability, at these concentrations, to loosen the cell wall directly, thus bypassing the action of auxin. The auxin-like effect of XG9 supports this idea. The failure of XG5 and Fuc→Gal→Glc to lose their anti-auxin activities at μM concentrations could be due to their inability to mimic auxin.

Xyloglucan oligosaccharides at 10^{-5} M: Loss of the growth promoting effect

Very high (herbicidal) levels of auxin might induce excessive levels of cellulase, and therefore of oligosaccharides. We have found that HPLC-purified XG7, XG9 and XG9n all lose some of their elongation-promoting activity when the concentration is raised to 10 μM (McDougall and Fry, 1990). It is very difficult to eliminate the possibility that the 10-μM solutions of the oligosaccharides used in these experiments are contaminated with low levels of a different, and possibly unrelated, growth inhibitor. Indeed, in the early stages of this work we found that certain crude samples of the oligosaccharides had the ability to kill the tissue, but only in the basal half of each stem segment. Killing was shown by staining with Evans' Blue (Anon, 1989). This effect, which had quite different symptoms from those induced by exogenous fungal cellulase, was eliminated if the xyloglucan oligosaccharide preperations were passed through a mixed-bed ion exchanger

before use in the bioassay. However, de-ionising did not prevent XG7, XG9 and XG9n losing their auxin-mimicking effect at 10 μM. The possibility that oligosaccharide fragments of the plant cell wall can exert phytotoxic effects, evoking the hypersensitive defence response, has been raised (Yamazaki, Fry, Darvill and Albersheim, 1983).

Discussion

Xyloglucan and its cellulase-generated oligosaccharide fragments, and recently cello-oligosaccharides, intervene in the action of auxin at three and possibly four different levels. This makes it possible to add considerably to the sophistication, or at least complexity, of the feedback control loop postulated earlier (Fry, 1985). At the architectural level, xyloglucan [which is present in the cell wall at a concentration of about 10 % (w/v)] is a prime candidate for the 'string' that must

Table 1. *Summary of the effects of progressively increasing cellulase action on xyloglucan metabolism and plant growth*

Cellulase action	Effect on XG degradation*	Result	Effect on growth
Zero	None	—	0
Low	Occasional single cleavages	Wall loosening	+ve
Moderate	Occasional double cleavages	nM XGOs formed [anti-auxin]	−ve
High	More double cleavages	μM XGOs formed [enhance *mid*-chain XG-cleavage → frequent single cleavages → wall loosening]	+ve
Very high	Frequent double cleavages	>10 μM XGOs formed [cytotoxic?]	−ve

**Single cleavage:*

⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦ →
↑

⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦+

⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦

**Double cleavage:*

⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦ →
↑ ↑

⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦+ ⑨+

⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨⑦⑨

XG = xyloglucan.
XGO = xyloglucan oligosaccharide.
⑦, ⑨ = repeating oligosaccharide units within xyloglucan chain.
↑ = site of attack of cellulase.

be cut in the cell wall to facilitate cell elongation in response to auxin or H^+. The enzyme that cuts it is cellulase, possibly working primarily as a transglycosylase rather than as a hydrolase. At the hormonal level ($\simeq 10^{-9}$ M), certain cellulase-generated oligosaccharides of xyloglucan which bear terminal α-L-fucose residues, and also certain cello-oligosaccharides, can antagonise the action of auxin and H^+. At the enzymic level ($\simeq 10^{-6}$ M), these and other cellulase-generated oligosaccharides of xyloglucan can promote the growth of pea stem segments, possibly by directly 'activating' the enzyme (cellulase) by which auxin and H^+ loosen the cell wall. The possible fourth level is that, at 10^{-5} M concentrations, the oligosaccharides lose their growth-promoting effect by acquiring a cytotoxic effect.

These four levels can be seen as the consequences of progressively more vigorous cellulase action on cell wall-bound polysaccharides. We suggest that each new level tends to act homeostatically, opposing the physiological trend set in train by the previous level (Table 1). The consequence could be a very gradually changing response to a broad range of signal intensities. It is of interest to consider whether this is related to the breadth of auxin's dose/response curve as a growth promoter.

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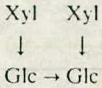
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Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants

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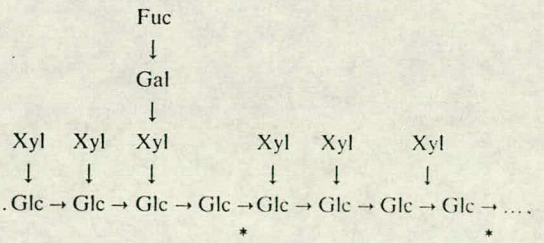
1. Cell-free extracts of all plants tested contained a novel enzyme activity (xyloglucan endotransglycosylase, XET) able to transfer a high-M_r portion from a donor xyloglucan to a suitable acceptor such as a xyloglucan-derived nonasaccharide ($Glc_4Xyl_3GalFuc$; XG9). 2. A simple assay for the enzyme, using [^{3}H]XG9 and based on the ability of the [^{3}H]polysaccharide product to bind to filter paper, is described. 3. The enzyme was highly specific for xyloglucan as the glycosyl donor, and showed negligible transglycosylation of other polysaccharides, including CM-cellulose. 4. The K_m for XG9 was 50 μM; certain other ^{3}H-labelled xyloglucan oligosaccharides also acted as acceptors, and certain non-radioactive xyloglucan oligosaccharides competed with [^{3}H]XG9 as acceptor; the minimum acceptor structure was deduced to be:



5. The pH optimum was approx. 5.5 and the enzyme was less then half as active at pH 7.0. The enzyme was slightly activated by Ca^{2+}, Mg^{2+}, Mn^{2+}, spermidine, ascorbate and 2-mercaptoethanol, and inhibited by Ag^+, Hg^{2+}, Zn^{2+} and La^{3+}. 6. XET activity was essentially completely extracted by aqueous solutions of low ionic strength; Triton X-100, Ca^{2+}, La^{3+}, and Li^+ did not enhance extraction. Negligible activity was left in the unextractable (cell-wall-rich) residue. 7. The enzyme differed from the major cellulases (EC 3.2.1.4) of pea in: (a) susceptibility to inhibition by cello-oligosaccharides, (b) polysaccharide substrate specificity, (c) inducibility by auxin, (d) requirement for salt in the extraction buffer and (e) activation by 2-mercaptoethanol. XET is therefore concluded to be a new enzyme activity (xyloglucan: xyloglucan xyloglucanotransferase; EC 2.4.1.—). 8. XET was detected in extracts of the growing portions of dicotyledons, monocotyledons (graminaceous and liliaceous) and bryophytes. 9. The activity was positively correlated with growth rate in different zones of the pea stem. 10. We propose that XET is responsible for cutting and rejoining intermicrofibrillar xyloglucan chains and that it thus causes the wall-loosening required for plant cell expansion.

INTRODUCTION

Xyloglucan is a structural polysaccharide of plant cell walls (Hayashi, 1989; Fry, 1989a). It is composed of a backbone of β-(1→4)-D-glucopyranose residues, about 75% of which have a single α-D-xylopyranose residue attached at O-6. Some of the xylose residues are β-D-galactopyranosylated at O-2, and (except in the storage xyloglucans of certain seeds, e.g. *Tropaeolum*) some of the galactose residues are α-L-fucopyranosylated at O-2. A repeating motif of xyloglucan appears to be



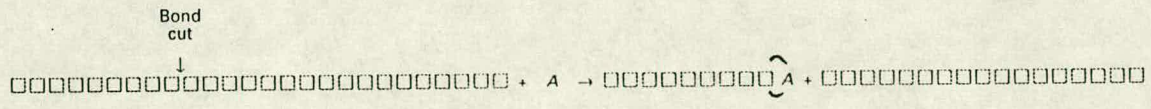
the linkages marked '*' being cellulase-labile. The galactose residues may also be *O*-acetylated.

Xyloglucan can hydrogen-bond to cellulose microfibrils (Hayashi *et al.*, 1987) and may cross-link them (Fry, 1989b; McCann *et al.*, 1990), restraining cell expansion. The enzymic cleavage of xyloglucan is thought to loosen the wall, enabling cell growth. For instance, it has been shown that xyloglucan decreases in mean M_r and is partially solubilized during auxin- or acid-induced growth (Labavitch & Ray, 1974; Gilkes & Hall, 1977; Nishitani & Masuda, 1983; Hayashi *et al.*, 1984; Wakabayashi *et al.*, 1991). In further support of this hypothesis, treatment of stem segments with xyloglucan-binding lectins or antibodies, which might be expected to restrict the access of an endogenous degradative enzyme to wall-bound xyloglucan, block auxin-induced growth (Hoson & Masuda, 1987, 1989).

The enzyme responsible for xyloglucan cleavage was assumed to be cellulase (Hayashi *et al.*, 1984; Fry, 1989b; McDougall & Fry, 1990), which hydrolyses the β-(1→4)-D-glucan backbone of xyloglucan. However, hydrolysis is irreversible whereas bond breakage during growth is reversible (Taiz, 1984), which argues against the involvement of cellulase-catalysed hydrolysis. A possible explanation of this discrepancy arises from the finding that xyloglucan is subject *in vivo* to endotransglycosylation, i.e., a xyloglucan chain (□□□□□...) can be cleaved and then transferred to a different (acceptor) chain (*A*) (Baydoun & Fry, 1989; Smith & Fry, 1991) (Scheme 1). Such cleavage could still loosen the wall, enabling growth, but would be followed by a

Abbreviations used: XET, xyloglucan endotransglycosylase; XG9 etc., xyloglucan-derived oligosaccharides (see Fig. 2); 2,4-D, 2,4-dichlorophenoxyacetic acid.

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Scheme 1.

repair step, as proposed by Albersheim (1974), so its loosening effect would be reversible.

The enzyme responsible for this endotransglycosylation is of considerable interest for studies of the mechanism and control of plant cell expansion. In the present paper we report the widespread occurrence in plants of a new enzyme activity, xyloglucan endotransglycosylase (XET), which catalyses this reaction. We show that it differs from cellulase and propose that it is central to the mechanism of plant growth.

EXPERIMENTAL

Plant material

Peas (*Pisum sativum*, cv Alaska) were soaked overnight in running tap water and then germinated in moist vermiculite at 25 °C in the dark. Analyses were performed on day 7 or 8, when the third internode was rapidly elongating. Tomato seedlings (cv. Ailsa Craig) were greenhouse-grown to a height of approx. 15 cm. Other plants were collected wild or from gardens in the vicinity of Edinburgh except *Marchantia*, which was collected by Loch Caolisport, Argyll.

Oligosaccharides

[1-^{3}H]XG9, i.e. XG9 labelled at position 1 of the reducing terminal glucose moiety (12 TBq/mol), was prepared as described previously (Smith & Fry, 1991). [1-^{3}H]XG7f ($Glc_3Xyl_2GalFuc$) and [1-^{3}H]XG5 ($Glc_2XylGalFuc$) were prepared from [1-^{3}H]XG9 as described by McDougall & Fry (1991) and purified by paper chromatography in ethyl acetate/acetic acid/water (10:5:6, by vol.). [^{3}H]XG2 was prepared by treatment of [*pentosyl*-^{3}H]-xyloglucan with 'Driselase' and purified by paper chromatography in ethyl acetate/pyridine/water (8:2:1, by vol.) (Fry, 1988). Non-radioactive XG9, XG9n and XG7 were prepared and h.p.l.c.-purified by the methods of McDougall & Fry (1990, 1991). XG3 was a generous gift from Dr. Y. Kato, Sendai, Japan. Cello-oligosaccharides and monosaccharides were from Sigma Chemical Co., Poole, Dorset, U.K.

Polysaccharides

Xyloglucan from *Tropaeolum* seeds and from *Rosa* cell suspension cultures was purified by Cu^{2+} precipitation (Rao, 1959). Rhamnogalacturonans were prepared from the walls of cultured *Acer* cells by the methods of Darvill *et al.* (1978) and McNeil *et al.* (1980). *Fagus* glucuronoxylan (Kohn *et al.*, 1986*a*) and *Secale* arabinoxylan (Kohn *et al.*, 1986*b*) were generous gifts from Dr. A. Ebringerová, Bratislava, Czechoslovakia. *Hordeum* arabinoxylan was as used by Ahluwalia & Fry (1986). Other polysaccharides were purchased from BDH, Sigma, Koch-Light and Bauer & Pfaltz.

Enzymes

Trichoderma cellulase and *Irpex lacteus* 'Driselase' were from Sigma Chemical Co. The 'Driselase' was partially purified as before (Fry, 1988).

Extraction of XET

Rapidly growing plant tissue (fresh or frozen) was used as the source of XET. For peas, the third (= uppermost) internodes were cut from 7–8-day etiolated seedlings, mixed with a little acid-washed sand, and ground with a pestle and mortar in ice-cold extractant [usually distilled water, though we have recently obtained higher activities using 10 mM-$CaCl_2$/10 mM-ascorbate/20 mM-Mes (Na^+), pH 6.0] at 1 ml/g fresh weight. The homogenate was centrifuged at 2000 *g* for 5 min, and the supernatant was used as the XET preparation.

Standard XET assay

All the experiments used the following basic methods except where otherwise stated. The reaction mixtures (total volume 40 μl) contained 2 mg of xyloglucan/ml, 1.4 kBq [^{3}H]XG9 (2.9 μM), 50 mM-Mes (Na^+), pH 6.0, and 10 μl of enzyme extract. After 1 h at 25 °C, the reaction was stopped by the addition of 100 μl of 20% (w/v) formic acid; the solution was dried on to a 5 cm × 5 cm square of Whatman 3MM filter paper, which was washed for at least 30 min in running tap water to remove unchanged [^{3}H]XG9. The square of paper was then redried at 60 °C, rolled into a cylinder with the loaded side facing outwards, placed in a 22 ml scintillation vial, soaked with approx. 2 ml of scintillant (0.5% 2,5-diphenyloxazole/0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene) and assayed for ^{3}H by scintillation counting. The counting efficiency was approx. 44% for [^{3}H]xyloglucan and approx. 7% for [^{3}H]oligosaccharides, presumably because the latter penetrated the paper fibres whereas the former coated them. Since most of the assays were conducted with oligosaccharide concentrations well below the K_m, product formation is reported as Bq of [^{3}H]polymer formed per kBq of [^{3}H]oligosaccharide supplied.

Assay of endotransglycosylation of polysaccharides other than xyloglucan

For the testing of donor substrate specificity, the reaction products (after addition of formic acid) were applied to 46 cm × 57 cm sheets of Whatman 3MM paper and chromatographed for 18 h in ethyl acetate/acetic acid/water (10:5:6, by vol.). Unchanged [^{3}H]XG9 had R_F approx. 0.1, whereas the polysaccharides were immobile (Fry, 1988); > 97% of the ^{3}H was accounted for in these two zones. Polymeric material (R_F = 0.00) was assayed for ^{3}H as already described and corrected for the value obtained in the absence of added polysaccharide.

Assay of protein

The protein content of plant extracts was assayed by the Coomassie Blue G dye-binding method as modified by Read and Northcote (1981).

Auxin induction of cellulases

Day-8 etiolated pea seedlings were sprayed with 0.1% 2,4-dichlorophenoxyacetic acid (2,4-D) in a solution containing 0.1% Tween 80 and 0.1 M-NaCl (pH adjusted to 7.0) and grown in the dark for a further 4 days. This caused the growing zone of the third internode to swell laterally whereas control stems (sprayed with Tween/NaCl alone) continued to elongate. Whole third internodes were excised from treated and untreated plants on day 12, and extracted with the buffer used by Wong *et al.* (1977) (20 mM-sodium phosphate, pH 6.2, containing 1% glucose and 5% glycerol) followed by the same buffer containing

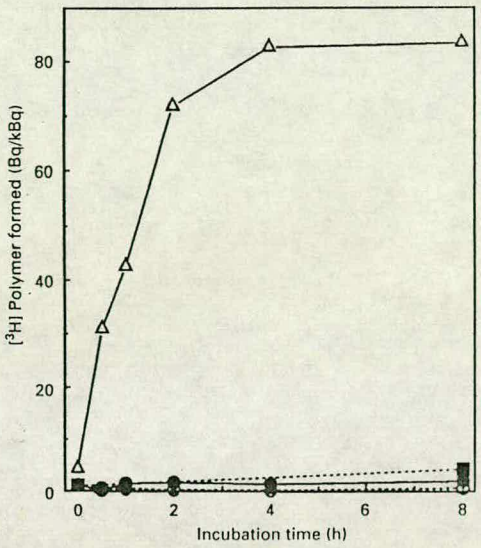


Fig. 1. Time-course for action of pea XET on xyloglucan + [^{3}H]XG9

The buffer was 50 mM-citrate + 50 mM-phosphate (Na^+). ○, pH 2.0; △, pH 6.0; ●, pH 6.0, enzyme boiled for 5 min before assay; ■, pH 6.0, no xyloglucan added.

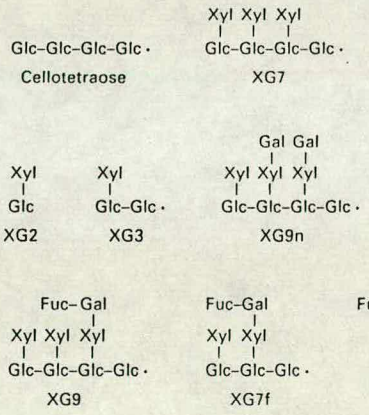


Fig. 2. Proposed structure of XG9 and related oligosaccharides

For further details, see Bauer *et al.* (1973), McDougall & Fry (1990, 1991) and Smith & Fry (1991). Glc· = reducing terminus.

1 M-NaCl. Each extractant was used at 1 ml/g initial fresh weight. For assay of cellulase (CM-cellulase), the enzyme extract (0.5 ml) was mixed with 2 ml of 0.8 % (w/v) CM-cellulose in the low-salt buffer and incubated at 25 °C. At intervals (0–3 h), approx. 1.2 ml of the reaction mixture was sucked into a 1 ml pipette and the time taken from the meniscus to flow from the 1.0 ml to the 0.5 ml mark was recorded. The initial mean flow time was 13.2 s.

RESULTS

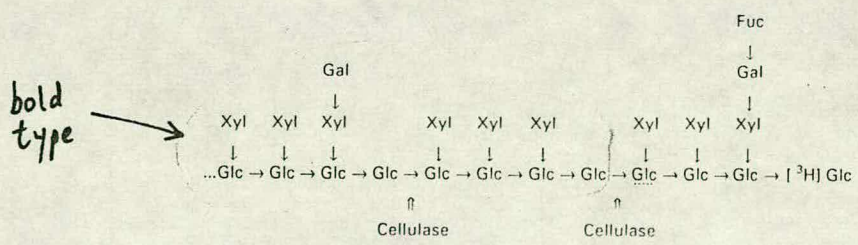
Demonstration of XET activity

XET activity was readily detected in pea stem homogenates (Fig. 1). XET was assayed by its ability to transfer part of a large (M_r approx. 2×10^5) non-radioactive xyloglucan molecule to a [^{3}H]nonasaccharide ([^{3}H]XG9; for structure, see Fig. 2), forming a [^{3}H]polymer (Scheme 1; A = [^{3}H]XG9). The [^{3}H]polymer produced in the reaction was distinguished from the [^{3}H]XG9 starting material in three ways: (1) the [^{3}H]polysaccharide bound tightly to cellulose (filter paper) during prolonged washing in water (e.g. Fig. 1), (2) it precipitated with ethanol (results not shown) and (3) it was immobile on paper chromatography (as in Table 4). For most of the following investigations, method (1) was used.

The [^{3}H]XG9 became the reducing terminus of the newly labelled polysaccharide, as demonstrated by the fact that its (reducing terminal) [1-^{3}H]glucose unit could be oxidized in warm NaOH to yield ^3H_2O and could be reduced by $NaBH_4$ to yield a [^{3}H]glucitol moiety (results not shown; cf. Smith & Fry, 1991). Thus the [^{3}H]XG9 was the acceptor for XET-catalysed transglycosylation and the polysaccharide was the donor. The ^{3}H could be released from the [^{3}H]polysaccharide, in the form of a product chromatographically identical with [^{3}H]XG9, by digestion with *Trichoderma* cellulase [endo-β-(1 → 4)-D-glucanase]. Since there are no cellulase-labile sites in XG9, this indicates that the non-radioactive portion of the polysaccharide (shown in bold in scheme 2) contributed a β-(1 → 4)- linkage to the product and thus that the acceptor hydroxy group of XG9 was O-4 of the glucose residue (underlined in Scheme 2) furthest from the reducing terminus. The enzyme assayed *in vitro* in this work thus corresponded closely to the characteristics of the endotransglycosylation *in vivo* of xyloglucans reported earlier (Smith & Fry, 1991).

Occurrence of XET in the plant kingdom

XET activity was extractable from the growing portions of all plants tested (Table 1). These included dicotyledons, graminaceous monocotyledons, a liliaceous monocotyledon, a moss and a liverwort (both sporophyte and gametophyte). XET thus seems likely to be present in all land plants. The high activity in the Gramineae is perhaps surprising in view of the relatively low xyloglucan content of these plants (for details, see Fry, 1988).



Scheme 1.

Table 1. Extraction of XET activity from growing plant tissues

The extractant used was 10 mM-$CaCl_2$/10 mM-sodium ascorbate/20 mM-sodium Mes, pH 6.0. Young rapidly growing portions of each plant were used. Each assay contained the quantity of protein (range 2.6–44.0 μg) extracted from 5 mg fresh weight of tissue. Values are corrected for the apparent rate of [^{3}H]polymer formation in enzyme-free blanks (11.8 ± 2.1 Bq/kBq per h per 5 mg fresh weight).

Plant	Organ extracted	XET activity (Bq/kBq per h)	
		Per 5 mg fresh wt.	Per μg of soluble protein
Bryophyta: Hepaticae			
Marchantia polymorpha (liverwort)	Seta	24.4	9.38
Marchantia polymorpha (liverwort)	Thallus	19.9	6.86
Bryophyta: Musci			
Mnium sp. (moss)	Gametophyte	64.5	18.4
Monocotyledons: Liliaceae			
Allium schoenoprasum (chives)	Peduncle	102	10.2
Monocotyledons: Gramineae			
Zea mays (maize)	Leaf + stem	161	10.4
Holcus lanatus (Yorkshire fog)	Leaf + stem	324	18.5
Bromus erectus (brome grass)	Leaf + stem	110	9.57
Dicotyledons			
Lupinus polyphyllus (lupin)	Leaf	55.1	3.44
Pisum sativum (pea)	Etiolated stem	97.1	2.21
Anthriscus sylvestris (cow parsley)	Leaf	33.7	2.25
Acer pseudoplatanus (sycamore)	1st year stem	39.4	6.06
Lycopersicon esculentum (tomato)	Leaf + stem	272	14.3
Taraxacum officinale (dandelion)	Peduncle	33.3	5.12

Enzymic characteristics

The enzyme was inactivated by boiling (Fig. 1). The reaction rate was fairly constant for the first 2 h, but then rapidly decreased; routine assays were conducted for 1 h (Fig. 1). The initial reaction rate was proportional to the amount of plant extract added (results not shown). The pH optimum was approx. 5.5, and the enzyme was less than half as active at pH 7.0 (Fig. 3). Non-radioactive XG9 decreased the incorporation of [^{3}H]-XG9 into xyloglucan (Fig. 4; see also Table 3) and the apparent K_m was 50 μM-XG9. The optimum xyloglucan concentration was approx. 2 mg/ml (approx. 10 μM); higher concentrations presumably competed with the [^{3}H]XG9 as an acceptor. XET activity was slightly enhanced by 1 mM-spermidine and 2-mercaptoethanol at various concentrations and 10 mM-Ca^{2+}, -Mg^{2+}, -Mn^{2+} and -ascorbate. It was partially inhibited by

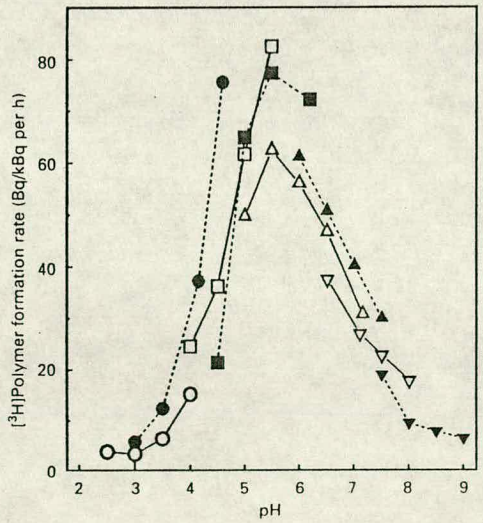


Fig. 3. pH-dependence of action of pea XET on xyloglucan + [^{3}H]XG9

The buffers used were (all 50 mM): ○, citrate; ●, formate; □, acetate; ■, phthalate; △, Mes; ▲, phosphate; ▽, Hepes (all Na$^+$ salts); or ▼, Tris (acetate salt).

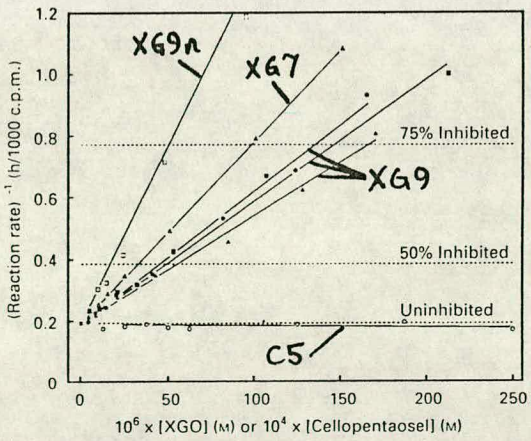


Fig. 4. Inhibition of the XET-catalysed incorporation of [^{3}H]XG9 into xyloglucan by added non-radioactive oligosaccharides

A pea stem extract was incubated with 2 mg of xyloglucan/ml + 2.2 μM-[^{3}H]XG9 either alone or supplemented with 0–220 μM-non-radioactive xyloglucan oligosaccharides (XGO) or with 0–25 mM-cellopentaose. The ordinate shows the reciprocal of the initial incorporation rate (h/1000 c.p.m.); the uninhibited rate was 5180 c.p.m./h. □, XG9n; △, XG7; ■, ●, ▲, three different samples of XG9; ○, cellopentaose. The zero oligosaccharide control value (D) shows the mean ± S.E.M. of ten determinations.

Table 2. Activators and inhibitors of XET

Pea stem extract was assayed for XET activity in the presence of the additives listed. The XET activity of the controls in this experiment was 37.4 ± 1.5 Bq/kBq per h. The results are means ± S.E.M. for 3–4 determinations.

Additive	Concentration (mM)	XET activity (% of control)
None	–	100 ± 4
$AgNO_3$	1	26 ± 2
$Hg(OAc)_2$	1	30 ± 3
$ZnCl_2$	10	49 ± 3
$LaCl_3$	10	74 ± 4
EDTA	10	82 ± 8
$CaCl_2$	10	126 ± 1
$MgSO_4$	10	127 ± 4
Ascorbic acid	10	106 ± 1
2-Mercaptoethanol	2.5	127 ± 4
2-Mercaptoethanol	5	133 ± 1
2-Mercaptoethanol	10	134 ± 1
2-Mercaptoethanol	20	132 ± 1
Spermidine	1	122 ± 4
Putrescine	1	104 ± 1
NaF	10	95 ± 4
Gluconolactone	10	98 ± 2

1 mM-Ag^+ and -Hg^{2+} and 10 mM-La^{3+} and -Zn^{2+}. It was unaffected by 10 mM-D-gluconolactone and by chelating agents (Table 2).

Acceptor substrate specificity

The acceptor substrate specificity was investigated in two ways (Table 3). First, several alternative [^{3}H]oligosaccharides were tested as potential acceptors in place of [^{3}H]XG9. Removal of the non-reducing terminal Xyl → Glc unit of [^{3}H]XG9 (by the method of McDougall & Fry, 1991) (yielding [^{3}H]XG7f; see Fig. 2) did not diminish acceptor activity, but removal of the next Xyl → Glc unit [yielding [^{3}H]XG5 ($Glc_2XylGalFuc$)] abolished activity, suggesting that a non-reducing terminal Xyl → Glc group was essential. The disaccharide [^{3}H]XG2 (α-D-Xyl*p*-(1 → 6)-D-Glc; isoprimeverose), however, was not an acceptor.

Second, various non-radioactive oligosaccharides were tested as competitors of the reaction with [^{3}H]XG9. Estimated I_{50} values were 19 μM-XG9n and 33 μM-XG7 (Fig. 4) and these oligosaccharides thus appeared to be better substrates than XG9 itself. Their activity shows that the fucose and galactose residues of XG9 were not required for acceptor activity. Cellopentaose did not compete at concentrations up to 25 mM (Fig. 4), nor did cellotetraose (equivalent to the Glc_4 backbone of XG9) at 5 mM (Table 3). Xylose, glucose, cellobiose, maltose, maltotetraose and XG3 (Xyl → Glc → Glc) also did not compete. Thus the simplest structural unit that we can propose for an acceptor is:

α-D-Xyl*p* α-D-Xyl*p*

↑ ↑

6 6

β-D-Glc*p*-(1 → 4)-β-D-Glc*p*-(1 → 4)-D-Glc

It is not yet clear whether both xylose residues are required.

Table 3. Acceptor substrate specificity of XET from pea stems

The [^{3}H]XG9 in the standard XET assay (using *Tropaeolum* xyloglucan as donor) was either supplemented by a non-radioactive oligosaccharide or replaced by a different [^{3}H]oligosaccharide (0.14–1.9 kBq per assay).

Acceptor substrate	Rate (Bq/kBq per h)
[^{3}H]XG9 ($Glc_4Xyl_3GalFuc$)	46.0
[^{3}H]XG9 + 5 mM-cellotetraose	53.8
[^{3}H]XG9 + 25 mM-cellopentaose	49.0
[^{3}H]XG9 + 50 mM-cellobiose	41.4
[^{3}H]XG9 + 10 mM-xylose	49.9
[^{3}H]XG9 + 130 μM-XG9 ($Glc_4Xyl_3GalFuc$)	12.7
[^{3}H]XG9 + 130 μM-XG9n ($Glc_4Xyl_3Gal_2$)	5.8
[^{3}H]XG9 + 130 μM-XG7 (Glc_4Xyl_3)	9.2
[^{3}H]XG2 [α-D-Xyl*p*-(1 → 6)-D-Glc]	0.4
[^{3}H]XG5 ($Glc_2XylGalFuc$)	2.8
[^{3}H]XG7f ($Glc_3Xyl_2GalFuc$)	51.2

Donor substrate specificity

Numerous polysaccharides were tested as potential donor substrates (Table 4). In this experiment, paper chromatography was used to separate the unchanged [^{3}H]XG9 from [^{3}H]polysaccharides (if any) because few polysaccharides bind to filter paper as tightly as xyloglucans. Nevertheless, xyloglucan was the only polysaccharide to act as an efficient donor substrate (Table 4). *Tropaeolum* seed xyloglucan (which lacks fucose) was some-

Table 4. Donor substrate specificity of XET from pea stems

Each polysaccharide was tested at 1.25 mg/ml using [^{3}H]XG9 as acceptor. All the polysaccharides formed clear solutions at pH 6.0 (those marked * by autoclaving for 15 min) except cellulose and chitosan (suspensions) and nigeran, the three xylans and *Hordeum* arabinoxylan (hazy solutions).

Substrate	Rate (Bq/kBq per h)
Blank (no added polysaccharide)	0.0
Cellulose [β-(1 → 4)-D-glucan], microgranular	0.1
CM-cellulose* (Na$^+$ salt)	1.0
Methylcellulose	−0.2
Xanthan gum* (*Xanthomonas campestris*)	1.1
Xyloglucan (*Tropaeolum* seed)	47.9
Xyloglucan (*Rosa* cultures)	24.0
Laminarin [β-(1 → 3)-D-glucan]	−0.4
β-(1 → 3), (1 → 4)-D-Glucan (*Hordeum*)*	−0.1
Lichenan* [β-(1 → 3), (1 → 4)-D-Glucan] (*Cetraria*)	−1.0
Dextran [α-(1 → 6)-D-glucan]	0.3
Nigeran* [α-(1 → 3), (1 → 4)-D-glucan]	−0.4
Starch* [α-(1 → 4), (1 → 6)-D-glucan]	0.0
Glucuronoxylan* (*Fagus* wood)	5.4
Xylan* [β-(1 → 4)-D-xylan] (*Avena*) (Sigma)	1.3
Xylan* (Pfaltz & Bauer)	−0.1
Xylan* (Koch Light)	1.0
Arabinoxylan* (*Secale*)	−0.6
Arabinoxylan* (*Hordeum*)	−0.4
Guar* [D-galacto-β-(1 → 4)-D-mannan]	0.1
Mannan (*Saccharomyces*)	0.0
Inulin* [β-(2 → 1)-D-fructan] (*Dahlia*)	−0.5
Chitosan	0.4
Polygalacturonic acid [α-(1 → 4)-D-galacturonan]	0.3
Pectin (*Citrus*)	0.9
Rhamnogalacturonan-I (*Acer* cell cultures)	1.8
Rhamnogalacturonan-II (*Acer* cell cultures)	0.4
α-(1 → 5), (1 → 3)-L-Arabinan (*Beta*)	0.2
β-(1 → 4)-D-Galactan	0.2
Arabinogalactan (*Larix*)	0.1

what better than *Rosa* callus xyloglucan (which is fucose-rich). No other β-glucans showed significant activity: cellulose, CM-cellulose, methylcellulose, xanthan, β-(1 → 3),(1 → 4)-D-glucan, and laminarin were all inactive. *Fagus* wood glucuronoxylan [a β-(1 → 4)-linked D-xylan with α-D-glucuronic acid and 4-*O*-methyl-α-D-glucuronic acid side chains] appeared to be 11% as effective as *Tropaeolum* xyloglucan, and several other xylan-related polysaccharides were therefore tested; however, three samples of β-(1 → 4)-D-xylan and two of arabinoxylan did not act.

The very high specificity of XET for xyloglucans resembles that of an endo-β-glucanase from *Tropaeolum* cotyledons (Edwards *et al.*, 1986), which also exhibits transglycosylase activity (C. Fanutti & J. S. G. Reid, personal communication).

Relationship with cellulase

XET differed from the major cellulases [endo-(1 → 4)-β-D-glucanases] of pea stems in several respects. First, cello-oligosaccharides that inhibit the hydrolysis of CM-cellulose by pea cellulase had no effect on XET (Table 3; Fig. 4). Cellopentaose at 2 mM gives 40–60% inhibition of pea cellulases (Wong *et al.*, 1977) but even 25 mM-cellopentaose did not affect XET.

Second, XET failed to transfer CM-cellulose or β-(1 → 3),(1 → 4)-D-glucan to [^{3}H]XG9 (Table 4), although these polysaccharides are efficiently hydrolysed by pea cellulases (Wong *et al.*, 1977).

Third, the auxin 2,4-D induced a large increase in concentrations of buffer- and salt-extractable cellulases, as reported by Hayashi *et al.* (1984), but caused some decrease in extractable XET activity (Table 5). XET extracted from 2,4-D-treated pea stems was inhibited by 50 μM-XG9n almost as effectively as was XET extracted from control stems (cf. Fig. 4), indicating that the lower apparent activity in the latter was not due to the presence of elevated concentrations of co-extracted xyloglucan oligosaccharides.

Fourth, a high-salt extract from 2,4-D-treated tissue that had been thoroughly pre-extracted with low-salt buffer contained low XET but high cellulase activity (Table 5).

Fifth, the XET activity of pea stem extracts was increased by approx. 30% on addition of 5–20 mM-2-mercaptoethanol (Table 2). We were unable to confirm the inhibitory effect of 2-mercaptoethanol on pea cellulases reported by Wong *et al.* (1977). Nevertheless, 2-mercaptoethanol did not promote cellulase activity whereas it did promote XET activity.

XET is not bound to cell walls

Most of the XET in pea stems was not ionically bound to the cell wall. Of several reagents that solubilize wall-bound enzymes (Fry, 1988), only 20 mM-$LaCl_3$ extracted any XET activity (a trace) after thorough pre-extraction with water (Table 6). Triton X-100 also failed to extract any additional activity, arguing against entrapment of XET within membranous vesicles.

Relatively little XET appeared to be covalently bound in the cell wall. The water-washed residue of pea stem homogenates showed little ability to incorporate [^{3}H]XG9 into either wall-bound or soluble xyloglucan (Table 6).

Table 5. XET and cellulase activity of extracts from pea stems grown with or without auxin

Pea seedlings were sprayed with or without 2,4-D and grown on for 4 days, and then the third internode was extracted sequentially with low- and high-salt buffers. The extracts were assayed for XET using xyloglucan + [^{3}H]XG9 and for cellulase using CM-cellulose.

Pretreatment	Extractant	XET (Bq/kBq per h)	Cellulase $\Delta\eta/t$* (s/h)
−2,4-D	Buffer	24.3	0.0
−2,4-D	Buffer + 1 M-NaCl	4.8	1.0
+2,4-D	Buffer	7.8	8.8
+2,4-D	Buffer + 1 M-NaCl	2.3	5.3

* $\Delta\eta$ = decrease in viscometer flow time (initially 13.2 s); t = incubation time.

Table 6. Extraction of XET from pre-extracted pea stems

Tissue from the third internode of day-8 etiolated pea stems was thoroughly pre-extracted by grinding and washing in distilled water, and then shaken gently for 3 h at 20 °C in the extractant indicated (1 ml/g original fresh weight). Buffer [20 mM-Mes (Na^+), pH 6.0] was present in all extractants. Controls showed that neither the XET-catalysed reaction nor the binding of [^{3}H]xyloglucan to filter paper was affected by these extractants at the final concentrations used. In the last two samples ('no extractant'), the wall-rich residue from tissue that had been thoroughly pre-extracted with water was resuspended in the assay solution (xyloglucan + [^{3}H]XG9) at 1 g initial fresh weight/ml; after incubation, the solution was tested for soluble [^{3}H]xyloglucan (solution) by the filter paper binding assay, and the insoluble residue (walls) was washed in water and assayed for bound ^{3}H.

Extractant	XET activity (Bq/kBq per h)
Control (not pre-extracted)	82.6
Buffer (Mes, pH 6.0)	0.0
1% Triton X-100	0.0
40 mM-$CaCl_2$	0.3
100 mM-$CaCl_2$	0.3
250 mM-$CaCl_2$	0.1
20 mM-$LaCl_3$	2.1
40 mM-$LaCl_3$	0.6
100 mM-$LaCl_3$	0.0
2.0 M-LiCl	0.5
2.7 M-LiCl	0.0
No extractant (solution)	0.2
No extractant (walls)	5.7

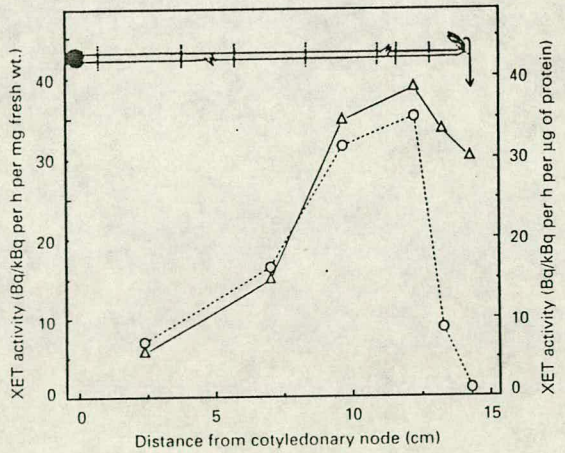


Fig. 5. Distribution of extractable XET activity along the pea stem

Day-7 etiolated pea seedlings were dissected into segments as indicated in the drawing and each segment was weighed and assayed for extractable XET and soluble protein. △—△, XET activity per mg fresh weight of tissue; ○ ○, XET activity per μg of extractable protein.

Correlations between XET activity and growth rate

We observed several correlations between extractable XET activity and growth rate, as expected if XET catalyses a wall-loosening reaction. First, XET activity was highest in the third (= fastest growing) pea internode, a short distance back from the apical bud (Fig. 5), and substantially lower in the first internode. This relationship was seen whether XET activity was expressed per unit fresh weight of tissue or per μg of extracted protein. Second, excised pea stem segments lost XET activity during an 18 h incubation, paralleling their fall in growth rate (K. F. Renwick & S. C. Fry, unpublished work). Third, the XET activity of cultured spinach cells peaked during the most rapid phase of cell expansion (R. C. Smith & S. C. Fry, unpublished work).

DISCUSSION

Xyloglucan-derived oligosaccharides can activate plant (but not fungal) 'cellulase' in a viscometric assay using xyloglucan (but not CM-cellulose) as substrate (Farkaš & Maclachlan, 1988; McDougall & Fry, 1990). The oligosaccharides (1–100 μM) were observed to cause an immediate but short-lived burst of xyloglucan depolymerization. A second burst occurred upon the addition of a second dose of oligosaccharide (McDougall & Fry, 1990), suggesting that the oligosaccharides exerted a stoichiometric rather than regulatory effect. In the light of the present results, it seems likely that the reaction detected viscometrically was not hydrolysis of the xyloglucan but XET-catalysed transglycosylation, with the oligosaccharides acting as acceptors. In support of this, the structural requirements for an oligosaccharide to act as an acceptor for XET (assayed radiochemically; Table 3) match closely the requirements for promotion of the enzymic depolymerization of xyloglucan (assayed viscometrically; E. P. Lorences & S. C. Fry, unpublished work).

According to this interpretation, in the absence of oligosaccharides the enzyme would catalyse an 'idling' reaction in which the new (potentially reducing) terminus of each cleaved xyloglucan chain (□□□□□□...) is transferred to the non-reducing terminus of another chain (■■■■■■...), resulting in no net change in mean M_r and therefore no loss of viscosity:

□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□ + ● ↔
□□□□□□□□□□□● + □□□□□□□□□□□□□□□□□□□□

more extensible. The simple assay developed for XET should facilitate future studies of its occurrence and function.

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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-β-(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 β-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 β-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 α-(1→4)-glucosyl bond in one starch molecule and then re-form a similar α-(1→4)-glucosyl bond with a different acceptor molecule (Pazur & Okada, 1968). Similarly, an endo-(1→3)-β-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 ^{3}H radioactivity; the pellet was re-suspended in water and assayed for ^{3}H 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 μl of 0.5% (w/v) *Tropaeolum* xyloglucan. To this was added 70 μl of Fehling's solution A [35% (w/v) potassium sodium tartrate tetrahydrate in 25% (w/v) KOH] followed by 70 μl 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 ^{3}H 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 ^{3}H 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 ^{3}H 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 μM-[*r.t.*-1-^{3}H]XG9 for up to 72 h. Samples (150 μl) 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 ^{3}H radioactivity.

RESULTS

Characterization of [*r.t.*-1-^{3}H]XG9

The ^{3}H-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 [^{3}H]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 [^{3}H]glucose and [^{3}H]glucitol respectively as sole radioactive product (results not shown), confirming that the ^{3}H was restricted to the reducing terminus. Driselase digestion of the ^{3}H-labelled oligosaccharide followed by paper chromatography yielded [^{3}H]glucose but no [^{3}H]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-^{3}H]XG9.

[^{3}H]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-^{3}H]XG9 for up to 72 h. If the XG9 were cleaved during the transglycosylation, the ^{3}H would be released as a smaller [^{3}H]oligosaccharide or [^{3}H]glucose, and not efficiently incorporated into polymer.

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

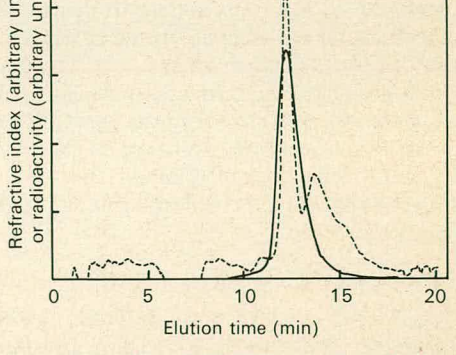


Fig. 1. H.p.l.c. analysis of [*r.t.*-1-^{3}H]XG9 on amino-substituted silica

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

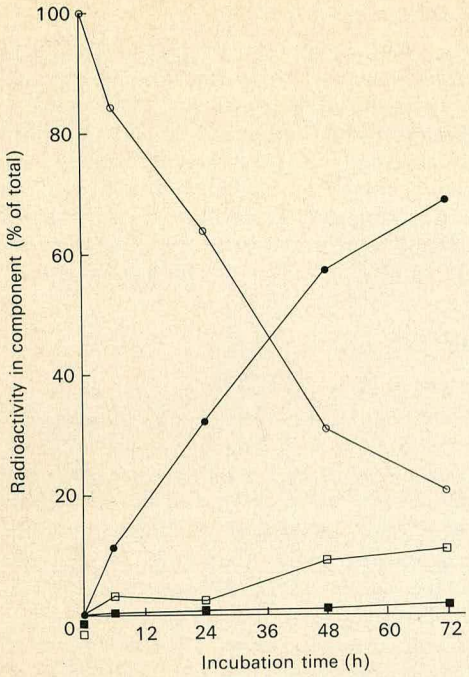


Fig. 2. Incorporation of ^3H from [*r.t.*-1-^3H]XG9 into fractions of spinach cell cultures

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.

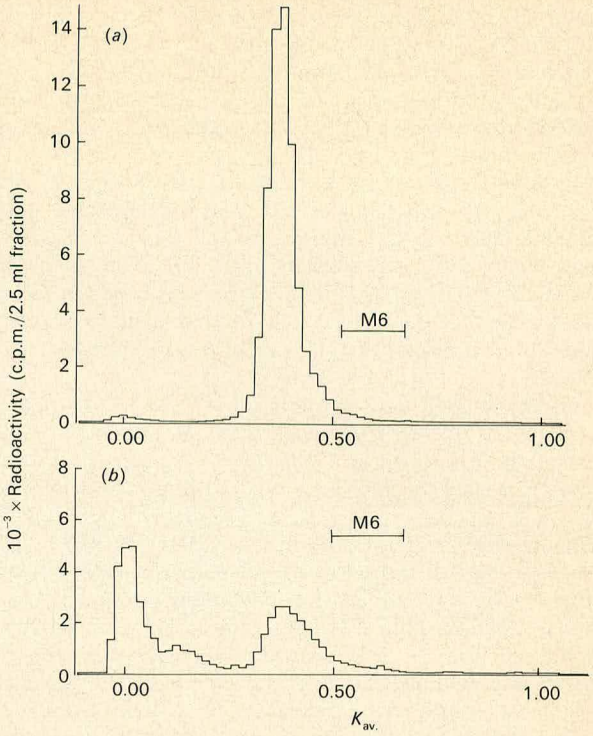


Fig. 3. G.p.c. of the products of incubating [*r.t.*-1-^3H]XG9 in spinach culture medium

(*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*).

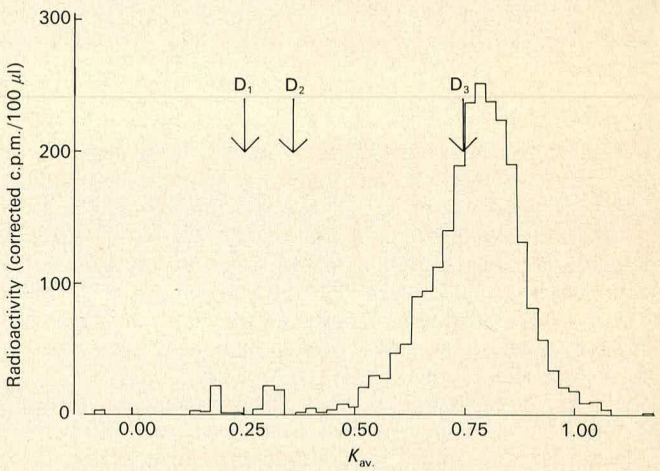


Fig. 4. G.p.c. of high-M_r transglycosylation products

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 5×10^6–40×10^6; $K_{av.}$ 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

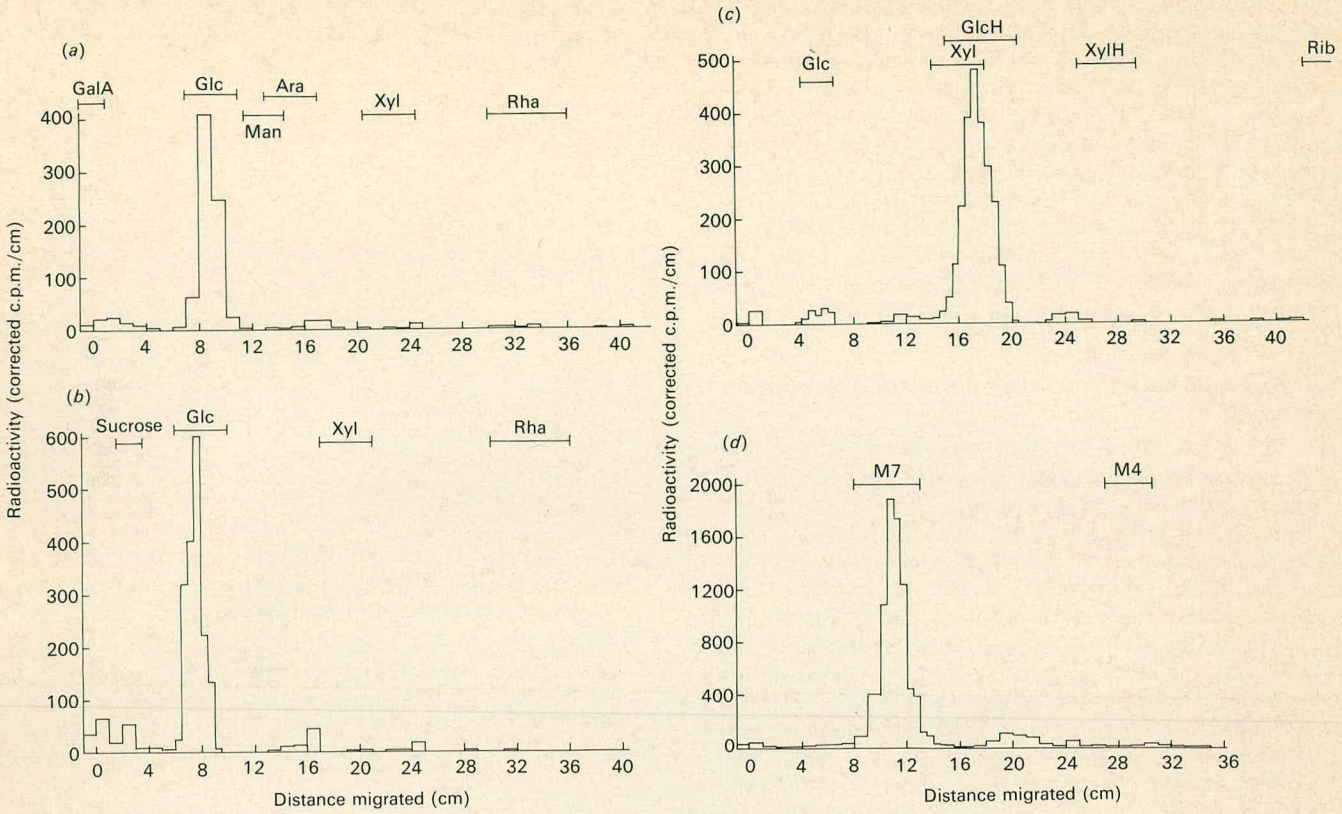


Fig. 5. Paper chromatography of high-M_r transglycosylation products after hydrolysis

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 ^{3}H migrated faster than M4. Positions of external markers are shown (GlcH, D-glucitol; XylH, xylitol; M7, maltoheptaose; M4, maltotetraose). Recovery of applied ^{3}H 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 ^{3}H radioactivity was confirmed to be present in the supernatant.

Precipitant used	Non-radioactive carrier used	Proportion of ^{3}H 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

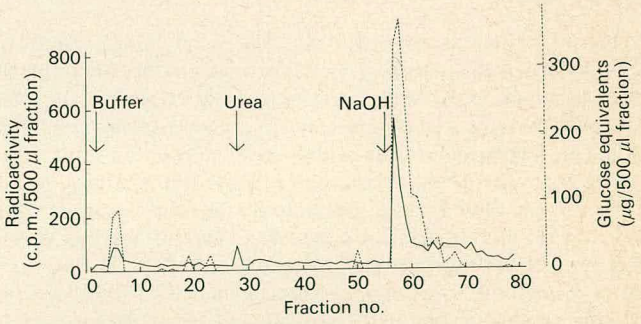


Fig. 6. Cellulose affinity chromatography of high-M_r transglycosylation products

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 ^{3}H from the column was 92.7% (70% of the applied ^{3}H eluted with NaOH).

that the polymer that acts as glycosyl donor probably contributes a β-(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 ^{3}H-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.

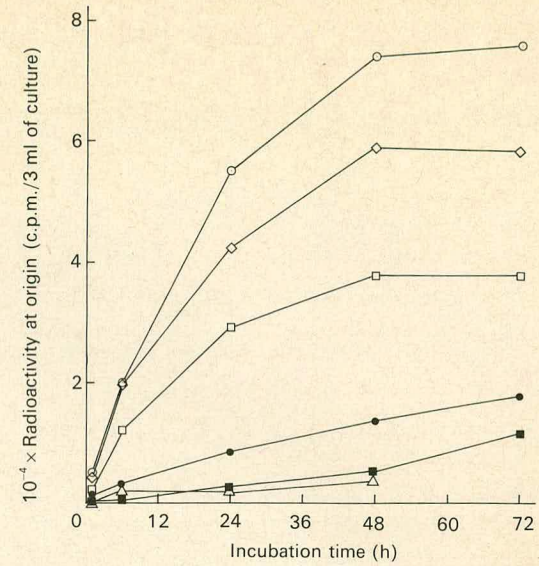


Fig. 7. Incorporation of ^{3}H from [*r.t.*-1-^{3}H]XG9 into soluble apoplastic polymer in cultures of six other species

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*).

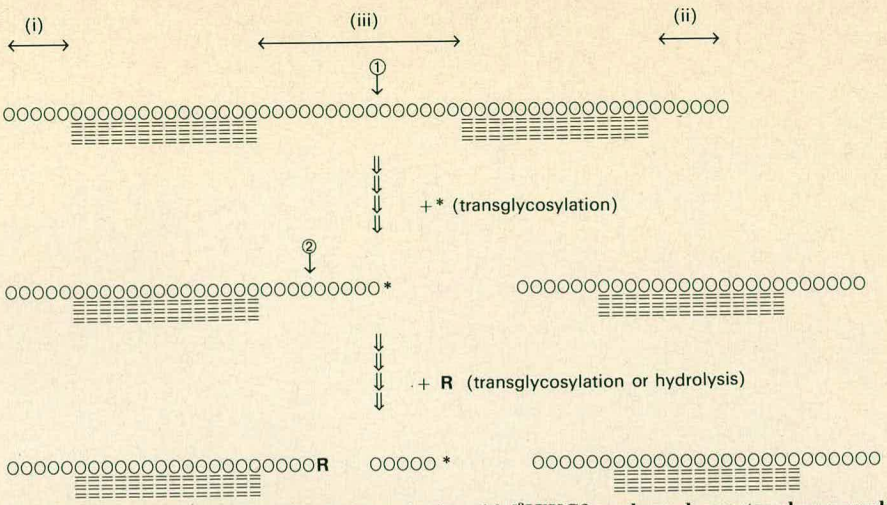
DISCUSSION

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-β-(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-β-(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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