Xyloglucan metabolism in the apoplast of suspension-cultured rose cells

James Thompson

A thesis presented in fulfilment of the requirements for the degree of Doctor of Philosophy, University of Edinburgh, 1996.
Declaration

I hereby declare that this thesis was composed by myself and the work contained herein to be my own work, except where otherwise indicated.

James Thompson

Edinburgh, 1996
Acknowledgements

I would like to thank Professor Stephen Fry for his help and advice throughout the lab work and the writing of this thesis.

I would also like to thank residents of lab 215, both past and present, for assistance (especially Janice Miller); and Dr. Rachel Smith in particular for preliminary development of the density-labelling strategy and advice on CsTFA gradient centrifugation conditions.
Abbreviations

Ara  Arabinose
BAW  Paper chromatography solvent; butan-1-ol/acetic acid/water (12:3:5, v/v/v)
BFA  Brefeldin A
cDNA  complementary DNA
CMC  Carboxymethylcellulose
CsTFA  Caesium trifluoroacetic acid
cXET  Cauliflower enzyme preparation containing XET activity
2,4-D  2,4-Dichlorophenoxyacetic acid
DCB  2,6-Dichlorobenzonitrile
d.p.  degree of polymerisation
EPyW  Paper chromatography solvent; ethyl acetate/pyridine/water (9:3:2, v/v/v; except where stated otherwise).
Fuc  Fucose
Gal  Galactose
Glc  Glucose
GPC  Gel permeation chromatography
HPLC  High performance liquid chromatography
IAA  Indole acetic acid
k_{av}  The volume in which a substance will elute from a GPC column, as a proportion of the total included volume
M_r  Relative molecular mass
PC  Paper chromatography
POPOP  1,4-bis-(5-phenyloxazol-2-yl)benzene
PPO  2,5-diphenyloxazole
psi  pounds per square inch
PyAW  volatile salts buffer; pyridine/acetic acid/water (1:1:23 or 1:1:98 v/v/v as stated in text)
$R_i$ The distance migrated by a substance on PC relative to the solvent front (origin = 0)

$rXG$ Xyloglucan extracted from suspension-cultured rose cells

$SCV$ Settled cell volume

$sXG$ Xyloglucan extracted from suspension-cultured spinach cells

$TFA$ Trifluoracetic acid

$tXG$ Tamarind seed xyloglucan (donated by Prof. J.S.G. Reid, University of Stirling)

$UV$ Ultra-violet

$V_i$ Included volume of GPC column

$V_o$ Void volume of GPC column

$WLF$ Wall-loosening-factor

$XET$ Xyloglucan endotransglycosylase

$XGase$ Xyloglucanase

$Xyl$ Xylose
Contents

Title page i
Declaration ii
Acknowledgements iii
Abbreviations iv
Contents vi
Abstract xii

1. Introduction 1

1.1 Primary cell walls of plants 1
  1.1.1 Cellulose 1
  1.1.2 Hemicelluloses 1
  1.1.3 Pectins 2
  1.1.4 Structural glycoproteins 2

1.2 Structural models of plant cell walls 3
  1.2.1 Covalent cross-links 3
  1.2.2 'Warp-weft' model 4
  1.2.3 Cellulose/xyloglucan network 4

1.3 Expansion of plant cells 5
  1.3.1 The Lockhart equation 5
  1.3.2 'Loosening' and 'tightening' of the cell wall 6

1.4 Xyloglucan 7
  1.4.1 Structure of xyloglucan 7
    1.4.1.1 Dicotyledonous xyloglucan 7
    1.4.1.2 Monocotyledonous xyloglucan 9
    1.4.1.3 Seed storage xyloglucan 9
  1.4.2 Structures of xyloglucan oligosaccharides 9
1.4.3 Biosynthesis of xyloglucan

1.4.4 Biological activity of xyloglucan oligosaccharides
  1.4.4.1 Anti-auxin activity
  1.4.4.2 Auxin-mimicking activity
  1.4.4.3 Effects on morphogenesis

1.4.5 Hydrogen-bonding of xyloglucan to cellulose

1.5 Transglycosylation
  1.5.1 Endo-transglycosylation of xyloglucan
  1.5.2 Mode of action of XET
    1.5.2.1 Enzymic modification of xyloglucan may affect the availability of XET substrates in the cell wall
  1.5.3 XET in plant cell expansion
  1.5.4 XET in the mobilisation of seed storage xyloglucan
  1.5.5 Assaying XET activity
    1.5.5.1 Paper-binding assay
    1.5.5.2 Viscosity assay
    1.5.5.3 Colorimetric assay
    1.5.5.4 Fluorescence-tagged xyloglucan
  1.5.6 Molecular biology of XET
    1.5.6.1 XET-related genes

1.6 Changes in xyloglucan Mr
  1.6.1 Decreases in xyloglucan Mr
    1.6.1.1 In response to auxin and low pH in vivo
  1.6.2 Increases in xyloglucan Mr

1.7 Aims and objectives

2. Materials and methods
  2.1 General methods
    2.1.1 Suspension-cultured rose cells
    2.1.2 Radiolabels
2.1.2.1 Radiolabelling rose suspension-cultures 25
2.1.2.2 Radiolabelling protoplast products 26
2.1.2.3 Radiolabelling xyloglucans for use as internal markers in density gradients 26
2.1.2.4 Use of radiolabels to develop density-labelling strategy 28

2.1.3 Fractionation of suspension-cultures 29
2.1.3.1 Extraction of culture-filtrates 29
2.1.3.2 Extraction of cell-contents 29
2.1.3.3 Extraction of hemicellulose from cell-wall preparations 30
2.1.3.4 Stability of xyloglucan in cell-wall extractant 30

2.1.4 Gel-permeation chromatography 30
2.1.4.1 Anthrone reagent 31
2.1.4.2 Calibration of the GPC column 31

2.1.5 'Driselase' 31
2.1.5.1 Purification of 'Driselase' 31
2.1.5.2 'Driselase' digestion of samples 32

2.1.6 Paper chromatography of 'Driselase' digests 32

2.1.7 Staining chromatograms 33
2.1.8 Radioactivity assay 33
2.1.9 Estimation of xyloglucan molecular weight 34
2.1.10 Density gradients 35
2.1.10.1 Preparation of density gradients 35
2.1.10.2 Fractionation of density gradients 36
2.1.10.3 Estimation of density of fractions 37
2.1.10.4 Scintillation counting of CsTFA fractions 37
2.1.10.5 Desalting of CsTFA fractions 37
2.1.10.6 Analysis of xyloglucan in density gradient fractions 37
2.1.10.7 Estimation of density of xyloglucan 38

2.2 Xyloglucan molecular weight changes 38
2.2.1 Relationship between molecular weight and density of xyloglucan

2.3 Protoplasts
   2.3.1 Preparation of protoplasts
   2.3.2 Recovery and analysis of protoplast products

2.4 Brefeldin A

2.5 Density labelling
   2.5.1 Protocol 1
   2.5.2 Protocol 2
   2.5.3 Protocol 3
   2.5.4 In vitro density assay
      2.5.4.1 Preparation of cauliflower XET extract
      2.5.4.2 XET activity assay
      2.5.4.3 Xyloglucanase activity assay
      2.5.4.4 Density-change assay

3. Results

3.1 General techniques
   3.1.1 Growth of suspension-cultured rose cells

3.1.2 Radiolabels
   3.1.2.1 Radiolabelling xyloglucans for use as internal markers in density gradients.
   3.1.2.2 Use of radiolabels to develop density-labelling strategy

3.1.3 Fractionation of suspension-cultures
   3.1.3.1 Stability of xyloglucan in extractant

3.1.4 Gel permeation chromatography
   3.1.4.1 Calibration of GPC column

3.1.5 Density gradients

3.2 Changes in molecular weight of xyloglucan
   3.2.1 Uptake and incorporation of the radiolabel
3.2.2 Molecular weight profiles 69
3.2.3 Discussion of results 72
3.2.4 Relationship between molecular weight and density of xyloglucan 73
  3.2.4.1 Molecular weight profile 73
  3.2.4.2 Density profiles 74

3.3 Protoplasts 78
  3.3.1 Molecular weight profiles 78
  3.3.2 Discussion of results 81

3.4 Brefeldin A 83
  3.4.1 Uptake and incorporation of the radiolabel 83
  3.4.2 Molecular weight profiles 85
  3.4.3 Discussion of results 86

3.5 Density labelling 87
  3.5.1 Protocol 1 87
    3.5.1.1 Uptake and incorporation of the radiolabel 87
    3.5.1.2 Density profiles 87
  3.5.2 Protocol 2 92
    3.5.2.1 Uptake and incorporation of the radiolabel 92
    3.5.2.2 Density profiles 92
  3.5.3 Protocol 3 97
    3.5.3.1 Uptake and incorporation of the radiolabel 97
    3.5.3.2 Density profiles 97
  3.5.4 Discussion of results 102
  3.5.5 In vitro XET assay by changes in density 106
    3.5.5.1 XET activity assay 106
    3.5.5.2 Xyloglucanase activity assay 106
    3.5.5.3 Molecular weight assay 106
    3.5.5.4 Density-change assay 106
    3.5.5.5 Discussion of results 112
4. Discussion

4.1 Changes in Mr of wall-bound xyloglucan
   4.1.1 In relation to growth-rate
   4.1.2 In relation to ageing of cell walls

4.2 Transglycosylation may integrate newly-secreted xyloglucan into the cell wall matrix
   4.2.1 Mechanism of action of XET in the incorporation of newly-secreted xyloglucan

4.3 Functions of XET in vivo
   4.3.1 XET may cut and re-form xyloglucan cross-links
   4.3.2 XET may 'loosen' the cell wall
   4.3.3 XET may 'tighten' the cell wall

4.4 Expansins may also modify the cellulose/xyloglucan network.

4.5 Conclusions

5. References

6. Publications
Abstract

Xyloglucan hydrogen-bonds to cellulose and has been proposed to form load-bearing cross-links between adjacent microfibrils in the primary cell walls of plant cells. Such cross-links may regulate wall extensibility and allow control of cell expansion. Changes in relative molecular mass (M_r) of xyloglucan have been correlated with wall extensibility in several studies. Endotransglycosylation of xyloglucan may cut and reform cross-links, enabling limited cell expansion through temporary loosening of the cell wall. An enzyme capable of catalysing such a reaction, xyloglucan endotransglycosylase (XET), has been reported previously.

A pulse-chase radiolabelling approach was used to follow the changes in M_r of a pool of radiolabelled wall-bound xyloglucan in the cell walls of suspension-cultured rose cells as the cells aged. This was done in rapidly-expanding cells and in slowly-expanding cells to study the effects of expansion rate on xyloglucan M_r. Radiolabelled xyloglucan extracted from the cell walls of rapidly-expanding cultures had a M_r consistently ~80000 lower than radiolabelled xyloglucan extracted from slowly-expanding cells. The relationship between xyloglucan M_r and expansion rate is discussed.

The radiolabelled xyloglucan in the cell wall extracts of both cultures decreased in M_r by ~40000 during the 7-day observation period, with 20-30% of the radiolabelled xyloglucan being lost from the cell wall. A similar amount of radiolabelled xyloglucan, with a mean M_r of ~39000, accumulated in the culture medium at the same time as the loss from the cell wall. This observation is proposed to be due to trimming of loose (i.e. not directly bound to microfibrils) sections of wall-bound xyloglucan from the cell wall and subsequent sloughing into the culture medium. The possible effects of trimming of xyloglucan on the extensibility of the cell wall are discussed.
Protoplasts isolated from suspension-cultured rose cells were used to determine the $M_r$ of newly-secreted xyloglucan. Brefeldin A was used to block temporarily the secretion of xyloglucan into the apoplast of suspension-cultured rose cells, allowing the changes in $M_r$ of xyloglucan to be followed during integration of newly-secreted xyloglucan into the cell wall matrix. Both experimental approaches suggested that low $M_r$ xyloglucan ($\leq 10000$) is secreted into the apoplast and undergoes a $\sim 20$-fold increase in $M_r$ soon after secretion, possibly by transglycosylation with wall-bound xyloglucan.

A dual-labelling technique which involved feeding a trace amount of radiolabelled precursor ($^{[3]}$H]arabinose) and a density-labelled carbon-source ($^{13}$C-enriched glucose) to suspension-cultured rose cells was developed. This technique was employed to investigate the occurrence of transglycosylation between wall-bound xyloglucan polymers in the cell walls of suspension-cultured rose cells. This mode of transglycosylation was not detected, although transglycosylation was detected between wall-bound xyloglucan and newly-secreted xyloglucan.

The mechanism of XET action in the integration of newly-secreted xyloglucan into the cell wall and the possible roles of XET in the cell wall are discussed. I suggest that xyloglucan is built up in the cell wall by annealing of smaller xyloglucan molecules, catalysed by a transglycosylase which may be an XET that preferentially attacks the donor substrate near to the reducing end. I also propose that incorporation of newly-secreted xyloglucan into the cell wall matrix may cause wall-loosening or wall-tightening depending on the type of xyloglucan molecule involved (e.g. cross-linking sections of xyloglucan or unbound sections of wall-bound xyloglucan containing either end of the xyloglucan chain) and the action of the transglycosylase involved (e.g. end-attacking or mid-chain attacking).
1. Introduction

1.1 Primary cell walls of plants

The primary cell walls of plants consist of a network of cellulose microfibrils embedded in a matrix of other cell wall polymers. These matrix polymers fall into three main categories: hemicelluloses, pectins and structural glycoproteins (Northcote, 1972; Preston, 1979; McNeil et al., 1984; Fry, 1986a).

1.1.1 Cellulose

Each cellulose molecule contains over 500 β-(1→4)-linked D-glucopyranose residues, forming a rigid ribbon-like structure which is stabilised by intramolecular hydrogen bonds. Cellulose molecules aggregate by intermolecular hydrogen bonding to form long microfibrils in primary cell walls of between 5-15 nm in diameter at a spacing of 20-40 nm apart (Monro et al., 1976; Brown, 1982; McCann et al., 1990). These semi-crystalline, rod-like structures are resistant to stretching and their orientation in the cell wall is thought to determine the direction of cell expansion (Green, 1980; Preston, 1982; McCann and Roberts, 1994). Cellulose microfibrils do not undergo substantial hydrolysis in the cell wall during cell expansion (Maclachlan, 1977), but can move apart or past one another (Preston 1982). The matrix polymers are thought to maintain the spacing of cellulose microfibrils via cross-links, preventing excessive separation during cell expansion and preventing hydrogen-bonding between microfibrils.

1.1.2 Hemicelluloses

Hemicellulosic polysaccharides are distinguished by their ability to hydrogen-bond to cellulose. They usually have a backbone of β-(1→4)-linked sugar residues (D-xylopyranose in the case of xylans and D-glucopyranose in the case of xyloglucans), often with short side chains attached. They may
form cross-links between microfibrils, either directly or via interaction with other matrix polymers. Xyloglucan (see section 1.4) is the dominant hemicellulose in the primary cell wall of dicots whereas xylans (e.g. arabinoxylan) are more prevalent in monocots.

1.1.3 Pectins

Pectic polysaccharides can be subdivided into neutral (e.g. arabinogalactans) and acidic pectins (e.g. rhamnogalacturonans and homogalacturonans). Acidic pectins contain a high proportion of galacturonic acid residues in their backbone, some of which are methyl-esterified. These pectins are highly hydrated owing to their negative charge and strongly bind cations; Ca\(^{2+}\) cross-links are thought to hold acidic pectins together as a gel in the cell wall matrix via formation of 'egg-box' junctions. Rhamnose residues in the backbone of rhamnogalacturonans introduce kinks and provide sites for covalent attachment of neutral pectins (for a review, see Jarvis, 1984).

1.1.4 Structural glycoproteins

Structural proteins in the walls of plant cells can be divided into five groups and can account for up to 10% of the dry weight of primary cell walls. The most extensively studied group are the extensins, these glycoproteins usually contain repeating sequences of amino acid residues with high proportions of hydroxyproline (up to 30%), serine and tyrosine. Short oligosaccharides are attached (via \(O\)-glycosylation) to hydroxyproline residues, often in a repeating block, to such an extent that carbohydrate can contribute over half the molecular weight of the glycoprotein (Darvill et al., 1980). Deposition of glycoproteins has been linked to wounding and infection responses, and to the transition between primary and secondary cell wall deposition (for a review, see Showalter, 1993).
1.2 Structural models of plant cell walls

Several models of the organisation of the different classes of cell wall components to form the structure of the cell wall have been put forward, some of which are described below.

1.2.1 Covalent cross-links

In 1973, Albersheim and co-workers proposed a structural model of primary cell walls based on the components they had characterised from suspension-cultured sycamore cell walls. This model consists of a monolayer of xyloglucan coating cellulose microfibrils via hydrogen-bonds; other linkages in the scheme are covalent. The reducing termini of xyloglucan are linked to neutral pectins; these are attached to acidic pectins which are themselves attached to hydroxyproline-rich proteins, forming a covalently bonded network containing all the major components of the cell wall matrix (Keegstra et al., 1973; Talmadge et al., 1973). The authors proposed that expansion was accommodated by acid-induced weakening of the hydrogen bonds between xyloglucan and cellulose microfibrils which allowed them to slip past each other and rejoin further along. It was later found that interactions between xyloglucan and cellulose are strengthened rather than weakened at low pH (Valent and Albershiem, 1974).

Although possible covalent linkages have been reported between xyloglucan and pectins (Bauer et al., 1973; Stevens and Selvendran, 1984abc; Chambat et al., 1984; O’Neill and Selvendran, 1985a; Redgewell and Selvendran, 1986; du Pont and Selvendran, 1987; Elrassi et al., 1991; Kato and Nevins, 1991; Renard et al., 1991, 1992; Fu and Mort, 1995), these are not considered to be abundant enough to account for the widespread network formation proposed in this model (Monro et al., 1976; Darvill et al., 1980; McNeil et al., 1984).
1.2.2 'Warp-weft' model

The 'warp-weft' model proposed by Lamport and Epstein in 1983 consists of a network of cellulose microfibrils interlaced with extensin forming loops (by polymerisation of tyrosine residues) around microfibrils. This network is suspended in a gel-like support formed by the other matrix polysaccharides. Extensin may physically entrap microfibrils and other matrix polysaccharides via loop formation.

Although isodityrosine and di-isodityrosine have been found in cell wall proteins, it is still unclear if these are part of intermolecular or intramolecular loops (Fry, 1982a; Brady et al., 1996), or are abundant enough to account for the strength of cell walls.

Another potential crosslink in the cell wall is diferulic acid formed by the dimerisation of ferulate groups attached to arabinose and galactose residues of pectins in the cell wall of some dicots, or to arabinose of arabinoxylan of Gramineae (Fry, 1982b, 1983, 1986a).

1.2.3 Cellulose/xyloglucan network

The cell wall in this model consists of cellulose microfibrils tethered by xyloglucan molecules supported in a matrix of other cell wall polymers (Hayashi and Maclachlan, 1984a; Fry, 1989a; Hayashi, 1989; Carpita and Gibeaut, 1993). Xyloglucan molecules are held in the wall by hydrogen-bonding to microfibrils (Monro et al., 1976; Chambat et al., 1984; Hayashi and Maclachlan, 1984a; Hayashi et al., 1987, 1994a; Levy et al., 1991); the pectins in the cell wall matrix form a highly hydrated gel (in the presence of calcium) which may support the cellulose/xyloglucan network.

Xyloglucan coats newly synthesised cellulose microfibrils throughout cell expansion and may help to prevent aggregation of microfibrils into larger bundles. However, there is more xyloglucan in the cell wall than can be accounted for by a simple monolayer coating microfibrils (Hayashi et al., 1987), therefore a portion (up to 95%, Baba et al., 1994) of the xyloglucan
must be free from the microfibrils. Theoretically, xyloglucan molecules are long enough (150-1500 nm) to form cross-links between neighbouring microfibrils which could prevent them moving apart (Hayashi and Maclachlan, 1984a; Fry, 1986a; Hayashi et al., 1987; Fry 1989ab; Hayashi, 1989). Xyloglucan cross-links have been observed between cellulose microfibrils in onion cell walls (McCann et al., 1990).

Expansion of the cell can be accommodated in this model via hydrolysis of the xyloglucan cross-links by cellulase, cutting and re-annealing of xyloglucan cross-links (by endo-transglycosylation), or by releasing the hydrogen-bonded segments from the microfibrils (Albersheim, 1976; Cleland, 1981).

1.3 Expansion of plant cells

Expansion of plant cells requires expansion of the cell wall; the driving force for this extension is the turgor pressure exerted on the cell wall. Expansion necessarily causes an increase in volume of the tissue which must be balanced by uptake of water into cells and an increase in solutes in the cells to maintain turgor (Salisbury and Ross, 1978; Cleland, 1971; Taiz, 1984; Cosgrove, 1986).

1.3.1 The Lockhart equation

A mathematical model to describe expansion of plant cells was put forward by Lockhart (1965) in which the rate of expansion (dV/dt), the rate of change in volume V with time t, is related to turgor pressure (P), the yield threshold (Y) of the cell wall (the turgor pressure above which irreversible expansion occurs), and the extensibility of the cell wall (m) as follows:

\[
\frac{dV}{dt} = m(P - Y)
\]

Application of this model to experimental results revealed that the parameters m and/or Y are variables, not constants. Step increases in turgor result in only a transient increase in the rate of expansion before returning to
the steady growth rate after about 15 min, not a permanent increase in the rate of expansion as predicted by the equation with \( m \) and \( Y \) as constants. In many cases, experiments have shown that plant cells respond to changes in turgor through changes in the yield threshold and/or extensibility of the cell wall to maintain a steady growth rate (Green et al., 1971; Cosgrove, 1986; Taylor and Davies, 1986; Pritchard et al., 1990, 1991; Passioura and Fry, 1992; Passioura, 1994) It is likely that expansion is controlled not by changing turgor, but by changing the properties of the cell wall (Pritchard et al., 1993).

1.3.2 ‘Loosening’ and ‘tightening’ of the cell wall

To allow controlled expansion in one direction, as in cell elongation, the cell wall must be subject to localised weakening or ‘loosening’ to allow cellulose microfibrils to move apart. This loosening process must also be reversible so that expansion does not continue, which would lead to the cell rupturing (Albersheim, 1976; Cleland, 1981). Therefore, there must be a ‘tightening’ of the cell wall after the required amount of expansion has been achieved.

If we assume from the cellulose/xyloglucan network model of the cell wall that xyloglucan cross-links form load-bearing tethers between microfibrils, then these cross-links are obvious targets for wall-loosening processes. Any mechanism by which these cross-links are removed will loosen the cell wall, allowing expansion to occur. Such mechanisms could include hydrolysis by cellulase, transglycosylation, and breaking of the hydrogen bonds onto microfibrils at either end of the cross-link (Albersheim, 1976; Cleland, 1981; Taiz, 1984; Fry 1989a; Hayashi, 1989).

A model in which xyloglucan cross-links exist in the cell wall in a ‘taut’ (load-bearing) or ‘relaxed’ state has been proposed (Passioura and Fry, 1992; Passioura, 1994). In this model, the load resulting from the effect of turgor pressure on the cell wall is shared amongst the load-bearing cross-links; if one or more of these ‘taut’ cross-links is removed the other load-bearing tethers will gradually be pulled off or ‘unzipped’ from the
microfibrils, due to breakage of hydrogen bonds, as the microfibrils move apart. This will continue until 'relaxed' cross-links become 'taut', as the separation of the microfibrils they are attached to increases, spreading the load between more 'taut' cross-links until there are a sufficient number to resist further separation of the microfibrils.

1.4 Xyloglucan

Xyloglucan is a major component of the primary cell walls of dicotyledonous plants where it typically constitutes 20-25% of the dry weight (McNeil et al., 1984; Fry, 1989a; Hayashi, 1989). It is less abundant in the primary cell walls of Graminaceous monocots (Kato and Matsuda, 1985), in which mixed-linkage β-(1→3),(1→4) glucans and xylans are predominant (Wada and Ray, 1978; Carpita, 1984). However, the ratio of xyloglucan to cellulose in monocot cell walls is similar to that in dicots (Hayashi, 1989). Xyloglucan is also found in the seeds of several plants as a storage polysaccharide which is depolymerised after germination (Edwards et al., 1985; Hensel et al., 1991).

1.4.1 Structure of xyloglucan

All forms of xyloglucan have a β-(1→4)-linked D-glucopyranose backbone (i.e. they are β-(1→4)glucans; Aspinall et al., 1969) of 200-3000 glucose residues (Hayashi et al., 1980; Nishitani and Masuda, 1982, 1983; Fry, 1989a, 1992), identical to the structure of cellulose, but with short oligomeric sidechains attached. It is a rigid rod-like molecule (O'Neill and Selvendran, 1983; Gidley et al., 1991) and is found throughout primary cell walls (Hayashi and Maclachlan, 1984a; Moore et al., 1986). The composition and distribution of the side-chains varies as described below.

1.4.1.1 Dicotyledonous xyloglucan

Xyloglucan from dicotyledonous plant cell walls has 60-75% of the glucose residues in the backbone substituted with α-(1→6)-linked D-
xylopyranose residues (Bauer et al., 1973; Kato and Matsuda, 1976; Hayashi et al., 1980; Joseleau and Chambat, 1984; Hayashi, 1989); these are often distributed in a repeating pattern of 3 consecutive xylosylated glucose residues followed by an unsubstituted glucose residue (Kooiman, 1961; Bauer et al., 1973; Kato and Matsuda, 1980ab; Hayashi and Maclachlan, 1984a). This is the repeating, cellotetraose-based, subunit of xyloglucan in most dicots. An exception to this general rule is xyloglucan from the Solanaceae such as Nicotiana which contain less xylose and resembles monocotyledonous xyloglucan (Mori et al., 1980).

Approximately 20-40% of the xylose residues are substituted with β-(1→6)-linked D-galactopyranose residues, which may be O-acetylated at O-6 and/or at O-3 or O-4 (York et al., 1988; Kiefer et al., 1989). Many of these galactose residues are substituted with α-(1→2)-linked L-fucopyranose residues (Bauer et al., 1973; Kato and Matsuda, 1980ab; Valent et al. 1980; O'Neill and Selvendran, 1983; Bacic et al., 1988). These are the 3 major sidechains which may be attached to the backbone of xyloglucan; in dicots, they are often distributed in a repeating pattern of an XXFG subunit followed by an XXXG subunit (e.g. pea stem xyloglucan, Hayashi and Maclachlan, 1984a).

Less common side-chains involve α-(1→2)-linked L-arabinofuranose residues attached to glucose residues with an unsubstituted xylose residue attached (Kiefer et al., 1990); or to α-(1→6)-linked D-xylopyranose residues (found in xyloglucan from the Solanaceae; Ring and Selvendran, 1981; Akiyama and Kato, 1982).

Other rare side-chains involve β-(1→2)-linked D-xylopyranose attached to a glucose residue which has an unsubstituted α-(1→6)-linked xylose residue attached; some of these β-(1→2)-linked xylose residues may have α-(1→3)-linked L-arabinofuranose residues attached (Hisamatsu et al., 1992).
1.4.1.2 Monocotyledonous xyloglucan

Xyloglucan present in the primary cell walls of Graminaceous monocots contains much less fucose (small amounts of a fucosylated oligosaccharide of xyloglucan have been identified in suspension-cultured cells of *Festuca*, a Graminaceous monocot; McDougall and Fry, 1994) and much less galactose than is found in xyloglucan from dicots. Graminaceous monocot xyloglucan is also less xylosylated than typical dicot xyloglucan so that there are more unsubstituted glucose residues (Labavitch and Ray, 1978; Shibuya and Misaki, 1978; Kato and Matsuda, 1980ab, 1985; Kato *et al.*, 1982ab).

Xyloglucan from non-Graminaceous monocots, including Liliaceae, Orchidaceae and Palmae, is very similar to typical dicotyledonous xyloglucan (Ohsumi and Hayashi, 1994ab). Some of the xylose residues may be feruloylated at O-4, e.g. in bamboo xyloglucan (Ishii *et al.*, 1990).

1.4.1.3 Seed storage xyloglucan

Plants such as tamarind (*Tamarindus*) and nasturtium (*Tropaeolum*) accumulate large amounts of xyloglucan (Kooiman, 1959; le Dizet, 1972) in the cotelydonly cells of their seeds (Reis *et al.*, 1987) where it functions as a storage polysaccharide which is degraded to provide energy and carbon skeletons for biosynthesis after germination. These xyloglucans contain glucose, xylose and galactose, often in a ratio of 3:2:1, but do not contain fucose (Kooiman, 1961). The variations in the ratio of component monosaccharides may reflect environmental influences on the biosynthesis of xyloglucan (Buckeridge *et al.*, 1992).

1.4.2 Structures of xyloglucan oligosaccharides

The xyloglucan backbone can be hydrolysed by fungal (e.g. *Trichoderma viride*) and some plant cellulases (EC. 3.2.1.4) which cleave after unsubstituted glucose residues (Wong *et al.*, 1977; Hayashi and Maclachlan, 1984a; Hayashi *et al.*, 1984). These cellulases can be used to release
oligosaccharides from xyloglucan preparations in vitro and have proved to be very useful in the determination of the structure of xyloglucans. Several xyloglucan oligosaccharides have been isolated and identified (Kooiman, 1961; 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; O'Neill and Selvendran, 1985b; Kiefer et al., 1990; Hisamatsu et al., 1991, 1992; Guillen et al., 1995; York et al., 1995). The similarity in structure of the major oligosaccharides (e.g. XXFG, XXXG and XXLG) indicates very precise biosynthesis of xyloglucan.

Other plant cellulases require more than one contiguous unsubstituted glucose residue in the backbone and will therefore cleave Graminaceous monocot xyloglucan and xyloglucan from Nicotiana to release mainly XXXG, XXG, XG, cellobiose, and glucose (Labavitch and Ray, 1978; Kato et al., 1982ab; Truelsen and Wyndaele, 1991). These cellulases will not degrade typical dicot xyloglucan or storage xyloglucans.
Figure 1.1 Structures of representative xyloglucan oligosaccharides. The nomenclature used throughout this thesis is that of Fry et al., 1993a. The structure of the repeating subunit of pea xyloglucan (XXFGXXXG; Hayashi, 1989) is also shown.

1.4.3 Biosynthesis of xyloglucan

All cell wall polysaccharides except cellulose and callose are synthesised within the Golgi body and transported to the cell wall via secretory vesicles. Biosynthesis of pectins occurs sequentially throughout the different compartments of the Golgi apparatus as the backbone is synthesised, methyl esterified and side-chains added to the rhamnose residues. In contrast, the biosynthesis of xyloglucan appears to commence in the trans cisternae of the Golgi apparatus (Moore et al., 1991; Driouich et al.,
Where the xyloglucan backbone is synthesised by a glucosyltransferase acting together with a xylosyltransferase, probably in a multi-enzyme complex (Hayashi and Matsuda, 1981abc; Gordon and Maclachlan, 1989). Galactosylation and fucosylation of xyloglucan occurs in the trans Golgi cisternae and the trans Golgi network (Zhang and Staehlin, 1992) onto pre-formed acceptor side-chains (Camirand and Maclachlan, 1986; Hanna et al., 1991; Maclachlan et al., 1992); fucosylation may be completed in the secretory vesicles (Brummel et al., 1990). These transferase activities appear to function independently of each, although fucosylation is stimulated by UDP-galactose in vitro (Gordon and Maclachlan, 1989). The fucosyltransferase has been proposed to contain a nonasaccharide (XLLG) binding site, and an octasaccharide (XXLG) site to be fucosylated (Maclachlan et al., 1992). Biosynthesis of xyloglucan may occur via a (possibly membrane bound) protein carrier covalently linked to the xyloglucan chain during synthesis (Campbell et al., 1988).

### 1.4.4 Biological activity of xyloglucan oligosaccharides

Many of the oligosaccharides released by cellulase hydrolysis of xyloglucan possess biological activity, i.e. they are oligosaccharins. Some of them may act as signalling molecules during expansion or fungal attack (McNeil et al., 1984; Fry, 1989a; Pavlova et al., 1996).

#### 1.4.4.1 Anti-auxin activity

The oligosaccharides XXFG and FG (see Figure 1.1 for structures) inhibit the auxin-stimulated elongation of pea stems at low optimal concentrations ($10^{-9}$ M and $10^{-8}$ M respectively); XXFG loses this activity at concentrations above $10^{-7}$ M and begins to stimulate expansion, whereas FG does not (York et al., 1984; McDougall and Fry, 1988, 1989ab; Augur et al., 1992; Hoson and Masuda, 1991a). Similar effects are observed on H⁺-stimulated elongation (Lorences et al., 1990), gibberrellin-induced elongation.
of pea stems (Warneck and Seitz, 1993) and in the fusicoccin-induced elongation of isolated pumpkin coleoptiles by synthetic FG (Pavlova et al., 1992). XXFG has been shown to be formed in vivo by suspension-cultured spinach cells (Fry, 1986b), from the degradation of xyloglucan (McDougall and Fry, 1991).

The structural requirement for this anti-auxin activity appears to be L-fucopyranosyl-α-(1-2)-D-galactose of the trisaccharide sidechain (McDougall and Fry, 1989ab). The action of an α-L-fucosidase could remove the anti-auxin activity of XXFG (and FG) and may prevent accumulation of XXFG in vivo (Farkas et al., 1991; Augur et al., 1993, 1995). The mechanism of this anti-auxin activity is unknown at present; given the low optimal concentrations and high structural specificity involved, it seems reasonable to predict that a protein receptor is involved, which may be bound to the plasma membrane (Fry et al., 1993b).

1.4.4.2 Auxin-mimicking activity

Some xyloglucan oligosaccharides can promote elongation of pea stems at a typical optimal concentration of $10^{-6}$ M (McDougall and Fry, 1990). The fucose residue is not required for this activity, but some or all of the XXXG structure is required, possibly with XXG as the minimum requirement. The order of effectiveness (XLLG > XXLG > XXXG > XXFG and FG not affective) as ‘auxin-mimics’ is the same as the order of effectiveness as acceptor substrates for xyloglucan endotransglycosylase (XET) which is discussed below (McDougall and Fry, 1990; Fry et al., 1992a; Lorences and Fry, 1993). It is likely that the growth-promoting effect of these oligosaccharides is due to them acting as acceptors for XET, causing loosening of the cell wall.
1.4.4.3 Effects on morphogenesis

The xyloglucan oligosaccharides XXFG and FG have been reported to exert various morphogenetic effects on cultured plant cells (Tran Thanh Van et al., 1985; Emmerling and Seitz, 1990; Pavlova et al., 1992).

1.4.5 Hydrogen-bonding of xyloglucan to cellulose

The structure of xyloglucan allows the β-(1→4)-glucan backbone to hydrogen-bond to the β-(1→4)-glucan chain of cellulose, the sidechains prevent xyloglucan from hydrogen-bonding to itself and influence the binding to cellulose; a xyloglucan with as few as five backbone glucose residues can bind to cellulose (Hayashi et al., 1994a; Vincken et al., 1995). It has been calculated that the fucose-containing sidechain has two interchangeable conformations, one of which promotes straightening of the neighbouring xyloglucan backbone and favours binding to cellulose, and another which is stabilised by a more relaxed backbone conformation associated with unbound sections of xyloglucan (Levy et al., 1991; Finckenstadt et al., 1995). Comparison of the binding efficiency of pea (fucosylated) and tamarind seed (non-fucosylated) xyloglucan oligomers with the same number of backbone glucose residues demonstrated that the fucosylated sidechain does contribute to cellulose binding (Hayashi et al., 1994b). However, the fucosylated sidechain is not necessary for cellulose-binding or cross-link formation (Whitney et al., 1995). A fucose-deficient mutant of Arabidopsis has been isolated which has more fragile cell walls than usual and stunted growth (Reiter et al., 1993).

1.5 Transglycosylation

Transglycosylation is the transfer of a glycosyl moiety from a donor substrate to an acceptor substrate. Transglycosylation reactions between polysaccharides are difficult to demonstrate in vivo because the end products are chemically identical to the initial reactants.
1.5.1 Endo-transglycosylation of xyloglucan

When \([\text{xylosyl-}^3\text{H}]XXFG\) and \([\text{fucosyl-}^3\text{H}]XXFG\) were supplied to suspension-cultured spinach cells, they became associated with polymeric material in the culture medium (Baydoun and Fry, 1989). Further investigation of this phenomenon led to the discovery of xyloglucan endotransglycosylase (abbreviated to XET; Smith and Fry, 1991, 1993; Nishitani and Tominaga, 1991, 1992; Farkas et al., 1992; Fry et al., 1992ab; Fanutti et al., 1993).

1.5.2 Mode of action of XET

Xyloglucan endotransglycosylase catalyses the cutting of a \(\beta(1\rightarrow 4)\) linkage between two glucose residues in the backbone of a xyloglucan polymer (the donor) and the transfer of the segment containing the newly-formed, potentially reducing glucose terminus onto the non-reducing terminal glucose residue of the backbone of another xyloglucan polymer or oligomer (the acceptor), joining the two with a \(\beta(1\rightarrow 4)\) linkage chemically identical to the one cut (Smith and Fry, 1991).

The minimum structural requirement for an acceptor substrate for XET appears to be XXG; GXG and XGG were not suitable acceptors for XET (Lorences and Fry, 1993). It is likely that XET activity is responsible for the reported stimulation of XGase/\(\beta\)-1,4-glucanase activities by xyloglucan oligosaccharides (Farkas and Maclachlan, 1988; McDougall and Fry, 1990; Maclachlan and Brady, 1992). Xyloglucan smaller than 10 kDa has been reported to be unsuitable as a donor substrate for XET purified from \textit{Vigna angularis} (Nishitani, 1992), this enzyme exhibited increased rates of transglycosylation with higher M, donor xyloglucan.
1.5.2.1 Enzymic modification of xyloglucan may affect the availability of XET substrates in the cell wall

Modifications such as removal of terminal xylose residues at the non-reducing end of xyloglucan oligomers or polymers could make them unsuitable as acceptor substrates for XET (e.g. converting XXFG- to GXFG- at the non-reducing end of a xyloglucan chain); α-xylosidases have been isolated (Koyama et al., 1981, 1983), some of which are reported to be xyloglucan oligosaccharide-specific (O’Neill et al., 1989; Fanutti et al., 1991; Chengappa et al., 1993) but which may release xylose residues from the non-reducing end of a xyloglucan chain. Suitability as an XET acceptor substrate could be restored by removal of the non-reducing terminal glucose residue (e.g. converting GXFG- to XFG- at the non-reducing end of a xyloglucan chain), by the action of a β-D-glucosidase. Also, sequential action of a α-L-fucosidase (Farkas et al., 1991; Augur et al., 1993, 1995) and a β-D-galactosidase (Edwards et al., 1988) could allow exo-hydrolysis to continue (e.g. converting FG- to LG- and LG- to XG- respectively). Combined action of these four enzyme activities could continuously remove and recreate acceptor substrates for XET (Lorences and Fry, 1993).

1.5.3 XET in plant cell expansion

Transglycosylation of xyloglucan may play an important role in the cutting and re-formation of xyloglucan cross-links between cellulose microfibrils during turgor-driven cell expansion. The cutting of cross-links may be responsible for ‘loosening’ of the cell wall, allowing microfibrils to move apart. The re-annealing step may lead to the ‘tightening’ of the cell wall to resist further separation of microfibrils (Fry et al., 1992b). XET may be responsible for the recycling of ‘taut’ cross-links to form new, ‘relaxed’ cross-links in the model of cell wall yielding described in section 1.3.2 (Passioura and Fry, 1992; Passioura, 1994), allowing the ‘creeping’ mechanism of

Levels of XET activity have been closely correlated to growth rate in many of the plant tissues studied (Fry et al., 1992a; Hetherington and Fry, 1993; Pritchard et al., 1993; Potter and Fry, 1993, 1994) although a causative relationship between increased XET activity and increased growth rate has not been established.

1.5.4 XET in the mobilisation of seed storage xyloglucan

High levels of XET activity have been found in the germinating seedlings of plants which use xyloglucan in their seeds as a storage polysaccharide. These XETs which mobilise seed xyloglucan also possess xyloglucan-specific endo-β-(1→4)-D-glucanase (XGase) activity, i.e. can use water as well as xyloglucan as an acceptor molecule (Edwards et al., 1986; Farkas et al., 1992; Fanutti et al., 1993). Xyloglucan endotransglycosylase acting in this way may therefore be sufficient to rapidly mobilise seed storage xyloglucan during germination without cellulase activity being required (Farkas et al., 1992).

1.5.5 Assaying XET activity

Several in vitro assays have been devised to detect XET activity as described below:

1.5.5.1 Paper-binding assay

This assay relies upon the ability of xyloglucan to hydrogen-bond to cellulose and the inability of xyloglucan oligosaccharides (of d.p. ≤10; Hayashi et al., 1994ab) to bind strongly to cellulose. Radiolabelled oligosaccharides are incubated with xyloglucan solution and the tissue extract to be assayed in buffer for a set time; the mixture is then loaded onto filter paper and allowed to dry before being washed in running tap water for
1 h or more. Radiolabel incorporated into xyloglucan remains bound to the paper and can be quantified by scintillation counting of the paper. Unreacted oligomer is removed by the washing step (Fry et al., 1992).

1.5.5.2 Viscosity assay

Mid-chain cleavage of xyloglucan polymers and transfer onto xyloglucan oligomers can lead to a decrease in the viscosity of xyloglucan solutions due to the depolymerisation of xyloglucan. Measurements of viscosity of a xyloglucan solution in the presence of xyloglucan oligosaccharides such as XXXG, at a constant temperature over a suitable timecourse, can therefore be used to detect (mid-chain attacking) XET activity in enzyme preparations (Lorences and Fry, 1993).

1.5.5.3 Colorimetric assay

This assay also relies upon the depolymerisation of xyloglucan catalysed by XET in the presence of suitable oligosaccharide acceptor substrates. In this assay, high molecular weight xyloglucan is able to form a blue-coloured complex by binding to iodine (used as an assay for xyloglucan; Kooiman, 1960). Low molecular weight xyloglucan (below 10 kDa) does not form this complex and depolymerisation can be assayed by measuring (spectrophotometrically) the loss of ability to form a blue complex.

This technique tends to be suitable only for enzyme preparations with very high XET activity such as seed storage xyloglucan mobilising XETs, which may also have hydrolase activity as mentioned earlier, owing to the high number of transglycosylase reactions required to decrease the molecular weight of xyloglucan to a level where iodine-complex formation is measurably affected (Sulova et al., 1995).

1.5.5.4 Fluorescence-tagged xyloglucan

In this method, the acceptor substrate is a xyloglucan oligosaccharide which has a fluorescent group covalently attached to the reducing terminus.
The xyloglucan, fluorescent oligosaccharide and enzyme preparation can be incubated in a buffer as before, then loaded onto paper and washed to remove unreacted oligosaccharide and illuminated with UV light to detect fluorescence-labelled xyloglucan formed by transfer of xyloglucan polymer onto fluorescence-labelled oligosaccharides catalysed by XET activity.

An alternative assay using fluorescence-labelled oligosaccharides is the 'dot-blot' assay in which the sample to be assayed for XET activity is applied to paper impregnated with xyloglucan and fluorescence-labelled oligosaccharide. The paper is then washed to remove unreacted oligosaccharide before observation under UV light to detect fluorescence-labelled xyloglucan formed via XET activity (Fry and Thompson, 1995).

The transglycosylation reaction can also be followed via changes in molecular weight (by GPC or HPLC) of the donor polymeric xyloglucan and of the acceptor oligosaccharide (detected using a fluorimeter; Nishitani, 1992).

1.5.6 Molecular biology of XET

Xyloglucan endotransglycosylases from nasturtium seed (Fanutti et al., 1993) and from Vigna angularis (Nishitani and Tominaga, 1992) have been purified and shown to be ~31-kDa glycosylated proteins. Their partial amino acid sequences have been used to identify cDNA copies of the corresponding gene transcripts for sequencing (nasturtium XET, de Silva et al., 1993; and Vigna EXT, Okazawa et al., 1993 respectively).

1.5.6.1 XET-related genes

By comparison with the nasturtium XET and Vigna EXT cDNA sequences and corresponding amino acid sequences deduced from them, several XET-related genes and proteins have been found.

In Arabidopsis thaliana, in addition to Arabidopsis EXT (Okazawa et al., 1993), there is an XET-related gene (TCH4), expression of which is regulated by auxin and brassinosteroids, and by environmental stimuli such as touch,
light levels, and temperature shock; recombinant TCH4 protein from *Escherichia coli* has also been shown to possess XET activity (Xu *et al.*, 1995). A gene expressed in *Arabidopsis thaliana* meristems (*meri-5*) also has homology to XET sequences and is expressed in rapidly expanding tissues (Medford *et al.*, 1991).

Other XET-related genes include:

- *wusl1005*, a flood-responsive gene from maize (Peschke and Sachs, 1994; Saab and Sachs, 1995);
- *BRUII*, a brassinosteroid up-regulated gene from soybean (Zurek and Clouse, 1994);
- *EXT* genes from tomato, soybean and wheat (Okazawa *et al.*, 1993);
- *NXGI* and *XETI*, nasturtium genes with mutually exclusive expression patterns (Rose *et al.*, 1996);
- an XET gene-family in tomato (de Silva *et al.*, 1994; Arrowsmith and de Silva, 1995).

### 1.6 Changes in xyloglucan Mr

#### 1.6.1 Decreases in xyloglucan Mr

Xyloglucan is likely to be the preferred substrate in the cell wall for cellulases (endo-β-(1→4)-D-glucanases) rather than cellulose owing to the structure of microfibrils; cellulose molecules in the microfibril will be much less accessible to cellulases than are xyloglucan molecules because of the semi-crystalline nature of the microfibrillar bundle (Gardner and Blackwell, 1974). Also, because of the intermolecular hydrogen bonding which reinforces the strength of microfibrils, a cut in the backbone of one cellulose molecule would not significantly decrease the strength of the microfibril; adjacent molecules would need to be cut in the same place, and many cuts would be required to cleave one microfibril completely.
Xyloglucan may be exposed to a wide variety of endo- and exohydrolases in the cell wall. However, because of the structure of xyloglucan, only a cellulase or a xyloglucan-specific endo-β-(1→4)-D-glucanase (XCase; Maclachlan and Brady, 1992, 1994) would be able to cause significant, fast degradation via hydrolysis of the xyloglucan backbone. Other enzymes (fucosidases, galactosidases and xylosidases; described in section 1.5.2.1) would be required to hydrolyse the remaining oligosaccharides, releasing the constituent monosaccharides. Xyloglucan in the cell wall may also be depolymerised by the action of xyloglucan endotransglycosylase which is discussed in section 4.1.2.

1.6.1.1 In response to auxin and low pH in vivo

It has been known for some time that plants respond to auxin and low pH with an increase in the turnover over xyloglucan in the cell wall, with a small proportion undergoing extensive degradation to low Mr products solubilised from the cell wall (Labavitch and Ray, 1974ab; Jacobs and Ray, 1975; Gilkes and Hall, 1977; Terry et al., 1981, 1982). The bulk of xyloglucan in the cell wall undergoes a rapid decrease in Mr during acid- and auxin-induced expansion (Sakurai et al., 1979; Nishitani and Masuda, 1981, 1982, 1983; Inouhe et al., 1984; Lorences et al., 1987, 1989; Lorences and Zarrar, 1987; Revilla and Zarrar, 1987; Arribas et al., 1991; Wakabayashi et al., 1991; Talbott and Ray, 1992).

Fucose-binding lectins and xyloglucan-binding antibodies inhibit auxin- and acid-induced expansion and the depolymerisation of xyloglucan (Hoson and Masuda, 1991b; Hoson et al., 1991).

These observations implicate xyloglucan in the mechanism of cell wall yielding in response to auxin and acid growth-promoting treatments; the increased growth rate following these treatments may be due to cellulase-(or xyloglucanase-) catalysed hydrolysis of xyloglucan in the cell wall, breaking crosslinks between microfibrils and weakening the cell wall.
Cellulases are found in the plant cell apoplast (Byrne et al., 1975; Wong et al., 1977; Wong and MacLachlan, 1979ab; Hatfield and Nevins, 1986, 1987), are synthesised de novo in response to auxin treatment (Verma et al., 1975; Hayashi and Ohsumi, 1994) and have enhanced activity at low pH (optimum pH 5.5 to 6.0; Hayashi et al., 1984).

1.6.2 Increases in xyloglucan Mr

Step increases in turgor promote a rapid increase in Mr of xyloglucan in the cell wall (Talbott and Ray, 1992); an increase in xyloglucan Mr also occurs on transition from a growth-promoting (acidic) pH to neutral pH (Nishitani and Masuda, 1982) and was also reported to be catalysed in vitro by an enzyme preparation from Vigna angularis (Nishitani and Tominaga, 1991).

Increases in molecular weight cannot be catalysed by cellulases but may result from the action of XET (see section 4.2.1). These observations provide strong circumstantial evidence of inter-polymeric transglycosylation between xyloglucan polymers in vivo, suggesting a role for XET in xyloglucan metabolism in the cell wall.
1.7 Aims and objectives

The aim of this work was to investigate the metabolism of xyloglucan and to determine the catalytic action of xyloglucan endotransglycosylase in the cell wall, using suspension-cultured rose cells.

The objectives are the work presented in this thesis were:

a). to examine the changes in molecular weight of a pool of wall-bound xyloglucan as cells age.

b). to relate the molecular weight of wall bound xyloglucan with the growth-rate of cells.

c). to study the mechanism of incorporation of newly-secreted xyloglucan into the cell wall matrix.

d). to demonstrate transglycosylation between xyloglucan polymers in the cell wall.

e). to investigate the role of XET in the cell wall in relation to xyloglucan metabolism.
2. Materials and methods

2.1 General methods

2.1.1 Suspension-cultured rose cells

Rose cell-suspension cultures (Rosa sp., 'Paul's Scarlet') were subcultured fortnightly by pipetting 5 ml of 14 day old culture into 50 ml of fresh sterile medium (Table 2.1) under sterile conditions (Fry and Street, 1980). The cultures were maintained at 25°C with constant rotation in a horizontal plane (100 rpm, 3 cm diameter orbit) and constant dim illumination (photon flux density of 2.5 μmol. m⁻². s⁻¹).

The medium was sterilised by autoclaving (121°C, 15 psi) 50-ml aliquots in 250 ml conical flasks stoppered with cotton wool and muslin bungs, capped with aluminium foil, for 20 min.

The growth of cells over a two week cycle was followed by leaving 5 ml of suspension culture to settle for 30 min in a measuring cylinder and measuring the volume of cells, expressed as a percentage of the total suspension volume.

2.1.2 Radiolabels

Radiolabelled sugars (D-[U-¹⁴C]glucose (300 MBq/μmol) were obtained commercially from Amersham International PLC, L-[1-³¹P]arabinose (97 MBq/μmol) and L-[1-³¹P]fucose (148 MBq/μmol) were custom-synthesised by Amersham by tritiation of L-arabinose and L-fucose supplied by S.C. Fry.

[¹⁴C]Acetic anhydride (20% w/w in toluene, 703 MBq/mmol) was also obtained from Amersham International PLC.
Table 2.1. Composition of rose culture medium.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in medium (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂·2H₂O</td>
<td>74</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>140.4</td>
</tr>
<tr>
<td>KCl</td>
<td>750</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>850</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>250</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>1</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.2</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>KI</td>
<td>0.1</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.02</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.01</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.02</td>
</tr>
<tr>
<td>FeCl₃·6H₂O</td>
<td>5.4</td>
</tr>
<tr>
<td>Na₂EDTA·2H₂O</td>
<td>7.4</td>
</tr>
<tr>
<td>2,4-D</td>
<td>1.0</td>
</tr>
<tr>
<td>Kinetin</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose (or glycerol), 20 g/l</td>
<td></td>
</tr>
</tbody>
</table>

Adjusted to pH 6.0 by dropwise addition of 1.0 M NaOH

2.1.2.1 Radiolabelling rose suspension-cultures

L-[1-³H]Arabinose was dried under vacuum to remove ethanol from the stock solution and the dried radiolabel was re-dissolved in 2 ml of rose medium and sterilised by autoclaving. This sterile solution of radiolabelled arabinose was then transferred aseptically to rose suspension-cultures to radiolabel polymers containing pentose sugar residues synthesised by suspension-cultured rose cells.
The uptake and incorporation of the radiolabel was followed by assaying \(^3\text{H}\) in aliquots of the three culture fractions (culture-filtrate, cell-contents, and cell-wall extracts) taken at each time point. \(^3\text{H}\) from L-[1-\(^3\text{H}\)]arabinose is incorporated into the xylose residue of isoprimeverose; \([\text{xylosyl-}^{3}\text{H}]\text{xyloglucan}\) was determined by assaying aliquots of each culture fraction at each time point for [\(^3\text{H}\)]isoprimeverose residues by 'Driselase' digestion, paper chromatography and scintillation counting the isoprimeverose spots.

2.1.2.2 Radiolabelling protoplast products

L-[1-\(^3\text{H}\)]Arabinose (1 MBq) was dried under vacuum to remove ethanol and re-dissolved in 2 ml of protoplast sedimenting buffer (see section 2.3.1). Isolated protoplasts were added to this solution and left for 3 h at 25°C, shaking gently, to radiolabel newly synthesised polymers containing pentose sugar residues synthesised by isolated protoplasts.

2.1.2.3 Radiolabelling xyloglucans for use as internal markers in density gradients

The most important feature of radiolabelled xyloglucan that is to be used as an internal marker in density gradients is that the radiolabel is not in the isoprimeverose residues. After 'Driselase' digestion, the internal marker can therefore be easily distinguished from [\(\text{xylosyl-}^{3}\text{H}\)]isoprimeverose formed by 'Driselase' digestion of [\(\text{xylosyl-}^{3}\text{H}\)]xyloglucan from the L-[1-\(^3\text{H}\)]arabinose radiolabelling step in the density-labelling protocols.

Preliminary experiments with [\(\text{fucosyl-}^{3}\text{H}\)]xyloglucan.

The uptake and incorporation of L-[1-\(^3\text{H}\)]fucose into alcohol-insoluble residues by various suspension-cultures (spinach, tomato, sycamore, glycerol-grown rose, and glucose-grown rose) was investigated to determine the most efficient cell source for the preparation of [\(\text{fucosyl-}^{3}\text{H}\)]xyloglucan. This was performed by adding 37 kBq of [\(^3\text{H}\)]fucose dissolved in 0.2 ml dH\(_2\)O to 5 ml of each culture at the rapid-growth stage.
A sample (50 μl) of each culture filtrate was removed at time points between 2 min and 6 h after addition of [³H]fucose, diluted with 450 μl of dH₂O and scintillation-counted to estimate uptake of the radiolabel. After 6 h of radiolabelling, the cells were killed by addition of 18 ml of absolute ethanol (giving a final concentration of 80% ethanol). The cells were then washed with 80% ethanol (6×5 ml) on glass-fibre filters using a Millipore multiple-sample vacuum filtration unit. The remaining alcohol-insoluble residues were dried by placing the filters and residues in scintillation vials and heating at 60°C for 30 min; the samples were left to cool for 30 min before addition of non-Triton scintillant (5 ml) and scintillation-counting to estimate incorporation of the radiolabel into cell-wall polymeric material.

[fucosyl-³H]xyloglucan from spinach.

Radiolabelled xyloglucan was obtained by feeding [³H]fucose to suspension-cultured spinach cells. The [³H]fucose (7.5 MBq) was dried under vacuum to remove ethanol from the stock solution, re-dissolved in 2 ml dH₂O and sterilised by autoclaving. This was then added to 25 ml of spinach suspension culture at 4 days after subculturing. The culture was left in normal growth conditions for 4 days to radiolabel xyloglucan (and other fucose-containing polymers) in the cell wall. The hemicellulose fraction was extracted from the cell-wall using the same method as for rose cells (section 2.1.3) and dried in 1-ml aliquots for use as an internal marker ([fucosyl-³H]sXG).

¹⁴C-Acetylated xyloglucan from tamarind-seed.

Tamarind-seed xyloglucan (tXG) was radiolabelled by acetylation with [¹⁴C]acetic anhydride to produce a tight-banding internal marker for use in density gradients. This was performed as follows:

- 12 ml of 1% (w/v) tXG in dH₂O was brought to pH 7.0 by slow addition of pyridine.
- 1.2 ml of acetic anhydride/collidine (1:2, v/v) was added slowly to avoid excessive heating, and left stirring vigorously for 2 h.
• 3.38 g NaOH (s) was added and left stirring for 30 min then neutralised with 5 ml glacial acetic acid, and dialysed at 4°C against dH₂O; the pre-acetylated tXG (ptXG) was then dried under vacuum.

(These steps acetylate any amine groups present (N-acetylation) and some sugar residues (O-acetylation; the O-acetylation is then reversed by the weak saponification step).

• 20 µl of [1-¹⁴C]acetic anhydride (in toluene, 1.85 MBq, 703 MBq/mmol) was added to 5 mg of ptXG in a capped 1.5-ml Eppendorf tube and left for 24 h to react.

• 1 ml of ice-cold pyridine/acetic acid/water (1:1:23) was added to the Eppendorf tube and the contents were immediately poured into 5 ml of cold Py/A/W (1:1:23) to stop the reaction. The Eppendorf tube was washed out with a further 4×1 ml of Py/A/W (1:1:23) and the washings were pooled and dried in 200-µl aliquots under vacuum for use as an internal marker (¹⁴C-acetylated tXG).

2.1.2.4 Use of radiolabels to develop density-labelling strategy

In order to determine the most efficient carbon source for the density-labelling experiments, the uptake and incorporation of [³H]galactose and [¹⁴C]glucose into alcohol-insoluble residues of both glycerol-grown and glucose-grown rose cells were compared. The radiolabels, [³H]galactose (2×925 kBq) or [¹⁴C]glucose (2×185 kBq), were dried under vacuum to remove ethanol and re-dissolved in 2 ml of dH₂O before being added to 50 ml of glycerol-grown or glucose-grown rose cell suspension-culture (4 days after subculturing).

Aliquots (2-ml) of each culture were removed at time points between 10 min and 6 h after addition of radiolabel and filtered through 'Polyprep' columns under suction, collecting the culture filtrates. The cells were suspended and washed in 80% ethanol (6×5 ml) on glass-fibre filters in a Millipore multiple-sample vacuum filtration unit. The alcohol-insoluble
residues were dried by transferring the filters and residues to scintillation vials and heating at 60°C for 30 min; the samples were left to cool for 30 min before addition of non-Triton scintillant (5 ml) and scintillation-counting to estimate incorporation of the radiolabel into cell-wall polymeric material.

The incorporation of glucose into 20% TCA-insoluble residues of glucose-grown rose cells was assayed by spiking 40 ml of 12C-glucose rose medium (0.5% w/v glucose), containing 5 g fresh weight of glucose-grown rose cells at 4 d after subculturing, with [14C]glucose (260 kBq) and assaying 2 ml of the culture, at time points between 2 min and 6 h after addition of the radiolabel, for 14C incorporated into 20% TCA-washed cell residues.

2.1.3 Fractionation of suspension-cultures

Suspension-cultures were fractionated as described below before further analysis.

2.1.3.1 Extraction of culture-filtrates

Suspension cultures were filtered through ‘Polyprep’ columns and washed with 4x5 ml of carbon-source free rose medium, pooling the filtrate and washings (culture-filtrate). Cells and culture filtrates were frozen at -70°C and stored at -20°C.

2.1.3.2 Extraction of cell-contents

Frozen cells were resuspended in sonication buffer (20 mM HEPES (Na+ salt), 40 mM NaF, 2% v/v Triton X-100, pH 7.0) at 50 mg fresh weight per ml of buffer and sonicated for 2 min at 18 μm amplitude in an MSE Soniprep 150 to rupture the cells and disrupt membranes, releasing the cell contents. The cell fragments were then immediately filtered and washed with 4x5 ml sonication buffer through ‘Polyprep’ columns, pooling the filtrates and washings (cell-contents). Cell-contents were frozen at -70°C and stored at -20°C.
2.1.3.3 Extraction of hemicellulose from cell-wall preparations

The cell-wall-rich residue from the sonication step was resuspended in 5 ml of hemicellulose extractant (6 M NaOH, 1% w/v NaBH₄) and left shaking for 24 h at 37°C. The suspension was then filtered and washed with 4×5 ml hemicellulose extractant in Polyprep' columns, pooling the filtrate and washings (cell-wall extract).

Cell-wall extracts were neutralised by slow addition of glacial acetic acid (avoiding excessive bubbling) before being desalted by extensive dialysis against distilled water at 4°C. Cell-wall extracts were frozen and stored at -20°C.

2.1.3.4 Stability of xyloglucan in cell-wall extractant

To assess the danger of the extraction method causing a breakdown of the xyloglucan backbone, the viscosities of 1% (w/v) solutions of tamarind-seed xyloglucan dissolved in 6 M NaOH + 1% NaBH₄ and incubated at temperatures of 4°C, 25°C, 37°C and 50°C were measured over a 6-day period. Viscosity was assayed by measuring the flow time in a fine-bore Ubbelohde viscometer after equilibrating the temperature of each solution to 25°C in a water bath.

2.1.4 Gel-permeation chromatography

Samples for gel-permeation chromatography were dried under vacuum; dried cell-contents extracts were washed by re-suspension and centrifugation in 2×1 ml of toluene to remove Triton X-100 (present in the sonication buffer and not removed by the drying step). The dried samples were re-dissolved in 1 ml of Py.A.W. (1:1:23, pH 4.7) and mixed with 1 ml internal marker mixture (0.04% w/v 5-MDa dextran, 0.04% w/v sucrose in Py.A.W. (1:1:23, pH 4.7)) before gently loading onto the surface of the gel (Sepharose CL-6B, equilibrated with Py.A.W. (1:1:23, pH 4.7), 80 ml bed volume in a 1.5 cm diameter column) and eluted with Py.A.W. (1:1:23, pH 4.7) with a flow rate of 7 ml/h. The sides of the column above the gel surface
were washed with 2 ml of elution buffer which was allowed to soak into the gel before attachment of the column to the buffer reservoir.

Fractions were collected (1.5 ml per fraction) using a BioRad model 2110 fraction collector and assayed using anthrone reagent to identify the void ($V_0$) and included ($V_i$) volumes of the gel from the elution peaks of dextran and sucrose respectively.

A small portion (200 μl) of each fraction between $V_0$ and $V_i$ was assayed for radioactivity to give an elution profile of the total radioactive material in the GPC runs. The remaining volume of each of these fractions was dried under vacuum for 'Driselase' digestion.

2.1.4.1 Anthrone reagent

To detect carbohydrates in GPC fractions, 0.2 ml of fraction was mixed with 0.4 ml anthrone reagent (0.2% w/v anthrone in concentrated H$_2$SO$_4$) and left to cool for 15 min before measuring $A_{600}$ (Dische, 1962).

2.1.4.2 Calibration of the GPC column

Calibration of the GPC column was performed by measuring the elution profiles (via anthrone reagent) of dextran sample solutions with known average molecular weights (249000, 74300 and 40200). The mean $k_{av}$ value for each elution profile was plotted against the corresponding log $M_r$ for each dextran to obtain a calibration curve for the Sepharose CL-6B gel permeation column showing the log-linear relationship between molecular weight and $k_{av}$ values.

2.1.5 'Driselase'

'Driselase' (from Sigma Chemical Co.) is a crude enzyme mixture extracted from the fungus Irpex; it contains laminarinase, xylanase, cellulase and other hydrolytic enzymes.

2.1.5.1 Purification of 'Driselase'

Crude 'Driselase' was partially purified by the following method:
5 g 'Driselase' was stirred for 15 min in ice-cold 50 mM acetic acid (pH adjusted to 5.0 with 1.0 M NaOH) to dissolve proteins.

- The suspension was centrifuged (10 min, 2,500 g, 4°C) to pellet undissolved contaminants and the supernatant was collected.
- Solid (NH₄)₂SO₄ was added slowly (26 g per 50 ml supernatant) with constant stirring on ice. The solution was left to stand at 0°C for 15 min to precipitate proteins.
- The suspension was centrifuged (10 min, 2,500 g, 4°C) to pellet precipitated proteins and the supernatant was rejected.
- The pellet was resuspended in fresh (NH₄)₂SO₄ solution (52% w/v) and re-centrifuged (10 min, 2,500 g, 4°C), rejecting the supernatant.
- The pellet was re-dissolved in 5 ml dH₂O and desalted on a Sephadex-G25 GPC column (800 ml bed volume, equilibrated and eluted with dH₂O, 4°C).
- The void volume, containing proteins (fast-eluting, brown material), was collected and frozen at -20°C before freeze-drying.

2.1.5.2 'Driselase' digestion of samples

Partially purified 'Driselase' was used at a concentration of 0.5% (w/v) in a buffer of pyridine/acetic acid/water (1:1:98, v/v/v, pH 4.7) containing the internal markers isoprimeverose, xylobiose, xylose, arabinose, fucose, glucose and galactose (0.5% w/v of each). This enzyme/marker mixture was added in 40-μl aliquots to dry samples (dried under vacuum in 1.5 ml Eppendorfs), agitated to dissolve the sample, then left at 37°C for 48 h. Digests were stopped by drying the samples onto Whatman 3MM chromatography paper.

2.1.6 Paper chromatography of 'Driselase' digests

Samples were applied in 40-μl aliquots onto sheets of Whatman 3MM chromatography paper (46x57 cm) and separated by descending
chromatography. A solvent system of ethyl acetate/pyridine/water (9:3:2, v/v/v, 20 h) was used to separate isoprimeverose from other cell wall digestion products. A solvent system of butanol/acetic acid/water (12:3:5, v/v/v, 16 h) was used to separate fucose from xylose. Chromatograms were left in a fume hood overnight to dry before staining.

2.1.7 Staining chromatograms

Dilute aniline hydrogen-phthalate (Table 2.2) was used to stain the paper chromatography markers; this enabled easy location of the internal markers without adversely affecting scintillation-counting efficiency due to chemical quenching. A range of concentrations was tested to compare visibility of the internal markers with scintillation-counting efficiency (data not shown).

Table 2.2. Composition of aniline hydrogen-phthalate stain.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>phthalic acid</td>
<td>1.6 g</td>
</tr>
<tr>
<td>diethyl ether</td>
<td>490 ml</td>
</tr>
<tr>
<td>acetone</td>
<td>490 ml</td>
</tr>
<tr>
<td>distilled water</td>
<td>20 ml</td>
</tr>
<tr>
<td>aniline</td>
<td>0.5 ml (added immediately before staining)</td>
</tr>
</tbody>
</table>

Dried chromatograms were dipped through dilute aniline hydrogen-phthalate stain in a trough, left to dry for at least 15 min, then heated at 105°C for 5 min to reveal the spots.

2.1.8 Radioactivity assay

Aqueous samples were assayed for radioactivity by mixing with 10 volumes of Triton scintillation cocktail (Table 2.3). Aqueous samples with a high salt concentration, such as CsTFA gradient fractions, were diluted prior
to addition of scintillant by adding 9 volumes of distilled water to 1 volume of sample. Samples on paper were soaked with 2 ml non-Triton scintillation cocktail (Table 2.4). Both sample types were assayed in plastic scintillation vials (22 ml capacity) in a Beckman LS5000CE scintillation counter.

Table 2.3. Composition of Triton scintillation cocktail.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPO</td>
<td>3.3 g</td>
</tr>
<tr>
<td>POPOP</td>
<td>0.33 g</td>
</tr>
<tr>
<td>toluene</td>
<td>667 ml</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>333 ml</td>
</tr>
</tbody>
</table>

Table 2.4. Composition of non-Triton scintillation cocktail.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPO</td>
<td>5.0 g</td>
</tr>
<tr>
<td>POPOP</td>
<td>0.5 g</td>
</tr>
<tr>
<td>toluene</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

2.1.9 Estimation of xyloglucan molecular weight

Each fraction of the xyloglucan GPC elution profile (determined by radioactive assay of the isoprimeverose spots from stained paper chromatographs) was assigned a $k_v$ value measured relative to the positions of the void and included volume markers and calculated as follows:

**Equation 2.1**

$$k_{av} = \frac{n - N_o}{N_i - N_o}$$

where:

$k_{av} = k_v$ value for fraction $n$;
\( N_v = \text{fraction number of the void volume, } V_o; \)
\( N_i = \text{fraction number of the included volume, } V_i. \)

Each fraction between \( k_{av} 0.05 \) and 0.8 was then assigned a molecular weight estimate based on the calibration curve for the GPC column derived from the elution profiles of dextran with differing average molecular weights. Very little \([\text{H}]\text{xyloglucan} \) (less than 3\%) eluted at \( k_{av} <0.05 \) or >0.8. The mean molecular weight of \([\text{H}]\text{xyloglucan} \) was calculated as follows:

**Equation 2.2**

\[
\overline{M_w} = \frac{\sum M_i R_i}{\sum R_i}
\]

where:

\( \overline{M_w} = \text{weight-averaged relative molecular mass of } [\text{H}]\text{xyloglucan}; \)
\( M_i = \text{assigned relative molecular mass for the } i\text{th fraction}; \)
\( R_i = \text{radioactivity (cpm) of } [\text{H}]\text{isoprimeverose in the } i\text{th fraction}. \)

### 2.1.10 Density gradients

The buoyant density of xyloglucan in hemicellulose extracts of cell-walls was estimated by centrifugation on caesium trifluoroacetate density gradients using the procedures described below:

#### 2.1.10.1 Preparation of density gradients

Radiolabelled hemicellulose extracts were dried under vacuum (\(~500 \text{ kcpm of extract}\)) and re-dissolved in 2 ml of pyridine/acetic acid/water buffer (1:1:23, pH 4.7). Dried internal-marker xyloglucan (section 2.1.2.3) was also re-dissolved in 1 ml of the same buffer (20 kcpm of \( ^{14}\text{C}-\text{acetylated xyloglucan} \) from tamarind-seed or 300 kcpm of \( [\text{fucosyl-}^{3}\text{H}]\text{xyloglucan} \) from spinach). Caesium trifluoroacetate solutions containing the sample and internal-marker xyloglucan were made up as shown below (Table 2.5).
Table 2.5. Composition of density gradients.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsTFA</td>
<td>8 ml</td>
</tr>
<tr>
<td>Py/A/W (1:1:23) containing sample and internal marker XG.</td>
<td>4.672 ml</td>
</tr>
<tr>
<td>pyridine</td>
<td>347 μl</td>
</tr>
<tr>
<td>acetic acid</td>
<td>347 μl</td>
</tr>
<tr>
<td>blue dextran (M, 2x10^6) (0.5% w/v in Py/A/W (1:1:23))</td>
<td>100 μl</td>
</tr>
</tbody>
</table>

This mixture has an initial density of 1.60 g/ml

This mixture was then transferred to 'Quickseal' polyallomer centrifuge tubes (from Beckman, 16x76 mm, 12.5 ml volume); pairs of tubes were balanced to within 1 mg and heat-sealed. The tubes were centrifuged in a Beckman 70.1 Ti fixed-angle titanium rotor at 45000 rpm (~100,000 g) for 100 h at 18°C in a Sorvall OTD ultracentrifuge. This formed a stable gradient from ~1.8 g/ml at the bottom of the tube to ~1.4 g/ml at the top of the tube. The centrifuge was stopped in reograd mode (no braking below 500 rpm) to give minimum disturbance of the gradients.

2.1.10.2 Fractionation of density gradients

Fractionation of density gradients was performed by downward displacement of the gradient by white mineral oil using a Perpex peristaltic pump (pumping at 13 ml/h). Fractions of the gradient were collected using a Biorad model 2110 fraction collector, collecting at 30 drops per fraction or at 1.1 min per fraction. Timewise collection gave the best fractionation of gradients; this was because the volume of drops changed along the gradient as surface-tension varied.
2.1.10.3 Estimation of density of fractions

The density of each fraction was estimated by weighing a volume of each fraction. Each fraction (including container) was placed on a balance (Mettler AE240) and the reading zeroed, 200 µl was then removed accurately by autopipette and the weight loss recorded to the nearest 0.1 mg.

2.1.10.4 Scintillation counting of CsTFA fractions

Each fraction was scintillation counted to give a profile of total radioactive material in each fraction. This step also revealed the position of the $^{14}$C-acetylated tamarind-seed xyloglucan internal marker by assaying $^{14}$C as well as $^3$H.

2.1.10.5 Desalting of CsTFA fractions

Fractions were desalted prior to 'Driselase' digestion on Bio-Gel P-2 columns (2 ml bed volume in 'Polyprep' columns) equilibrated and eluted with Py/A/W buffer (1:1:23, pH 4.7). Each fraction (100 µl) was applied to the surface of the gel and allowed to run into the gel before the sides of the columns were washed with 200 µl of elution buffer. This was allowed to run to the gel surface before addition of a further 300 µl of elution buffer which was allowed to run to the gel surface. The desalting columns were then placed above Eppendorf tubes before addition of a further 500 µl of elution buffer, eluting and collecting material in the void volume (polymeric material). The columns were then washed with 3×1 ml of elution buffer, eluting and rejecting the included volume material (CsTFA), before re-using the columns. This method was observed to give good separation of blue dextran from cobalt chloride (pink) when loaded together in solution.

2.1.10.6 Analysis of xyloglucan in density gradient fractions

The void volume of each fraction from the desalting step was dried under vacuum and digested with 'Driselase' to release [xylosyl-$^3$H]isoprimeverose from [xylosyl-$^3$H]xyloglucan (the test sample) and [$^3$H]fucose from [fucosyl-$^3$H] xyloglucan (internal marker, if included). These
digests were then paper chromatographed, stained and the isoprimeverose spots (and fucose spots) scintillation counted (sections 2.1.5 to 2.1.8 for details) to reveal the profiles of radiolabelled sample xyloglucan (and \([fucosyl-3^3]sXG\)).

2.1.10.7 Estimation of density of xyloglucan

The profiles of radiolabelled sample xyloglucan and internal marker xyloglucan were plotted using a graph-drawing package (SigmaPlot) on a computer. A built-in curve-fitting procedure was used to fit a Gaussian curve to the distribution of each xyloglucan (sample and internal marker); the results of this procedure were used to calculate the mean fraction number for each xyloglucan. This information was then used in conjunction with the density measurements to calculate a mean density for the sample xyloglucan (see section 3.1.5).

2.2 Xyloglucan molecular weight changes

Suspension-cultured rose cells at day 4 and day 9 after subculturing were radiolabelled by addition of L-[1-3H]arabinose (12 MBq, section 2.1.2.1) to 55 ml of suspension culture. The culture was mixed well and divided into 10 sterile containers (4 ml in each, 50 ml capacity) and left under normal growth conditions.

One container was removed at time intervals ranging from 1 h to 7 days after addition of the radiolabel and each culture was fractionated into culture filtrate, cell contents, and cell wall extracts as described in section 2.1.3. The molecular weight of the xyloglucan in each extract was analysed as described in sections 2.1.4 to 2.1.9.

2.2.1 Relationship between molecular weight and density of xyloglucan

Fractions containing high-Mr xyloglucan from gel permeation chromatography of a radiolabelled cell-wall hemicellulose extract were
pooled and the buoyant density of the xyloglucan was analysed as described in section 2.1.10. The same was performed for fractions containing low-M\textsubscript{x} xyloglucan and the results were compared.

2.3 Protoplasts

Protoplasts were radiolabelled by the addition of L-[\textsuperscript{1-\textsuperscript{3}}H]arabinose (1 MBq, section 2.1.2.2) in order to assess the molecular weight of newly-secreted xyloglucan; in whole cells, new xyloglucan is rapidly incorporated into the cell wall after secretion, making it impossible to estimate the molecular weight of xyloglucan as it emerges from the protoplasm. An experiment was designed to overcome this problem by removing the cell wall so that the newly-secreted, radiolabelled xyloglucan in the protoplast medium would reflect the molecular weight of newly-secreted xyloglucan as it is secreted into the cell wall of whole cells.

2.3.1 Preparation of protoplasts

Protoplasts were isolated from suspension-cultured rose cells taken at 4 days after subculturing. 100 mg (fresh weight) of cells was transferred into a 25-ml capacity conical flask; the cell walls were removed by gently shaking the cells (60 rpm, 3 cm diameter orbit) in 1 ml of protoplast isolation buffer (Table 2.6) containing 2\% (w/v) purified 'Driselase' at 33°C for 2 h. Ice cold protoplast sedimenting buffer (12 ml, Table 2.7) was added before pelleting the protoplasts by centrifugation (50 g, 10 min). The supernatant was discarded and the pellet re-suspended in 5 ml cold protoplast floating buffer (Table 2.8) mixed with 5 ml cold protoplast sedimenting buffer; this suspension was centrifuged (50 g, 10 min) and the top 2 ml of supernatant was transferred to a clean centrifuge tube. Cold protoplast sedimenting buffer (10 ml) was added and mixed gently before centrifugation (50 g, 5 min). The pellet was resuspended and re-centrifuged in 10 ml cold protoplast sedimenting buffer twice before resuspending the purified protoplasts in 2 ml sedimenting buffer containing L-[\textsuperscript{1-\textsuperscript{3}}H]arabinose (1 MBq,
section 2.1.2.2). This was left for 3 h at 25°C, shaking gently, to radiolabel newly-synthesised polysaccharides.

2.3.2 Recovery and analysis of protoplast products

Radiolabelled products secreted into the protoplast medium were recovered by filtering the protoplast suspension through 'Polyprep' columns and washing with 4x1 ml of protoplast sedimenting buffer, pooling the filtrate and washings. The protoplasts were then lysed by suspending in 3 ml cold distilled water, leaving for 5 min, then filtering and washing with 3x1 ml cold distilled water to recover soluble intraprotoplasmic products.

The molecular weight of newly-synthesised xyloglucan in each fraction was assessed by gel-permeation chromatography, 'Driselase' digestion, paper chromatography and scintillation-counting of isoprimeverose as described in sections 2.1.4 to 2.1.9.

Table 2.6. Composition of protoplast isolation buffer.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per 100 ml (concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂.2H₂O</td>
<td>103 mg (7.0 mM)</td>
</tr>
<tr>
<td>NaH₂PO₄.2H₂O</td>
<td>11 mg (0.7 mM)</td>
</tr>
<tr>
<td>MES</td>
<td>58.5 mg (3.0 mM)</td>
</tr>
<tr>
<td>sorbitol</td>
<td>6.37 g (0.35 M)</td>
</tr>
<tr>
<td>mannitol</td>
<td>6.37 g (0.35 M)</td>
</tr>
<tr>
<td>pH adjusted to 5.3 with 10 mM KOH added dropwise.</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.7. Composition of protoplast sedimenting buffer.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per 100 ml (concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂·2H₂O</td>
<td>103 mg (7.0 mM)</td>
</tr>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>11 mg (0.7 mM)</td>
</tr>
<tr>
<td>MES</td>
<td>58.5 mg (3.0 mM)</td>
</tr>
<tr>
<td>sorbitol</td>
<td>9.0 g (0.49 M)</td>
</tr>
<tr>
<td>pH adjusted to 6.0 with 0.5 M KOH added dropwise.</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.8. Composition of protoplast floating buffer.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per 100 ml (concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂·2H₂O</td>
<td>103 mg (7.0 mM)</td>
</tr>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>11 mg (0.7 mM)</td>
</tr>
<tr>
<td>MES</td>
<td>58.5 mg (3.0 mM)</td>
</tr>
<tr>
<td>sucrose</td>
<td>18 g (0.53 M)</td>
</tr>
<tr>
<td>pH adjusted to 6.0 with 0.5 M KOH added dropwise.</td>
<td></td>
</tr>
</tbody>
</table>

2.4 Brefeldin A

Brefeldin A is a fungal antibiotic which disrupts vesicle transport between Golgi stacks and the plasma membrane and inhibits secretion of polysaccharides into the cell wall. It was used in this experiment to cause an accumulation of radioactively labelled xyloglucan within suspension-cultured rose cells to enable the molecular weight of newly synthesised, intraprotoplasmic xyloglucan to be estimated before secretion into the apoplast and incorporation into the cell wall.

- Rose suspension cultures at 7 d after subculturing (2 flasks, 55 ml each) were used; Brefeldin A was added to one flask (770 mg
dissolved in 2 ml rose medium, sterilised by autoclaving) to a final concentration of 48 µM and the other flask was left as a control culture. The two cultures were left shaking gently for 15 min.

- L-[1-3H]arabinose was added to each culture (4 MBq, dried under vacuum to remove ethanol, dissolved in 2 ml rose medium, sterilised by autoclaving).

- 5 ml of each culture was removed at time points between 15 min and 24 h after addition of the radiolabel. These culture extracts were then fractionated as described in section 2.1.3 and the molecular weight of xyloglucan in each fraction was estimated as described in sections 2.1.4 to 2.1.9.

2.5 Density labelling

To investigate the hypothesis that XET catalyses interpolymeric xyloglucan endo-transglycosylation in plant cell walls, a dual-labelling strategy was developed involving density-labelling and radiolabelling. In this approach suspension-cultured rose cells synthesise a pool of density-labelled xyloglucan and a pool of non-density-labelled (normal) xyloglucan in the same cell wall. By radiolabelling one of these pools of xyloglucan, interpolymeric xyloglucan endo-transglycosylation between the two pools can be followed via changes in density of the radiolabelled xyloglucan.

2.5.1 Protocol 1

The results from preliminary radiolabelling experiments described in section 2.1.2.4 suggested that the best density-labelling results would be achieved by feeding glycerol-grown rose cell suspension cultures with [13C]glucose. This was performed as follows:

- Glycerol-grown rose cell suspension-cultures (2x55 ml) at 4 d after subculturing were filtered on sterile muslin, washed with carbon-
source free sterile rose medium (3x200 ml) and gently squeezed to remove excess liquid.

- 5 g fresh weight of cells was transferred to sterile $[^{13}\text{C}]$glucose or $[^{12}\text{C}]$glucose rose medium (40 ml, 0.5% w/v glucose) and left under normal growth conditions for 20 min to deplete endogenous $^{12}\text{C}$ pools.

- Radiolabelled precursor was then added (24 MBq of L-[1-$^{3}\text{H}$]arabinose, section 2.1.2.1) and mixed well.

- The cultures were dispensed in 4-ml aliquots into sterile ‘Sterilin’ pots (50 ml capacity).

- One pot of each culture ($^{12}\text{C}$ and $^{13}\text{C}$) was harvested at time points between 1 h and 7 d after addition of the radiolabel.

- The cultures were fractionated and the hemicellulose was extracted from the cell-wall-rich fraction (section 2.1.3).

- The buoyant density of xyloglucan in cell-wall hemicellulose extracts was then estimated (section 2.1.10) with [fucosyl-$^{3}\text{H}$]sXG as an internal marker.

### 2.5.2 Protocol 2

Protocol 1 gave a lower than expected density of density-labelled [$^{3}\text{H}$]xyloglucan. It was assumed that this was due to the cells having a store of $[^{12}\text{C}]$glycerol within the cells, or not depleting the endogenous $^{12}\text{C}$ pools within the 20 min allowed. The experiment was therefore modified to use glucose-grown rose cells, allowing 1 h to deplete endogenous $^{12}\text{C}$ pools, as described below:

- Glucose-grown rose cell suspension-cultures (2x55 ml) at 4 d after subculturing were filtered on sterile muslin, washed with carbon-source free sterile rose medium (3x200 ml) and gently squeezed to remove excess liquid.
5 g fresh weight of cells was transferred to sterile $^{13}$C glucose or $^{12}$C glucose rose medium (40 ml, 0.5% w/v glucose) and left under normal growth conditions for 1 h to deplete endogenous $^{12}$C pools.

Radiolabelled precursor was then added (12 MBq of L-[1-3H] arabinose per culture, section 2.1.2.1) and mixed well.

The cultures were dispensed in 4-ml aliquots into sterile 'Sterilin' pots (50 ml capacity).

One pot of each culture ($^{12}$C and $^{13}$C) was harvested at time points between 1 h and 7 d after addition of the radiolabel.

The cultures were fractionated and the hemicellulose was extracted from the cell-wall-rich fraction (section 2.1.3).

The buoyant density of xyloglucan in the cell-wall hemicellulose extracts was then estimated (section 2.1.10) with $^{14}$C-acetylated txG as an internal marker.

### 2.5.3 Protocol 3

Protocols 1 and 2 gave very similar results. To test the hypothesis that the lower than expected density of density-labelled [3H]xyloglucan was due to transglycosylation of newly-secreted xyloglucan with older, cell-wall bound xyloglucan, further modifications to the protocol were devised as described below:

- Glucose-grown rose cell suspension-culture (55 ml) at 14 d after subculturing were filtered on sterile muslin, washed with carbon-source free sterile rose medium (3×200 ml) and gently squeezed to remove excess liquid.

- 2 g fresh weight of cells was transferred to sterile $^{13}$C glucose or $^{12}$C glucose rose medium (50 ml, 1.0% w/v glucose) and left under normal growth conditions for 7 d to deplete endogenous $^{12}$C pools and to saturate the cell-wall with $^{13}$C-polysaccharides.
• Radiolabelled precursor was then added (12 MBq of L-[1-3H]arabinose per culture, section 2.1.2.1) and mixed well, the cultures were left for 6 h to radiolabel newly-synthesised polysaccharides.

• The cultures were filtered and washed with carbon-source free rose medium; 10 g fresh weight of cells was transferred into [12C]glucose rose medium (50 ml, containing 5 mM unlabelled L-arabinose) and mixed well.

• The cultures were dispensed in 4-ml aliquots into sterile 'Sterilin' pots (50 ml capacity).

• One pot of each culture (12C and 13C) was harvested at time points between 1 h and 7 d after change of the carbon source to 12C-glucose.

• The cultures were fractionated and the hemicellulose was extracted from the cell-wall-rich fraction (section 2.1.3).

• The buoyant density of xyloglucan in the cell-wall hemicellulose extracts was then estimated (section 2.1.10) with 14C-acetylated tXG as an internal marker.

2.5.4 In vitro density assay

The ability of XET to catalyse interpolymeric xyloglucan transglycosylation was investigated in vitro using the methods described below:

2.5.4.1 Preparation of cauliflower XET extract

A crude enzyme extract containing XET activity was prepared from cauliflower florets as follows:

• Fresh cauliflower florets and stem tissue (no green tissue) were finely grated to obtain 200 g of tissue.
• The grated tissue was homogenised by blending ('Atomix', full speed) in 300 ml of ice-cold succinate buffer (Table 2.9) for 15 s, inverted, then blended for a further 30 s.

• The homogenate was left to stand (0°C, 2 h) with occasional mixing before being strained through muslin (4 layers) and squeezed by hand to extract the maximum volume of juice.

• This juice was centrifuged (2,500 g, 30 min, 4°C); the supernatant was poured through 'Miracloth' (4 layers) and dialysed against succinate buffer (3×500 ml, 3×6 h).

• The dialysed extract (cXET) was dispensed in 10-ml aliquots into scintillation vials and frozen at -70°C.

Table 2.9. Composition of succinate buffer.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>succinic acid</td>
<td>41 g</td>
</tr>
<tr>
<td>NaOH (s)</td>
<td>21 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>980 ml</td>
</tr>
<tr>
<td>pH adjusted to 5.5 with 10 M NaOH (dropwise)</td>
<td></td>
</tr>
<tr>
<td>dH₂O added to final volume of 1 litre.</td>
<td></td>
</tr>
</tbody>
</table>

2.5.4.2 XET activity assay

The activity of XET in the cauliflower extract was assayed as described below:

• 20 μl of tXG solution (0.3% w/v, containing 48.6 kcpm [3H]XLLGol (20 MBq/μmol; donated by S.C.Fry), unbuffered) was mixed with 10 μl of a 1 in 10 dilution of cXET extract (in succinate buffer) in duplicate.

• The assay mixture was incubated in a water bath (25°C) for 20 min and the reaction was stopped by addition of formic acid (100 μl, 30% v/v).
• The incubation products were dried onto Whatman 3MM chromatography paper (4x6 cm) and the paper was washed under running tap water for 1 h.

• The paper was dried and assayed for $^3$H (section 2.1.8) to estimate the amount of radioactivity incorporated into xyloglucan.

This experiment was repeated with 3 incubation times (20 min, 40 min, & 1 h, 4 replicates each) to obtain a time-course for the reaction.

2.5.4.3 Xyloglucanase activity assay

The cXET preparation was assayed viscometrically for xyloglucan-hydrolysing activity as follows:

• 4 ml of tXG solution (1% w/v in dH$_2$O) was mixed with 0.7 ml cXET extract (or 0.7 ml succinate buffer as control) and incubated at 25°C in a water bath.

• The viscosity of this solution was assayed at 25°C by measuring the flow time in a fine-bore 0.2 ml glass pipette at time points between 2 min and 1 h after addition of the enzyme (or buffer).

2.5.4.4 Density-change assay

The ability of the cXET extract to alter the distribution of density in a mixture of $[^{13}$C]$\text{xyloglucan}$ and $[^{12}$C]$\text{xyloglucan}$ was assayed in vitro as follows:

• $[^{13}$C, xylosyl-$^3$H]$\text{xyloglucan}$ from rose (~300 kcpm, 0.25 mg glucose equivalents as determined by anthrone assay, from 12-h time point of protocol 3, section 2.5.3) was mixed with $[^{12}$C, fucosyl-$^3$H]$\text{sXG}$ (section 2.1.2.3, ~300 kcpm, 0.50 mg glucose equivalents as determined by anthrone assay) in a total volume of 0.2 ml (dissolved in dH$_2$O) in duplicate.

• 55 μl of succinate buffer was added.

• 45 μl of cXET extract was added (or 45 μl boiled cXET extract (100°C, 1 h) as control).
• The assay mixtures were incubated in a water bath (25°C) for 1 h and the reaction was stopped by addition of formic acid (1 ml, 30% v/v).

• 160 µl of each assay mixture was removed for GPC analysis (section 2.1.4) and the remainder was dried under vacuum for density analysis (section 2.1.10) with 14C-acetylated tXG as an internal marker.
3. Results

3.1 General techniques

3.1.1 Growth of suspension-cultured rose cells

The growth curves for glucose-grown and glycerol-grown rose cell-suspension cultures are shown in Figure 3.1.1; both cultures had a lag phase of ~3 days. The growth stage continued until ~10 days after subculturing, after which growth slowed down and became stationary. This graph demonstrates the similarity of the growth curves between the two cultures. The rose cells were transferred to a medium with glycerol as the sole carbon source with the assumption that the cells would become partially starved of carbon and would therefore be more efficient in the uptake and incorporation of $^{13}$C-glucose in the density-labelling experiments. In fact, these rose cells quickly adapted to utilising glycerol and grew at a rate similar to glucose-grown cells, showing no signs of carbon starvation.
Figure 3.1.1 Growth of glucose-grown (closed circles) and glycerol-grown (open circles) rose cell suspension cultures.

3.1.2 Radiolabels

3.1.2.1 Radiolabelling xyloglucans for use as internal markers in density gradients.

In the survey of cell-suspension cultures, spinach gave the most efficient uptake (Figure 3.1.2) and incorporation of $^{3}$Hfucose ($\sim$22% incorporation into alcohol-insoluble residues within 6 h). The other cultures tested (including glucose- and glycerol-grown rose cells; results not shown) showed negligible uptake of the $^{3}$Hfucose and negligible incorporation
(<0.5%). Therefore, it was decided to use spinach cultures in the preparation of [fucosyl-\(^3\)H]xyloglucan for use as an internal marker in density gradients.

The molecular weight profile of the \(^3\)H-labelled material extracted from the cell-walls of suspension-cultured spinach cells fed with [\(^3\)H]fucose is shown in Figure 3.1.3; the higher molecular weight material yielded [\(^3\)H]fucose upon enzymatic degradation with 'Driselase', whereas the lower molecular weight material was either resistant to enzymatic degradation by 'Driselase' or hydrolysed to release a component which was immobile on paper chromatography in the butanol/acetic acid/water (12:3:5, 16 h) solvent system used.

![Graph showing uptake of [\(^3\)H]fucose from culture media by three suspension-cultured cell lines: sycamore (circles), spinach (squares), and tomato (triangles).](image-url)
Figure 3.1.3 Molecular weight profile of total $^3$H-labelled material extracted from the cell-walls of suspension-cultured spinach cells supplied with $[^3H]$fucose precursor (solid line); the red dotted line represents the profile of $[^3H]$fucose released from the total material by 'Driselase' digestion; the blue dotted line represents the profile of $^3$H-labelled material with $R_f=0$ on paper chromatography after 'Driselase' digestion of the total material.
3.1.2.2 Use of radiolabels to develop density-labelling strategy

Glycerol-grown rose cells were much more efficient than glucose-grown cells at incorporating radioactivity from $[^{3}\text{H}]$galactose (Figure 3.1.4) and $[^{14}\text{C}]$glucose (Figure 3.1.5) into alcohol-insoluble residues. $[^{14}\text{C}]$Glucose was taken up very rapidly by the glycerol-grown rose cells and the maximum incorporation of radioactivity into alcohol-insoluble residues was reached by 2 h after addition of the radiolabel.

These results suggested that the most efficient incorporation of $^{13}\text{C}$ into polymeric material in the density-labelling experiments would be achieved by feeding glycerol-grown rose cells with $[^{13}\text{C}]$glucose. However, the density-labelling experiments do not involve the uptake of trace amounts of glucose from glycerol-rose medium; the culture medium in the density-labelling step must contain a high concentration of $[^{13}\text{C}]$glucose and no $^{12}\text{C}$ source to give effective density-labelling of polymers.

Figure 3.1.6 shows the ability of glucose-grown rose cells to incorporate radioactivity from $[^{14}\text{C}]$glucose into 20% TCA-insoluble cell residues in the presence of 0.5% w/v $[^{12}\text{C}]$glucose. This assay provides a prediction of the efficiency of $^{13}\text{C}$ incorporation in the density-labelling experiments because the experimental conditions are closely related. This experiment also provides an estimate of the lag-time ($\leq$20 min) between addition of $[^{13}\text{C}]$glucose and incorporation of $^{13}\text{C}$ into polymeric material at a steady rate.
Figure 3.1.4 Incorporation of $^3$H from [$^3$H]galactose into alcohol-insoluble residues of glucose-grown (closed circles) and glycerol-grown (open circles) rose cells.
Figure 3.1.5 Incorporation of $^{14}$C from $[^{14}$C$]$glucose into alcohol-insoluble residues of glucose-grown (closed circles) and glycerol-grown (open circles) rose cells.
Figure 3.1.6 Incorporation of $^{14}$C from $[^{14}$C]glucose into 20% TCA-insoluble residues of glucose-grown rose cells; the radiolabel was added to cells suspended in $^{13}$C-glucose rose medium (50 ml, 0.5% w/v glucose).
3.1.3 Fractionation of suspension-cultures

3.1.3.1 Stability of xyloglucan in extractant

To assess the danger of the extraction procedure causing breakdown of the xyloglucan backbone in the cell-wall hemicellulose extracts, viscosities of tamarind-seed xyloglucan solutions (and carboxymethylcellulose solutions) were assayed (at 25°C) after incubation in extractant at temperatures ranging from 4°C to 50°C (Figure 3.1.7 a and b).

There was little change in viscosity of the xyloglucan solutions over the 6 day period and very little difference in the viscosity of xyloglucan solutions incubated at 25°C and at 37°C (Figure 3.1.7a). Therefore, the use of a 24-h extraction at 37°C should not alter the molecular weight distribution of xyloglucan in the hemicellulose extracts to an appreciable degree and should cause less degradation of the xyloglucan than a prolonged extraction at 25°C.

Figure 3.1.7b shows that incubation temperature had a very similar effect on the viscosity of carboxymethylcellulose solutions as it had on xyloglucan solutions.

The harsh conditions are required to extract the majority of the hemicellulosic fraction from the cell wall but if they cause a breakdown of the xyloglucan backbone then this will also affect the molecular weight distribution of xyloglucan. Extraction of hemicellulose by 6 M NaOH at 25°C takes 14 days to complete but by raising the temperature to 37°C this can be accomplished in 24 hours (Edelmann and Fry, 1992a).
Figure 3.1.7 Effect of incubation at 4°C (circles), 25°C (squares), 37°C (upward-pointing triangles) or 50°C (downward-pointing triangles) on viscosity of:

a) tamarind-seed xyloglucan (1% w/v dissolved in extractant (6 M NaOH + 1% NaBH₄)); flow time of extractant incubated at the same temperatures shown by open symbols.

b) carboxymethylcellulose (1% w/v in extractant (6 M NaOH + 1% NaBH₄))

All flow times were measured at 25°C.
3.1.4 Gel permeation chromatography

3.1.4.1 Calibration of GPC column

Figure 3.1.8 Calibration curve for Sepharose CL-6B GPC column; prepared from elution profiles of dextran (249 kDa, 74.3 kDa, and 40.2 kDa). Manufacturer's data state that this gel-type gives log-linear separation of dextran between 10 kDa and 1 MDa.
3.1.5 Density gradients

The profile of total radioactivity in each density gradient was plotted (see Figure 3.1.9 and Figure 3.1.10 for examples). The profiles of \(^{3}
\text{H}\)xyloglucan and density along each density gradient were also plotted (see Figure 3.1.11 and Figure 3.1.12 for examples).

A Gaussian curve was fitted to the raw data profiles of \(^{3}\text{H}\)xyloglucan and internal marker profiles using a scientific graph-drawing computer software package (Sigmaplot) (see Figure 3.1.13 for an example). The computer-generated fitted curves were transformed into straight-line plots as follows:

\[
t = \sqrt{\log\left(\frac{c}{C}\right)}
\]

where:
- \(t\) = value of transformed data;
- \(c\) = predicted \(^{3}\text{H}\)xyloglucan cpm from fitted curve;
- \(C\) = maximum predicted \(^{3}\text{H}\)xyloglucan cpm from fitted curve.

These straight-line transformations were then plotted as shown in Figure 3.1.14; the equation of each line was used to calculate the fraction number corresponding to the peak of the fitted curves. The density profiles were also plotted (Figure 3.1.14) and linear regressions were fitted; the equations of these lines were used to calculate the average densities of \(^{3}\text{H}\)xyloglucan in the 'heavy' and control gradients from the fraction number corresponding to the peak of each fitted curve.

To plot the results of two density gradients ('heavy' and control gradients) on one graph to enable a visual comparison of the \(^{3}\text{H}\)xyloglucan profiles, the internal marker profile from the control gradient was shifted sideways so that the peak coincided with the peak of the internal marker profile of the 'heavy' gradient. The \(^{3}\text{H}\)xyloglucan profile of the control gradient was shifted to the same extent as the internal marker profile to give
a true representation of the difference in density profiles of $[^3H]$xyloglucan between the 'heavy' gradient and the control gradient at each time point (Figure 3.1.13).

Figure 3.1.9 Profile of $^3$H-labelled material from a $[^{13}C]$glucose-fed culture (protocol 3, 1 d after transfer to $^{12}C$-glucose medium) shown by closed symbols, profile of $^{14}$C-acetylated tamarind-seed xyloglucan shown by open symbols (internal marker xyloglucan).
Figure 3.1.10 Profile of $^3$H-labelled material from a $^{14}$C-glucose-fed culture (protocol 3, 1 d after transfer to $^{12}$C-glucose medium) shown by closed symbols, profile of $^{14}$C-acetylated tamarind-seed xyloglucan shown by open symbols (internal marker xyloglucan).
Figure 3.1.11 Profile of $[^{13}\text{C}, ^{3}\text{H}]$xyloglucan (via isoprimeverose residues) from a $[^{13}\text{C}]$glucose-fed culture (protocol 3, 1 d after transfer to $^{12}\text{C}$-glucose medium) shown by closed symbols; density estimates of each fraction shown by open symbols.
Figure 3.1.12 Profile of [\textsuperscript{12}C, \textsuperscript{3}H]\text{xyloglucan} (via isoprimeverose residues) from a \textsuperscript{12}C-glucose-fed culture (protocol 3, 1 d after transfer to \textsuperscript{12}C-glucose medium) shown by closed symbols; density estimates of each fraction shown by open symbols.
Figure 3.1.13 Graph showing the density profiles of [H]xyloglucan from a ‘heavy’ gradient (closed circles, fitted Gaussian curve shown by solid line) and from a control gradient (open circles, fitted Gaussian curve shown by broken line); also showing the density profile of the internal marker in the ‘heavy’ gradient (small circles, fitted Gaussian curve shown by dotted line).
Figure 3.1.14 Graph showing density plots and transformation of fitted curves to give straight line plots; index as shown above.
3.2 Changes in molecular weight of xyloglucan

To investigate the changes in molecular weight of xyloglucan which occur as cells age, a pulse-labelling experiment was devised to compare the changes occurring during rapid cell expansion to those during slow cell expansion. The aim of this was to determine whether or not xyloglucan endotransglycosylase could have a role to maintain a constant xyloglucan chain length within primary cell walls during the normal growth cycle.

3.2.1 Uptake and incorporation of the radiolabel

Radioactivity was rapidly incorporated from \(^{3}\text{H}\)arabinose into hemicellulose, cell contents, and culture filtrate fractions of the rose cell suspensions (Figure 3.2.1 a & b); over 95% of the radiolabel was taken up by the cells within 3 h.

\(^{3}\text{H}\)Xyloglucan rapidly appeared in the hemicellulosic fraction, with maximum incorporation at 3 to 6 h after addition of the \(^{3}\text{H}\)arabinose (Figure 3.2.1 c & d). Therefore, the experiment was a pulse-chase type with a population of labelled xyloglucan molecules being followed as it aged in the cell wall. There was an initial fast accumulation of \(^{3}\text{H}\)xyloglucan in the culture filtrate for the first 3 h after addition of the \(^{3}\text{H}\)arabinose as reported before (Edelmann and Fry, 1992b); this accumulation then continued at a slower rate over the rest of the timecourse.
Figure 3.2.1 Incorporation of $^3$H from $[^3$H]arabinose into hemicellulose extracts (circles), culture filtrates (squares), and cell contents (triangles) of suspension-cultured rose cells:

a) total $[^3$H], $[^3$H]arabinose added 4 days after subculturing.

b) total $[^3$H], $[^3$H]arabinose added 9 days after subculturing.

c) $[^3$H]xyloglucan, $[^3$H]arabinose added 4 days after subculturing.

d) $[^3$H]xyloglucan, $[^3$H]arabinose added 9 days after subculturing.

In b and d the effect of a repeated hemicellulose extraction is also shown (open circles).
3.2.2 Molecular weight profiles

The GPC elution profiles of total $^3$H-labelled material in hemicellulose extracts from rose cells radiolabelled at 4 d after subculturing typically had a large peak around $k_{av}$ 0.40 and a smaller peak around $k_{av}$ 0.78 (Figure 3.2.2a, as an example). Driselase digestion of the first peak released mainly $[^3]$H]arabinose and $[^3]$H]isoprimeverose (the latter from xyloglucan), with smaller amounts of $[^3]$H]xylose and $[^3]$H]xylobiose (from xylans). The material in the second peak proved to be mostly resistant to Driselase digestion but some $[^3]$H]arabinose was released by the Driselase treatment. The remaining Driselase-resistant material was hydrolysed by treatment with 2 M TFA (1 h, 120°C) which released $[^3]$H]arabinose and a small amount of $[^3]$H]xylose (data not shown). It was decided not to follow the changes in molecular weight of polymers other than xyloglucan because:

- a) the arabinose profile is not diagnostic of a single polymer (unlike the isoprimeverose profile) as it reflects the profile of all polymers containing arabinose residues which are released by Driselase (e.g. arabinogalactans, arabinomannans).
- b) although the xylose/xylobiose profile is diagnostic of xylans, there is too little radioactivity in xylose and xylobiose residues to enable accurate calculations of molecular weight.

The GPC elution profile of $[^3]$H]xyloglucan in these hemicellulose extracts typically had one peak centred between $k_{av}$ 0.33-0.38 (Figure 3.2.2c).

The GPC elution profile of total $^3$H-labelled material in hemicellulose extracts from rose cells radiolabelled at 9 d after subculturing (Figure 3.2.2b, as an example) typically had a large peak around $k_{av}$ 0.36 and a smaller peak around $k_{av}$ 0.78. The GPC elution profile of $[^3]$H]xyloglucan in these hemicellulose extracts typically had one peak centred between $k_{av}$ 0.27-0.30 (Figure 3.2.2d).
Figure 3.2.2 GPC elution profiles of hemicellulose extracts:--
a) total $[^3H]$, 3 d after addition of the radiolabel; cells radiolabelled at 4 d after subculturing.
b) total $[^3H]$, 2 d after addition of the radiolabel; cells radiolabelled at 9 d after subculturing.
c) $[^3H]$xyloglucan, 3 d after addition of the radiolabel; cells radiolabelled at 4 d after subculturing.
d) $[^3H]$xyloglucan, 2 d after addition of the radiolabel; cells radiolabelled at 9 d after subculturing.
Figure 3.2.3 GPC elution profiles of [\textsuperscript{3}H]xyloglucan:

a) in culture filtrate, 6 h after addition of [\textsuperscript{3}H]arabinose; radiolabel added at 9 d after subculturing.
b) in culture filtrate, 4 d after addition of [\textsuperscript{3}H]arabinose; radiolabel added at 9 d after subculturing.
c) in cell contents, 2 h after addition of [\textsuperscript{3}H]arabinose; radiolabel added at 4 d after subculturing.
d) in cell contents, 3 d after addition of [\textsuperscript{3}H]arabinose; radiolabel added at 4 d after subculturing.
Figure 3.2.4 Changes in molecular weight of xyloglucan extracted from cell-walls of rose cells radiolabelled at 4 d (closed circles) and 9 d (open circles) after subculturing.

3.2.3 Discussion of results

The amount of wall-bound \(^{3}H\)xyloglucan in each population decreased by approximately 30% over the observation period, with a similar amount accumulating in the culture filtrates (Figure 3.2.1 c & d). This \(^{3}H\)xyloglucan had a lower molecular weight (~39 kDa; Figure 3.2.3 a & b as examples) than xyloglucan in the hemicellulose extracts and may represent xyloglucan sloughed or trimmed from the cell wall by the action of cellulase or xyloglucan endotransglycosylase which will be discussed later. \(^{3}H\)Xyloglucan present in cell contents extracts had a very low molecular
weight (≤10 kDa, Figure 3.2.3 c & d as examples); however, the fact that there was very little radioactivity in these samples raised concerns over the validity of this observation. The molecular weight of newly-synthesised xyloglucan was investigated further as described in sections 3.3 and 3.4.

Figure 3.2.2 c & d are examples of the molecular weight profiles of xyloglucan in hemicellulose extracts at one time point of each experiment. The molecular weight of [3H]xyloglucan at each time point was calculated as described in section 2.1.9, the results of this are shown in Figure 3.2.4.

Similar changes in molecular weight of [3H]xyloglucan occurred during rapid and slow cell expansion. Both populations of wall-bound [3H]xyloglucan appeared to lose approximately 40 kDa between 0 h and 48 h after addition of the radiolabel (Figure 3.2.4).

There was a clear difference between the two populations of xyloglucan in terms of their absolute molecular weights. Xyloglucan extracted from rapidly expanding cells had a molecular weight consistently ~80 kDa lower than that extracted from slowly expanding cells.

3.2.4 Relationship between molecular weight and density of xyloglucan

3.2.4.1 Molecular weight profile

[3H]Xyloglucan is a high molecular weight component of the total 3H-labelled material in the hemicellulose extracts as shown in Figure 3.2.2; by pooling GPC column fractions, the xyloglucan elution profile was split into ‘large’ and ‘small’ xyloglucan fractions (Figure 3.2.5). These two pools were then run on density gradients to see if there was a difference in density between high M, xyloglucan and low M, xyloglucan which could reflect a difference in structure (e.g. degree of polymerisation, composition of sidechains etc.).
3.2.4.2 Density profiles

The density profiles of 'large' xyloglucan (Figure 3.2.6) and 'small' xyloglucan (Figure 3.2.7) were very similar. Both profiles had the same average density, when compared relative to the position of the internal marker xyloglucan, which suggests that the xyloglucan in the two pools was structurally identical.

The only noticeable difference between the two profiles is that the 'small' xyloglucan did not band as tightly as 'large' xyloglucan on the density gradients; this is due to the physical process of band formation in density gradients. High Mr material forms narrower bands because it migrates towards its buoyant density in the density gradient faster than low Mr material; it is also less prone to widening of the band by diffusion down the concentration gradient away from its buoyant density.
Figure 3.2.5 Molecular weight profile of $^3$H-labelled hemicellulose (solid line) and xyloglucan (dotted line): The broken lines indicate the pooling of GPC column fractions into 'large' and 'small' xyloglucan pools (graph shows hemicellulose extracted from suspension-cultured rose cells 1 d after addition of the [$^3$H]arabinose; radiolabelled added at 9 d after subculturing).
Figure 3.2.6 Density profile of 'large' hemicellulose (solid line) and xyloglucan (dotted line); the density is indicated by the broken line.
Figure 3.2.7 Density profile of 'small' hemicellulose (solid line) and xyloglucan (dotted line); the density is indicated by the broken line.
3.3 Protoplasts

Protoplasts isolated from suspension-cultured rose cells were fed \[^{3}\text{H}]\text{arabinose}\) in an attempt to radiolabel newly-secreted xyloglucan and estimate the molecular weight of xyloglucan before it is incorporated into the cell-wall-matrix of whole cells.

3.3.1 Molecular weight profiles

The molecular weight profiles shown in Figure 3.3.1 suggested that xyloglucan synthesised by protoplasts and secreted into the medium had a very low molecular weight (< 10 kDa) in comparison to the molecular weight of xyloglucan extracted from the cell-walls of whole suspension-cultured cells (~180 kDa). The high amount of \[^{3}\text{H}]\text{arabinose}\) in the included-volume fractions may be due, in part, to unused precursor remaining in the medium.

The results from the GPC analysis of the cell contents (Figure 3.3.2) were much the same, with \[^{3}\text{H}]\text{isoprimeverose}\) only being released from material in the included-volume fractions, suggesting a molecular weight for intracellular xyloglucan of ≤1 kDa.
Figure 3.3.1 Protoplast products in culture filtrate; culture filtrate was fractionated on the Sepharose CL-6B GPC column, 200 μl of each fraction was assayed for total $^3$H in the culture filtrate, 1 ml of each fraction was dried and subjected to 'Driselase' digestion and PC in butanol/acetic acid/water (12:3:5, 16 h) and assayed for $^3$H in isoprimeverose (red line), arabinose (blue line) and PC-immobile material (green line, material at origin).
Figure 3.3.2 Protoplast products in cell contents; cell contents were fractionated on the Sepharose CL-6B GPC column, 200 µl of each fraction was assayed for total $^3$H in the cell contents, 1 ml of each fraction was dried and subjected to 'Driselase' digestion and PC in butanol/acetic acid/water (12:3:5, 16 h) and assayed for $^3$H in isoprimeverose (red line), arabinose (blue line) and PC-immobile material (green line, material at origin).
Figure 3.3.3 GPC profile of \([xylosyl-^3H]\)xyloglucan synthesised by protoplasts (blue line) in culture filtrate spiked with spinach \([fucosyl-^3H]\)xyloglucan (red lines, 150 kcpm). The solid red line indicates the GPC profile of the spinach xyloglucan prior to the 6 h incubation with the protoplasts, and the broken line shows the GPC profile of the spinach xyloglucan after the 3 h incubation with the protoplasts.

3.3.2 Discussion of results

The low molecular weights of xyloglucan shown above may be due to degradation of the xyloglucan, either by ‘Driselase’ that had not been removed during the rigorous washing of the protoplasts, or by enzymes (e.g. cellulase, XET) from the protoplasts themselves. This was investigated by repeating the experiment and spiking the protoplast medium during the 3 h radiolabelling step with spinach \([fucosyl-^3H]\)xyloglucan and observing the
change in molecular weight of this control xyloglucan after incubation with
the protoplast suspension. The results of this (Figure 3.3.3) show that there
was moderate degradation of the spinach [fucosyl-3H]xyloglucan ($M_w$
decreased from 220,000 to 51,000) in the protoplast medium, some of which
may be due to transglycosylation with smaller xyloglucan polymers or
oligomers catalysed by XET secreted into the medium by the protoplasts.
However, the xyloglucan secreted by the protoplasts showed a much lower
molecular weight than the spinach xyloglucan after the 3 h incubation;
explanations of this observation include:
a). the xyloglucan synthesised by protoplasts experienced greater
exposure to degradative conditions than the spinach xyloglucan
(*ie.* degradation started within the protoplasm, perhaps by
'Driselase' enzymes internalised by the protoplasts).
b). the xyloglucan synthesised by protoplasts is more susceptible to
degradation than the spinach xyloglucan.
c). the xyloglucan synthesised by protoplasts has a lower initial
molecular weight than the spinach xyloglucan.

Given the experimental difficulties encountered using protoplasts, it
was decided to develop a different approach to estimate the molecular
weight of newly-secreted xyloglucan using Brefeldin A as described below.
The results of the Brefeldin A experiments support the explanation that
newly-synthesised xyloglucan has a lower initial molecular weight than the
spinach xyloglucan.
3.4 Brefeldin A

Brefeldin A (BFA) is a fungal antibiotic which disrupts vesicle transport between Golgi stacks and prevents secretory vesicles fusing with the plasma membrane. It inhibits the secretion of cell wall polysaccharides and glycoproteins (Driouich et al., 1993b) and also inhibits the expansion of plant cells (Schindler et al., 1994; Edelmann and Volkmann, 1996).

Brefeldin A was used in this experiment to cause an accumulation of radioactively labelled xyloglucan within cells in order to determine the molecular weight of newly synthesised xyloglucan before it is secreted and incorporated into the cell wall.

3.4.1 Uptake and incorporation of the radiolabel

Brefeldin A interfered with the ability of the cells to take up [³H]arabinose from the culture medium (Figure 3.4.1), although >60% of the radiolabelled precursor was taken up by the cells within 4 hours.

Secretion of radiolabelled material into the cell-wall was effectively blocked for at least 8 hours after addition of BFA (Figure 3.4.2). By 24 hours there was appreciable recovery of the exocytosis pathway and radiolabelled material was incorporated into the cell wall and the culture filtrate.

If the Brefeldin A were inhibiting the incorporation of radiolabelled polymeric material into the cell-wall-matrix then we should have observed an increased incorporation of radiolabelled polymeric material into the culture filtrate; this was not seen which suggests that the Brefeldin A was blocking secretion of radiolabelled polymeric material from the cells. This is supported by the observation that radiolabelled material accumulated within the cells after application of BFA.
Figure 3.4.1 Uptake of [3H]arabinose from culture medium by cells in the presence (closed symbols) or absence (open symbols) of 48 μM Brefeldin A.
Figure 3.4.2 Incorporation of radioactivity into cellulose-binding material in cell-wall hemicellulose extracts (squares), culture filtrates (circles), and cell-contents (triangles) of rose cell-suspension cultures in the presence (closed symbols) or absence (open symbols) of 48 μM Brefeldin A.

3.4.2 Molecular weight profiles

The radiolabelled xyloglucan in the cell-contents extracts at 4 h after addition of the radiolabel in the presence of BFA had a much lower molecular weight than the radiolabelled xyloglucan in the cell-wall hemicellulose extract taken at 24 h after addition of the radiolabel (Figure 3.4.3).
Figure 3.4.3 Molecular weight profiles of $[^3]$Hxyloglucan in cell-contents (solid line, 4 h after addition of radiolabel) and cell-wall hemicellulose extracts (dotted line, 24 h after addition of radiolabel) of Brefeldin A treated cells; the $M_r$ indicators represent the typical elution positions of markers with the molecular weights shown.

### 3.4.3 Discussion of results

The results suggest that xyloglucan is secreted into the apoplast as a low molecular weight polymer and processed in the apoplast to form a high molecular weight polymer; this processing could involve transglycosylation onto older, wall-bound xyloglucan. This mechanism would also incorporate newly-secreted xyloglucan into the cell-wall-matrix, which is known to happen very rapidly after secretion into the apoplast (Edelmann and Fry, 1992b).
3.5 Density labelling

3.5.1 Protocol 1

3.5.1.1 Uptake and incorporation of the radiolabel

Radioactivity was rapidly incorporated from \([^3\text{H}]\text{arabinose}\) into all three culture fractions analysed (Figure 3.5.1.1). The initial (fast) decrease in radioactivity in the culture filtrate represents the uptake of the radiolabel from the culture medium into the cells.

The incorporation of radioactivity into xyloglucan in each culture fraction was also analysed (Figure 3.5.1.2). The radiolabel evidently rapidly infiltrated the UDP-pentose pools in the cell, from which \([1-[^3\text{H}]\text{xylose}\) residues were rapidly incorporated into isoprimeverose residues of newly-synthesised xyloglucans.

3.5.1.2 Density profiles

The \(([^{13}\text{C}, ^3\text{H}])\)- and \(([^{12}\text{C}, ^3\text{H}])\)-labelled xyloglucans obtained from protocol 1 differed in density less than expected (Figure 3.5.1.3), presumably because the rose cells inadequately depleted their endogenous \(^{12}\text{C}\) pools in the 20 min prior to addition of the radiolabel. It is known that cells can accumulate large amounts of glycerol without adversely affecting their water potential. It was decided to repeat the experiment using glucose as the \(^{12}\text{C}\)-carbon source instead of glycerol to avoid this problem (see protocol 2).

In protocol 1, the \([^{12}\text{C}, ^3\text{H}]\text{xyloglucan}\) showed very little change in density with time (Figure 3.5.1.4). The \([^{13}\text{C}, ^3\text{H}]\text{xyloglucan}\) showed an increase in density with time, not a decrease as would be expected from transglycosylation of \([^{13}\text{C}]\text{xyloglucan}\) with \([^{12}\text{C}]\text{xyloglucan}\) polymers in the cell wall. Possible explanations of this are discussed in section 3.5.4.
Figure 3.5.1.1. Incorporation of radioactivity from $^3$H-arabinose into each of the culture fractions in protocol 1; cell-wall hemicellulose extract shown by squares, culture filtrate shown by circles, and cell-contents shown by triangles. The horizontal bars represent the availability to the cells of $^{12}$C-glucose (green), $^3$H-arabinose (red), and $^{13}$C-glucose (blue).
Figure 3.5.1.2. Incorporation of radioactivity from $[^3]$H]arabinose into xyloglucan in each of the culture fractions of protocol 1; cell-wall hemicellulose extract shown by squares, culture filtrate shown by circles, and cell-contents shown by triangles. The horizontal bars represent the availability to the cells of $[^{12}]$C]glucose (green), $[^3]$H]arabinose (red), and $[^{13}]$C]glucose (blue).
Figure 3.5.1.3. An example of the density profiles obtained by protocol 1 (data from 1-d time point): density profile of rose $[^{13}\text{C}]$xyloglucan shown by closed circles, density profile of rose $[^{12}\text{C}]$xyloglucan shown by open circles. The dotted line indicates the position of the internal marker (spinach $[^{fucosyl-3}\text{H}]$xyloglucan).
Figure 3.5.1.4. Changes in densities of $[^3\text{H}]$xyloglucan from cell-walls of $[^{13}\text{C}]$glucose-fed rose cells (closed circles), and from $[^{12}\text{C}]$glucose-fed rose cells (open circles) in protocol 1.
3.5.2 Protocol 2

3.5.2.1 Uptake and incorporation of the radiolabel

Radioactivity was rapidly incorporated from \[^{3}\text{H}]\text{arabinose}\) into the cell-wall hemicellulose fraction, culture filtrate fraction and the cell contents (Figure 3.5.2.1). Only 7% of the added radioactivity was present in the culture filtrate at 1 h after addition of the radiolabel.

The \(^{3}\text{H}\) from \[^{3}\text{H}]\text{arabinose}\ was incorporated into isoprimeverose residues of xyloglucan at a rapid rate, with maximal incorporation into xyloglucan in the cell-wall hemicellulose extract at 4 to 8 h after addition of the radiolabel (Figure 3.5.2.2).

3.5.2.2 Density profiles

The difference in density between \(^{13}\text{C}, \(^{3}\text{H}\)- and \(^{12}\text{C}, \(^{3}\text{H}\)-labelled xyloglucan (Figure 3.5.2.3) was similar to that of protocol 1, indicating that the results from protocol 1 were not due to incorporation of both \(^{12}\text{C}\) and \(^{13}\text{C}\) simultaneously into the \[^{3}\text{H}]\text{xyloglucan}\ during synthesis as previously suggested. It may instead be due to post-synthetic incorporation of \[^{12}\text{C}\]xyloglucan into the \[^{13}\text{C}, \(^{3}\text{H}\]xyloglucan via transglycosylation in the cell wall.

The \[^{12}\text{C}, \(^{3}\text{H}\]xyloglucan showed very little change in density over 7 days (Figure 3.5.2.4). The \[^{13}\text{C}, \(^{3}\text{H}\]xyloglucan showed an increase in density, not the decrease in density which would have been expected if cell-wall-bound \[^{13}\text{C}, \(^{3}\text{H}\]xyloglucan had undergone transglycosylation with pre-existing cell-wall-bound \[^{12}\text{C}, \(^{1}\text{H}\]xyloglucan. Possible explanations for this will be discussed in section 3.5.4.
Figure 3.5.2.1. Incorporation of radioactivity from [3H]arabinose into each of the culture fractions in protocol 2; cell-wall hemicellulose extract shown by squares, culture filtrate shown by circles, and cell-contents shown by triangles. The horizontal bars represent the availability to the cells of [12C]glucose (green), [3H]arabinose (red), and [13C]glucose (blue).
Figure 3.5.2.2. Incorporation of radioactivity from $[^{3}\text{H}]$arabinose into xyloglucan in each of the culture fractions of protocol 2; cell-wall hemicellulose extract shown by squares, culture filtrate shown by circles, and cell-contents shown by triangles. The horizontal bars represent the availability to the cells of $[^{12}\text{C}]$glucose (green), $[^{3}\text{H}]$arabinose (red), and $[^{13}\text{C}]$glucose (blue).
Figure 3.5.2.3. An example of the density profiles obtained by protocol 2 (data from 10-h time point): density profile of rose $[^{13}\text{C}]\text{xyloglucan}$ shown by closed circles, density profile of rose $[^{12}\text{C}]\text{xyloglucan}$ shown by open circles. The dotted line indicates the position of the internal marker ($^{14}\text{C}$-acetylated tamarind-seed xyloglucan).
Figure 3.5.2.4. Changes in densities of $[^3]$H]xyloglucan from cell-walls of $[^{13}]$C]glucose-fed rose cells (closed circles), and from $[^{12}]$C]glucose-fed rose cells (open circles) in protocol 2.
3.5.3 Protocol 3

3.5.3.1 Uptake and incorporation of the radiolabel

The \(^{3}\)H-arabinose was very rapidly exhausted from the culture medium with >95% being removed from the medium by 1 h after addition to the cultures (Figure 3.5.3.1).

The rapid incorporation of \(^{3}\)H from \(^{3}\)H-arabinose into each culture fraction is shown in Figure 3.5.3.2; \(^{3}\)H-arabinose was not detectable in the culture filtrate (data not shown), confirming that it had been removed in the radiolabelling step and by the subsequent washing of the cells. The \(^{3}\)H from \(^{3}\)H-arabinose was rapidly incorporated into cellulose-binding material (Figure 3.5.3.3), most of which is xyloglucan (data not shown).

3.5.3.2 Density profiles

The difference in density between xyloglucan from the cell-walls of \(^{13}\)C-glucose-fed rose cells and that from \(^{12}\)C-glucose-fed rose cells using protocol 3 (Figure 3.5.3.4) was two-fold greater than that of previous experiments (protocols 1 & 2).

The \(^{12}\)C, \(^{3}\)H-xyloglucan showed very little change in density as it 'aged' in the cell wall (Figure 3.5.3.5). The \(^{13}\)C, \(^{3}\)H-xyloglucan decreased in density with time as predicted by the hypothesis that newly-secreted xyloglucan is incorporated into the cell wall matrix by transglycosylation onto older, cell-wall-bound xyloglucans.
Figure 3.5.3.1. Uptake of radiolabel from the culture filtrate in protocol 3; total $^3$H shown by circles, $[^3]$H]arabinose remaining in the culture medium shown by squares, $^3$H-labelled polymeric material accumulating in the culture filtrate shown by triangles.
Figure 3.5.3.2. Incorporation of $^3$H into each culture fraction of protocol 3; cell-wall hemicellulose extract (pre-dialysis) shown by squares, cell-wall hemicellulose extract (post-dialysis) shown by diamonds, culture filtrate shown by circles, and cell-contents shown by triangles. The horizontal bars represent the availability to the cells of $^{13}$C-glucose (blue), $[^3]$H-arabinose (red), and $^{12}$C-glucose (green).
Figure 3.5.3.3. Incorporation of $^3$H into cellulose-binding material (CBM) in protocol 3; $^3$H-CBM in cell-wall hemicellulose extract (predialysis) shown by squares, $^3$H-CBM in cell-wall hemicellulose extract (post-dialysis) shown by diamonds, $^3$H-CBM accumulated in culture filtrate shown by circles, and $[^3]$H-CBM in remaining in cell-contents shown by triangles. The horizontal bars represent the availability to the cells of $[^{13}]$C-glucose (blue), $[^3]$H-arabinose (red), and $[^{12}]$C-glucose (green).
Figure 3.5.3.4. An example of the density profiles obtained by protocol 3 (data from 12-h time point): density profile of rose $^{13}$C xyloglucan shown by closed circles, density profile of rose $^{12}$C xyloglucan shown by open circles. The dotted line indicates the position of the internal marker ($^{14}$C-acetylated tamarind-seed xyloglucan).
3.5.4 Discussion of results

In all three density-labelling protocols, the rapid labelling of xyloglucan in the cell wall enabled a pulse-chase type of experiment and allowed the density-changes in cell-wall-bound xyloglucan to be followed as the radiolabelled pool of xyloglucan 'aged' in the cell wall. There was very little radiolabelled xyloglucan in the cell-contents at any time-point, suggesting that newly-synthesised xyloglucan was rapidly secreted into the apoplast, where the majority became incorporated into the cell-wall-matrix.
A small proportion of this newly-secreted xyloglucan appeared to pass through the cell-wall without becoming bound to it, accumulating in the culture filtrate. This accumulation of radiolabelled xyloglucan in the culture filtrate continued over the time-course, probably due to sloughing of cell-wall-bound xyloglucan into the medium.

The $^{12}\text{C}, {^3}\text{H}]$xyloglucan in all three protocols showed no measurable change in density over the observation period and provided an essential control experiment for the density-labelled xyloglucan results.

The observation that the density of $[^3\text{H}]$xyloglucan increased in protocols 1 & 2 showed that wall-bound $[^3\text{H}]$xyloglucan did not undergo transglycosylation with older, wall-bound $[^{12}\text{C}]$xyloglucan to a measurable degree (see Figure 3.5.4.1) as this would have caused a decrease in density of the $[^3\text{H}]$xyloglucan. The observed increase in density of the wall-bound $[^3\text{H}]$xyloglucan suggested that it was being transglycosylated with newly-secreted $[^{13}\text{C}]$xyloglucan. This mechanism may be responsible for the rapid incorporation of radiolabelled xyloglucan into the cell wall matrix. Also, if the newly-secreted $[^{13}\text{C}, {^3}\text{H}]$xyloglucan was incorporated into the cell wall in this manner, then the wall-bound $[^3\text{H}]$xyloglucan would be $[^{12}\text{C}, {^{13}}\text{C}, {^3}\text{H}]$xyloglucan and not simply $[^{13}\text{C}, {^3}\text{H}]$xyloglucan.

In protocols 1 & 2, decreases in density of the wall-bound $[^{12}\text{C}, {^{13}}\text{C}, {^3}\text{H}]$xyloglucan due to interpolymeric transglycosylation with older, wall-bound $[^{12}\text{C}]$xyloglucan may be outweighed (and therefore not observed) by increases in density caused by the incorporation of newly-secreted $[^{13}\text{C}]$xyloglucan.
Protocol 3 was designed to test the theory that newly-secreted xyloglucan was incorporated into the cell wall by transglycosylation with older, wall-bound xyloglucan. The decrease in density of $^{13}$C, $^3$H]xyloglucan in protocol 3 suggests that newly-secreted $^{12}$C]xyloglucan was incorporated into the cell wall matrix via transglycosylation with older, wall-bound $^{13}$C, $^3$H]xyloglucan, causing the observed decrease in density of the $^{13}$C, $^3$H]xyloglucan (see Figure 3.5.4.2). The increased difference in densities between $^{12}$C] and $^{13}$C]xyloglucan in this protocol as compared to protocols 1 & 2 also supports this mechanism; the $^3$H]xyloglucan extracted from the cell walls of density-labelled cells in protocols 1 & 2 would already have been transglycosylated onto older, cell-wall-bound $^{12}$C]xyloglucan in the process of being incorporated into the cell wall matrix and would therefore be $^{12}$C, $^{13}$C, $^3$H]xyloglucan, with a decreased density prior to extraction and analysis.
Figure 3.5.4.2 Diagrammatic representation of transglycosylation between wall-bound $[^{13}\text{C}, ^{3}\text{H}]$xyloglucan (blue) and newly-secreted $[^{12}\text{C}]$xyloglucan (green).
3.5.5 *In vitro* XET assay by changes in density

3.5.5.1 XET activity assay

The crude cauliflower enzyme extract contained a high level of XET activity, catalysing the incorporation of ~20% of the radiolabelled xyloglucan oligosaccharide ([3H]XLLGol) into polymeric xyloglucan during a 1 h incubation (Figure 3.5.5.1).

3.5.5.2 Xyloglucanase activity assay

The viscometric assay demonstrated that there was a slight decrease in viscosity of the xyloglucan solution during incubation with cXET extract (Figure 3.5.5.2).

3.5.5.3 Molecular weight assay

The molecular weight profiles of the [13C, 3H]xyloglucan and the [12C, 3H]xyloglucan were very similar (Figure 3.5.5.3). Incubation with cXET extract did not cause a noticeable change in the molecular weight profiles of either xyloglucan (data not shown).

3.5.5.4 Density-change assay

Rose [13C, xylosyl-3H]xyloglucan (0.25 mg glucose equivalents) plus spinach [12C, fucosyl-3H]xyloglucan (0.5 mg glucose equivalents) were incubated for 1 h with cXET or with boiled cXET. The density profile of [fucosyl-3H]xyloglucan (Figure 3.5.5.4) did not show a significant change in density on incubation with cXET extract. However, the density profile of [xylosyl-3H]xyloglucan (Figure 3.5.5.5) showed a decrease in density on incubation with cXET extract.
Figure 3.5.5.1. Time course of XET activity in the cauliflower extract; incorporation of $[^3\text{H}]XLL\text{Gol}$ into polymeric xyloglucan, data points represent the mean of 4 replicates, error bars show standard errors.
Figure 3.5.5.2. Viscosity time-course assay of xyloglucanase activity; xyloglucan incubated with cXET extract (closed symbols) or with buffer (control, open symbols). No oligomeric acceptor substrate was added.
Figure 3.5.5.3. Molecular weight profiles of rose $[^{13}\text{C},\text{xylosyl}^{3}\text{H}]\text{xyloglucan}$ (solid line) and spinach $[^{12}\text{C},\text{fucosyl}^{3}\text{H}]\text{xyloglucan}$ (dotted line) on the Sepharose CL-6B GPC column.
Figure 3.5.5.4 *In vitro* XET density-change assay, density profiles of [*fucosyl-3H*]xyloglucan. Rose [*13C, xylosyl-3H*]xyloglucan plus spinach [*12C, fucosyl-3H*]xyloglucan were incubated for 1 h with cXET (closed symbols, solid line represents fitted curve) or with boiled cXET (open symbols, broken line represents fitted curve). The dotted line indicates the position of the internal marker (*14C*-acetylated tamarind-seed xyloglucan).
Figure 3.5.5.5. *In vitro* XET density-change assay, density profiles of [*xylosyl-*^3^H]xyloglucan. Rose [*¹³C, xylosyl-*^3^H]xyloglucan plus spinach [*¹²C, fucosyl-*^3^H]xyloglucan were incubated for 1 h with cXET (closed symbols, solid line represents fitted curve) or with boiled cXET (open symbols, broken line represents fitted curve). The dotted line indicates the position of the internal marker (*¹⁴C-acetylated tamarind-seed xyloglucan).
3.5.5.5 Discussion of results

The decrease in viscosity of the xyloglucan solution could be due to xyloglucanase activity in the cXET extract; it could also be due to XET activity altering the distribution of molecular weights of the xyloglucan which would not affect the mean molecular weight but may affect the viscosity of the solution. A high xyloglucanase activity would have led to a large decrease in molecular weight (and viscosity) of the xyloglucan; this would have increased the width of band formed on the density gradients. The broadening of the density-labelled xyloglucan band illustrated in Figure 3.5.5.5 is not extensive enough to affect the analysis of the data.

Transglycosylation between \[^{12}\text{C},\,\text{fucosyl-}^3\text{H}]\text{xyloglucan}\) and \[^{13}\text{C},\,\text{xylosyl-}^3\text{H}]\text{xyloglucan}\) would not be detected by the GPC analysis because the two xyloglucan pools had very similar M, profiles before the incubation with cXET. The action of XET would merge the GPC profiles of the two pools towards their combined average molecular weight which would result in a profile very similar to the two initial profiles.

A change in molecular weight of the two pools of xyloglucan after incubation with cXET extract could have been due to one, or a combination, of the following mechanisms:

a). endotransglycosylation between two pools of xyloglucan with different molecular weight profiles, increasing or decreasing the molecular weight of each pool towards the average of the two pools;

b). hydrolysis of the xyloglucan backbone by xyloglucanases in the cXET extract, decreasing the molecular weight of each pool;

There was no appreciable change in either of the molecular weight profiles after incubation with cXET extract for 1 h, indicating a lack of any significant xyloglucan-hydrolysing activities. This result also suggests that the broadening of the band of density-labelled xyloglucan in Figure 3.5.5.5 is
due to a wider range of density (after transglycosylation with non-density-labelled xyloglucan), and not due to wider banding of lower molecular weight xyloglucan.

The shift in the density profile of \([xylosyl-^3H]\)xyloglucan on incubation with cXET (Figure 3.5.5.5) represents a decrease in buoyant density from 1.61 g per ml to 1.60 g per ml, presumably owing to transglycosylation between rose \([^{13}C, \ xylosyl-^3H]\)xyloglucan and spinach \([^{12}C, \ fucosyl-^3H]\)xyloglucan polymers, catalysed by cauliflower XET \textit{in vitro}.\)
4. Discussion

4.1 Changes in Mr of wall-bound xyloglucan

4.1.1 In relation to growth-rate

Each glucose residue in the backbone of a xyloglucan molecule contributes ~0.515 nm to the length of the backbone (Taylor and Atkins, 1985) and accounts for ~300 Da of the molecular weight of the molecule when sidechains and their frequency of occurrence are considered. Therefore, the initial Mr of newly-deposited xyloglucan in rapidly-expanding and slowly-expanding cells (160000 and 240000 respectively, if the GPC calibration is accurate) represent respective chain lengths of ~275 nm and ~410 nm. The ~80000 difference in Mr (if the GPC calibration is accurate) between xyloglucan extracted from slowly expanding cell walls and xyloglucan extracted from rapidly expanding cell walls translates to a difference in length of ~140 nm. This observation is in agreement with the model of intermicrofibrillar xyloglucan cross-links affecting cell wall extensibility; an increase in the length of xyloglucan will increase the chances of that xyloglucan forming intermicrofibrillar cross-links by enabling each xyloglucan molecule to come into contact with more microfibrils. An increase in the number of cross-links will decrease the extensibility of the cell wall and lead to a decreased rate of expansion. Similar correlations between xyloglucan molecular weight and growth rate have been reported in the comparison between upper and lower sections of gravitropically responding pea stems (Talbott and Pickard, 1994), and in response to exogenous auxin and acidic pH (see section 1.6.1).

4.1.2 In relation to ageing of cell walls

The decrease in molecular weight of wall-bound [3H]xyloglucan by approximately 40 kDa and concomitant loss of 20-30% of [3H]xyloglucan
from the cell wall as the cells age coincides with an accumulation of a similar amount of [3H]xyloglucan in the culture medium, which has a molecular weight of ~39 kDa. This sloughing of xyloglucan from the cell wall into the culture medium may be due to trimming of sections of wall-bound xyloglucan which are not directly hydrogen-bonded to cellulose by cellulase or XET action (see Figure 4.1). An XET-catalysed trimming mechanism, cutting within sections of wall-bound xyloglucan which are not directly hydrogen-bonded to cellulose and using XXFG as the acceptor substrate would have very similar structural consequences for the cell wall as the action of cellulase (see Figure 4.1b).

The age-related trimming of xyloglucan molecules observed in this work may reflect a transition of the xyloglucan from a load-bearing stratum of the cell wall to a non-load-bearing region. Trimming of unbound sections of wall-bound xyloglucan molecules may remove existing xyloglucan cross-links and could decrease the ability to form new cross-links, reducing the load-bearing ability of the cell wall in that area.
Figure 4.1 Diagrammatic representation of the different types of unbound xyloglucan in cell walls. Cutting of xyloglucan chains at the positions marked by scissors in (a) by cellulase or by XET using a xyloglucan oligosaccharide as the acceptor substrate will lead to trimming, as shown in (b). Cellulose microfibrils are represented by thick black lines; dimensions are not to scale.

4.2 Transglycosylation may integrate newly-secreted xyloglucan into the cell wall matrix

Newly-secreted xyloglucan was observed in this work to undergo an increase in molecular weight from \( \leq 10 \) kDa to \( \sim 200 \) kDa on incorporation into the wall-bound xyloglucan fraction in the cell wall (see sections 3.3 and 3.4); similar results were reported for pea stem segments (Talbott and Ray, 1992). This suggests that glycosidic bond formation is occurring between
xyloglucan molecules during integration into the cell wall matrix, possibly by the action of xyloglucan endotransglycosylase.

Integration of newly-secreted xyloglucan into the cell wall matrix is known to be rapid, occurring within minutes of secretion (Edelmann and Fry, 1992b). Also, integration can still occur for at least 24 h after cellulose deposition has been inhibited by DCB treatment (Edelmann and Fry, 1992c); therefore, hydrogen-bonding to newly-deposited cellulose microfibrils is not the only available mechanism of integration of newly-secreted xyloglucan into the cellulose/xyloglucan network. However, prolonged inhibition of cellulose synthesis does result in a decrease in the amount of xyloglucan in the cell wall, with xyloglucan accumulating in the culture medium (Shedletzky et al., 1990, 1992). Reassembly of cell walls and the incorporation of newly-synthesised xyloglucan into the forming cell wall by isolated protoplasts is reported to be promoted by addition of xyloglucan to the medium (Hayashi et al., 1986).

The combination of these observations and the results of the density-labelling work described in this thesis (see section 3.5.4) provides strong evidence for transglycosylation onto older, wall-bound xyloglucan playing a role in the integration of newly-secreted xyloglucan into the cell wall.

The ~50% increase in the initial Mr of newly-deposited xyloglucan from cells pulse-labelled at 9 d after subculturing as compared to cells pulse-labelled at 4 d after subculturing observed in this work (see section 3.2) may be due to incorporation of newly-secreted segments of xyloglucan into the cellulose/xyloglucan network by a transglycosylation reaction; this would result in a gradual increase of the Mr of wall-bound xyloglucan as the cells aged. The length of xyloglucan molecules in cell walls of onion has been reported to exhibit a periodicity of ~30 nm (McCann et al., 1992), equivalent to ~17 kDa of xyloglucan; this may be because xyloglucan in the cell wall is built up from blocks of smaller xyloglucan molecules or may be due to a
periodicity in the distribution of cellulase or XET attack sites along the xyloglucan backbone.

The role of cellulose deposition should not be overlooked in the incorporation of newly-secreted xyloglucan into the cell wall matrix. Although cellulose deposition is not necessary for the incorporation of xyloglucan into the cell wall matrix, the cell wall becomes more extensible during inhibition of cellulose synthesis by DCB treatment (Edelmann and Fry, 1992c). Also, the integration of newly-secreted xyloglucan into the tightly wall-bound xyloglucan fraction has been described as a biphasic process, with the majority becoming firmly wall-bound (extractable with 6 M NaOH) very soon after secretion into the apoplast and the remainder becoming firmly wall-bound at a slower rate (Edelmann and Fry, 1992b). Therefore, transglycosylation of newly-secreted xyloglucan with older, wall-bound xyloglucan may complement direct hydrogen-bonding onto newly-deposited microfibrils and may result in cross-link formation and wall tightening.

4.2.1 Mechanism of action of XET in the incorporation of newly-secreted xyloglucan

It is likely that the most accessible substrates for XET in the cell wall are sections of xyloglucan which are not directly hydrogen-bonded to cellulose microfibrils, such as unbound sections of wall-bound xyloglucan (which may be cross-linking sections of xyloglucan or which may contain either the reducing or the non-reducing end of a xyloglucan chain), xyloglucan oligosaccharides (released by cellulase hydrolysis of xyloglucan or by the action of XET) and newly-secreted xyloglucan (see Figure 4.1a).

Some enzyme preparations which can be assayed for XET activity by the paper-binding method fail to cause an appreciable decrease in viscosity in the viscometric assay (e.g. XET in rose cell-suspension culture medium
and XET extracted from cauliflower florets). Presumably, these XETs do not cut xyloglucan mid-chain to a measurable extent (and therefore do not depolymerise xyloglucan), but 'prefer' to cut the xyloglucan backbone near to the ends (Fry and Thompson, 1995). A possible transglycosylation reaction, catalysed by an enzyme preparation from *Vigna* epicotyls, has been reported to cause an increase in the molecular weight of a xyloglucan fraction *in vitro* (Nishitani and Tominaga, 1991) which could be due to action of an end-acting XET. However, this xyloglucan fraction became insoluble and was therefore removed from further reactions, this could have biased the overall reaction towards production of high molecular weight xyloglucan.

Cutting of a xyloglucan chain near to the reducing end followed by transfer onto the non-reducing end of another xyloglucan chain (see Figure 4.2) would be a more efficient mechanism for incorporation of newly-secreted xyloglucan than repeated cutting near the non-reducing end of a xyloglucan chain and transfer onto the non-reducing end of another xyloglucan chain (see Figure 4.3), although both could result in the incorporation of newly-secreted xyloglucan (as donor or acceptor in the reaction) into the cell wall matrix with a concomitant increase in M_r of newly-secreted xyloglucan.
Figure 4.2 Diagrammatic representation of the action of a reducing end attacking XET in interpolymeric transglycosylation. Repeated action with a xyloglucan oligosaccharide as the acceptor will only switch the subunit at the reducing end of the xyloglucan chain. Each circle represents one cellotetraose-based subunit of xyloglucan, with the reducing end to the right-hand side; the donor is on the left and the acceptor on the right.

Figure 4.3 Diagrammatic representation of the repeated action of a non-reducing end attacking XET in interpolymeric transglycosylation. Each circle represents one cellotetraose-based subunit of xyloglucan, with the reducing end to the right-hand side; the donor is on the left and the acceptor on the right.
Low M, xyloglucan (below ~10 kDa) is reported to be a poor donor substrate for Vigna XET (Nishitani, 1992); this work has demonstrated that newly-secreted xyloglucan has a low M, (≤10 kDa; see sections 3.3 and 3.4); therefore, newly-secreted xyloglucan may not act as a donor for XET in the cell wall to a large extent and may be incorporated into the cell wall matrix by acting as an acceptor for end-acting XETs. If newly-secreted xyloglucan acts as the acceptor in the majority of XET-catalysed reactions, then incorporation of the newly-secreted xyloglucan into the cell wall and concomitant increase in molecular weight of the wall-bound xyloglucan can only occur if a reducing-end attacking XET is involved (see Figure 4.4).

![Diagram](image)

*Figure 4.4 Diagrammatic representation of reducing-end attacking XET action in interpolymeric transglycosylation with wall-bound xyloglucan as the donor (on the left) and newly-secreted xyloglucan as the acceptor (on the right); sections of xyloglucan directly bound to cellulose are underlined. Lengths of molecules are not shown to scale. Each circle represents one cellotetraose-based subunit of xyloglucan, with the reducing end to the right-hand side.*

### 4.3 Functions of XET *in vivo*

XET-related gene families have been discovered in tomato and Arabidopsis (Arrowsmith and de Silva, 1995; Xu *et al.*, 1995). These genes may encode XETs with differing sites of action (*e.g.* cell wall region-specific, cell-specific, tissue-specific and organ-specific XETs or wall-bound XETs). The expression of these genes may be developmentally and/or environmentally regulated to differing degrees.

The XETs encoded by these genes may have different substrate specificities; it is known that some seed XETs can use water as well as xyloglucan as the acceptor (Farkas *et al.*, 1992; Fanutti *et al.*, 1993). The
diversity of XET genes may reflect different XETs with different specificities for the site of attack on the donor substrate (e.g. end attacking or mid-chain attacking) as discussed above (see section 4.2.1).

4.3.1 XET may cut and re-form xyloglucan cross-links

Mid-chain cleavage followed by transfer onto the non-reducing end of an unbound section of wall-bound xyloglucan may convert 'taut' cross-links into 'relaxed' cross-links (see Figure 4.5), this mechanism could give limited loosening of the cell wall and allow 'creep' extension of the cell wall (see section 1.3.2). The acceptor may be newly-secreted xyloglucan which has become hydrogen-bonded to a newly-deposited cellulose microfibril, or older, wall-bound xyloglucan.

![Diagram of xyloglucan cross-links and microfibril movement](image)

*Figure 4.5 Diagrammatic representation of a mechanism by which XET action could break and reform cross-links, allowing 'creep' extension.*

4.3.2 XET may 'loosen' the cell wall

It has been suggested that xyloglucan breakdown (in response to exogenous auxin or low pH) may be an important wall loosening process,
but may not be the only mechanism of wall loosening (Bret-Harte and Talbott, 1993). The mechanism behind endogenous growth may be different from the mechanism promoted by addition of high levels of auxin; the \( M_r \) of xyloglucan along cucumber stems during endogenous growth was demonstrated not to change with extensibility (Wakabayashi et al., 1993). During auxin-induced elongation of maize coleoptiles, 'osmiophilic particles' (OPs) have been observed to accumulate; these have been proposed to contain a 'wall loosening factor' (WLF; Kutschera et al., 1987; Schopfer, 1991). OPs have been observed to increase in number at the upper (growth-inhibited) section during shoot gravitropism and in response to Brefeldin A treatment of rye coleoptiles (Edelmann and Sievers, 1995; Edelmann and Volkmann, 1996). Brefeldin A has also been reported to cause an accumulation of densely-packed vesicles containing large amounts of xyloglucan (Driouich et al., 1993b). Therefore, the WLF may be xyloglucan; it is known that growth requires continued deposition of cell wall polymers (except cellulose; Brummell and Hall, 1985; Kutschera and Briggs, 1987) and incorporation of newly-secreted polysaccharides has been indirectly related to growth (Hoson and Masuda, 1992).

Mid-chain cleavage of a xyloglucan cross-link followed by transfer onto the non-reducing end of a xyloglucan oligosaccharide or of an unbound, newly-secreted xyloglucan polymer would cause a loosening of the cell wall (see Figure 4.6). This process may be responsible for the auxin-mimicking effect of xyloglucan oligosaccharides which can act as acceptors for XET (see section 1.4.4.2). This mechanism may also explain the promotion of loosening of the cell wall during DCB treatment observed by Edelmann and Fry (1992c); during inhibition of cellulose synthesis there will be fewer binding sites for newly-secreted xyloglucan, causing the majority of newly-secreted xyloglucan to remain unbound, increasing the availability of potentially wall-loosening XET acceptor substrates.
**Figure 4.6** Diagrammatic representation of a mechanism by which XET action could promote 'loosening' of the cell wall.

4.3.3 XET may 'tighten' the cell wall

Cleavage of an unbound (non-crosslinking) section of wall-bound xyloglucan either mid-chain or near the reducing end followed by transfer onto the non-reducing end of an unbound section of wall-bound xyloglucan would cause direct cross-link formation (see Figure 4.7). This type of mechanism would increase the number of cross-links in the cell wall. The acceptor may be newly-secreted xyloglucan which has become hydrogen-bonded to a newly-deposited cellulose microfibril, or older, wall-bound xyloglucan.

If this mechanism is catalysed by reducing-end attacking XET, and the acceptor molecule is an unbound xyloglucan molecule, then the increase in molecular weight of the wall-bound xyloglucan will increase the probability of each xyloglucan molecule encountering two or more microfibrils. This will increase the chances of cross-link formation; also, the wall-bound
xyloglucan could form more cross-links per molecule and has the potential to form longer cross-links, perhaps between non-adjacent microfibrils.

Figure 4.7 Diagrammatic representation of a mechanism by which XET action could promote 'tightening' of the cell wall.

4.4 Expansins may also modify the cellulose/xyloglucan network.

In addition to hydrolysis catalysed by β-1,4-glucanases and endotransglycosylation catalysed by XETs, the cellulose/xyloglucan network may be subject to modification by a recently discovered family of non-glycosylated wall-bound proteins named expansins. Expansins have been found in many plant tissues from several dicots and Graminaceous monocots; the genes encoding expansins are members of a highly-conserved multigene family. These proteins have the ability to loosen cell walls (and filter paper) when added in vitro despite having no hydrolytic or transglycosylase activity. It has been proposed that expansins loosen cell walls gradually by disrupting hydrogen-bonds between cellulose microfibrils and other cell wall polysaccharides, with 'junction zones' between microfibrils and xyloglucan (or between microfibrils and mixed-
linkage β-(1→3),(1→4)-glucans in the grass family) being the most likely targets (McQueen-Mason et al., 1992, 1993; Li et al., 1993; Fry, 1994; McQueen-Mason and Cosgrove, 1994, 1995; Taiz, 1994; McQueen-Mason, 1995; Shcherban et al., 1995). This mode of action of expansins would allow cross-linking sections of xyloglucan to be lengthened without any breakage, allowing limited cell expansion. Expansins could also remove cross-links, promoting cell wall loosening, if they release the end of a xyloglucan tether from the cellulose microfibril it was formerly bound to; the cross-link may be re-formed by XET acting as described in section 4.3.3.

4.5 Conclusions

The balance between the localised secretion of xyloglucan and deposition of microfibrils into the cell wall may affect the extensibility of the cell wall by altering the ratio of wall-loosening to wall-tightening XET acceptor substrates. The balance between mid-chain attacking and end-attacking XET actions may provide another control point for cell expansion. Cellulase action (or possibly mid-chain attacking XET using xyloglucan oligosaccharides as the acceptor) may play a role in the trimming of non-bound sections of wall-bound xyloglucan, decreasing the load-bearing potential of older layers of the cell wall. Exo-hydrolysis (e.g. by α-xylosidase) at the non-reducing end of xyloglucan would provide yet another control point by affecting their suitability as acceptor substrates for XET (see section 1.5.2.1). Other enzymes, such as cellulases and expansins may act together with XET in modifying the cellulose/xyloglucan network.

These factors, either alone or in combination, would provide a very subtle and complex mechanism for the regulation of cell expansion as would be expected for such a fundamental process. The effects of exogenous auxin and changes of turgor on xyloglucan metabolism in the cell wall may represent only the extremes of plant responses.
5. References


xyloglucan 4-β-D-glucosyltransferase and 6-α-D-xylosyltransferase. *Journal of Biological Chemistry* 256:11117-11122.


pea epicotyl membranes. *Archives of Biochemistry and Biophysics* **294**:200-205.


Shibuya, N. and Misaki, A. (1978). Structure of hemicellulose isolated from rice endosperm cell wall: Mode of linkages and sequences in
xyloglucan, β-glucan and arabinoxylan. *Agricultural and Biological Chemistry* **42**:2267-2274.


6. Publications

Poster presented at the 3rd Scottish Cell Wall meeting, held on the 8th of April, 1993 at Stirling University.

**Changes in molecular weight distribution of xyloglucan in suspension-cultured Rose cells with age of cell.**

*James Thompson*

*Centre for Plant Science, Daniel Rutherford Building, King's Buildings, University of Edinburgh. EH9 3JH.*

**Background.** Xyloglucan is a hemicellulosic polysaccharide which constitutes 20-25% of the dry weight of primary cell walls of Dicotyledons. It is therefore an important structural component.

Xyloglucan tightly binds to cellulose via hydrogen bonds. Each xyloglucan molecule could form a cross-link between cellulose microfibrils in the primary cell wall, thereby resisting cell expansion.

Previous work has shown that the bulk of xyloglucan in the cell wall decreases in molecular weight during acid- or auxin-stimulated growth. These effects were studied over a short timescale and the changes in xyloglucan M, which occur over a longer period without such treatments are now being investigated.

**Method.** [3H]Arabinose was added to suspension-cultured rose cells at 9 days after subculturing. Samples were removed at several time points up to 7 days after addition of the tracer. Hemicellulose was extracted from these samples in 6M NaOH at 37°C for 24h. This crude xyloglucan preparation was then fractionated according to M, by gel permeation chromatography on Sepharose CL-6B gel eluted with pyridine/acetic acid/water (1:1:23).

The resulting fractions of each hemicellulose extract were dried under vacuum and subjected to enzymic digestion using purified Driselase, followed by paper chromatography to separate the products. To identify the whereabouts of xyloglucan in the original fractions, the product XG2 (D-xylopyranosyl-α-(1→4)-glucose was scintillation counted (as well as other products).

XG2 only arises from Driselase digestion of xyloglucan so any shift in the profile of XG2 in the fractions between different samples reflects a change in M, of xyloglucan in the time between those samples being taken.

Previous work has shown that >95% of the [3H]arabinose is taken up within 2h after its addition, so this experiment is a pulse-chase type in which the xyloglucan is monitored as it ages in the cell wall.

**Results.** Preliminary results from this experiment suggest that the molecular weight of xyloglucan in the primary cell wall of rose cells decreases as the age of the cells increases. The results also show that incorporation of [3H] into xyloglucan reaches a peak at 6h after addition of [3H]arabinose, then the label is gradually lost from xyloglucan in the cell wall until at 7 days only 50% of the maximum amount of [3H]xyloglucan initially incorporated is still present in the cell wall.

**References.**

Xyloglucan metabolism in the primary cell walls of suspension-cultured rose cells.

J.E. Thompson & S.C. Fry, Centre for Plant Science, University of Edinburgh.

Xyloglucan is the major hemicellulosic polysaccharide in the primary cell walls of Dicotyledons. It is well established that the molecular weight of the bulk of xyloglucan in cell walls decreases during acid or auxin-induced cell growth. A role for xyloglucan in cell growth has been suggested in which xyloglucan molecules form tethers between cellulose microfibrils in the cell wall, resisting turgor-driven cell expansion.

To investigate the constitutive changes in molecular weight distribution of xyloglucan molecules as they 'age' in the primary walls of living cells, a pulse-chase radiolabelling method was used to create a 3H-labelled population of xyloglucan molecules in suspension-cultured rose cells. The fate of these xyloglucan molecules could then be followed as the cells aged over the subsequent 7 days. Most growth of the rose cells occurred during the period between 4 and 9 days after subculturing. By pulse-labelling different cell cultures at 4d and 9d after subculturing, the changes in molecular weight distribution of xyloglucan molecules occurring during rapid and slow cell expansion could be compared.
Application of a density labelling technique to detect transglycosylation between xyloglucans in muro

James E. Thompson and Stephen C. Fry
ICMB, The University of Edinburgh, King’s Buildings, Edinburgh. EH9 3JH, UK.

Background. Xyloglucan hydrogen-bonds to cellulose and is presumed to form cross-links between cellulose microfibrils. Breakage and regeneration of these tethers could provide the reversible wall weakening required for cell expansion. This is a proposed role of xyloglucan endo-transglycosylase (XET), an enzyme that can cut the backbone of a xyloglucan molecule and transfer the newly-formed, potentially reducing terminus to the non-reducing terminus of the backbone of another xyloglucan chain [1,2]. XET activity has been demonstrated in vitro, but the major obstacle to demonstrating polymer-to-polymer transglycosylation in muro is that the products of such a reaction may be identical to the reactants. Density labelling can overcome this kind of problem [3].

Data. In experiment 1, suspension-cultured rose cells were transferred to and maintained in a medium containing [13C]glucose as the only carbon source; 1 h after this transfer, a pulse of [3H]arabinose was supplied. This produced a pool of wall-bound [13C,3H]xyloglucan that was distinguishable from [12C]xyloglucan by isopycnic centrifugation. Transglycosylation between the wall-bound [13C,3H]xyloglucan and the older, wall-bound [12C,1H]xyloglucan would decrease the density of the radiolabelled xyloglucan. No such decrease in density occurred between 1 h and 7 days after the addition of [3H]arabinose.

In experiment 2, the cells were fed [13C]glucose for 1 week, the last 6 h of which included a pulse of [3H]arabinose, followed by a chase with [1H]arabinose and [12C]glucose. Transglycosylation between the wall-bound [13C,3H]xyloglucan chains and the newly secreted, non-bound [12C,1H]xyloglucan would decrease the density of the radiolabelled xyloglucan. Such a decrease was observed.

Conclusion. In cultured rose cells little or no transglycosylation occurs between pairs of wall-bound xyloglucan chains. However, transglycosylation may accompany, and is proposed to cause, the integration of new xyloglucan into the cell wall, perhaps simultaneously breaking tethers.

Acknowledgement. This research was supported by the European Commission.

Paper presented at the 7th Cell Wall Meeting, held from the 25th to the 29th of September, 1995 in Santiago de Compostela, Spain.

Xyloglucan endotransglycosylase (XET): A novel “dot-blot” assay and evidence for the site of attack on the donor substrate

Stephen C. Fry and James E. Thompson
ICMB, The University of Edinburgh, Mayfield Road, Edinburgh EH9 3JH, U.K.

Background. XET is widespread in plant cell walls [1]. It cuts the backbone of a donor xyloglucan chain and joins the new potentially reducing terminus to an acceptor substrate. Density-labelling experiments, in which [13C]glucose and [31-1]arabinose were fed to cultured rose cells, demonstrated interpolymeric transglycosylation in vivo between newly-secreted xyloglucan chains and existing wall-bound chains [2], suggesting a role for XET in the assembly of new xyloglucan into the cell wall. For transglycosylation to operate efficiently in “assembly” mode, XET would have to cause maximal integration, with minimal concomitant excision, of xyloglucan. This could be achieved by preferential cleavage of the donor substrate close to its reducing terminus. We have therefore explored the pattern of attack of XET along the backbone of its donor substrate.

Data. Dot-blot test for XET activity. A novel XET dot-blot was devised using paper impregnated with xyloglucan and a fluorescent derivative of XLLG. A 4-μl droplet of test solution was pipetted on to the paper, which was incubated under humid conditions for 1 h, then washed to remove unreacted fluorescent XLLG; remaining paper-bound (high-Mr) fluorescent material indicated XET activity. The assay was amenable to the assay of crude plant extracts and column fractions and to tissue-printing. Crude extracts of a wide range of plant materials had high XET activity according to the dot-blot; XET from rose cell culture spent medium was selected for further work.

Site of attack on donor substrate. Rose XET was incubated with either (a) [rt-3H]-xyloglucan + XLLG01, or (b) xyloglucan +[rt-3H]XLLG01. Concentrations of reactants in (a) and (b) were identical. Initial rates of (a) production or (b) consumption of 3H-labelled, Glc4-based oligosaccharides were monitored by TLC. In assay (b), consumption of 1 mol of XLLG01 in was assumed to reflect the cleavage of 1 mol Glc4 repeat-units in the xyloglucan. Assay (b) showed that an average Glc4 repeat-unit in the donor substrate had a probability of 0.84% h⁻¹ of being cleaved. Assay (a) showed that one particular Glc4 repeat-unit (that adjacent to the rt) had a probability of 0.48% h⁻¹ of being cleaved; the rate of release of Glc8-based oligosaccharides in (a) was lower still.

Conclusion. The average cleavable bond in xyloglucan was slightly more susceptible to cleavage by rose XET than were the two cleavable bonds nearest to the rt. It thus remains unclear how XET may drive the net integration of newly secreted xyloglucan into the cell wall fabric.

Acknowledgement. Supported by the European Commission.


Abbreviations. rt= reduced or reducing terminus; XLLG = a xyloglucan-derived nonasaccharide (Glc4 Xyl3 Gal2); XLLG01 = NaBH4-reduced XLLG.