Evolution and Diversity of the Primary Cell Wall in Green Plants

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This thesis has been composed by myself and the work, of which it is a record, has been carried out by myself. All sources of information have been specifically acknowledged by means of a reference.

Zoë A. Popper, Edinburgh 2001.

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To my parents,
with love

Abstract

Evolution has a major influence on the plant cell wall and variation in primary cell wall (PCW) composition is known to exist between different angiosperm taxa. The PCWs of lower land plants have not been well studied. It is of interest to see what changes have taken place in PCW composition during plant evolution.

One of the main qualitative variables within angiosperms is the presence of mixed-linkage β-(1→3),(1→4)-glucan (MLG) in gramineous monocots and apparent absence from non-gramineous monocots and dicots. Anomalously, the presence of MLG had been reported in the dicot *Phaseolus aureus*. In the present work, MLG was not detected (< 0.02% w/w of the cell wall) in *P. aureus* hypocotyls or in PCWs of all non-gramineous land plants tested (dicots, monocots, pteridophytes and bryophytes). One exception was *Flagellaria guineensis*, a close relative of the gramineous monocots, which on licheninase digestion produced the characteristic tri- and tetrasaccharide of MLG. MLG is therefore restricted to the Poaceae and some closely related members of the Poales.

Xyloglucan was found in all land plants tested including bryophytes. Methylation analysis had indicated some of the glycosyl linkages typically found in xyloglucan are present in a cell wall polymer from the charophyte *Nitella*. However, Driselase-digestible xyloglucan was not detected (< 0.01% w/w of the cell wall) in *Chara*, a charophyte, thought to be closely related to land plants. In addition, LCWs of *Chara*, two other charophytes and *Ulva lactuca* were not digested to xyloglucan-derived oligosaccharides by cellulase or xyloglucan-specific endoglucanase. Land plants are thought to have originated from a single species of charophyte: it is therefore likely the putative ancestor, unlike other charophytes, had xyloglucan in its cell walls.

The lycopodiophyte LCW composition differed from that of the euphyllophytes. 3-*O*-Methyl-D-galactose was isolated from LCWs of the lycopodiophytes, a monophyletic group, but was not detected at comparable levels in any other land plants.

Bryophyte LCWs contained higher concentrations of pectic polysaccharides (indicated by high concentrations of uronic acids) than LCWs of the remaining land plants. It was also found that the LCW of the vegetative tissues of leptosporangiate ferns, gymnosperms and angiosperms contained a far lower concentration of mannose residues than the bryophytes, lycopodiophytes, equisetophytes and psilotophytes.

Variation in cell wall composition appears to be particularly pronounced between monophyletic groups of plants and may correlate with changed ecological pressures.

Abbreviations

AIR	Alcohol-insoluble residue
Ara	L-Arabinose
B:A:W	Butanol:acetic acid:water, either 12:3:5, 3:1:1 or 2:1:1 as stated
B:Py:W	Butanol:pyridine:water 4:3:4
CDTA	*trans*-1,2-Diaminocyclohexane-*N,N,N',N'*-tetraacetic acid
DHA	3-Deoxy-D-*lyxo*-2-heptulosaric acid
DMSO	Dimethylsulphoxide
DP	Degree of polymerisation
$EDTANa_2$	Ethylenediamine-tetraacetic acid disodium salt
EDTAFeNa	Ethylenediamine-tetraacetic acid ferric-sodium salt
E:Py:W	Ethyl acetate:pyridine:water, either 10:4:3 or 8:2:1 as stated
E:Py:A:W	Ethyl acetate:pyridine;acetic acid:water, 5:3:1:1
Fuc	L-Fucose
FW	Formula weight
g	Grams
g	Gravity
G2	Cellobiose [β-D-glucopyranosyl-(1→4)-D-glucose]
Gal	D-Galactose unless stated L-Gal
GalA	D-Galacturonic acid
Glc	D-Glucose
GlcA	D-Glucuronic acid
GulA	D-Guluronic acid
HPLC	High-pressure liquid chromatography
IdA	D -Iduronic acid
IP	Isoprimeverose [α-D-xylopyranosyl-(1→6)-D-glucose]
KDO	3-Deoxy-D-*manno*-2-octulosonic acid
Lam2–Lam6	Laminarin oligosaccharides (laminaribiose to laminarihexaose)
LCW	Leaf cell wall
m_{GlcA}	Mobility on PE with respect to GlcA

M2–M7	Maltose oligosaccharides (maltose to maltoheptaose)
Man	D-Mannose
ManA	D-Mannuronic acid
MeGlcA	4-*O*-MethylGlcA
min	Minutes
MLG	Mixed-linkage glucan
MLG3–MLG6	Mixed-linkage glucan oligosaccharides
Mya	Million years ago
$N_{2(l)}$	Liquid nitrogen
NMR	Nuclear magnetic resonance
PAD	Pulsed amperometric detector
PC	Paper chromatography
PC(a)	Paper chromatography (ascending)
PC(d)	Paper chromatography (descending)
PE	High voltage paper electrophoresis
R_F	$\frac{\text{Distance moved by compound}}{\text{Distance moved by solvent front}}$
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan-II
Rha	L-Rhamnose
Rib	D-Ribose
R_T	Retention time
SDS	Sodium dodecyl sulphate
t	Time
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
UV	Ultraviolet
XEG	Xyloglucan–specific endoglucanase
XET	Xyloglucan endotransglycosylase
Xyl	D-Xylose
Xyl2	Xylobiose [β-D-xylopyranosyl-(1→4)-D-xylose]

Adaptation	Genetic modification of an organism or feature of an organism that makes it better adapted to a particular environment.
Angiosperms	Flowering plants excluding the gnetophytes. Angiosperms are monophyletic and defined by a suite of characters described in the introduction.
Anthophytes	Vascular plants that bear flowers. Extant anthophytes are angiosperms and gnetophytes.
Autapomorphy	A character state which uniquely defines a taxon.
Bryophytes	Mosses, liverworts and hornworts.
Clade	A phylogenetic lineage of related taxa originating from a common ancestral taxon.
Embryophyte	A member of those plant groups in which an embryo, dependent at least at first on the parent plant, is formed. The extant embryophytes are the byrophytes and vascular plants.
Euphyllophytes	All tracheophytes excluding the lycophytes.
Geological time periods	See Table G1
Gymnosperms	Cycads, conifers, ginkgos and gnetophytes.
Homology	Similarity due to a shared evolutionary history.
Homoplasy	Similarity which has resulted from convergent evolution and therefore reached by independent (not evolutionarily related) lineages.
Inverted repeats	Two regions of DNA that are identical but read in opposite directions. They are not necessarily transcribed.

Long branch attraction	A situation in which strongly unequal evolutionary rates can cause parsimony to fail. Unusually long branches, on a cladogram, tend to connect together whether or not they are closely related. This is because there may be many random changes which can obscure the changes relating to characters which indicate common ancestry.
Molecular systematics	The use of DNA, RNA and protein sequences to infer relationships among organisms.
Monocolpate	(Pollen grain) having one colpus (germinal aperture), synonymous with monoaperturate. These pollen grains are also monosulcate having only one groove.
Monophyletic	(A taxon) arising from diversification of a single ancestor. Only taxa that contain all the descendants of a common ancestor are monophyletic.
Neoteny	Arresting of the normal development of cells except those of the germ line. This results in a sexually mature organism with juvenile characteristics. Large morphological changes can arise from relatively small genetic changes.
Ontogeny	All the changes that occur during the life history of an organism.
Paraphyletic	(A taxon or taxa) originating from and including a single stem species (known or hypothetical) but excluding one or more smaller clades nested within it. An example of a paraphyletic group is the gymnosperms. It is commonly accepted that the angiosperms arose from a gymnosperm ancestor. The gymnoperms do not contain all the descendants of a common ancestor and is therefore paraphyletic.

Parsimony	Ockham's razor stated that you should not generate a hypothesis more complex than the data demand. Parsimony methods aim to give the simplest explanation of the data. Evolutionary trees generated by parsimony methods do not necessarily give the correct phylogeny.
Phylogeny	Evolutionary history.
Sister group	A species, or higher monophyletic taxon, believed to be the closest geneological relative of a given taxon excluding the species ancestral to both taxa. Two taxa forming sister groups are therefore thought to share an ancestral species not shared by any other taxon.
Streptophytes	All living green plants belong to one of two major taxa: the Streptophyta and the Chlorophyta. The streptophytes are a monophyletic group composed of the charophytes (Figure 1.2) and the embryophytes. Lemieux et al. (2000) found molecular evidence which indicated that the Prasinophyceae are actually a paraphyletic assemblage of green algae which only superficially superficially resemble each other. *Mesostigma viridae* (Figure 1.2) was found to predate the evolutionary divergence of green plants into the streptophytes and chlorophytes.
Systematics	The study of biological diversity.
Taxon	Any group of organisms to which any rank of taxonomic name is applied.
Tracheophytes	Vascular embryophytes. There are about 250 000 species.
Tricolpate	(Pollen grain) having three germinal apertures.

Table G1: Geological time periods

Era	Period	Period and Epoch (beginning)	Characteristic plants
Cenozoic	Quaternary	Recent 0.1 Mya	
		Pleistocene 1.9 Mya	Extinction of many temperate tree genera
	Tertiary	Pleiocene 5.1 Mya	Spread of grasslands
		Miocene 25 Mya	Establishment of present forests and grasslands
		Oligocene 38 Mya	Temperate forests at middle latitudes
		Eocene 55 Mya Paleocene 65 Mya	Widespread occurrence of many now relict taxa — *Metasequoia* and many woody angiosperms
Mesozoic	Cretaceous	144 Mya	Angiosperms
	Jurassic	213 Mya	Age of cycads
	Triassic	248 Mya	Diversification of conifers and ferns
Paleozoic	Permian	286 Mya	Rise of conifers — extinction of tree lycophytes
	Carboniferous	360 Mya	Forests of tree ferns, tree lycopods, sphenopsids, clamites and ferns
	Devonian	408 Mya	First seed plants at end of Devonian
	Silurian	428 Mya	Simple vascular plants
	Ordovician	505 Mya	Spores of first possible land plants
	Cambrian	590 Mya	Multicellular marine algae abundant
Precambrian		~ 4,600 Mya	Definite evidence of algae 3,500 Mya

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1 Introduction

Stebbins (1992) stated that 'even the most superficial survey of ecological plant anatomy points to the adaptive importance of cell wall differentiation'. It is largely undisputed that land plants evolved from green algae. It is thought that green algae had existed for 85 million years before the land plants evolved (Cassab 1998). One of the most notable changes that occurred was the development of different kinds of cell walls (Cassab 1998) whose evolutionary history remains a major evolutionary challenge (Kieliszewski and Lamport 1994).

Evidence that a primary cell wall can adapt in a genetically stable way is provided by tobacco cells grown under high saline or drought-mimicking conditions. Their cell walls alter so that they have less cellulose relative to xyloglucan and altered pectic constituents (Iraki et al. 1989a, b, c).

Major agents of evolutionary diversification are mutations of genes that control the growth, size and form of cells, via the "action of [the encoded] proteins on cell walls" (Stebbins 1992). Such proteins would include XETs and expansins. However, a complete description of enzymes involved in cell wall biosynthesis has not been obtained owing to many problems with the purification of membrane-bound polysaccharide synthases so that there are few characterised that can be linked unequivocally with the synthesis of a cell wall polysaccharide (Burton et al. 2000). Owing to some redundancy of the genetic code not all mutations in nucleic acid sequence are expressed as changes in amino acids (amino acids are coded for by codons which consist of three nucleotide residues and in some cases a change in one or even two nucleotides does not alter the amino acid coded for) ; fewer cause major changes in protein performance. Therefore, few mutations will give rise to a change in the phenotype of the plant. Consequently genotype may be less informative for biological interpretations than phenotype (O'Brien and Clegg 1993).

1.1 Cell wall function

1.1.1 Introduction

In general, cells have adopted two strategies for dealing with the osmotic problems associated with living in an aqueous environment (Gerhart and Kirschner 1997). Plant and bacterial cells have either retained or secondarily evolved semi-inextensible cell walls that resist the turgor pressure generated by osmosis. Wall-less animal cells can pump water out of the cell at the rate at which it enters to prevent the cells bursting.

The cellulose-rich cell wall, present in green plants including some phytoplankton, was first described in the seventeenth century and was considered to be an inert skeleton. However, the cell wall has since been shown to be fundamentally involved in many plant processes (Goldberg 1995). The cell wall is dynamic and continually modified by enzyme action during growth, development, environmental stresses and infection (Cassab 1998). Cell walls are involved in support, structure, growth, multicellularity, differentiation and defence and are therefore likely to be under rigorous selection pressure.

1.1.2 Multicellularity and differentiation

Cell wall biosynthesis and differentiation are accurately regulated in a temporal, spatial and developmental manner. The cell wall governs the shape and size of the cell and fastens it to its neighbours. A correlation has been found between an increased cell size and increased ecological diversity in related groups (Stebbins 1992). Stebbins (1992) postulates that alterations in the cell wall composition may have been involved in bryophyte diversification and have played a leading role in the evolution of leptosporangiate ferns from the eusporangiate ferns.

Multicellularity results when two or more neighbouring cells adhere, interact and physiologically communicate. It increases the total surface area of cell membranes for a given size of organism, increasing the ability to exchange chemicals between the plant and its external environment. The apoplastic pathway, made up of the

network of plant cell walls, is important in transport of materials around the plant. The infrastructure of plant cell walls also provides support. Diversity of cell shape, based on wall growth, is great among leaves. It serves functions such as maximising exposure to light, giving protection from overheating, allowing abscission and dormancy under unfavourable conditions, and enabling recovery from insect damage and grazing by large animals (Stebbins 1992). Morphogenesis in plants is the result of differential growth of the organs at the level of cell walls (Kaplan and Hageman 1991). Multicellularity also allows some cells to become specialised at specific tasks. Arabinogalactan proteins present in the cell wall are thought to play a major role in cell–cell interactions and have been shown to have a morphoregulatory role in bryophyte (Basile 1980, Basile and Basile 1983, 1987) and higher plant development (Kreuger and van Holst 1996). The metabolism of some cell wall polysaccharide components may be altered by gravity. Under hypergravity the activity of xyloglucan-degrading enzymes is decreased owing to an increase in the apoplastic pH (Soga et al. 2000). A transition from an aquatic to terrestrial environment could affect cell wall metabolism.

1.1.3 Defence

Multicellularity increases competitiveness. The increased size conferred on a plant by being multicellular means that herbivory will not always result in complete consumption of the plant. Under pathogen attack affected cells can be isolated and sacrificed. Cell walls provide a physical barrier to bacterial, viral and fungal pathogens. However, many pathogens secrete enzymes capable of degrading cell wall components. Those which degrade polysaccharides are among the most specific. Many are only able to hydrolyse a glycosidic bond between two specific monosaccharides and often even then the bond needs to be between two particular carbon atoms and of a particular anomeric configuration. This means that changes that affect one type of bond, especially since many polysaccharides are made up of repeating units, can alter the susceptibility of a polysaccharide to enzymic degradation. There is a wide variety of plant cell wall polysaccharide structure and pathogen-resistant varieties may be selected for on the basis of specific wall polysaccharide constituents (Albersheim et al. 1969). For example, there is

evidence that MLG has antiviral activity which is active early in the process of viral replication (Stübler and Buchenauer 1996).

1.2 Cell wall type

1.2.1 Primary cell wall

The primary cell wall, typically 0.1 μm to 10 μm thick, is deposited during growth in cell surface area. The primary cell wall defines the cell shape and thereby contributes to the structural integrity of the entire plant. New walls are usually formed soon after mitosis, dividing the mother cell in two. The siting of a new wall is directed, in charophycean algae and land plants, by the phragomoplast, a cluster of microtubules. At maturity some common cell types (parenchyma and collenchyma) frequently only have a primary cell wall.

The primary cell wall is biphasic, being composed of crystalline cellulosic microfibrils which lie in the plane of the cell surface and are embedded in a matrix of gel-like non-cellulosic polysaccharides and glycoproteins (Fry 1988). According to current cell-wall models the cellulosic framework is interconnected with hemicellulosic polysaccharides, such as xyloglucan, MLG and arabinoxylan, forming a cellulose–hemicellulose network (Carpita and Gibeaut 1993). Xyloglucan and MLG have been shown to bind to cellulose (Mishima et al. 1998). The cellulose–hemicellulose network coexists with another network that consists of the pectic polysaccharides homogalacturonan, RG-I and RG-II. The structural inextensibility and strength of the wall are thought to depend on the integrity of the cellulose–hemicellulose network. Recent evidence indicates that hemicellulose–pectin linkages may also be important components in the structural inextensibility of the wall. About 30% of the total xyloglucan extracted from the cell walls of suspension-cultured rose cells has been found to be anionic, a property not induced by the extraction procedure (Thompson and Fry 2000). Arabinanase, galactanase and endopolygalacturonase digestion converted appreciable proportions of the anionic xyloglucan to neutral material showing about 30% of xyloglucan in cell walls of suspension-cultured rose cells exists covalently linked to acidic pectins (Thompson and Fry 2000). Enzyme-catalysed modification of the hemicellulose is

considered to be essential for wall expansion during cell growth (Talbott and Ray 1992).

The composition and structure of the primary cell wall of higher plants studied so far appear to be comparable although not identical (Albersheim 1976). The primary cell wall of bryophyte gametophytes is likely to be homologous to that of tracheophyte sporophytes as they both respond in a similar way to auxin and ethylene (Basile 1990, Thomas et al. 1984). Both liverwort (*Marchantia polymorpha*) and moss gametophyte (*Mnium hornum*) tissues have been shown to have XET activity at levels comparable with those found in angiosperm sporophyte tissue (Fry et al. 1992).

1.2.2 Secondary cell wall

The secondary cell wall, if present, is laid down internally to the primary cell wall once cell growth has ceased. The composition and ultrastructure, in higher plants, varies from one cell type to another as well as between plant species. This therefore makes it unsuitable for comparative evolutionary studies. Many secondary cell walls are lignified, for example xylem cells, which increases wall strength. However, the primary cell wall can also contain lignin as lignification begins in the primary cell wall and extends inward to the secondary cell wall (Franz and Blaschek 1990).

1.2.3 No cell wall

All living green plants belong to one of two major phyla: the Streptophyta (Bremer 1985) (the land plant lineage) and the Chlorophyta (Sluiman 1985) (the lineage of green algae which diverged before the land plant lineage). Genetic data suggest that *Mesostigma*, an alga in the family Prasinophyceae, is the earliest diverging extant member of the Streptophyta (Lemieux et al. 2000). Members of the Prasinophyceae possess scales on their cell surface instead of a cell wall (Becker et al. 1995). In some more advanced genera, *Tetraselmis* and *Scherffelia*, the scales fuse to form a theca or cell wall (Becker et al. 1995). Thecae and scales both consist of acidic polysaccharides (70% of dry mass), glycoproteins (10% dry mass) and cellulose

(20% dry mass) (Becker et al. 1996, Domozych et al. 1991). The acidic polysaccharides are characterised by high amounts of unusual 2-keto-sugar acids: 3-Deoxy-L-*manno*-2-octulosonic acid (KDO), 3-Deoxy-5-*O*-methyl-L-*manno*-2-octulosonic acid and 3-Deoxy-D-*lyxo*-2-heptulosaric acid (DHA) (Becker et al. 1996). KDO and DHA have also been isolated from RG-II, a minor component of the angiosperm primary cell wall (York et al. 1995).

1.3 Components of the primary cell wall of seed plants

During mitosis the calcium salts of pectins are deposited in the cell plate forming the middle lamella which cements the cells together (Brett and Waldron 1996, Knox et al. 1990). The primary cell wall originating from both the daughter cells is laid on to the middle lamella and surrounds the protoplast. The primary cell wall is composed of a cellulose–xyloglucan network which makes up approximately 50% of the wall dry mass, pectic polysaccharides (approximately 30% of the wall dry mass) and structural proteins (Carpita and Gibeaut 1993). The pectins in some species may be cross-linked to other pectins and non-cellulosic polysaccharides by ester linkages with di(hydroxycinnamic acids) such as diferulic acid (Fry 1986). The primary cell wall is highly hydrated, being approximately 60% water (fresh weight) (Franz and Blaschek 1990).

1.3.1 Cellulose

Payen first described cellulose in the cell wall in 1842. It has since been found in some fungi, bacteria and animals. No other cell wall polysaccharide is known to be so widespread. Fundamentally the primary cell wall is a network of cellulose microfibrils, each of which is several dozen linear chains of (1→4) linked β-D-glucose residues (0.15–7.2 μm chain length; Franz and Blaschek 1990), hydrogen-bonded to form long semi-crystalline strings that wrap around each cell. The microfibrils are 5–15 nm wide and are spaced 20–40 nm apart (McCann et al. 1990). Glucose residues are in the chair formation, increasing the hydrophobicity of the polymer (Franz and Blaschek 1990). The primary cell wall is about 20% (dry mass) cellulose and the secondary cell wall can be up to 50% cellulose (dry mass).

Specialised cell walls, such as those of cotton 'fibres', can be up to 98% (dry mass) cellulose.

1.3.2 Mixed-linkage glucan

1.3.2.1 Introduction

MLGs were first noted by Kjeldahl (1881). They were later described as β-amylan which was 'isolated with some difficulty from some barley's' (O'Sullivan 1882).

1.3.2.2 Structure, organisation and solubility

About 90% of the MLG chain consists of β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranose (MLG3) and β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-β-D-glucopyranose (MLG4) residues joined by (1→4)-linkages (Woodward et al. 1983). However, blocks of up to 14 adjacent β-(1→4)-linkages have been reported from barley MLG (Gómez et al. 1997b). A member of the family of the fresh water green algae, Xanthophyceae (*Monodus subterraneus*), has an MLG with longer stretches of 1→4 linkages as a principal cell wall polysaccharide (Beattie and Percival 1962, Ford and Percival 1965, Stone and Clarke 1992). The β-(1→4)-linked stretches are fairly rigid, but the β-(1→3)-linkages give some flexibility and an irregular shape to the polymer. It is thought that this enables the molecule to be soluble in water. Up to 65% of barley MLG is extractable into water at 65°C (Anderson et al. 1978). However, cold water is not a good extractant for MLG (Gómez et al. 1997a). Forrest and Wainwright (1977) found there were three types of MLG, varying in their extractibility, present in barley endosperm. Differences in observed extractablilities may be accounted for by different arrangements of β-(1→3)-linkages (Forrest and Wainwright 1977). All three types of MLG were reported to be covalently bound to protein (Forrest and Wainwright 1977). The polymer is reported to have a molecular weight in the range 150,000–300,000 (Woodward et al. 1983). It may form labile aggregates with other MLG molecules, cellulose microfibrils or unsubstituted regions of arabinoxylan and has been found bound to pectin (Woodward et al. 1983; Gómez et al. 1997a).

1.3.2.3 Function

Proportions of MLG present vary developmentally. MLG is a major component of mature starchy endosperm walls, e.g. 75% of the dry mass, and a minor component of mature vegetative cell walls, e.g. 3% of the dry mass (Bacic et al. 1988). The highest concentrations of MLG in coleoptiles are reached during expansion growth (Brown et al. 1997). Both MLG concentration and degree of polymerisation appear to decrease with maturity as does the ratio of (1→3) to (1→4) linkages (Buchala and Wilkie 1970, 1973). In view of location and developmental stage at which it is found both storage and structural roles are suggested for MLG (Wada and Ray 1978). A reduction in concentration of MLG correlates to cell growth and it is likely MLG is involved in growth. Proteins secreted in response to the action of the growth hormone auxin may cause modifications in cell wall polymers leading to wall loosening and allowing expansion growth. The action of auxin enhances MLG degradation (Sakurai et al. 1979; Yamamoto et al. 1980). MLG breakdown is often associated with cell wall loosening (Hoson and Masuda 1995). In gramineous monocots MLGs are among the most prominent matrix polysaccharides to undergo auxin-induced turnover. Concanavalin A and specific anti-MLG antibodies have been shown to suppress auxin-induced elongation of grass segments (Inouhe and Nevins 1991, Hoson et al. 1992, Hoson and Masuda 1995). The appearance of MLG during cell expansion, the association of hydrolysis of MLG *in muro* and acceleration of its hydrolysis by growth regulators all imply direct physical involvement of the polymer in growth (Carpita and Gibeaut 1993).

1.3.2.4 Taxonomic distribution

MLG is interesting taxonomically as, apart from a few anomalies, it appears to be confined to gramineous monocots. It is even present in the Bambusoideae, a subfamily of grasses which are markedly different from the other subfamilies as their stems are distinctly wood-like and perennial and their deciduous leaves survive more than a year (Wilkie and Woo 1976). Smith and Harris (1999) isolated MLG from the cell walls of five Poales families: Anarthiaceae, Centrolepidaceae, Ecdeiocoleaceae, Flagellariaceae and Poaceae. Therefore MLG is not unique to the Poaceae but is present in other closely related taxa. MLG has been controversially

reported in two dicots, regenerating tobacco protoplasts and 3-day-old mung beans. However, the reported presence in tobacco (Hensel and Franz 1988), although in a rapidly growing system, seems unlikely as the authors suggest laminaribiose and glucose produced by digestion of the cell wall with a specific β-glucanase, licheninase (E.C.3.2.1.73), are indicative of MLG. The enzyme, however hydrolyses a (1→4)-linkage where the D-glucose residue is substituted at position 3. Hydrolysis of MLG with licheninase should therefore yield the oligosaccharides MLG3 and MLG4. However, licheninase is often contaminated by amylase and whilst amylase cannot hydrolyse MLG it may digest starch in the cell wall preparation, although not to laminaribiose. Although MLG was reported from 3-day-old mung beans (Franz 1972, Buchala and Franz 1974) later studies on 2-day-old mung beans failed to confirm these results (Kato and Masuda 1976). This and the absence of MLG in the related *Vicia rosea* (Takeuchi and Komamine 1981) make reports of its presence in mung beans controversial. However, MLG may fail to be found owing to its solubility, so it may be lost in a solvent, or owing to the short time in development at which it may be present.

In addition to its presence in gramineous monocots, MLG is unambiguously present in *Monodus subterraneus* (a freshwater green alga) and occurs in the thallus of several lichens, such as *Cetraria islandica* (Clarke and Stone 1963). It is unknown whether the MLG in lichens is derived from the algal or fungal component.

1.3.3 Xyloglucan

1.3.3.1 Structure and Function

Xyloglucans were first found as amyloids in the cell walls of plant seeds (e.g. *Tamarindus* and *Tropaeolum*), where they function as storage polysaccharides (Kooiman 1957, Le Dizet et al. 1987). The importance of xyloglucan to the cell is possibly indicated by the universality of occurrence in the primary cell walls of seed plants (Hayashi 1989). Xyloglucan was originally thought to be confined to dicots but has now been described in many monocots, including rice (Shibuya and Misaki 1978), and in a gymnosperm (Andrew and Little 1997). Xyloglucan may have an important role in auxin-induced cell wall loosening. Auxin was reported to have no

effect on arabinoxylans (Inouhe et al. 1984). However, Darvill reported (1978) an auxin-induced pH shift could result in activation of the enzymes which break linkages connecting glucuronoarabinoxylan with other hemicelluloses and proteins. Xyloglucan is suggested to function in cell enlargement during growth (Hayashi 1989) and cell enlargement involves the activity of XET which is able to cut and rejoin intermicrofibrillar xyloglucan chains (Fry et al. 1992, Thompson et al. 1997). During growth of the primary cell wall there is a significant decrease in xyloglucan content and some xyloglucan biodegrades rapidly after acid or auxin treatment (Labavitch 1981).

Xyloglucans contain D-glucose, D-xylose and D-galactose, often in a molar ratio of approximately 4:3:1. It has been suggested that variations in ratio of the component monosaccharides may reflect environmental influences on the biosynthesis of xyloglucan (Buckeridge et al. 1992). All forms of xyloglucan have a (1→4)-linked β-D-glucopyranose backbone (Aspinall et al. 1969) of 200–3000 glucose residues long (Hayashi et al. 1980, Nishitani and Masuda 1982, Fry 1992). The backbone of xyloglucan is therefore qualitatively identical to cellulose. However, the xyloglucan backbone has side-chains.

The structure of xyloglucan allows the β-(1→4)-glucan backbone to hydrogen bond to the β-(1→4)-glucan backbone of cellulose. However, xyloglucan's side-chains prevent it from hydrogen-bonding to itself and influence the binding to cellulose (Hayashi et al. 1994, Vinken et al. 1995). The chemical structures of xyloglucan side-chains can be rigorously established by analysis of the xyloglucan oligosaccharides that are generated upon endoglucanase digestion of the polymer. Xyloglucans bind to cellulose *in vitro* in a pH-dependent manner (the bonds are stable below pH 6, but unstable above pH 6) suggesting the formation of hydrogen bonds is involved in the association of these polymers (Hayashi et al. 1987). Owing to differences in extractability of xyloglucan Pauly et al. (1999) suggested that there are likely to be three xyloglucan domains in most dicot primary cell walls. One domain cross-links cellulose microfibrils and includes any exposed loops and tails that extend away from the microfibril. This domain can be digested with

xyloglucan-specific endoglucanase (Pauly et al. 1999) and is covalently attached to a second domain, xyloglucan which is non-covalently bound to the surface of cellulose microfibrils. The second domain is extracted by alkali (e.g. NaOH) owing to the close association of the xyloglucan and cellulose. A third domain is entrapped within the cellulose and can be released by cellulase (Pauly et al. 1999). Edelmann and Fry (1992) showed that all the xyloglucan in the primary cell wall can be extracted with 6 M NaOH.

1.3.3.2 Taxonomic distribution and variation

The composition and distribution of xyloglucan side-chains (Figure 1.1) varies with the source of the xyloglucan and multiple forms of xyloglucan may be present in a species (Hayashi 1989). The proportion of xyloglucan present also varies with source. Generally xyloglucans comprise 20–25% of the dry mass of the primary cell walls of dicots and 2–5% in gramineous monocots (Fry 1989). However, Hayashi (1989) suggests that the relative amount of xyloglucan in dicot and gramineous monocot primary cell walls may be equivalent since there are fewer xylose residues in the gramineous monocot xyloglucan and less cellulose in gramineous monocot cell walls.

The xyloglucans from dicot cell walls often contain L-Fuc α-linked to the 2-position of the Gal residues. α-L-Ara may sometimes be present in small quantities. Xyloglucans of the Solanaceae are different from most other dicot xyloglucans in containing α-L-Ara linked to the Xyl residues at position 2, containing little Fuc and the glucan backbone being less substituted with Xyl residues (Eda and Katô 1978, Ring and Selvendran 1981, Akiyama and Katô 1982). A xyloglucan has been isolated from the legume *Hymenaea courbaril* which differs from typical tamarind xyloglucan in that when it is hydrolysed half of the oligosaccharides produced are XXXXG although some of the Xyl residues may be substituted with Gal (Buckeridge et al. 1997). Another legume, pea, appears to have a variation in its xyloglucan structure as it is made up of the repeating oligosaccharide XXFGXXXG (Hayashi 1989). The storage xyloglucans found in dicot seeds contain no Fuc (Kooiman 1961).

Xyloglucans from gramineous monocots contain little terminal Fuc (McDougall and Fry 1994). Gramineous monocot xyloglucans also contain less Xyl and much less Gal than xyloglucans extracted from dicots (Labavitch and Ray 1978, Hayashi 1989). However, fewer monocot xyloglucans have been studied and the xyloglucans present in the cell walls of most non-graminaceous monocots may be like those found in dicot cell walls. This is supported by the similarities to dicot xyloglucans, both structural and percent cell wall content, of xyloglucans found in onion (Liliaceae) and other non-gramineous monocots (including the Orchidaceae and Palmae) (Mankarios et al. 1980, Redgwell and Selvendran 1986, Ohsumi and Hayashi 1994ab).

Xyloglucans have also been isolated from gymnosperm suspension cultures (Thomas et al. 1987) and seedlings (Acebes et al. 1993, Andrew and Little 1997). In general gymnosperm xyloglucans appear to be similar to dicot xyloglucans both in their structure and in their degree of association with the cell wall.

It has been reported that the green alga *Ulva lactuca* may contain a Fuc-lacking xyloglucan in its cell walls (Lahaye et al. 1994). Enzymic hydrolysis of the xylogucan from *Ulva lactuca* with Finizyme, a commercial enzyme preparation containing β-(1→4)-xylanase, cellulase, laminarinase and licheninase activities, released the trisaccharide β-D-xylopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-D-glucopyranose and xylotriose; in higher plant xyloglucans Xyl is present in its α-anomer and is (1→6) linked to the xyloglucan backbone (Lahaye et al. 1994). If xyloglucan is present in *Ulva lactuca* it appears to be very different from typical angiosperm xyloglucans.

Xyloglucan has been reported present in the charophycean green algae *Chara* and *Nitella*, which are thought to be among the green algae most closely related to land plants (Morrison et al. 1993, Anderson and King 1961a, b). Anderson and King (1961a, b) suggested xyloglucan may be present in the cell walls of *Chara* and *Nitella* on the basis of acid hydolysis of sequential cell wall extracts. Uronic acid was found to be present in the hemicellulose fractions as well as Xyl, Glc, Gal, Man

and Ara. Anderson and King (1961b) compared the total hemicellulose content of *Chara* and *Nitella* cell walls to the total hemicellulose content of gramineous monocot cell walls (including wheat straw and corn cob); they recorded the total hemicellulose content of the charophycean cell wall to be 13–26% w/w compared to 32–42% w/w in gramineous monocots. It is now known the xyloglucan content of the primary cell walls is 2–5% of the dry mass in comparison to 20–25% in dicots (Hayashi 1989). Therefore if xyloglucan is present in the cell walls of the charophycean green algae it is likely to be at a much lower concentration than found in land plants. Morrison et al. (1993) reported a xyloglucan extracted predominantly in the 1 M KOH fraction of a sequential cell wall extraction from *Nitella*; usually xyloglucan is extracted predominantly in the 4 M KOH extract of sequential cell wall extractions (Kakegawa et al. 2000). The 4 M KOH extract from *Nitella* cell walls was rich in Glc and Man and Morrison et al. (1993) suggested that glucomannan was more tightly bound to cellulose than xyloglucan was bound to cellulose. Morrison et al. (1993) claimed the presence of xyloglucan in the 1 M KOH extract of *Nitella* cell walls on the basis of methylation analysis which showed the fraction to contain 4-Glc and/or 4,6-Glc, 2-Xyl and/or 4-Xyl, 2-Gal and t-Fuc. It was not clear whether the method of methylation analysis used to identify the glycosidic linkages was able to distinguish between 2-Xyl and 4-Xyl and the relative proportions of each type of linkage were not given. It is unusual that no terminal Gal or Xyl was reported as they are found in typical angiosperm xyloglucans. If xyloglucan is present in the cell walls of the charophycean green algae it appear to differ from typical angiosperm xyloglucans in structure and composition as well as degree of association cellulose. It is of interest to compare these algal xyloglucans with those present in land plants. The presence of xyloglucan has not been recorded in lower land plants and needs investigation.

Figure 1.1: Structure of some common xyloglucan-derived oligosaccharides (Fry et al. 1993).

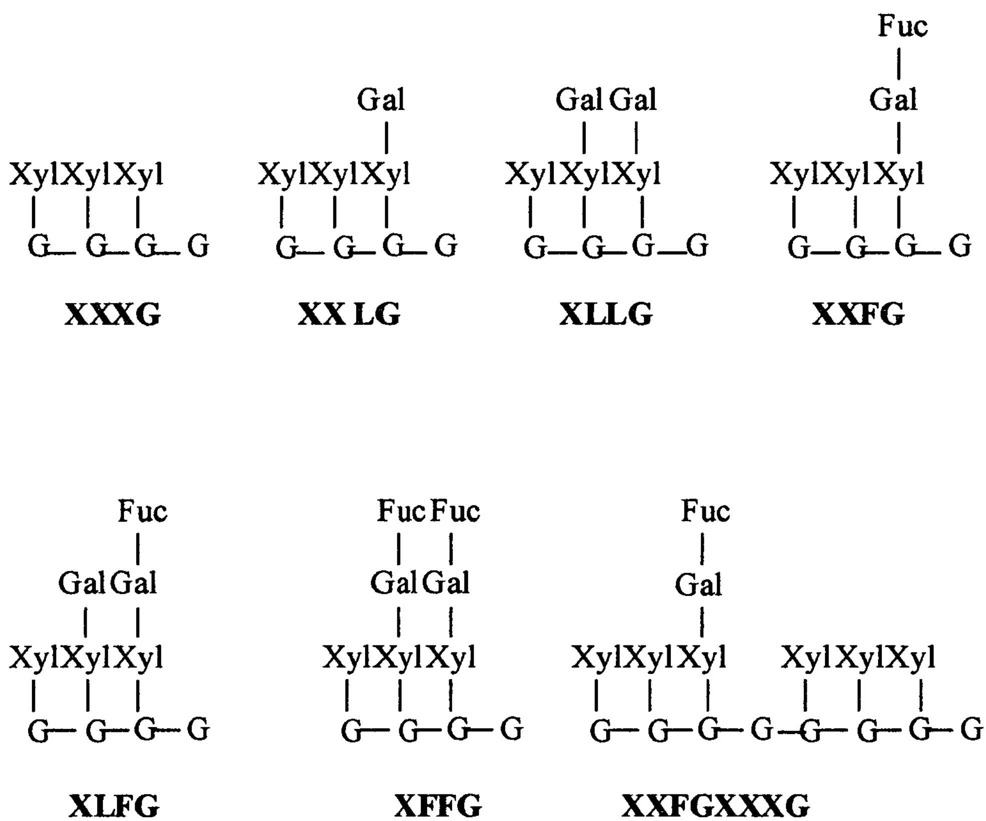
Key to abbreviated names

G = β-D-Glc

X = α-D-Xyl-(1→6)-β-D-Glc

L = β-D-Gal-(1→2)-α-D-Xyl-(1→6)-β-D-Glc

F = α-L-Fuc-(1→2)-β-D-Gal-(1→2)-α-D-Xyl-(1→6)-β-D-Glc



1.3.4 Mannans

1.3.4.1 Mannan

Pure mannan is similar to cellulose in ultrastructure as both occur as crystalline microfibrils as structural components of some cell walls. It is a homopolymer of (1→4)-β-D-mannopyranose residues. Microfibrils of mannan have been observed in ivory nut endosperm and date seeds.

1.3.4.2 Galactomannans

Galactomannans are often found as storage polysaccharides in the endosperm of certain plants such as palm, date and coffee beans (Millane and Hendrixson 1994). Galactomannans are formed by a linear β-(1→4)-mannosyl chain branched with α-(1→6)-galactosyl units. Diverse degrees of substitution with galactose are found in different galactomannans.

1.3.4.3 Glucomannans

Glucomannan molecules are thought to be co-occur with cellulose in the cell wall (Millane and Hendrixson 1994). Glucomannans are linear polymers similar in structure to galactomannans except that some of the mannose residues are replaced, in an apparently random fashion, by 4-linked β-D-glucose residues. Glucomannans often contain a small number of D-galactose terminal side-chains (2–6% per main chain residue) as well as varying degrees of acetylation (glucomannans from pine are about 50% acetylated; Andrew and Little 1997). Glucomannans have been isolated from many monocots including the leaves of *Aloe arborescens* (Wozniewski et al. 1990) and bamboo shoots (Edashige and Ishii 1998) and they make up over 90% of the walls of *Asparagus officinalis* endosperm (Goldberg et al. 1992,1992) and *Lilium testaceum* bulbs (Wozniewski et al. 1992).

1.3.4.4 Galactoglucomannans

Galactoglucomannans are made up of (1→4)-linked alternating β-D-mannopyranose and β-D-glucopyranose residues with the mannose substituted at O-6 by α-D-galactopyranose or 2-*O*-β-D-galactopyranose-α-D-galactopyranose side chains.

Galactoglucomannans are major components of the cell walls of the woody tissues of both angiosperms and gymnosperms, but have also been reported in the primary cell walls of angiosperm and gymnosperm cambial tissues, suspension-cultured tobacco cells (Eda et al. 1985), and secondary cell walls from the stem tissues of the aquatic moss *Fontinalis antipyretica* (Geddes & Wilkie 1971, 1972) and the fern *Pteridium aquilinum* (Bremner and Wilkie 1971). Galactoglucomannans of monosaccharide composition Gal:Glc:Man of about 1:1:3 have been found in gymnosperms seedlings and are water-soluble (Andrew and Little 1997). Galactoglucomannans are reported to occur in the primary cell walls of *Nicotiana tabacum* and *Rubus fruticosus* (Akiyama et al. 1983, Eda et al. 1985, Cartier et al. 1988). They differ from those of secondary cell walls by containing almost equal amounts of galactose, glucose and mannose. They appear to have a backbone of alternating (1→4)-β-D-mannopyranose and (1→4)-β-D-glucopyranose residues substituted with α-galactose residues at the *O*-6 position of the mannose residues. Galactoglucomannan containing fewer galactose residues and (2 mol%) terminal arabinose residues has been isolated from *Nicotiana plumbaginifolia* cell suspension cultures (Sims et al. 1997). Galactoglucomannans are found as storage polysaccharides in the leguminous seed endosperms (Reid 1985).

1.3.5 Xylans

1.3.5.1 Xylan

Typical gymnosperm and dicot xylans consist of a β-(1→4)-linked Xyl backbone with a single α-GlcA or 4-*O*-methyl-α-GlcA residue attached to the O-2 position of the Xyl residues (Shatalov et al. 1999). Xylans of similar structure have been isolated from *Osmunda cinnamomea*, a primitive fern (Timell 1962). However, there can be variation in monosaccharide residues that are found as side chains and in overall degree of branching. A xylan has been isolated from the dicot, *Eucalyptus globulus*, which has side chains of 4-*O*-methyl-GlcA attached to the O-2 position of Xyl; some of the 4-*O*-methyl-GlcA residues themselves are substitued at O-2 with α-D-Gal residues (Shatalov et al. 1999). α-L-Ara residues are attached to the 0-3 position of the Xyl residues which make up the backbone of dicot and non-gramineous monocot xylans.

Arabinoxylans from the Gramineae have complex feruloyated and acetylated side chains attached to position 5 of the α-L-arabinofuranose residues (Wende and Fry 1997a, b). Glucuronoarabinoxylans are present in the primary and secondary walls of gramineous monocots and are made up of a backbone of β-(1→4)-linked Xyl residues which are substituted with single α-L-Ara residues at the carbon 3 position of Xyl and either α-GlcA or 4-*O*-methyl-α-GlcA at the carbon 2 position of Xyl (Shibuya et al. 1983). Roughly 78% of the Xyl residues in rice coleoptiles are substituted with Ara (Shibuya and Misaki 1978). Ara is reported to be attached to O-2 position of Xyl in gramineous monocots and the O-3 position of Xyl in dicots (Bacic et al. 1988). The results of Darvill et al. (1980) suggest that 11.5–20% of the residues constituting the backbone of glucuronoarabinoxylans extracted from *Acer* are substituted monocot glucuronoarabinoxylans where nearly all of the Xyl residues are substituted (Bacic et al. 1988). The degree of substitution affects the ability of the polymers to bind together.

1.3.5.2 Mixed-linkage xylans

Xylans have been reported from members of the Rhodophyta (e.g. *Palmeria palmata*) which are very different from those reported for land plants. They have a mixed β-(1→3),(1→4)-linked xylan backbone (Painter 1983, Howard 1957).

1.3.6 Pectic polysaccharides

Pectic polysaccharides are a class of structurally complex polysaccharides that occur in the matrix of the primary cell walls of land plants. Pectic polysaccharides consist of distinct structural domains that may or may not be covalently linked together. These domains include rhamnogalacturonan I, rhamnogalacturonan II, homogalacturonan and xylogalacturonan. Pectic polysaccharides are implicated in the regulation of cell wall ion status, cell wall porosity, cell–cell adhesion and expansion, cell proliferation and cell differentiation (Willats et al. 1999).

1.3.6.1 Rhamnogalacturonan I

RG-I consists of heteropolymers of repeating (1→2)-α-L-Rha-(1→4)-α-D-GalA disaccharide units with side chains of Ara, Fuc and Gal (Lau et al. 1985). RG-I has a high ratio of Rha:GalA and a high overall neutral sugar content. A number of the GalA residues may be methyl or acetyl esterified. RG-I has a molecular weight of about 200000 and constitutes 7% of the dry weight of the *Asparagus officinalis* seedling primary cell wall (Goldberg et al. 1994).

1.3.6.2 Rhamnogalacturonan II

RG-II is complex and contains the greatest diversity of sugars and linkage structures of any biological polymer. In particular, as well as more common sugars (GalA, GlcA, Ara, Rha, Gal, Fuc) RG-II contains the relatively rare sugars 2-*O*-methyl-L-fucose, 2-*O*-methyl-D-xylose, D-apiose, 3-carboxy-5-deoxy-L-xylose (aceric acid), 2-keto-3-deoxy-D-*manno*-octulosonic acid (KDO) and 3-deoxy-D-*lyxo*-heptulosonic acid (DHA). Aceric acid has not been detected in any other polysaccharide (Spellman et al. 1983). Unusual linkages are also found in RG-II; 3-linked rhamnosyl, 2-linked glucosyl and 3,4-linked fucosyl groups have not been detected in any other primary cell wall polysaccharides (Darvill et al. 1978). RG-II exists predominantly as a dimer that is cross-linked by a borate-diol ester (Ishii et al. 1999) and therefore exhibits molecular weights of 5000 and 10000. Immunocytology has indicated that RG-II is not present in the middle lamella but is concentrated near to the plasma membrane (Matoh et al. 1998).

RG-II, with little variation in structure, has been found in at least 24 species of gramineous monocots, other monocots and dicots (Matoh et al. 1998). RG-II found in a gymnosperm, Douglas fir, has a similar sugar composition to angiosperm RG-II (Thomas et al. 1987). Although RG-II is a fairly minor component of the cell wall (about 3% dry mass total wall composition in dicots and less than 1% in gramineous monocots) it is potentially important to cell wall structure. The unusual sugars and linkages found in RG-II render it undigestible to most potential pathogens. Boron, an essential micronutrient, binds exceptionally strongly to certain apiose residues of RG-II (Kaneko et al. 1997). Carrot cells grown in the absence of borate rupture

within 4 days (Loomis and Durst 1992) although the boron can be replaced by germanium. Bamboo shoot cell walls contain eight times less boron than dicot cell walls and this is correlates with a reduced RG-II content.

1.3.6.3 Homogalacturonan

Homogalacturonan makes up 57–69% of extractable pectin in suspension-cultured sycamore cells (O'Neill et al. 1990) and is made up of helical polymers of (1→4)-α-D-galactosyluronic acid of about 200 galacturonic acid residues and 100 nm long. When de-esterified, homogalacturonan can associate via divalent calcium ions. This leads to gel formation and is important in cell–cell adhesion (Jarvis 1984).

1.3.6.4 Xylogalacturonan

Xylogalacturonan is a modified form of homogalacturonan with α-D-xylose substituted at the O-2 or O-3 positions. It is not present in as large quantities in the cell wall as the other pectic domains. A xylogalacturonan has been reported present in the highly branched regions of apple fruit pectin and in a gymnosperm (pollen from mountain pine) (Schols et al. 1995).

1.3.6.5 Apiogalacturonan

Apiogalacturonan has been identified as the major cell wall polysaccharide in vegetative tissues of the marine monocot *Heterozostera tasmanica* (Webster and Stone 1994a, b, Ovodova et al. 1968) and an aquatic duckweed, *Lemna minor* (Beck 1967, Cheng and Hart 1997, Hart and Kindel 1970). Typically apiose forms about 15% of the total sugar composition of these cell walls (Webster and Stone 1994b). These plants form a group of aquatic plants that diverged early in monocot evolution.

1.4 Land plant evolution

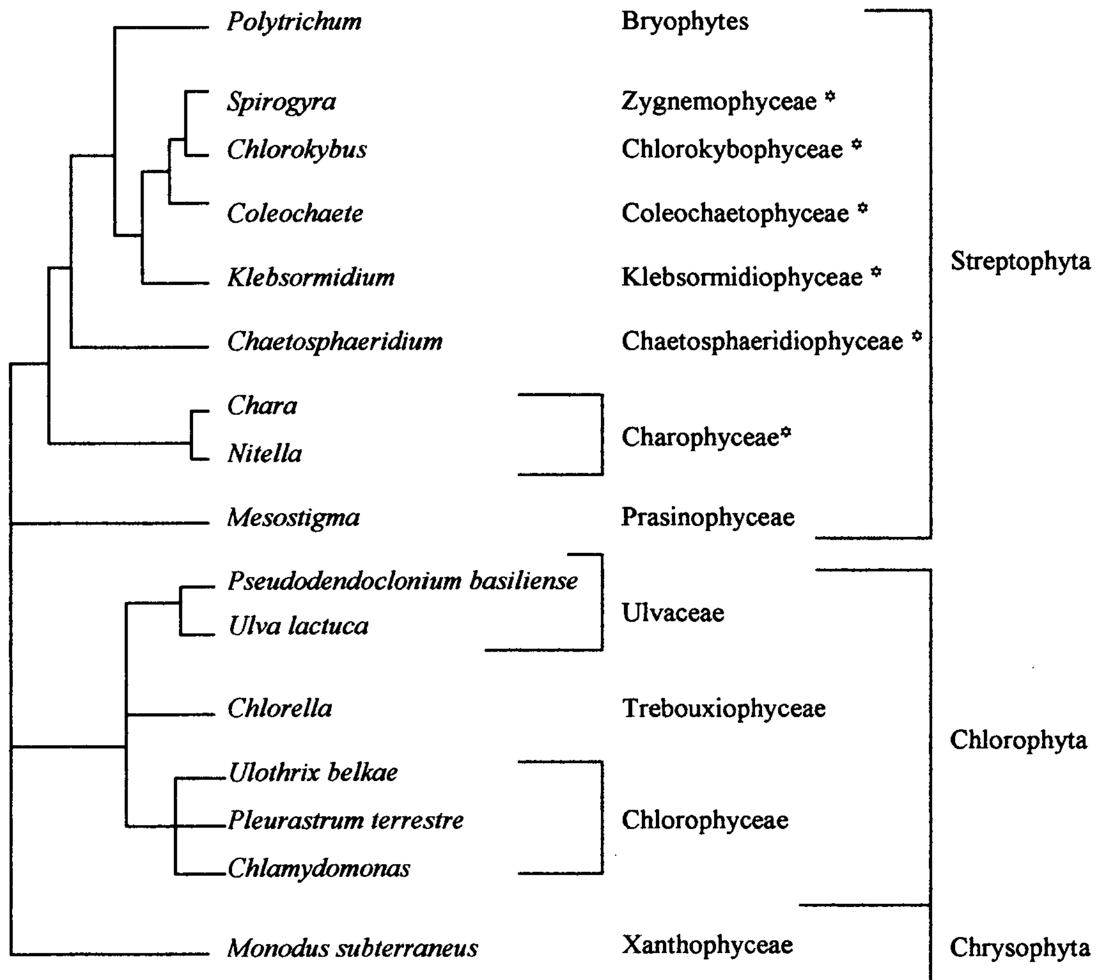
1.4.1 Charophyceae and origin of the land plants

Land plant monophyly is strongly supported by comparative morphology (Graham 1993, Mishler et al. 1994, Hiesel et al. 1996, Kenrick and Crane 1997a, Bremer et al. 1987), 18-*S* rRNA sequences and mitochondrial DNA gene sequences (Kranz 1995, Hiesel et al. 1996). There is now no dispute that the common ancestor of land plants would be classified with the charophycean green algae; however, rDNA sequence data currently available are unable to resolve which extant alga is most closely related to the bryophytes (Figure 1.2). Although it had previously been thought that *Chara* or *Nitella* may have been the common ancestor of land plants complete small-subunit rRNA gene sequences suggest that *Klebsormidium* and *Coleochaete* have a closer affinity to land plants than do *Chara* and *Nitella* (Kranz et al 1995). Features of cell division (Pickett-Heaps and Marchant 1972, Pickett-Heaps 1976, Bhattacharya 1998) and the presence of cutin, sporopollenin, phenolic compounds (generally absent from green algae) and the glycollate oxidase pathway of photorespiration (Raven 1991) also indicate the charophycean ancestry of the land plants. An alternative explanation, which is not currently favoured (Oiso et al. 1999), is that charophycean green algae share common features with land plants as they may have evolved from land plants and represent a return to an aquatic environment.

A group II intron is a portion of non-coding DNA within a gene. It is transcribed but subsequently auto-spliced from the primary transcript to produce mature RNA. Group II introns are found in the $tRNA^{Ala}$ and $tRNA^{Ile}$ genes of chloroplasts of all land plants whereas eubacteria and the chloroplasts of most algae have uninterrupted genes. The group II introns have been found in *Coleochaete*, *Nitella* and *Spirogyra* (Manhart and Palmer 1990) suggesting a closer relationship with land plants than other green algae. *Chara* and *Nitella* have been hypothesised as the closest to land plants as their gross morphology is similar to some land plants. However, gross morphological characters may appear similar but have evolved via different evolutionary pathways. Sequence data for 5-*S* rRNA also indicate a close relationship between *Nitella* and land plants (Van de Peer et al. 1990). Owing to a

large evolutionary gap there are a greater number of mutations between the Charophyceae and the bryophytes than between the remaining charophytes and the bryophytes (the charophytes are more closely related to each other and have more molecular and morphological similarities to each other than they do to the bryophytes); this may cause artificial grouping of the Charales with land plants (a phenomenon called long branch attraction) (Kranz and Huss 1996). Complete small-subunit rRNA gene sequences suggest *Klebsormidium* and *Coleochaete* have a closer affinity to land plants than do *Chara* and *Nitella* (Kranz et al. 1995). There is fossil evidence that *Parka*, a plant ecologically and morphologically similar to *Coleochaete*, existed at around the same time as the origin of land plants which suggests that the land plants evolved from a plant similar to *Coleochaete* (Graham 1982). Among the Charophyceae, *Coleochaete* is unique and similar to embryophytes in that its zygote is retained by the parent plant and undergoes further development before it is released (Graham 1984). Figure 1.2. shows a resolved phylogeny for the green algae mentioned in this section, which places *Coleochaete*, *Klebsormidium* and *Chaetosphaeridium* close to the base of land plants. Such a placing is the most commonly resolved phylogeny from both molecular and morphological data.

Figure 1.2. Phylogeny of 18-*S* rDNA data for algae and bryophytes. Adapted from Sluiman and Guihal (1999).



Key

* indicates Charophytes

1.4.2 Bryophytes

The transition from an aqueous to an, initially low-competition, gaseous medium exposed plants to new physical conditions that resulted in selection for key physiological and structural changes. Early plant terrestrialisation is predicted to have rapidly filled all niches where water availability was unlimited (Bateman et al. 1998), therefore increasing competition and potentially selecting for turgor-stabilised upright stems and decreased dependence on water availability. Land plants are thought to share one common ancestor and this idea is supported by chloroplast tRNA data (Manhart and Palmer 1990). Graham et al. (1994) reported that the shared autapomorphies of the embryophyte clade are: a conserved size and architecture of the plastid genome, preprophase wall localisation site defined by a band of microtubles, the presence of an apical meristem having more than two cutting faces caused by successive asymmetric divisions being preceded by an angular change in the mitotic spindle, features of sperm development, the presence of a multicellular embryo nutritionally and developmentally dependent on female gametophytic tissues, more than one spore mother cell generated per zygote, the presence of a multicellular sporophytic generation and resistant-walled (sporopollenin-invested) spores. Phylogenetic studies predict that early land plants were small and morphologically simple. This hypothesis is supported by early fossils which strongly resemble mosses and liverworts (Kenrick and Crane 1997b).

Bryophytes (hornworts, liverworts and mosses) are supported as monophyletic, and basal to the land plants, by both molecular and morphological data (Manhart and Palmer 1990). However, relationships among living groups of bryophytes are uncertain, possibly owing to their rapid radiation. One hypothesis resolves liverworts as basal to the other bryophytes on the basis of molecular and morphological data (Graham 1993, Mishler et al. 1994, Hiesel et al. 1996, Kenrick and Crane 1997a, Bremer et al. 1987), with either mosses or hornworts as the living sister group to tracheophytes. Liverworts themselves may be paraphyletic with Marchantiales basal and Jungermanniales more closely related to hornworts or mosses (Arrington et al. 1997). Among land plants only the hornworts have chloroplasts with pyrenoids, a character shared with some green algae (Doyle

1970). In a few species of the vascular genus *Selaginella* (e.g. *S. willenowii*) the cells of the upper epidermis of the leaf contain a single cup-shaped chloroplast similar to that of the hornwort *Anthoceros* but lacking a pyrenoid (Bell and Hemsley 2000). Alternative hypotheses of land plant evolution place hornworts, on the basis of morphological characters as well as both mitochondrial and nuclear gene data, as a monophyletic group basal to the rest of the embryophytes (Hedderson et al. 1996, Mishler and Churchill 1996, Bopp and Capesius 1996, Renzaglia et al. 2000). Monophyly of the mosses is well supported and the Sphagnales and Andreales are consistently resolved as basal groups (Bopp and Capesius 1996, Hedderson et al. 1996).

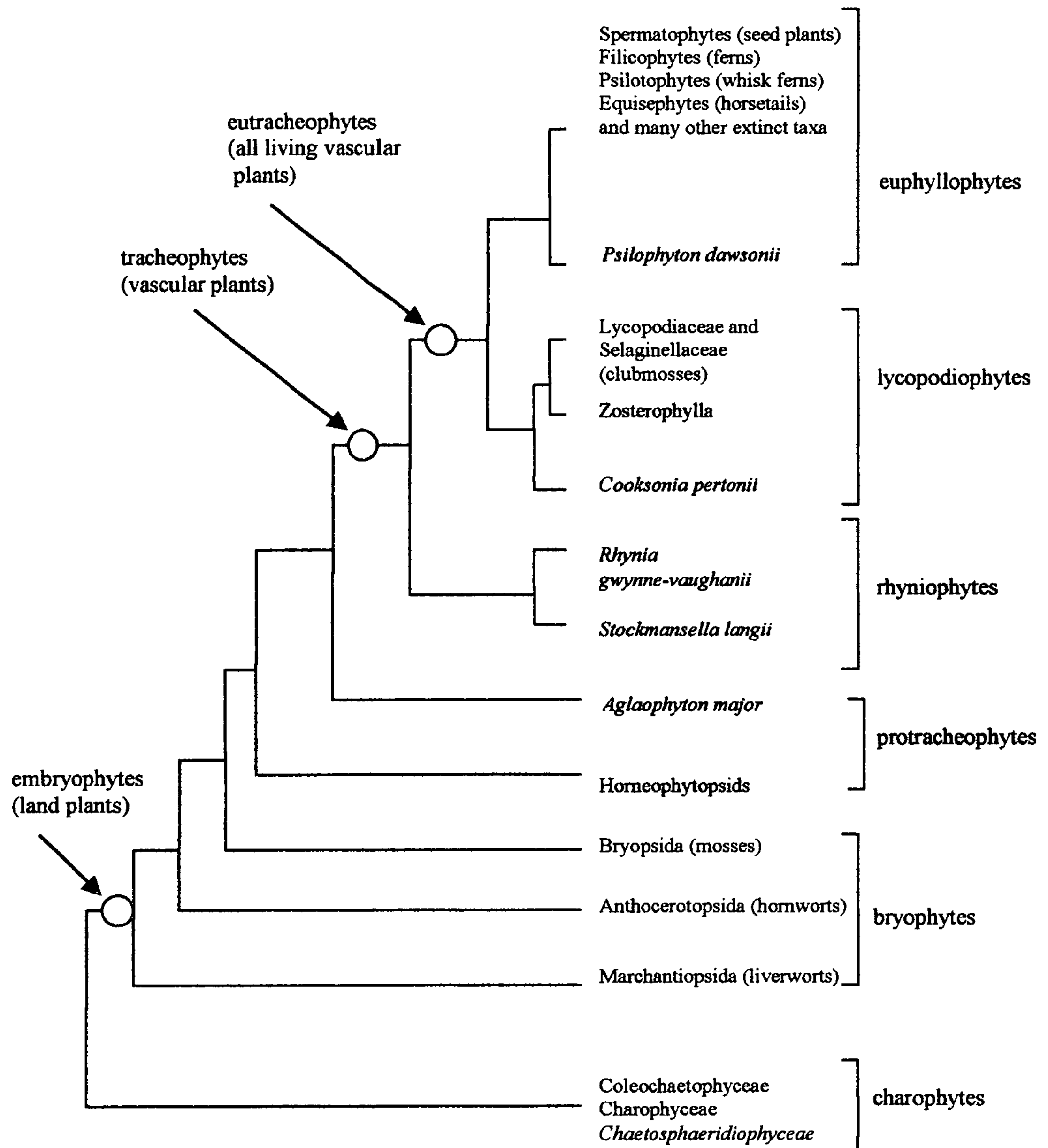
1.4.3 Pteridophytes

Within the existing land plants there are substantial morphological gaps. Many fossil plants exist which are placed between the bryophytes and tracheophytes (Figure 1.3). The tracheophytes are thought to have evolved about 420 million years ago with the earliest members superficially resembling the modern *Psilotum* (Psilotaceae). Fossil evidence also indicates that some lineages of lycopodiophytes have undergone mass extinction, which would account for the morphological gap which exists between the extant lycopodiophytes (*Lycopodium*, *Selaginella* and *Isoetes*) and the monophyletic euphyllophytes (horsetails, *Psilotum*, *Tmesipteris*, ferns and seed plants) (Kenrick and Crane 1997b, Stewart 1981) (Figure 1.3). The lycopodiophytes form a distinctive, basal, monophyletic clade within the eutracheophytes and the major clades evolved during the Devonian (408–360 million years ago) (Bateman et al. 1998). The placement of *Equisetum* in the fern lineage is supported by an analysis combining *rbc*L and morphological data (Pryer et al. 1995) and mitochondrial DNA sequence data (Duff and Nickrent 1999). The extant euphyllophytes all have a unique 30-kb inversion in their chloroplast genome, which is shared by *Psilotum* suggesting that it is within the euphyllophyte clade (Raubeson and Jansen 1992). *Psilophyton* is placed in the euphyllophytes on the basis of anatomical and morphological characters (Edwards 1993, Ewbank et al. 1996). On the basis of data from complete small-subunit rRNA gene sequences, three chloroplast genes (*rbc*L, 16-*S* rRNA and *atp*B) and two mitochondrial genes

(*cox*3 and *nad*5), *Psilotum* has been shown to be closely related to ferns in the Ophioglossales (Kranz et al. 1995, Manhart 1994, 1995, Wolf 1997, Malek et al. 1996, Vangerow 1999). Ferns are divided into two groups: eusporangiate (where the sporangium wall has two or more cell layers) and leptosporangiate (where the sporangium wall has just one cell layer). The majority of plants referred to as ferns belong to the leptosproangiate group, which is monophyletic and, as well as the character of sporangium thickness, is defined by four unique characters: annulus, sporangial stalk, vertical first zygotic division and primary xylem with scalariform pits. The eusporangiate condition characterises lycopodiophytes, equisetophytes and psilotophytes as well as ferns in the Ophioglossaceae and Marattiaceae, which are thought to be among the more primitive fern members (Smith 1995, Pryer et al. 1995). Among the leptosporangiate ferns ten families are generally recognised as being primitive: Osumdaceae, Schizaeaceae, Gleicheniaceae, Matoniaceae, Dipteridaceae, Plagiogyriaceae, Loxomataceae, Hymenophyllaceae, Dicksoniaceae and Cycanthaceae. The more advanced fern families are considered to be monophyletic and include Dennstaeditaceae, Pteridaceae, Vittariaceae, Polypodiaceae, Guammitideceae, Thelypteridaceae, Dryopteridaceae and Blechnaceae (Smith 1995, Pryer et al. 1995).

Figure 1.3: Relationships among land plants (adapted from Kenrick and Crane 1997). Fossil taxa are shown in blue.

Not drawn to scale



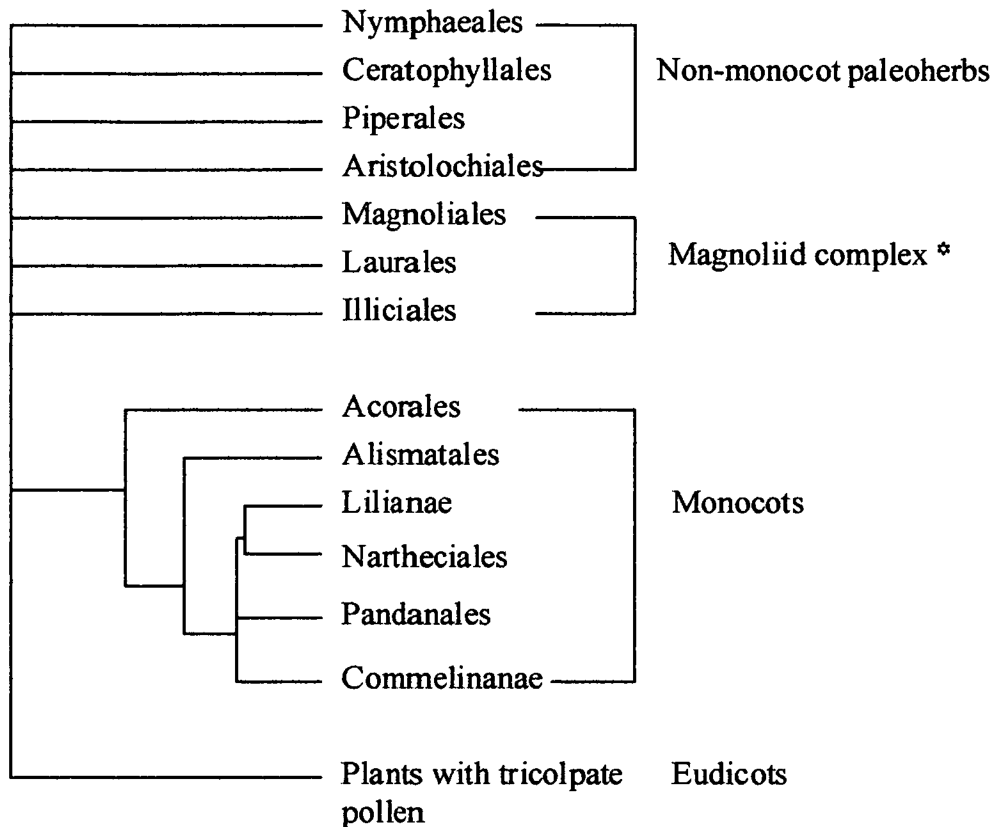
1.4.4 Seed plants

No other extant lineage of land plants is thought to have shared a common ancestor with angiosperms for over 350 million years. Darwin (1879) stated that 'The rapid advance, as far as we can judge, of all the higher plants within recent geological times, is an abominable mystery'; this is still true today. In addition to angiosperms, extant seed plants comprise four different groups of gymnosperms: conifers, cycads, gnetophytes and ginkgo. Seed plants are a monophyletic group (Crane 1985). On the basis of morphological characters (flower-like appearance of reproductive structures, vessels in secondary xylem, and a kind of double-fertilisation) gnetophytes have been considered as the sister group of the angiosperms. However, molecular evidence provided by developmental genes suggests that the gnetophytes are more closely related to the conifers than to flowering plants (Winter et al. 1999). New studies on the supposed shared morphological characters also suggested that these characters are of independent origin (Winter et al. 1999). Owing to the close relationship between angiosperms and gymnosperms, Doyle and Donoghue (1986) suggested that it is likely that angiosperm floral organs have direct homologues in related groups. Recently it has been shown that flowers may have evolved from male gymnosperm cones as they are both developmentally regulated by B- and C-type MADS-box genes (Frohlich 1999, 2001, Engström et al. 2001, Sundström et al. 1999). Takhtajan (1969) suggested that angiosperms could have evolved from gymnosperms by phylogenetic shifting of juvenile features to later stages of ontogeny. Relationships among the seed plants are not completely resolved on the basis of present data even when fossil taxa are considered (Doyle and Donoghue 1992, Soltis et al. 1999, Kenrick 1999, Qiu et al. 1999). The first putative angiosperm fossils are from 200 million years ago (Crane 1993). The Bennetitales (extinct Triassic–Cretaceous seed plants) and Gnetales are potential angiosperm sister groups. Both the Bennetitales and Gnetales were present by the Triassic, which implies that the lineage leading to the angiosperms must have diverged by at least 230 million years ago (Crane 1993). Triaperturate pollen is diagnostic of the eudicots which includes about 70% of extant angiosperm species. There is a good pollen record and the pollen record suggests that the eudicots are unlikely to have existed before about 120 million years ago (Crane 1993).

The angiosperms are monophyletic. They are united by nine apomorphies: sieve tubes and companion cells derived from the same initials, stamens with two lateral pairs of pollen sacs, closed carpels with stigmatic pollen germination, hypodermal endothecium in the anther, lack of laminated endoexine, megaspore wall without sporopollenin, three-nuclear male gametophyte without prothallials or a sterile cell, megagametophyte with eight nuclei and double fertilisation with associated endosperm formation. However, when these characters are removed from phylogenetic analyses the angiosperms remain clustered (Doyle and Donoghue 1986). It is suggested that the evolution of vessels and enclosure of the carpel contributed to diversification of the angiosperms (Mathews and Donoghue 1999).

Most current hypotheses of angiosperm evolution recognise two large clades, monocots and eudicots, embedded within a poorly defined basal assemblage of magnoliid dicots (Magnoliidae) and non-monocot paleoherbs (Figure 1.4). Molecular data (*rbc*L) indicate the earliest angiosperms to be the Magnoliidae which is composed of *Amborella* and the Illiciales–Trimeniaceae–Austrobaileya (ITA group), with *Amborella* as sister to all other angiosperms (Qiu et al. 1999, 1993); analysis of duplicate phytochrome genes give a similar result (Mathews and Donoghue 1999). The monocots are thought to be most closely related to the non-monocot paleoherbs which consist of the Nymphaeales, Aristolochiales, Piperales and Ceratophyllales. The magnoliid dicots and non-flowering seed plants have monosulcate pollen which suggests the magnoliid dicots may be basal to the angiosperms. The Magnoliidae and monocots both use the tyrosine-pathway for synthesising aromatic cyanogenic compounds whereas dicots use the phenylalanine-pathway (Qiu et al. 1993).

Figure 1.4: Relationships among extant angiosperms (adapted from Judd et al. 1999)

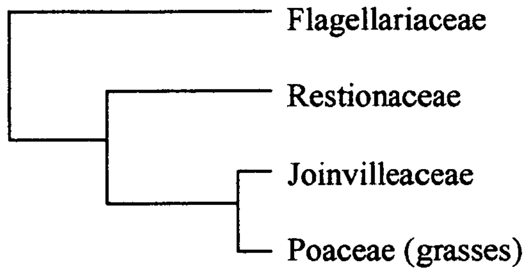


* *Amborella* thought to be one of the earliest diverging members of the Magnoliid complex.

Monocots are believed to share a single common ancestor, *Acorus calamus* being a member of the oldest extant lineage (Duvall et al. 1993, Chase et al. 1993). *Acorus calamus* was classified with the Araceae because of superficial morphological similarities but has recently been placed in monogeneric family (Grayum 1987). *Acorus* has a stout rhizome, linear leaves with parallel veins and many hermaphrodite flowers which are closely packed together and produce berries. The monocots diversified rapidly. Cretaceous fossils of modern-looking ginger fruits, palm leaves and stalks have been found and by the early Tertiary many monocot groups had differentiated (Herendeen and Crane 1995). The Poales are thought to

be one of the most derived groups of monocots. Within the Poales, Flagellariaceae is thought to have diverged before Restionaceae and Joinvilleaceae is immediately basal to the Poaceae (grasses) (Linder and Kellogg 1995, Duvall et al. 1993) (Figure 1.5).

Figure 1.5: Relationships of the closest relatives of the Poaceae



1.5 The primary cell wall of green plants

From studies so far the cell walls of ferns, liverworts and charophycean algae are thought to resemble those of seed plants, but have been investigated in less detail. The cell wall of non-charophycean algae differ widely from each other and those of land plants.

1.5.1 Green algae

Green algal cell walls show a great deal of variation. This variation has been used as an important character in algal classifications (Stace 1989). It is thought that in green algae the cell wall may have had independent origins in at least three lines of evolution (Domozych et al. 1980).

The cell walls of the Chlamydomonaceae (primitive, single-celled members of the Chlorophyta) have been shown to differ greatly from higher plant cell walls. Roberts (1974) proposed a model whereby the primitive algal cell wall is composed of several layers of hydroxyproline-rich glycoproteins and contains no cellulose.

The major sugars present in the cell wall are arabinose and galactose with mannose, xylose and fucose present in smaller quantities (Roberts 1974).

In contrast to the cell walls of the chlorophycean algae, the charophycean cell wall is more similar to the land plant cell wall with respect to polysaccharide composition, uronic acid, pentose and rhamnose content (Domozych et al. 1980). Some algae in the Charophyceae (*Klebsormidium flaccidum*), Chlorophyceae (*Ulothrix belkae* and *Pleurasrum terrestre*) and Ulvaceae (*Pseudendoclonium basiliense*) (Figure 1.2) have been shown to contain cellulose and hydroxyproline (Domozych et al. 1980). However, *Chara* and *Nitella* (Charophyceae) cell walls lack detectable hydroxyproline (Gotelli and Cleland 1968, Kieliszewski and Lamport 1994). X-ray diffraction patterns of alkali-insoluble and enzyme-resistant polymers extracted from *Ulva lactuca* were interpreted as indicating the presence of a xylose-containing glucan (Lahaye et al. 1994). The charophycean (*Chara foetida*) cell wall has been thought to contain cellulose (Zacharias 1890, Morikawa 1974) but has also been reported as lacking cellulose (Kreuger 1970). Morikawa (1974) reported the *Nitella* cell wall to be made up of 23–27% pectin, 37–43% hemicellulose and ~ 36% cellulose. Morikawa (1974) reported that the main sugar residues present in the hemicellulose fraction were either xylose or mannose (the method that Morikawa used to identify the sugar residues was not capable of differentiating between xylose and mannose) and arabinose; glucose, galactose and rhamnose were present in minor amounts. Fucose has also been reported present in *Nitella* cell wall polysaccharides (Métraux 1982). Morrison et al. (1993) reported the main hemicelluloses present in *Nitella* to be xyloglucan, glucomannan and rhamnogalacturonan, based on methylation analysis of sequentially extracted fractions. The reported presence of xyloglucan in *Nitella* (Morrison et al. 1993) was inferred from the presence of 2- and/or 4- linked xylose residues (4-linked xylose is a xylose residue which has something attached to position 4). 4-Linked Xyl is not normally found as a component of xyloglucan. Many methods of methylation analysis cannot distinguish 2-linked Xyl from 4-linked Xyl because xylitol is symmetrical. It is not known whether the method of methylation analysis used by Morrison et al. (1993) was capable of distinguishing between the two reported

xylose linkages. From the way in which Morrison et al. (1993) reported the sugar linkages present in *Nitella* hemicellulose it was not possible to infer the relative amounts of any of the sugar linkages. Anderson and King (1961a, b) reported the cell wall composition of *Chara* and *Nitella* to be similar to that of land plants, but having more pectin and less hemicellulose (13% compared with 38% in gramineous monocots).

1.5.2 Lower land plants

A survey of bryophyte taxa showed that, similarly to charophycean green algae, they have vegetative cell walls that have a specific type of fluorescence (blue-white in UV excitation and yellow-green in violet irradiation) and are resistant to acetolysis and decay (Kroken et al. 1996). Deposition of autofluorescent wall compounds (thought to be lignin-like) in response to desiccation occurs in a variety of charophytes (Kroken et al. 1996). *Coleochaete* is unique among charophytes, but similar to all bryophtyes in that sexual reproduction induces autofluorescence in cell walls of well-hydrated tissues at the placental junction. The bryophytes do not contain lignin as defined in angiosperms and gymnosperms, but produce other types of polyphenolic compounds (Ligrone et al. 2000, Erickson and Miksche 1974). Bryophyte cell walls have been shown to contain lignans (Chodat and Cortesi 1939). Lignans are short polymers which are composed of the same types of monomers present in lignin. Lignans are water-extractable and are thought to indicate that bryophytes may have a partially developed phenylpropanoid pathway that could have served as the basis for evolution of lignin in the ancestors of higher land plants (Kroken et al. 1996). It has been suggested that lignans may have had a role as antimicrobial agents or as a screen against UV light (Delwiche et al. 1989; Raven 1997). The lignans may have been secondarily recruited as a structural cell wall component, extensive polymerisation making them water insoluble.

Mannuronic acid is reported to have been isolated from the cell walls of gametophytic tissues of the liverwort *Riccardia* (Das and Rao 1966). Das and Rao (1963) reported the presence of mannuronic acid residues in *Riccardia* walls on the basis of R_F values on PC and staining with ammoniacal silver nitrate ('yellowish-

red'), ammonium vanadate and *p*-anisidine stains. Unfortunately Das and Rao (1963) did not report the presence of either GalA or GlcA in liverworts. However, GalA is commonly found in high concentrations in the cell walls of angiosperms, gymnosperms and ferns (Edsashige and Ishii 1996, Jarvis et al. 1988). Most studies on the uronic acid content of bryophyte cell walls do not identify which uronic acids are present (Thomas 1977, Thomas et al. 1984). I could not find any references reporting the presence of either GlcA or GalA in bryophyte cell walls. Liverworts broadly resemble gymnosperms and dicots in their cell wall sugar residue composition, the major neutral monosaccharides being Glc, Gal and Man; lower concentrations of Xyl, Ara, Fuc and Rha are also present (Thomas 1977; Thomas et al. 1984).

The cell walls of some mosses (Rhacocarpaceae) are specially adapted to rapidly absorb atmospheric water. The ultrastructure of the leaves is sponge-like, reticulate and highly porous, the leaf ultrastucture acting to insulate the protoplast from water loss (Edelmann et al. 1998). These cell walls are composed of lignin-like polymers, hemicellulose and cellulose in a ratio of 9:8:5 (Edelmann et al. 1998). The lignin-like polymers were reported to resemble lignin except they are based on cinnamyl residues without methoxyl groups. The lignin-like polymers are found in the same regions of the cell wall as cellulose and hemicellulose (Edelmann et al. 1998). Sphagnum acid has been isolated from *Sphagnum* and is absent from other moss genera as well as hornworts and liverworts (Rudolph and Samland 1985, Rasmussen et al. 1995). Sphagnum acid is extractable in ethanol (and is therefore likely to be absent from my samples, which are AIR's). Other bryophyte walls contain phenolic compounds (Czapek 1899), for example sphagnum acid (Rudolph and Samland 1985). It is thought that these phenolic compounds may give *Sphagnum* tissues some resistance to decomposition. Galactoglucomannans have been isolated from an aquatic moss which are similar in structure to those from angiosperms and gymnosperms (Geddes and Wilkie 1971). Pectic polysaccharides are major components of the cell walls of dicots and gymnosperms and there is evidence that the cell walls of fern stems are rich in pectins (White et al. 1986).

1.5.3 Gymnosperms

Relatively few species of gymnosperm have had their primary cell walls characterised. However, the gymnosperm cell wall appears to be closely similar to the dicot cell wall with differences being quantitative rather than qualitative. Gymnosperms have lower levels of extensins and higher levels of pectic arabinans than dicots (Bacic et al. 1988). Kieliszewski and Lamport (1994) showed close similarities between dicot and gymnosperm extensins but these differed greatly from gramineous monocot extensins. The results of Kieliszewski and Lamport (1994) suggest that dicots may have evolved from basal gymnosperms as isodityrosine was not found in the cell walls of an advanced gymnosperm (Douglas fir) but was found in all dicots and a putatively basal gymnosperm (*Ginkgo*). However, isoditryosine has been reported, in small but measurable quantities, from suspension-cultured cells of the relatively advanced gymnosperm *Pinus sylvestris* (Scots pine) (Fry 1982). Edashige and Ishii (1996) reported that walls of suspension-cultured cells of the gymnosperm *Cryptomeria japonica* have a high mannose residue concentration in comparison to angiosperms.

1.5.4 Angiosperms

The growth physics of all angiosperms is thought to be similar since they respond to the same classes of growth regulator in the same tissue-specific way. Usually the angiosperms lay down new cellulose microfibrils during elongation (Brett and Waldron 1990). However, there are some differences in composition which relate to specific taxa.

1.5.4.1 Dicots

The non-cellulosic polysaccharide compositions of all dicots are thought to be similar (Harris 2000); however, variation in composition is relatively well documented.

Dicots (with the exception of the Caryophyllales) and non-commelinoid monocots typically have a much lower level and complexity of simple phenolics associated with their cell walls, in the absence of pathogen attack, than commelinoid monocots

(Mølgaard 1985, Micard 1997). The primary cell walls of the Caryophyllales *s. l.* exhibit autofluorescence under UV and are rich in ferulic acid (Fry 1979; Hartley and Harris 1981). The Caryophyllales are well defined both chemically and morphologically. Most have free or basal placentation in a compound ovary, betalain rather than anthocyanin pigments and anomalous secondary thickening in roots. They may represent a lineage that diverged early and accumulated many autapomorphies, including ferulic acid-rich primary cell walls. It is reported that ferulic acid in these dicots forms esters with arabinose and galactose residues (Fry 1982 and 1983, Micard et al. 1997) and is therefore linked differently from the ferulic acid found in gramineous monocots. The ferulic acid in gramineous monocots is linked to arabinoxylans (Harris et al. 1997).

Galactomannans are present in higher amounts with higher mannose:galactose ratios in the Caesalpinioideae than the other two Leguminosae families (Mimosoideae and Faboideae). Differences in mannose:galactose ratios between *Trigonella cretica* and other members of the tribe Trifoleae led to Reid and Meier (1970) suggesting a revision of the taxonomic position of this species.

Xyloglucans from the Solanaceae (which includes tobacco, tomatoes and potatoes) differ from those of other dicots in the branching pattern of their xylosyl substituents; XXXG and XXFG are absent (Vincken et al. 1997). Xyl residues are 2-*O*- substituted predominantly with α-L-Ara in tobacco or with α-L-Ara and β-D-Gal in potato and tomato (York et al. 1996). Olive fruits (*Olea europea* cv. koroneiki) have a xyloglucan which is similar to that found in the Solanaceae (Vierhuis et al. 2001). The Solanaceae and Lamiales (which includes the Oleaceae) are closely related (Judd et al. 1999). A xyloglucan has been isolated from the legume *Hymenaea courbaril* which differs from typical tamarind xyloglucan in that when it is hydrolysed half of the oligosaccharides produced have the core structure XXXXG (with some of the Xyl residues substituted with Gal) (Buckeridge et al. 1997). Another legume, pea, appears to have a variation in its xyloglucan structure as it is made up of the repeating oligosaccharide XXFGXXXG (Hayashi 1989).

1.5.4.2 Monocots

Jarvis et al. (1988) considered the primary cell wall composition of the gramineous monocots to be more like the secondary cell wall composition of dicots. The cell wall composition of the Poaceae was once assumed to be representative of all monocots. However, the Poaceae only represent 16% of all monocot species (Cronquist 1988) and their wall composition has been found to differ from that of most other monocots (Harris 2000). It was discovered relatively recently (Mankarios 1980, Ishii 1982) that onion, a lilioid monocot, has a similar cell wall composition to dicots. Some plants, such as pineapple, appear to fall into an intermediate category having cell walls which are like those of dicots in level of xyloglucan present (20–25% of the dry mass of the cell wall, as found in dicots, in comparison to the 2–5% found in gramineous monocots) and absence of MLG, but with pectic polysaccharides which resemble those of gramineous monocots (Smith and Harris 1995).

The monocots can be split into two groups based on the presence or absence of ester-linked ferulic acid in their primary cell walls. These two groups have been shown to have very different polysaccharide compositions (Harris et al. 1997). The group which have ester-linked ferulic acid in their cell walls are identical to the commelinoid group identified by *rbc*L gene data (Chase et al. 1993). The commelinoid group have glucuronoarabinoxylans present in their cell walls, to which ferulic acid is attached. Glucuronoarabinoxylans are also present in dicots but they are present in lower concentrations, are less branched and are not feruloylated.

The primary cell walls of the Poaceae and other families in the Poales usually contain only small amounts of pectic polymers (Shibuya et al. 1983) and variable amounts of MLG. MLG has been found in cell walls of the Arundinoideae, Bambusoideae, Chloridoideae, Panicoideae and Pooideae subfamilies of the Poaceae (Nevins et al. 1978) and is absent from the primary cell walls of other commelinoid members such as the Arecales (e.g. *Cocos*, coconut and *Phoenix*, date palm), Bromeliales (e.g. *Ananas comosus*, pineapple), Philydrales (e.g.

Angioxanthos, kangaroo paw), Commelinales (e.g. *Tradescantia*), Typhales (e.g. *Typha*, cat tail), Juncales (e.g. *Juncus*, rushes) and Zingiberales (e.g. *Zingiber*, ginger, *Curcuma*, turmeric and *Amomum*, cardamon). However, all non-Poaceae members of the commelinoid group have an overall polysaccharide composition which appears to be intermediate between the Poales and non-commelinoid monocots. The non-commelinoid monocot cell wall appears to be similar to that of dicots. An exception is the Araceae whose primary cell walls contain ferulic acid but whose overall polysaccharide composition is similar to non-commelinoids. The Araceae have been resolved as the basal clade in the commelinoid monocots on the basis of *rbc*L data (Chase et al. 1993).

The Poaceae also differ in their xyloglucan content and composition. The Poaceae primary cell wall is composed of 1–5% xyloglucan compared to about 20% in dicots (Bacic et al. 1988). Poaceae xyloglucan contains less xylose, much less galactose and very much less fucose than typical dicot xyloglucan (Hayashi 1989, Vinken 1997, McDougall and Fry 1994).

The Gramineae, Cyperaceae, Juncaceae and Restionaceae have a low galacturonan content (less than 5% dry mass of the cell wall), other monocots have intermediate levels, and lower moncots, dicots and gymnosperms have a high content (more than 10% dry mass) (Jarvis et al. 1988).

1.6 Use of cell wall components as taxonomic markers

The cell wall composition of algae has been used as an important taxonomic marker. The main structural polysaccharide has been shown to vary between taxa. Some algae, the Charophyceae and closely related species, have cellulose as the main structural polymer whereas other algae may have glucomannans, galactans or xylans (van den Hoek 1981).

In general, cell wall characters have been little used as taxonomic markers in land plants. However, variation in cell wall composition for a few taxa has been recorded and in some cases has been used to delimit taxa.

Occurrence and concentration of specific arabinogalactan proteins have been studied in seven leafy liverworts and have been found to vary with taxa (Basile et al. 1989). It is thought (Basile et al. 1989) that arabinogalactan proteins may be a useful character for systematics of the liverworts although the composition of some AGP fractions may be conserved in closely related taxa.

Cell wall characters have been used to delimit four species of the leafy liverwort genus *Odontoschisma* (Szwykowsk and Buczkowska 1999). However, the characters used were staining properties of the leaf blade cell walls and cell wall fluorescence as seen under UV light. These characters did not seem particularly distinct but were surprisingly stated as being more reliable for defining these species even when compared to ecological and morphological characters.

Galactomannans are important storage polysaccharides in seeds of the Leguminosae and have been used taxonomically at the level of subfamily (Buckeridge et al. 1995) and species (Reid and Meier 1970). Brazilian members of the Rubiaceae differ in the phytoalexin-eliciting abilities of their pectic polysaccharides: it has been suggested that this may be due to differences in methyl-esterification of pectins in their cell walls (Braga and Dietrich 1998, Braga et al. 1998).

Gramineous monocots and related orders have primary cell walls which are rich in esterified phenolic acids such as ferulic acid, *p*-hydroxybenzoic acid and *p*-coumaric acid. They give a negative phloroglucinol–HCl test for lignin, but show green fluorescence, in the presence of alkali, under UV light because of the feruloyl esters (Harris and Hartley 1976). It has been shown that several genera previously classified as lilioids, like commelinoids, show this type of green fluorescence under UV light (Harris and Hartley 1976). Rudall and Caddick (1994) placed the Australian genera *Dasypogon, Kingia* and *Calectasia*, which had obscure affinities

within the monocots, but exhibit green wall-fluorescence under UV light, within the commelinoid clade. Commelinoids can be distinguished from non-Commelinoids on the basis of (a) the presence of a starchy endosperm, (b) silica bodies in the epidermis, (c) the absence of calcium oxalate raphide crystals. Closer examination of *Dasypogon*, *Kingia* and *Calectasia* showed that they had these morphological characters, which distinguished them as being commelinoids. The term commelinoid as used by Chase et al. (1993) describes the large terminal monocot clade that includes many 'non-petaloid' groups (grasses, sedges and rushes) and also the palms and gingers. The non-commelinoid group may represent a paraphyletic grade and includes all the basal monocot groups (aroids, alismatids and lilioids).

1.7 Statement of plan

The main aim of my thesis is to evaluate the evolution of the primary plant cell wall. In particular I am interested in how plant cell wall polysaccharides may have altered in composition and structure and to see whether major changes in cell wall composition, if any, relate to specific evolutionary events such as terrestrialisation. I was interested to see when, and possibly why, specific components known in the cell walls of dicots first become evident.

In order to do this I studied AIRs of plants which I selected on the basis of land plant phylogenetic studies (Chase et al. 1993, Judd et al. 1999). I particularly concentrated on lower land plants as little was known about their cell wall composition. Within the angiosperms I investigated the cell walls of basal groups, including the Illiciales–Trimeniaceae–*Austrobaileya* group as again little was known about their cell wall composition. I also investigated the cell wall composition of three charophycean green algae (*Klebsormidium*, *Coleochaete* and *Chara*), because of their proposed close relationship to the ancestors of land plants, to see how similar their cell walls are to those of land plants.

2 Materials and Methods

2.1 Materials

2.1.1 Chemical reagents

All chemical reagents used were purchased from Fisher chemicals (Fisher Scientific), Sigma-Aldrich (Poole, United Kingdom), BDH AlanaR Chemicals Ltd (Poole, United Kingdom) or Labscan Ltd. Analytical Chemicals (Dublin, Ireland) unless otherwise stated. All water used was deionised water unless otherwise stated.

2.1.2 Plants selected

Material examined is listed in Table 2.1. Much of the material was collected from the living collection at the Royal Botanic Garden, Edinburgh, UK (RBGE; accession number given where available). Otherwise material was collected by myself or kindly donated by names indicated. In the case of bryophytes voucher specimens were kept and identification was checked by David Long (RBGE). The youngest available plant material was collected. For the majority of tracheophyta the plant tissue collected was unexpanded leaves. The exceptions to this were *Phaseolus aureus* L. (hypocotyls) and *Secale cereale* L., *Triticum aestivum* L., *Hordeum vulgare* L. and *Avena sativa* L. (coleoptiles) as MLG had been reported to be at its greatest concentration in these tissues (Buchala and Franz 1974, Franz 1972, Luttenegger and Nevins 1985, Brown et al. 1997). For the Bryophyta the youngest possible gametophyte leaves were selected as sporophyte tissues are not abundant enough to be used and primary cell wall composition is thought to similar in all tissues (Brett and Waldron 1996).

Table 2.1: Plant material selected for study

Chlorobiota (Green algae) (Sluiman and Guihal 1999)	RBGE accession number (E) or collector
Chlorophyta Ulvophyceae *Ulva lactuca* L.	 North Berwick (Z. Popper)
Charophyta Charophyceae *Chara corallina* (Klein ex Willd. Em. R.D.W.) Coleochaetophyceae *Coleochaete scutata* Brébisson Klebsormidiophyceae *Klebsormidium flaccidum* (Kützing) Silva, Mattox et Blackwell	 York University (Dale Sanders). CCAP 414/1 RBGE (Hans Sluiman)
Embryobiota (Land plants)	
Bryophyta (Watson 1984). Bryopsida (Musci) Sphagnidae Sphagnales *Sphagnum palustre* L. (*S.cymbifolium* (Ehrh.)Hedw.) *Sphagnum molle* Sull. Andreaeidae Andreaeles *Andrea rupestris* Hedw. (*A. petrophila* Ehrh.) Bryidae Polytrichales *Polytrichum formosum* Raddi. Dicranales *Dicranum scoparium* Hedw. Bryales *Mnium hornum* Hedw. *Philonotis fontana* (Hedw.) Brid.	 Cairngorms (Z. Popper) Cairngorms (Z. Popper) Cairngorms (W.M.M. Eddie) Roslin Glen (Z. Popper & D. Long) Roslin Glen (Z. Popper & D. Long) Roslin Glen (Z. Popper & D. Long) Cairngorms (Z. Popper)

Rhizomnium punctatum (Hedw.) Kop. (*Mnium punctatum* Hedw.)	Cairngorms (Z. Popper)
Isobryales	
Neckera complanata (Hedw.) Hüben	Roslin Glen (Z. Popper & D. Long)
Hookeriales	
Hookeria lucens (Hedw.) Sm. *Pterygophyllum lucens* (Hedw. Brid.)	Mull (Z. Popper)
Thuidiales	
Thuidium tamariscinum (Hedw.) B., S.& G	Roslin Glen (Z. Popper & D. Long)
Plagiothecium undulatum (Hedw.) B., S.& G.	Roslin Glen (Z. Popper & D. Long)
Hypnum cupressiforme Hedw.	Roslin Glen (Z. Popper & D. Long)
Hepaticae (Liverworts)	
Anthocerotales	
Anthoceros caucasicus C. Spr.	Azores (Rene Schumacker)
Marchantiales	
Lunularia cruciata (L.) Dum. Ex Lindb.	Balerno (P.M.Smith)
Marchantia polymorpha L.	Edinburgh (Z. Popper)
Metzgeriales	
Pellia epiphylla (L.) Corda	Roslin Glen (Z. Popper & D. Long)
Jungermanniales	
Ptilidiineae	
Trichocolea tomentella (Ehrh.) Dum.	Appin (D.S.Rycroft 99062)
Lepidoziineae	
Lepidozia reptans (L.) Dum.	Milngavie (D.S.Rycroft)
Jungermanniineae	
Nardia scalaris (Schrad.) Gray (*Alicularia scalaris* (Schrad. Corda)	Cairngorms (Z. Popper)

Marsupella emarginata var. aquatica (Lindb.) Dum.	Cairngorms (Z. Popper)
Plagiochila asplenioides (L.) Dum. (P. asplenioides *var. major* Nees)	Cairngorms (Z. Popper)
Lophocolea bidentata (L.) Dum.	Edinburgh (Z. Popper)
Scapania undulata (L.) Dum.	Cairngorms (Z. Popper)
Pleuroziineae	
Pleurozia purpurea Lindb.	Cairngorms (Z. Popper)
Porellineae	
Porella cordaeana (Hüb.) Moore (*Madotheca cordaeana* (Hüb.) Dum.)	Milngavie (D.S.Rycroft 99068)
Porella sp.	Cairngorms (Z. Popper)
Tracheophyta (Vascular plants) Judd *et al.* (1999)	
Lycopodiophytes	
Lycopodiaceae	
Lycopodium pinifolium Blume	19835037 (E)
Huperzia selago (L.) Bernh. Ex. Schrank & Mart.	Cairngorms (Z. Popper)
Diphasiatrum alpinum (L.) Holub.	Cairngorms (Z. Popper)
Selaginellaceae	
Selaginella apoda (L.)Spring.	19677705 (E)
Selaginella davidii Franch	19782510 (E)
Selaginella erythropus Spring	19715473 (E)
Selaginella helvetica Link	19699941 (E)
Selaginella martensii Spring	Edinburgh University
Selaginella pallescens (C.Presl.) Spring	19697710 (E)
Equisetophytes	
Equisetaceae	
Equisetum debile Roxb. Ex. Vaucher	19731694 (E)
Psilotophytes	
Psilotaceae	
Psilotum nudum (L.) P.Beauv.	Edinburgh University
Filicophytes (Ferns)	
Eusporangiate ferns	
Ophioglossales	
Ophioglossum vulgatum L.	19695522 (E)

Marattiales	
Marattiaceae	
Marattia fraxinea Sm.	19697183 (E)
Leptosporangiate ferns	
Osmundaceae	
Osmunda regalis L.	19578631 (E)
Osmunda banksifolia (Presl.)Kuhn	19940406 (E)
Todea barbara (L.) T. Moore	19652792 (E)
Dryopteriodeae	
Dryopteris affinis (Lowe) Fraser-Jenk	19900786 (E)
Dryopteris crispifolia Rasbach & al.	19920813 (E)
Polystichium aculeatum (L.)Schott	19942883 (E)
Asplenioideae	
Asplenium australassium (J.Sm.)Hook.	19933661 (E)
Asplenium bulbifera G. Forst.	19933663 (E)
Nephrolepis lauterbachii H. Christ.	19933715 (E)
Davallia fejeensis (Burm.f.)Mett.	19902432 (E)
Onoclea sensiblis L.	19662802 (E)
Phyllitis scolopendrum L.	19731529 (E)
Blechnoideae	
Blechnum spicant (L.) Roth	Cairngorms (Z. Popper)
Cycatheoideae	
Dicksonia blumei (Kunze) Moore	19530207 (E)
Cibotium barometz (L.) J.Sm.	19696413 (E)
Salviniales	
Salvinia auriculatea Aubl.	19830813 (E)
Azolla carolinana Willd.	19933668 (E)
Marsileales	
Marsilea quadrifolia L.	19710423 (E)
Dennistaedtioideae	
Pteridium aquilinum (L.)Kuhn	Roslin Glen (Z. Popper)
Histiopteris incisa (Thunb.) F.Donn.Sm.	19691200 (E)
Schizaeaceae	
Lygodium japonica (Tmb.)Sw.	19732455 (E)
Anemia	19933657 (E)
Adiantaceae	
Pellaea falcata (R.Br.) Fée	19895049 (E)
Polypodiaceae	
Platycerium bifurcatum (Car.) C.Chr.	19734554 (E)

Seed plants	
Gymnosperms	
Cycads	
Zamiaceae	
Encephalartos altensteinii Lehm	19754185 (E)
Conifers	
Pinaceae	
Pinus sylvestris L.	Cairngorms (Z. Popper)
Gnetophytes	
Ephedraceae	
Ephedra likigeensis var. mairei (Florin) L.K.Fu & Y.F.Yu	19943963 (E)
Gnetaceae	
Gnetum gnemon L.	19902511 (E)
Gnetum indicum Merr.	19550226 (E)
Gnetum montana Markgr.	19791010 (E)
Angiosperms (Chase et al.1993)	
Paleoherbs	
'Non-monocot Paleoherbs'	
Nymphaeales	
Nymphaceae	
Nymphaea colorata Peter	19972169 (E)
Austrobaileyaceae	
Austrobaileya scandens C.T. White	19973060 (E)
Aristochiales	
Aristolochia arborea Linden	19696194 (E)
'Magnoliid complex'	
Magnoliales	
Magnoliaceae	
Magnolia × soulangiana Soul.-Bud.	19351039 (E)
Liriodidendron tulipifera L.	19687757 (E)
Annonaceae	
Annona crassilora Mart.	19981727 (E)
Laurales	
Lauraceae	
Cinnamomum loureirii Nees	19100029 (E)
Monimiaceae	
Hedycarya arborea J.R. & J.G.A. Forst	19900026 (E)
Hernandiaceae	
Hernandia cordigera Vieill.	19696964 (E)
Winteraceae	
Drimys lanceolata (Poir.)Baill.	19531038 (E)

Chloranthaceae	
Chloranthus glaber Makino	19696408 (E)
Calycanthaceae	
Calycanthus floridus var. laevigatus L.	19687220 (E)
Illiciales	
Illicium verum Hook.f.	19741584 (E)
Schizandraceae	
Schizandra rubiflora Rehder & E.H.Wilson	19091003 (E)
Monocots	
Acorales	
Acoraceae	
Acorus calamus L.	Edinburgh (Z. Popper)
Alismatales	
Araceae	
Lemna sp.	Edinburgh (Z. Popper)
Hydrocharitaceae	
Vallisneria spiralis L.	19697868 (E)
Aponogetonaceae	
Aponogeton distachyos L.	19931562 (E)
Lilianae	
Alliaceae	
Allium porrum L.	Edinburgh (Z. Popper)
Marantaceae	
Calathea zebrina (Sims.)Lindl.	19696326 (E)
Commelinanae	
Commelinaceae	
Callisia repens L.	19831987 (E)
Carex elata All.	19850773 (E)
Cyanotis longifolia Wight *var. longifolia*	19672908 (E)
Dichorisandra thyrsifolia J.C.Mikan	19644295 (E)
Geogenthus undatus (K.Koch & Linden) Mildbr. & Strauss	19696892 (E)
Palisota albertii L. Gentil	19140061 (E)
Siderasis fuscata (Lodd.) H.E. Moore	19633223 (E)
Tradescantia longipes E.S. Anders & R.E. Woodson	19802706 (E)
Juncales	
Juncaceae	
Juncus effusus L.	Cairngorms (Z. Popper)
Cyperaceae	
Cyperus esculentus L.	19960902 (E)
Cyperus papyrus L.	20000863 (E)

Poales	
Poaceae	
Secale cereale L	PBI
Triticum aestivum L.	PBI
Zea mays L.	PBI
Hordeum vulgare L.	PBI
Avena sativa L.	PBI
Restionaceae	
Restio tetraphyllus Labill.	19812071 (E)
Elegia capensis (Burm.f.)Schelpe	19860037 (E)
Flagellariaceae	
Flagellaria guineensis Schum.	19720171 (E)
Tricolpates (Eudicots)	
Basal tricolpates	
Ranunculales	
Helleborus argutifolius Viv.	19780406 (E)
Core tricolpates	
Caryophyllanae	
Caryophyllales	
Caryophyllaceae	
Spinacia oleracea (L.)T.Moore	Johnsons seeds, W.W. Johnson & Son Ltd, England
Polygonaceae	
Fallopia japonica (Houtt.)Ronse Deraene	Roslin Glen (Z. Popper)
Rosid Clade	
Eurosids I	
Urticaceae	
Urtica dioica L.	North Berwick (Z. Popper)
Cucurbitales	
Cucumis sativus L.	Edinburgh University
Leguminaceae	
Phaseolus aureus L.	Sainsbury's

2.2 Algal culture

Coleochaete scutata Brébisson was purchased from CCAP (Culture Collection of Algae & Protozoans, Windermere, United Kingdom). Cultures were maintained by subculturing monthly. The culture was divided between four flasks containing fresh Jaworski's medium (Table 2.2). All manipulations were carried out aseptically. The cultures were keep at room temperature (15–20°C) by a window but out of direct sunlight.

Jaworski's medium (Table 2.2) is commonly used in the culture of green, freshwater algae. It is made up as described in Table 2.2 and 250-ml aliquots were poured into conical flasks. Flasks were sealed with cotton wool and muslin bungs and covered with a double layer of aluminium foil before being autoclaved (121°C, 15 psi, 19 min).

Table 2.2: Jaworski's medium (Jaworski 1988)

Component	**Molarity (µM)**
$Ca(NO_3)_2.4H_2O$	85
KH_2PO_4	90
$MgSO_4.7H_2O$	200
$NaHCO_3$	190
EDTAFeNa	6
$EDTANa_2$	6
H_3BO_3	40
$MnCl_2.4H_2O$	7
$(NH_4)_6Mo_7O_{24}$	0.8
cyancobalamin	0.03
thiamine HCl	0.2
biotin	1.6
$NaNO_3$	900
$Na_2HPO_4.12H_2O$	200

Final pH = 7.5

Klebsormidium flaccidum (Kützing) Silva, Mattox et Blackwell was a kind gift from Hans Sluiman (RBGE). It was cultured in the same way as the *Coleochaete scutata* Brébisson but, as recommended by Hans Sluiman (RBGE), Bold's Basal Medium (Table 2.3) was used instead of Jawarski's medium.

Table 2.3: Bold's basal medium (Bold 1942)

Component	Molarity (μM)
$NaNO_3$	300
$MgSO_4.7H_2O$	30
NaCl	40
K_2HPO_4	30
KH_2PO_4	130
$CaCl_2.2H_2O$	17
$ZnSO_4.7H_2O$	30
$MnCl_2.4H_2O$	7
$(MoO_3)Na_2MoO_4.2H_2O$	3
$CuSO_4.6H_2O$	6
$Co(NO_3)_2.6H_2O$	1.7
H_3PO_3	180
$EDTANa_2$	134
KOH	600
$FeSO_4.7H_2O$	18
Concentrated H_2SO_4	590

Final pH = 6.6

2.3 Plant culture

Dry mung bean seeds (*Phaseolus aureus* L.) were purchased from Sainsbury's, imbibed for 16 h and then grown in the dark on wet tissue paper for various time periods. Hypocotyls were then harvested and frozen.

Secale cereale L., *Triticum aestivum* L., *Hordeum vulgare* L., *Avena sativa* L. and *Zea mays* L. grains were purchased from PBI (Plant Breeding International, Cambridge, United Kingdom). Seeds were sown in vermiculite and grown in the dark at room temperature for 8–10 d before harvesting coleoptiles.

2.4 Preparation of cell walls

Plant material was frozen at –80°C as soon as possible after collection. This was done to stop enzyme action and synthesis of callose which is often produced as a defence response (Iglesias and Meins 2000, Vleeshouwers et al. 2000)

Initially cell walls were extracted by homogenising roughly 30 g fresh weight in 1.5% SDS, 5 mM sodium metabisulphite, 20 mM HEPES to rupture cell membranes and extract cytoplasmic proteins. The homogenate was then filtered on nylon gauze and the residue washed and homogenised in $N_{2(l)}$. A small amount of the residue was stained with Evan's blue (0.25% w/v for 5 min) and examined under the microscope to check protein was no longer present at detectable levels. Any remaining protein was extracted by stirring for 2 h in phenol:acetic acid:water :2:1:1. This was then filtered and in initial work the residue washed in 90% DMSO and stirred for 16 h in 90% DMSO. A small sample of the residue was stained with potassium iodide (0.33% I_2, 0.67% KI) and examined under the microscope to check that starch was no longer present at detectable levels. The residue was then washed in ethanol and dried. The activity reported by Megazyme for α-amylase in the licheninase preparation is less than 0.0005 units/mg of protein compared to the licheninase activity of 118 units/mg of protein. Had it had greater amylase activity it would have been able to hydrolyse starch to give appreciable amounts of maltotriose and maltotetraose, which might have co-chromatographed with the licheninase digestion products of mixed-linkage glucan. However, I later found that the enzymic contaminants, notably α-amylase, of the licheninase preparation were not capable of hydrolysing starch detectably from either a monocotyledon, wheat, or a dicotyledon, potato. It was therefore decided that making AIR preparations, although not resulting in starch and protein removal, would be acceptable, and indeed preferable as it would reduce the chances of losing hemicellulose from the wall preparations. Mixed-linkage glucan (MLG) is soluble in hot water, up to 65% of barley endosperm MLG being extracted by water at 65°C, and in DMSO and can therefore potentially be lost during some cell wall preparation methods (Anderson et al. 1978). AIR does not suffer from potential loss of MLG as the preparation of AIR does not involve water or DMSO instead 70% ethanol at 55°C is used.

2.4.1 Preparation of alcohol-insoluble residue (AIR)

To prepare alcohol insoluble residues (AIRs) roughly 30 g fresh weight plant material was blended in an MSE Atomix blender with 150 ml 70% ethanol in two 1-min bursts. The suspension was then filtered on Miracloth (Calbiochem). The residue was ground to a fine powder, in $N_{2(l)}$, with a ceramic pestle and mortar (both of which had been precooled to −80°C). The fine powder was transferred to a 250-ml conical flask with 150 ml 70% ethanol and heated at about 55°C for 5 d to solubilise any sugars. The suspension was then filtered and repeatedly washed with 70% ethanol until the filtrate was colourless. The residue was washed with acetone to remove water and left to dry at room temperature.

2.4.2 Cell wall extractions

A method adapted from Morrison et al. (1993) was used to prepare a hemicellulose-rich extract from *Chara corallina* (klein ex Willd. Em. R.D.W.). *Chara* cells had the protoplasm squeezed out and were then frozen at −20°C then ground to a fine powder in $N_{2(l)}$. The cells were washed in 60% ethanol and further ground in 1% SDS, 5 mM sodium metabisulphite, 20 mM HEPES. The residue was then washed in 60% ethanol to remove the SDS. The cell wall preparation was washed for 1 d in each of the following: 50 mM CDTA with 50 mM sodium acetate, pH 6.5, 50 mM Na_2CO_3 with 20 mM $NaBH_4$, 1 M KOH with 10 mM $NaBH_4$, 4 M KOH with 10 mM $NaBH_4$. After each extraction the residue was washed in water and the washings were added to the extract. The extracts were then neutralised, dialysed and freeze dried before further analysis.

2.5 Hydrolyses

2.5.1 Enzymic hydrolysis

Enzyme hydrolysis is a good way to degrade polysaccharides as it can be extremely specific when pure enzyme preparations are available, e.g. in the case of licheninase, or a mixture of enzymes, such as those present in Driselase, may together yield informative products.

2.5.1.1 Licheninase digestion

The enzyme licheninase (EC 3.2.1.73; (1→3,(1→4)-β-D-glucan 4-glucanohydrolase) from *Bacillus subtilis* was purchased from Megazyme (Megazyme International, Bray Business Park, County Wicklow, Ireland). With a high degree of specificity, licheninase cleaves a β-D-glucopyranosyl-(1→4) linkage when it is preceded by a β-D-glucopyranosyl-(1→3) linkage (Parrish et al. 1960). Licheninase is therefore able to yield β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-D-glucose (MLG3), β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-D-glucose (MLG4) and larger oligosaccharides composed of longer stretches of β-D-glucopyranosyl-(1→4) linkages from MLG. Owing to the high degree of specificity of licheninase the oligosaccharides produced by its activity can be used to indicate the presence of MLG.

Licheninase was supplied in 3.2 M ammonium sulphate. The presence of this concentration of salt would prevent the digestion products from developing properly on TLC. The ammonium sulphate was therefore removed by dialysis of the enzyme preparation against water (0.5% chlorobutanol precipitates ammonium sulphate rapidly) at 4°C for 2 h. The enzyme was then frozen at –20°C.

Prior to digestion by licheninase the hemicellulose present in 10 mg of AIR was swollen and solubilised by heating at 120°C for 2 h in 1.85 ml collidine acetate buffer (100 mM collidine, 0.5% chlorobutanol, adjusted to pH 7 with acetic acid) in a tightly sealed Sarstedt tube (Sarstedt microtubes, Aktiengesellschaft & Co. Nümbrecht). Sarstedt tubes were used as they seal securely. Initially screw-cap Pyrex tubes were used but they had to be cleaned in chromic acid as polysaccharides are difficult to remove from pyrex. After cooling, 0.5 ml of the suspension was set aside as a control and 10 units of dialysed licheninase was added to the remainder. After 24 h at room temperature the reaction was stopped by addition of formic acid to a final concentration of 15%. The digest was then dried and redissolved in 350 μl water to concentrate the digestion products. Aliquots of

2.5 μl was then applied to a silica gel TLC plate (see 2.6.5) developed in B:A:W 3:1:1 and visualised with thymol–sulphuric acid reagent (as described 2.7.1.2).

2.5.1.2 Driselase digestion

Driselase is a mixture of endo- and exo- enzymes from a basidiomycete fungus (*Irpex lacteus*). This mixture of enzymes is highly active on plant cell walls (Fry 1988). Exo-hydrolases attack the non-reducing ends of polysaccharides, usually cleaving off single specific sugars. The exo-hydrolases reported to be present in Driselase are: α-D-galactopyranosidase, β-D-galactopyranosidase, β-D-glucopyranosidase, α-D-mannopyranosidase, β-D-mannopyranosidase, α-L-arabinofuranosidase, β-D-xylopyranosidase, α-L-fucopyranosidase and cellulose-cellobiohydrolase. Endo-hydrolases cleave specific glycosidic linkages within a polysaccharide chain. Endo-hydrolases reported to be present in Driselase are: β-D-galactanase, α-L-arabinanase, pectinase [α-(1→4)-D-galacturonase], β-D-mannanase, xylanase [β-(1→4)-D-xylanase] and cellulase [1→4–(1→3),(1→4)-β-D-glucan 4-glucanohydrolase] (Fry 1988). Driselase is able to digest completely xyloglucan to the disaccharide α-D-xylopyranosyl-(1→6)-D-glucose (isoprimeverose) because it lacks α-xylosidase but is rich in cellulase, α-L-fucosidase, β-D-galactopyranosidase and it possesses a β-glucosidase which tolerates the presence of an α-xylose on the 6 position of the otherwise non-reducing terminal β-glucose residue. Isoprimeverose (IP) can be used to indicate the presence of xyloglucan.

Before Driselase can be used it must be freed of non–enzymic components. The partial purification was carried out at 4°C and all buffers used were pre-chilled to minimise loss of enzyme activity. Driselase (10 g) was stirred for 15 min in 100 ml 50 mM acetate buffer (Na^+, pH 5.0). After centrifugation (10 min, 2500 *g*) 26 g ammonium sulphate was added per 50 ml supernatant with constant stirring. The solution was then allowed to stand for 15 min at 0°C before being centrifuged (10 min, 2500 *g*) and the supernatant rejected. The pellet was redissolved in 100 ml fresh 52% w/v ammonium sulphate solution. The pellet formed after a further

centrifugation step was dissolved in 20 ml water and desalted on a Sephadex G–25 column. The resulting enzyme mixture was freeze dried (Modulyo freeze drier) and stored at –20°C ready for use.

Before AIR was treated with Driselase it was pretreated to make the hemicelluloses more accessible to the enzymes. AIR (20 mg) was shaken at 37°C for 16 h with 10 ml 6 M NaOH containing 1% $NaBH_4$. The suspension was then neutralised, whilst being kept chilled, with an excess of acetic acid. The whole suspension was then dialysed for 24 h (Visking tubing , Medicell International Ltd., 239 Liverpool Road, London, N1 1LX; Size 5, 19.0 mm diameter, molecular weight cut-off 12–14000). The suspension was freeze-dried. After drying of the sample, 1 ml 0.1 M TFA was added and heated to 85°C for 1 h to hydrolyse furanosyl linkages. For removal of the TFA the samples were then dried in a Speed Vac (Savant Speed Vac concentrator and refrigerated condensation trap, Stratech Scientific, London; Genevac CVP100 pump), redissolved in water and redried.

Driselase digestion was carried out at room temperature for 2 d. Driselase, 1% w/v, was made up in buffer (1:1:98 pyridine:acetic acid:0.5% chlorobutanol, pH 4.7) and 0.5 ml was added to the pretreated AIR derived from 20 mg AIR. A Driselase-only control was always carried out at the same time because Driselase undergoes some autolysis on incubation to produce mannose. After 2 d the digestions were stopped by addition of formic acid to a final concentration of 15% v/v then dried and redissolved in 300 µl water. The sample was separated into neutral and acidic fractions (2.6.1) before analysis. Samples were for PC and TLC were stored at 4°C and those for HPLC were stored at –20°C.

2.5.1.3 Cellulase digestion

Cellulase [EC 3.2.1.4; 1→4-(1→3),(1→4)-β–D-glucan 4-glucanohydrolase], isolated from *Trichoderma longibrachiatum*, was purchased from Megazyme. Cellulase cleaves non-substituted β–D–glucosyl-(1→4)-linkages. It can therefore cleave roughly one in every four glucosyl bonds in tamarind xyloglucan. A range of oligosaccharides is released. The hepta–, octa– and nonasaccharides indicate the

presence of a typical xyloglucan. It was found that owing to the structural similarity of glucose and xylose the enzyme preparation has an intrinsic and substantial xylanase activity.

Before treatment with cellulase the AIRs were treated to remove cellulose. AIR (0.1 g) was shaken (37°C, 16 h) in 10 ml 6 M NaOH containing 1% $NaBH_4$ (Fry 1988). The suspension was then filtered on Miracloth to remove the cellulose before neutralisation of the filtrate with an excess of acetic acid. During neutralisation the sample was kept on ice in a fume hood and the acetic acid was added slowly as H_2 evolves rapidly causing vigorous bubbling. The solution was then dialysed overnight against running tap water and freeze dried. AIR from members of the Poaceae was treated with licheninase and dialysed to remove MLG before cellulase digestion.

The alkali–soluble polymers (0.01 g) were dissolved in 1 ml buffer (50 mM acetate buffer, Na^+, pH 4.7). Cellulase (2.5 units) was then added and the digest was incubated at room temperature for 3 d. Digestion was stopped by loading of the products on to a silica gel TLC plate.

2.5.1.4 Xyloglucan–specific endoglucanase (XEG)

Xyloglucan–specific endoglucanase [E.C.3.2.1.4] isolated from *Aspergillus aculeatus* (Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd) specifically digests xyloglucan (Pauly et al. 1999). I found the preparation to have cellulase and xylanase activity because digestion products were yielded from the following substrate; methyl-cellulose, cellohexaose, MLG and xylan extracted from oat spelts. However, the cellulase and xylanase activities were considerably less than those of the cellulase preparation (Megazyme).

AIR (1 mg) was incubated in 0.5 ml 50 mM acetate, Na^+, pH 3 containing 0.1% XEG. The mixture was incubated at room temperature and the reaction was complete within 5 min. The reaction was stopped by loading onto a silica gel TLC

plate which was then run in B:A:W 2:1:1 and stained to visualise the digestion products.

2.5.1.5 Galactose oxidase digestion

Galactose oxidase [E.C.1.1.3.9; D-galactose:oxygen 6-oxido-reductase] isolated from *Dactylium dendroides* is able to oxidise D-galactose but has no affinity for L-galactose. It is reported to have a high affinity for 3-*O*-methyl D-galactose (Schlegel et al. 1968, Cleveland et al. 1975). This enzyme was used to indicate whether the 3-*O*-methyl galactose purified from *Selaginella apoda* was in the D or L form. D-Galactose and L-galactose (0.5 mg of each) and 3-*O*-methyl galactose (0.2 mg) were incubated in 100 µl 100 mM potassium phosphate buffer pH 6.0 with 10 units of catalase and 15 units of galactose oxidase. At various time points 20 µl from each incubation was loaded on to a cellulose TLC plate which was then run in B:A:W 2:1:1 followed by a second run of E:P:W 10:4:3 in the same direction. The oxidation products were stained with aniline hydrogen–phthalate. D–Galactose was characterised by the appearance of oxidation products.

2.5.2 Acid hydrolysis

Polysaccharides can be completely hydrolysed to their monosaccharide constituents by acid hydrolysis. This does not give any indication of the linkages between monosaccharides but the monosaccharides present and their relative amounts may indicate which polysaccharides are present. Trifluoroacetic acid (TFA) is used as it is a strong, volatile acid and can be easily removed by drying.

AIR (20 mg) was dissolved in 1 ml 2 M TFA in a Sarstedt tube and incubated for 1 h at 120°C to give complete hydrolysis. For removal of the TFA the samples were dried in the Speed Vac, redissolved in 1 ml water and redried. The products released by TFA hydrolysis were separated into neutral sugars and sugar acids before further analysis (2.6.1).

Under milder conditions (0.1 M TFA, 85°C, 1 h) acid hydrolysis can be used to treat AIR samples before treatment by Driselase. In this case the TFA hydrolysis

increases the number of sites for some of the enzymes present in Driselase by breaking almost all of the arabinofuranosyl linkages and therefore removing most of the side-chains from xylans.

2.6 Separation of sugars

2.6.1 Ion-exchange chromatography

Ion-exchange chromatography separates sugars on the basis of charge. There are two types: anion-exchange chromatography, in which the resin has a greater affinity for acidic or negatively charged sugars, and cation-exchange, in which the resin has a greater affinity for positively charged molecules.

The anion exchange resin used was Dowex 1 X 4–200 strongly basic anion-exchanger for chloride ions. This was treated so that it became a formate ion-exchanger as the formic acid eluted is volatile and can be removed by drying. The Dowex resin was washed in 0.5 M NaOH to remove any impurities and then washed twice with 0.5 M formic acid. This was followed by a wash in 2 M sodium formate and finally the resin was washed in the running buffer (10 mM pyridinium formate, pH 5.5). All washes were for 1 h. Columns of bed-volume 1.5 ml were then set up in Pasteur pipettes that had glass wool at the base to hold the resin.

To optimise separation of neutral and acidic sugars the sugar acid lactones, which are neutral, had to be delactonised before separation. Delactonisation was achieved by alkali treatment of the sugars. The pH indicator alizarin red was added to the hydrolysis products. Aliquots of freshly made 0.1 M NaOH were then added to the sample until the colour change brick-red to violet was observed. This indicated that the pH had been adjusted to 13. Delactonisation at pH 13 takes about eight seconds. After conversion of the lactones to acids the pH had to be returned to neutral before the sample could be applied to the columns. The pH indicator bromocresol green was added to the sample and 0.1 M formic acid was added until the colour change blue to green was observed indicating that the solution was about pH 7. The sample was then made up to 1 ml with the running buffer and loaded onto the Dowex 1 formate column. The first 4 ml was eluted with 10 mM pyridinium formate, pH 5.5,

as the neutral fraction. The acidic fraction was then eluted with 4 ml of pyridine:formic acid:water 1:1:23 adjusted to pH 5.5 with pyridine. The pH indicators were easily removed as both bound irreversibly to the Dowex resin. To avoid contamination fresh columns were used for each sample. Both the neutral and acidic fractions were then dried in the Speed Vac. The acidic fraction was dissolved in 100 µl ready for analysis. Sodium salts from the delactonisation process eluted in the neutral fraction and was removed by cation-exchange chromatography before analysis. Sodium salts at this concentration would prevent samples from developing properly in paper chromatography.

The cation exchange residue used was Dowex 50W X 8 100–200 hydrogen form (Supelgo Park, Bellefonte, PA, USA). The Dowex resin was prepared by shaking for 1 h in 1 M HCl and then rinsing with water until the filtrate was neutral (tested using universal indicator paper). Columns of 1.5 ml bed volume were prepared in Pasteur pipettes as previously described.

The neutral fraction was redissolved in 100 µl of water and loaded on to the cation exchange columns. The sample was eluted with water and the first 1.5 ml was collected. This was then dried and redissolved in 100 µl water ready for analysis.

2.6.2 Gel-permeation chromatography

Gel-permeation chromatography was used to separate oligosaccharides of different sizes and to isolate specific mono- and oligosaccharides. Gel-permeation chromatography resolves compounds on the basis of their molecular weight. Large compounds such as a polysaccharide travel relatively fast through the gel matrix as they travel around the gel beads. Smaller compounds, such as monosaccharides, travel much more slowly through the matrix as they can enter pores in the beads and so have further to travel. A column was calibrated by using blue dextran to mark the void and either cobalt chloride or glucose to mark the included volume. Oligosaccharides (DP 2–12) were separated using a Bio-Gel P-2 column (Bio-Gel P-2 gel fractionation range 100–1800, Bio-Rad laboratories) with a bed volume of 140 ml. The sample size was always kept to below 5% of the bed volume to ensure

good resolution. The running buffer was pyridine:acetic acid:0.5% chlorobutanol 1:1:23 which had been degassed and eluted fractions were collected using an automated fraction collector (Bio-Rad model 2110 fraction collector). Monosaccharides were separated from contaminating proteins and polysaccharides using a Bio-Gel P-2 column of bed volume 25 ml. A column of Sephadex G-25 of bed volume 600 ml was used to purify the enzymes that make up Driselase from the other constituents of Basidiomycetes.

2.6.3 High-pressure liquid chromatography (HPLC)

HPLC gives good resolution of sugars. Advantages of HPLC are that it can be very sensitive and the amount of sugar can be quantified. Disadvantages of HPLC are that many samples cannot be run at the same time. During HPLC sugars are separated by passing through a column packed with small particles giving a large surface area. A Dionex gradient pump module pressurises the eluents with helium as high pressure is required to maintain the solvent flow and helium is both inert and has low solubility in the solvents used for HPLC.

Dionex HPLC was used consisting of CarboPak PA1 anion-exchange column (4 mm internal diameter, 250 mm long) and detected with a PAD. The flow rate was always 1.0 ml/min at room temperature and 20-μl samples in H_2O were injected through a 100-μl loop. Before HPLC all samples were filtered (Nalgene 4-mm syringe filters, acetate membrane, pore size 0.45 μm). Three methods were used to separate sugars: method 1 to separate monosaccharides, disaccharides and uronic acids (2.6.3.1), method 2 to separate MLG-derived oligosaccharides (2.6.3.2) and method 3 to separate xyloglucan-derived oligosaccharides (2.6.3.3).

2.6.3.1 HPLC method 1: separation of monosaccharides, disaccharides and uronic acids.

Gradients were linear between all specified time points: t = 0 min, 20 mM NaOH 100 mM NaOAc; t = 1.8 min, 20 mM NaOH 100 mM NaOAc; t = 1.81 min 100% H_2O; t = 30 min 100% H_2O. There was an isocratic gradient of 30 mM NaOH 150 mM NaOAc from t = 40 min to t = 75 min. This was followed by a further isocratic

gradient of 0.8 M NaOH from t = 76 min to t = 81 min. From t = 82 min to t = 90 min there was an isocratic gradient of 20 mM NaOH 100 mM NaOAc. This method took 90 min per sample.

2.6.3.2 HPLC method 2: separation of MLG-derived oligosaccharides.

A method was adapted from Gibeaut and Carpita (1993) to separate the oligosaccharides produced by licheninase action on MLG. The solvents used were A = 0.5 M NaOH / 0.5 M NaOAc, B = 0.5 M NaOH. The gradients used were linear between all specified time points: t = 0 min, 100% B; t = 21 min, 88% B, t = 31 min, 65% B; t = 41 min, 25% B; t = 44 min, 0% B; t = 64 min, 0% B; t = 65 min, 100% B; t = 75 min, 100% B. This method took 75 min per sample.

2.6.3.3 HPLC method 3: separation of xyloglucan–derived oligosaccharides

The solvents used were A = 100 mM NaOH with 50 mM NaOAc. B = 100 mM NaOH with 100 mM NaOAc. From time point 0 to 40 min the solvent system was a linear gradient of 100% A. From t = 40 min to t = 41 min the solvent increased from 0% to 100% B with a linear gradient. From t = 41 min to t = 46 there was an isocratic gradient of 100% A. This method took 46 min per sample.

2.6.4 Paper chromatography (PC)

PC is one of the oldest chromatographic methods. It is cheap and effective. However, its main advantage is that it gives good resolution of ten common monosaccharides (GalA, Gal, Glc, Man, Ara, Xyl, Fuc, Rib, Rha and MeXyl). It also has a better tolerance of impurities than most other systems (Fry 1988). PC has good sensitivity, 0.1 μg of arabinose by staining (Fry 1988). Another advantage is that one cannot lose the original sugars provided that the solvent front remains on the paper. Solvent systems used for PC are described in Table 2.4. After chromatography the paper was hung in the fume hood to dry. Residual traces of a volatile solvent such as E:Py:W were effectively removed.

The stationary phase is a sheet of paper (Whatman No. 1 for small samples and Whatman 3MM for large samples). When samples were loaded as spots, less than

0.4 mg sugar was loaded per spot on Whatman No. 1 paper and less than 1 mg was loaded on Whatman 3MM. The sheets of paper were 46 × 57 cm but the width was sometimes reduced if fewer samples were loaded.

2.6.4.1 Descending paper chromatography

In PC(d) the stationary phase is hung from a glass trough containing solvent (the mobile phase). This was held in an air-tight glass chromatography tank (Panglas Shandon 500 Chromatank). The sheets of paper were hung from the trough with their long edges vertical. The samples were loaded onto the paper 9 cm from the short edge. As the solvent moves down the paper by capillary action different compounds in the sample move at characteristic speeds relative to the solvent front (R_F). Some sugars have a low R_F value and were separated more effectively if the solvent was allowed to drip off the bottom short edge (which was serrated so the solvent dripped off evenly). Samples were loaded as spots parallel with the top short edge. The spots were about 1.5 cm in diameter and standard markers were loaded 1.5 cm apart centre to centre whereas samples were loaded at least 2.5 cm apart centre to centre. The marker mixture lactose, Rha, Man, Fuc, Gal, Ara, Rib and maltose was always loaded (28.5 μl each).

2.6.4.2 Ascending paper chromatography

For PC(a) the paper (20 cm × 20 cm) was hung between two glass rods which were held together at either end and to the sides of an airtight glass chromatography tank (Shandon Chromatank) by rubber tubing. The samples were loaded on to the paper as spots (0.5 cm diameter). Spots were loaded 3 cm parallel to the bottom edge.

2.6.4.3 Preparative paper chromatography

Samples of a specific compound of interest may be prepared by either PC(a) or PC(d). In both cases the sample was loaded as a streak parallel with the short side of the paper. The PC was run in the solvent which separated it most effectively from any other components of the sample. After separation, thin vertical bands either side of the streak were cut and stained to identify the zone containing the compound of interest. The zone of interest was cut out and folded into the barrel of

a disposable 5-ml syringe. The syringe was hung vertically in a centrifuge tube and wet with water. The sample of interest was washed off the paper by centrifuging for 5 min at 2500 *g*. The paper was then rewetted and centrifuged 3–5 times (Eshdat and Mirelman 1972).

Table 2.4: Solvent systems for PC

Solvent system	Separation
B:A:W, 12:3:5, 16 h	monosaccharides, oligosaccharides and uronic acids
E:Py:W, 8:2:1, 18 h	monosacharides
B:A:W, 12:3:5, 16 h; then E:P:W, 8:2:1, 18 h in the same dimension.	clearest separation of monosaccharides and oligosaccharides; however, some of the faster-moving oligosaccharides were lost
E:Py:W, 10:4:3, 16 h	good separation of monosaccharides and disaccharides
80% Phenol, 12 h	this was used only for PC(a) and is good at resolving methyl and deoxy monosaccharides from non-substituted monosaccharides
B:Py:W, 4:3:4, 16 h	used to separate aldobiouronic acids from xylose and uronic acids

2.6.5 Thin-layer chromatography (TLC)

The stationary phase for TLC was a thin layer (0.2 mm thick) of either cellulose powder or silica-gel bound to a plastic plate 20 × 20 cm. All TLC plates used were purchased from Merck (KgaA, 64271 Darmstadt, Germany) as these gave the best resolution. Samples were loaded as 1-µl spots positioned 5 mm apart centre to centre, 2 cm horizontally from the bottom edge. The plate was placed in a closed tank (Shandon TLC Chromatank) with about 75 ml of solvent. Silica-gel is better for non-polar compounds. For TLC, samples have to be free of contaminants as they can interfere with the way the sample runs. However, TLC is very sensitive. The limit of detection of Ara is 0.08 µg by aniline hydrogen-phthalate staining (Fry 1988) and 25 ng by thymol–sulphuric acid staining (Jork et al. 1994).

The solvent system used for TLC depended on whether it was a cellulose or a silica-gel layer. TLC solvent systems are described in Table 2.5. In all cases the solvent front was run until it reached 1 cm from the top of the plate.

Table 2.5: Solvent systems used for TLC

Solvent system	Type of TLC plate used	Separation
B:A:W, 2:1:1	Silica-gel	good separation of oligosaccharides but the solvent separates to form a band where MLG3 and MLG4 run, which would obscure their detection
B:A:W, 3:1:1	Silica-gel	oligosaccharides moved slower in this solvent than the previous solvent but were still well resolved. The solvent separates to form a band but this does not run to the same place as MLG3 and MLG4.
Propan-1-ol:Nitromethane:water, 5:2:3	Silica-gel	separates large oligosaccharides but monosaccharides are not resolved
Propan-1-ol:Methanol:water, 2:1:1	Silica-gel	gives rapid separation of mono- and oligosaccharides but some streaking is observed
B:A:W, 2:1:1, followed by E:Py:W, 10:4:3 in the same dimension	Cellulose	good separation of monosaccharides
E:Py:A:W, 5:3:1:1	Cellulose	good separation of mono- and oligosaccharides

2.6.6 High-voltage paper electrophoresis (PE)

PE separates charged molecules with relation to their relative charge to mass ratio (Wright and Northcote 1975). Samples were dried onto Whatman No. 1 paper as spots spaced at 2 cm centre to centre and 12 cm from the cathode end. A coloured marker of Orange G (for PE at pH 3.5) or Orange G and methyl green (PE pH 2.0) was also loaded so that the electrophoresis could be visually monitored. The loaded paper was laid onto a sheet of glass and wetted with buffer delivered from a pipette. The origin was raised between two glass rods and wetted last. The paper was then blotted to remove excess buffer. The paper was suspended in a chromatography

tank filled with a coolant of white spirit. The top of the paper was held in a trough of 250 ml buffer and a platinum cathode. The bottom dipped into another layer of buffer and the platinum anode. Coils with running tap water kept the system below 30°C during a run. For paper electrophoresis at pH 3.5 the buffer was acetic acid:Py:W, 10:1:189, v/v/v. The paper was wetted with half-strength running-buffer. Typical running conditions for Whatman No. 1 paper of width 38 cm were 3 kV 100 mA for 1.5 h. For paper electrophoresis at pH 2.0 the buffer was acetic acid:formic acid:water 35:1:355, v/v/v. The paper was wetted with full-strength running buffer. Typical running conditions for Whatman No. 1 paper of width 38 cm were 2.5 kV 50 mA for 1 h. For paper electrophoresis at pH 9.4 the buffer was 200 ml 0.1 M Boric acid, 113.5 ml 0.1 M NaOH, pH adjusted to 9.4. The paper was wetted with full-strength running buffer. Typical running conditions for Whatman No. 1 paper of width 38 cm were 3 kV 100 mA for 3 h.

Preparative PE was used to purify a compound of interest in a similar way to preparative PC (2.6.4.3).

2.7 Identification of sugars

2.7.1 Staining

The choice of stain was dependent on whether a silica-gel or cellulose was to be stained. PCs and PEs were dipped through a stain in a trough. TLCs were dipped vertically into a tank (Desaga 124162 and 124171) of stain. After staining, chromatograms were recorded by scanning (Microtek ScanMaker 6400 XL), photography or colour photocopying.

2.7.1.1 Celluose chromatograms

There are two preparations that can be used to stain sugars and uronic acids on cellulose (TLC, PC or PE). Aniline hydrogen-phthalate is less sensitive than silver nitrate. However, aniline hydrogen-phthalate has the advantage that it can give an indication of the type of sugar. Hexoses are stained brown, pentoses pink-purple and uronic acids are stained orange.

Aniline hydrogen-phthalate

Staining was carried out in a fume hood. Immediately before use 0.5 ml of aniline was added to 100 ml of the stock solution. If aniline comes in contact with skin it must be thoroughly washed with soapy water. Aniline must not be inhaled. The stock solution was 0.1 M phthalic acid, 49% v/v diethyl ether, 49% v/v acetone and 2% v/v water. The PC, PE or TLC was wetted with the stain and allowed to dry for 5 min in the fume hood. It was then heated for 5 min at 105°C. The detection limit was approximately 0.4 µg Ara per spot on PC (Fry 1988).

Silver nitrate

Sugars were stained with silver nitrate followed by dipping through three stain solutions with 15 min drying between.

Solution 1 — 5 mM $AgNO_3$ in acetone. Water was added drop-wise until the precipitate dissolved.

Solution 2 — 0.125 M NaOH in ethanol.

Solution 3 — 10% Sodium thiosulphate.

The paper was dipped through solution 2 twice to increase the intensity of staining. Immediately after dipping through solution 3 the chromatograms were placed into a sink of water for 2 h. The chromatograms were kept out of direct sunlight during the staining as bright light can cause darkening of the background. If the paper had been developed in a borate containing solvent solution 2 was modified to 2% NaOH, 4% pentaerythritol in 80% ethanol.

2.7.1.2 Silica-gel chromatograms

Aniline hydrogen-phthalate can be used to stain silica gel. However, it was not as sensitive as the orcinol and thymol–sulphuric stains. Orcinol and thymol–sulphuric stains produce specific colours for each monosaccharide. However, thymol–sulphuric can also differentiate between GalA and GlcA. TLC plates were dipped into a tank of stain and dried in the fume-hood. After drying TLC plates were incubated for 5 min at 105°C. The orcinol stain was made up of 2% w/v orcinol, 10% v/v sulphuric acid and 90% v/v ethanol. The thymol–sulphuric stain (Jork et al

1994) was made up of 6 mM thymol (5-methyl-2-isopropylphenol), 5% v/v sulphuric acid and 95% v/v ethanol.

2.7.2 Autoradiography

To help confirm the identity of a sugar an internal marker of authentic ^{14}C-sugar, of an amount too small to stain, was added to the sample before chromatography. After sugar separation the chromatogram was placed in a cassette (Dupont SR–397 30 × 40 cm). X–ray film was placed on top of the chromatogram (Cronex X–ray film 30 X 40 cm Sterling diagnostic imaging, Inc. Newark, DE 19714; U.S.A) and three corners of the chromatogram were marked with spots of radioactive ink. The spots of ink were used later to line up the chromatogram and X-ray film. After 2 weeks' storage in the dark the X-ray film was developed. The chromatogram was then stained and identification of the sugar could be verified by precise co-chromatography of radioactive and non-radioactive material.

2.7.3 NMR

NMR was kindly carried out and the spectra interpreted by Dr. Ian H. Sadler (National Ultra High Field NMR Centre, Department of Chemistry, Joseph Black Building, The University of Edinburgh). The NMR spectra were measured on deuterium oxide solutions using a Varian INOVA 600 MHz spectrophotometer operating at 599.9 MHz for protons and 150.9 MHz for ^{13}C nuclei.

The 2-D gradient selected COSY proton spectrum was obtained using the sequence reported by von Kienlin et al. (1991): D1--90°--t1--zgrd--90°--AQ with D1 = 1.5 s, AQ = 0.646 s, zgrd = 1 gauss/cm, 0.002 s. Other parameters were SW = 1586 Hz, 2K data points, 512 increments each with 16 transients per FID were used. The data were processed using sine-bell squared functions in both dimensions with zero filling in of the F1-data to 2K data transformation resulting in digital resolution of 1.5 Hz/pt.

The 2-D proton detected one-bond ^{1}H-^{13}C correlation (HMQC) spectrum was obtained by the method of Summers et al. (1986): D1--90°(^{1}H)--D2--180°(^{1}H);

180°(^{13}C)--D2--90°(^{1}H)--D3--90°(^{1}H)--D2--90°(^{13}C)--t_1/2--180°(^{1}H)--t_1/2--90°(^{13}C)--D2--AQ. The delays used were D1 = 1.6 s (presaturation), D2 = 3.7 ms (1/2$^{1}J_{CH}$) and D3 = 600 ms (to minimise signals from protons bonded to ^{12}C nuclei). The experiment was preceded by 64 dummy scans to establish thermal equalibrium. A 4-step phase cycle (hypercomplex acquisition) was used with ^{13}C broad band decoupling during acquisition of the proton signals. Other parameters were SW(^{1}H) = 4632 Hz, 2K data points, SW(^{13}C) = 18000 Hz, AQ = 0.408 s, 200 increments each with 4 transients per FID. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1 data from 200 W to 1024 W before transformation.

The 2-D proton detected gradient selected long-range ^{1}H-^{13}C correlation (HMBC-g) was obtained using the method of Ruiz-Cabello et al. (1992): D1--90°(^{1}H)--D2--90°(^{13}C)--D3--90°(^{13}C)--zgrd1--t_1/2--180°(^{1}H)--t_1/2--zgrd1--90°(^{13}C)--zgrd2--AQ. The delays were D1 = 1.4 s, D2 = 3.7 ms (1/2$^{1}J_{CH}$) and D3 = 55 ms (optimised for signals from protons with couplings to carbon of about 9 Hz), zgrad1 = 4 gauss/cm, 0.002 s, zgrad2 = 2 gauss/cm, 0.002 s. A 16-step phase cycle (hypercomplex acquisition) was used with no ^{13}C broad band decoupling during the acquisition of the proton signals. Other parameters were SW(^{1}H) = 2500 Hz; 2K data points; SW(^{13}C) = 15000 Hz, AQ = 0.410 s; 128 increments each with 192 transients per FID. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1 data from 200 W to 1024 W before transformation.

2.8 Preparation of standards

2.8.1 4-*O*-Methyl glucuronic acid and aldobiouronic acid

4-*O*-Methylglucuronoxylan (2%) from beech wood was heated for 1 h at 120°C in 2 M TFA. The TFA was then removed by drying, resuspending in water and redrying. 4-*O*-Methylglucuronic acid and aldobiouronic acid were separated from the neutral sugars (notably xylose) using anion exchange chromatography (2.6.1). Fractions containing xylose eluted first with 10 mM formic acid after which fractions containing uronic acids were eluted with pyridinium formate pH 5.5

(Py:formic acid:0.5%, chlorobutanol 1:1:23). Aldobiouronic acid and 4-*O*-methyl glucuronic acid were separated by preparative PE at pH 3.5 (2.6.6). 4-*O*-Methyl glucuronic acid had a slightly higher mobility than GlcA at pH 3.5 whereas aldobiouronic acid had a lower mobility than GalA in this separation system ($\underline{m}_{GlcA}$ GalA was 0.82, $\underline{m}_{GlcA}$ 4-*O*-Methyl glucuronic acid was 1.05 and $\underline{m}_{GlcA}$ for aldobiouronic acid was 0.76). It was also found that with aniline hydrogen-phthalate the aldobiouronic acid stained pink whereas monomeric uronic acids stained orange. The purified markers were analysed by PE followed by PC at 90°, and by TLC. Further hydrolysis of the aldobiouronic acid in 4 M TFA showed that it was made up of xylose and 4-*O*-methyl glucuronic acid; GalA was also present in the 4-*O*-methyl glucuronoxylan preparation.

2.8.2 MLG oligosaccharide series

The MLG series was prepared by licheninase digestion of a commercial extract of MLG from barley. The digest was loaded on to a Bio-Gel P-2 column bed-volume 140 ml fractions of 1 ml were collected. Samples of 1 μl from each fraction were loaded on to a TLC plate which was then run in BAW 2:1:1 and stained with thymol–sulphuric stain to indicate which oligosaccharides were present in which fraction. Fractions containing MLG3, MLG4, MLG5 and MLG6, respectively were then pooled. The oligosaccharides were stored either at 4°C (for TLC) or at –20°C (for HPLC).

2.8.3 Laminarin oligosaccharide series

Laminarin from *Laminaria digitata* was subjected to mild TFA hydrolysis (0.1 M TFA, 80°C, 1 h). Lam2 to Lam6 were separated in the same way as the MLG oligosaccharides (2.8.2).

2.8.4 Xylose oligosaccharide series

Xylose, xylobiose, xylotriose and xylotetraose were produced by Driselase digestion of commercial xylan from birchwood. The oligosaccharides and monosaccharide were not separated.

2.8.5 Isoprimeverose

Isoprimeverose was produced by Driselase digestion of a 1% solution of tamarind xyloglucan and purified by preparative paper chromatography (2.6.4.3).

2.8.6 Mannuronic acid and Guluronic acid

ManA and GulA were prepared by complete TFA hydrolysis (2M TFA 1h 120°C) of 0.6 ml sodium alginate. After removal of TFA the hydrolysate was resuspended in 0.6 ml of water.

2.8.7 Iduronic acid

IdA was prepared by complete TFA hydrolysis of 6 mg of chondroitin sulphate B. After removal of TFA the hydrolysate was resuspended in 0.6 mg of water.

2.9 Sodium borohydride reduction

$NaBH_4$ reduction followed by acid hydrolysis can be used to identify the reducing end of a disaccharide by conversion of the disaccharide to an alditol and a monosaccharide. About 0.1 mg sugar was dissolved in 0.2 ml 0.5 M $NaBH_4$ dissolved in 1 M ammonia and incubated at 25°C for 4 h. Any excess $NaBH_4$ was destroyed by the addition of 30 µl acetic acid. The solution was then passed through a 1.5-ml Dowex 50 H^+ column and eluted with 3 ml water. This removed ammonium. The sample was then dried, redissolved in 0.1 ml methanol/acetic acid, 10:1, and redried. This step was repeated six times. The sample was then TFA-hydrolysed. After removing TFA the products were loaded on to cellulose TLC.

2.10 Quantification of sugars

Sugars were quantified using the PAHBAH test (Lever 1972). Immediately before use 40 ml 0.5 M NaOH was added to 10 ml of 5% p-hydroxybenzoic acid hydrazide in 0.5 M HCl. To 0.25 ml aqueous sample 0.75 ml of the above solution was added. The mixture was incubated in a boiling water bath for 5 min. After cooling the A_{410} was read on the spectrophotometer (Cecil 8000 series). The results were compared with a standard curve for Glc.

2.11 Identification of tannins

Tannins were identified by the method of Fry (1988). AIR, 0.01g, was heated in a Sarstedt tube (without the top on) to 95°C for 1 h in 1 ml butanol:HCl 19:1 v/v. Tannins were identified by their A_{550} maximum values in relation to a control. To read the A_{550} values the samples had to be diluted 1 ml of reaction to 2 ml H_20.

3 Results

3.1 Mixed-linkage glucan

3.1.1 Introduction

Until recently it was thought that MLG was confined to gramineous monocots, the Poaceae. MLG is present in the Bambusoideae, a tribe of grasses which are very different from other subfamilies because its members have wood-like stems, are perennial and have deciduous leaves which survive more than one year (Wilkie and Woo 1976). Smith and Harris (1999) isolated MLG from five families within the Poales: Anarthriaceae (0 and 0.2% w/w MLG/cell wall in two samples of *Anarthria prolifera*), Centrolepidaceae (*Centrolepis strigosa*, 0.5% w/w MLG/cell wall), Ecdeiocoleaceae (0.3 and 1.7% w/w MLG/cell wall in two samples of *Ecdeiocolea monostachya*), Flagellariaceae (*Flagellaria indica*, 3.2% w/w MLG/cell wall) and Poaceae (1.8 (*Lolium multiflorum*) and 8.8% (*Lolium perenne*) w/w MLG/cell wall). The presence of MLG in the Restionaceae, the remaining family of Poales, was found to be variable (Smith and Harris 1999). Smith and Harris (1999) reported that they could not detect MLG in two members of the Restionaceae, *Chondropetalum tectorum* and *Ischyrolepis subverticellata*. However, MLG was detected at trace concentrations in *Restio tetraphyllus* and 0.1% w/w total cell wall polysaccharide composition in *Leptocarpus similis* (Smith and Harris 1999), both members of the Restionaceae. MLG is therefore not unique to the Poaceae but is present in other members of the Poales.

MLG has been reported to occur in the regenerating walls of isolated tobacco protoplasts: Hensel and Franz (1978) digested tobacco protoplasts with licheninase and identified the products as being laminaribiose and glucose. However, digestion of MLG with licheninase yields MLG3, MLG4 and traces of higher members of this homologous series; glucose and laminaribiose are not produced (Woodward et al. 1983). Licheninase preparations are often contaminated with amylase. Amylase, if present in high concentration, is capable of digesting any contaminating starch in the cell wall preparations, although it does not digest starch to laminaribiose. It seems possible that Hensel and Franz (1978) mistook maltose for laminaribiose.

MLG has also been reported from 3-day-old mung bean (*Phaseolus aureus*, legume) cell walls (Franz 1972, Buchala and Franz 1974). Buchala and Franz (1974) reported that methylation and periodate oxidation studies showed that a glucan was present in mung bean hypocotyls containing (1→3) and (1→4) linked D-Glc residues in the molar ratio 1.0:1.7. However, Kato and Masuda (1976) did not detect MLG in the cell walls of 2-day-old mung beans. Additionally Takeuchi and Komamine (1981) did not detect MLG in the cell walls of another legume, *Vicia rosea*.

A polymer similar to MLG has been reported present in the xanthophycean alga *Monodus subterraneus*. However, the polymer present in *M. subterraneus* has longer stretches of (1→4)-linked Glc than are found in MLG as described from the gramineous monocots (Beattie and Percival 1962). A closely related but non-identical polysaccharide, lichenin, has been isolated from the thallus of the lichen *Cetraria islandica* (Clarke and Stone 1963). It is not known whether lichenin is synthesised by the fungal or algal component of *C. islandica*. The phycobiont component of *C. islandica* is *Trebouxia* (Trebouxiophyceae), a member of the Chlorophyta which molecular data suggest is a sister group to the Ulvophyceae (McCourt 1995).

3.1.2 Detection of MLG

MLG is digested by licheninase to yield the characteristic oligosaccharides MLG3, MLG4 and smaller amounts of higher members of this homologous series. These oligosaccharides are clearly separated by silica gel TLC developed in B:A:W 3:1:1. Commercially purified cellulose (1→4-linked β-Glc) and wheat and potato starch (1→4-linked α-Glc) were not digested by the licheninase enzyme preparation as the concentration of amylase in the preparation was less than 0.0005 units per mg of protein in comparison to a licheninase activity of 118 units per mg of protein. The oligosaccharides MLG3 and MLG4, produced by licheninase digestion of MLG, can act as an indicator for the presence of MLG. AIR preparations from the gramineous monocots, rye, maize and barley gave a clear positive for the presence of MLG (Figure 3.1.1).

Commercially purified barley MLG was digested with licheninase. The MLG oligosaccharides produced were partially purified by gel-permeation chromatography on a Bio-Gel P-2 column, 140 ml. The oligosaccharides were clearly separated by HPLC using a method adapted from Gibeaut and Carpita (1993) (Figure 3.1.2). The retention times of MLG oligosaccharides are reported in Table 3.1.1. I used both TLC and HPLC to survey and verify the occurrence of MLG in land plants.

Figure 3.1.1: The licheninase digestion products of the AIRs of gramineous monocots and the green alga *Ulva lactuca* separated by silica gel TLC developed in B:A:W 3:1:1 and stained with aniline hydrogen-phthalate.

Scale = 110%

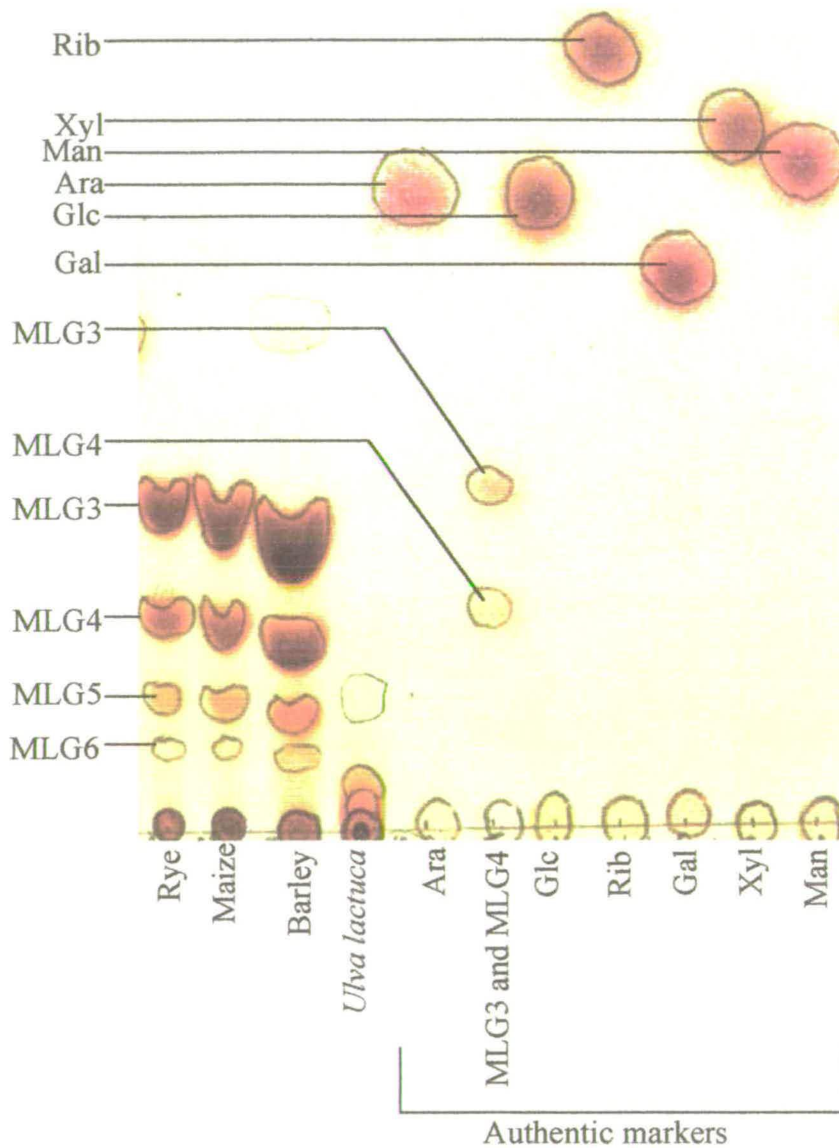


Figure 3.1.2: HPLC trace of MLG oligosaccharides produced by licheninase digestion of commercial barley MLG and partially purified by gel permeation chromatography on Bio-gel P-2.

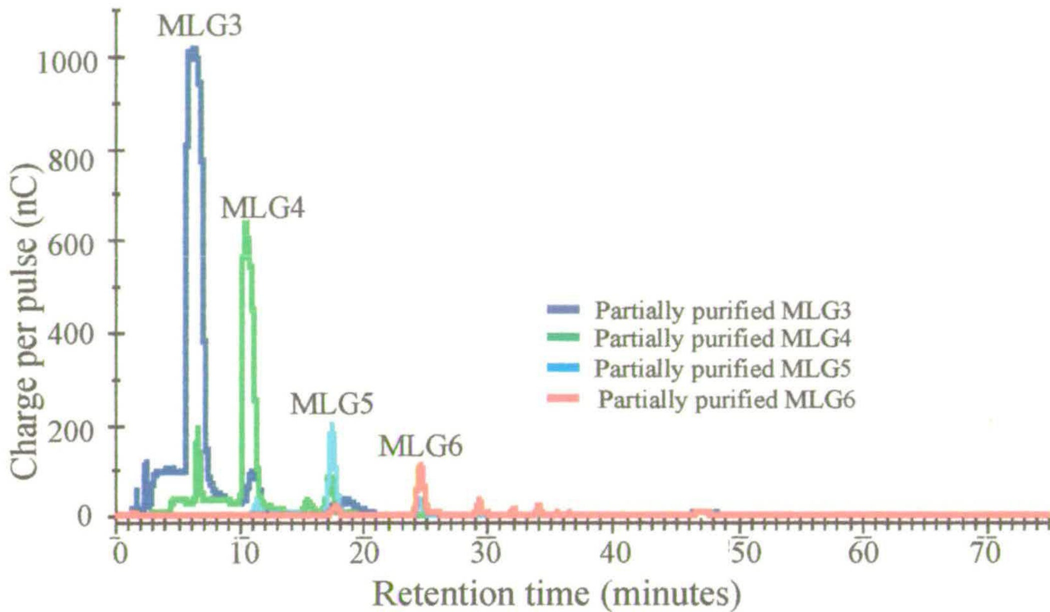


Table 3.1.1: Retention times of MLG oligosaccharides on HPLC.

Oligosaccharide	Retention time (minutes)*
MLG3	6.5–10
MLG4	11–15
MLG5	16.5–18
MLG6	24–25.5
MLG7	28–29.5
MLG8	31.5–32.5
MLG9	34–34.5

* The range represents the results of samples run on different days and the broadness of the peak.

By the HPLC method of Gibeaut and Carpita 1993, it was seen that in some traces the peaks for the MLG oligosaccharides were sharp. However, in other traces the

peak for MLG3 was broader and started to separate into two peaks. Different concentrations of MLG oligosaccharides were run and it was found that the shape of the peak was affected by MLG oligosaccharide concentration. At very high concentrations (2% solution of MLG prior to digestion) the peak representing MLG3 was seen as two overlapping peaks. I therefore took precautions to load relatively low overall concentrations of AIR digestion products.

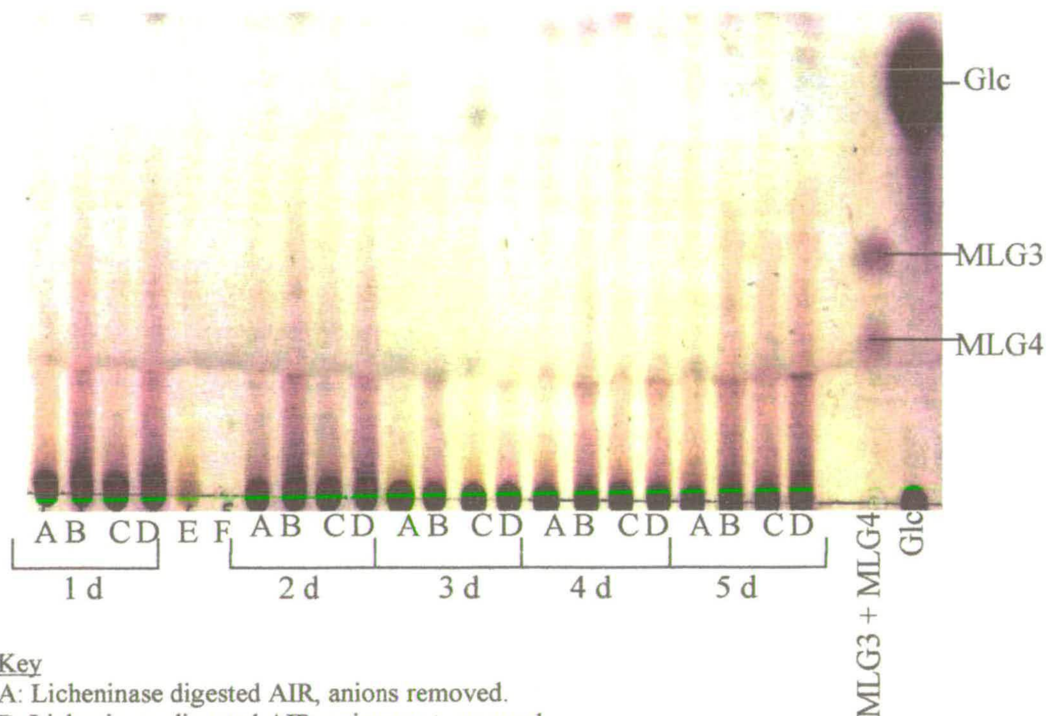
3.1.3 Attempts to detect MLG in *Phaseolus aureus* AIR

MLG has previously been reported in 3-day-old *Phaseolus aureus* (mung beans) (Franz 1972, Buchala and Franz 1974). These reports were controversial as MLG has not been detected in any other dicots. Therefore it was important to establish whether MLG is present in mung beans. Kato and Masuda (1976) did not detect MLG in 2-day-old mung beans. However, MLG can fail to be detected in purified cell walls owing to its high solubility in water. Additionally in gramineous monocots the concentration of MLG present in the cell wall varies developmentally. Owing to the high solubility of MLG in water, cell walls were prepared as AIRs and not washed in aqueous buffers. To allow for the possibility that MLG concentration in mung bean cell walls, if present, may vary developmentally, as is found in gramineous monocot cell walls, AIRs were prepared from mung bean hypocotyls 1, 2, 3, 4, 5, 7 and 19 d after germination. AIRs prepared from mung beans (1–5, 7 and 19 d) were licheninase-digested. The characteristic oligosaccharides, MLG3 and MLG4, produced by licheninase digestion of MLG were not detected either by TLC (Figure 3.1.3) or HPLC (Figure 3.1.6).

Figure 3.1.3: Silica gel TLC, developed in B:A:W 3:1:1 and stained using the thymol stain, of the licheninase digestion products of mung bean AIR prepared from hypocotyls 1–5 d post-germination.

Scale = 60%

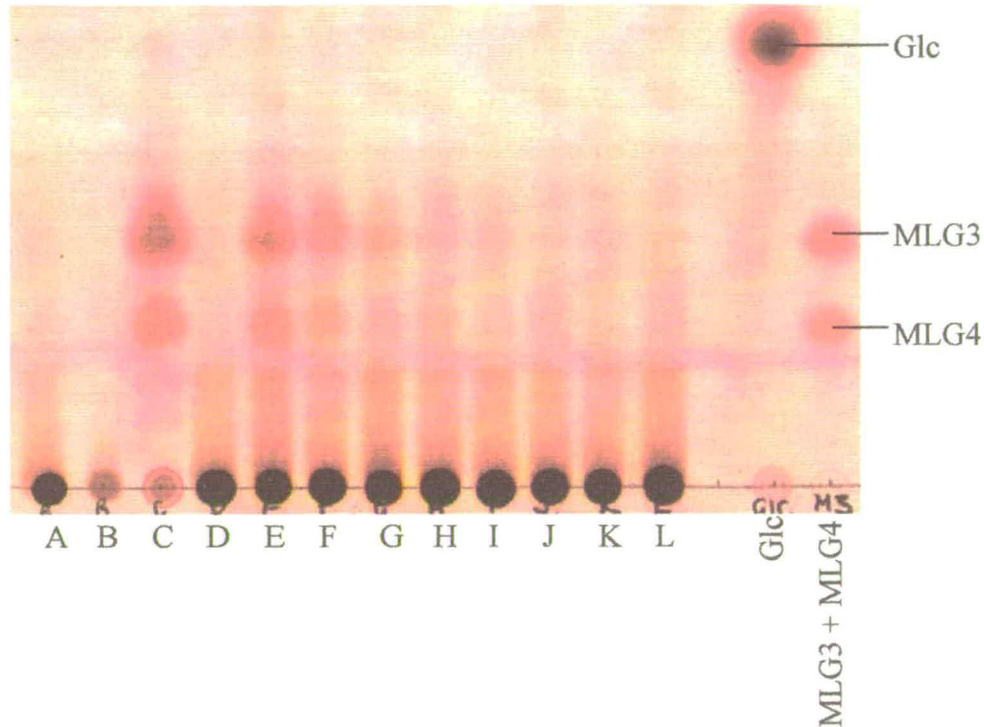
This is a scan of a photocopy and did not reproduce particularly well. However, in the original it was clear that there were no spots which ran with the same R_f values as MLG3 or MLG4 in any of the tracks.



Key
A: Licheninase digested AIR, anions removed.
B: Licheninase digested AIR, anions not removed.
C: AIR (no licheninase), anions removed.
D: AIR (no licheninase), anions not removed.
E: Licheninase + buffer only, anions removed.
F: Licheninase + buffer only, anions not removed.

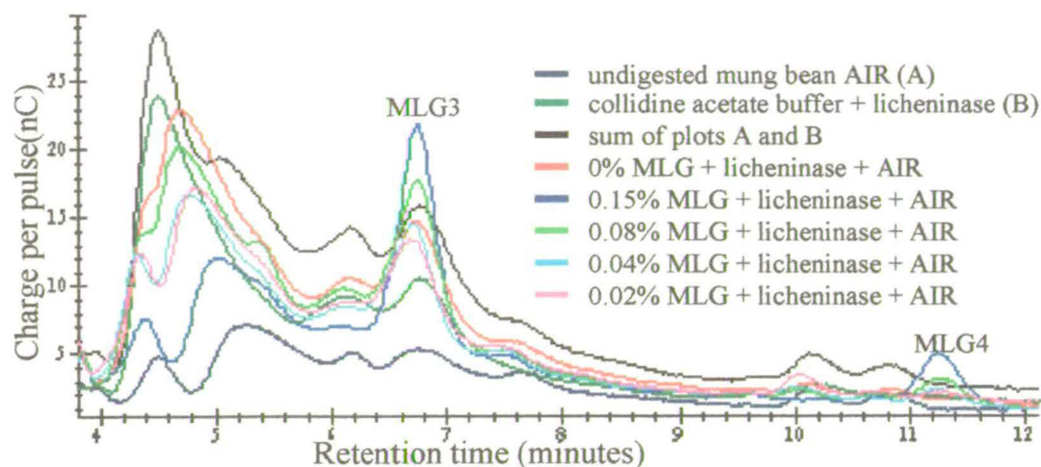
It is possible that the presence of mung bean AIR blocks the action of licheninase on MLG. I therefore added known concentrations of commercial MLG to AIR prepared from 3-day-old mung beans prior to licheninase digestion. It was found that MLG digestion products could be detected by TLC to a concentration of 0.01 % w/w MLG/mung bean AIR (Figure 3.1.4) and by HPLC to a concentration of 0.08% w/w MLG/mung bean AIR HPLC (Figure 3.1.5).

Figure 3.1.4: Detection of MLG by licheninase digestion in the presence of AIR produced from 3-day-old mung-bean hypocotyls. Digestion products were run on a silica gel TLC developed in B:A:W 3:1:1 and stained with thymol stain.
Scale = 55%



Key
A: Undigested AIR prepared from 3-day-old mung bean hypocotyls (no licheninase added)
B: Buffer + licheninase (no AIR)
C: 1.25% MLG in buffer + licheninase
D: Licheninase + AIR (no MLG)
E: 1.25% MLG w/w MLG/AIR + licheninase
F: 0.6% MLG w/w MLG/AIR + licheninase
G: 0.3% MLG w/w MLG/AIR + licheninase
H: 0.15% MLG w/w MLG/AIR + licheninase
I: 0.08% MLG w/w MLG/AIR + licheninase
J: 0.04% MLG w/w MLG/AIR + licheninase
K: 0.02% MLG w/w MLG/AIR + licheninase
L: 0.01% MLG w/w MLG/AIR + licheninase

Figure 3.1.5: HPLC detection of MLG digestion products formed in the presence of mung bean AIR.



Both traces produced from undigested AIR and a sample containing buffer and licheninase gave peaks in the MLG3 region of 5 and 10 nC per pulse respectively (Figure 3.1.5). Addition of these peaks resulted in a trace peaking at 15 nC per pulse, similar to that of the sample containing licheninase, mung bean AIR and buffer. This peak is closely similar to the trace resulting from a sample containing mung bean AIR and 0.02% MLG w/w AIR/MLG (Figure 3.1.5). Therefore, it is impossible to detect MLG in the presence of mung bean AIR to a concentration of 0.08% w/w AIR/MLG by HPLC.

In summary MLG was not present in mung bean AIR to a concentration of 0.08% as detected by HPLC. However, TLC was slightly more sensitive and it was possible to conclude by this method that MLG was not present in mung bean AIR to a concentration of 0.01%.

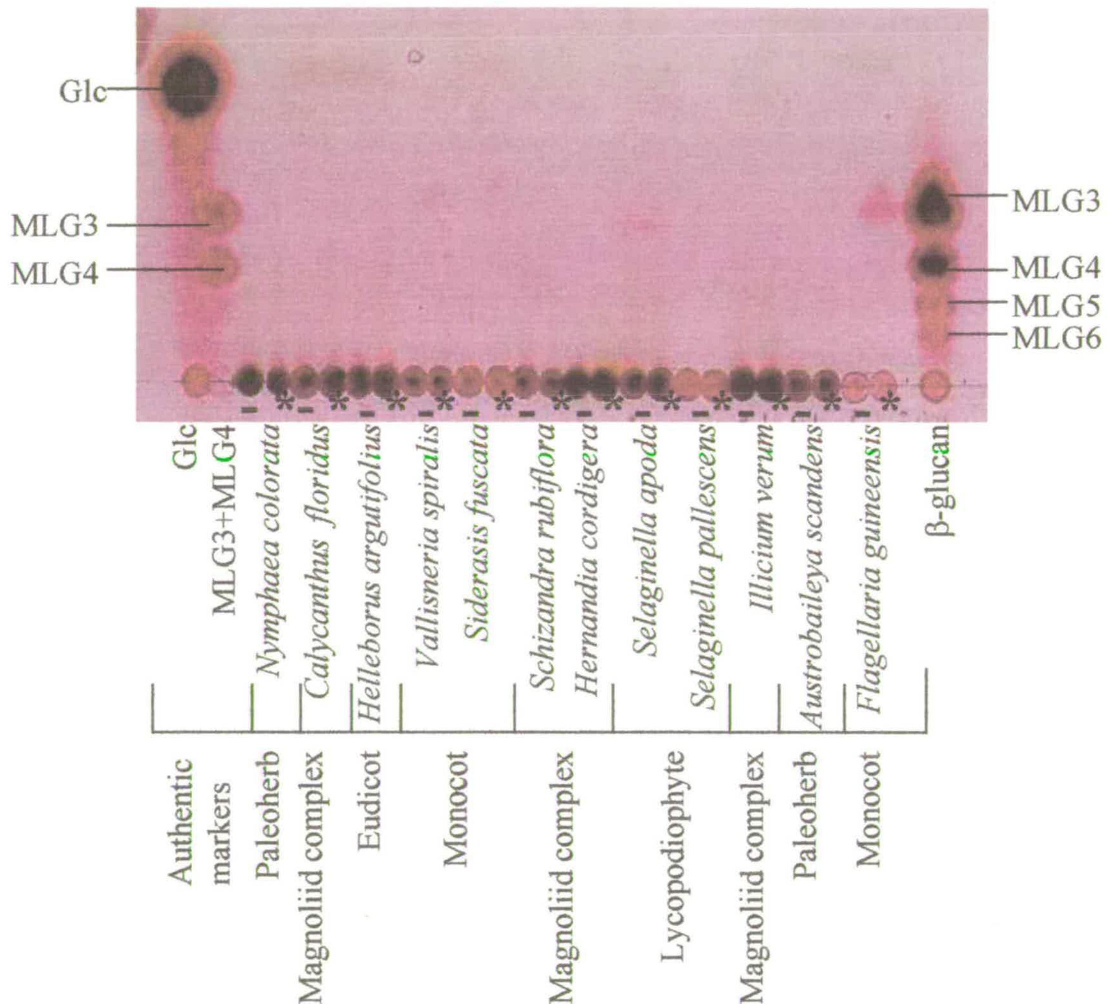
3.1.4 Survey of MLG in land plants

After establishing that MLG does not appear to be present in the cell walls of *Phaseolus aureus*, a dicot, I surveyed a wide spectrum of other land plants for the presence of MLG (Table 3.1.2). It is of interest to investigate which plants may have first started synthesising MLG in their cell walls. It is thought that MLG may be restricted to the Poaceae and other members of the Poales (Smith and Harris 1999). Within the monocots I concentrated my investigation on members of the super-order Commelinanae, a monophyletic group which includes the Poales (Judd et al. 1999, Chase et al. 1993). I also investigated gymnosperms and basal members of the angiosperms for the occurrence of MLG as the cell wall composition of the primitive angiosperms is not known (Figure 3.1.6).

Figure 3.1.6: Licheninase digests of various angiosperms AIRs. Digestion products were loaded on silica gel TLC, developed in B:A:W 3:1:1 and stained with thymol stain.

Scale = 55%

Key
* licheninase added
- control (no licheninase added), buffer + AIR only.



Spots appeared to be present in *Vallisneria spiralis*, *Siderasis fuscata* and *Selaginella apoda* which had similar R_f values to MLG3 (Figure 3.1.6). However, these spots were present in both the control and the licheninase digests, therefore they were not digestion products. In addition the digests were repeated and the spots were not visible. Similarly to Smith and Harris (1999) I detected MLG in AIR

from *Flagellaria guineensis*, a primitive member of the Poales (Figure 3.1.7). However, MLG appeared to be absent from the more advanced member of the Poales, *Elegia capensis* (Restionaceae) (Table 3.1.2). I confirmed these results by HPLC (Figures 3.1.7 and 3.1.8).

Figure 3.1.7: HPLC trace of licheninase digest of *Flagellaria guineensis* AIR.

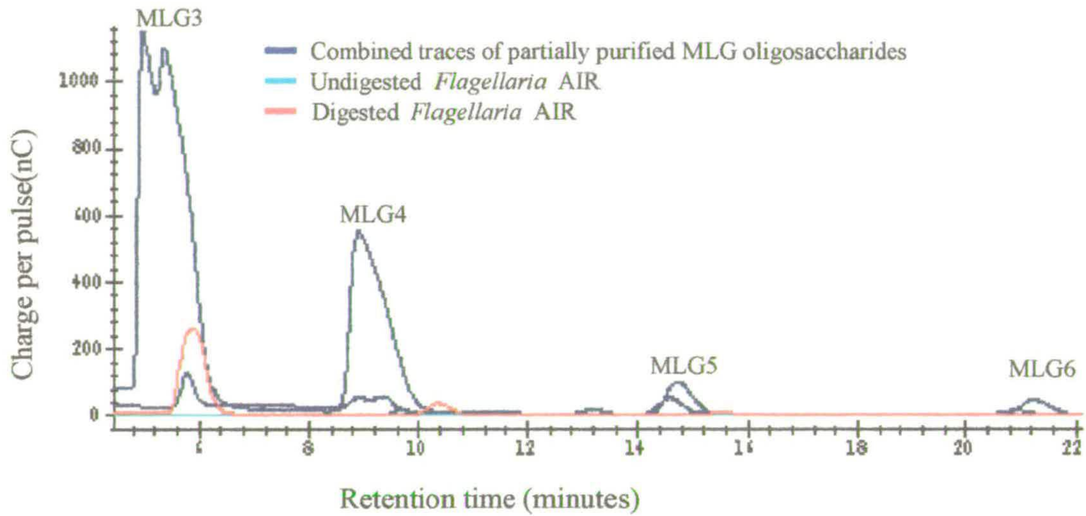
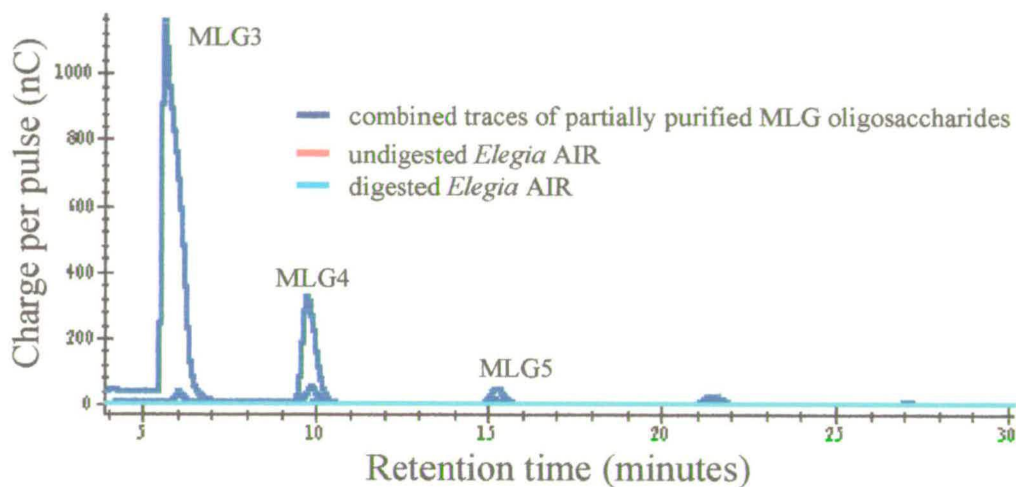


Figure 3.1.8: HPLC trace resulting from the licheninase digest of *Elegia capensis* AIR. The equivalent of 400 μg of AIR was injected.

The red line indicating the trace for undigested *Elegia* AIR is not visible because at this magnification the trace for licheninase digested *Elegia* AIR exactly overlies it. However, at greater magnification some noise is visible in the traces for both digested and non-digested *Elegia* AIR. However, at the higher magnification the MLG oligosaccharide peaks are no longer clear.



Molecular data suggest *Acorus calamus* is the most primitive extant monocot (Chase et al. 1993, Judd et al. 1999). The cell wall of the non-gramineous monocots has a similar composition to the dicot cell wall. MLG was not detectable in a licheninase digestion of *Acorus calamus* (Figure 3.1.9 and Figure 3.1.10).

Figure 3.1.9: Licheninase digestion of AIR of three species of monocots run as silica gel TLC, developed in B:A:W 3:1:1 and stained with thymol.

Scale = 85%

Key
* licheninase added
- control (no licheniase added), buffer + AIR only.

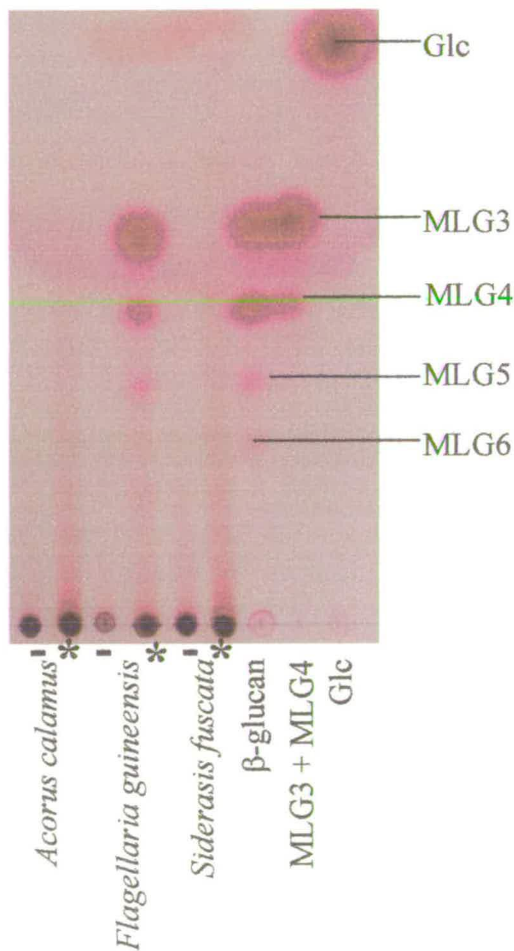
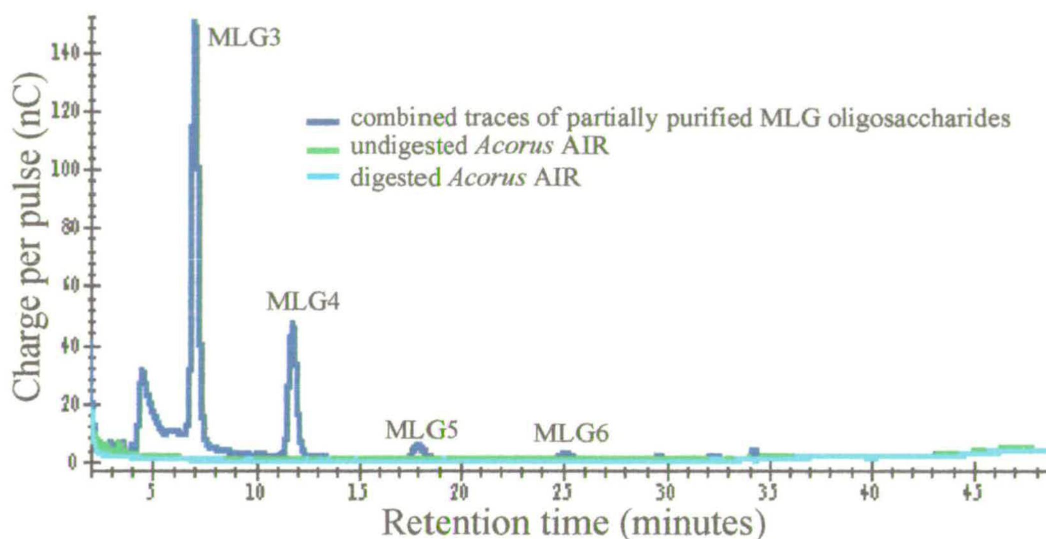


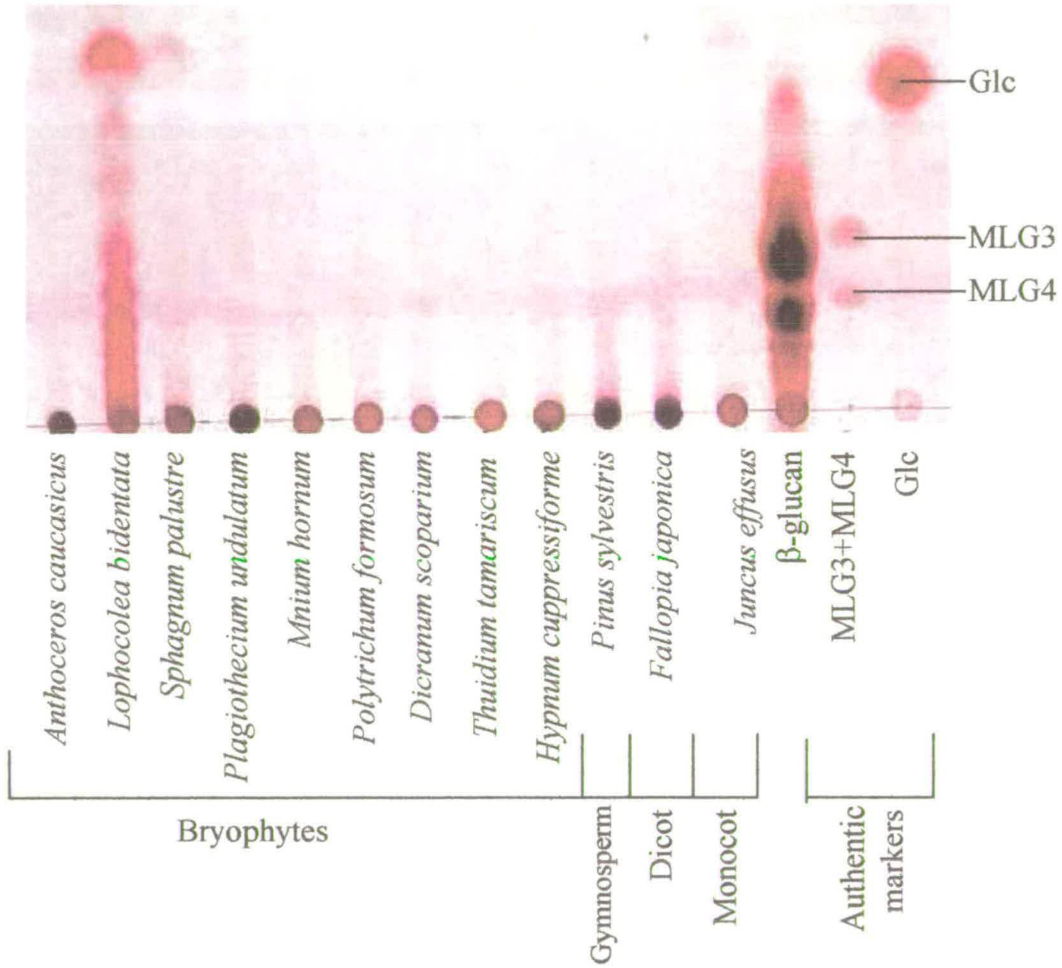
Figure 3.1.10: HPLC of licheninase digest of *Acorus calamus*. The equivalent of 400 µg of AIR was injected.



The cell wall polysaccharide composition of ferns and bryophytes has not been well studied. Therefore, it is not known whether MLG is present in their cell walls. MLG was not detectable in licheninase digests of AIRs prepared from fern cell walls (Table 3.1.2). AIR prepared from the leafy liverwort *Lophocolea bidentata* appeared to contain a licheninase-digestible polysaccharide (Figure 3.1.12). However, the licheninase-digestible polysaccharide in *L. bidentata* was not always detectable. I detected digestion products 5 times in a total of 7 replicates of the licheninase digest of *L. bidentata*. I detected digestion products in 5 out of 7 digests of an AIR sample prepared from *L. bidentata* collected in May and no digestion products in 4 licheninase digests of AIR prepared from *L. bidentata* collected in July. In addition licheninase digestion of *L. bidentata*, *S. palustre* and *J. effusus* produced spots on TLC (Figure 3.1.11) which had a higher R_f value than Glc. I do not know what these spots are. However, they do not correlate with the presence of MLG oligosaccharides in licheninase digested AIR.

Figure 3.1.11: Licheninase digests of bryophyte AIRs. Digestion products were loaded on silica gel TLC, developed in B:A:W 3:1:1 and stained with thymol.

Scale = 60%



The green alga, *Ulva lactuca*, appeared to contain a licheninase-digestible polysaccharide which differed markedly from MLG found in gramineous monocots (Figure 3.1.1). *Ulva lactuca* is a member of the Chlorophyta. Although the licheninase-digestible polysaccharide present in *Ulva lactuca* was very different from barley MLG, it is possible that it is present in many algae as a similar polysaccharide has been reported from the Xanthophyta (Beattie and Percival 1962). However, it is not known whether an MLG-like polysaccharide is present in the Charophyceae, the green algae most closely related to land plants. I therefore licheninase-digested AIR prepared from the charophyte *Coleochaete scutata*. No licheninase digestion products were detected in *Coleochaete scutata* AIR (Figure 3.1.12).

Figure 3.1.12: Licheninase digest of *Coleochaete scutata* loaded on silica gel TLC, developed in B:A:W 3:1:1 and stained with thymol.

Scale = 85%

Key
* licheninase added
- control (no licheninase added), buffer + AIR only.

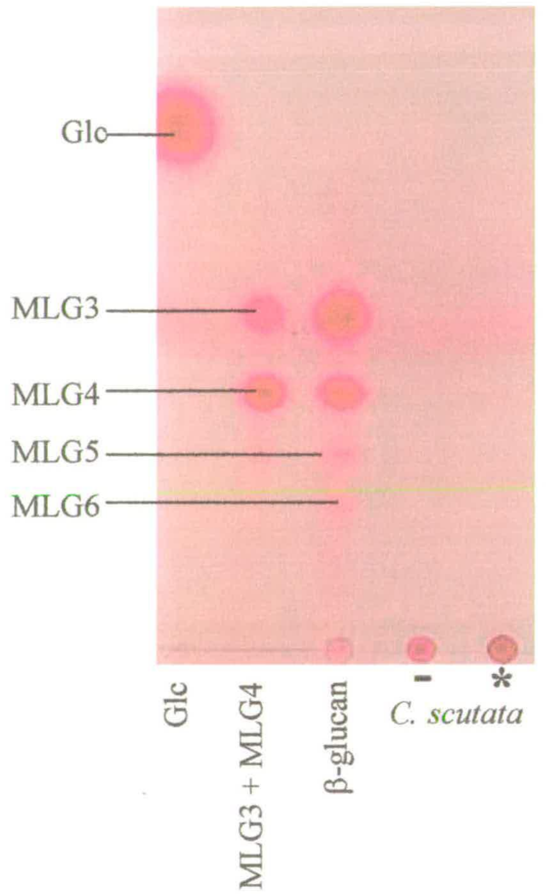


Table 3.1.2: Survey of MLG presence in land plants.

Species	MLG
Chlorobiota	
Chlorophyta	
Ulvophyceae	*
Ulva lactuca	
Charophyta	
Chara corallina	-
Coleochaetophyceae	
Coleochaete scutata	-
Embryobiota	
Bryophyta	
Bryopsida	
Sphagnum palustre	-
Sphagnum molle	-
Andrea rupestris	-
Polytrichum formosum	-
Dicranum scoparium	-
Mnium hornum	-
Philonotis fontana	-
Rhizomnium punctatum	-
Hookeria lucens	-
Thuidium tamariscinum	-
Plagiothecium undulatum	-
Hypnum cupressiforme	-
Hepaticae	
Anthoceros caucasicus	-
Lunularia cruciata	-
Pellia epiphylla	-
Trichocolea tormentella	-
Lepidozia reptans	-
Nardia scalaris	-
Marsupella emarginata var. aquatica	-
Plagiochila asplenioides	-
Lophocolea bidentata	**
Scapania undulata	-
Pleurozia purpurea	-
Porella cordaeana	-

Tracheophyta	
Lycopodiophytes	
Lycopodium pinifolium	-
Huperzia selago	-
Diphasiastrum alpinum	-
Selaginella apoda	-
Selaginella erythropus	-
Selaginella pallescens	-
Equisetophytes	
Equisetum debile	-
Psilotophytes	
Psilotum nudum	-
Filicophytes	
Eusporangiate ferns	
Marattia fraxinea	-
Leptosporangiate ferns	
Osmunda regalis	-
Todea barbara	-
Dryopteris crispifolia	-
Asplenium australassium	-
Nephrolepis lauterbachii	-
Onoclea sensiblis	-
Phyllitis scolopendrum	-
Salvinia auriculatea	-
Seed plants	
Gymnosperms	
Encephalartos altensteinii	-
Pinus sylvestris	-
Gnetum gnemon	-
Gnetum indicum	-
Gnetum montanta	-
Angiosperms	
Non-monocot Paleoherbs	
Nymphaea colorata	-
Austrobaileya scandens	-
Magnoliid complex	
Hernandia cordigera	-
Drimys lanceolata	-
Calycanthus floridus	-
Schizandra rubiflora	
Illicum verum	-
Monocots	
Acorus calamus	-
Lemna sp.	-
Vallisneria spiralis	-
Calathea zebrina	-

Callisia repens	-
Cyanotis longifolia	-
Dichorisandra thyrsifolia	-
Geogenthus undatus	-
Pallisota albertii	-
Siderasis fuscata	-
Juncus effusus	-
Cyperus esculentus	-
Cyperus papyrus	-
Secale cereale	+
Triticum aestivum	+
Zea mays	+
Hordeum vulgare	+
Avena sativa	+
Elegia capensis	-
Flagellaria guineensis	+
Eudicots	
Helleborus argutifolius	-
Spinacia oleraceae	-
Fallopia japonica	-
Phaseolus aureus	-

Key

+ MLG present

- MLG not detected

* Licheninase-digestible polysaccharide present.

** Licheninase-digestible polysaccharide present in some samples.

3.1.5 Partial characterisation of the licheninase digestible polymer from *Ulva lactuca*

A licheninase-digestible polysaccharide was detected in *Ulva lactuca* AIR (Figure 3.1.1). It appeared to differ greatly from MLG as on TLC the oligosaccharides had a lower R_F value than MLG6. However, MLG oligosaccharides with a DP greater than 6 are not detected by TLC in this solvent system as they are not mobile, the spots which stain at the origin may partly consist of MLG oligosaccharides with a DP of greater than 6. Gel-permeation chromatography on a Bio-Gel P-2 column (volume 140 ml) suggested that the oligosaccharides produced by licheninase digestion of *U. lactuca* had a DP of between 6 and 9. MLG oligosaccharides with a DP of greater than 6 can be detected by HPLC (Figure 3.1.14). On HPLC the licheninase digestion products of *U. lactuca* eluted with a similar retention time to MLG7, 8 and 9 (Figures 3.1.13. and 3.1.14). No digestion products eluted with a retention time similar to MLG3 and MLG4, the major oligosaccharides produced by licheninase digestion of barley MLG (Figure 3.1.13).

Figure 3.1.13: HPLC of the licheninase digestion products of *U. lactuca*

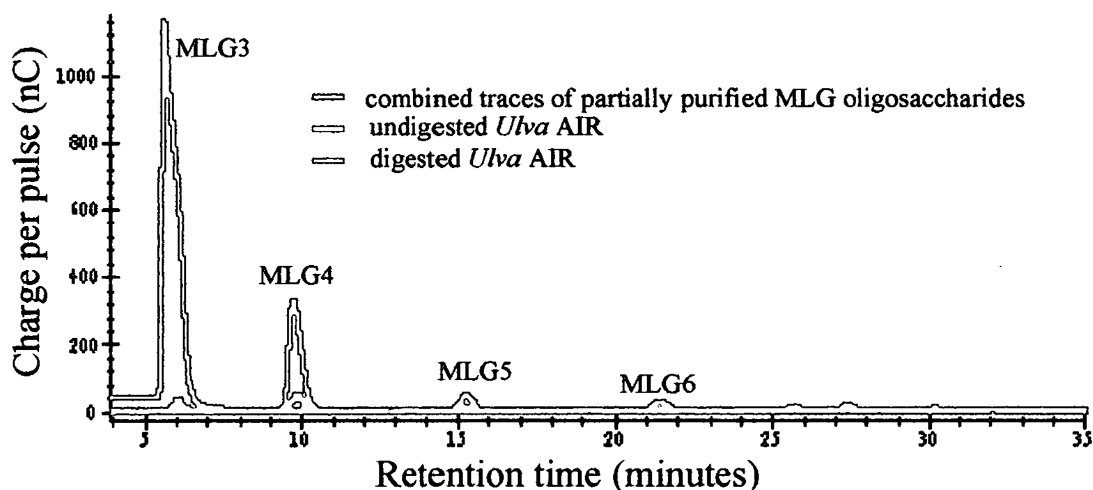
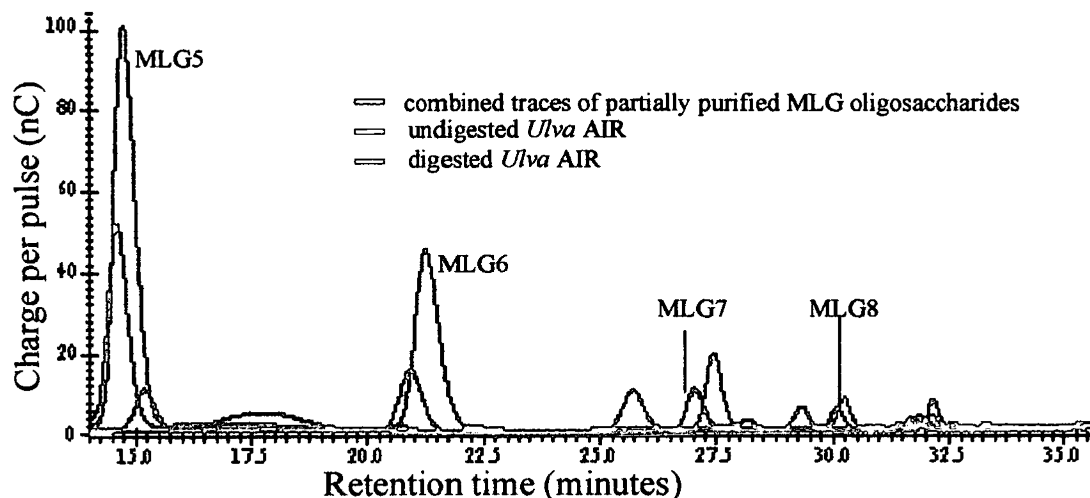


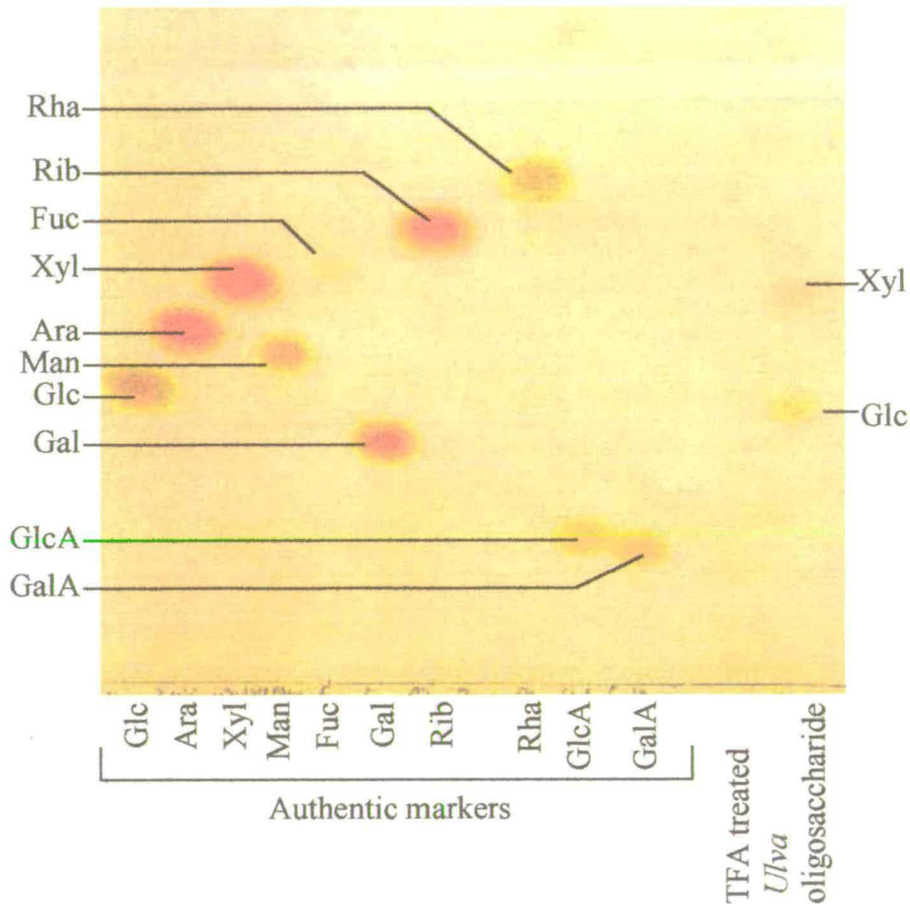
Figure 3.1.14: HPLC of the licheninase digestion products of *U. lactuca* enlargement of retention times 14–35 minutes.



I tried to characterise the licheninase-digestible polysaccharide from *U. lactuca* by TFA hydrolysing the major licheninase digestion products which were partially purified by gel-permeation chromatography. TFA hydrolysis of the oligosaccharides indicated Xyl as well as Glc was present in the licheninase digestible polysaccharide from *U. lactuca* (Figure 3.1.15).

Figure 3.1.15: TFA hydrolysis products of the major oligosaccharides produced by licheninase digestion of *U. lactuca* and partially purified by gel permeation chromatography. Loaded as cellulose TLC, developed in B:A:W 3:1:1 followed by E:Py:W 10:4:3 and stained with aniline hydrogen-phthalate.

Scale = 50%



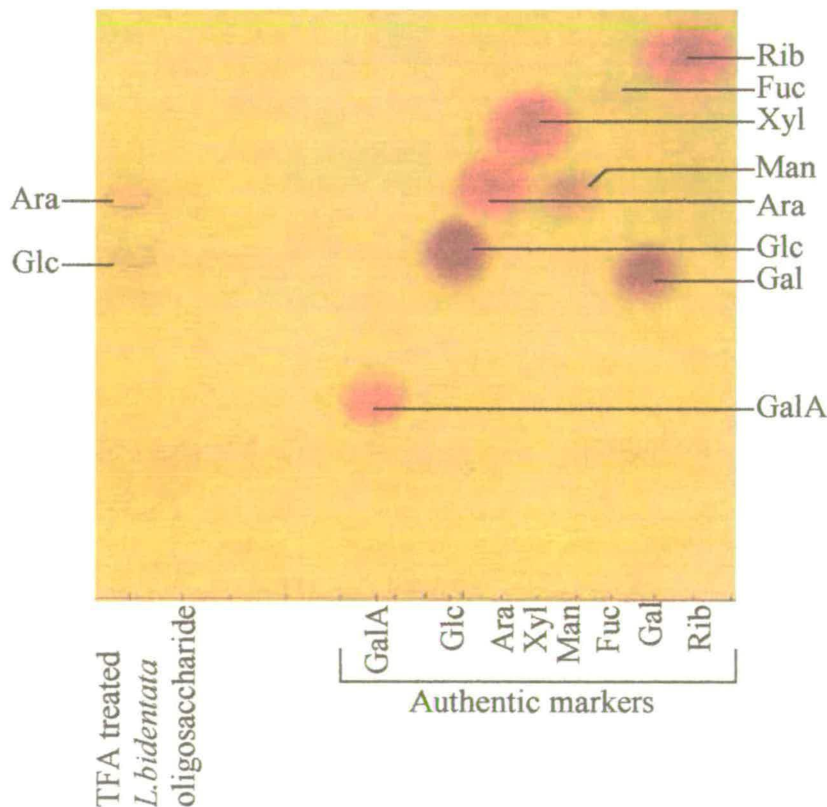
The licheninase-digestible polysaccharide present in *U. lactuca* was also found to be digestible with cellulase and the xyloglucan-specific endoglucanse XEG (see Section 3.2).

3.1.6 Partial characterisation of the licheninase-digestible polymer from *Lophocolea bidentata*

A licheninase-digestible polysaccharide appeared to be present in the leafy liverwort *L. bidentata* (Figure 3.1.11). However, this polysaccharide differed greatly from barley MLG as the licheninase digestion products did not have similar R_F values to the MLG oligosaccharides on TLC. The *L. bidentata* oligosaccharides were separated by gel-permeation chromatography (Bio-Gel P-2, 140 ml) and the fractions subjected to TFA hydrolysis. TFA hydrolysis of the licheninase digestion products of *L. bidentata* showed the polymer was composed of Ara and Glc (Figure 3.1.17).

Figure 3.1.16: TFA hydrolysed licheninase-digestion products of *L. bidentata* run as cellulose TLC, developed in B:A:W 3:1:1, E:Py:W 10:4:3 and stained with aniline hydrogen-phthalate.

Scale = 50%



3.1.7 Discussion

MLG was not detectable in mung bean AIR to a concentration of 0.01% w/w MLG/AIR. I did not detect MLG in any dicots, gymnosperms or ferns. My results confirm the report by Smith and Harris (1999) that MLG is present in the AIR of *Flagellaria guineensis* (Poales, Flagellariaceae). I did not find MLG present in the AIR of *Elegia capensis* (Poales, Restionaceae). However, Smith and Harris (1999) found concentrations of MLG in different species of Restionaceae to vary from undetectable to 0.1% w./w MLG/total cell wall composition. The Flagellariaceae and Restionaceae are members of the Poales. Molecular and morphological evidence suggest the Flagellariaceae to be more primitive than the Restionaceae (Kellogg and Linder 1995). MLG appears to be absent in all vascular plants apart from members of the Poales. The presence of MLG in all members of the Poales with the exception of some species of Restionaceae (Smith and Harris 1999) suggests that MLG evolved at the same time as the Poales.Another possibility is that MLG was present in the extinct ancestor of the Poales. MLG could once have been more widespread in the monocots and subsequently have been lost from all monocots except the Poales. However, this theory is likely to require multiple losses of MLG and is not the most parsimonious explanation for MLG distribution. The variability in occurrence and concentration of MLG in the Restionaceae indicates that the ability to synthesise MLG has been lost by some members of this family. It is interesting that among the Poales the Poaceae contain the highest concentration of MLG in their cell walls. The Poaceae include many major food crops including rice, wheat, barley, oats and rye. Evolution of the cereals has to a large extent included selection by man. It is possible that man may have selected for the increased concentrations of MLG found in the Poaceae.

All bryophytes appeared to lack MLG. The licheninase-digestible polysaccharide found in *L. bidentata* differed greatly from barley MLG in its composition as it contained Ara as well as Glc. The oligosaccharide digestion products were bigger than the characteristic MLG oligosaccharides. The licheninase-digestible polysaccharide was not consistently found in *L. bidentata*. It is possible that this was because of seasonal variation in cell wall polysaccharide composition. The

possibility of polysaccharides occurring seasonally could be investigated in samples collected at various times of year. Chlorophytes potentially contain various licheninase-digestible polysaccharides. It is possible the May collected *L. bidentata* sample was contaminated with an alga capable of synthesising a licheninase digestible polysaccharide. The presence of the licheninase-digestible polysaccharide in *L. bidentata* is unlikely to be due to contamination with gramineous monocot MLG as the licheninase-digestion pattern, as seen by TLC, and monosaccharide composition were different. No other leafy liverwort was found to contain a licheninase-digestible polysaccharide. Therefore the licheninase-digestible polysaccharide found in *L. bidentata* appears to be unique to *L. bidentata*.

The chlorophyte *Ulva lactuca* contained a licheninase-digestible polysaccharide which was very different from barley MLG. The main digestion products were much larger than the MLG oligosaccharides, DP 6–9 in comparison with DP3–5. The polysaccharide was also found to be composed of Xyl in addition to Glc and was digestible with cellulase and the xyloglucan-specific endoglucanase XEG. Lahaye et al. (1994) have identified a polysaccharide from *U. lactuca* which is reported to have some similarities to xyloglucan. The polysaccharide was reported to contain Glc (72.9% w/w total polysaccharide) and Xyl (9.7% w/w total polysaccharide), with GalA, Rha and sulphate groups present in trace concentrations (Lahaye et al. 1995, Ray and Lahaye 1995). Digestion of the *Ulva* polysaccharide with the commercial enzyme preparation Finizyme, which contains xylanase, cellulase and licheninase activities, released Glc, Xyl, G2 and the trisaccharides Xyl3 and β-D-Xyl-(1→4)-β-D-Glc-(1→4)-D-Glc (Ray and Lahaye 1995). This suggests that although the polysaccharide contains Xyl and Glc it differs from xyloglucan and MLG both in monosaccharide composition and in the linkages between the monosaccharides. The licheninase-digestible polysaccharide present in *U. lactuca* could be futher characterised by digestion with a variety of different exo-acting enzymes. It is possible that the polysaccharide described by Lahaye et al. (1995) may contain some 1→3 linkages which would make it licheninase digestible.

Molecular evidence suggests *Trebouxia*, the phycobiont component of the lichen *Cetraria islandica*, is closely related to *Ulva* (McCourt 1995). It is possible that *Trebouxia* is responsible for the production of lichenin. Immunological evidence indicates that *Trebouxia* can also occur as a free-living alga (Mukhtar et al. 1994). It should therefore be possible to isolate the alga and investigate whether it can synthesise lichenin or a similar cell wall polysaccharide. The polysaccharide composition of the chlorophytes appears to be diverse and to differ greatly from that of land plants. However, since I did not detect digestion products from licheninase-treated *Chara* and *Coleochaete* AIR (Figure 3.1.12 and Table 3.1.2), my results suggest the cell walls of the charophytes do not contain a licheninase-digestible cell wall polysaccharide and may be more similar in composition to land plant cell walls.

3.2 Xyloglucan

3.2.1 Introduction

3.2.1.1 Occurrence of xyloglucan

The importance of xyloglucan is suggested by the universality of occurrence in the primary cell walls of seed plants (Hayashi 1989). Xyloglucan was originally thought to be confined to dicots but has now been described in many monocots including rice (Shibuya and Misaki 1978) and in a gymnosperm (Andrew and Little 1997). The composition and distribution of xyloglucan side-chains (Figure 1.1) varies with source of xyloglucan and multiple forms may be present in a species (Hayashi 1989). The proportion of xyloglucan present also varies with source. Generally xyloglucan makes up 20–25% dry mass of the primary cell walls of dicots and 2–5% dry mass of the primary cell walls of gramineous monocots (Fry 1989). However, Hayashi (1989) suggests that the relative amount of xyloglucan in dicots and monocots may be equivalent as fewer xylose residues are present in gramineous monocot xyloglucan and less cellulose is present in the primary cell walls of gramineous monocots.

It is not known whether xyloglucan is present in the cell walls of ferns or bryophytes. The cell wall composition of these groups of land plants is not well studied, probably because they do not form major agricultural crops. However, the cell wall of bryophytes is of interest as they are widely accepted to represent the earliest diverging land plants.

Owing to the large size of *Chara* and *Nitella* cells they are often used as models for plant growth and physiology. *Chara* cell walls contain cellulose (Zacharias 1890) of a similar DP to the cellulose found in cotton cell walls (El Amin 1955a). Morikawa (1974) reported that hemicellulose makes up 37–43% of the *Nitella* cell wall. The hemicellulose fraction was reported to contain Glc and Gal in equal concentrations and to have slightly higher concentrations of Ara and either Xyl or Man (Morikawa 1974). Xyl and Man did not appear to be resolved by the PC solvent used by Morikawa (1974). Métraux (1982) reported that *Nitella* cell walls consist of 30% neutral sugars, principally Glc, Xyl, Man, Gal, Ara, Fuc and Rha.

He found that the Xyl and Glc concentrations followed a similar pattern during cell growth; both decreased in total dry weight of the cell wall from 6% in cells 1–10 mm in length to 2% in cells 50–80 mm in length. *Nitella* cells are most rapidly growing when they are between 1 and 10 mm in length (Métraux 1982, Morrison et al. 1993). Anderson and King (1961b) reported that the cell wall of *Chara* consists of 13% hemicellulose w/w. They suggested that the concentration of hemicellulose in *Chara* was comparable with the hemicellulose concentration in land plants. However, all the land plants investigated by Anderson and King (1961b) were gramineous monocots and the majority were straw and therefore no longer growing. Anderson and King (1961b) reported the concentration of hemicellulose in oat coleoptiles to be 38%. Therefore Anderson and King (1961b) compared the hemicellulose content of *Chara* with land plants now known to have relatively low xyloglucan concentrations and found that the overall hemicellulose content was roughly three times lower. El Amin (1955b) isolated a hemicellulose fraction from *Chara* cell walls which was composed of 85% Glc, 10.5% Xyl, 2.5% Ara and 2% GlcA. The hemicellulose fraction stained purple/blue with iodine and was hydrolysed by saliva (El Amin 1955b) which together with the high Glc content suggests that the majority of this 'hemicellulose' is starch. Morrison et al. (1993) stated that the primary and secondary cell walls of *Nitella* are similar in composition. A hemicellulose fraction extracted by 1 M KOH from the primary cell walls of *Nitella* was found to contain 10% of the total cell wall carbohydrate (Morrison et al. 1993). The hemicellulose was found to be composed of 10% w/w Fuc, 21.7% w/w Xyl, 10.4% w/w Gal and 45.8% w/w Glc residues (Morrison et al. 1993). Dicot xyloglucan has a similar monosaccharide composition. However, linkage analysis determined the presence of 4-linked Glc and 4,6-linked Glc, 2 and/or 4-linked Xyl, 2-linked Gal and terminal Fuc (Morrison et al. 1993). The methods used to determine glycosyl linkages do not appear to have been able to distinguish between 2-linked Xyl and 4-linked Xyl. No terminal Xyl or Gal was reported. If a xyloglucan had been present it is likely that terminal Xyl and Gal would have been recorded.

3.2.1.2 Variation in xyloglucan structure.

Dicot xyloglucans have L-Fuc α-linked to the 2 position of Gal residues. α-L-Ara may be present in small quantities linked to the 2-position of Gal. Xyloglucans of the Solonaceae differ from tamarind xyloglucan as α-L-Ara is linked to Xyl at position 2, little Fuc is present and the glucan backbone is less substituted with Xyl residues (Eda and Kato 1978, Ring and Selvendran 1981, Akiyama and Kato 1982). Variation is also exhibited in legume xyloglucans. When *Hymenaea courbaril* xyloglucan is hydrolysed half of the oligosaccharides produced are based on XXXXG although some Xyl residues of this repeat unit may be substitued with Gal (Buckeridge et al. 1997). Xyloglucans from pea, another legume, also have longer stretches of unsubstituted Xyl than is typically found and are made up of repeating units of XXFGXXXG (Hayashi 1989). However the majority of pea xyloglucan is still based on XXXG. Gramineous monocot xyloglucans have very little terminal Fuc (McDougall and Fry 1984), and less Xyl and much less Gal (Labavitch and Ray 1978) than dicot xyloglucans. Xyloglucans present in the non-gramineous monocots onion (Liliaceae), Orchidaceae and Palmae are similar to dicot xyloglucans in structure and proportion of the cell wall (Mankarios et al. 1980, Redgwell and Selvendran 1986, Ohsumi and Hayashi 1994ab). Xyloglucans extracted from gymnosperm suspension cultures (Thomas et al. 1987) and seedlings (Acebes et al. 1993, Andrew and Little 1997) are similar to dicot xyloglucans.

Ulva lactuca is reported to contain a Fuc-lacking 'xyloglucan' (Lahaye et al. 1994). Enzymic hydrolysis of this 'xyloglucan' released the trisaccharides β-D-xylose-(1→4)-β-D-glucosyl-(1→4)-D-glucose and xylotriose. In higher plant xyloglucans Xyl is present in its α-anomer and is 1→6 linked to the xyloglucan backbone. Therefore the *Ulva* polysaccharide is not xyloglucan as described from angiosperm and gymnosperm cell walls.

3.2.2 Identification of xyloglucan

I digested tamarind xyloglucan with Driselase (2.8.5). The digestion products were developed by preparative PC (2.6.4.3). Isoprimeverose was isolated and used as a standard marker in subsequent HPLC and PC.

Hemicelluloses were alkali extracted (2.5.1.2) prior to Driselase digestion then developed by PC. This allowed detection of reducing sugars. Detection was possible at lower concentrations of reducing sugars by observation under a 366-nm UV lamp (Table 3.2.1). Initially, complete TFA hydrolysis of bryophyte AIRs yielded more Xyl than could be seen as IP, Xyl or Xyl2 in the Driselase digests. I therefore pretreated AIRs with 1 M TFA, 80°C, 1 h, prior to Driselase digestion. This made the cell wall hemicelluloses more accessible to enzymic digestion. It is possible that something is present in bryophyte AIRs which makes them less easily digestible with Driselase. I used the PAHBAH test (2.10) to ascertain the concentration of reducing sugars in AIRs which had been subjected to complete TFA hydrolysis and those that had been Driselase digested with prior mild TFA treatment and found them to be comparable.

Table 3.2.1: Detection of isoprimeverose after developing Driselase digest by PC in B:A:W 12:3:5, E:Py:W 8:2:1 on Whatman 3mm paper and staining with aniline hydrogen-phthalate.

% solution of xyloglucan (5 μl loading)	Detectability after staining with aniline hydrogen-phthalate	Detectability after staining with aniline hydrogen-phthalate and observation under UV
1.000	+++	+++
0.500	+++	+++
0.250	++	+++
0.125	++	++
0.060	+	++
0.030	+	++
0.015	±	+
0.008	±	±
0.004	–	±
0.002	–	±
0.001	–	±

Key
+++ Strongly detectable
++ clearly detectable
+ detectable
± faintly detectable
– not clearly detectable

I also used HPLC to detect isoprimeverose in my samples (Figure 3.2.1). This also allowed me to identify monosaccharides released by Driselase digestion (Figure 3.2.2).

Figure 3.2.1: Detection of isoprimeverose by HPLC.

The isoprimeverose standard also contains Gal. It is clearly seen that Driselase digestion of xyloglucan resulted in a peak in the isoprimeverose region at about 47 min.

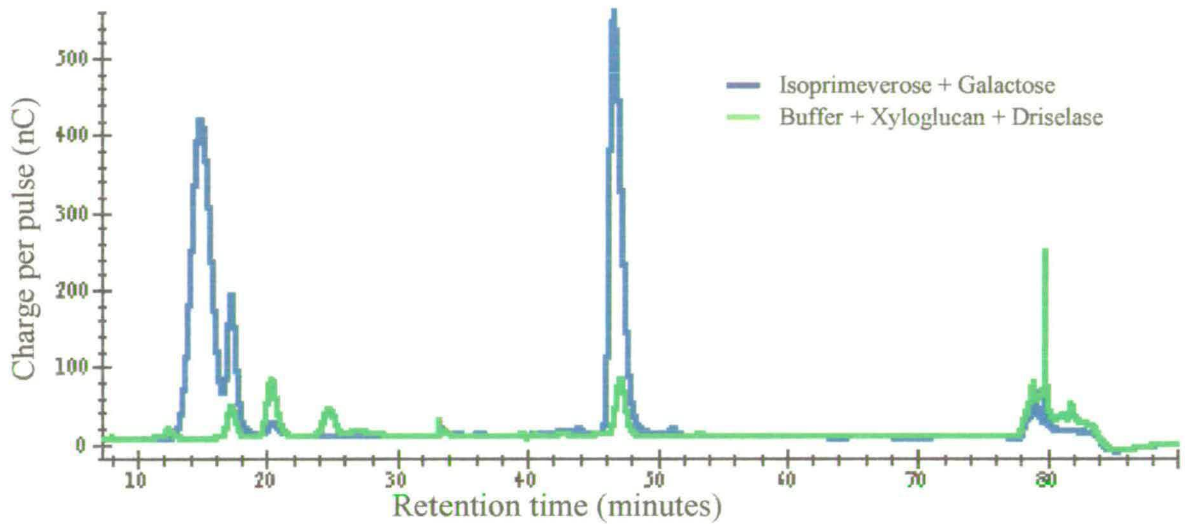
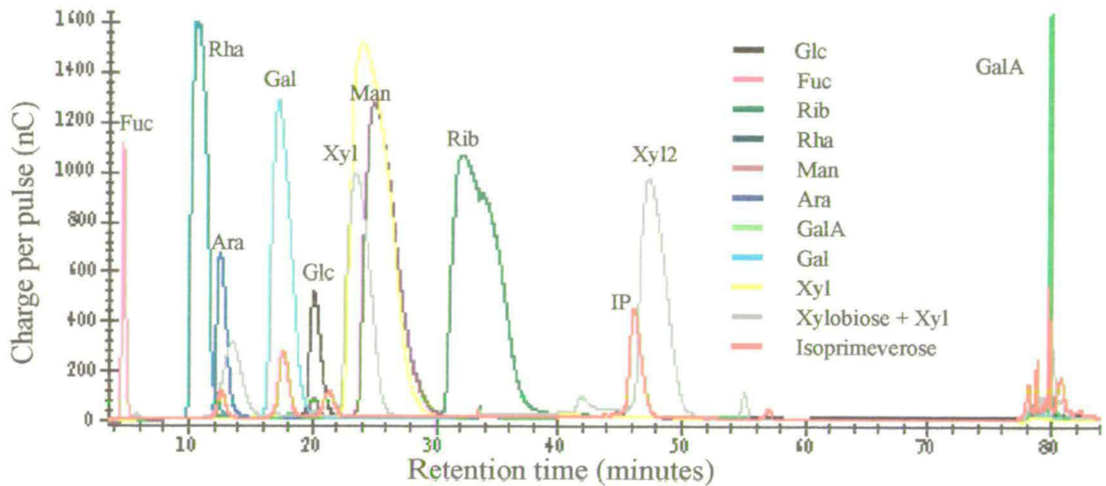


Figure 3.2.2: Detection of isoprimeverose, xylobiose and common cell wall monosaccharides by HPLC.

Isoprimeverose eluted from the HPLC column just before xylobiose at the concentration in this trace. However, at slightly lower concentrations the peaks were more defined and overlapped less. This effect could also be seen with Xyl and Man. At high concentration Man eluted at the same time as Xyl (yellow trace) whereas at lower concentration the peaks were more clearly distinguished (Xyl grey trace).



3.2.3 Attempt to detect xyloglucan in the charophycean green algae

I prepared AIR from *Chara corallina* kindly donated by Prof. Dale Sanders, University of York. AIR was prepared from cells which were 5 mm or less in length and therefore still growing rapidly. The fastest-growing cells in *Nitella* are 1–10 mm in length (Métraux 1982, Morrison et al. 1993).

I could not detect IP by HPLC or PC of Driselase-digested alkali-extract from *Chara corallina* AIR (Figures 3.2.3 and 3.2.4). It is possible that there is something present in *Chara* cell walls which prevents xyloglucan being Driselase-digested. I therefore added a known concentration of xyloglucan to *Chara* AIR prior to Driselase digestion. By PC (viewed under UV) I could detect IP in Driselase-digested AIR samples which contained exogenous xyloglucan to a level of 0.01% w/w xyloglucan/AIR (Figure 3.2.3). By HPLC exogenous xyloglucan could be detected in *Chara* AIR to a level of 0.02% w/w xyloglucan/AIR (Figure 3.2.5). However, I could not detect IP in Driselase digests of *Chara* which did not contain exogenous AIR. Monosaccharides were visible in the non-digested *Chara* AIR (Figure 3.2.3). It was found that these monosaccharides were released by the mild TFA pretreatment of the AIR. It appeared that the alkali extract of *Chara* cell walls was not digested by Driselase to produce IP. Therefore it seems unlikely that xyloglucan, as defined from angiosperms, is present in *Chara* cell walls.

Figure 3.2.3: PC of Driselase digest of *Chara corallina* AIR.

PC developed in B:A:W 12:3:5 and stained with aniline hydrogen-phthalate. This image is a scan of a photograph. The photograph was exposed for 32 s. During exposure the PC was illuminated for 8 s each side with UV. IP was still detectable when as little as 0.01% w/w exogenous xyloglucan was added to *Chara* AIR.

Scale = 30%.

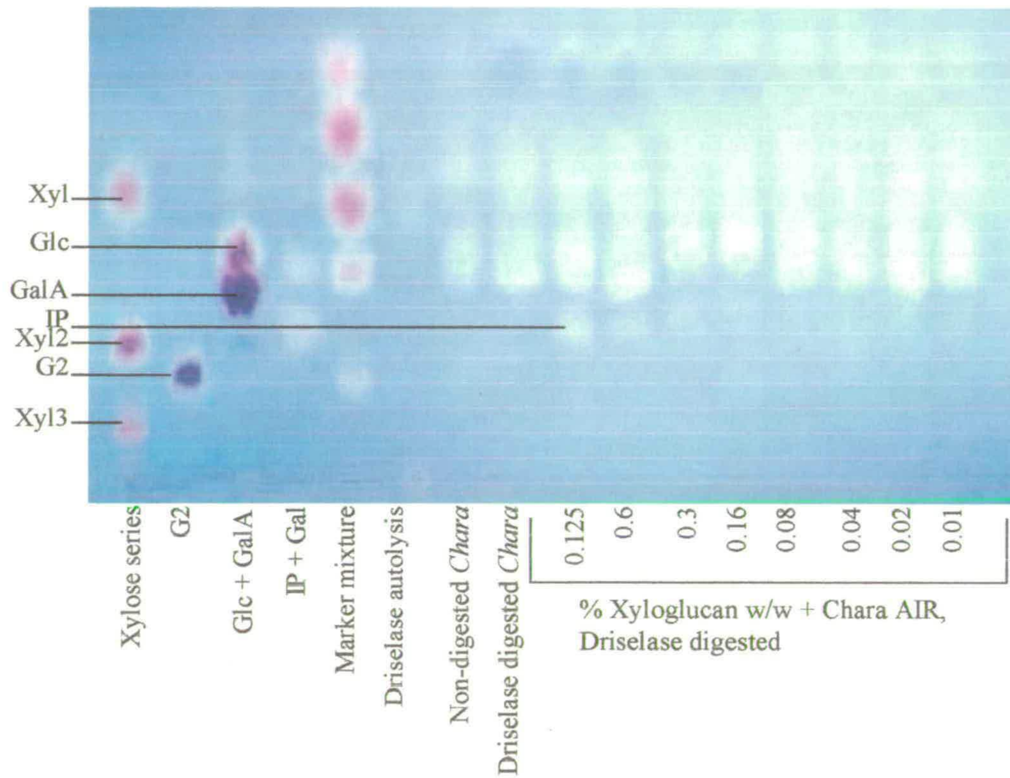


Figure 3.2.4: HPLC of Driselase digest of *Chara* AIR.

It is clearly seen that isoprimeverose is not released by Driselase treatment of *Chara* AIR. However, many monosaccharides are released including Gal, Glc, Xyl and Man.

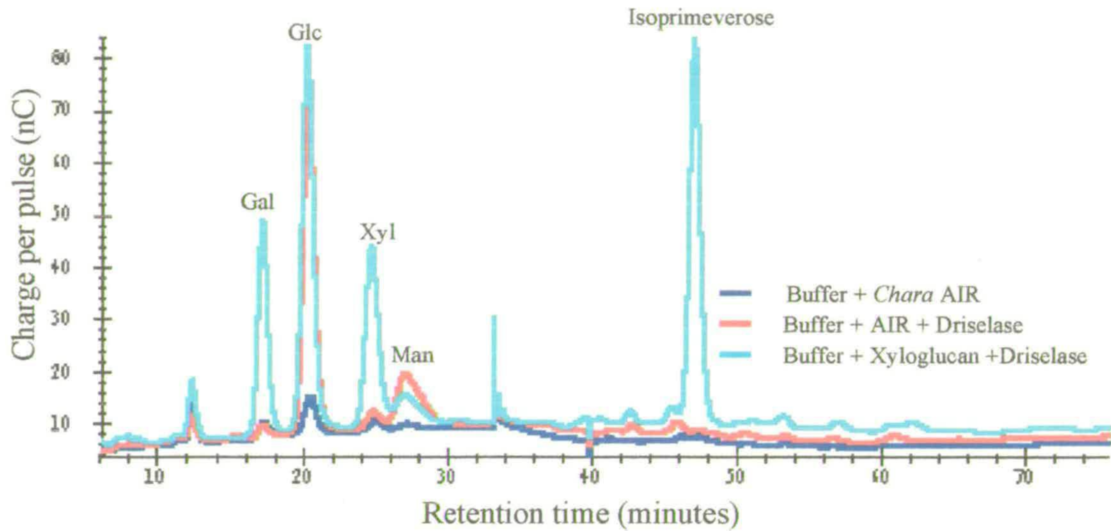
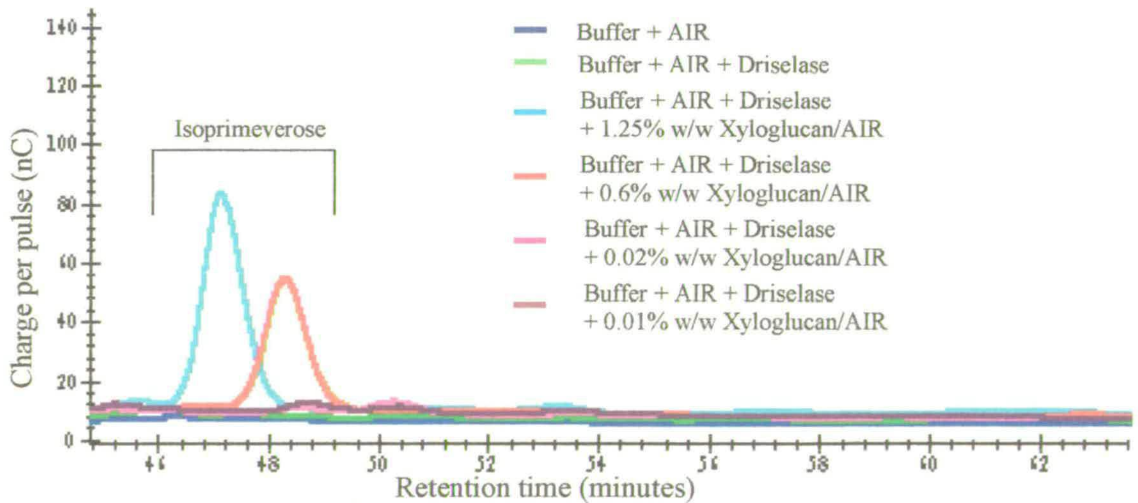


Figure 3.2.5: HPLC of Driselase digest of *Chara* AIR + added exogenous xyloglucan.

IP can clearly be detected in all traces. At higher concentrations of xyloglucan the peak is shifted slightly to the left.

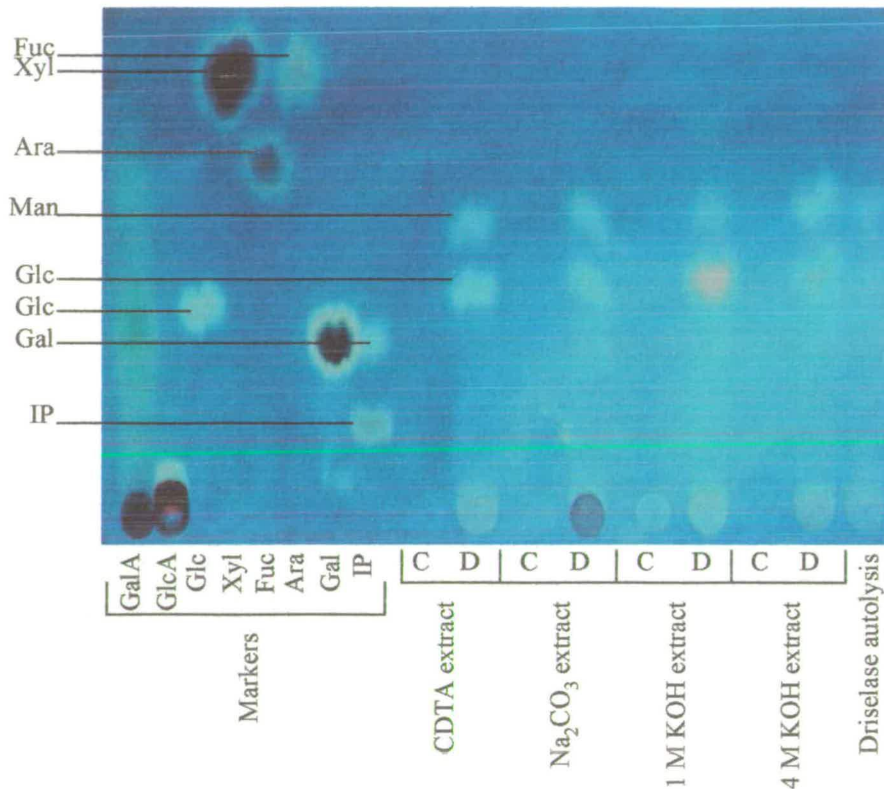


Morrison et al. (1993) detected some of the components of xyloglucan in the 1 M KOH extract of *Nitella* cell walls. I carried out on *Chara* a sequential extraction (section 2.4.2) adapted from Morrison et al. (1993). The different extracts were then treated with Driselase as before. I could not detect IP by PC (Figure 3.2.6) or HPLC (Figures 3.2.7, 3.2.8, 3.2.9 and 3.2.10) in any of the extracts including the 1 M extract, which Morrison et al. (1993) had found to contain the highest concentration of hemicellulose. Driselase digestion of the CDTA extract of *Chara* AIR did not release IP. In angiosperm sequential extracts the CDTA extract is rich in pectic polysaccharides (Figure 3.2.7). Driselase digestion of the Na_2CO_3 extract of Chara AIR did not release IP (Figure 3.2.8). The 1 M KOH extract of angiosperm cell walls is normally hemicellulose-rich and if xyloglucan had been present it is likely that it would have been present in this extract. Morrison et al. (1993) found 1 M KOH to extract hemicelluloses from *Nitella*, which is closely related to *Chara*, that contained some of the same residues as xyloglucan. However, Driselase digestion of the 1 M KOH extract of *Chara* did not yield detectable IP (Figure 3.2.9). The main digestion product appears to be Glc. The 4 M KOH extract of angiosperm cell walls is normally hemicellulose rich and if xyloglucan had been present it is likely that it would have been present in this extract. Driselase digestion of the 4 M KOH extract of *Chara* did not yield detectable IP (Figure 3.2.10). The major digestion products appear to be Glc and Xyl.

Figure 3.2.6: PC of Driselase digests of *Chara* polysaccharide fractions prepared by the method of Morrison et al. (1993).

The PC was developed in E:Py:W 8:2:1 18 h and stained with aniline hydrogen-phthalate. This image is a scan of a photograph. During the 40 s exposure of the film, the PC was illuminated for 10 s from each side with 366-mn UV. Driselase autolysis releases some Man.

Scale = 30%



Key

C Control — the polysaccharide was suspended in the same buffer as the Driselase digest for 2 d.

D Driselase of *Chara* extract.

Figure 3.2.7: HPLC trace of Driselase digest of CDTA-extract of *Chara* AIR.

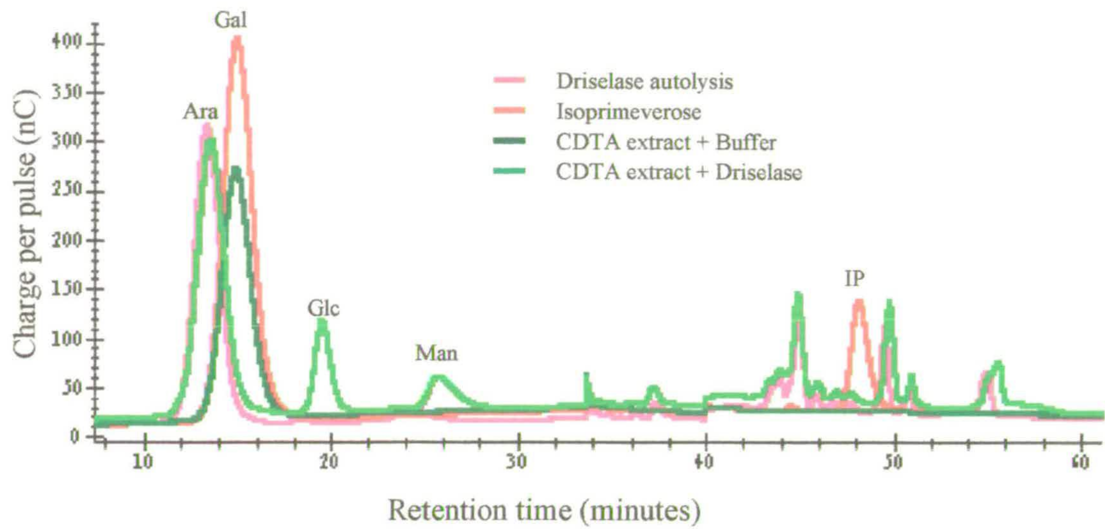


Figure 3.2.8: HPLC trace of Driselase-digest of Na_2CO_3-extract of *Chara* AIR.

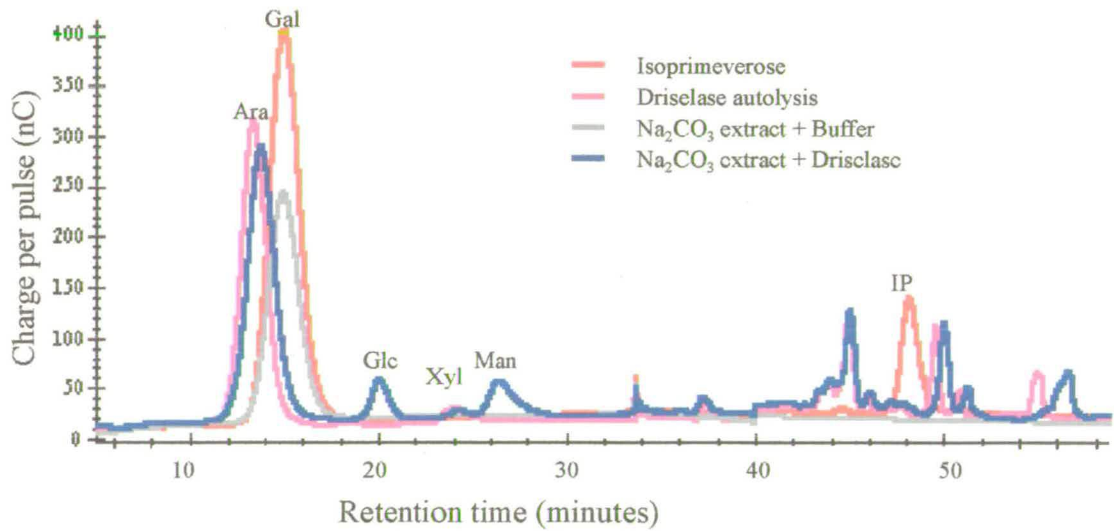


Figure 3.2.9: HPLC trace of Driselase digest of 1 M KOH-extract of *Chara* AIR.

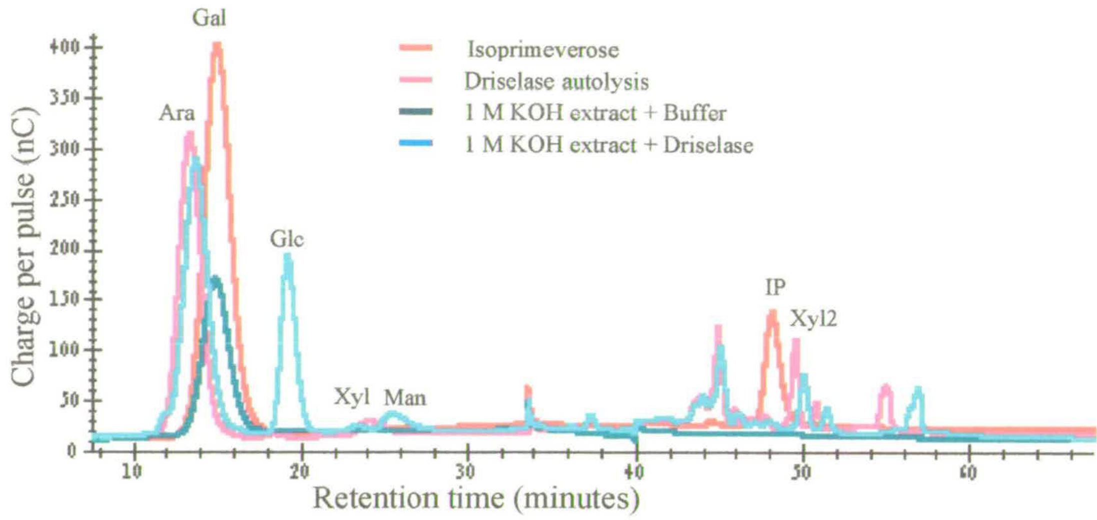
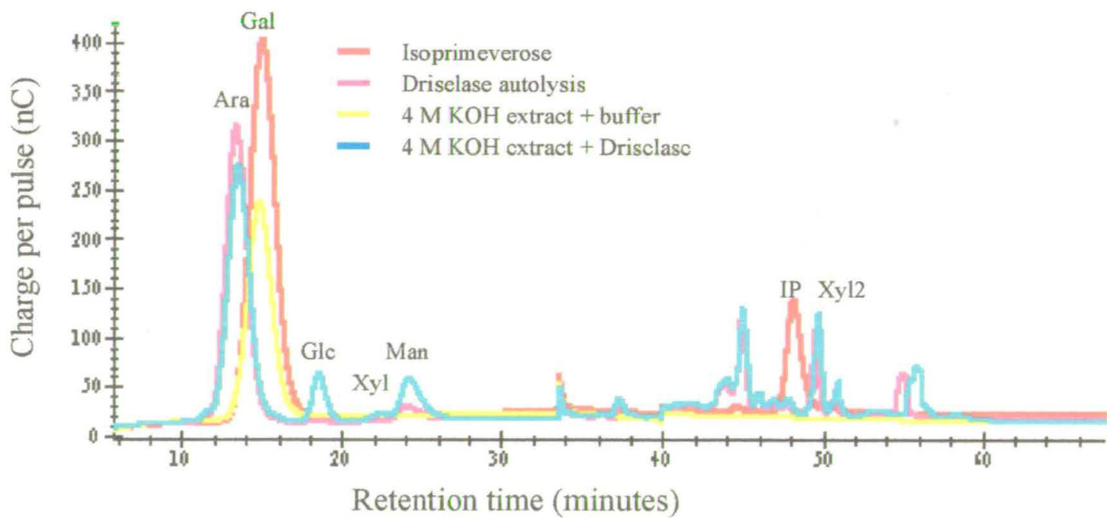
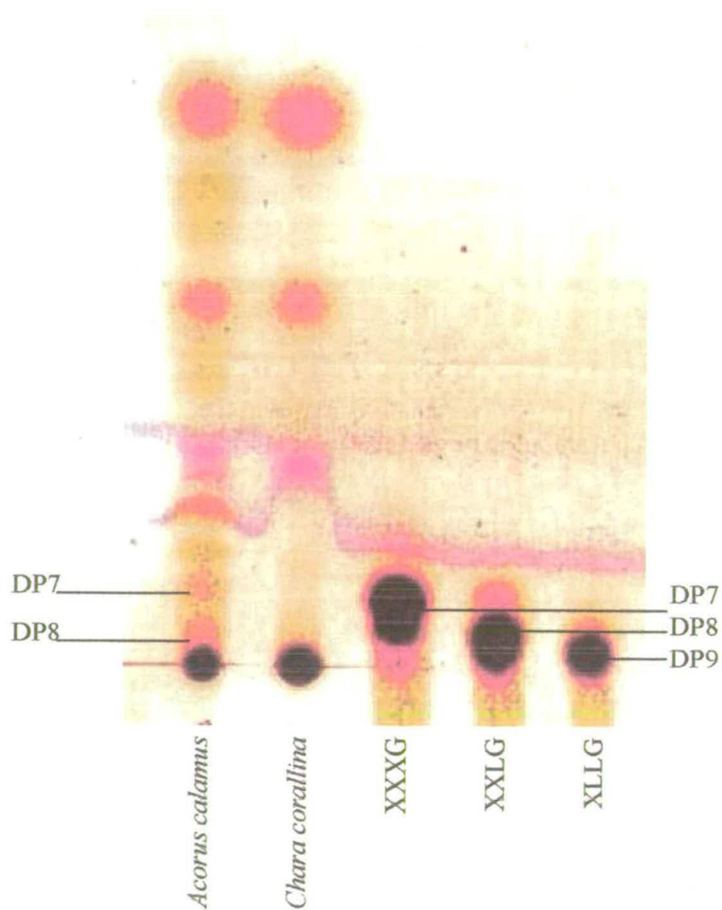


Figure 3.2.10: HPLC trace of Driselase digest of 4 M KOH-extract of *Chara* AIR.



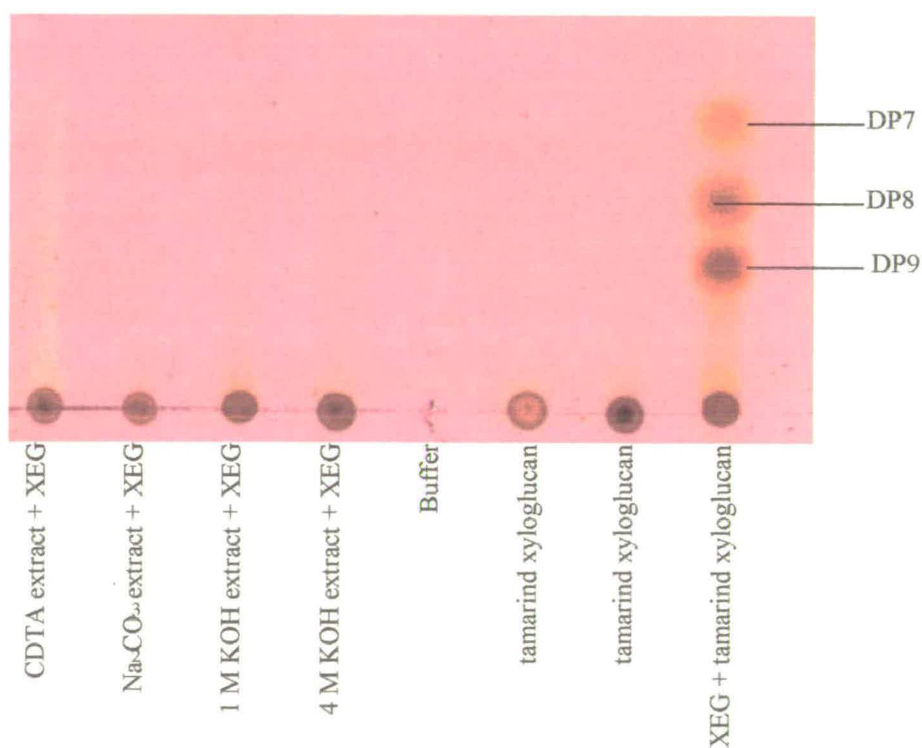
Cellulase digestion releases oligosaccharides from xyloglucan. The major oligosaccharides are usually of 7, 8 or 9 sugar residues although this varies depending on the structure of xyloglucan. A small percentage of legume xyloglucan is typically digested to oligosaccharides containing more sugar residues (Hayashi 1989). However, the major products are XXXG and XXFG. The release of oligosaccharides containing 7, 8 or 9 residues is characteristic of xyloglucan and can be used to indicate the likely presence of xyloglucan. Cellulase digestion of *Chara corallina* AIR did not result in the release of characteristic xyloglucan oligosaccharides (Figure 3.2.11). However, cellulase was able to digest some of the hemicelluloses present in *Chara* AIR. It is likely that the cellulase was able to digest xylan present in *Chara* AIR as the digestion products with the highest R_F value produced by cellulase digestion of *Chara* and *Acorus* (Figure 3.2.11) appeared to be similar to the products produced by the action of cellulase on xylan (Figure 3.2.19). *Acorus calamus* AIR was clearly digested by cellulase to produce products of DP 7 and 8 (Figure 3.2.11). Two further digestion products were present, which had a higher R_F value. The digestion product with the highest R_F value appears to correspond to Xyl2 and is a product of the digestion of xylan. There do not appear to be any digestion products of *Chara corallina* which correspond to xyloglucan oligosaccharides of DP 7–9 (Figure 3.2.11). However, cellulase is able to digest *Chara* AIR and the two spots with higher R_F values appear to be digestion products of xylan (Figure 3.2.11).

Figure 3.2.11: Cellulase digest of *Chara corallina* AIR on silica gel TLC in B:A:W 2:1:1. The TLC plate was stained with thymol.



It is possible that even though cellulase was able to digest xylans present in *Chara* AIR it was not able to digest xyloglucan. Therefore I digested sequential extracts of *Chara* with the xyloglucan-specific endoglucanase XEG. XEG has a much lower xylanase activity than the cellulase preparation (2.5.1.4). XEG digestion of the sequential extracts of *Chara* did not result in the production of any oligosaccharides with similar R_F values to xyloglucan oligosaccharides of DP 7–9 (Figure 3.2.12). Tamarind xyloglucan was rapidly digested by XEG to XXXG, XXLG and XLLG (Figure 3.2.12).

Figure 3.2.12: Digestion products of the action of XEG on *Chara* extracts prepared by the method of Morrison et al. (1993). Products were developed on silica gel TLC in B:A:W 2:1:1 and stained with thymol.
Scale = 100%
The streaking seen in the CDTA extract is due to the presence of some CDTA which remained in the preparation after dialysis for 72 hours.



Chara is a charophycean green alga and is closely related to land plants. However, other charophycean green algae may be more closely related to land plants. Therefore I investigated both *Klebsormidium flaccidum* and *Coleochaete scutata* for the presence of xyloglucan. It was also of interest to ascertain whether non-charophycean green algae have xyloglucan in their cell walls. I therefore also investigated *Ulva lactuca* for the presence of a xyloglucan with a similar structure to xyloglucan characterised from angiosperms.

Driselase digestion of *Coleochaete scutata* AIR did not produce IP detectable by PC or HPLC (Figure 3.2.14). The major digestion product of Driselase on *C. scutata* appeared to be Glc. In addition very little Xyl was released by the complete TFA hydrolysis of *C. scutata* AIR (Section 3.4). The action of Driselase on *Ulva lactuca* AIR did not produce any detectable IP (Figure 3.2.14). Digestion of *U. lactuca* AIR with Driselase yielded Glc, Xyl and what appears to be Xyl3 (Figure 3.2.14). However, the peak which appears to be Xyl3 could also include the trisaccharide Xyl-Glc-Glc which has been reported to occur in *Ulva* (Lahaye et al. 1994) In addition, cellulase digestion of *Ulva lactuca* did not yield any products with similar R_F values to XXXG, XXLG or XLLG (Figure 3.2.15). Cellulase was capable of digesting hemicelluloses present in *Ulva* AIR but most of the products appeared to be derived from a xylan or xylan-like polymer (Figure 3.2.15). Tamarind xyloglucan was clearly digested by XEG to XXXG, XXLG and XLLG. However, no digestion products were produced by the action of XEG on *K. flaccidum* (Figure 3.2.13).

Cellulase digestion of *Ulva lactuca* AIR yielded products with relatively high R_F values (Figure 3.2.15). These digestion products appeared to arise by cellulase action on xylan. No digestion products were produced by the action of cellulase on *U. lactuca* that had similar R_F values to the xyloglucan-derived oligosaccharides XXXG, XXLG and XLLG (Figure 2.3.15). Xyloglucan-derived oligosaccharides are seen in cellulase digests of AIRs of land plants (Figure 3.2.15). The spots for *Lycopersicon esculentum* and *Trichocolea tormentella* are very faint. However, there are products in the *Lycopersicon* digest which have similar R_F values to

XXXG and XXLG and in *Trichocolea* there are digestion products with a similar R_F value to XXXG (Figure 3.2.15).

Figure 3.2.13: XEG digestion of *Klebsormidium flaccidum* AIR. Digestion products were loaded on silica gel TLC, developed in B:A:W 2:1:1 and stained with thymol.
Scale = 150%

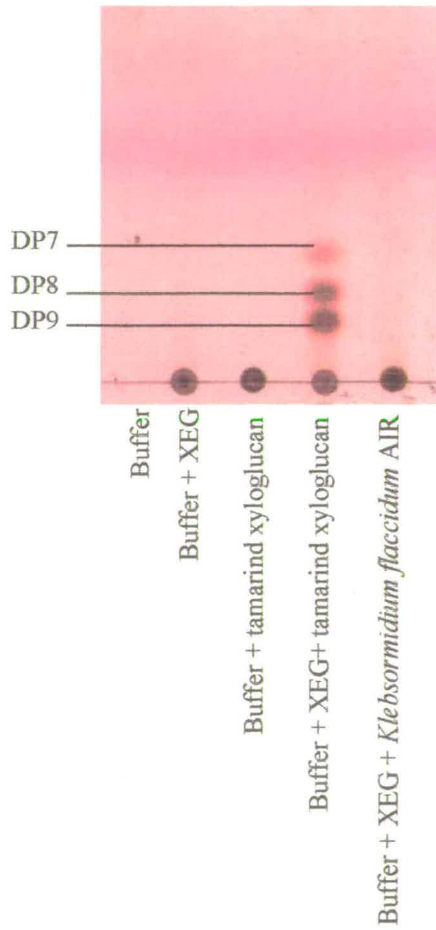


Figure 3.2.14: HPLC trace of Driselase digestion products of *Ulva lactuca* and *Coleochaete scutata* AIR.

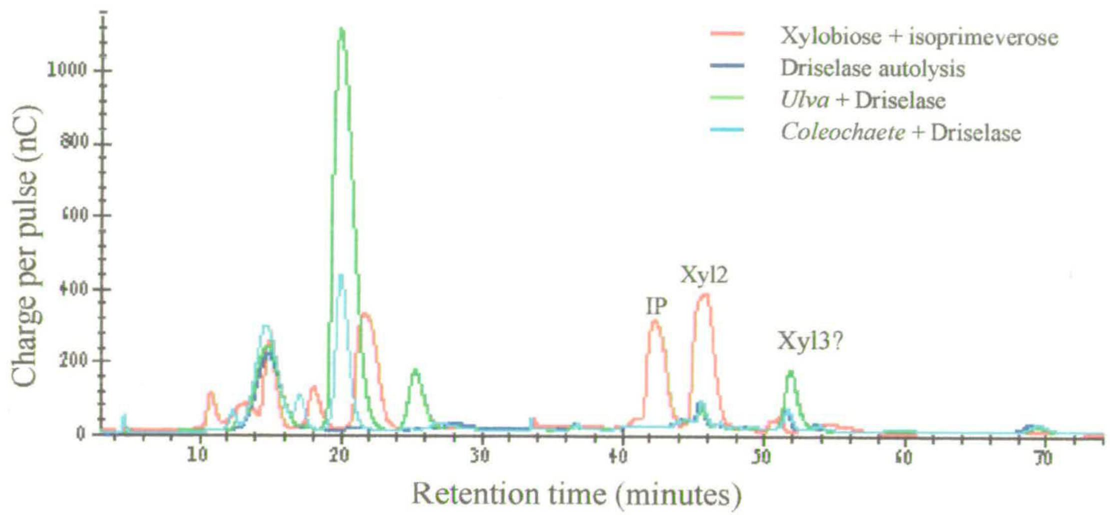
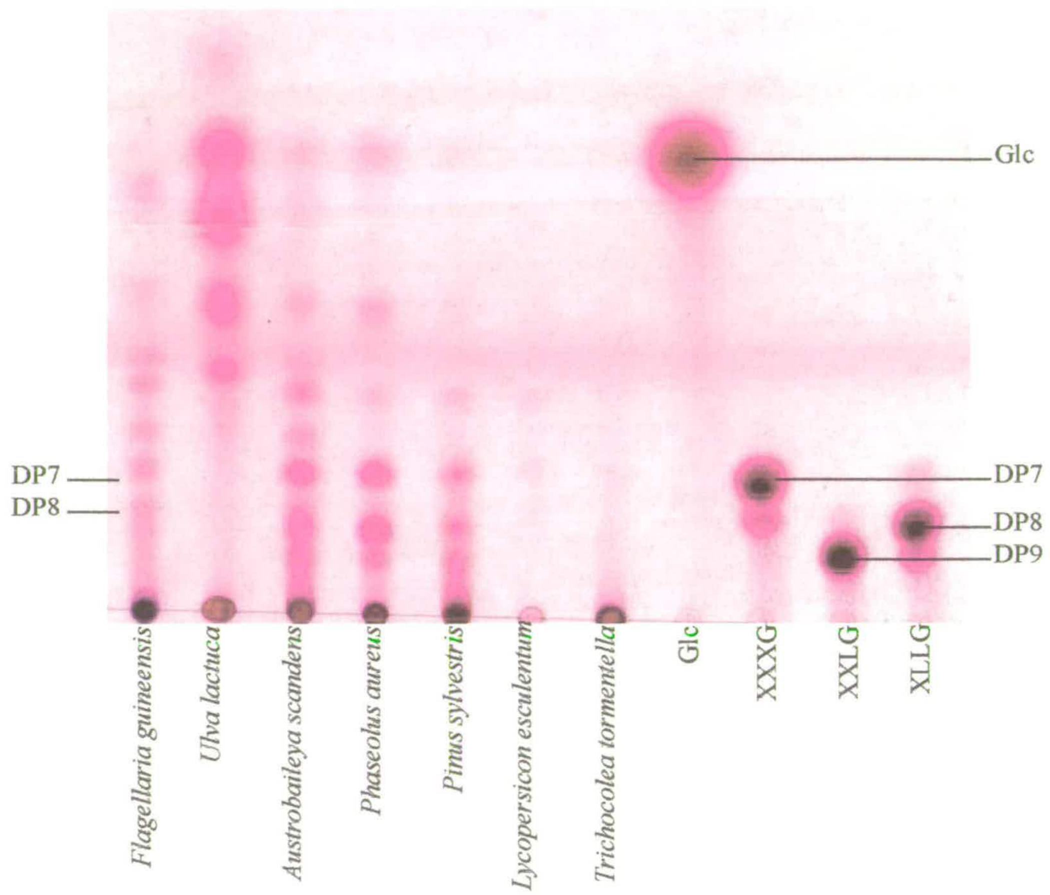


Figure 3.2.15: Cellulase digestion of AIR from *Ulva lactuca* ands diverse land plants. Products developed on silica gel TLC in B:A:W 2:1:1 and stained with thymol.

Scale = 200%.



3.2.4 Survey of xyloglucan occurrence in land plants

I investigated a wide variety of land plants for the presence of xyloglucan. I found that xyloglucan was present in all land plants investigated but appeared to be absent from the charophycean green algae (Figures 3.2.16, 3.2.17 and 3.2.18 and Table 3.2.2). IP can be clearly distinguished in all traces as eluting just before Xyl2. The concentration in *Blechnum* is very low in comparison to the others (grey trace) but still a distinguishable peak of 100 nC per pulse (Figure 3.2.16). The traces produced by the monocots *Flagellaria guineensis* and *Siderasis fuscata* appeared to give a lower peak for IP but a slightly higher peak for Xyl2 than the rest of the angiosperms (Figure 3.2.17). The dicot *Helleborus argutifolius* appeared to have the highest concentration of IP but had a lower or similar concentration of Xyl2 lower than or similar to that of the rest of the angiosperms (Figure 3.2.17). IP is detectable in *Pellia epiphylla*, *Rhizomnium punctata*, *Philonotis fontana*, *Scapania undulata*, *Marsupella emarginata* (Figure 3.2.18). IP is not so clearly detectable in the remaining bryophytes (Figure 3.2.18). These bryophytes appear to have been less well digested by the Driselase treatment as Man and Ara are present in far lower concentration than the bryophytes in which IP can be seen more clearly (Figure 3.2.18).

Figure 3.2.16: HPLC trace of the Driselase digestion products of AIR from *Blechnum* (a fern), lycopodiophytes (*Selaginella*, *Huperzia* and *Diphasiastrum*) and an equisetophyte.

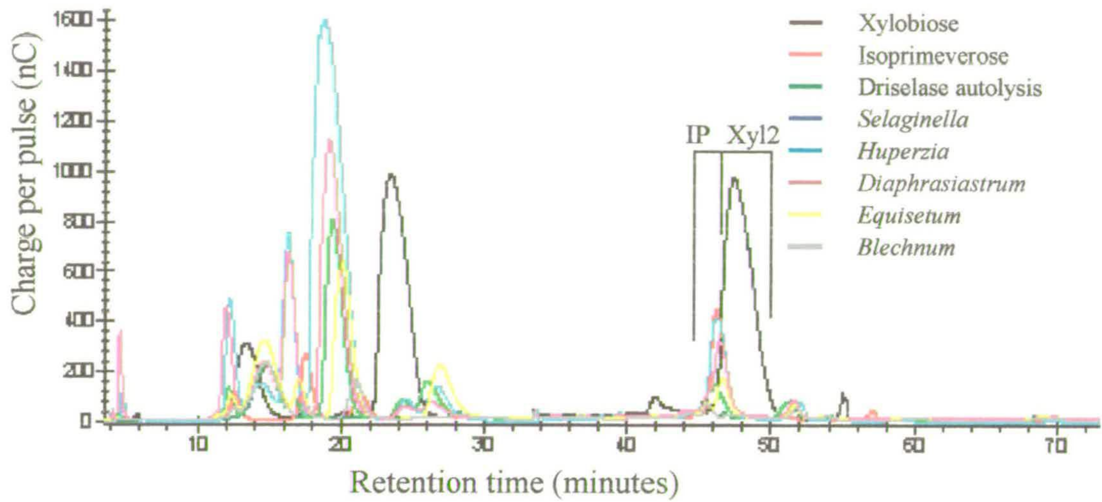


Figure 3.2.17: HPLC trace of the Driselase digestion products of AIR from a variety of angiosperms.

The two peaks isoprimeverose and xylobiose do not line up very well. In particular the peaks for *Siderasis* and *Flagellaria* do not line up well with those for the rest of the angiosperms. The main reason for this may be that *Siderasis* and *Flagellaria* were run on different days to the rest of the samples. However, the rest of the peaks in these two samples line up better with the peaks for the rest of the angiosperms. Therefore these peaks may also have shifted slightly to the left due to the higher concentration of xylobiose in *Siderasis* and *Flagellaria*.

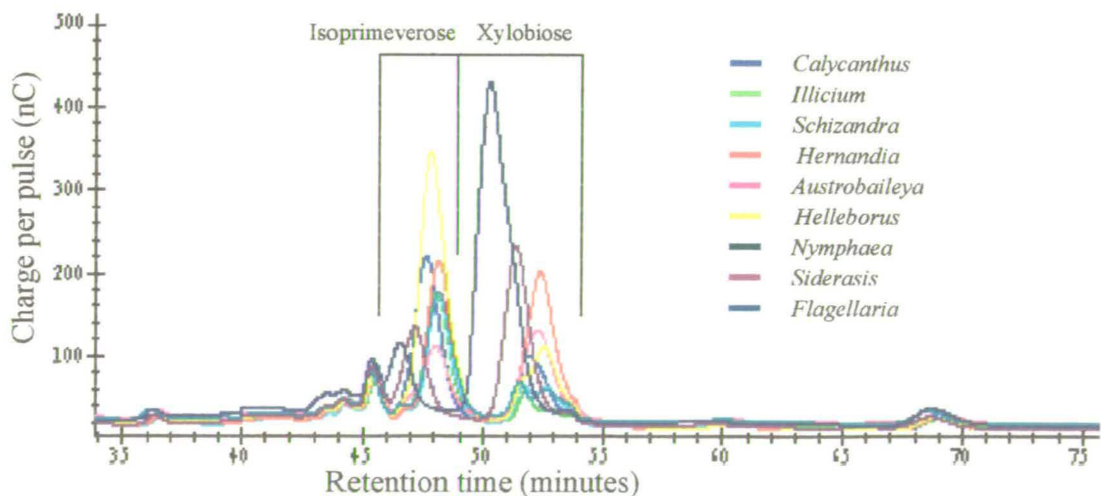


Figure 3.2.18: PC of Driselase digests of AIR of various bryophytes.

PC developed in B:A:W 12:3:5, E:Py:W 8:2:1 and stained with aniline hydrogen-phthalate. This image is a scan of a photograph. The photograph was exposed for 32 s. During exposure the PC was illuminated for 8 s each side with 366-nm UV. Scale = 30%.

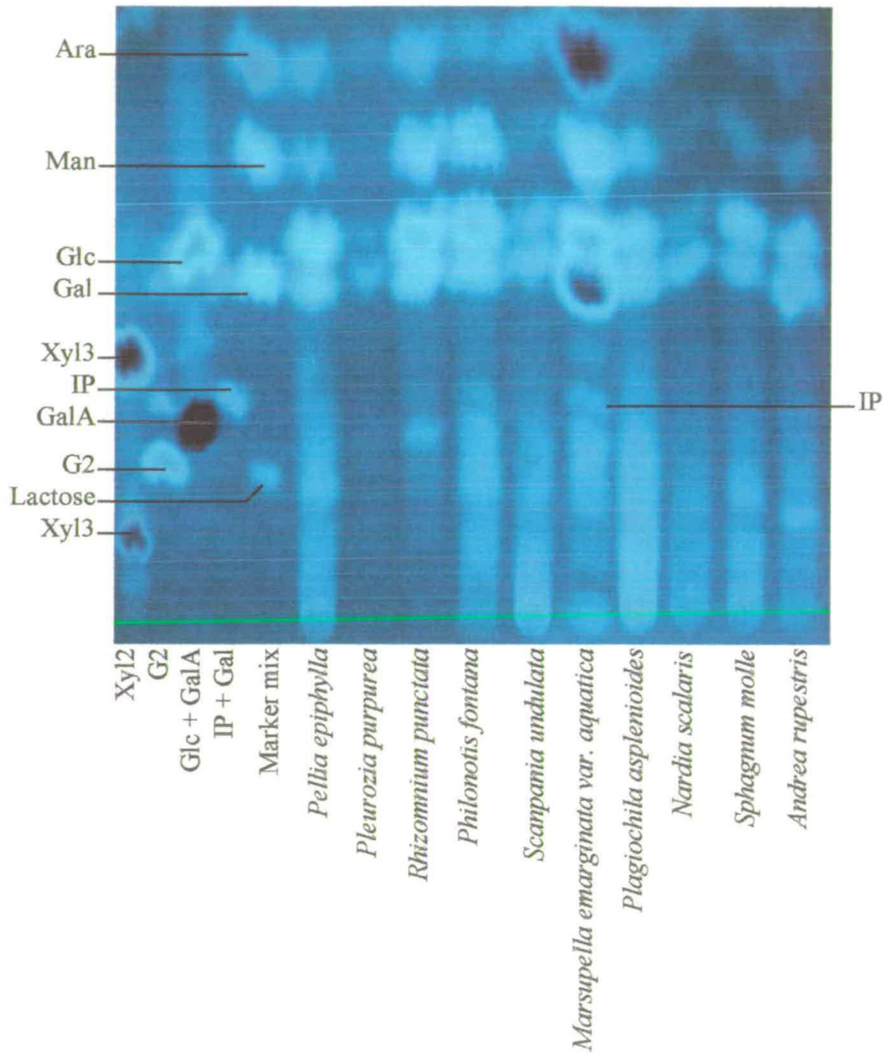


Table 3.2.2: Presence of xyloglucan in land plants as indicated by Driselase, cellulase and XEG digestion of AIR.

Species	Xyloglucan presence
Chlorobiota	
Chlorophytes	
Ulva lactuca	*
Charophytes	
Coleochaete scutata	□
Klebsormidium flaccidum	□□
Chara corallina	**
Embryobiota	
Bryophytes	
Sphagnum molle	+
Sphagnum palustre	+
Andrea rupestris	+
Lunularia cruciata	+
Dicranum scoparium	+
Mnium hornum	+
Philonotis fontana	+
Rhizomnium fontana	+
Thuidium tamariscinum	+
Plagiothecium undulatum	+
Hypnum cupressiforme	+
Anthoceros caucasicus	+
Trichocolea tormentella	+
Lepidozia reptans	+
Nardia scalaris	+
Marsupella emarginata var.aquatica	+
Plagiochila asplenioides	+
Lophocolea bidentata	+
Scapania undulata	+
Pleurozia purpurea	+
Porella coredaeana	+

Tracheophyta	
Lycopodiophytes	
Lycopodium pinifolium	+
Huperzia selago	+
Diphasiastrum alpinum	+
Selaginella apoda	+
Selaginella erythropus	+
Selaginella pallescens	+
Equisetophytes	
Equisetum debile	+
Psilotophytes	
Psilotum nudum	+
Filicophytes	
Eusporangiate ferns	
Marattia fraxinea	+
Leptosporangiate ferns	
Osmunda regalis	+
Todea barbara	+
Dryopteris crispifolia	+
Asplenium australassium	+
Nephrolepis lauterbachii	+
Onoclea sensiblis	+
Phyllitis scolopendrum	+
Salvinia auriculatea	+
Blechnum spicant	+
Seed Plants	
Gymnosperms	
Encephalartos altensteinii	+
Pinus sylvestris	+
Gnetum indicum	+
Gnetum montana	+
Gnetum gnemon	+

Angiosperms	
Nymphaea colorata	+
Austrobaileya scandens	+
Hernandia cordigera	+
Calycanthus floridus	+
Illicium verum	+
Schizandra rubiflora	+
Drimys lanceolata	+
Calathea zebrina	+
Callisia repens	+
Cyanotis longifolia	+
Dichorisandra thyrsifolia	+
Geogenthus undatus	+
Siderasis fuscata	+
Palisota albertii	+
Juncus effusus	+
Cyperus esculentus	+
Cyperus papyrus	+
Elegia capensis	+
Flagellaria guineensis	+
Fallopia japonica	+
Helleborus argutifolius	+

Key

*	Digestible with XEG and cellulase but not to produce xyloglucan-derived oligosaccharides. IP not observed after Driselase digestion
**	Digestible with cellulase but not to xyloglucan-derived oligosaccharides. Not digestible with XEG. IP not observed after Driselase digestion.
□	IP not observed after Driselase digestion; other methods not tested.
□□	Not digestible with XEG to yield xyloglucan-derived oligosaccharides; other methods not tested.
+	Digested with XEG and cellulase and IP yielded after Driselase digestion.

3.2.5 Xyloglucan structure

Cellulase digestion can give information about the structure of xyloglucan. AIR from a variety of angiosperms was cellulase-digested (Figures 3.2.19 and 3.2.20). This does give some information about xyloglucan structure. However, it was also found that the cellulase preparation had xylanase and licheninase activities (Figure 3.2.20). The digestion products originating from the licheninase activity of the cellulase preparation were eliminated by pretreatment of the AIR with licheninase and removal of the licheninase digestion products by dialysis (Figure 3.1.20). The xylanase activity of cellulase may be due to the inability of cellulase to distinguish between Glc and Xyl.

It was still possible to see variation in xyloglucan structure as the xyloglucan-derived oligosaccharides are relatively large and therefore have low R_F values on TLC whereas xylan-derived oligosaccharides are smaller had have higher R_F values. It was possible to see that generally the monocots e.g. *Calathea zebrina*, *Avena sativa*, *Cyperus papyrus* and *Cyperus esculentus* (Figure 3.2.19) and *Elegia capensis* and *Siderasis fuscata* (Figure 3.2.20), had a lower concentration of xyloglucan-derived oligosaccharide of DP9 than the dicots. The exception to this was *Allium porrum* which appeared to have similar concentrations of xyloglucan-derived oligosaccharides to the dicots.

Figure 3.2.19: Cellulase-digestion products of AIR of a variety of angiosperms. Digests were loaded on silica gel TLC, developed in B:A:W 2:1:1 and stained with thymol.

Scale = 170%

It was found that cellulase also had xylanase activity. The digestion products are different colours. The xylan derived oligosaccharides stained purple whereas the xyloglucan-derived oligosaccharides stained pink. However, the two colours are not clearly distinguishable, especially if some of the products have closely similar R_F values.

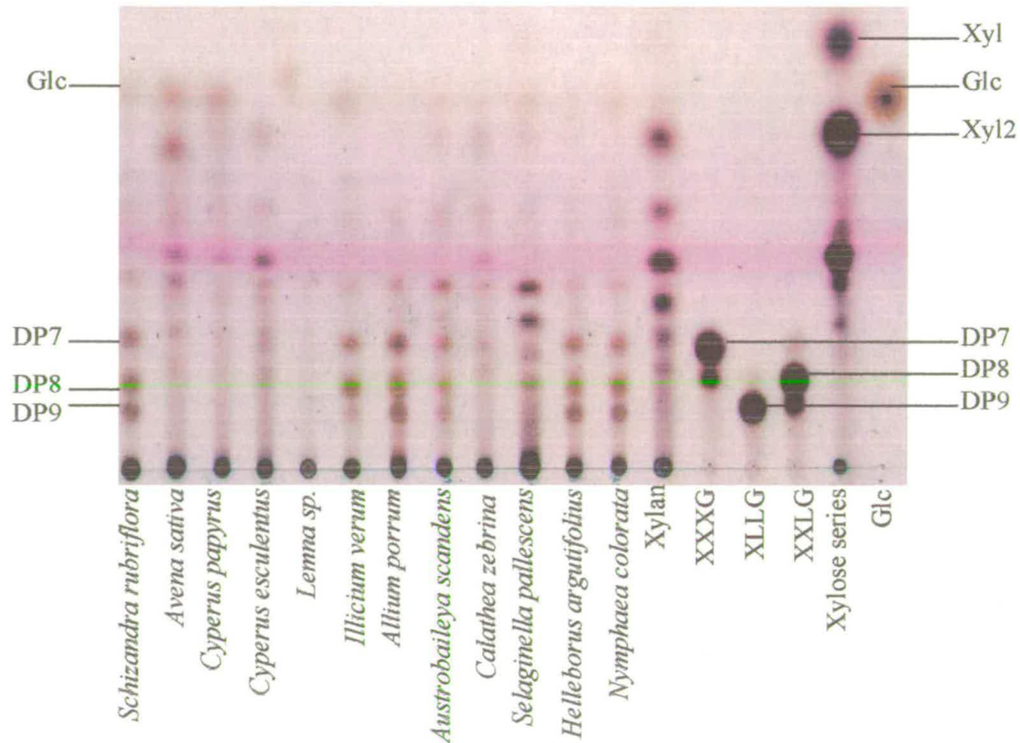
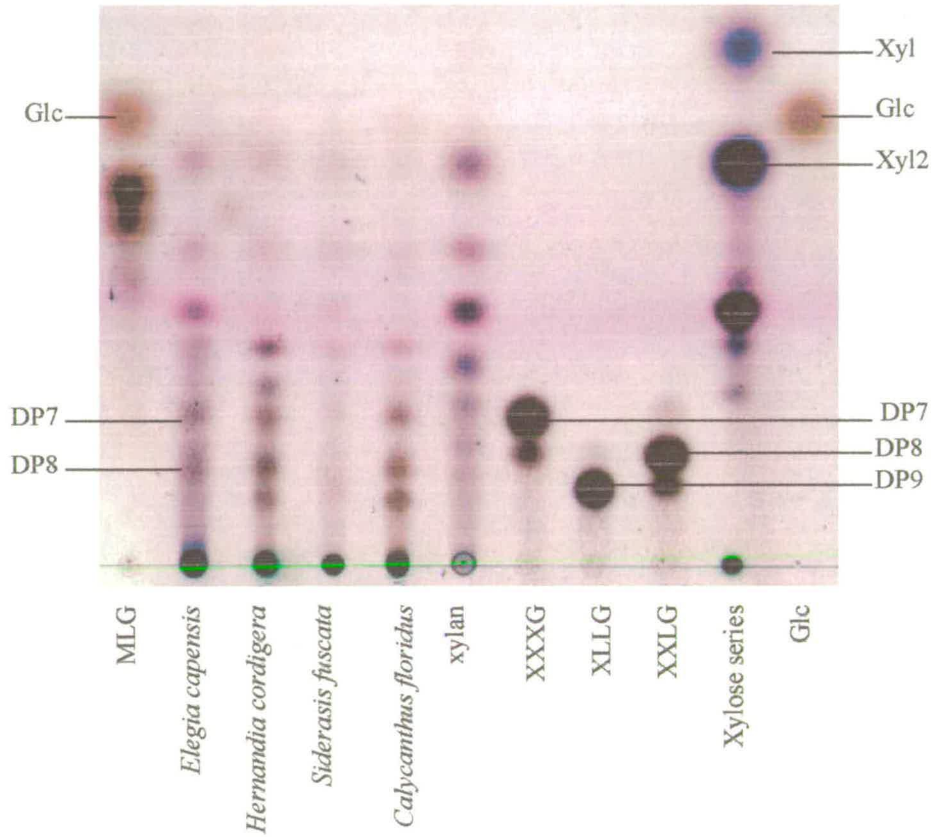


Figure 3.2.20: Cellulase digestion products of xylan, MLG and AIRs of a variety of angiosperms. Digestion products were loaded on silica gel TLC, developed in B:A:W 2:1:1 and stained with thymol.

Scale = 60%



Owing to the high xylanase activity of cellulase Novo Nordisk kindly gave me a sample of the xyloglucan-specific endoglucanase, XEG. XEG has very little xylanase and licheninase activity and is capable of hydrolysing xyloglucan to oligosaccharides in 5 minutes. The XEG enzyme requires a G (xyloglucan terminology, Fry et al. 1993) reducing terminus and an X non-reducing terminus in order to hydrolyse the substrate. Therefore XXGGXXGG will be hydrolysed to XXGG and not a mixture of XXG, GXXG and XXGG (personal communication; Markus Pauly; Novo Allé, 2880 Bagsvaerd, Denmark). XEG therefore digests xyloglucan in a similar way to cellulase but with greater specificity.

I used XEG on AIR from a variety of land plants to try to gain information about the xyloglucan structure (Figures 3.2.21 and 3.2.22). All XEG digests of land plants released some xyloglucan-derived oligosaccharides but the amounts were very variable. The bryophytes and some ferns, psilotophytes, equisetophytes and lycopodiophytes yielded xyloglucan oligosaccharides but in very low concentrations (Figure 3.2.21). It is possible that the low concentrations of xyloglucan found in the bryophytes is due to a reduction in digestibility of the AIR. These AIR were not subjected to mild TFA hydrolysis prior to XEG treatment. The AIRs of bryophytes were found to contain high concentrations of uronic acids (Section 3.6) and 2-*O*-MeXyl (Section 3.4). Bryophyte cell walls may contain high concentrations of pectic polysaccharides reducing the accessibility of xyloglucans to XEG.

Ulva lactuca AIR was digested by XEG (Figure 3.2.22). Some of the digestion products had similar R_F values to xyloglucan-derived oligosaccharides of DP7–9 whereas others had much higher R_F values. It appears that a polymer similar but not identical to xyloglucan is present in *U. lactuca*. This may be the hemicellulose described by Lahaye et al. (1994).

Figure 3.2.21: XEG digestion products of a AIR from a variety of land plants. Digestion products were loaded on silica gel TLC, developed in B:A:W 2:1:1 and stained with thymol.

Scale = 60%

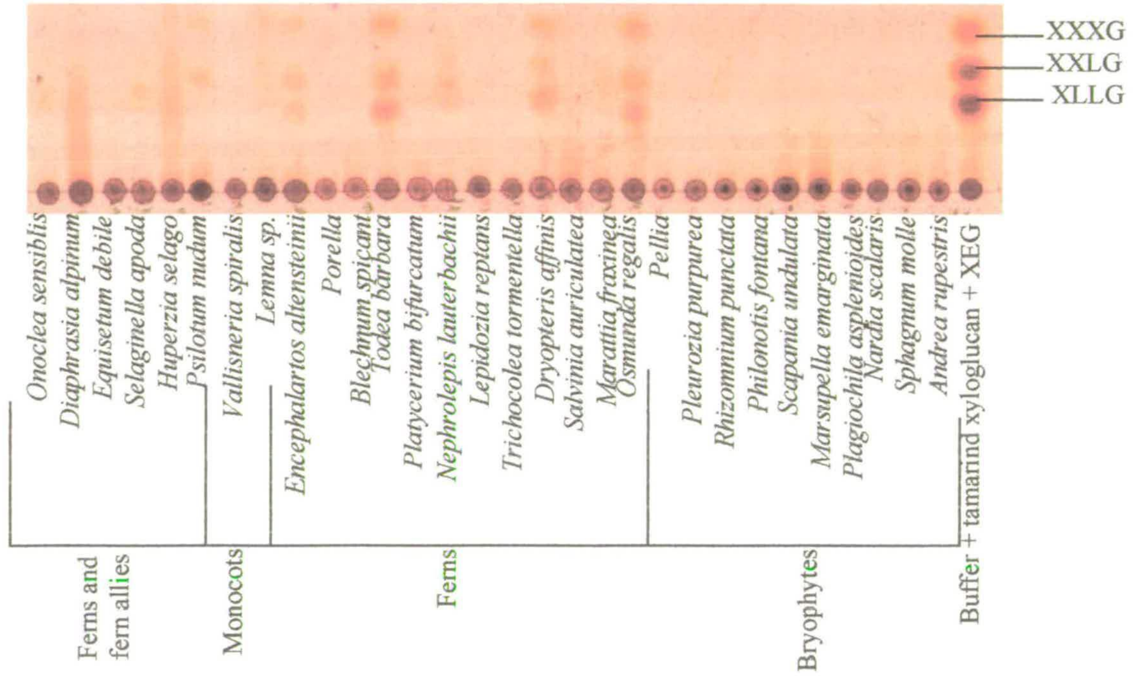
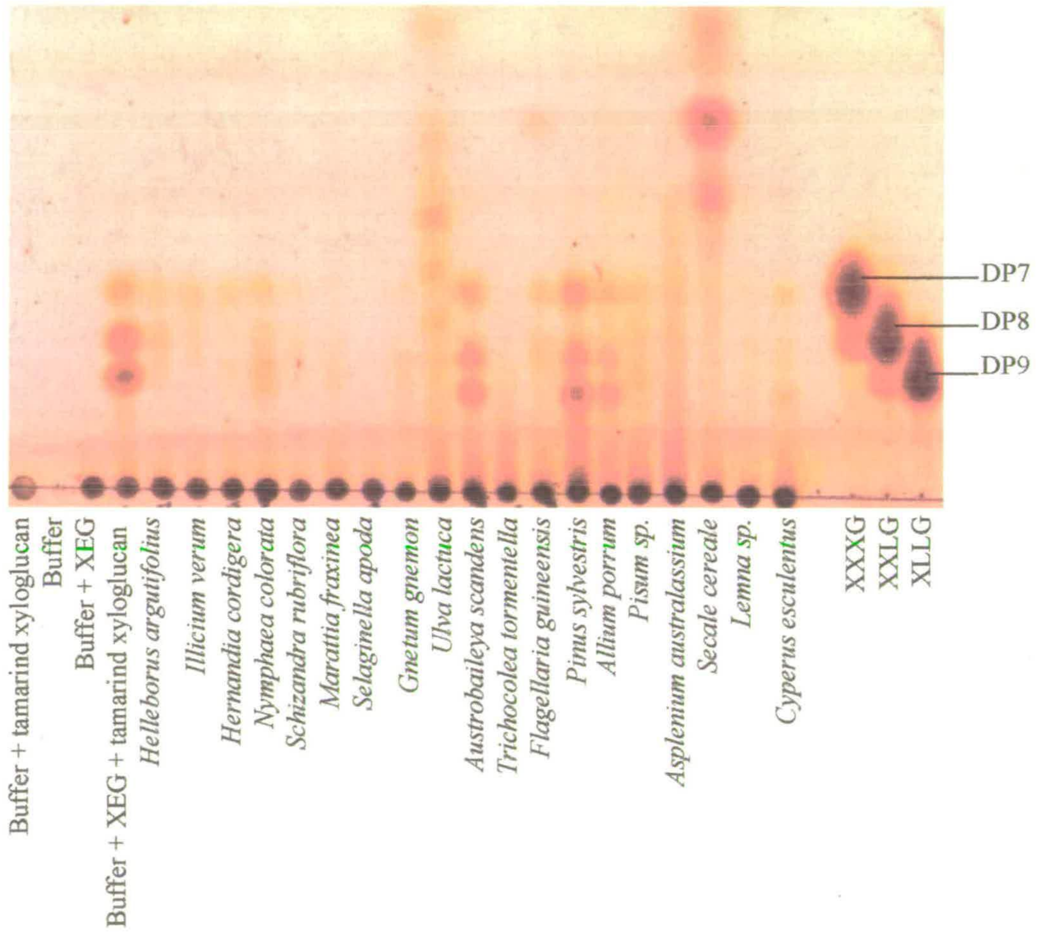


Figure 3.2.22: XEG digestion products of AIR of a variety of land plants on silica gel TLC, developed in B:A:W 2:1:1 and stained with thymol.
Scale = 60%



3.2.6 Discussion

Xyloglucan appears to be present in all land plants. However, xyloglucan appeared to be present in the bryophytes at far lower concentrations than in the tracheophytes. This result may be due to the presence of high concentrations of pectic polysaccharides present in the bryophyte cell wall (Sections 3.4 and 3.6) reducing the Driselase digestibility of bryophyte AIR. This could be tested by carrying out XEG digests on mild TFA-pretreated NaOH extracts from bryophyte AIR. However, IP is seen in PCs of Driselase-digested AIR.

The charophycean green algae and *Ulva lactuca* (a chlorophycean green alga) appeared to lack xyloglucan. Driselase-digestible xyloglucan is not present in *Chara* AIR to a concentration of 0.01% w/w xyloglucan/AIR. It is possible that xyloglucan is present but below the level of detection. If this is the case it is unlikely to be a biophysically significant cell wall component. Another explanation is that xyloglucan may be present in *Chara* cell walls but something else was present in the cell wall which prevented digestion of the xyloglucan. However, I found that exogenous xyloglucan was digested to IP in the presence of *Chara* AIR. A further possibility is that *Chara* cell walls may contain a xyloglucan which differs significantly from the xyloglucans of angiosperms. The structure of *Chara* xyloglucan may protect the α-D-Xyl-(1→6)-D-Glc linkage. However, XEG was unable to digest *Chara* polysaccharide fractions and cellulase digestion resulted in products which appeared to be derived from xylan. It therefore seems likely that xyloglucan is absent from *Chara* cell walls. A Xyl- and Glc-containing polysaccharide may be present in *Chara* cell walls as both Xyl and Glc were found in complete TFA hydrolysates of *Chara* AIR (Section 3.1.4). If a polymer containing Xyl and Glc is present in the *Chara* cell wall it is possible that the α-D-Xyl-(1→6)-D-Glc linkage is not present. In this case the polysaccharide would not bexyloglucan. A polymer has been characterised from *U. lactuca* which contains β-D-Xyl-(1→4)-β-D-Glc and which has been described as a xyloglucan (Lahaye et al. 1994). However, it lacks the α-D-Xyl-(1→6)-D-Glc linkage which is a xyloglucan defining linkage present in angiosperm xyloglucan. The α-D-Xyl-(1→6)-D-Glc

linkage is resistant to digestion by Driselase. β-D-Xyl-(1→4)-β-D-Glc may be Driselase-resistant. It is possible that the same linkage present in *U. lactuca* cell walls is also present in the charophycean green algae.

There are significant similarities between the land plants and the charophycean green algae. Charophycean green algae are the closest relatives of land plants. Some charophycean green algae are thought to be more closely related to land plants than *Chara*. It is possible that the charophycean green algal ancestor of land plants contained xyloglucan in its cell walls. Xyloglucan may have acted as a pre-adaptation to a non-aquatic environment. Xyloglucan appears to be present in all land plants and therefore be important to land plant cell growth. The early charophycean green algae, including the ancestors of *Chara*, may have had xyloglucan in their cell walls and the genes resposible for xyloglucan assembly were either lost or mutated in later diverging members of the charophycean green algae. This scenario seems less likely than others I have discussed as xyloglucan appears to be absent from chlorophycean green algae and widely divergent members of the charopycean green algae. Xyloglucan may have evolved by mutations in the genes which are responsible for the formation of the Xyl- and Glc-containing polymer in *U. lactuca*.

Variation exists in xyloglucan concentration. The euphyllophytes, excluding the Commelinae and some ferns, have similarly high xyloglucan concentrations in their cell walls. The bryophytes and the Commelinae have much less xyloglucan in their cell walls. Xyloglucan structure varies within the angiosperms (Hayashi 1989). However, most xyloglucans appear to be composed of repeating units of 7, 8 or 9 sugar residues. Hayashi suggested that *Pisum sativum* xyloglucan was composed of the repeating oligosaccharide XXFGXXXG. XEG digestion of *Pisum sativum* (Figure 3.2.22) AIR generated the oligosaccharides XXXG, XXLG and XLLG. The concentration of each repeating unit shows variation, with xyloglucan-derived oligosaccharides of DP7 and 8 being present as a higher percentage of the xyloglucan molecule in the Commelinae. The paleoherb, *Illicium verum*, appears to share the same proportions of xyloglucan oligosaccharides as the Commelinae. The

remaining angiosperms, including the remaining paleoherb; *Nymphaea colorata*, *Austrobaileya scandens*, *Hernandia cordigera*, *Calycanthus floridus* and *Schizandra rubriflora*, have equal proportions of the xyloglucan oligosaccharides. The ferns and gymnosperms also have similar concentrations of xyloglucan oligosaccharides. The bryophytes appear to have XXXG only.

3.3 3-*O*-Methyl-D-galactose

3.3.1 Introduction

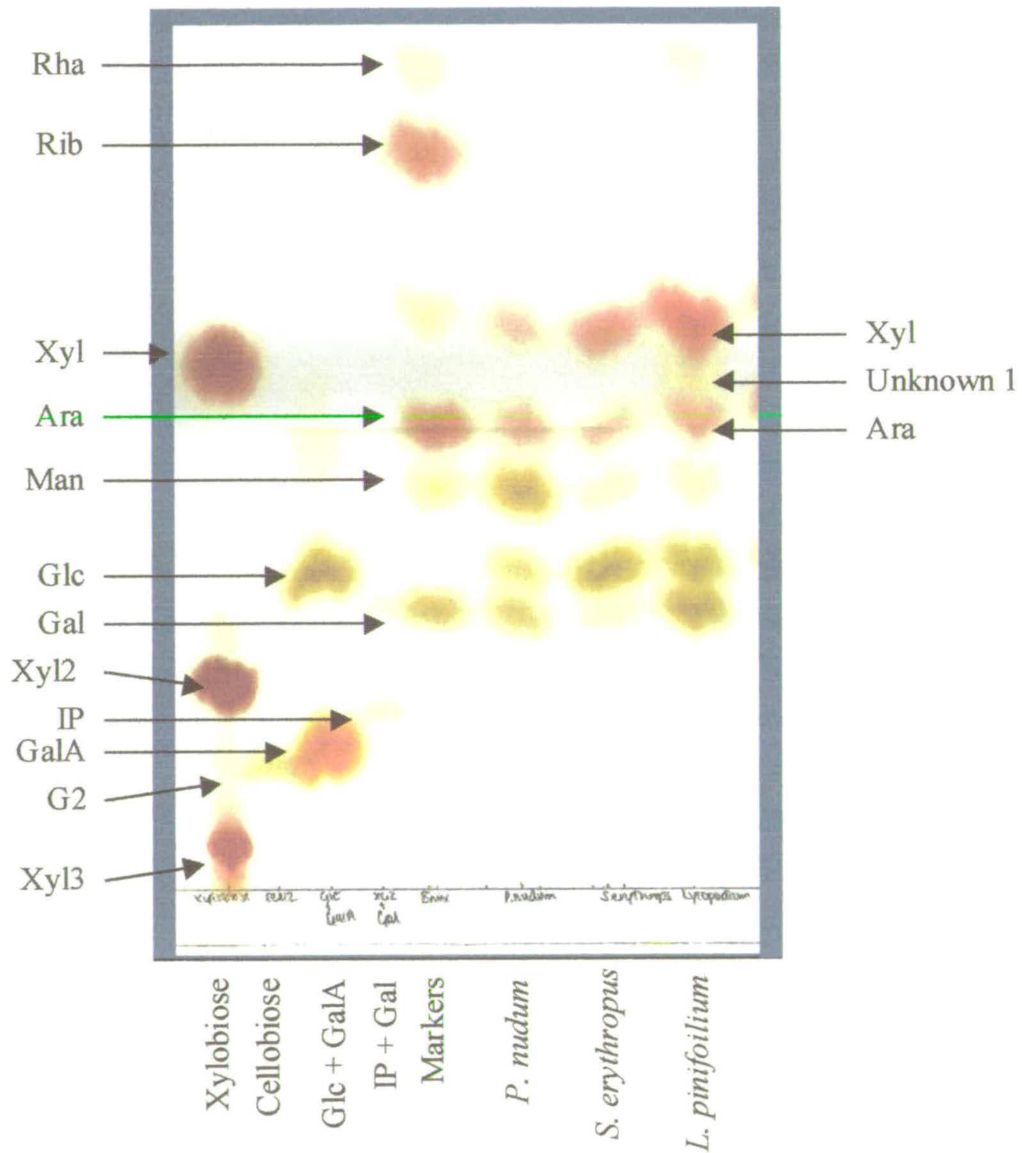
PC of the neutral fraction of TFA hydrolysis products of *Selaginella erythropus* Spring. and *Lycopodium pinifolium* Blume gave a yellow-staining (aniline hydrogen-phthalate) sugar which ran between xylose and arabinose (in E:Py:W 8:2:1 and B:A:W 12:3:5 followed by E:Py:W 8:2:1). The colour of staining suggested that unknown 1 was a hexose. However, no common hexose sugars run to this position in these solvents (Figure 3.3.1). Unknown **1** is not visible in *S. erythropus* in Figure 3.3.1 as this figure is a scan of a photocopy and did not reproduce well. However, unknown **1** is clearly visible in *Selaginella apoda* (L.) Spring (Figure 3.3.8.b).

A second unknown sugar (unknown **2**) was present in *L. pinifolium* only. This sugar stained red with aniline hydrogen-phthalate so is likely to have been a pentose. Unusually for a pentose unknown **2** ran to a position beyond rhamnose in the solvents used (E:Py:W 8:2:1 and B:A:W 12:3:5 followed by E:Py:W 8:2:1). In Figure 3.3.1 unknown **2** ran off the end of the paper. I decided to purify and further characterise unknown 1 as its presence in two lycopodiophytes and apparent absence from other land plants potentially made it of evolutionary interest. I purified unknown **1** from *Selaginella apoda* (L.) Spring. as it is closely related to *S. erythropus* and I had a greater quantity of *S. apoda* AIR.

Figure 3.3.1 Neutral fraction of TFA hydrolysis products of two lycopodiophytes and *P. nudum* run in B:A:W 12:3:5 (16 h) followed by E:Py:W 8:2:1 (18 h) showing the presence of unknown **1**. Unknown 2 ran off the end of the PC in this solvent system.

Stain = Aniline hydrogen-phthalate.

Scale = 10%



3.3.2 Purification

[^{14}C]Ara was added as an internal marker to the complete hydrolysis products (TFA 2 M 1 h) of 1 g *S. apoda* AIR and the mixture developed by PC(d) in E:Py:W (18 h). An autoradiogram of the PC was used to help locate unknown 1, which would run to the area immediately beyond [^{14}C]Ara. Vertical strips from each side of the PC were cut and stain to identify the presence and location of unknown **1**. The zone containing unknown **1** was cut out (care was taken to avoid as much Ara and Xyl as possible) and unknown **1** was eluted from the paper with water. Further purification was necessary as unknown **1** was not completely separated from Ara and Xyl by the PC(d). Many methods were tested in an attempt to separate unknown **1** from the two pentoses (Table 3.3.1).

Table 3.3.1: R_F values of Xyl, Ara and unknown 1 in the solvent systems used.

	R_F value in solvent							
	1	2	3	4	5	6	7	8
Ara	0.28	0.29	0.12	0.52	0.21	0.75	0.58	0.46
Xyl	0.38	0.21	0.13	0.41	0.15	0.79	0.49	0.37
Unknown 1	0.24	0.18	(-)	(-)	0.12	(-)	0.80	0.70
Comments			(s)	(s)	(s)			

Solvent system 1: Silica gel TLC, B:A:W 3:1:1

Solvent system 2: Silica gel TLC, B:A:W 4:1:1

Solvent system 3: Silica gel TLC, Butanone:W 85:7

Solvent system 4: Silica gel TLC, E:Py:A:W 5:3:1:1

Solvent system 5: Silica gel TLC, Propan-2-ol:ammonia:W 8:1:1

Solvent system 6: Silica gel TLC, Propan-2-ol:nitromethane:W 5:2:3

Solvent system 7: PC(a), Phenol 80% (w/w)

Solvent system 8: Cellulose TLC, Phenol 80% (w/w)

(-): unrecordable because unknown **1** streaked or ran in solvent system with a similar R_F value to Ara or Xyl.

(s): streaked

The method which separated unknown **1** most clearly from arabinose and xylose was cellulose, both TLC and PC(a), in 80% (w/w) phenol. I therefore used preparative PC(a) in 80% phenol to purify unknown **1**. On drying, the sugar was slightly coloured and therefore was likely to contain additional contaminants, possibly from the cellulose. To eliminate these contaminants I loaded the sugar onto a 25-ml Bio-Gel P-2 column. An aliquot of each fraction was spotted on paper and stained with aniline hydrogen-phthalate. The fractions containing unknown **1** were pooled and dried.

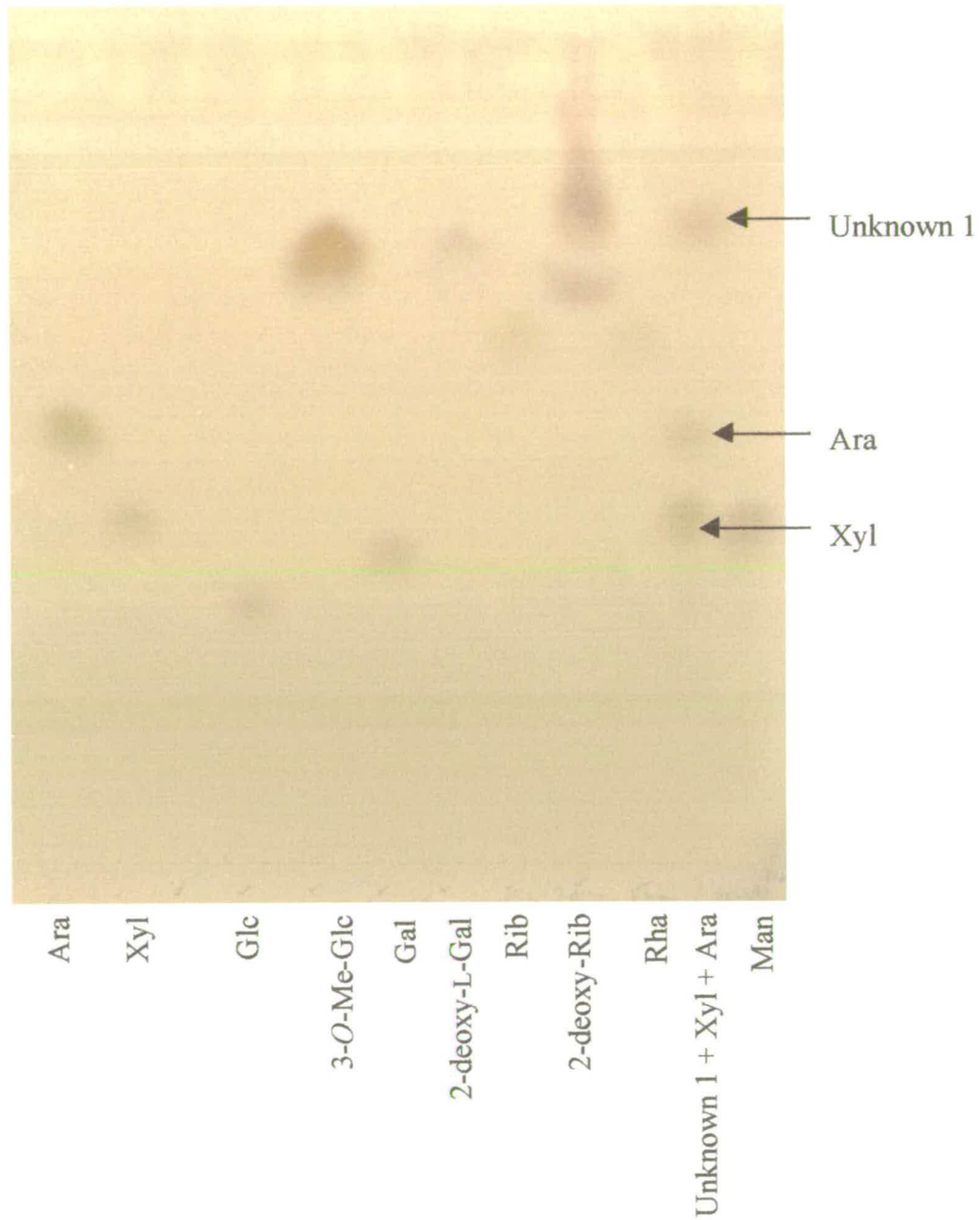
3.3.3 Chromatographic comparison with authentic *O*-methyl and deoxy-sugars

In an attempt to characterise unknown **1** it was run on cellulose TLC in 80% phenol with several methyl and deoxy sugars (Figure 3.3.2). In this system unknown **1** had a similar R_F value to 3-*O*-methylglucose, 2-deoxygalactose and 2-deoxyribose, but stained brown as opposed to pink-red.

Figure 3.3.2: Cellulose TLC in 80% (w/w) phenol of unknown **1** and several authentic *O*-methyl and deoxy sugar markers.

Stain = Aniline hydrogen-phthalate

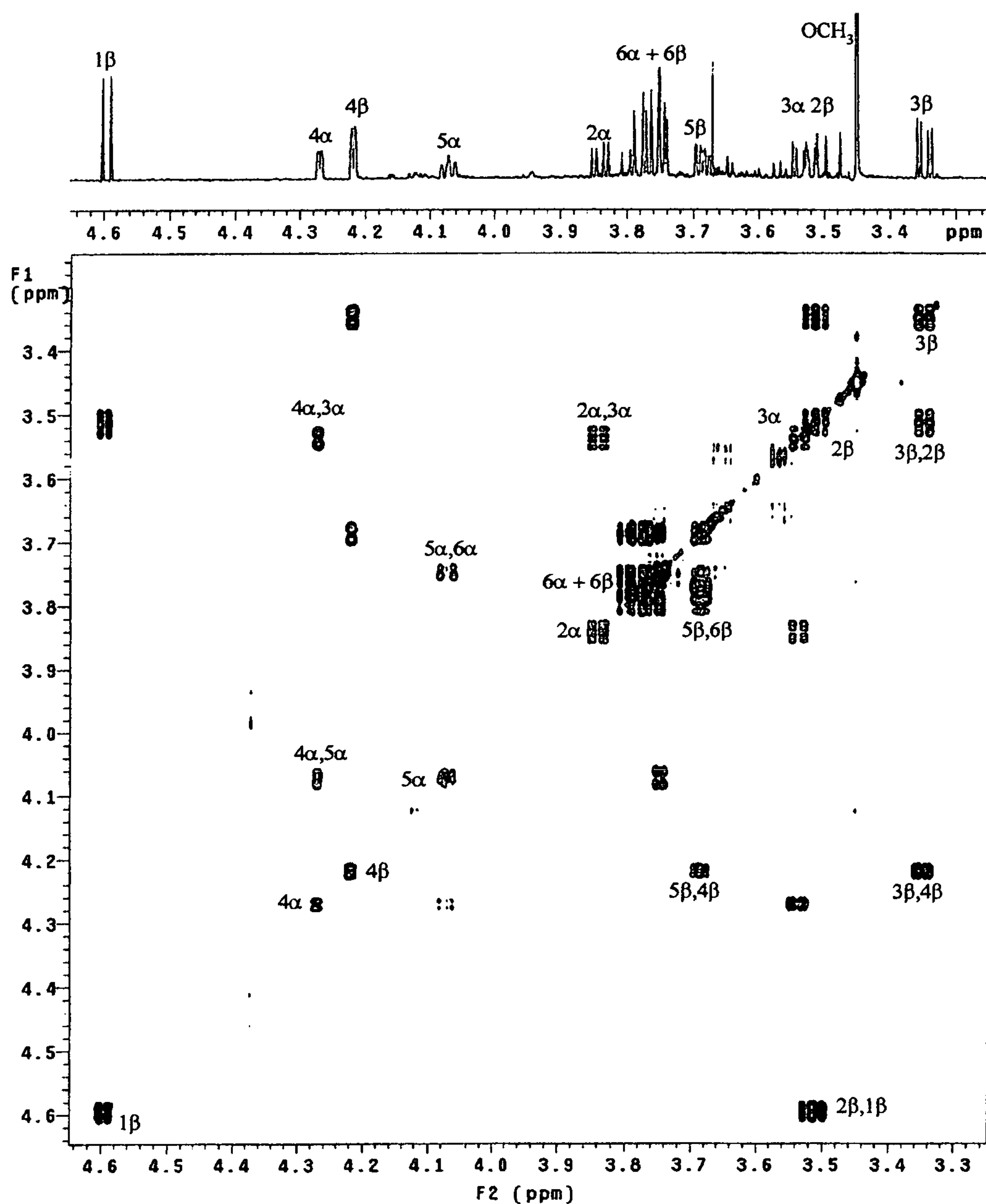
Scale = 75%



3.3.4 Nuclear magnetic resonance

The N.M.R. spectra of 100 μg of purified unknown **1** were kindly obtained and interpreted by Dr. I. H. Sadler (National Ultra High Field N.M.R. Centre, Department of Chemistry, The Joseph Black Building, University of Edinburgh). The standard 1-D single-pulse 600-MHz proton spectrum of 100 μg of purified unknown **1** (Figure 3.3.3.a), all OH groups having exchanged in D_2O used as the solvent and excluding the residual DHO signal after suppression, is reasonably well resolved. The doublets at 4.60δ and 5.26δ suggest the presence of the α and β anomers of a glucose or galactose derivative. The sharp signal at 3.45δ suggests the presence of one methoxy group. Further examination of this spectra confirms the presence of the requisite number of signals and their assignment follows immediately from the 2D-COSY spectrum (Figure 3.3.3.b). Significant overlap occurs only forthe C-6 protons. In the β-anomer the C-6 protons have different chemical shifts, each appears as a doublet of doublets. In the α-anomer the C-6 proton signals produced a single doublet because the signals coincided so that the coupling between them is not seen. One peak of the doublet for the C-6 of the α-anomer virtually coincides with one peak from one of the C-6 protons of the β-anomer. The coupling constants of each multiplet, measured from the 1-D spectrum (Table 3.3.2) are consistent only with a galactose derivative.

Figure 3.3.3: NMR spectra of 3-*O*-methylgalactose (a) 1-D single pulse 600 MHz proton NMR spectrum (b) 2-D gradient selected proton COSY NMR spectrum.



The C-13 chemical shifts, obtained from a 2-D 1-bond carbon–proton shift (HMQC) correlation spectrum, are similar to those for the epimers of galactose. However, the C-13 chemical shift for C-3 is about 10 ppm higher than is found for galactose. This is consistent with methylation of the hydroxyl at C-3. The site of methylation is confirmed by correlation between the methyl protons and C-3 of each epimer in the 2-D long range carbon–proton (HMBC) shift correlation spectrum. The ^{1}H and ^{13}C spectral data of the individual components are given in table 3.3.2.

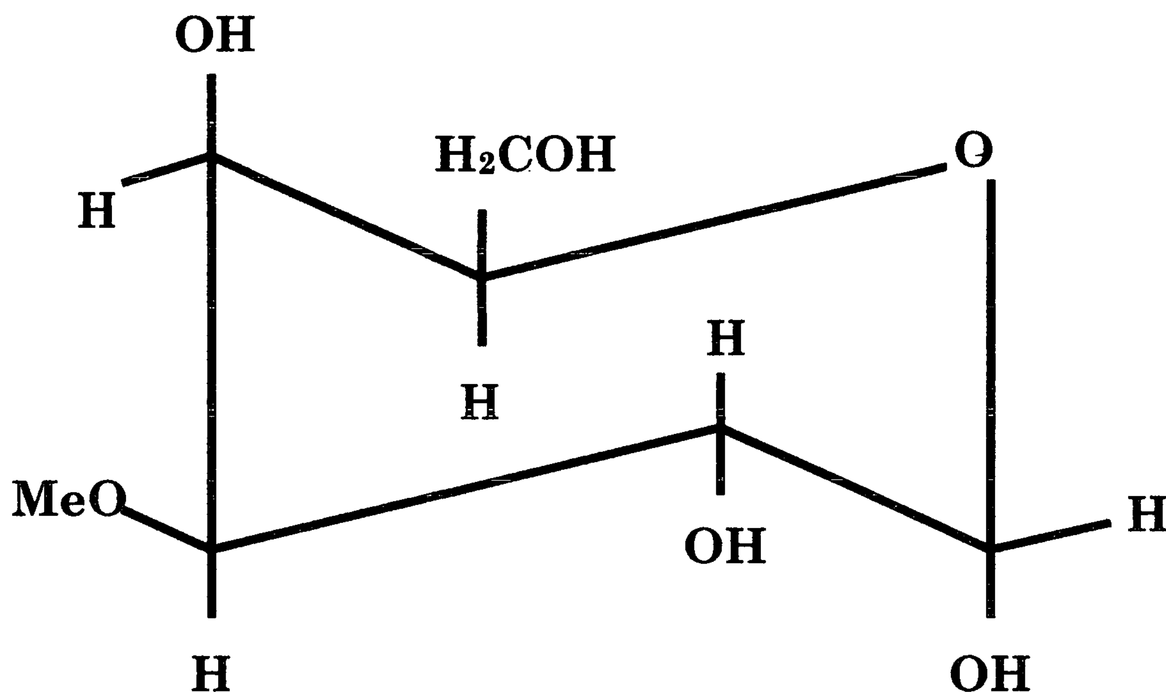
Table 3.3.2: ^{1}H and ^{13}C N.M.R. spectral data of 3-*O*-methylgalactose

Site	δ_C	δ_H	multiplicity	J_{HH} (Hz)
β-Galactose				
1	99.2	4.60	(d)	7.9
2	73.6	3.52	(dd)	9.9, 7.9
3	84.7	3.35	(dd)	9.9, 3.3
4	67.0	4.22	(dd)	3.3, 0.9
5	77.8	3.69	(ddd)	7.7, 4.4, 0.9
6	63.8	3.76	(dd)	11.6, 4.4
		3.80	(dd)	11.6, 7.7
O-Methyl	58.8	3.45	(s)	
α-Galactose				
1	94.9	5.26	(d)	4.0
2	70.1	3.84	(dd)	10.2. 4.0
3	81.2	3.54	(dd)	10.2, 5.2
4	67.6	4.27	(dd)	5.2, 1.3
5	73.0	4.08	(ddd)	6.7, 5.8, 0.9
6	64.0	3.74	(d)	Not measurable
O-Methyl	58.8	3.45	(s)	

Key: d = doublet, dd = doublet of doublets, s = singlet

In conclusion, the spectral data show unambiguously that, apart from a few unidentified minor impurities, unknown **1** was a mixture of the α and β anomers of 3-*O*-methylgalactose (Figure 3.3.4).

Figure 3.3.4: The structure of 3-*O*-methylgalactose



3.3.5 Determination of enantiomeric form of 3-*O*-methylgalactose

All monosaccharides (except for dihydroxyacetone) contain one or more asymmetric carbon atoms. They are therefore chiral molecules and will rotate plane-polarised light. This allows the molecule to exist as two stereoisomers, the D- and L-enantiomers (D and L refer to the configuration of the carbons). D- and L-enantiomers of a monosaccharide exhibit identical chemical and physical properties, apart from chirality and chemical interaction with other chiral compounds. In the cell wall D-galactose and L-galactose are synthesised from different precursors. D-Galactose is synthesised from UDP-D-Glc and L-galactose is synthesised from GDP-D-Man (Feingold and Avigad 1980). The majority of galactose found in the cell wall is the D-enantiomer. However, the D-Gal:L-Gal ratio has been reported at ~7.3:1 in cultured sycamore cells (Roberts and Harrer 1973) and ~70:1 in cultured

spinach cells (Baydoun and Fry 1987). Much of the L-galactose appears to be pectic, but L-galactose residues do not appear to be confined to any one particular polymer (Baydoun and Fry 1987). L-Gal has been reported in some xyloglucans (Zablakis et al. 1996) and as a component of RG-II (Hantus et al. 1997). It was therefore of interest to find out whether 3-*O*-methylgalactose was a derivative of D-Gal or L-Gal.

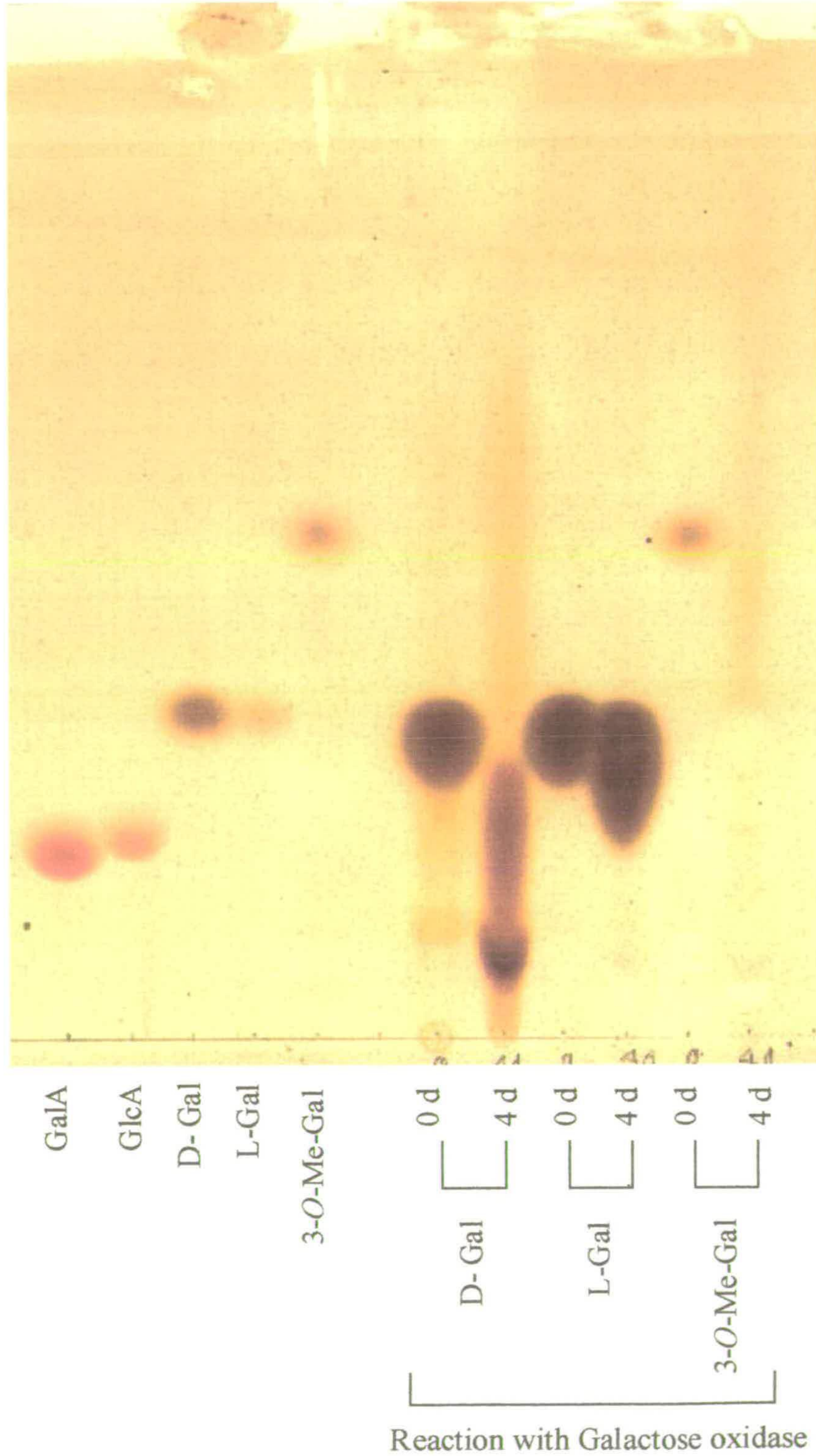
D-Galactose oxidase is reported to oxidise the —CH_2OH group of D-galactose and some of its derivatives but not L-galactose (Schlegel et al. 1968). Galactose oxidase still has activity on D-galactose even when it has undergone methylation at positions 1, 2 or 3 (Schlegel et al. 1968). In fact the relative rate of reaction of galactose oxidase for 3-*O*-methyl-D-galactose is reported to be almost twice that for D-galactose (Avigad et al. 1962). Galactose oxidase is unable to oxidise L-galactose or 4-*O*-methyl-D-galactose. D-Galactose oxidase can therefore be used to distinguish 3-*O*-methyl- D-galactose from 3-*O*-methyl-L-galactose.

I confirmed that the commercial sample of galactose oxidase could oxidise D- but not L-Gal. I also found that Galactose oxidase was able to oxidise 3-*O*-methylgalactose (unknown 1) over 4 d (Figure 3.3.5). The substrates D-Gal and 3-*O*-methylgalactose were completely destroyed within 4 d, yielding multiple reaction products. In addition, during incubation the reaction mixtures containing D-galactose or 3-*O*-methylgalactose with galactose oxidase turned yellow (probably owing to the formation of unstable aldehyde products) whereas L-galactose with galactose oxidase remained very pale brown. Together these results show that unknown 1 isolated from *S. apoda* was 3-*O*-methyl-D-galactose.

Figure 3.3.5 Effect of D-galactose oxidase on unknown **1**, D-Gal and L-Gal. The three potential substrates were incubated with the enzyme for 0 d or 4 d as indicated, then analysed by cellulose TLC in B:A:W 3:1:1, E:Py:W 10:4:3.

Stain = Aniline hydrogen-phthalate

Scale = 100%



3.3.6 Occurrence of 3-*O*-methyl-D-galactose in land plants

3-*O*-Methyl-D-galactose was isolated from *S. apoda* and had been observed to be present in *S. erythropus* and *L. pinifolium*. This sugar thus appeared to be present in lycopodiophytes. In addition, I had not noted 3-*O*-methyl-D-galactose in PCs of TFA-treated AIRs from any other land plants. To ascertain the distribution of 3-*O*-methyl-D-galactose more clearly I ran 2-dimensional PC(a)s of complete TFA hydrolysates of ferns, lycopodiophytes and the monocot *Cyanotis longifolia* (Figure 3.3.6.a to Figure 3.3.6.q). In addition I ran 2-dimensional PC(a)s of of complete TFA hydrolysates of bryophytes and charophycean green algae.

The results indicate 3-*O*-methyl-D-galactose is present in all lycopodiophytes tested, both homosporous (*Lycopodium*, *Diphasiastrum* and *Huperzia*) and heterosporous (*Selaginella*). Typical yields (estimated from staining intensity relative to authentic 3-*O*-Methyl-D-galactose) were in the order of 800 mg of 3-*O*-Methyl-D-galactose per g of AIR. 3-*O*-Methyl-D-galactose was undetectable in all euphyllophytes tested (including a equisetophyte, a psilotophyte, a euspeorangiate fern and several leptosporangiate ferns, gymnosperms and angiosperms). It was also undetectable in the walls of bryophytes and charophycean green algae which are thought to be relatively closely related to the hypothetical, extinct, green alga from which all land plants evolved (Table 3.3.3).

Table 3.3.3: Occurrence of 3-*O*-methyl-D-galactose in acid hydrolysates of AIR from a range of plants.

Species	3-*O*-methyl-D-galactose
Charophycean algae	
Klebsormidium flaccidum	-
Chara corallina	-
Coleochaete scutata	-
Bryophytes	
Hornwort	
Anthoceros caucasicus	-
Thalloid liverwort	
Pellia epiphylla	-
Leafy liverwort	
Marsupella emarginata var aquatica	-
Trichocolea tormentella	-
Porella	-
Moss	
Sphagnum molle	-
Andrea rupestris	-
Hypnum cuppressiforme	-
Lycopodiophytes	
Homosporous	
Lycopodium pinifolium	+
Huperzia selago	+
Diphasiastrum alpinum	+
Heterosporous	
Selaginella apoda	+
Selaginella erythropus	+
Equisetophytes	
Equisetum debile	-
Psilotophytes	
Psilotum nudum	-

Ferns Eusporangiate *Marattia fraxinea*	-
Leptosporangiate	
Osmunda regalis	-
Onoclea sensiblis	-
Salvinia auriculata	-
Platycerium bifurcatum	-
Todea barbara	-
Phyllitis scolopendrum	-
Asplenium australassium	-
Blechnum spicant	-
Dryopteris crispifolium	-
Gymnosperms	
Gnetum gnemon	-
Angiosperms	
Cyanotis longifolia	-

+ indicates 3-*O*-methyl-D-galactose detectable after 2-dimensional PC as shown in Figure 3.3.6

Figure 3.3.6 Presence of 3-*O*-methyl-D-galactose in complete TFA hydrolysates from a range of land plants. The TFA hydrolysates were run by 2D PC(a). The PC(a)s were developed in B:A:W 12:3:5 in one direction (vertically on diagrams) and then 80% (w/w) phenol at 90° to the first solvent (horizontally on diagrams). The PC(a)s were stained with aniline hydrogen-phthalate.

Figure 3.3.6.a Presence of 3-*O*-methyl-D-galactose (unknown **1**) and unknown **2** of *Lycopodium pinifolium* (lycopodiophyte).

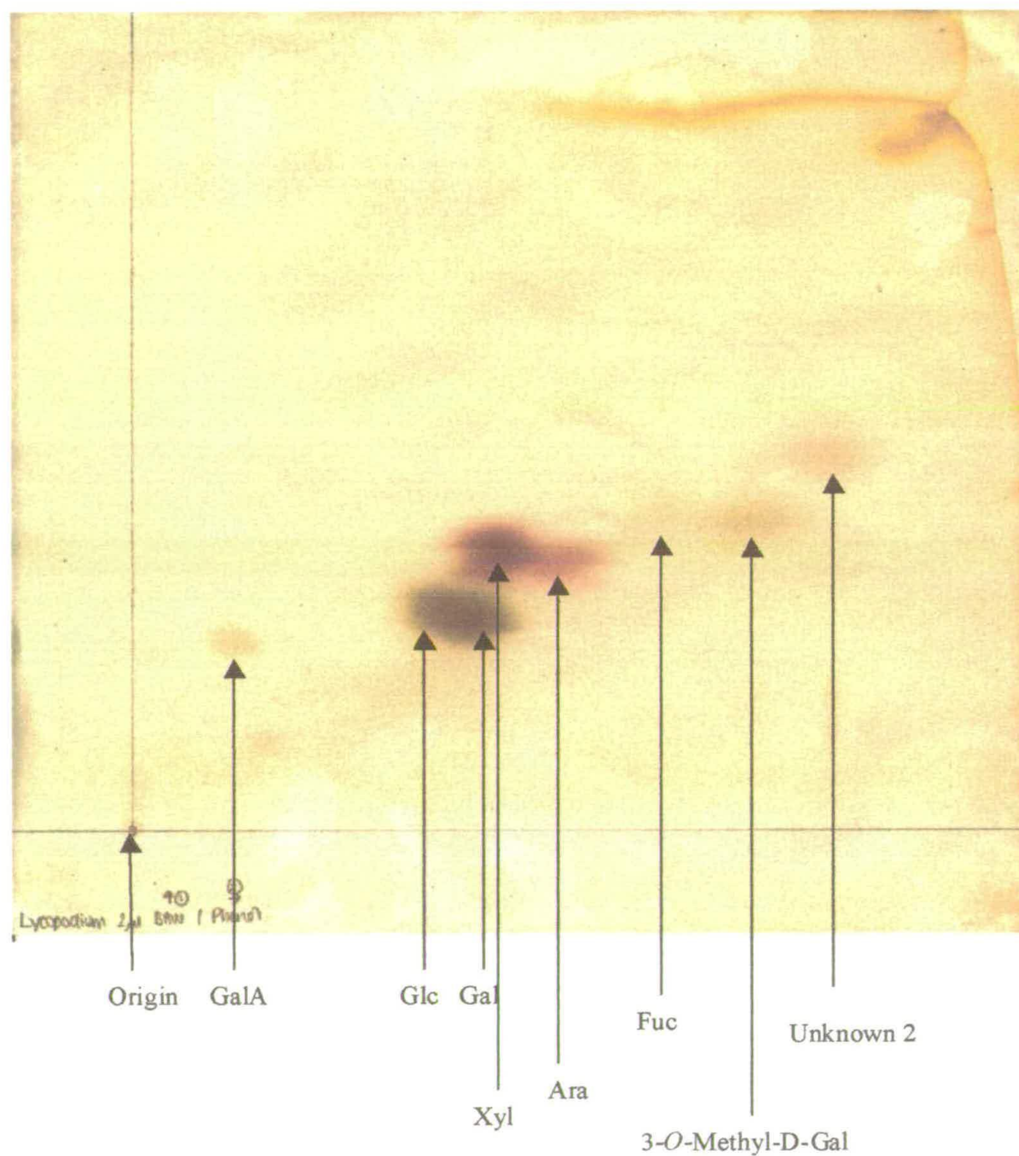


Figure 3.3.6.b: *Selaginella apoda* (lycopodiophyte). Arrow indicates 3-*O*-MeGal.

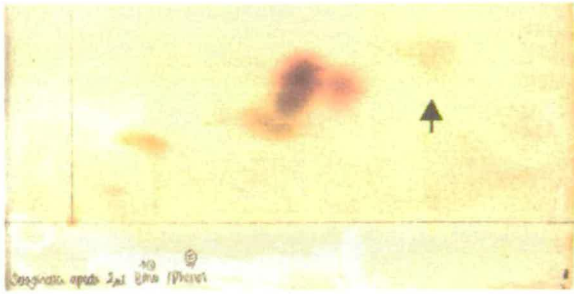


Figure 3.3.6.c: *Huperzia selago* (L.) Bernh. Ex. Schrank & Mart. (lycopodiophyte). Arrow indicates 3-*O*-MeGal.

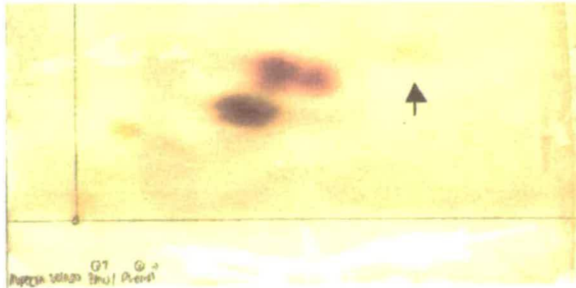


Figure 3.3.6.d: *Diphasiastrum alpinum* (L.) Holub. (lycopodiophyte). Arrow indicates 3-*O*-MeGal.

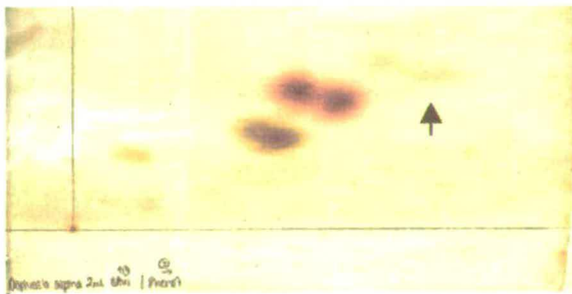


Figure 3.3.6.e: *Equisetum debile* Roxb. Ex. Vaucher (euphyllophyte; equisetophyte).

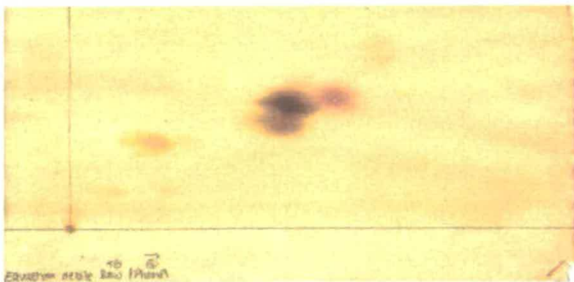


Figure 3.3.6.f: *Psilotum nudum* (L.) P. Beauv. (euphyllophyte; psilotophyte).

Figure 3.3.6.g: *Marattia fraxinea* Sm. (euphyllophyte; eusporangiate fern).

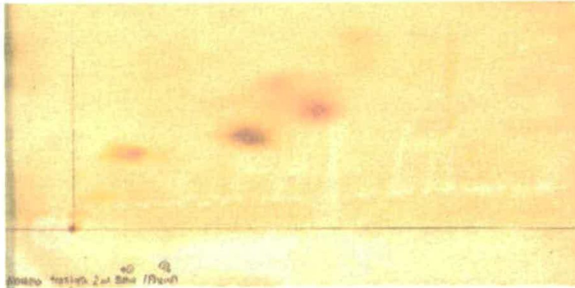


Figure 3.3.6.h: *Osmunda regalis* L. (euphyllophyte; leptosporangiate fern).

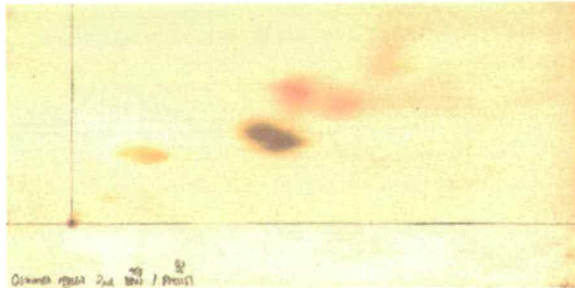


Figure 3.3.6.i: *Onoclea sensiblis* L. (euphyllophyte, leptosporangiate fern).

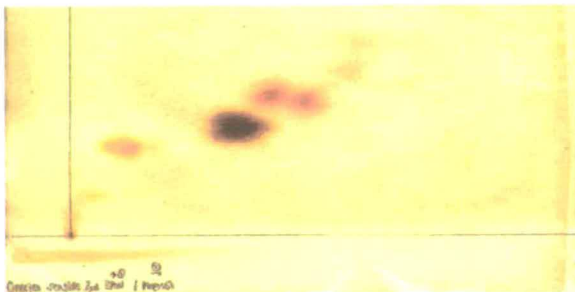


Figure 3.3.6.j: *Salvinia auriculata* Aubl. (euphyllophyte, leptosporangiate fern).

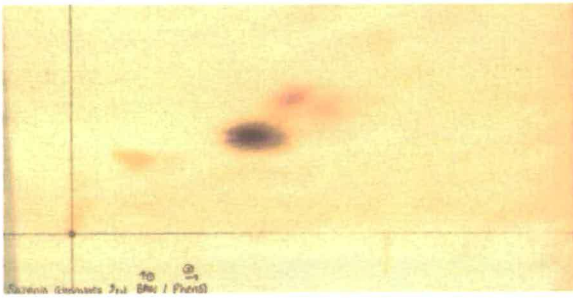


Figure 3.3.6.k: *Platycerium bifurcatum* (Car.) C. Chr. (euphyllophyte; leptosporangiate fern).

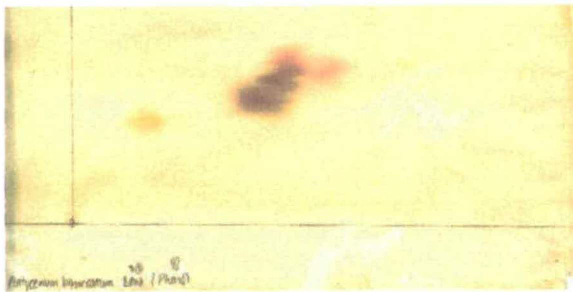


Figure 3.3.6.l: *Todea barbara* (L.) T. Moore (euphyllophyte; leptosporangiate fern).

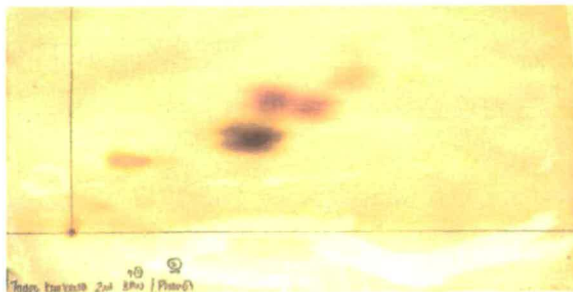


Figure 3.3.6.m: *Phyllitis scolopendrum* L. (euphyllophyte; leptosporangiate fern).

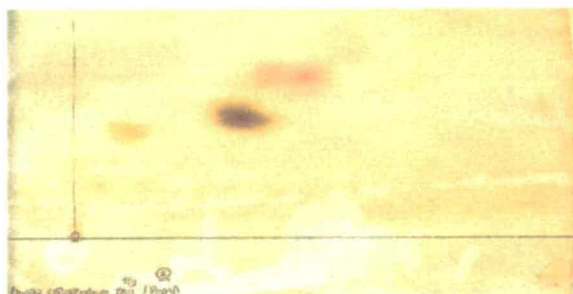


Figure 3.3.6.n: *Asplenium australassium* (J. Sm.) Hook. (euphyllophyte; leptosporangiate fern).

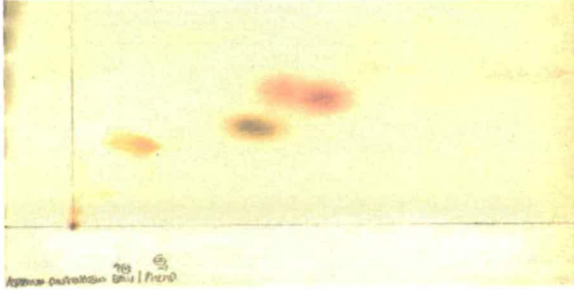


Figure 3.3.6.o: *Blechnum spicant* (L.) Roth. (euphyllophyte; leptosporangiate fern).

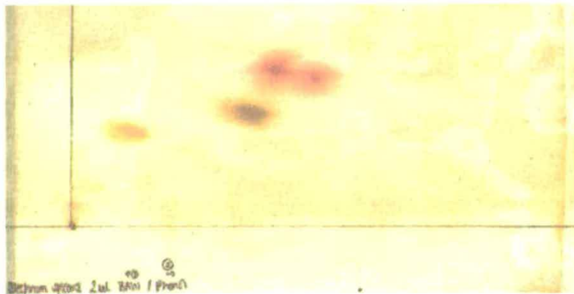


Figure 3.3.6.p: *Dryopteris crispifolia* Rasbach & al. (euphyllophyte; leptosporangiate fern).

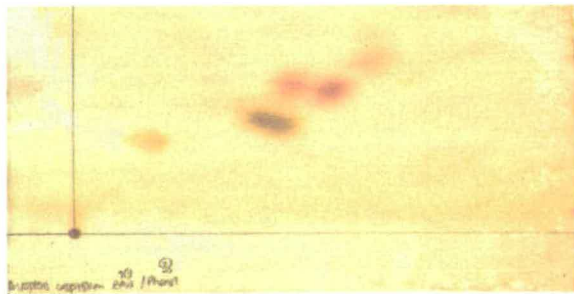
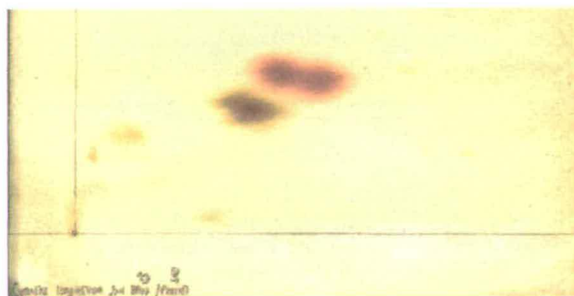


Figure 3.3.6.q: *Cyanotis longifolia* var. longifolia (euphyllophyte; angiosperm).



3.3.7 Discussion

The presence of 3-*O*-methyl-D-galactose in lycopodiophytes, both homosporous and heterosporous, supports the proposed monophyly of the lycopodiophytes (Bateman 1986). Extant lycopodiophytes are the relicts of a much more diverse group of plants which are now extinct. Lycopodiophytes are morphologically different from the bryophytes and euphyllophytes; this may be explained by the mass extinction of taxa which may have been intermediate between lycopodiophytes and the bryophytes or euphyllophytes. Morphological features suggest that the lycopodiophytes evolved from ancestral plants between the bryophytes and the euphyllophytes. My results suggest that this major evolutionary step was accompanied by the adoption of 3-*O*-Methyl-D-galactose as a significant component of the leaf cell wall.

3-*O*-Methyl-D-galactose was not detectable in either *Equisetum* or *Psilotum*. This may indicate that *Equisetum* and *Psilotum* are more closely related to the ferns than to the lycopodiophytes. Molecular sequence data have shown *Equisetum* and *Psilotum*, but not the lycopodiophytes, to be closely related to eusporangiate ferns within the euphyllophyte clade (Pryer et al. 1995, Duff and Nickrent 1999, Raubeson and Jansen 1992, Kranz et al. 1995, Manhart 1994, 1995, Wolf 1997, Malek et al. 1996, Vangerow 1999).

3-*O*-Methyl-galactose has been isolated, in trace amounts, from an extracellular polysaccharide exuded by members of the Cryptophyceae, division Cryptophyta (Cryptomonads) (Paulsen et al. 1992). There are 12 genera of Cryptophyceae and all are aquatic. There are about 100 marine and 100 freshwater species. They frequently have red photosynthetic pigments. Members of the Cryptophyceae are responsible for the spring algal blooms seen in the North Sea. Most are free-swimming but one member of the Cryptophyceae has been found as an endosymbiont in a marine cilliate in a complex is known as *Mesodinium rubrum*. Since within the lycopodiophytes only *Isoetes*, which I did not analyse, is aquatic they are unlikely to have any cryptophycean endosymbionts (Dr. D.G. Mann, Royal Botanic Garden Edinburgh; personal communication). 3-*O*-Methyl-galactose has

also been isolated from polymers present in the aquatic green alga *Chlorella vulgaris* (Ogawa et al. 1994). *Chlorella* are also primarily free-swimming alga and are again unlikely to form a symbiotic relationship with terrestrial lycopodiophytes.

Among the fungi, 3-*O*-methyl-D-galactose has been detected as a polysaccharide component in *Armillaria mellea* (Bouveng et al. 1967) and a 3-*O*-methylgalactose (enantiomer not determined) in *Lamperomyces japonicus* (Fukuda and Hamada 1978). 3-*O*-Methyl-D-galactose has also been reported as a component of haemocyanin, a glycoprotein from snails (Hall et al. 1977).

3-*O*-Methyl-D-galactose has been reported to constitute about 5% of the total neutral sugar residues in the soluble extracellular polysaccharides produced by the freshwater red alga *Porphyridium aerugineum* (Percival and Foyle 1979). Methylgalactoses are present in the rhodophyte genera, *Geldiliella* and *Gracilaria*, from which agar is extracted. However, the *O*-methyl groups are attached to positions 4 (Chiovitti et al. 1995) or 6 (Matsuhiro and Urzua 1991, Givernaud et al. 1999, Mollet et al. 1998, Mollion et al. 1992) and there are no records of 3-*O*-methylgalactose from these algae. It is therefore highly unlikely that 3-*O*-methyl-D-galactose occurred in my lycopodiophyte samples as a contaminant from laboratory agar. The lycopodiophytes were collected from different places (Royal Botanic Garden, Edinburgh; Daniel Rutherford Building Greenhouse; and near the top of Cairngorm); it is therefore unlikely that they had a common contaminant.

Pectic polysaccharides from slippery elm (*Ulmus fulva*; Hirst et al. 1951) and *Ulmus glabra* bark (Barsett and Paulsen 1991) contain 3-*O*-methyl-D-galactose. These polymers have a backbone of 4-linked α-D-galactopyranosyluronic acid interspersed with 2-linked α-L-rhamnopyranose residues. Some of the rhamnose residues are substituted on O-4 with short side-chains containing 3-*O*-methylgalactose, galactose and/or GlcA. It is possible that a similar polymer may occur in the lycopodiophytes as Rha and GalA were present in the TFA hydrolysates of the lycopodiophytes. However, if this is the case it is likely that this polymer would be more widely distributed as elm trees and lycopodiophytes are not closely related. In addition,

Rha and GlcA occur in pectic polymers in most higher plant cell walls. At present it is not known what 3-*O*-methyl-D-galactose is linked to in the lycopodiophyte cell wall.

3-*O*-Methyl-D-galactose was also detected as a component of a polysaccharide extracted from sassafras (*Sassafras albidum*) twigs (Springer et al. 1965), and a 3-*O*-methylgalactose (enantiomer not determined) was found in hydrolysates of the leaves of various dicot leaves (Bacon and Cheshire 1971). The yield from sweet-chestnut leaves was 11 mg of 3-*O*-methyl-D-galactose (and an unknown associated impurity) per 100 mg AIR (Bacon and Cheshire 1971), a very low concentration in comparison with the high levels detected in lycopodiophytes in our survey.

In conclusion, the adoption of 3-*O*-methyl-D-galactose as a major component of the leaf wall appears to be an autapomorphy of the lycopodiophytes, acquired early during their evolution from hypothetical ancestral tracheophytes, intermediate between the bryophytes and the euphyllophytes, and retained by all extant lycopodiophytes.

3.4 Unidentified sugars

3.4.1 Observation of unknown sugars

While attempting to delimit the presence of 3-*O*-methylgalactose, I carried out 2-dimensional PC(a) (in B:A:W 12:3:5 followed by 80% w/w phenol at 90° to the first solvent) of the complete TFA hydrolysis (2 M TFA, 1 h, 120°C) products of bryophyte and charophycean green algae AIRs. This method of PC gives a clear separation of many of the monosaccharides present in the plant cell wall. On staining with aniline hydrogen-phthalate three previously unidentified sugars (**3**, **4** and **5**) were recorded (Figure 3.4.1).

Unknown **3** stained brown with aniline hydrogen-phthalate suggesting that it was likely to be a hexose derivative. Unknown **3** had a particularly low R_F value in both solvent systems (B:A:W 12:3:5, 80% w/w phenol) and could be a dimer with a hexose residue at its reducing end and containing a bond which was particularly resistant to TFA hydrolysis. I am not certain whether 'unknown **3**' is the same compound in the hydrolysates of all species tested; more work would need to be done to test this (3.4.5).

It was initially suggested unknown **3** may be sulphated owing to its low R_F value in both solvent systems used. Sulphated sugars are found in the chlorophycean algae (Lahaye et al. 1994) and sulphated extra-wall mucopolysacchaccharides have been reported to be exuded from bryophyte rhizoids (Ea 1989). Ea (1989) reported that the exudate from bryophyte rhizoids stained red with periodic acid–Schiff reagent, indicating that it was a non-cellulosic polysaccharide. However, periodic acid–Schiff reagent will stain most carbohydrates including starch. Ea reported the polysaccharide to be sulphated on the basis of staining pink with toluidine blue. However, toluidine blue will also stain pink DNA, RNA and carboxylated and phosphorylated sugars. I therefore carried out PE at pH 2.0 (Figure 3.4.2): some of the TFA hydrolysis products from *Anthoceros caucasicus* migrated to a similar position to uronic acids but stained brown with aniline hydrogen-phthalate unlike the orange observed with uronic acids, possibly indicating the presence of an acidic hexose; a spot of brown-staining neutral sugars remained near the origin. However,

there did not appear to be a spot which ran with Glc-6-P or GalNAc.SO_4 (*N*-acetyl-D-glucosamine sulphate). It therefore seems unlikely that unknown **3** is a sulphated hexose. Unknown **3** was also unlikely to be a sugar amine; although there was a streak on PE (Figure 3.4.2) which ran towards GlcN and GalN, the streak was not well defined and is therefore likely to consist of a graduated series of cationic compounds. It therefore seems unlikely that unknown **3**, whivh appears to be a single major compound, is a sugar amine.

A sample of *Anthoceros caucasicus* AIR was treated with 2 M TFA for 2 h and run by preparative PC in B:A:W 12:3:5 for 18 h. The partially-purified unknown **3** was then subjected to further TFA hydrolysis and run by PE pH 3.5 (Figure 3.4.3). The products were only GlcA, neutral sugars and unchanged **3**. A further portion of the TFA hydrolysate of unknown **3** was run by PC in E:Py:W (Figure 3.4.4). This showed the only neutral product to be Gal. Unknown **3** is therefore an oligoccharide containing GlcA and Gal. Owing to the acid resistance of the disaccharide it can be inferred that GlcA is likely to be at the non-reducing terminus. Unknown **3** is likely to be a disaccharide and not an oligosaccharide with a higher DP as the products of partial acid hydrolysis were unknown **3**, Gal and GlcA. A larger oligosaccharide would be expected to yield a variety of shorter oligosaccharides on partial acid hydrolysis. These oligosaccharides would probably have been separated from unknown **3**, GlcA and Gal on PE pH 3.5 (Figure 3.4.3) or PC in E:Py:W 8:2:1 (Figure 3.4.4).

Unknown **5** stained pink with aniline hydrogen-phthalate and is likely to be an *O*-methyl or deoxypentose; it had a similar R_F value (in the two solvent systems used) to a standard of 2-*O*-methylxylose (Table 3.4.1). Unknown **5** differed from unknown **2** (Chapter 3.3) as it had a higher R_F value in both solvent systems. It is therefore possible that unknown **5** is 2-*O*-methylxylose.

Unknown **4** stained brown with aniline hydrogen-phthalate suggesting it was likely to be a hexose derivative. It had a higher R_F value than unknown **5** suggesting it may be a deoxymethylhexose as it is unlikely that an *O*-methylhexose would have a

higher R_F value than an *O*-methylpentose. Although Rha and Fuc used to be described as methylpentoses they are now described as deoxyhexoses and, like hexoses, they stain brown with aniline hydrogen-phthalate. The solvent system 80% w/w phenol carries *O*-methylhexoses to roughly the same position as 2-*O*-methylxylose on PC(a); Table 3.4.1 thus suggests unknown 4 may be an *O*-methylhexose or an *O*-methyldeoxyhexose.

Other unidentified sugars were also noticed but did not appear to be present in particular groups of plants. The taxa in which the unknown sugars were present may indicate an evolutionary change in the cell wall as they appeared to be present in phylogenetically related plants.

Figure 3.4.1: 2-Dimensional PC(a) in B:A:W 12:3:5 (vertical on image) followed by 80% w/w phenol (horizontal) of the complete TFA hydrolysis products of *Coleochaete* AIR.

Stain — Aniline hydrogen-phthalate

Scale — 75%

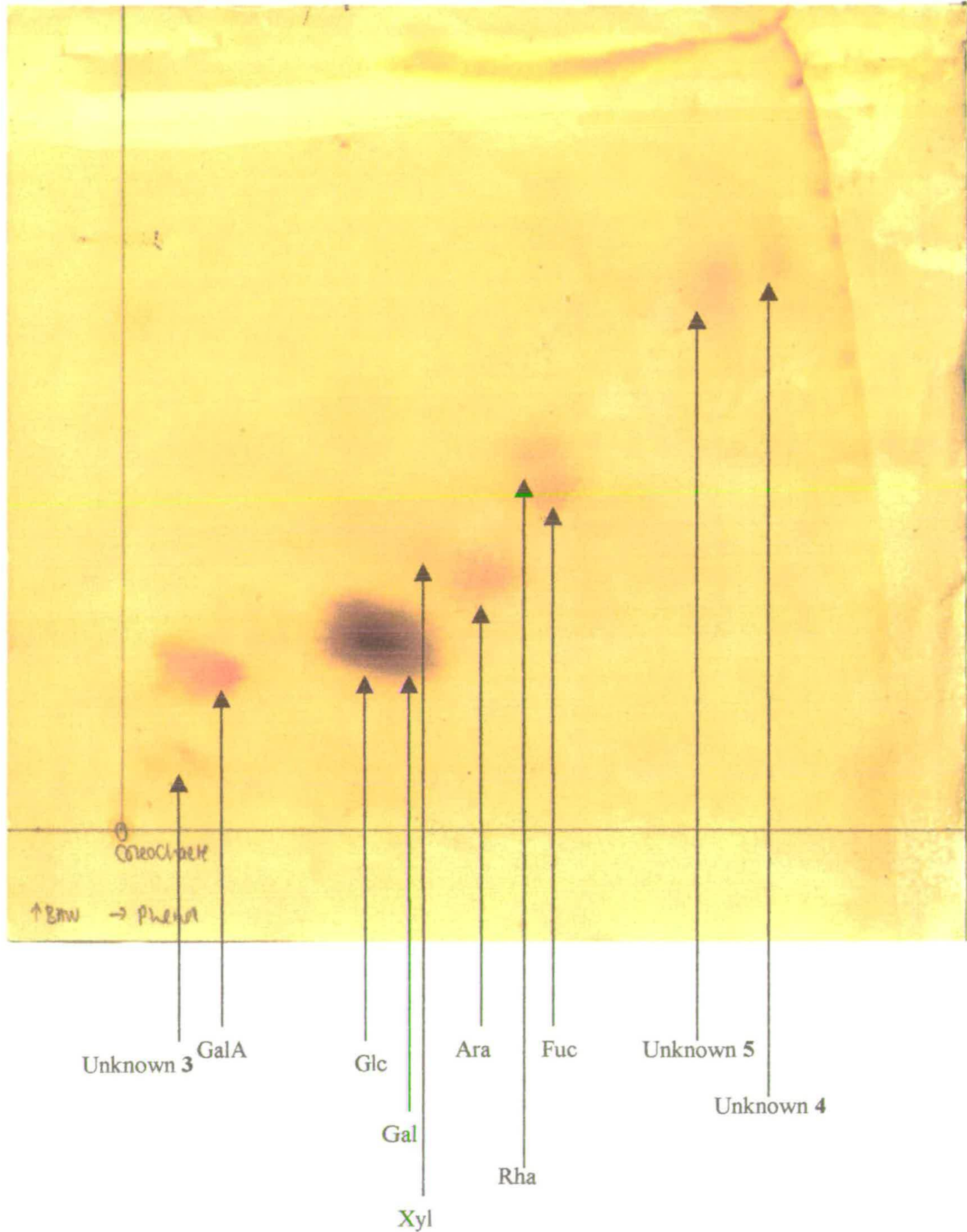


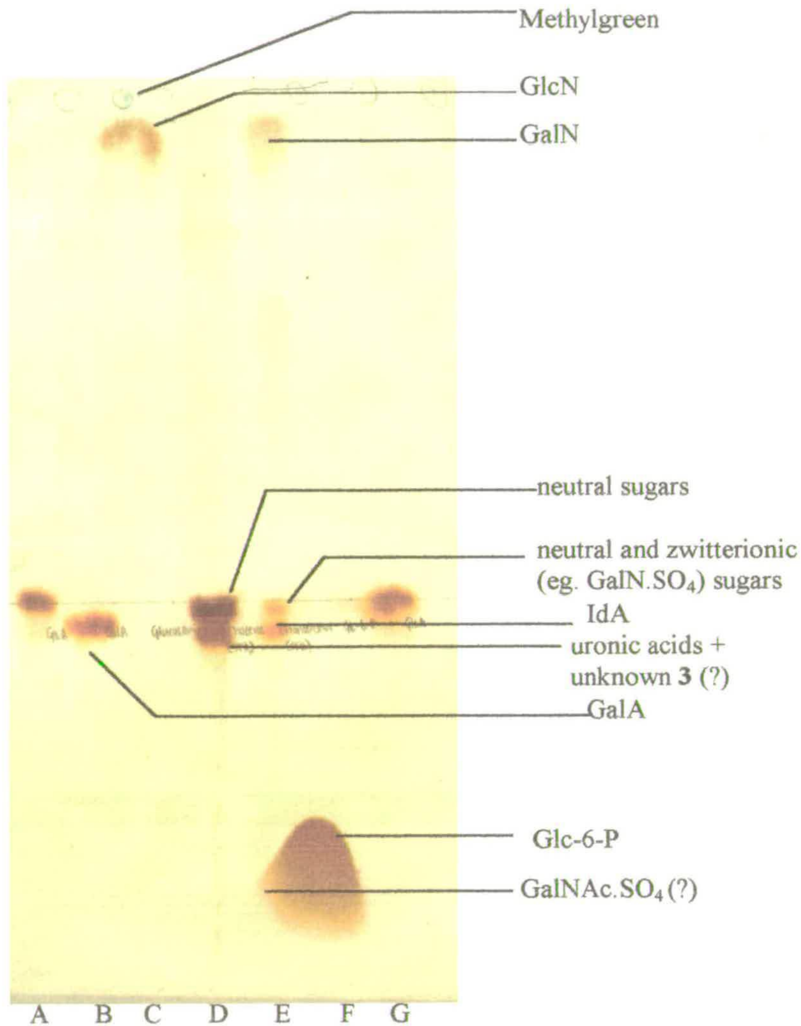
Table 3.4.1: R_F values of common cell wall sugars and unidentified sugars in B:A:W 12:3:5 and 80% phenol w/w.

Sugar (unknown)	Colour on staining with aniline hydrogen-phthalate	Type of sugar suggested	R_F value in B:A:W 12:3:5	R_F value in 80% w/w phenol
3	brown	hexose	0.09	0.09
4	brown	hexose	0.69	0.89
5	pink	pentose	0.69	0.82
Ara	pink	pentose	0.32	0.48
Xyl	pink	pentose	0.36	0.40
2-*O*-MeXyl	pink	pentose	0.68	0.80
GalA	orange	uronic acid	0.20	0.13
Man	brown	hexose	0.27	0.40
Rha	brown	hexose	0.48	0.56
Rib	pink	pentose	0.42	0.58
Fuc	brown/yellow	hexose	0.43	0.59
Gal	brown	hexose	0.23	0.37
Glc	brown	hexose	0.25	0.30

Figure 3.4.2: PE pH 2.0 of the complete TFA hydrolysate of *Anthoceros caucasicus* AIR.

Stain = aniline hydrogen-phthalate

Scale = 30%



Key

A = Glucuronolactone

B = GalA

C = D-Glucosamine (GlcN).

D = complete TFA hydrolysis products of *Anthoceros caucasicus* AIR

E = complete TFA hydrolysis products of chondroitin sulphate B: IdA, galactosamine 4-sulphate, GalN (D-galactosamine) and GalNAc.SO_4.

F = Glc-6-P

G = Glucuronolactone

Figure 3.4.3: PE pH 3.5, 2.0 kV, 1 h, of partially purified unknown **3** from *Anthoceros caucasicus* AIR subjected to severe TFA hydrolysis.

Stain = Aniline hydrogen-phthalate

Scale = 45%

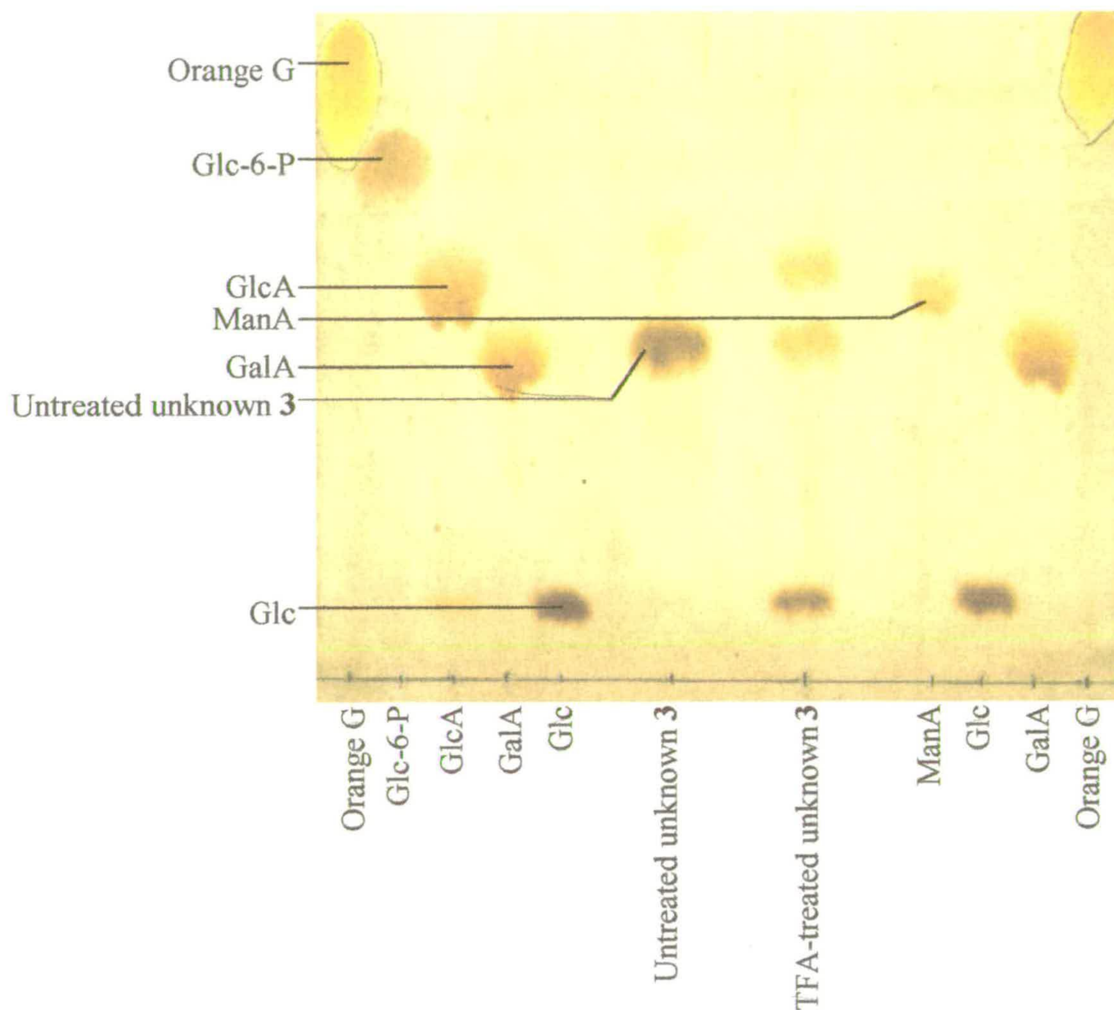
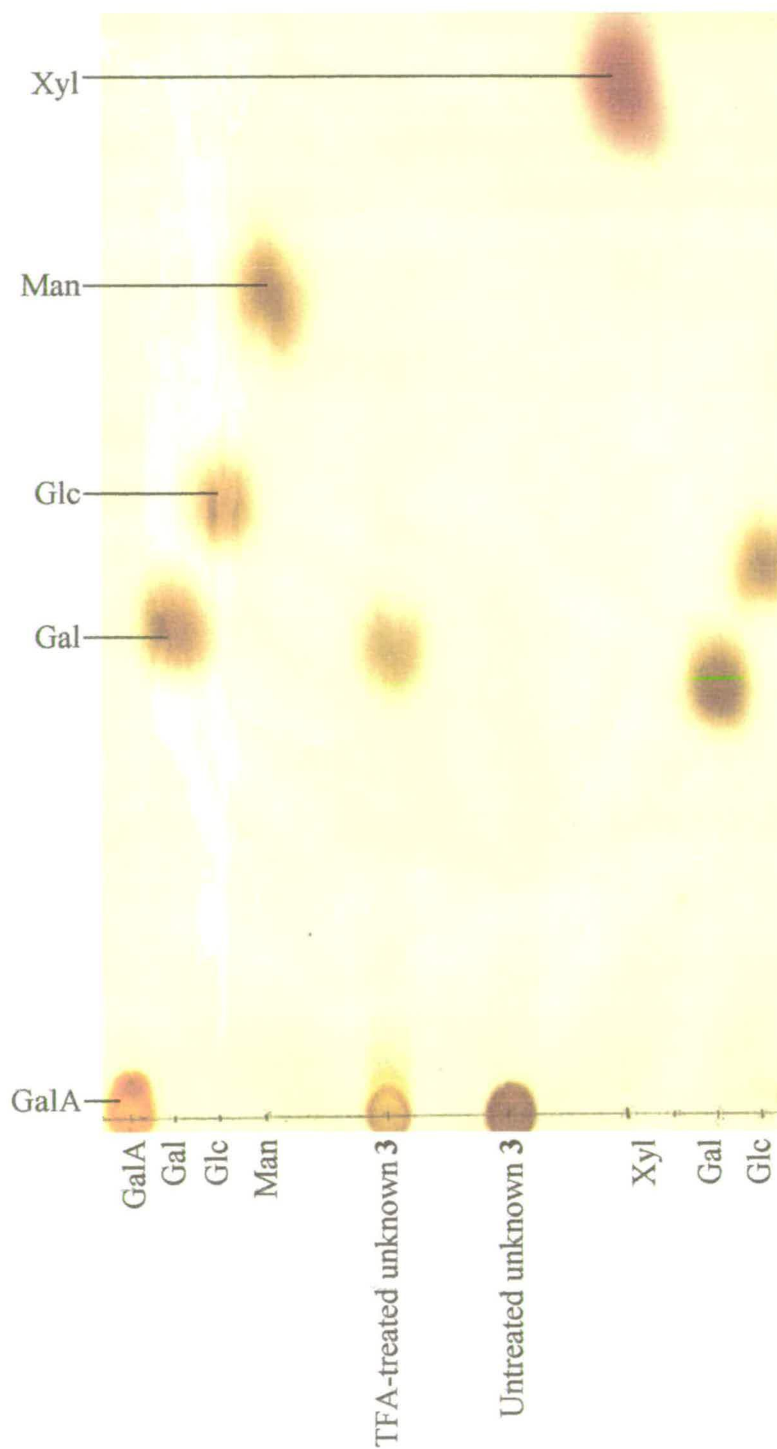


Figure 3.4.4: TFA-treated unknown 3 run by PC in E:Py:W 8:2:1, 42 h.

Stain = Aniline hydrogen-phthalate

Scale = 50%



3.4.2 Distribution of unknown sugars

Unknown **5** was found in all bryophytes, charophycean green algae and homosporous lycophytes investigated but was not detectable in *Selaginella apoda* (the only heterosporous lycophyte investigated) or any other vascular plants investigated. Unknown **4** was found in *Anthoceros caucasicus* and all the charophycean green algae investigated but was not detected in any other streptophytes investigated. Unknown **3** was found in *Anthoceros caucasicus* and was not found in any other streptophytes investigated (Figure 3.4.1, Figure 3.4.5.a–c and Table 3.4.2). TFA hydrolysates of AIRs of *Coleochaete scutata* and *Trichocolea tormentella* both contained a compound with a similar R_F value to unknown **3** but it was present at a lower proportion of total sugars.

Table 3.4.2: Distribution of unknown sugar residues in the AIRs of lower land plants.

Species	Unknown 3	Unknown 4	Unknown 5
	(GlcA→Gal)	(*O*-Methyl-deoxyhexose?)	(2-*O*-MeXyl?)
Charophycean algae			
Klebsormidium flaccidum	–	+	+
Chara corallina	–	+	+
Coleochaete scutata	*	+	+
Bryophytes			
Hornwort			
Anthoceros caucasicus	+	+	+
Thalloid liverwort			
Pellia epiphylla	–	–	+
Leafy liverwort			
Marsupella emarginata var aquatica	–	–	+
Trichocolea tormentella	*	–	+
Porella	–	–	+
Moss			
Sphagnum molle	–	–	+
Andrea rupestris	–	–	+
Hypnum cuppressiforme	–	–	–
Lycopodiophytes			
Lycopodium pinifolium	–	–	+
Selaginella apoda	–	–	–
Huperzia selago	–	–	+
Diphasiastrum alpinum	–	–	+
Equisetophytes			
Equisetum debile	–	–	–
Psilotophytes			
Psilotum nudum	–	–	–

Ferns			
Marattia fraxinea	–	–	–
Osmunda regalis	–	–	–
Onoclea sensiblis	–	–	–
Salvinia auriculata	–	–	–
Platycerium bifurcatum	–	–	–
Todea barbara	–	–	–
Phyllitis scolopendrum	–	–	–
Asplenium australassium	–	–	–
Blechnum spicant	–	–	–
Dryopteris crispifolium	–	–	–
Gymnosperms			
Gnetum gnemon	–	–	–
Angiosperms			
Cyanotis longifolia	–	–	–

Key

– undetectable

+ sugar present

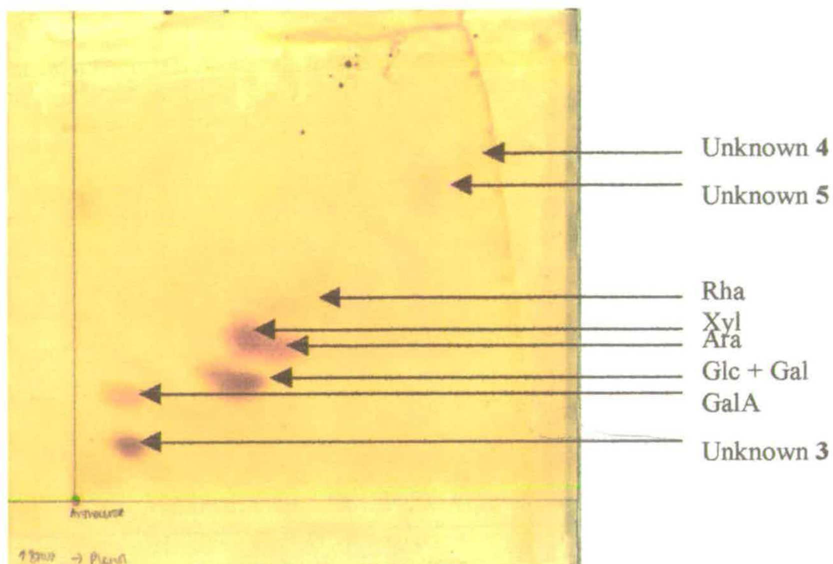
* sugar with similar R_F value but not confirmed to be identical with that from *Anthoceros caucasicus*.

Figure 3.4.5.a: 2-Dimensional PC(a) in B:A:W 12:3:5 (vertical on image) followed by 80% w/w phenol (horizontal) of the complete TFA hydrolysis products of (a) *Anthoceros caucasicus* (hornwort), (b) *Trichocolea tormentella* (leafy liverwort) and (c) *Sphagnum molle* (moss) showing the presence of unknown sugars.

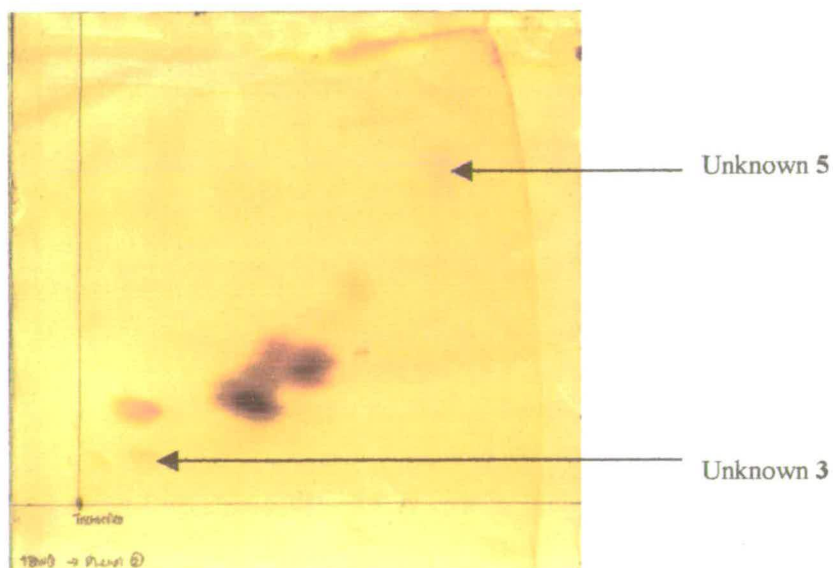
Stain — Aniline hydrogen-phthalate

Scale — 30%

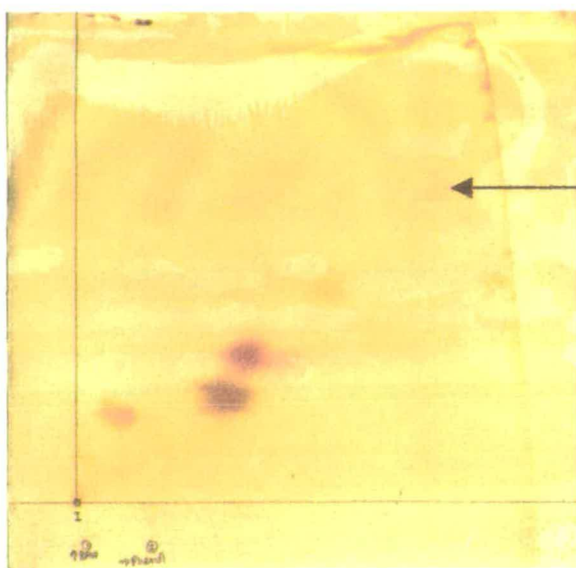
(a)



(b)



(c)



Unknown **5**

3.4.3 Observations on known plant cell wall monosaccharides

This method of developing complete TFA hydrolysates of AIRs allowed observation of common monosaccharides of cell wall polysaccharides in addition to the unknown sugars. In particular *Coleochaete scutata* AIR appeared to have very few Xyl residues present in comparison to Ara residues. However, *Chara* and *Klebsormidium* AIR both had more Xyl residues than Ara residues present. All the charophycean green algae had a relatively large amount of rhamnose and fucose present (Figure 3.4.1). Most of the common plant cell wall monosaccharides were present in the TFA hydrolysates of bryophyte AIR. These included Xyl=Glc>Ara=Man=Gal>GalA>Rha=Fuc.

3.4.4 Discussion

The possible presence of the acidic unknown **3**, in *Coleochaete scutata* and *Trichocolea tormentella*, in trace concentrations, and presence in *Anthoceros caucasicus*, in much larger concentrations, is interesting as *Coleochaete scutata* is considered by some researchers (Kranz et al. 1995, Graham 1982) to be more closely related to bryophytes than other members of the charophycean green algae and because hornworts and liverworts are considered by most researchers to be among the more basal members of the embryophyte lineage (Hedderson et al. 1996, Mishler and Churchill 1996, Bopp and Capesius 1996, Renzaglia et al. 2000).

To test whether 'compound **3**'-spots observed in *Anthoceros caucasicus, Coleochaete scutata* and *Trichocolea tormentella* are the same they could have been loaded together on a range of chromatograms to see if the three different spots had similar R_F values. Unknown **3** (from all three sources) could be purified and subjected to more severe TFA hydrolysis conditions; this would reveal whether it is a dimer and whether it is composed of the same sugar residues in all three organisms. Unknown **3** observed in *Anthoceros caucasicus* was shown to be a GlcA→Gal disaccharide. To find out the linkage between the two monosaccharides either N.M.R or methylation analysis could be employed. Potentially unknown **3** could be non-hornwort derived. The hornwort genera *Anthoceros* and *Phanoceros* are reported to have cavities in their thalli, some containing colonies of

cyanobacteria from the genus *Nostoc* (Watson 1984). Partial acid hydrolysis of *Nostoc commune* is reported to yield the oligosaccharides GlcA-(1→4)-6-*O*-methylGlc-(1→4)-Gal-(1→4)-Glc-(1→4)-Xyl and GlcA-(1→4/6)-Glc-(1→4)-Gal-(1→4)-Glc-(1→4)-Xyl (Brull et al. 2000). GlcA-(1→4)-6-*O*-methylGlc and GlcA-(1→4/6)-Glc are probably relatively resistant to severe acid hydrolysis. However, *Nostoc commune* is also reported to secrete a polysacccharide which contains *N*-acetylglucosamine, glucosamine and galactosamine (Hill et al. 1994). These sugar amines if present would have been separated from other sugars present in the TFA hydrolysate of *A. caucasicus* by PE pH 2.0. PE did not clearly show the presence of sugar amines. Additionally unknown **3** is a disaccharide composed of GlcA and Gal. This disaccharide has not been reported present in *Nostoc* polysaccharides. It is therefore likely that the sample did not contain high concentrations of *Nostoc*. Unknown **3** was present in high concentrations as judged by staining. It is therefore likely that unknown **3** was derived from *Anthoceros*. Unknown **4** and **5** are unlikely to be non-plant cell wall derived as they are present in liverworts and mosses as well as hornworts. Mosses and liverworts do not commonly contain colonies of cyanobacteria (Watson 1994).

The presence of unknown **5** (possibly 2-*O*-methylxylose) in substantial amounts in all charophycean green algae, bryophytes and homosporous lycophytes investigated suggests that unknown **5** may have been present in the common ancestor of all of these plants; a common ancestor of these plants would be placed within the charophycean green algae. Unknown 5 may be present in other organisms. If unknown **5** is 2-*O*-methylxylose it has not been reported except as a minor component of RG-II. RG-II is present as a minor pectic polysaccharide with what appears to be a highly conserved structure in at least 24 species of gramineous monocots, other monocots, dicots (Matoh et al. 1998) and a gymnosperm (Thomas et al. 1987). RG-II constitutes about 3% of the dry mass of the primary cell wall in dicots and less than 1% in gramineous monocots (Matoh et al. 1998). However, by the techniques used in the present work, unknown **5** was not detectable in the tracheophytes other than the homosporous (i.e., the more primitive) lycopodiophytes. Potentially RG-II may be present in the cell walls of lower land

plants at a greater concentration than in the tracheophytes (excluding the homosporous lycopodiophytes). Alternatively methylxylose may be present in other polymers than RG-II or lower-plant RG-II may have more methylxylose than has been reported in higher-plant RG-II.

Unknown **4**, possibly methylfucose was present in detectable quantities in the charophycean green algae and the hornwort. This suggests that these plants are more closely related to each other than to the rest of the plants investigated. This is interesting as the hornworts have been considered to be among the more basal members of the embryophyte lineage (Hedderson et al. 1996, Mishler and Churchill 1996, Bopp and Capesius 1996, Renzaglia et al. 2000). If unknown **4** is 2-*O*-methylfucose it has been reported to be a minor component of RG-II. These results suggest that RG-II may be present in the bryophytes in higher concentrations than in higher land plants. The identity of unknowns **4** and **5** could be tested by NMR of purified samples.

Xylose residues were present as a major component of the cell wall in all the land plants investigated. However, the TFA hydrolysate of *Coleochaete scutata* AIR contained very little xylose. Two major groups of xylose-containing hemicelluloses in the cell wall are xyloglucan and the xylans. The low proportion of xylose residues found in *Coleochaete scutata* AIR correlates with results which indicate that a xyloglucan similar to those found in angiosperms may not be present in *Coleochaete scutata* (Chapter 3.2). However, *Chara corallina* and *Klebsormidium flaccidum*, also charophycean green algae, contained a greater concentration of Xyl residues than Ara residues in their AIRs; in this respect *Chara* and *Klebsormidium* cell wall components are more similar to those of embryophytes than to those of *Coleochaete*.

3.5 Mannans

3.5.1 Introduction

In the cell wall mannose residues are present within mannans, glucomannans and galactoglucomannans with lower concentrations of mannose residues present in many wall enzymes such as peroxidases. The neutral fraction of complete TFA hydrolysates (2 M TFA, 1 h, 120°C) were run by PC in B:A:W 12:3:5, E:Py:W 8:2:1 and B:A:W 12:3:5 followed by E:Py:W 8:2:1. These PCs gave information about the distribution and relative quantities of common cell wall monosaccharide residues. In particular I noticed the concentration of mannose residues was higher in the bryophytes, lycopodiophytes, psilotophytes and equisetophytes than it was in the green algae, leptosporangiate ferns, gymnosperms and angiosperms (Figures 3.5.1, 3.5.2, Table 3.5.1).

3.5.2 Relative amount of mannose-containing polysaccharides in the primary cell wall

Mannose residues were found to be present in particularly high concentrations in the bryophytes, lycopodiophytes, psilotophytes and equisetophyes (Figures 3.5.1, 3.5.2, Table 3.5.1).

Table 3.5.1: Relative content of mannose released by TFA hydrolysis of AIRs from land plants and green algae.

AIR source	Mannose content (TFA hydrolysate)
<u>Chlorobiota (Green algae)</u>	
Ulvophyceae	
Ulva lactua L.	±
Charophyceae	
Chara corallina (Klein ex Willd. Em. R.D.W.)	±
<u>Embryobiota (land plants)</u>	
Bryophyta	
Bryopsida (Mosses)	
Sphagnum palustre L. (S. cymbifolium (Ehrh.) Hedw.)	++
Sphagnum molle Sull.	+
Andrea rupestris Hedw. (*A. Petrophila* Ehrh.)	+
Polytrichum formosum Raddi	+++
Dicranum scoparium Hedw.	++
Mnium hornum Hedw.	++
Philonotis fontana (Hedw.) Brid.	++
Rhizomnium punctata (Hedw.) Kop. (*Mnium punctata* Hedw.)	+++
Hookeria lucans (Hedw.) Sm. (*Pterygophyllum lucens* (Hedw. Brid.))	+++
Thiudium tamariscinum (Hedw.) B., S. and G.	++
Plagiothecium undulatum (Hedw.) B., S. and G.	++
Hypnum cupressiforme Hedw.	++
Hepaticae	
Anthocerotales (hornworts)	
Anthoceros caucasicus Spring	+

Marchantiales (thalloid liverworts)	
Lunularia cruciata (L.) Dum. ex. Lindb.	++
Metzgeriales	
Pellia epiphylla (L.) Corda	+
Jungermanniales (leafy liverworts)	
Trichocolea tomentella (Ehrh.) Dum.	+++
Lepidozia reptans (L.) Dum.	+++
Nardia scalaris (Schrad.) Gray (*Alicularia scalaris* (Schrad. Corda))	+
Marsupella emarginata var. aquatica (Lindb.)Dum.	++
Plagiochila asplenioides (L.) Dum. (*P. asplenioides var. major* Nees)	+++
Lophocolea bidentata (L.) Dum.	++
Scapania undulata (L.) Dum.	++
Pleurozia purpurea Lindb.	+++
Porella cordaeana (Hüb.) Moore (*Madotheca cordaeana* (Hüb.) Dum.	+++
Tracheophyta (Vascular plants)	
Lycopodiophytes	
Lycopodium pinifolium Blume	++
Huperzia selago (L.) Bernh. ex. Schrank and Mart.	++
Diphasiastrum alpinum (L.) Holub.	+
Selaginella apoda (L.) Spring	++
Selaginella pallescens (C.Presl.) Spring	++
Selaginella erythropus Spring	++
Equisetophytes	
Equisetum debile Roxb. ex Vaucher	+++
Psilotophytes	
Psilotum nudum (L.) P. Beauv.	+++

Filicophytes	
Eusporangiate ferns	
Marattia fraxinea Sm.	++
Leptosporangiate ferns	
Osmunda regalis L.	±
Todea barbara (L.) T. Moore	±
Dryopteris crispifolia Rasbach et al.	±
Asplenium australassium (J.Sm.) Hook.	±
Nephrolepis lauterbachii H. Christ	+
Onoclea sensiblis L.	±
Phyllitis scolopendrum L.	±
Blechnum spicant (L.) Roth.	±
Salvinia auriculatea Aubl.	±
Spermatophyta (Seed plants)	
Gymnosperms	
Cycads	
Encephalartos altensteinii Lehm.	++
Conifers	
Pinus sylvestris L.	++
Gnetophytes	
Gnetum indicum Merr.	±
Gnetum monotana Markgr.	±
Gnetum gnemon L.	±
Angiosperms	
Non-monocot Paleoherbs	
Nymphaea colorata Peter	+
Austrobaileya scandens C. T. White	++

'Magnoliid complex'	
Hernandia cordigera Vieill.	±
Illicium verum Hook. f.	±
Schizandra rubiflora Rehder and E. H. Wilson	±
Calycanthus floridus var. laevigatus L.	±
Drimys lanceolata (Poir.) Baill.	±
Monocots	
Calathea zebrina (Sims.) Lindb.	±
Callisia repens L.	±
Cyanotis logifolia var. longifolia Wight	±
Dichorisandra thyrsifolia J. C. Mikan	±
Geogenthus undatus (K. Koch and Linden) Mildbr. and Strauss	±
Palisota albertii L. Gentil	±
Siderasis fuscata (Lodd.) H. E. Moore	±
Juncus effusus L.	±
Cyperus esculentus L.	±
Cyperus papyrus L.	±
Elegia capensis (Burm. f.) Schelpe	±
Flagellaria guineensis Schum.	±
Tricolpates (Eudicots)	
Helleborus argutifolius Viv.	±
Fallopia japonica (Houtt.) Ronse Deraene	±

Key

+++ high

++ medium

\+ low

± trace

Figure 3.5.1: PC (B:A:W 12:3:5 16 h) of complete TFA hydrolysis products of various tracheophytes AIRs.

Stain = Aniline hydrogen-phthalate

Scale = 20%.

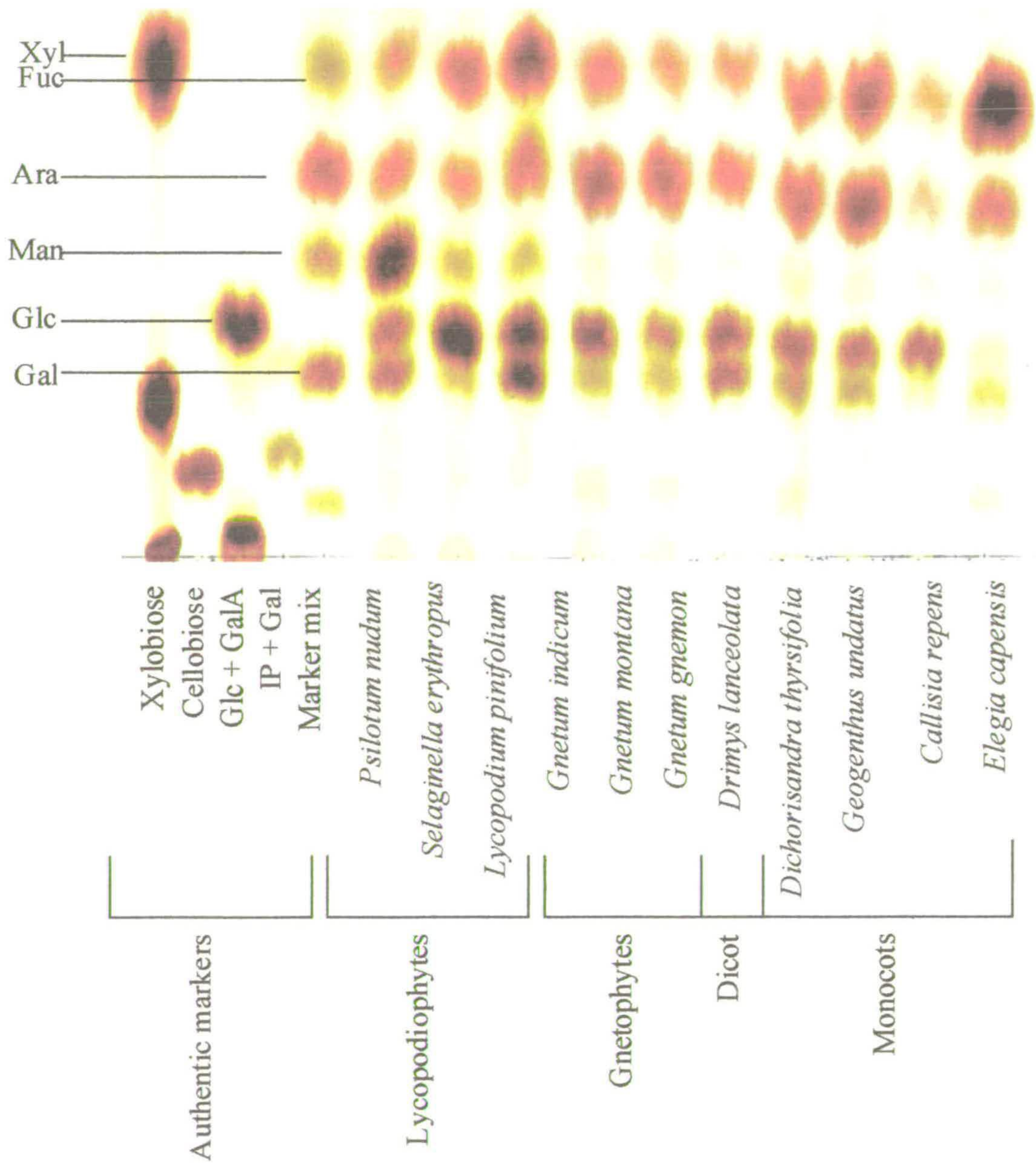
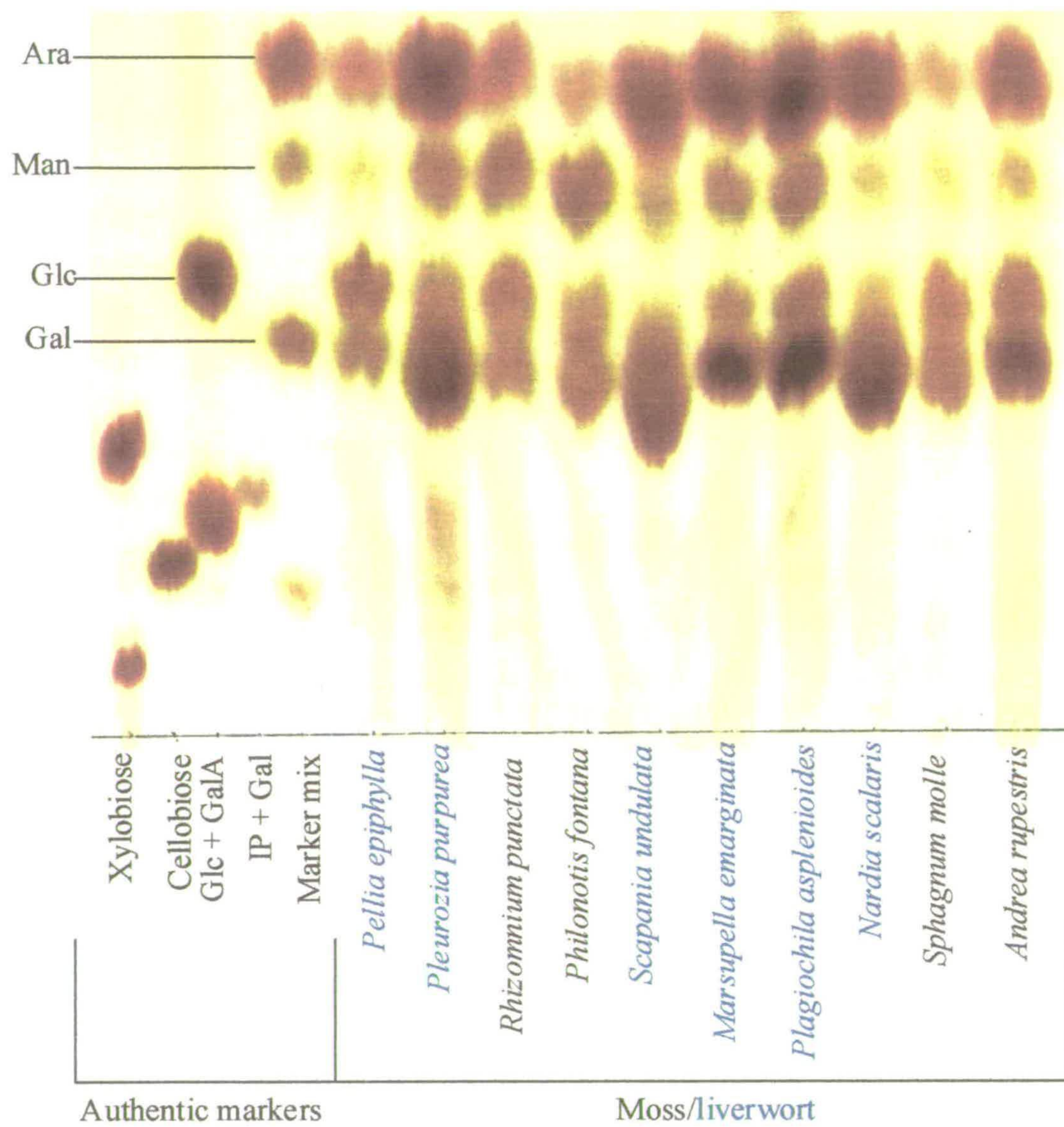


Figure 3.5.2: PC (B:A:W 12:3:5 16 h, E:Py:W 8:2:1 18 h) of complete TFA hydrolysis products of various bryophyte AIRs.

Stain = Aniline hydrogen-phthalate

Scale = 20%.



3.5.3 A mannose-containing disaccharide

The neutral fraction of a Driselase digest from *Psilotum nudum* gave a large spot which stained brown with aniline hydrogen-phthalate and had an R_{Glc} value similar to that of cellobiose in the solvent systems used (B:A:W 12:3:5 16 h, B:A:W 12:3:5 16 h followed by E:Py:W 8:2:1 18 h, E:Py:W 8:2:1 18 h) (Figure 3.5.3). Cellobiose stains brown with aniline hydrogen-phthalate, as do all reducing disaccharides of glucose; however, cellobiose is usually completely digested by Driselase to glucose. The *Psilotum* disaccharide was purified by preparative PC and run alongside glucose disaccharide markers kojibiose (α-1$\rightarrow$2), nigerose (α-1$\rightarrow$3), maltose (α-1$\rightarrow$4), isomaltose (α-1$\rightarrow$6), sophorose (β-1$\rightarrow$2), laminaribiose (β-1$\rightarrow$3), cellobiose (β-1$\rightarrow$4), gentiobiose (β-1$\rightarrow$6) and trehalose (α-1$\leftrightarrow$1-α). In all solvent systems used (PC in B:A:W 12:3:5 16 h, E:Py:W 8:2:1 18 h, E:Py:W 10:4:3 24 h, B:A:W 12:3:5 16 h followed by E:Py:W 8:2:1 18 h and borate-PE pH 9.4, 3 kV, 3 h) the disaccharide had R_{Glc} or m_{Glc} values similar to those of cellobiose and kojibiose (Table 3.5.2).

Figure 3.5.3: PC (B:A:W 12:3:5 16 h) of Driselase digestion products of *Psilotum nudum* and *Selaginella erythropus* AIR.

Stain = Aniline hydrogen-phthalate

Scale = 8%.

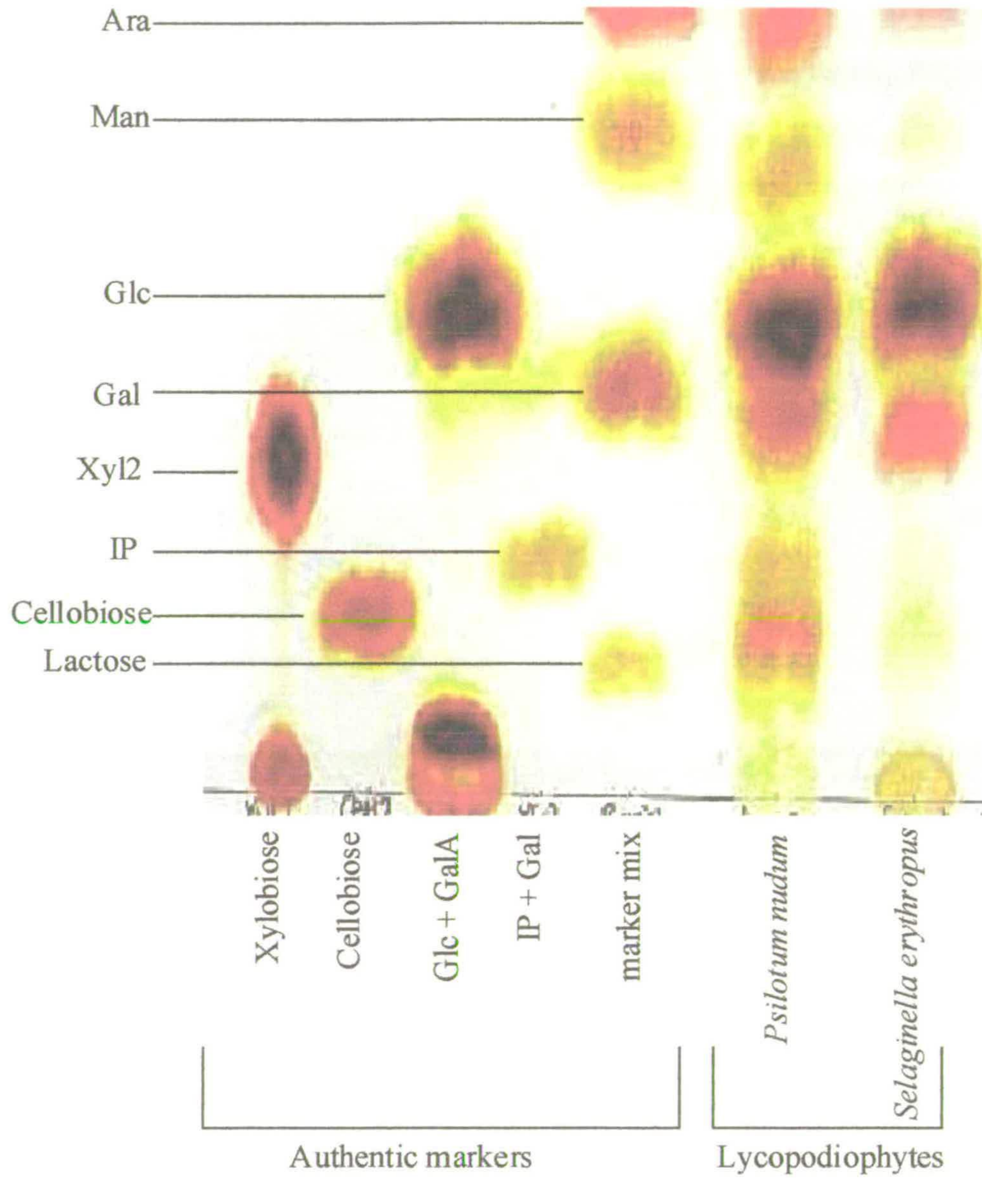


Table 3.5.2: Chromatographic and electrophoretic properties of *Psilotum nudum* and glucose disaccharides in various solvent systems.

Disaccharide	R_{Glc} value in solvent system				Distance moved from origin (cm) in electrophoretic system
	1	2	3	4	5
P. nudum disaccharide	0.55	0.34	0.52	0.38	10.5
Kojibiose	0.53	–	0.51	–	12.5
Nigerose	–	–	0.64	–	–
Maltose	–	–	0.57	–	–
Isomaltose	–	–	0.42	–	–
Sophorose	–	–	0.56	–	–
Laminaribiose	–	–	0.69	–	–
Cellobiose	0.49	0.34	0.53	0.39	11.0
Trehalose	–	–	0.45	–	–
Gentiobiose	–	–	0.40	–	–

Key

Solvent system 1 = PC B:A:W 12:3:5, 16h

Solvent system 2 = PC E:Py:W 8:2:1, 18 h

Solvent system 3 = PC E:Py:W 10:4:3, 24 h

Solvent system 4 = B:A:W 12:3:5, 16 h followed by E:Py:W 8:2:1, 18 h

Electrophoretic system 5 = borate-PE pH 9.4, 3 kV, 3 h. I did not use an immobile marker (such as 2,3,4,6-tetra-*O*-methylglucose) to account for movement of sugars away from the origin by electroendoosmosis.

– = unrecorded (these markers were not loaded as only kojibiose and cellobiose ran with an R_{Glc} similar to that of the *Psilotum* disaccharide in solvent system 3).

In an attempt to identify the reducing end of the *Psilotum* disaccharide I treated the purified disaccharide with sodium borohydride to reduce the reducing end to glucitol or mannitol. I then treated the disaccharide with TFA to release the monosaccharide components. The TLC of the hydrolysis products was stained with aniline hydrogen-phthalate showing that the hydrolysis products included both Glc and Man (Figure 3.5.4). The reduction was the repeated for 16 h instead of 4 h; the detectable hydrolysis products were still Glc and Man (glucitol and mannitol are not detectable with aniline hydrogen-phthalate stain).

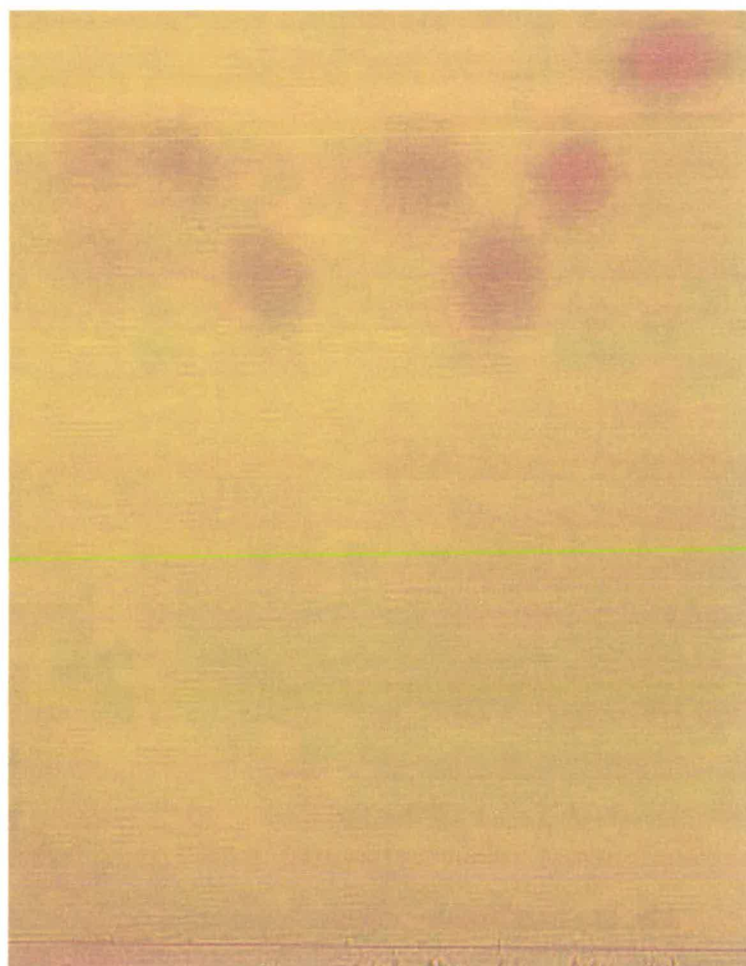
TFA hydrolysis of the disaccharide released mannose as well as glucose (Figure 3.5.4). Mannose and allose are known to run with similar R_{Glc} values in various solvent systems. I confirmed that it was mannose rather than allose released from the *Psilotum* disaccharide by TFA hydrolysis (Table 3.5.3).

Table 3.5.3: R_{GLC} value of mannose and allose on PC

Sugar	R_{Glc} value in solvent system	
	E:Py:W 8:2:1 48 h	E:Py:W 8:2:1 67 h
'mannose' from *P.nudum* disaccharide	1.32	1.36
Mannose	1.33	1.36
Allose	1.22	1.31

Figure 3.5.4: Cellulose TLC in B:A:W 3:1:1, E:Py:W 10:4:3, of the complete TFA hydrolysate of *Psilotum nudum* disaccharide ($NaBH_4$ treated 16 h).
Stain = Aniline hydrogen-phthalate

Scale = 105%



3.5.4 Discussion

Glucomannans have been found as a major hemicellulose component in the secondary cell walls of the gymnosperms (10% total cell wall w/w) and a minor hemicellulose component in the secondary cell walls of dicots (3–5% total cell wall w/w) (Matheson 1990). They have been reported to constitute roughly 15% of the total secondary cell walls of a fern, *Pteridium aquilinum* (Bremner and Wilkie 1970). My results suggest that the primary cell wall of euphyllophytes contains much less mannose than that of the secondary cell wall.

Edashige and Ishii (1996) reported that glucomannan was a major component (10.6% total cell w/w) present in suspension-cultured cells of the gymnosperm *Cryptomeria japonica*. The high percentage of glucomannan recorded present in the suspension-cultured cells of *Cryptomeria japonica* (Edashige and Ishii 1996) is consistent with my observation that the complete TFA hydrolysate of AIR from young gymnosperm (*Pinus sylvestris*) tissue had a greater total content of mannose residues than the complete TFA hydrolysates of leptosporangiate ferns and angiosperms. Among the gymnosperms I looked at the mannose residue content in the complete TFA hydrolysate of a cycad (*Encephalartos*), a conifer (*Pinus*) and a gnetophyte (*Gnetum*). The cycad and conifer had similar and fairly high concentrations of mannose residues in their AIRs. The gnetophyte had a concentration of mannose residues which was lower than found in the other gymnosperms and more similar to that found in the leptosporangiate ferns and angiosperms (Table 3.5.1). Although gnetophytes had previously been thought to resemble more closely angiosperms than the rest of the gymnosperms, molecular evidence provided by developmental genes suggests that the gnetophytes (gymnosperms) are more closely related to the conifers than to flowering plants (Winter et al. 1999). New studies on the supposed shared morphological characters between gnetophytes and angiosperms also suggest that these characters are of independent origin (Winter et al. 1999). The primary cell wall of vegetative tissues of leptosporangiate ferns, gymnosperms and angiosperms contained far less mannose than the bryophytes, lycopodiophytes, equisetophytes and psilotophytes. It is possible that the primary cell wall composition of the vegetative tissues of

bryophytes, lycopodiophytes, equisetophytes and psilotophytes is similar to that of the secondary cell wall of leptosporangiate ferns, gymnosperms and angiosperms.

I conclude that either the sodium borohydride was unable to reduce the reducing end of the disaccharide or two disaccharides were present which had similar R_F values, one disaccharide having Glc as the reducing end and the other having Man as the reducing end. The latter explanation seems to be more likely as the borohydride was not completely consumed during either the 4 h or the 16 h incubation (it fizzed rapidly on neutralisation of the solution). Driselase contains an endo-β-D-mannanase and an endo-β-D-glucanase. Enzymic hydrolysis of a glucomannan with an endo-β-D-mannanase is reported to yield Glc-Glc-Man, Glc-Man and mannobiose as major products (Goldberg et al. 1991). However, enzymic hydrolysis of a glucomannan with endo-β-D-glucanase is much less selective and gives mixtures of oligosaccharides that contain both mannose and glucose at the reducing end. The major components yielded by endo-β-D-glucanase digestion of a glucomannan, extracted from *Asparagus officinalis* seeds, are Glc-Man and Man-Glc. It is therefore likely that the disaccharide which I isolated from *Psilotum* was actually a mixture of Glc-Man and Man-Glc produced by endo-β-D-glucanase action on a glucomannan. The relatively high quantity of the mannose-containing disaccharides is reflected by the large amount of mannose residues released from *Psilotum* AIR on TFA hydrolysis. It is therefore likely that a glucomannan forms a quantitatively important part of the primary cell wall in *Psilotum nudum*.

3.6 Uronic acids

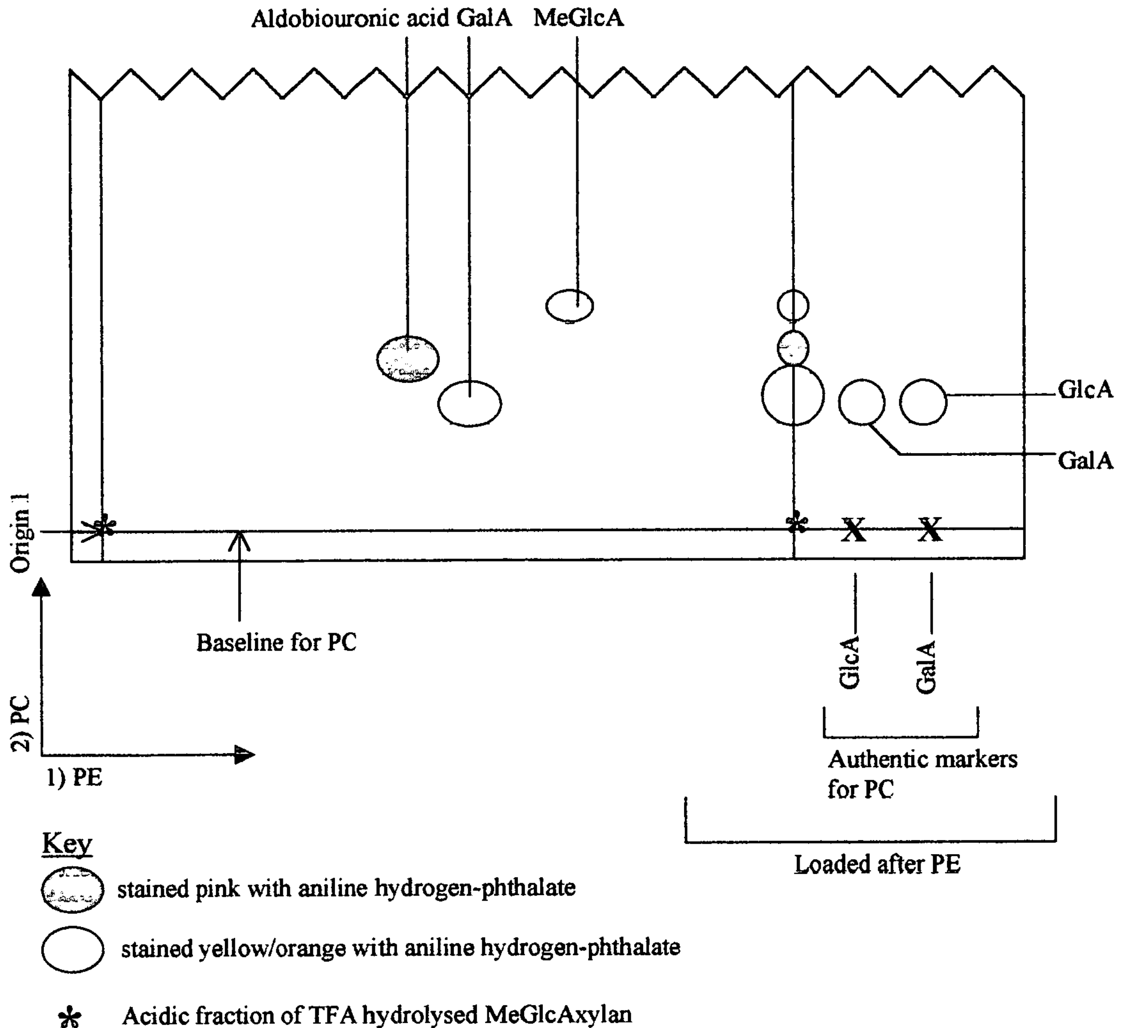
3.6.1 Introduction

Within the primary cell wall uronic acid residues are found as constituents of xylans, pectins and glucuronomannans. Typical gymnosperm and dicot xylans may be substituted with GlcA or MeGlcA on the O-2 of the Xyl residues (Shatalov et al. 1999). The backbone of RG-I is made up of repeating (1→2)-α-L-Rha-(1→4)-α-D-GalA disaccharide units (Lau et al. 1985). RG-II has both GalA and GlcA residues present. However, the cell wall polymer which contains the highest concentration of uronic acids is the pectic polysaccharide homogalacturonan. Homogalacturonan is made up of (1→4)-α-D-GalA residues. Mannuronic acid has been reported as the major uronic acid residue in the cell walls of the liverwort *Riccardia* (Das and Rao 1963, 1966a, b). Das and Rao (1963) reported the presence of mannuronic acid in *Riccardia* on the basis of R_F values on PC and staining with ammonical silver nitrate, ammonium vanadate and *p*-anisidine stains. They reported that on staining with ammoniacal silver nitrate mannuronic acid stained a characteristic yellowish-red; however, I have found that mannuronic acid, from a TFA hydrolysate of alginate, stains the usual dark brown with silver nitrate (Figure 3.6.2). Alginates are unbranched polymers composed of β-D-mannuronic acid and α-L-guluronic acid. Alginates are cell wall pectic polysaccharides commonly found in the Phaeophyceae (brown algae) (Panikkar and Brasch 1996, Miller 1996) and bacteria (Rehm et al. 1996) and have also been reported to occur in some members of the Rhodopyceae (red algae) (Usov et al. 1995). Lahaye et al. (1999), using N.M.R. spectroscopy, have identified iduronic acid residues attached to ulvan (a cell wall polysaccharide) in the green alga *Ulva lactuca*. Iduronic acid is most commonly found as a component of chondroitin B sulphate, which is a constituent of mammalian collagen.

3.6.2 Fractionation of uronic acids

Authentic MeGlcA and aldobiouronic acid were purified from the complete TFA hydrolysis products of a commercial preparation of 4-*O*-methylglucuronoxylan (beech wood) (2.8.1). The TFA hydrolysate was separated into neutral and acidic fractions by anion exchange-chromatography and the acidic fraction was run by PE pH 3.5, 2.75 kV, 80 min in one direction followed at 90° by PC B:A:W 12:3:5, 5 h. Before PC markers of GalA and GlcA and the acidic fraction of the TFA hydrolysate were added to the PC origin as external markers. The PE/PC was then stained with aniline hydrogen-phthalate. The aldobiouronic acid stained pink. TFA hydrolysis of the aldobiouronic acid confirmed that it was made up of MeGlcA and Xyl; it is likely that the Xyl was at the reducing terminus. It was found that the acidic fraction of the TFA hydrolysis products of 4-*O*-methylglucuronoxylan contained MeGlcA, aldobiouronic acid and GalA (Figure 3.6.1).

Figure 3.6.1: Two-dimensional separation (PE/PC) of the complete TFA hydrolysis products of beechwood 4-*O*-methylglucuronoxylan. PE was at pH 3.5, 2.75 kV, 80 min followed by PC B:A:W 12:3:5, 5 h at 90° to the first system. The sugars were stained with aniline hydrogen-phthalate.



The complete TFA hydrolysis products of AIRs were run by PE pH 3.5, which gave good separation of GlcA, GalA, ManA, GulA, IdA and an aldobiouronic acid (Table 3.6.1). On PE pH 3.5 GlcA and MeGlcA had closely similar though not identical mobilities (Table 3.6.1).

Table 3.6.1: m_{GlcA} values for a variety of uronic acids and an aldobiouronic acid on PE pH 3.5, 3 kV, 1.5 h

Uronic acid	m_{GlcA}	Colour stained with aniline hydrogen-phthalate
4-*O*-MeGlcA	1.05	orange
GlcA	1.00	orange
ManA	0.92	orange
IdA	0.87	orange
GalA	0.82	orange
GulA	0.78	orange
aldobiouronic acid	0.76	pink

3.6.3 Occurrence of uronic acids

PE pH 3.5 indicated that the major uronic acid present in TFA hydrolysates of all land plants and charophycean green algae was GalA (Table 3.6.2 and Figure 3.6.2). The presence of GalA in the TFA hydrolysates was supported not only by m_{GlcA} values but also by co-electrophoresis of an internal marker of authentic [^{14}C]GalA. The charophycean green algae and some of the more primitive bryophytes contained more GlcA than the rest of the land plants (Table 3.6.2 and Figure 3.6.2). To verify that it was GlcA and not MeGlcA, as they have closely similar though not identical mobilities on PE at pH 3.5, co-electrophoresis with an internal marker of [^{14}C]GlcA was checked. This showed that an internal marker of [^{14}C]GlcA precisely co-electrophoresed with some of the uronic acid residues in the TFA hydrolysates (data not shown). Co-electrophoresis of an internal marker of [^{14}C]GalA and GalA present in my TFA hydrolysates was carried out in the same way (Figure 3.6.3). Precise co-electrophoresis of [^{14}C]GalA with the majority of the uronic acid residues in my TFA hydrolysates confirmed that the majority of uronic acid residues in my AIRs were GalA. It was found that the primitive bryophytes contained GlcA in much higher concentrations than the majority of land plants and MeGlcA was not present in detectable concentrations.

Table 3.6.2: GlcA and GalA content of TFA hydrolysates

AIR source	Amount of GalA and GlcA relative to each other	
	GalA	GlcA
Green algae		
Chlorophytes		
Ulva lactuca	++	++
Charophytes		
Chara corallina	++	+
Land plants		
Bryophytes		
Bryopsids (mosses)		
Sphagnum palustre	++	++
Mnium hornum	++	–
Thuidium tamariscarum	++	–
Plagiothecium undulatum	++	–
Dicranum scoparium	++	–
Hypnum cupressiforme	+	–
Hornworts		
Anthoceros caucasicus	+++	++
Thalloid liverworts		
Lunularia cruciata	++	+
Leafy liverworts		
Lophocolea bidentata	++	++
Tracheophyta (vascular plants)		
Lycopodiophytes		
Selaginella erythropus	++	–
Selaginella pallescens	++	–
Psilotophytes		
Psilotum nudum	++	–

Filicophytes (Ferns)		
Eusporangiate ferns		
Marattia fraxinea	++	–
Leptosporangiate ferns		
Osmunda regalis	++	–
Phyllitis scolopendrium	++	–
Nephrolepis lauterbachii	++	–
Onoclea sensiblis	++	–
Todea barbara	++	–
Dryopteris crispifolia	++	–
Asplenium australassium	++	–
Salvinia auriculata	++	–
Seed Plants		
Gymnosperms		
Cycads		
Encephalartos altensteinii	++	–
Conifers		
Pinus sylvestris	+	–
Gnetophytes		
Gnetum gnemon	++	–
Gnetum montana	++	–
Gnetum indicum	++	–
Angiosperms		
Non-monocot paleoherbs		
Nymphaea colorata	++	+
Austrobaileya scandens	++	+

Magnoliid complex		
Illicium verum	++	–
Schizandra rubiflora	++	+
Calycanthus floridus	++	+
Drimys lanceolata	++	–
Hernandia cordigera	+	–
Monocots		
Juncus effusus	++	+
Cyanotis longifolia	++	+
Cyperus esculentus	++	+
Cyperus papyrus	+	+
Palisota albertii	++	+
Dichorisandra thyrsifolia	+	±
Calathea zebrina	+	±
Callisia repens	++	±
Elegia capensis	++	±
Flagellaria guineensis	++	±
Siderasis fuscata	++	±
Tricolpates (Eudicots)		
Helleborus argutifolius	++	–
Fallopia japonica	++	–

Key

+++ large amount detectable

++ medium amount detectable

+ small amount detectable

± trace amount detectable

– not detectable by methods used.

Figure 3.6.2: PE pH 3.5, 2.75 kV, 1.5 h of the acidic fraction of TFA hydrolysates of bryophytes.

Stain = Silver nitrate

Scale = 30%

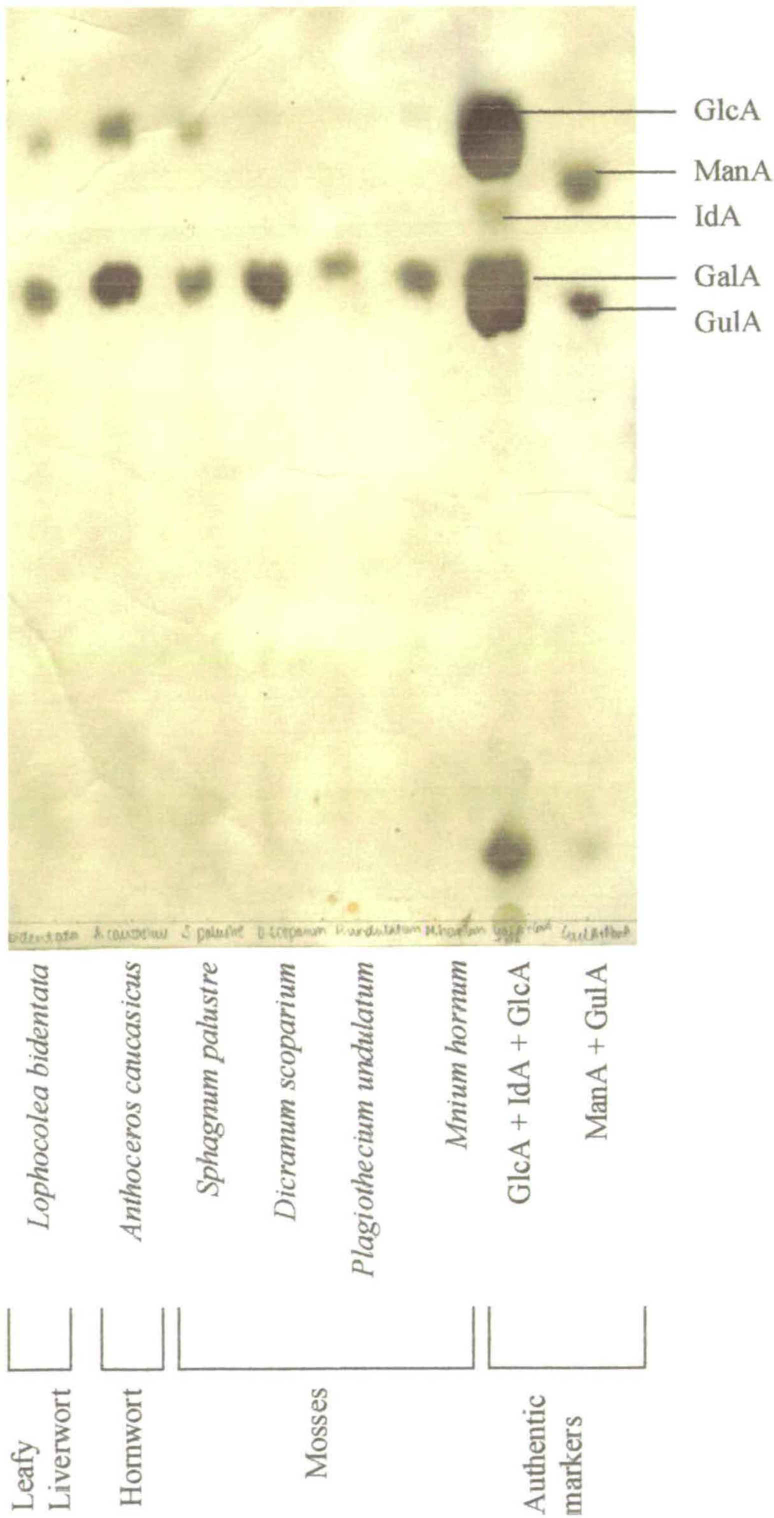


Figure 3.6.3: PE pH 3.5, 2.75 kV, 1.5 h of the acidic fraction of TFA hydrolysates of bryophytes. An internal marker of [^{14}C]GalA was added to each hydrolysate before loading on to the PE.

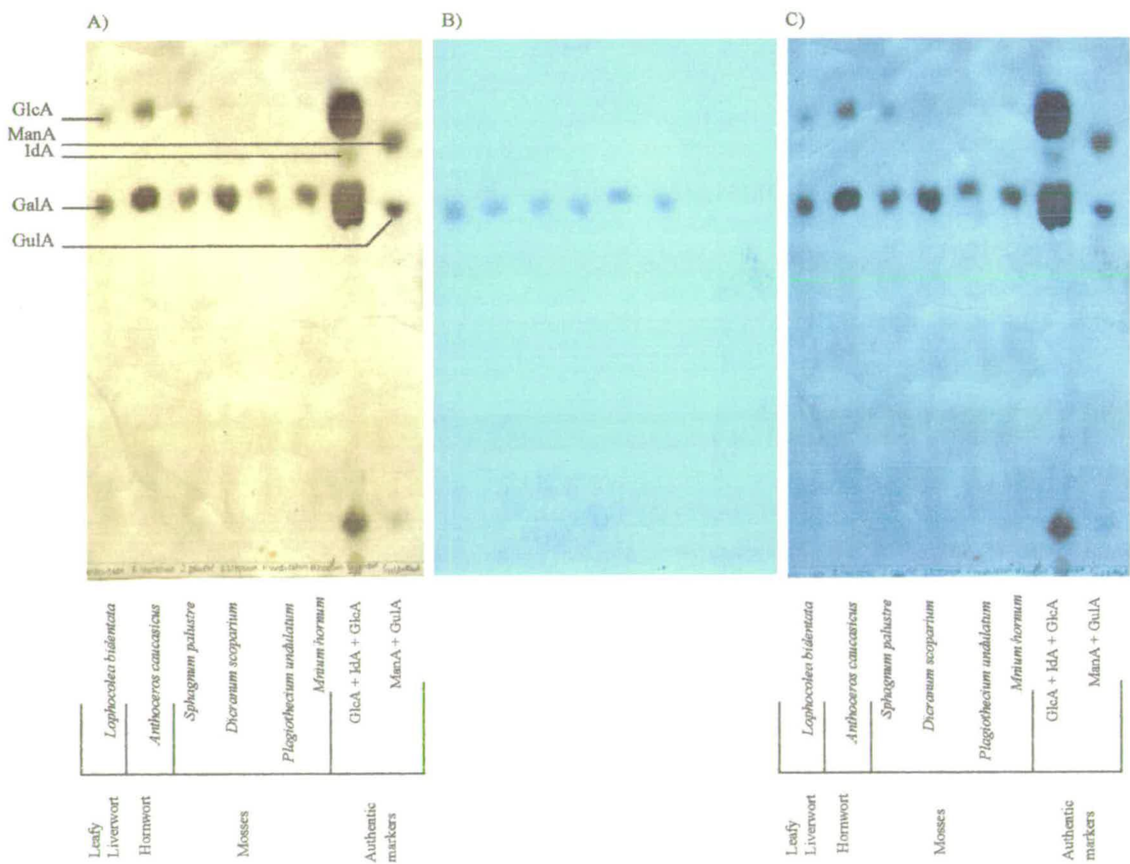
Scale = 20%

Key

A) PE stained with silver nitrate.

B) Autoradiogram of PE.

C) Autoradiogram laid on PE showing precise co-electrophoresis of [^{14}C]GalA and the uronic acid with the lowest R_F value in the TFA hydrolysates.



3.6.4 Discussion

My results suggest that among the land plants there are more GlcA residues present in those bryophyte taxa that are generally thought of as more primitive: the liverworts, hornworts and *Sphagum* (Bopp and Capesius 1996; Hedderson et al. 1996). The green algae, *Ulva* and *Chara*, were also found to have high concentrations of GlcA residues present in their cell walls. Vascular land plants, and the more advanced bryophytes, appeared to have a much lower concentration of GlcA residues in their cell walls. It is possible the reduction in GlcA concentration between the more primitive (liverworts, hornworts and *Sphagnum*) and the more advanced bryophytes may correlate with a change in habitat; the more advanced bryophytes tend to live in drier habitats. Iraki et al. (1989abc) reported that the cell walls of tobacco cells grown under drought-mimicking conditions had decreased concentrations of pectic polysaccharides with altered constituents. Among the angiosperms the monocots and non-monocot paleoherbs appear to have higher concentrations of GlcA present in their cell walls than the rest of the angiosperms. It is thought that the non-monocot paleoherbs and monocots are more closely related to each other than they are to the rest of the angiosperms (Judd et al. 1999).

The presence of GalA in high or medium concentrations appears to be ubiquitous in the cell walls of land plants. The uronic acid composition of the majority of land plants appears to be highly conserved. Those bryophytes and algae that have relatively high concentrations of GlcA in their cell walls still have high concentrations of GalA residues. It is likely that GalA is important to land plant primary cell wall function. However, variation in overall uronic acid residue concentration is reported to vary within the monocots (Jarvis et al. 1988). Jarvis et al. (1988) reported that the concentration of uronic acid residues is lower in the Commelinanae than the rest of the monocots. It would therefore appear GalA and therefore pectic polysaccharides are less important to the primary cell wall function of the Commelinanae. Reduction in the concentration of GalA residues could in fact be an advantage for the Commelinanae as it may be one of the factors which makes it difficult for *Agrobacterium tumefaciens* to attach to monocot cell surfaces (Rao et al. 1982, Jarvis et al. 1988).

I did not detect MeGlcA in the cell walls of any of the plants I investigated. The MeGlcA which I prepared from beechwood 4-*O*-methylglucuronoxylan had a slightly higher *m* value than GlcA on PE pH 3.5 (Table 3.6.1); it is likely that MeGlcA was present below the level of detection by the methods I used. 4-*O*-Methylglucuronoxylan is known from xylem (secondary cell walls) and may be present at much lower concentrations in primary cell walls. Darvill et al. (1980) reported that in suspension-cultured sycamore cells MeGlcA constitutes 4.9% of the monosaccharide residues present in GAX and that GAX constitutes 5% total cell wall dry weight. Therefore, the primary cell walls of dicots contain roughly 0.25% MeGlcA. This correlates with the results of Harris et al. (1997), who reported that the unlignified cell walls of dicots and some monocots (including the Liliales and other monocots whose cell walls have relatively low GAX concentrations) contain 0.3–0.8% MeGlcA w/w of the cell wall. Similar to the Poaceae, the remaining Poales and the Commelinanae have higher concentrations of GAX present in their primary cell walls. However, Harris et al. (1997) reported the concentration of MeGlcA in this group to be 0.2–0.8% w/w of the cell wall. This would mean the MeGlcA concentration of gramineous monocot GAX would have to be lower than the MeGlcA concentration of dicot GAX. I did not investigate gramineous monocot cell walls for the presence of MeGlcA. However, I did look at other members of the Poales (*Flagellaria* and *Elegia*) that are reported to have comparable GAX concentrations (Harris et al. 1997). Therefore, if MeGlcA was present in the AIRs I investigated, it is likely that it was at concentrations below the level of detection by the methods I used.

Lahaye et al. (1999) reported the presence of iduronic acid residues in ulvan, a cell wall polysaccharide extracted from the marine green alga *Ulva lactuca*. I did not detect iduronic acid residues in the cell walls of *Ulva* but Lahaye et al. (1999) detected iduronic acid in only trace concentrations.

ManA has been reported as the major uronic acid in the cell walls of the leafy liverwort *Riccardia* (Das and Rao 1963, 1966a). However, I did not detect ManA in the TFA hydrolysates from liverworts; I detected both GlcA and GalA residues in

the cell walls of thalloid and leafy liverworts. Das and Rao (1963, 1966a, b) do not report the presence of either GlcA or GalA in the cell walls of *Riccardia*, but reported that GlcA had a similar R_F value to the putative ManA found in their sample and to an authentic sample of ManA in the solvent systems used (in E:Py:A:W 5:5:1:3 the R_F value of GlcA was 17.0, that of authentic ManA was 18.5 and that of the uronic acid in their sample was 18.2). Das and Rao (1966b) also report that in the solvent system they used to separate GlcA and ManA the authentic sample of ManA 'gave rise to a characteristically elongated spot' whereas GlcA 'exhibited a discrete heart-shaped spot'. However, Das and Rao (1966b) report that their ManA co-chromatographed with authentic ManA. It is possible that ManA is present in the cell walls of liverworts but is below the limit of detection of the methods I used. It is also possible that the presence of ManA is not a common feature of liverworts but is restricted to the leafy liverwort *Riccardia* and the thalloid liverwort *Riccia* investigated by Das and Rao (1966b). However, it is surprising Das and Rao (1966a, b) reported the presence of ManA but did not detect the presence of GalA, which I found to be present at high concentration in all land plants. It is therefore possible that ManA is not present in liverwort cell walls and Das and Rao (1966a,b) mistook GalA or GlcA for ManA.

It appears that the major uronic acid residue present in all plant cell walls is GalA. In addition GlcA residues are present in high concentrations in the charophycean green algae and those members of the bryophytes widely considered to be the most primitive. The concentration of uronic acids, and therefore potentially pectic polysaccharides, appears to be higher in the bryophytes than the rest of the land plants. Reduction in pectic polysaccharide may be an adaptation to a drier habitat. Among the angiosperms the Commelinanae have a greatly reduced uronic acid concentration. It has been suggested that this may reduce the incidence of infection by *Agrobacterium tumefaciens* (Rao et al. 1982, Jarvis et al. 1988). Therefore reduction in uronic acid residue content may be an adaptive advantage.

3.7. Tannins

3.7.1 Introduction

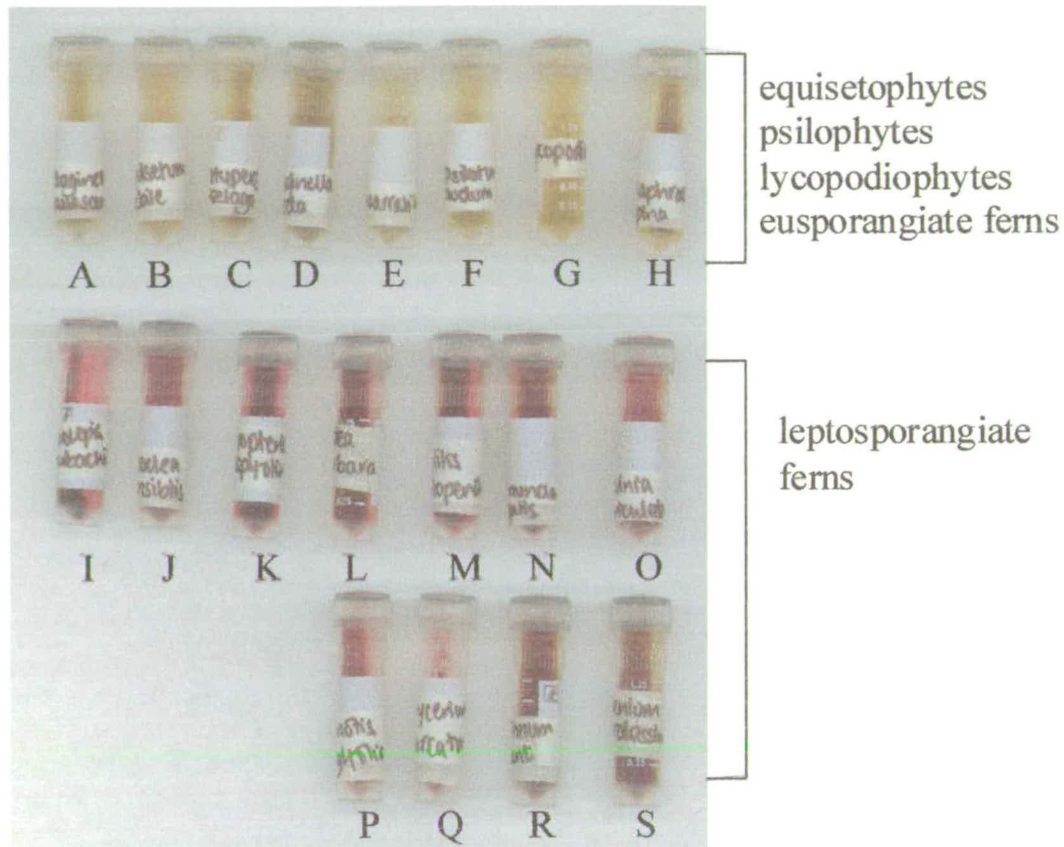
Tannins are secondary metabolites. It was thought by some workers that they are waste products (Muller 1969). However, many secondary metabolites are thought to have or have had important biological functions (Fraenkel 1959). Tannins are thought to be highly effective at repelling microbes and predators owing to their astringency, which makes them unpalatable to animals by precipitation of salivary proteins and impedes invasion of plant tissue by parasitic organisms by imobilising extracellular enzymes (Bate-Smith 1954). It has also been suggested that tannins may exert a growth regulatory function by inhibiting growth caused by gibberellins (Green and Corcoran 1975). Condensed tannins or proanthocyanidins are nonhydrolyzable and are composed of a polymer of flavan-3-ol in which the interflavan bonds are most commonly C-4 to C-8 but in which some C-4 to C-6 bonds may be present (Haslam 1977). Proanthocyanidins are products of the flavonoid pathway of biosynthesis (polyketide and cinnamate) (Haslam 1974). Proanthocyanidins and the hydrolysable gallo- and ellagitannins are degraded by acid in the presence of oxygen to anthocyanidins which can be detected by chromatography, colour or fluorescence under UV light (gallic acid, violet; ellagic acid, 'soft' violet) (Haslam 1981). Bate-Smith (1962) studied the distribution of phenolics in land plant, making use of the acid lability of the proanthocyanidins. Bate-Smith (1962) established that tannins are not regularly found in great quantities in fungi, algae, mosses, liverworts and grasses but are of significance in many dicotyledons. Bate-Smith (1977) concluded that proanthocyanidins are present in relatively primitive vascular plants such as ferns and gymnosperms and could be found in both immature and senescent leaves (Bate-Smith 1954). Other studies have shown (Thompson et al. 1972, Haslam 1977) that several plant families are able to synthesise different types of tannin. Bate-Smith (1977) stated that proanthocyanidins first appear, from the phylogenetic point of view, as plants develop a vascular character although he discounted a connection of proanthocyanidin production and lignification. However, although proanthocyanidins were generally found in ferns, Bate-Smith and Learner (1954) did not find them in the Selaginellaceae, Psilotaceae or Lycopodiaceae.

Synthesis of complex polyphenols has been shown to occur in the rough and smooth endoplasmic reticulum of *Pinus elliotti* tissue cultures (Baur and Walkinshaw 1974). Electron microscopy showed substantial tannin deposits within the vacuole and surrounding the plasma membrane (Baur and Walkinshaw 1974). It is not known whether tannins are wall components in living cells and it is possible they bind there after preparation of AIRs. However, proanthocyanidins have been found covalently-linked to polysaccharides in the conifer *Larix gmelini* (Shen et al. 1986); this probably includes those of the cell wall (Haslam 1981).

3.7.2 Observation of proanthocyanidins in AIRs

Whilst carrying out TFA hydrolyses to study monosaccharide composition of my AIRs I noticed many of my samples became red on heating whereas other samples remained yellow. The distribution of samples becoming red on TFA hydrolysis was interesting as it appeared to be confined to the ferns, gymnosperms, and angiosperms. I therefore looked more closely at the distribution of colouring on TFA hydrolysis and found that the equisetophytes, psilotophytes, lycopodiophytes and the eusporangiate fern, *Marattia fraxinea*, did not turn red on TFA hydrolysis; all leptosporangiate ferns studied became red on acid hydrolysis (Figure 3.7.1). Boyle (1840, reported in Haslam, 1977) reported that 'very many plant tissues gave rise to a deep red colour — later shown to be that of the pigment cyanidin — when treated with acid'.

Figure 3.7.1: Distribution of red coloration of AIRs (among ferns and fern allies) on TFA hydrolysis



Key

A *Selaginella pallescens* (C.Presl.) Spring. (Selaginellaceae)
B *Equisetum debile* Roxb. Ex. Vaucher (Equisetaceae)
C *Huperzia selago* (L.) Bernh. Ex. Schrank & Mart. (Lycopodiaceae)
D *Selaginella apoda* (L.) Spring. (Selaginellaceae)
E *Marattia fraxinea* Sm. (Marattiaceae — eusporangiate fern)
F *Psilotum nudum* (L.) P. Beauv. (Psilotaceae)
G *Lycopodium pinifolium* Blume (Lycopodiaceae)
H *Diphasiastrum alpinum* (L.) Holub. (Lycopodiaceae)
I *Nephrolepis lauterbachii* H. Christ. (Asplenioideae — leptosporangiate fern)
J *Onoclea sensiblis* L. (Asplenioideae — leptosporangiate fern)
K *Dryopteris crispifolia* Rasbach et al. (Dryopteriodeae — leptosporangiate fern)
L *Todea barbara* (L.) T. Moore (Osmundaceae — leptosporangiate fern)
M *Phyllitis scolopendrum* L. (Asplenioideae — leptosporangiate fern)
N *Osmunda regalis* L. (Osmundaceae — leptosporangiate fern)
O *Salvinia auriculatea* Aubl. (Salviniales — leptosporangiate fern)
P *Cyanotis longifolia* Wight (Commelinaceae — monocot)
Q *Platycerium bifurcatum* (Car.) C. Chr. (Polypodiaceae — leptosporangiate fern)
R *Blechnum spicant* (L.) Roth (Blechnoideae — leptosporangiate fern)
S *Asplenium australassium* (J. Sm.) Hook. (Asplenioideae — leptosporangiate fern)

3.7.3 Identification of proanthocyanidins in AIRs

In order to confirm whether the red coloration of AIRs observed on TFA hydrolysis was due to proanthocyanidins I carried out an assay for the presence of anthocyanidins (2.11, Fry 1988). I obtained a positive result for the presence of anothocyanidins in the leptosporangiate ferns. However, a positive result for the presence of anthocyanidins in the Selaginellaceae, Lycopodiaceae, Equisetaceae, Psilotaceae and the eusporangiate fern, *Marattia fraxinea*, was not obtained.

3.7.4 Discussion

My results indicate that proanthocyanidins are present in leptosporangiate ferns, gymnosperms and angiosperms and absent from the lycopodiophytes, psilotophytes, equisetophytes and the eusporangiate fern *Marattia fraxinea*. These results are similar to those obtained by Bate-Smith and Learner (1954) with the exception that they reported the presence of a 'moderate[ly]' strong positive reaction for the presence of anthocyanidin in *Marattia fraxinea*. However, Bate-Smith and Learner (1954) found all other ferns, gymnosperms and angiosperms to produce a 'exceedingly' or 'very' strong positive reaction for the presence of anthocyanidin. The psilotophytes and equisetophytes, like *Marattia fraxinea*, exibit the eusporangiate condition. The vascular plants (tracheophytes) form a well-supported monophyletic group and this group includes the lycopodiophytes, psilotophytes, equisetophytes and leptosporangiate ferns as well as eusporangiate ferns, gymnosperms and angiosperms (Judd et al. 1999). It is therefore possible that production of proanthocyanidins may have evolved at the same time as the leptosporangiate condition rather than the vascular condition as proposed by Bate-Smith (1977).

4 Discussion

My results indicate that major changes in leaf cell wall composition tend to accompany major evolutionary changes for example the transition from an aquatic to a terrestrial environment. It therefore appears that the chemical diversity of plant cell walls is much greater than that of angiosperm cell walls. More research needs to be done to understand more fully the cell walls of lower land plants.

A major event in plant evolution was the transition from an aquatic to a non-aquatic environment. Plants survive, grow and reproduce on land because they are able to resist desiccation. Within the aquatic environment carbon dioxide, oxygen and light are more available near the water surface, so early aquatic plants would have thriven in shallower water which potentially dried out seasonally. The first land plants are likely to have resembled the charophycean algal colonies which still exist today. I found that all land plants tested, including the bryophytes, have xyloglucan in their primary cell walls. Xyloglucans contain D-glucose, D-xylose and D-galactose, often in a molar ratio of approximately 4:3:1. However, the TFA hydrolysate of *Coleochaete scutata* AIR yielded a very low concentration of xylose residues showing that xyloglucan could not have been a major component. Nevertheless, TFA hydrolysates of *Chara* and *Klebsormidium* AIR had a concentration of xylose residues equal to that found in land plants. In this respect *Chara* and *Klebsormidium* cell wall components are more similar to those of embryophytes than to those of *Coleochaete*. The presence of xyloglucan can be indicated by the presence of isoprimeverose after Driselase digestion. The results of Driselase digestion indicate xyloglucan may not be present in the charophycean green algae. Driselase digestion of *Chara* and *Coleochaete* AIR did not yield isoprimeverose. This indicates that a Driselase-digestible xyloglucan is not to be present. In addition, digestion with xyloglucan-specific endoglucanase (XEG) did not yield xyloglucan-derived oligosaccharides. It therefore seems likely that xyloglucan is not present in any of the charophycean green algae tested.

This conclusion conflicts with previous reports which suggest that xyloglucan is present in the charophycean cell wall. Anderson and King (1961b) reported that *Chara* had a similar cell wall composition to the land plants. However, this comparison was based on the relative concentrations of the main classes of cell wall components (hemicellulose, pectic polysaccharides and cellulose) present in *Chara* and a variety of land plants. All the land plants included in the comparison were gramineous monocots, which are now known to have a lower xyloglucan concentration than non-gramineous monocots, dicots and gymnosperms. In addition, all the land plants contained a hemicellulose concentration which was three-fold greater than that reported for *Chara* (Anderson and King 1961b). The results of Anderson and King (1961b) indicate that *Chara* cell walls contain a hemicellulose fraction. This hemicellulose fraction was not characterised and the presence of hemicellulose in *Chara* cell walls does not prove the presence of xyloglucan. Morrison et al. (1993) reported that the 1 M KOH extract of *Nitella* contained xyloglucan. The extracted polysaccharide was composed of 10% Fuc, 21.7% Xyl, 10.4% Gal and 45.8% Glc residues w/w (Morrison et al. 1993). Dicot xyloglucan has a similar monosaccharide composition. However, methylation analysis revealed that 4-linked Glc, 4,6-linked Glc, 2- and/or 4- linked Xyl, 2-linked Gal and terminal Fuc residues were present in the *Nitella* polysaccharide. If xyloglucan with a similar structure to that from angiosoperm cell walls had been present, terminal Xyl and possibly terminal Gal residues would also have been present. The results of Morrison et al. (1993) do not conclusively prove the presence of xyloglucan in *Nitella* cell walls; many of the sugar linkages indicated to be present could result from the presence of other hemicelluloses such as xylans and possible contamination with starch.

All land plants ultimately descend from a common algal ancestor from which the modern Charophyceae are also descended (Pickett-Heaps 1976, Mattox and Stewart 1984, Graham 1993). It is possible that the early charophycean green algae had xyloglucan in their cell walls but it was subsequently lost from its algal descendants. A polymer has been recorded from *Ulva lactuca* which has some structural similarities to xyloglucan (Lahaye et al. 1994). Enzymic hydrolysis (with the

commercial β-D-glucanase preparation Finizyme) of this polysaccharide releases the trisaccharides β-D-xylosyl-(1→4)-β-D-glucosyl-(1→4)-D-glucose and xylotriose (Lahaye et al. 1994). In xyloglucan extracted from higher plants, xylose is present as its α-anomer and is 1→6-linked to the glucan backbone. Therefore the polymer described by Lahaye et al. (1994) is not a xyloglucan as the term is generally used in angiosperms and gymnosperms. It is possible that the presence of xyloglucan was a pre-adaptive advantage to colonisation of a non-aquatic environment.

To test further for the presence of xyloglucan in the charophycean green algae it could be interesting to look at the cell walls of additional charophycean green algae such as *Spirogyra*. It is possible that xyloglucan was present in the cell walls of charophycean green algae at a very low concentration. If charophycean green algae metabolise Ara in the same way as land plants [^{3}H]Ara could be fed to algal cultures. This may radiolabel pentose- containing polysaccharides in algal cell walls. AIRs could be digested with Driselase, cellulase and XEG to look for the presence of isoprimeverose and xyloglucan-derived oligosaccharides. However, xyloglucan (as defined by the release of isoprimeverose on Driselase-digestion) was not present in charophycean algal cell walls to a concentration of 0.01% w/w xyloglucan/AIR. It would also be of interest to identify whether XET is present in algal cell walls. Some XETs have endoglucanase as well as transglycosylase activity (Fry 1995). The transglycosylase activity may not have been the original role of the enzyme. Algal genomes could be probed for a gene sequence that has a high homology with the gene sequence for XET. Potentially xyloglucan could be present in charophycean cell walls but be substituted with sugar residues which are indigestible by cellulase, XEG and the enzymes present in Driselase. Anti-xyloglucan antibodies could be developed to help identify the presence of xyloglucan. However, the anti-xyloglucan antibodies would have to be reactive only with isoprimeverose α-D-Xyl-(1→6)β-D-Glc. Anti-xyloglucan antibodies have been reported which recognise the terminal fucosylated residue of the trisaccharide side-chain of xyloglucan (Zhang and Staehelin 1992) and another has been developed which is reactive with MLG, RG-I and xyloglucan (Lynch and Staehelin 1992). Additionally anti-xyloglucan antibodies have been developed which

specifically recognise isoprimeverose (Sone et al. 1989). Anti-xyloglucan antibodies therefore exist at present which are specific enough to identify the presence of xyloglucan in the charophytes.

The first land plants are commonly thought to have resembled members of the extant bryophytes. The hornworts and liverworts cling to the moist substrates on which they grow. This maximises the surface area through which water can be absorbed and minimises exposure to moving and therefore drying air. In a high-competition environment where all suitable niches are colonised it is likely that any mutations resulting in an increased height were an advantage. In the absence of specialised conducting tissues, height is physiologically limited by the time it takes water molecules to diffuse passively through or between cells and reach aerial portions of the plant. It takes approximately 1 s for 50% of water molecules to diffuse passively through a living cell 50 μm in length (Nobel 1983). The time taken for water molecules to diffuse through a string of cells placed end on end is relatively long. Therefore, some kind of conducting tissue is a physiological necessity for the continued vertical growth of land plants. Mosses are the first land plants which have both apical cell division and xylem. The water-conducting tissue, hydrom, of mosses is stated to be homologous with the xylem tissue of vascular plants (Scheirer 1980, Mishler and Churchill 1984, 1985, Mishler et al. 1994). Mosses are thought to be more closely related to the tracheophytes than are the rest of the bryophytes (Mishler et al. 1994). TFA hydrolysates of the AIR of various green plants revealed that the charophytes and bryophytes had a higher uronic acid residue concentration than the rest of the land plants. Additionally, the more primitive members of the extant bryophytes were found to have high concentrations of GlcA residues in their cell walls whereas all other land plants had high concentrations of GalA and very few GlcA residues present. It is likely that the higher concentrations of uronic acid residues present in the charophytes and bryophytes reflects a higher concentration of acidic polysaccharides. A higher concentration of acidic polysaccharides in a cell wall would make it more ionic and therefore more inclined to take up water than neutral polymer networks which are less electrolytic (Ryden et al. 2000). The higher the ionic strength of the cell wall

the greater the osmotic pressure of the cell wall becomes and the lower the turgor pressure that can be generated (Jarvis and McCann 2000). A cell growing in water does not need to generate high turgor pressures for support. In a non-aquatic environment a cell whose wall has a lower osmotic pressure can generate greater turgor pressure to support the tissue. A cell whose wall contains a relatively low concentration of acidic polysaccharides is therefore better adapted to a terrestrial environment in that it can generate greater turgor pressure for a given cell-sap osmotic pressure, giving increased support and potential to drive growth of the cell. It is reported that tobacco cells grown under drought-mimicking conditions have reduced concentrations of pectic polysaccharides in their cell walls (Iraki et al. 1989a, b, c). Additionally the pectic polysaccharides had an altered monosaccharide composition (Iraki et al. 1989a, b, c). Mosses that are generally considered to be more advanced (Bopp and Capesius 1996; Hedderson et al. 1996) are more drought-tolerant and their cell walls appear to have a lower uronic acid concentration than the rest of the bryophytes, potentially indicating a lower pectic polysaccharide concentration (Section 3.6). The reduction in acidic polysaccharide concentration may be a response to decreased water availability.

The tracheophytes evolved about 420 million years ago and are united by the presence of tracheids and a dominant branched sporophytic generation. Extant tracheophytes are a monophyletic group. The unidentified sugars **3**, **4** and **5** do not appear to be present in high concentration in the tracheophytes. Although I did not fully characterise **4** and **5**, they appear to be an *O*-methylpentose and an *O*-methylhexose respectively. It is possible that they are 2-*O*-methylxylose and 2-*O*-methylfucose, which have been recorded as minor components of RG-II. This would again suggest that a decrease in the pectic polysaccharide concentration is linked with the evolution of the tracheophytes. It is of interest to identify **4** and **5**. They could be purified by PC and subjected to N.M.R. Unknown **3** noted in *Anthoceros caucasicus* is interesting as it is a disaccharide composed of GlcA and Gal which is relatively resistant to severe acid hydrolysis. The disaccharide could be further characterised by N.M.R. or methylation analysis to reveal what linkages

are present. Treatment with galactose oxidase would tell us which enantiomer of Gal is present.

The lycopodiophytes are a distinct monophyletic group within the tracheophytes (Bateman 1986). Extant lycopodiophytes are the relicts of a much more diverse group of plants which are now extinct. Morphological features suggest that the lycopodiophytes evolved from ancestral plants between the bryophytes and euphyllophytes. My results suggest that this major evolutionary step was accompanied by the adoption of 3-*O*-methyl-D-galactose as a significant component of the primary cell wall. 3-*O*-Methyl-D-galactose was not detectable in either *Equisetum* or *Psilotum*. This is consistent with the view that *Equisetum* and *Psilotum* are more closely related to the ferns than to the lycopodiophytes. Molecular sequence data have shown *Equisetum* and *Psilotum*, but not the lycopodiophytes, to be closely related to eusporangiate ferns within the euphyllophyte clade (Pryer et al. 1995, Duff and Nickrent 1999, Raubeson and Jansen 1992, Kranz et al. 1995, Manhart 1994, 1995, Wolf 1997, Malek et al. 1996, Vangerow 1999). The molecular data correlate with my finding that tannins were undetectable in eusporangiate ferns, *Equisetum* and *Psilotum* but present in the leptosporangiate ferns.

3-*O*-Methyl-D-galactose has previously been found within a pectic polysaccharide associated with slippery elm bark (Hirst et al. 1951, Barsett and Paulsen 1991). To discover what functions and possible importance 3-*O*-methyl-D-galactose may have in the cell wall it is important to find out what type of polysaccharide it is part of. The mannose content of the lycopodiophytes was relatively high. Galactose constitutes 20% of a typical angiosperm galactoglucomannan and galactoglucomannans constitute less than 10% of the angiosperm primary cell wall (Cartier et al. 1988, Goldberg et al. 1992). Thus the Gal of galactoglucomannan, even if it was all methylated, would not account for the high concentration of 3-*O*-methyl-D-galactose found in lycopodiophyte primary cell walls; if lycopodiophytes have less than 10% galactoglucomannan and if lycopodiophyte galactoglucomannan has 20% galactose. It is possible 3-*O*-methyl-D-galactose is a component of a

polysaccharide specific to the lycopodiophytes. The monosaccharide 2-*O*-methylxylose (possibly unknown 4) appeared to be present in particularly high concentration in lower land plant cell walls; 3-*O*-methyl-D-galactose could be an RG-II component present only in the lycopodiophytes.

To find out what type of polysaccharide 3-*O*-methyl-D-galactose is part of sequential extraction of lycopodiophyte AIR could be carried out. Each extract could then be subjected to complete TFA hydrolysis and the monosaccharides separated by two-dimensional PC(a). However, this would not reveal what 3-*O*-methyl-D-galactose is attached to. Mild TFA hydrolysis to produce disaccharides which could then be fully TFA-hydrolysed may reveal what monosaccharides 3-*O*-methyl-D-galactose is linked to. Enzyme digestion may also be able to tell us more about the 3-*O*-methyl-D-galactose containing polysaccharide. Driselase-digestion will probably not yield free 3-*O*-methyl-D-galactose but would possibly yield short oligosaccharides with 3-*O*-methyl-D-galactose at the non-reducing end; these could be isolated and further characterised by N.M.R. A useful tool to help in characterising the polysaccharide containing 3-*O*-methyl-D-galactose would be radiolabelling of the methyl groups by feeding lycopodiophyte tissues with [*Me*-^{14}C]methionine (Miller at al. 1994).

Evolution of the leptosporangiate ferns is associated with rearrangements of the chloroplast and mitochondrial genomes and the acquistion of many morphological characters: the annulus, the sporangial stalk, a vertical first zygotic division and the presence of primary xylem with scalariform thickening and bordered pits. The primary cell wall also appears to have undergone major changes in composition. Mannose residues are present in high concentrations in bryophytes, lycopodiophytes, equisetophytes, psilotophytes and eusporangiate ferns. The leptosporangiate ferns, gymnosperms and angiosperms have a substantially lower concentration of mannose residues in their cell walls. I also found that acquisition of tannins was associated with the leptosporangiate ferns, gymnosperms and angiosperms but not the eusporangiate ferns, psilotophytes, equisetophytes and

lycopodiophytes. The presence of tannins is thought to reduce herbivory and microbial attack (Bate-Smith 1954).

Within the angiosperms the main qualitative difference in cell wall composition is the presence of MLG in the Poales and its absence from all other land plants. It has been considered that MLG was restricted to the Poaceae (Kato and Masuda 1976). Smith and Harris (1999) isolated MLG from five families within the Poales: Anarthriaceae (0 and 0.2% w/w MLG/cell wall in two samples of *Anarthria prolifera*), Centrolepidaceae (*Centrolepis strigosa*, 0.5% w/w), Ecdeiocoleaceae (0.3 and 1.7% w/w in two samples of *Ecdeiocolea monostachya*), Flagellariaceae (*Flagellaria indica*, 3.2% w/w) and Poaceae (1.8 (*Lolium multiflorum*) and 8.8% (*Lolium perenne*) w/w). The presence of MLG in the Restionaceae, the remaining family of Poales, was found to be variable (Smith and Harris 1999). Smith and Harris (1999) reported that they could not detect MLG in two members of the Restionaceae, *Chondropetalum tectorum* and *Ischyrolepis subverticellata*. However, MLG was detected at trace concentrations in *Restio tetraphyllus* and 0.1% w/w in *Leptocarpus similis* (Smith and Harris 1999), both members of the Restionaceae. My results confirm those of Smith and Harris (1999). However, neither Smith and Harris (1999) nor I have found MLG outside the Poales. This suggests that the polysaccharide, MLG, may have evolved at the same time as the Poales. Alternatively it was present in other members of the Commelinanae but was subsequently lost. It is of interest that within the Poales the Poaceae have the greatest concentration of MLG in their cell wall, other members of the Poales having MLG present in traces or at most at half the concentration found in the Poaceae. Evolution within some species of the Poaceae has been heavily influenced by selective breeding by man. It is possible that man has selected for the high concentration of MLG found in some species of the Poaceae. The physical characteristics (e.g. viscosity) of MLG are reported to be highly heritable (Greenberg 1977). High levels of MLG could potentially lead to increased endospermic carbohydrate and therefore increased survival of seedlings. A possible advantage of high MLG concentration in the endosperm in preference to starch is

that MLG has been shown to have anti-viral activity (Stübler and Buchenauer 1996).

Among the angiosperms all non-Poales Commelinanae have a greatly reduced uronic acid concentration and no MLG. It has been suggested that reduced uronic acid concentration may reduce the incidence of infection by *Agrobacterium tumefaciens* (Rao et al. 1982, Jarvis et al. 1988). Therefore reduction in uronic acid residue content may be an adaptive advantage. Additionally plants living in a more arid environment may adapt by decreasing the concentration and altering the composition of pectic polysaccharides. Iraki et al. (1989a, b and c) recorded a decrease in the concentration of pectic polysaccharides in tobacco cells grown under drought-mimicking conditions. It is therefore possible that the evolution of the Commelinanae was associated with adaptation to a particularly arid environment.

Conclusion

This thesis shows that the cell wall composition differs between monophyletic groups of plants. In particular, 3-*O*-methyl-D-galactose uniquely occurs in high concentrations in lycopodiophyte cell walls. Bryophyte cell walls have high concentrations of acidic polysaccharides and those bryophytes considered to be among the more primitive were unusual in having high concentrations of GlcA residues in their cell walls. In addition, unknown sugar residues were observed to occur in bryophyte cell walls. It is of special interest to investigate the bryophyte cell wall as it may reveal information about the ecological pressures experienced by the first land plants. It was found that xyloglucan is present in all land plants but appears to be absent in the cell walls of the charophycean green algae. This suggests that the acquisition of xyloglucan was a pre-adaptive advantage for the colonisation of land. The euphyllophytes are a large monophyletic group. All euphyllophytes, including ferns but excluding the monophyletic Poales, have a qualitatively similar cell wall composition. However, the eusporangiate ferns, equisetophytes and psilotophytes contain higher concentrations of mannose residues than the leptosporangiate ferns, gymnosperms and angiosperms. Among the

angiosperms the Poales are thought to be among the more recently evolved (Kellogg and Linder 1995). The Poales contain MLG, which appears to be absent in all other land plants. It is possible that the variation observed in cell wall composition is related to the ecological pressures experienced during evolution of the taxon. My results suggest that there is still a lot of research to be done before we can adequately describe the primary cell wall of land plants.

5 References

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6 Appendix

Poster presentation: Scottish cell wall group meeting 8-9th April 1998 (Dundee University and the Scottish Crop Research Institute).

Primary cell wall composition in relation to the evolution of green plants

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Introduction

Although differences in cell wall composition are one of the main tools used in algal classification, cell wall characters are only infrequently used for classifying land plants [1]. The responsibility of the cell wall for multicellularity, differentiation and support and its involvement in plant growth and defence lead it to have a major influence on plant adaptation and evolutionary diversification [2]. Among Angiosperms the primary cell walls of gramineous monocots are significantly different from both non-gramineous monocots and dicots [3]. The main qualitative difference is the presence of mixed-linkage glucans (MLG's) in gramineous monocots. They are thought to be absent in non-gramineous monocots and dicots. Anomolously, MLG has been reported present in *Phaseolus aureus* and tobacco cell walls [4,5,6]. Little is known about the chemical composition of the primary cell walls of lower land plants.

Methods

Plant cell walls were purified from young expanding tissues after freezing at -70°C [7]. MLG was then swelled or solubilised by heating at 120°C for 2 h in collidine buffer pH 7.0. The soluble material was then hydrolysed, for 8 h at 20°C, using licheninase and the products were analysed by thin layer chromatography and paper electrophoresis in borate.

Results

Rye coleoptiles are a classic source of MLG. A clear positive was obtained for the presence of MLG in rye cell walls. This is indicated by the presence of a trisaccharide and a tetrasaccharide after digestion with licheninase.

Discussion

The method developed is suitable for screening for the presence of MLG. A variety of plants will be looked at including *Phaseolus aureus* of different ages and non-angiosperm land plants with the aim of elucidating the evolution of the cell wall.

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Poster presentation: 8th International Cell Wall Group Meeting 1-5th September 1998 (John Innes Centre, Norwich).

Evolution of plant cell wall polysaccharides: algae to angiosperms.

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Introduction

The cell wall has a major influence on evolution and cell wall characters are used extensively in algal classifications [1]. However, despite variation within angiosperm cell wall composition it is not used in the classification of angiosperms. Little is known about the chemical composition of the primary cell wall of the lower land plants. One of the main qualitative differences within angiosperms is the presence of mixed linkage glucan (MLG) in gramineous monocots and apparent absence from both dicots and non-gramineous monocots. Anomolously the presence of MLG has been reported in regenerating tobacco protoplasts and rapidly growing *Phaseolus aureus* hypocotyls [2,3]. The present work was therefore undertaken to re-assess the presence of MLG in non-gramineous plants.

Results and Discussion

Rye coleoptile walls, a classic source of MLG, gave a clear positive indicated by the presence of a characteristic tri and tetrasaccharide after digestion with licheninase. *Phaseolus aureus* hypocotyls at different ages post germination were examined: a clear positive for the presence of MLG was not obtained. The green seaweed *Ulva lactuca* did not give the characteristic tri and tetrasaccharide, but was digested by licheninase. It yielded two oligosaccharides suggesting the presence of an MLG with a greater percentage of 1,4 linkages. Studies of green land plants, including bryophytes, pteridophytes and gymnosperms, will be reported with the aim of elucidating the evolution of MLG in the cell wall.

Acknowledgement: This work is funded by a BBSRC studentship.

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Poster presentation: Scottish cell wall group meeting 30-31st March 1999 (Glasgow University and Paisley University).

Is mixed-linkage glucan unique to gramineous monocots?

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Introduction

One of the main qualitative differences within angiosperm primary cell walls isthe presence of mixed linkage glucan (MLG) in gramineous monocots (Class Poales, Tribe Poaceae) and apparent absence from both dicots and non-gramineous monocots. Although MLG had previously been thought absent from other members of the class Poales it has recently been reported as present [4]. There are conflicting reports of MLG in the rapidly growing hypocotyls of the dicot *Phaseolus aureus* (mung bean) [1,2,3]. Controversy may have arisen as even in gramineous monocots levels of MLG are tightly linked with developmental stage. MLG is also soluble in boiling water and some methods of cell wall purification subject material to boiling water.

Results and Discussion

Rye coleoptile cell wall, a classic source of MLG, gave a clear positive for the presence of MLG. This was indicated by the presence of a characteristic tri- and tetrasaccharide after digestion with licheninase. *Phaseolus aureus* hypocotyls at different ages, post germination, were examined. The characteristic tri- and tetrasaccharide were clearly not present when examined by TLC. However, if authentic MLG was added to *Phaseolus aureus* alcohol-insoluble residue, before digestion by licheninase, the tri- and tetrasaccharide were clearly detected by TLC to a minimum concentration of 0.02% w/w of the cell wall. Analysis of the products of *Phaseolus aureus* digestion by licheninase by HPLC show that if MLG is present in the primary cell walls it is at levels of less than 0.01% w/w. The presence of MLG in other angiosperms and lower land plants will be investigated by our methods.

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Seminar presentation: Scottish cell wall group meeting 3-4th April 2000 (Edinburgh University).

Evolution and diversity of plant cell walls

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Introduction

The plant cell wall has a major influence on evolution and cell wall characters are used extensively in algal classifications [4]. Variation in primary cell wall composition is known to exist between different angiosperm taxa. However, the cell walls of the lower land plants have not been well studied. It is of interest to see what changes have taken place in cell wall composition during plant evolution. One of the main qualitative differences within angiosperms is the presence of mixed-linkage glucan (MLG) in gramineous monocots and its apparent absence from non-gramineous monocots and dicots. Anomalously, the presence of MLG has been reported in the dicot, *Phaseolus aureus* (mung bean) [1]. Xyloglucan is present in both monocot and dicot cell walls. Although it is not known whether xyloglucan is present in lower land plants, the enzyme xyloglucan endotransglycosylase (XET) has been found in the liverwort *Marchantia polymorpha* [3]. The present work was therefore undertaken to reassess the presence of MLG in non-gramineous plants and to characterise further the chemical composition of lower land plant primary cell walls.

Results

Rye coleoptiles, a classic source of MLG, gave a clear positive indicated by the presence of a characteristic tri- and tetrasaccharide (MLG3 and MLG4) after digestion with licheninase. *Phaseolus aureus* hypocotyls at different ages post-germination were examined: if MLG is present it is at a level of less than 0.02% w/w of the cell wall. Hemicelluloses in the cell walls of a seaweed, *Ulva lactuca*, and a leafy liverwort, *Lophocolea bidentata*, were digestible by licheninase. The licheninase-digestible hemicellulose from *U. lactuca* contained xylose as well as glucose and appeared to have longer stretches of β-(1→4)-glucose than is found in gramineous monocot MLG. The hemicellulose from *L. bidentata* contained the monosaccharides arabinose and glucose. Hemicelluloses from *Flagellaria guineensis*, a close relative of the gramineous monocots, was digested by licheninase to produce the characteristic oligosaccharides MLG3 and MLG4. The presence of the disaccharide isoprimeverose after digestion by Driselase is a good indication of xyloglucan presenbce: a clear positive was obtained for all land plants studied. *Chara*, a green alga, is thought to be closely related to land plants; however, xyloglucan was not detected in *Chara* cell walls to a level of 0.01% w/w of the cell wall.

Discussion

It is interesting that although *Chara* has strong affinities with land plants it appears to have no xyloglucan present in its cell walls. Land plants are thought to have originated from a single species of charophycean green alga. Because of the presence of xyloglucan in all land plants it is likely that the ancestor would also have had xyloglucan in its cell walls. Perhaps xyloglucan conferred an adaptive advantage in a non-aquatic environment. Molecular evidence suggests that the gramineous ,monocots are a recently evolved monophyletic group [2]. MLG appears to have evolved at the same time as gramineous monocots because if its absence from close allies of the graminous monocots (the Restionaceae, sedghes and rushes).

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Seminar presentation: Scottish cell wall group meeting 10th April 2001 (Stirling University).

3-*O*-Methyl-D-galactose residues in lycophyte cell walls

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Abstract

Acid hydrolysis of cell wall-rich material from young leaves of the lycophyte *Selaginella apoda* (L.) Spring yielded substantial amounts of 3-*O*-methyl-D-galactose (1) in addition to the usual major monosaccharides (glucose, galactose, arabinose, xylose and galacturonic acid). The yield of **1** approximately equalled that of galacturonic acid. **1** was identified as 3-*O*-methylgalactose by its ^{1}H- and ^{13}C-NMR spectra, and shown to be the D-enantiomer by its susceptibility to D-galactose oxidase. **1** was detected in acid hydrolysates of the alcohol-insoluble residues from young leaves of all lycophytes tested, both homosporous (*Lycopodium*, *Huperzia* and *Diaphasiastrum*) and heterosporous (*Selaginella*). It was not detectable in the charophyte green algae *Coleochaete scutata*, *Chara coralina* or *Klebsormidium flaccidum*, any bryophytes [a hornwort (*Anthoceros*), four liverworts and three mosses], or any euphyllophytes [a psilopsid (*Psilotum*), a horsetail (*Equisetum*), eusporangiate and leptosporangiate ferns, the gymnosperm *Gnetum* and diverse angiosperms]. A high content of 1 is thus an autapomorphy (unique character) of the lycophytes.

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3-*O*-Methyl-D-galactose residues in lycophyte primary cell walls

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Abstract

Acid hydrolysis of cell wall-rich material from young leaves of the lycophyte *Selaginella apoda* (L.) Spring yielded substantial amounts of 3-*O*-methyl-D-galactose (**1**) in addition to the usual major monosaccharides (glucose, galactose, arabinose, xylose and galacturonic acid). The yield of **1** approximately equalled that of galacturonic acid. Compound **1** was identified as 3-*O*-methylgalactose by its ^{1}H and ^{13}C NMR spectra, and shown to be the D-enantiomer by its susceptibility to D-galactose oxidase. Compound **1** was detected in acid hydrolysates of the alcohol-insoluble residues from young leaves of all lycophytes tested, both homosporous (*Lycopodium*, *Huperzia* and *Diphasiastrum*) and heterosporous (*Selaginella*). It was not detectable in the charophyte green algae *Coleochaete scutata*, *Chara coralina* or *Klebsormidium flaccidum*, any bryophytes [a hornwort (*Anthoceros*), four liverworts and three mosses], or any euphyllophytes [a psilopsid (*Psilotum*), a horsetail (*Equisetum*), eusporangiate and leptosporangiate ferns, the gymnosperm *Gnetum*, and diverse angiosperms]. A high content of **1** is thus an autapomorphy of the lycophytes. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Selaginella apoda*; *S. erythropus*; *Lycopodium pinifolium*; *Huperzia selago*; *Diphasiastrum alpinum*; Lycophytes; Euphyllophytes; Pteridophytes; Primary cell walls; Polysaccharides; 3-*O*-Methyl-D-galactose; NMR spectroscopy; HMBC; HMQC

1. Introduction

Within dicots and non-graminaceous monocots, the primary cell wall has a relatively constant sugar composition, major monosaccharide residues being Glc, Gal, Ara, Xyl, GalA, Rha, Fuc, GlcA and Man (McNeil et al., 1984; Fry, 1988; Brett and Waldron 1996). Graminaceous primary walls contain the same monosaccharide residues but usually have more Xyl and less Gal, Ara and Fuc (Burke et al., 1974; Carpita, 1996). Gymnosperms are also similar to dicots, but tend to be richer in Man residues (Edashige and Ishii, 1996). Despite these quantitative differences, no qualitative differences in the monosaccharide composition of primary cell walls have been noted between different groups of seed plants.

Few studies have been undertaken of cell wall composition in lower land plants (Chodat and Cortesi, 1939; Edelmann et al., 1998), probably because they are not major world crops. Among bryophytes (non-vascular land plants), liverworts broadly resemble gymnosperms in their cell wall sugar residue composition, the major neutral monosaccharide residues being Glc > Gal > Man > Xyl ≈ Ara > Fuc ≈ Rha (Thomas, 1977; Thomas et al., 1984). Uronic acid residues, stated to be mannuronic acid, are also present (Das and Rao, 1967).

We have not found any reports specifically dealing with the composition of the primary cell wall of pteridophytes (non-seed vascular plants), although a few reports on their secondary walls have appeared (Timell, 1962; Bremner and Wilkie, 1971). Modern pteridophytes are the few surviving progeny of a much greater diversity of extinct taxa, representing the explosive evolutionary experimentation which occurred among land plants in the Silurian (Raven, 1993; Kenrick and Crane, 1997). Since the primary cell wall plays several essential biological roles, including the regulation of cell expansion, tissue cohesion, defence (e.g. against microbes), ion-exchange, and the production of oligosaccharins (Fry, 1988; Kaplan and Hageman, 1991; Brett and Waldron, 1996; Stübler and Buchenauer, 1996; Goldberg, 1995; Cassab, 1998), we suggest that the demands placed on it, and thus its optimal

Abbreviations: AIR, alcohol-insoluble residue; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; PC, paper chromatography

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composition, may have changed rapidly during periods of rapid plant evolution. It is certainly the case that some green algae have cell wall compositions very different from those of seed plants (Mackie and Preston, 1974; Roberts, 1974; Domozych et al., 1980).

We are, therefore, documenting the evolution of the primary cell wall in land plants. DNA sequence data (Raubeson and Jansen, 1992; Manhart, 1994, 1995; Kranz et al., 1995; Pryer et al., 1995; Malek et al., 1996; Wolf, 1997; Duff and Nickrent, 1999, Vangerow et al., 1999) have recently confirmed the morphology-based view that the lycophytes (club-mosses) diverged very early from the ancestors of the euphyllophytes (= plants with 'true' leaves, extant euphyllophytes being all seed-plants as well as non-lycophyte pteridophytes). Indeed, the lycophytes show a relatively close relationship to the bryophytes. In the present paper we report on an unusual chemical feature in the primary cell walls of the lycophytes, which is apparently related to their isolated evolutionary position.

2. Results

2.1. An unusual sugar residue in *Selaginella* cell walls

Acid hydrolysis of the alcohol-insoluble residue (AIR; i.e. cell wall-rich material) from young leaves of *Selaginella apoda* followed by paper chromatography (PC) yielded an unfamiliar monosaccharide (**1**) in addition to the major monosaccharides characteristic of the cell wall polysaccharides of seed plants. In chromatography system 2, **1** migrated between Ara and Xyl. However, unlike these pentoses, **1** stained yellow-brown with aniline hydrogen-phthalate, suggesting a hexose derivative.

Larger amounts of **1** were partially purified by preparative PC (in system 2) of the acid hydrolysate obtained from 1 g of *S. apoda* AIR. Of several PC and TLC systems tested for their ability to separate **1** from the last traces of Ara and Xyl (Table 1), preparative PC in system 3 was selected, followed by gel-permeation chromatography on Bio-Gel P-2. On TLC (cellulose) in system 3, cmpd **1** exhibited an unusually high R_F, similar to that of authentic 3-*O*-methylglucose and 2-deoxygalactose; it was well resolved from the common hexoses and pentoses. R_F values (cellulose TLC, system 3) were Glc, 0.30; Gal, 0.36; Man, 0.39; Xyl, 0.37; Ara, 0.46; Rib 0.63; 6-deoxymannose (= rhamnose), 0.61; 2-deoxygalactose, 0.73; 3-*O*-methylglucose, 0.72; cmpd **1**, 0.70.

2.2. Identification of 3-O-methylgalactose by NMR spectroscopy

The standard 1D single-pulse 600-MHz proton NMR spectrum of 100 μg of purified unknown **1** in D_2O as solvent is reasonably well resolved (Fig. 1a) The doublets at 4.60δ and 5.26δ suggest the presence of the α and β anomers of a glucose or galactose derivative and the sharp signal at 3.45 suggest the presence of one methoxy group. Careful examination of this spectrum confirms the presence of the requisite number of signals and their assignment follows immediately from the 2D-COSY spectrum (Fig. 1b). Significant signal overlap occurs only for the C-6 protons. In the β-anomer the C-6 protons have different chemical shifts, and each appears as a doublet of doublets. In the α-anomer the C-6 proton signals coincide so the coupling between them is not seen resulting in a single doublet. One peak of the doublet for the C-6 of the α-anomer virtually coincides with one peak from one of the C-6 protons of the β-anomer. The coupling constants of each multiplet, measured from the 1-D spectrum (Table 2) are consistent only with a galactose derivative.

The ^{13}C chemical shifts, obtained from a 2D 1-bond carbon–proton shift (HMQC) correlation spectrum, are similar to those for the anomers of galactose with the exception of that for C-3, which is about 10 ppm higher. This is consistent with methylation of the hydroxyl at C-3. The site of methylation is confirmed by correlation between the methyl protons and C-3 of each anomer in the 2D long range carbon–proton (HMBC) shift correlation spectrum. The ^{1}H and ^{13}C spectral data of the individual components of **1** are given in Table 2.

In conclusion, the spectroscopic data show unambiguously that, apart from a few unidentified minor impurities, **1** was a mixture of the α and β anomers of 3-*O*-methylgalactose.

2.3. Determination of enantiomeric form

Plant cell wall polysaccharides contain both D- and L-Gal residues, which are biosynthetically derived from UDP-D-Glc (via UDP-D-Gal) and from GDP-D-Man (via GDP-L-Gal), respectively (Feingold and Avigad, 1980). The D-Gal:L-Gal ratio has been reported at ~7.3:1 in cultured sycamore cells (Roberts and Harper, 1973) and ~70:1 in cultured spinach cells (Baydoun and Fry, 1988). D-Gal, the more abundant enantiomer, is found in pectins, xyloglucans and arabinogalactan-proteins. L-Gal has been reported in the side-chains of xylans (Whistler and Corbett, 1955) and some xyloglucans (Zablakis et al., 1996; Hantus et al., 1997). It was, therefore, of interest to determine whether **1** was a derivative of D- or L-Gal.

D-Galactose oxidase uses O_2 to oxidise the $-CH_2OH$ group of D-Gal and some of its derivatives, but not of L-Gal. Derivatives of D-Gal that can be oxidised by this enzyme include the 2- and 3- but not 4-*O*-methyl monoethers (Avigad et al., 1962; Schlegel et al., 1968; Cleveland et al., 1975). D-Galactose oxidase can therefore be used to distinguish 3-*O*-methyl-D-galactose from 3-*O*-methyl-L-galactose.

Table 1
R_f values of Xyl, Ara and 1 in various solvent systems

Compound	R_F value in system[a]							
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*
Ara	0.28	0.21	0.12	0.41	0.15	0.75	0.58	0.46
Xyl	0.38	0.29	0.13	0.52	0.21	0.79	0.49	0.37
1	0.24	0.18	(s)[c]	(s)	0.12 (s)	(u)[b]	0.80	0.70

[a] Chromatographic systems used were: *a*, TLC (silica-gel) in BuOH–HOAc–H_2O (3:1:1 by vol.); *b*, TLC (silica-gel) in BuOH–HOAc–H_2O (4:1:1 by vol.); *c*, TLC (silica-gel) in MeEtCO–H_2O (85:7 by vol.); *d*, TLC (silica-gel) in EtOAc–Py–HOAc–H_2O (5:3:1:1 by vol.); *e*, TLC (silica-gel) in *i*-PrOH–NH_4OH–H_2O (8:1:1 by vol.); *f*, TLC (silica-gel) in *i*-PrOH–nitromethane–H_2O (5:2:3 by vol.); *g*, PC (Whatman No. 1) in PhOH–H_2O (4:1, w/w); *h*, TLC (cellulose) in PhOH–H_2O (4:1, w/w).
[b] (u) = Unrecordable (did not separate from Ara and Xyl).
[c] (s) = Streaked.

We confirmed that the commercial sample of galactose oxidase was able to oxidise D- but not L-Gal; it also oxidised cmpd **1**. The substrates (D-Gal and **1**) were partially destroyed within 1 h; oxidation was complete within 4 days, yielding multiple reaction products (Fig. 2). In contrast, the enzyme had no effect on L-Gal, despite the incubation continuing up to 4 days (Fig. 2). During the 4-day incubation, reaction mixtures containing D-Gal or **1** turned yellow (presumably owing to the formation of unstable aldehyde products) whereas the L-Gal reaction mixture did not change colour.

Together the results show that **1**, isolated from *S. apoda*, was 3-*O*-methyl-D-galactose.

2.4. *Occurrence of 3-O-methylgalactose in various green plants*

To ascertain the distribution of 3-*O*-methylgalactose among land plants we analysed acid hydrolysates of AIR from young shoot tissue (leaves and/or immature stems) of various pteridophytes and the thalli of various bryophytes and algae by 2D PC (exemplified by Fig. 3). 3-*O*-Methylgalactose was present in all lycophytes tested, both homosporous and heterosporous (Table 3). Typical yields (estimated from staining intensity relative to authentic 3-*O*-methylglucose) were in the order of 5–10 mg of 3-*O*-methylgalactose per g of AIR. 3-*O*-Methylgalactose was, however, undetectable in all euphyllophytes tested: a psilopsid, a horsetail, eusporangiate and leptosporangiate ferns and a gymnosperm (Table 3) and numerous angiosperms (data not shown). 3-*O*-Methylgalactose was also undetectable in all bryophytes tested: three mosses, four liverworts and a hornwort (Table 3). In addition, it was undetectable in walls of the charophytes *Coleochaete*, *Chara* and *Klebsormidium* — green algae which are thought (Kenrick and Crane, 1997) to be relatively closely related to the hypothetical, extinct, green alga from which all land plants evolved.

3. Discussion

The presence of 3-*O*-methylgalactose in both homosporous and heterosporous lycophytes supports the proposed (Bateman, 1996; Kranz and Huss, 1996) monophyly of the lycophytes. Extant lycophytes are the relicts of a much more diverse group of extinct lycophytes. Morphological features suggest that lycophytes evolved from ancestral plants between the bryophytes and the euphyllophytes. Our findings suggest that this phase of very rapid evolutionary experimentation was accompanied by the adoption of 3-*O*-methyl-D-galactose as a major component of the primary cell wall.

3-*O*-Methyl-D-galactose was not detectable in either *Equisetum* or *Psilotum*, supporting the view that these plants are more closely related to the ferns than to the lycophytes. Gene sequence data have shown *Equisetum* and *Psilotum*, but not the lycophytes, to be within the euphyllophyte clade (Robeson and Jansen, 1992; Manhart, 1994, 1995; Kranz et al., 1995; Pryer et al., 1995; Malek et al., 1996; Wolf, 1997; Duff and Nickrent, 1999; Vangerow et al., 1999).

A 3-*O*-methylgalactose (enantiomer not determined) has been detected, in trace amounts, in an extracellular polysaccharide exuded by a soil-dwelling *Cryptomonas* sp. (Paulsen et al., 1992). *Cryptomonas* is a member of the Cryptophyta, a small group of biflagellate, eukaryotic algae which frequently have red photosynthetic pigments. Most are free-swimming but one member of the Cryptophyceae has been found as an endosymbiont in a marine cilliate as a complex known as *Mesodinium rubrum*. Since the lycophytes analysed by us are not aquatic, they are unlikely to have any cryptophycean endosymbionts in their shoot tissues (Dr. D.G. Mann, RBGE, personal communication).

3-*O*-Methyl-D-galactose has also been isolated from polymers present in certain strains of the aquatic green alga *Chlorella vulgaris* (Ogawa et al., 1994). *Chlorella* spp. are primarily free-swimming and unlikely to form a symbiotic relationship with terrestrial lycophytes.

3-*O*-Methyl-D-galactose has been reported to constitute about 5% of the total neutral sugar residues in the soluble extracellular polysaccharides produced by the freshwater red alga *Porphyridium aerugineum* (Percival and Foyle, 1979). Methylgalactoses are also present in the rhodophyte genera *Geldiliella* and *Gracilaria*, from which agar is extracted. However, in agar the *O*-methyl groups are attached to galactose at position 4 and/or 6 (Matsuhiro and Urzua, 1991; Mollion, 1992; Chiovitti et al., 1995, Mollet et al., 1998; Givernaud et al., 1999). Therefore, it is highly unlikely that 3-*O*-methyl-D-galactose occurred in our lycophyte samples as a contaminant from laboratory agar. The lycophytes were collected from diverse sites (Table 3); it is, therefore, unlikely that they had a common contaminant.

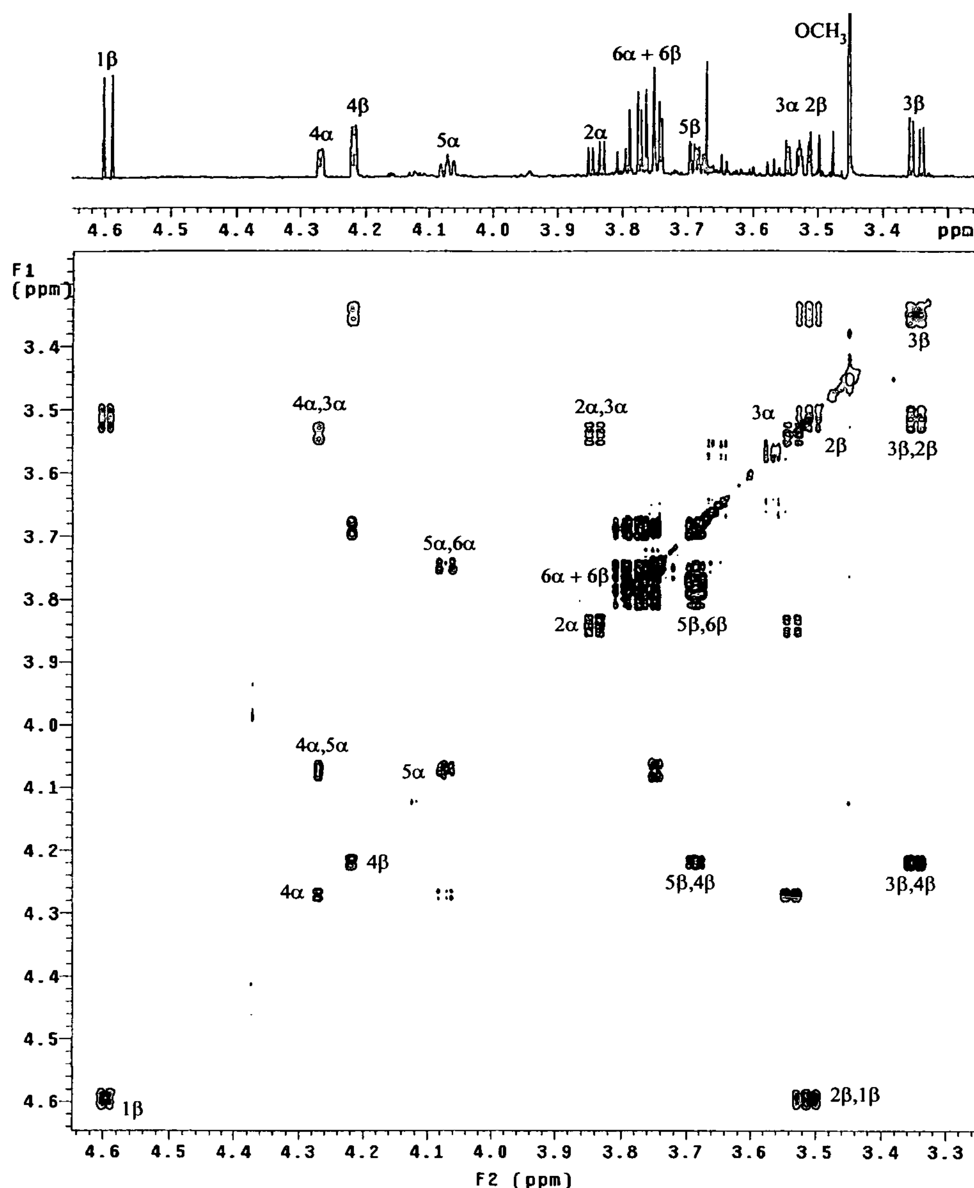

Fig. 1. NMR spectra of 3-*O*-methylgalactose (**1**): (a) 1D single-pulse 600 MHz proton NMR spectrum over the region 3.25δ–4.65δ [i.e. excluding the H-1α resonance at 5.26δ]; (b) 2D gradient selected proton COSY NMR spectrum over the same region as in (a) [i.e. excluding the H-1α diagonal resonance and the H-1α, H-2α off-diagonal resonances].

Among the fungi, 3-*O*-methyl-D-galactose has been detected as a polysaccharide component in *Armillaria mellea* (Bouveng et al., 1967) and a 3-*O*-methylgalactose (enantiomer not determined) in *Lamperomyces japonicus* (Fukuda and Hamada, 1978). 3-*O*-Methyl-D-galactose has also been reported as a component of haemocyanin, a glycoprotein from snails (Hall et al., 1977).

3-*O*-Methyl-D-galactose has been reported previously in land plants. For example, pectic polysaccharides from elm bark contain 3-*O*-methyl-D-galactose (Hirst et al., 1951; Barsett and Paulsen, 1985, 1991). These polymers have a backbone of 4-linked α-D-galacturonic acid interspersed with 2-linked α-L-rhamnose residues; some of the latter are substituted on O-4 with short side-chains containing 3-*O*-methylgalactose, galactose and/or glucuronic acid. It is possible that a similar polymer may occur in lycophytes since both rhamnose and galacturonic acid were detected in the hydrolysates of lycophyte AIRs. However, elm trees and lycophytes are not closely related and at present it is not known in which polysaccharide(s) 3-*O*-methyl-D-galactose occurs in the lycophyte cell wall.

3-*O*-Methyl-D-galactose was also detected as a component of a polysaccharide extracted from sassafras (*Sassafras albidum*) twigs (Springer et al., 1965), and a 3-*O*-methylgalactose (enantiomer not determined) was found in hydrolysates of the leaves of various dicot trees (Bacon and Cheshire, 1971). The yield from sweet-chestnut

Table 2
^{1}H and ^{13}C NMR spectral data of 3-*O*-methylgalactose (**1**)

Site	δ_C	δ_H	Multiplicity[a]	J_{HH} (Hz)
β-Galactose				
1	99.2	4.60	(*d*)	7.9
2	73.6	3.52	(*dd*)	9.9, 7.9
3	84.7	3.35	(*dd*)	9.9, 3.3
4	67.0	4.22	(*dd*)	3.3, 0.9
5	77.8	3.69	(*ddd*)	7.7, 4.4, 0.9
6	63.8	3.76	(*dd*)	11.6, 4.4
		3.80	(*dd*)	11.6, 7.7
O-Methyl	58.8	3.45	(*s*)	
α-Galactose				
1	94.9	5.26	(*d*)	4.0
2	70.1	3.84	(*dd*)	10.2, 4.0
3	81.2	3.54	(*dd*)	10.2, 3.2
4	67.6	4.27	(*dd*)	3.2, 1.3
5	73.0	4.08	(*ddd*)	6.7, 5.8, 0.9
6	64.0	3.74	(*d*)	nm[b]
O-Methyl	58.8	3.45	(*s*)	

[a] Key: *d* = doublet, *dd* = doublet of doublets, *ddd* = doublet of doublet of doublets, *s* = singlet.
[b] nm = Not measurable.

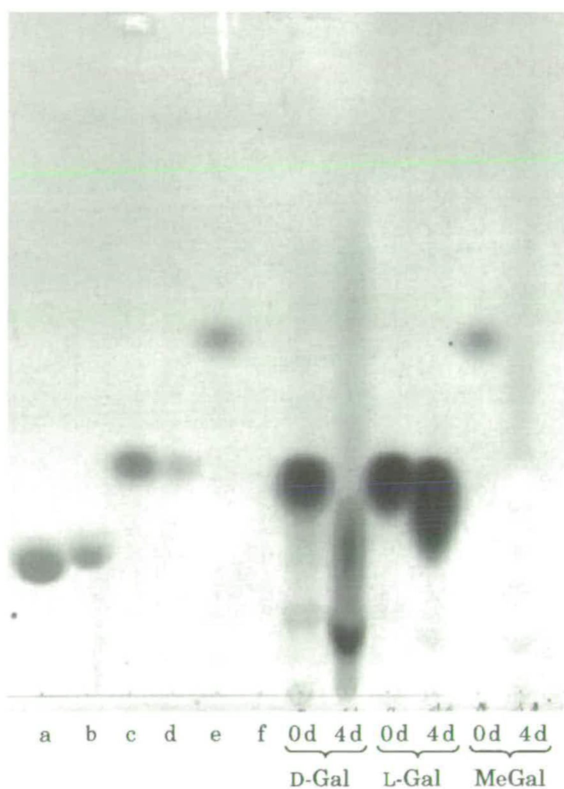

Fig. 2. Effect of D-galactose oxidase on D-Gal, L-Gal and 3-*O*-methylgalactose (**1**; MeGal) from *Selaginella apoda*. The three potential substrates were incubated with the enzyme for 0 or 4 days, as indicated, then analysed by TLC on cellulose in system 4 and stained with aniline hydrogen-phthalate. Markers used were: (a) D-GalA; (b) D-GlcA; (c) D-Gal; (d) L-Gal; (e) compound (**1**); (f) blank.

leaves was 11 mg of 3-*O*-methyl-D-galactose per 100 g AIR (Bacon and Cheshire, 1971), a very low concentration in comparison with the high levels detected in lycopod AIRs in our survey.

Fig. 3. Two-dimensional paper chromatogram showing 3-*O*-methylgalactose in an acid hydrolysate of AIR from *Lycopodium pinifolium* Blume. The PC was developed in system 1 (bottom to top, as photographed) followed by system 3 (left to right). Staining was with aniline hydrogen-phthalate. The spot marked '?' is unidentified: it was clearly distinguishable from rhamnose (not detectable in Fig. 3), which had an R_F higher than that of fucose in system 1 but approximately equal to that of fucose in system 3. Sugar '?' stained red with aniline hydrogen-phthalate and so is likely to have been a pentose derivative.

In conclusion, the adoption of 3-*O*-methyl-D-galactose as a quantitatively appreciable component of the primary cell wall appears to be an autapomorphy (a character which uniquely defines a taxon; Hennig, 1966) of the lycophytes that was acquired early during their evolution from hypothetical extinct ancestral land plants intermediate between the bryophytes and the euphyllophytes, and that has been retained by all extant lycophytes.

4. Experimental

4.1. Source of plant material

Sources of botanical material are listed in Table 3.

Table 3
Occurrence of 3-*O*-methylgalactose (MeGal) as a major component in acid hydrolysates of AIR from a range of lower plants

Classification		Species	MeGal[a]	Source[b]
Algae	Charophytes	*Chara coralina* (Klein ex Willd. Em. R.D.W.)	–	Culture, D. Sanders
		Coleochaete scutata Brébisson	–	CCAP 414/1
		Klebsormidium flaccidum (Kützing) Silva, Mattox et Blackwell	–	Culture, H. Sluiman
Bryophytes	Hornwort	*Anthoceros caucasicus* Spring.	–	Faial Island (R. Schumacker)
	Thalloid liverwort	*Pellia epiphylla* (L.) Corda	–	Roslin Glen
		Marsupella emarginata var *aquatica* (Lindb.) Dum.	–	Cairngorms
	Leafy liverworts	*Porella* sp.	–	Cairngorms
		Trichocolea tormentella (Ehrh.) Dum.	–	Glen Creran (D.S. Rycroft)
		Andrea rupestris Hedw. (*A. petrophila* Ehrh.)	–	Cairngorms (W.M.M. Eddie)
	Mosses	*Hypnum cupressiforme* Hedw.	–	Roslin Glen
		Sphagnum molle Sull.	–	Cairngorms
Lycophytes	Homosporous	*Lycopodium pinifolium* Blume	+	19835037(E)
		Huperzia selago (L.) Bernh. Ex. Schrank and Mart.	+	Cairngorms
		Diphasiastrum alpinum (L.) Holub.	+	Cairngorms
	Heterosporous	*Selaginella apoda* (L.) Spring.	+	19677705(E)
		Selaginella erythropus Spring.	+	19715473(E)
		Selaginella pallescens (C. Presl.) Spring.	+	19697710(E)
Euphyllophytes	Horsetail	*Equisetum debile* Roxb. Ex. Vaucher	–	19731694(E)
	Psilopsid	*Psilotum nudum* (L.) P. Beauv.	–	DRB
	Eusporangiate fern	*Marattia fraxinea* Sm.	–	19697183(E)
	Leptosporangiate ferns	*Osmunda regalis* L.	–	19578631(E)
		Onoclea sensiblis L.	–	19662802(E)
		Salvinia auriculata Aubl.	–	19830813(E)
		Platycerium bifurcatum (Car.) C. Chr.	–	19734554(E)
		Todea barbara (L.) T. Moore	–	19652792(E)
		Phyllitis scolopendrum L.	–	19731529(E)
		Asplenium australassium (J. Sm.) Hook.	–	19933661(E)
		Blechnum spicant (L.) Roth.	–	Cairngorms
		Dryopteris crispifolia Rasbach et al.	–	19920813(E)
	Gymnosperm	*Gnetum gnemon* L.	–	19902511(E)

[a] + Indicates 3-*O*-methylgalactose detectable after 2-dimensional PC as shown in Fig. 3.

[b] Accession numbers followed by (E) refer to material from the Royal Botanic Garden, Edinburgh (RGBE); CCAP = Culture Collection of Algae and Protozoa, Ambleside, Cumbria; DRB = glasshouses of the Daniel Rutherford Building, The University of Edinburgh. Additional material was kindly provided by Dr. D. Long and Dr. H. Sluiman (RBGE), Dr. D.S. Rycroft (Glasgow University), Dr. R. Schumacker (University of Liege), Dr. W.M.M. Eddie (University of Texas, Austin) and Prof. D. Sanders (University of York). Other material was collected by Z.A.P. Collection sites were: Cairngorm Hills (57°05–10′ N, 3°35–45′W.), Roslin Glen (55°51′ N, 3°10′ W), Glen Creran (56°36′ N, 5°12′ W) and Faial Island (38°40′ N, 28°40′ W).

4.2. Preparation and hydrolysis of AIR

About 30 g fr. wt of (fresh or frozen) young plant material was blended (two 1-min bursts) in an MSE 'Ato-Mix' with 150 ml 70% ethanol. The suspension was filtered through Miracloth (Calbiochem) and the residue ground to a fine powder in liquid N_2 with a ceramic pestle and mortar (both pre-cooled to −80°C). The powder was transferred to a flask with 150 ml 70% ethanol and incubated at 55°C for 5 days to solubilise free sugars. The suspension was then filtered again and repeatedly washed with 70% ethanol until colourless. The residue was finally washed in acetone and dried.

A portion (20 mg) of each AIR sample was subjected to hydrolysis in 1 ml 2 M TFA at 120°C for 1 h. The hydrolysate was dried in vacuo and re-dissolved in 50 µl water, of which 2 µl was subjected to 2-dimensional PC.

4.3. Paper chromatography and TLC

Analytical PC was on Whatman No. 1 paper; preparative PC was on Whatman 3MM. One-dimensional PC was by the descending method; 2-dimensional PC was by the ascending method. Solvent systems used were (1), BuOH–HOAc–H_2O (12:3:5 by vol.); (2), as system 1 for 16 h followed, in the same dimension, by EtOAc–pyridine–H_2O (8:2:1 by vol.) for 18 h; (3), PhOH–H_2O (4:1, w/w); (4), BuOH–HOAc–H_2O (2:1:1 by vol.) followed, in the same dimension, by EtOAc–pyridine–H_2O (10:4:3 by vol.); others are indicated in Table 1. TLC was on Merck silica-gel or microcrystalline cellulose. Chromatograms were stained with aniline hydrogen-phthalate; faint spots were more readily visible by their fluorescence when viewed under a 366-nm UV lamp (Fry, 1988).

4.4. NMR methods

The NMR spectra were measured on D_2O solutions using a Varian INOVA 600-MHz spectrometer operating at 599.9 MHz for protons and 150.9 MHz for ^{13}C nuclei.

The 2D gradient selected COSY proton spectrum was obtained using the sequence (von Kienlin, 1991): D1–90°–t1–zgrd–90°–zgrd–AQ with D1 = 1.5 s, AQ = 0.646 s, zgrad = 1 gauss/cm, 0.002 s. Other parameters were SW = 1586 Hz, 2K data points, 512 increments each with 16 transients per FID. The data were processed using sine-bell squared functions in both dimensions with zero filling of the F1-data to 2K data transformation resulting in digital resolution of 1.5 Hz/pt.

The 2-D proton detected one-bond ^1H–^{13}C correlation (HMQC) spectrum was obtained using the sequence (Summers et al., 1986): D1–90°(^1H)–D2–180°^1H); 180°(^{13}C)–D2–90°(^1H)–D3–90°(^1H)–D2–90°(^{13}C)–t_1/2–180°(^1H)–t_1/2–90°(^{13}C)–D2–AQ. The delays used were D1 = 1.6 s (pre-saturation), D2 = 3.7 ms ($1/2^1J_{CH}$) and D3 = 600 ms (to minimise signals from protons bonded to ^{12}C nuclei). The experiment was preceded by 64 scans to establish thermal equilibrium. A 4-step phase cycle (hypercomplex acquisition) was used with ^{13}C broad band decoupling during acquisition of the proton signals. Other parameters were SW(^1H) = 4632 Hz, 2K data points, SW(^{13}C) = 18000 Hz, AQ = 0.408 s, 200 increments each with 4 transients per FID. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1 data from 200 to 1024 W before transformation.

The 2D proton detected gradient selected long-range ^1H–^{13}C correlation (HMBC-g) spectrum was obtained using the sequence (Ruiz-Cabello et al., 1992): D1–90°(^1H)–D2–90°(^{13}C)–D3–90°(^{13}C)–zgrd1–t_1/2–180°(^1H)–t_1/2–zgrad1–90°(^{13}C)–zgrd2–AQ. The delays used were D1 = 1.4 s, D2 = 3.7 ms ($1/2^1J_{CH}$) and D3 = 55 ms (optimised for signals from protons with couplings to carbon of ca. 9 Hz), zgrad1 = 4 gauss/cm, 0.002 s, zgrad 2 = 2 gauss/cm, 0.002 s. A 16-step phase cycle (hypercomplex acquisition) was used with no ^{13}C broad band decoupling during the acquisition of the proton signals. Other parameters were SW(^1H) = 2500 Hz; 2K data points; SW(^{13}C) = 15,000 Hz, AQ = 0.410 s; 128 increments each with 192 transients per FID. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1 data from 200 to 1024 W before transformation.

4.5. Oxidation with D-galactose oxidase

D-Galactose (0.5 mg), L-galactose (0.5 mg) or cmpd **1** (trace) was incubated in an open vial containing 100 µl of 100 mM P_i (K^+) buffer, pH 6.0, containing 0.5% chlorobutanol, 15 'Sigma Units' of D-galactose oxidase (EC 1.1.3.9; from *Dactylium dendroides*) and 10 Units of bovine liver catalase (both enzymes from Sigma Chemical Co., Poole, UK). The K_m of galactose oxidase for D-galactose is reported to be 0.24 M (Avigad et al., 1962). One 'Sigma Unit' of D-galactose oxidase is the activity which will produce a ΔA_{425} of 1.0 per min in a reaction volume of 3.4 ml with D-Gal as substrate in the presence of a peroxidase–*o*-tolidine system to detect the H_2O_2 generated. After incubation for 1 h to 4 days at 20°C, 20-µl samples were analysed by TLC on cellulose in system 4.

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