CHARACTERISATION OF CELLULOSE BIOSYNTHESIS IN *ARABIDOPSIS THALIANA*

BY

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DECLARATION

The work reported in this thesis was performed
by the author,
except where due reference and acknowledgment is given,
and has not been submitted for any previous
degree, or diploma.

Liangcai Peng

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ABSTRACT

Biochemical analyses of cell wall polysaccharides has shown that several temperature-inducible radial swelling mutants (*rsw1, rsw2, rsw3* and *rsw5*) of *Arabidopsis thaliana* have defects in genes involved in cellulose biosynthesis.

All the mutants and wild type grown at 21 °C for 5 days have broadly similar growth. At the restrictive temperature (31 °C) for 5 days, *rsw1, rsw2* and *rsw3* show radial swelling with small differences in the extent of swelling and in other aspects of growth. In contrast, the wild type does not swell at 31 °C and *rsw5* only swells strongly on media where sucrose replaces glucose as the carbon-supply. The radial swelling phenotype is consistent with the mutants having defective cell walls.

All mutants grown at 31 °C for 5 days have 35-56% less cellulose in their roots and 50-62% less cellulose in their shoots than the wild type grown at 31 °C. Rw values (the ratio of cellulose production at 31 °C : cellulose production at 21°C) also indicate that all mutants are strongly inhibited in cellulose biosynthesis at 31 °C in both root and shoot, whereas cellulose production is stimulated in the wild type at 31 °C.

A large quantity of β-1,4-glucan fails to form crystalline cellulose microfibrils in shoot tissue of *rsw1*, *rsw2* and *rsw3* and is recovered as poorly-crystalline (or non-crystalline) cellulose from fractions containing pectins and hemicelluloses. Only very small amounts of extra β-1,4-glucan are found in the acid-soluble fraction of root cell walls, but a lot of extra carbon is found as α-1,4-glucan (starch) in mutants at 31 °C. Apparently, the excess carbon not used for cellulose biosynthesis in mutants at 31 °C is converted into non-crystalline cellulose in shoots, and is partially stored as starch in roots.

β-1,4-Glucan can not be purified by the same methods from *rsw5* and wild type at 31 °C and can not be purified from any plants grown at 21 °C. The interactions of non-crystalline cellulose with pectins, hemicellulose and cellulose are discussed.

The non-cellulosic wall polysaccharides of the mutants are similar to those of the wild type in monosaccharide composition and glycosidic linkage pattern as shown by methylation analysis. Moreover, the cellulose reductions in mutants at 31 °C do not greatly affect the total levels of non-cellulosic polysaccharides in the wall except perhaps for small differences in the proportions of different types of hemicelluloses.

With the reduction of glucose in the cellulose of mutants at 31 °C, the distribution of glucose was greatly altered only with respect to non-crystalline cellulose in shoots and starch in roots with little change in levels in other wall matrix polysaccharides. This also suggests that decreased cellulose did not change the deposition of wall matrix polymers.

The data support the view that the mutations are in genes involved in cellulose biosynthesis.

LIST OF ABBREVIATIONS

A_{396}	absorbance at 396 nm
AA	alditol acetate
ADP	adenosine diphosphate
Api	D-apiose
Ara	L-arabinose
C-	carbon-linked
c-di-GMP	cyclic diguanylic acid
CTAB	cetyltrimethylammonium bromide
D- or L-	optical isomer
Da	Dalton
DCB	2,6-dichlorobenzonitrile
df	degree of freedom
DMSO	dimethylsulphoxide
DP	degree of polymerisation
EDTA	ethylenediaminetetraacetate
EI-MS	electron impact mass spectrometry
f	furanose
Fuc	L-fucose
Gal	D-galactose
GalA	D-galacturonic acid
GAX	glucuronoarabinoxylan
GC/MS	gas chromatography-mass spectrometry
GDP	guanosine diphosphate
Glc	D-glucose
GlcA	D-glucuronic acid
GTP	guanosine 5'-triphosphate
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate
IS	internal standard
kDa	kilodalton
m-	meta

Man	D-mannose
MS	mass spectra
MWCO	molecular weight cut off
N-	nitrogen-linked
ND	not detected
o-	ortho
O-	oxygen-linked
p	pyranose
p-	para
PMAA	partially methylated alditol acetate
psi	pounds per square inch
R_f	chromatographic mobility relative to solvent front
RG	rhamnogalacturonan
Rha	L-rhamnose
RRT	relative retention time
Rw	ratio of carbohydrates extracted from tissue grown at 31 °C, to that extracted from tissue grown at 21 °C.
s	second
SD	standard deviation
TFA	trifluoroacetic acid
UDP	uridine 5'-diphosphate
UroA	uronic acid
v	volume
w	weight
XG	xyloglucan
Xyl	D-xylose

LIST OF ABBREVIATIONS

FOR PMAAs

For PMAAs of the Hexoses (Hex, hexitol: Glc, glucitol; Gal, galactitol; Man, mannitol)

2,3,4,6-Me_4 Hex	1,5-di-*O*-acetyl-(1-deuterio)-2,3,4,6-tetra-*O*-methyl hexitol
2,4,6-Me_3 Hex	1,3,5-tri-*O*-acetyl-(1-deuterio)-2,4,6-tri-*O*-methyl hexitol
3,4,6-Me_3 Hex	1,2,5-tri-*O*-acetyl-(1-deuterio)-3,4,6-tri-*O*-methyl hexitol
2,3,4-Me_3 Hex	1,5,6-tri-*O*-acetyl-(1-deuterio)-2,3,4-tri-*O*-methyl hexitol
2,3,6-Me_3 Hex	1,4,5-tri-*O*-acetyl-(1-deuterio)-2,3,6-tri-*O*-methyl hexitol
2,6-Me_2 Hex	1,3,4,5-tetra-*O*-acetyl-(1-deuterio)-2,6-di-*O*-methyl hexitol
4,6-Me_2 Hex	1,2,3,5-tetra-*O*-acetyl-(1-deuterio)-4,6-di-*O*-methyl hexitol
3,6-Me_2 Hex	1,2,4,5-tetra-*O*-acetyl-(1-deuterio)-3,6-di-*O*-methyl hexitol
2,4-Me_2 Hex	1,3,5,6-tetra-*O*-acetyl-(1-deuterio)-2,4-di-*O*-methyl hexitol
3,4-Me_2 Hex	1,2,5,6-tetra-*O*-acetyl-(1-deuterio)-3,4-di-*O*-methyl hexitol
2,3-Me_2 Hex	1,4,5,6-tetra-*O*-acetyl-(1-deuterio)-2,3-di-*O*-methyl hexitol
2-Me Hex	1,3,4,5,6-penta-*O*-acetyl-(1-deuterio)-2-*O*-methyl hexitol
3-Me Hex	1,2,4,5,6-penta-*O*-acetyl-(1-deuterio)-3-*O*-methyl hexitol
4-Me Hex	1,2,3,5,6-penta-*O*-acetyl-(1-deuterio)-4-*O*-methyl hexitol
6-Me Hex	1,2,3,4,5-penta-*O*-acetyl-(1-deuterio)-6-*O*-methyl hexitol
Hex-$(OAc)_6$	1,2,3,4,5,6-hexa-*O*-acetyl-(1-deuterio)-hexitol
myo-inositol-Ac_6	1,2,3,4,5,6-hexa-*O*-acetyl-myo-inositol

For PMAAs of the Pentoses (Pent, pentitol: Xyl, xylitol; Ara, arabinitol)

2,3,5-Me_3 Pent	1,4-di-*O*-acetyl-(1-deuterio)-2,3,5-tri-*O*-methyl pentitol
2,3,4-Me_3 Pent	1,5-di-*O*-acetyl-(1-deuterio)-2,3,4-tri-*O*-methyl pentitol
2,4-Me_2 Pent	1,3,5-tri-*O*-acetyl-(1-deuterio)-2,4-di-*O*-methyl pentitol
2,3-Me_2 Pent	1,4,5-tri-*O*-acetyl-(1-deuterio)-2,3-di-*O*-methylpentitol

3,4-Me_2 Pent	1,2,5-tri-*O*-acetyl-(1-deuterio)-3,4-di-*O*-methyl pentitol
2-Me Pent	1,3,4,5-tetra-*O*-acetyl-(1-deuterio)-2-*O*-methyl pentitol
3-Me Pent	1,2,4,5-tetra-*O*-acetyl-(1-deuterio)-3-*O*-methyl pentitol
4-Me Pent	1,2,3,5-tetra-*O*-acetyl-(1-deuterio)-4-*O*-methyl pentitol
Pent-$(Ao)_5$	1,2,3,4,5-penta-*O*-acetyl-(1-deuterio)-pentitol

For PMAAs of the 6-Deoxy-Hexoses (Deo, 6-deoxy-hexitol: Fuc, fucitol; Rha, rhamnitol)

2,3,4-Me_3 Deo	1,5-di-*O*-acetyl-(1-deuterio)-2,3,4-tri-*O*-methyl-6-deoxy-hexitol
2,3-Me_2 Deo	1,4,5-tri-*O*-acetyl-(1-deuterio)-2,3-di-*O*-methyl-6-deoxy-hexitol
2,4-Me_2 Deo	1,3,5-tri-*O*-acetyl-(1-deuterio)-2,4-di-*O*-methyl-6-deoxy-hexitol
3,4-Me_2 Deo	1,2,5-tri-*O*-acetyl-(1-deuterio)-3,4-di-*O*-methyl-6-deoxy-hexitol
2-Me Deo	1,3,4,5-tetra-*O*-acetyl-(1-deuterio)-2-*O*-methyl-6-deoxy-hexitol
3-Me Deo	1,2,4,5-tetra-*O*-acetyl-(1-deuterio)-3-*O*-methyl-6-deoxy-hexitol
4-Me Deo	1,2,3,5-tetra-*O*-acetyl-(1-deuterio)-4-*O*-methyl-6-deoxy-hexitol
Deo-$(OAc)_5$	1,2,3,4,5-penta-*O*-acetyl-(1-deuterio)-6-deoxy-hexitol

Deduced linkages are abbreviated by prefixing the monosaccharide abbreviation with numbers to indicate the linkage or with a "t" to indicate the terminal or non-reducing end of the polysaccharide chains. The suffix "*p*" or "*f*" indicates pyranose or furanose ring form, respectively.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION

Cellulose is the most abundant naturally occurring organic polymer and is the major polysaccharide component of most plant cell walls. In the cell wall, cellulose is embedded in a matrix of two major types of polysaccharide: pectins and hemicelluloses. These each comprise several individual polysaccharides such as xyloglucan (XG), glucuronoarabinoxylan (GAX; (1,3), (1,4)-β–glucan), etc, in the case of hemicelluloses (Duffus and Duffus, 1984; Dahlgren et al, 1985; Bacic et al, 1988; Fry, 1988; Kennedy et al, 1989).

Understanding cellulose biosynthesis is important scientifically, because cellulose influences the mechanical properties of the cell wall and controls many of the distinctive feature of plant morphogenesis. It also has potential industrial importance in the cotton and forest products industries.

However, the extraordinary complexity of cell wall structure and the diversity of its functions during plant cell growth and development have contributed to limiting knowledge of cell wall formation (Seitz and Emmerling, 1990). In particular, the difficulty of isolating and purifying functional cellulose synthase has greatly limited understanding of cellulose biosynthesis in plants (Blaschek et al, 1983; Delmer, 1987; Tarchevsky and Marchenko, 1991; Gibeaut and Carpita, 1994; Delmer and Amor, 1995; Kudlicka et al, 1995; Kawagoe and Delmer, 1998).

In this study, attempts to understand cellulose biosynthesis are made using several temperature-sensitive *Arabidopsis thaliana* mutants which are believed to be defective in cellulose production (Arioli et al, 1995). Comparison of the biochemical and genetic characteristics of the mutants and *Arabidopsis* wild type will help in understanding the biosynthesis of cellulose and other wall polysaccharides during wall formation.

1.2 GENERAL FEATURES OF PLANT CELL WALLS

The cell wall is the layer of structural material which surrounds the plant cell and is external to the protoplast. It is a product of cell metabolism and is composed mainly of microfibrils embedded in a gel-like matrix of polysaccharides, with smaller amounts of glycoproteins and phenolic compounds. The microfibrils are cellulose while the polysaccharides of the matrix are heteropolysaccharides which include pectins and hemicelluloses, etc (Elbein, 1980; Duffus and Duffus, 1984; Dahlgren et al, 1985; Reese, 1988; Showalter, 1993; Eleftheriou, 1994).

A cell wall rich in carbohydrates distinguishes plants from animals cells. It determines the size, shape and form of plant cells and strictly influences the structural and mechanical properties of the cells. The plant cell wall can be divided into primary and secondary cell walls (Bacic et al, 1988). Synthesis of cell wall is initiated as cell plates during cell division and is characterised by the deposition of matrix polysaccharides (Aspinall, 1980; Hall et al, 1982). As the cells continue to grow, further accumulation of matrix polysaccharides together with microfibrils, forms the primary cell wall. The adjacent cell walls are separated from each other by a layer called the middle lamella, in which pectin is a major component (Jarvis, 1984; Fry, 1988). After differentiation has started, secondary wall is formed and located between the cell membrane and the primary wall (Roberts, 1989 and 1990). In some mature plants, it is difficult to discern the primary cell wall from the very thick secondary wall (Hall et al, 1982; Stevens and Selvendran, 1984; Bacic et al, 1988).

A typical primary wall is about 0.1 μm thick with a biphasic structure (rigid microfibrils and gel-like matrix). The microfibrils lie parallel to the plane of the cell surface and their orientation within that plane has been suggested to determine the direction of cell elongation (elongation perpendicular to the microfibrils) in some cells (eg. Wardrop et al, 1979). New microfibrils are

deposited with a uniform orientation (typically perpendicular to the principal axis of cell elongation) and old microfibrils, according to the multinet growth hypothesis, are passively reorientated by cell expansion (Richmond, 1983; Lamport and Epstein, 1983; Neville, 1985; Fry, 1988). In other cells, however, the orientation of microfibril deposition changes rhythmically so that the wall has a helicoidal structure (Vian and Roland, 1987). Although new microfibrils are deposited parallel to cortical microtubules (Bolwell, 1993), there is no direct evidence showing how microtubules govern microfibril orientation (Seagull, 1990; Emons et al, 1992).

In growing primary walls, water comprises 65% of total wall fresh weight, and almost all exists in the matrix (Northcote, 1972). The primary walls of dicotyledons have extensive similarities in polysaccharide composition. Typically these walls contain a high proportion of pectic polysaccharides (Aspinall and Jiang, 1974; deVries et al, 1983; Jarvis, 1984), relatively small amount of hemicellulosic polysaccharides, such as XG and GAX (Bauer et al, 1973; Hayashi, 1989; Carpita and Gibeaut, 1993), with 9-40% cellulose (Aspinall, 1953, 1959, 1967, 1980 and 1982; Jeffries, 1963; Kennedy et al, 1989; Zablackis et al, 1995). Grasses in contrast have much smaller amounts of pectins and xyloglucan, and substantial amounts of alternative polymers [(1,3), 1,4)-β-glucan and GAXs] and similar quantities of cellulose (James et al, 1985; Dahlgren et al, 1985; Carpita and Gibeaut, 1993). The alternative polymers in grasses presumably have the same functions as pectins and xyloglucan in dicotyledons, because the primary walls of grasses and dicotyledons have similar general characteristics, especially similar responses to growth-controlling agents (Darvill et al, 1980; Zhong and Lauchli, 1993).

The secondary wall layer is often much thicker and harder than the primary wall (reviewed by Bacic et al, 1988) and has a distinctive polysaccharide composition (reviewed by Aspinall, 1980; Fry, 1988) and morphology

(Shafizadeh and McGinnis, 1971; Brett and Waldron, 1990). It has a higher proportion of cellulose and a lower proportion of matrix material than the primary wall (Northcote, 1972; Darvill et al, 1980; Chesson et al, 1985; Dahlgren et al, 1985) leading to a much lower water content. Lignin may be deposited in the secondary wall, although lignification often begins in the primary walls, or middle lamellae (Northcote, 1972; Hoson, 1991). In lignified secondary walls, 4-*O*-methylglucuronoxylans are major matrix materials in Angiosperms, and galactomannans and glucomannans are mainly present in Gymnosperms (Northcote, 1972). But in the unlignified secondary walls of the cells of cotyledons, the matrix mainly contains mannans, galactomannans and glucomannans (Bacic et al, 1988; Duchesne and Larson, 1989).

The life cycle of the flowering plant involves a complex series of processes in growth and differentiation. Throughout its life, the plant requires the cell wall to provide mechanical strength to construct the plant body and provide protection against the environment (Darvill et al, 1980; Labavitch, 1981; Bishop and Ryan, 1987; Anderson and Beardall, 1991). Meanwhile, the cell wall must allow the plant to grow by weakening at appropriate times. When plant cells differentiate, dramatic changes occur in their cell walls and each differentiated cell forms its characteristic cell wall (Sakurai, 1991). The plant cell changes its cell wall architecture during growth and development through the synthesis and degradation of wall polysaccharides. Changes of chemical components in the cell wall result not only from synthesis and degradation as introduced in Sections 1.5 and 1.6, but also from shifts in the molecular-weight distribution for some component polysaccharides (Thornber and Northcote, 1962; Labavitch, 1981; Hall et al, 1982; Northcote, 1985). Alterations in the chemical structure of the cell wall change its physical properties (Brett and Waldron, 1990).

1.3 THE NATURE OF MAJOR WALL POLYSACCHARIDES

Some polysaccharides contain a single type of monosaccharide subunit and are termed homoglycans, or homopolymers, while other polysaccharides with more than one type of monosaccharide subunit are called heteroglycans (Duffus and Duffus, 1984). The degree of polymerisation (DP) indicates the number of monosaccharide subunits present and varies for different polysaccharides (Aspinall, 1980). In the plant cell wall, the non-cellulosic polysaccharides are further divided into two groups: acidic polysaccharides (e.g. pectins and GAXs) and neutral polysaccharides [e.g. XG, β-(1,3; 1,4)-glucan, and callose; Northcote, 1972; Wada and Ray, 1978; Dahlgren et al, 1985; York et al, 1985; Carpita and Kanabus, 1988].

1.3.1 Cellulose

1.3.1.1 Definition of Cellulose

Cellulose is the fibrillar component of higher plant walls, varying in amount from approximately 2-4% in cereal endosperm walls to approximately 94% in the secondary walls of cotton seed hairs (Fry, 1988; Kennedy et al, 1989). It is a high molecular weight polymer of D-glucopyranose resides containing as many as 14,000 subunits that are joined by β-1,4-glucosidic linkages (Harland, 1959; Duffus and Duffus, 1984). The polymers are flat and extended ribbons in which the hydroxyl at C-3 is hydrogen bonded to the ring oxygen of a monosaccharide in an adjacent chain. In the cell walls, this intermolecular hydrogen bonding between parallel chains leads to a compact, tightly bonded structure that is fibrous, insoluble in aqueous solvents and mechanically strong (Duchesne and Larson, 1989; Hatakeyama, 1989; Sugiyama et al, 1991a).

The width and DP of the cellulose microfibrils are highly variable according to the source and age of the plant tissue, and the DP of glucan chains in microfibrils has been best characterised in walls of developing cotton seed hairs (Harland, 1959; Zhu et al, 1984; Zhong and Lauchli, 1993). From X-ray

diffraction, electron microscopy and chemical studies, the microfibrils are crystalline β-1,4-glucan (Woods, 1959; Marrinan, 1959; Sotton, 1980). However, complex cellulose microfibril and diverse crystalline sizes are found in nature (Woods, 1959; Hatakeyama, 1989; Roberts, 1989 and 1990; Kuga et al, 1993; Lee et al, 1994).

1.3.1.2 Heterogeneity of Cellulose

It has been suggested that cellulose microfibrils may have structural heterogeneity (reviewed by Brett and Waldron, 1990): (1) Chemical analyses of the cellulose fraction of cell walls (obtained after all other wall polysaccharides were removed) indicated that the crystalline β-1,4-glucan (microfibrils) may contain other sugars (mannose and xylose). (2) The microfibrils observed from X-ray diffraction were rather thinner than ones seen by electron microscopy, which suggests that the microfibrils may consist of a crystalline β-1,4-glucan core surrounded by a non-crystalline or poorly crystalline layer or possibly some crystalline "elementary fibrils" lying side by side. (3) Less crystalline regions occasionally interrupted the central crystalline structure, suggesting that the non-glucose residues (mannose and xylose) may exist in the less crystalline regions. (4) Disruption of microfibrils by strong acids supports the idea that less crystalline regions occur in the microfibrils. Moreover, some chemicals (dyes and fluorescent brighteners) alter the microfibril pattern and an amorphous material is deposited in the wall (Robinson and Quader, 1981; Quader et al, 1983; Richmond, 1983; Haigler and Chanzy, 1988; Knight et al, 1993). The amorphous material is presumed to be a poorly-crystalline β-1,4-glucan (reviewed by Quader, 1991). In the wall of *Phytophthora*, a poorly crystalline β-1,4-glucan was also observed using X-ray diffraction (Bartnicki-Garcia, 1966; Burnett and Trinci, 1979). Various models of microfibril substructure have been proposed to account for amorphous and crystalline regions in native cellulose (reviewed by Shafizadeh and McGinnis, 1971; French, 1987; Blanton, 1987;

Haigler, 1991). These models are mainly based on three concepts: (1) continuous glucan chains which pass through alternating crystalline and para-crystalline regions along the length of the fibril; (2) crystallites which alternate with paracrystalline regions throughout the width of the fibril; (3) an overall continuous lattice which is interrupted by distortions (Haigler, 1987).

1.3.1.3 Crystallite Pattern of Cellulose

A given crystalline form of a material always gives the same type of diffraction pattern. The fractions of crystalline and non-crystalline materials can be determined by various diffraction methods (French, 1987). The crystallite size, shape and perfection of cellulose are characterised using X-ray diffraction. Electron diffraction is especially used to study small crystals of short chain polysaccharides (Hebert et al, 1973) and neutron diffraction has advantages for single-crystal studies of cellulose. In general, there are four major crystalline lattices for celluloses termed cellulose I, II, III, and IV which have different histories. Each allomorph is identified by its unit cell constants rather than any other criterion (Woods, 1959; French, 1987; Kennedy et al, 1989).

Native celluloses from many different sources all show a similar diffraction pattern, due to a common structure referred to as cellulose I (Chanzy, 1979; Sugiyama et al, 1991b). Although clear differences in perfection and angular orientation of the crystallites are still observed between different samples, they all have the same major diffraction maxima and the same proportion of intensity. The crystallographic form of cellulose synthesised by plants and bacteria is cellulose I (Leppard and Colvin, 1978; Lee et al, 1994). The individual chains within the microfibrils in native cellulose are all parallel, but adjacent microfibrils can be antiparallel to each other (Haigler, 1987). Cellulose II is formed when cellulose I is dissolved and precipitated, or treated with a concentrated alkaline as a swelling agent and washed with water (mercerisation). The transformation from I to II is irreversible. Only one report indicates that a

short chain cellulose II can be changed to cellulose I at high temperature (VanderHart and Atalla, 1983). Thus, cellulose I is the most common crystalline form and cellulose II is the most stable allomorph. Cellulose II is believed to consist of antiparallel chains. Antiparallel chains are also reported for mercerised cotton (Kolpak et al, 1978) and for Fortisan rayon, the most crystalline regenerated cellulose known (Sarko et al, 1976). Although mercerisation can transform cellulose I to cellulose II without dissolving the material, the mechanism of conversion from parallel to antiparallel remains obscure. The studies just described suggest that the process of conversion requires adjacent microfibrils and intermixes individual chains from different microfibrils (Kolpak et al, 1978). It is further assumed by French (1987) that the swollen microfibrils involved in the conversion from native to mercerised cellulose allow chains to intermix and form a structure of lower energy.

After cellulose samples are swollen with amines or liquid ammonia and subsequently have the swelling agent removed anhydrously, cellulose III is formed. It is a very stable allomorph with subclasses III_I and III_{II} resulting from use of different starting materials (cellulose I and II; Sarko et al, 1976). Samples subjected to high temperature form cellulose IV. Like cellulose III, it also has two subclasses (IV_I and IV_{II}) depending on their sources (Hayashi et al, 1975).

1.3.1.4 Accessibility of Cellulose

Chemical methods may provide additional information beyond that coming from physical measurements such as X-ray diffraction (reviewed by Rowland and Bertoniere, 1987). The physical and chemical characteristics of native celluloses, for example, point to crystallinity decreasing and accessibility increasing in a sequence such as: *Valonia ventricosa*, ramie, cotton, and rayon. Many treatments have been employed to measure the variation in accessibility of different cellulosic substrates including moisture regain (Jeffries, 1963), deuteration (Mann, 1963), acid hydrolysis (Section 1.4.3), periodate oxidation

(Hay et al, 1965). Each type of accessibility measurement shows its own characteristic deviation from the values detected by X-ray diffraction. Generally, X-ray diffraction for crystallinity measurement of cellulose is one way to characterise a solid state having ordered regions above a minimum size, but chemical treatments provide other ways to probe the availability of internal surface, volumes, or hydroxyl groups, with each agent treatment having its own characteristic size, swelling, and penetrating capability (Rowland and Bertoniere, 1987).

1.3.2 Hemicelluloses

Hemicelluloses are polysaccharides containing a diversity of different monosaccharide subunits. They can be effectively dissolved in different concentrations of alkali. The main types of hemicelluloses are described below (Northcote, 1972; Bauer et al, 1973; Aspinall, 1959 and 1980; Carpita, 1984).

1.3.2.1 Xyloglucan

XG are neutral polysaccharides that have a linear extended backbone of β-1,4-linked D-glucopyranosyl residues identical to cellulose. These are substituted at the C6 hydroxyl with α-D-xylopyranosyl residues (McNeill et al, 1984; Fry 1988; Hayashi, 1989; Zablackis et al, 1995). Some heterogeneity exists in their fine structure particularly regarding the substituents on the xylopyranosyl residues, which include β-D-galactopyranosyl-(1,2)-, α-L-arabinofuranosyl-(1,2)- and α-L-fucopyranosyl-(1,2)-β-D-galactopyranosyl-(1,2)- side chains. From the form and pattern of XG, at least three types of XG have been recognised based on the fundamental heptasaccharide unit structure and the addition of mono- and disaccharide substituents (York et al, 1984; McDougall and Fry, 1991; Zablackis et al, 1995). In dicotyledonous plants, a hepta-saccharide is found as a structural unit common to all xyloglucans which contains cellotetraosyl residues substituted through the C6 hydroxyl on three residues by single xylopyranosyl residues. The molecular weights of xyloglucans

in different plants range from 7,600 to 180,000 Da (Bacic et al, 1988). They are thought to interact with other wall components either by noncovalent interactions (hydrogen bonding to cellulose) (Levy et al, 1991) or perhaps covalent interactions with pectic polysaccharides (Mori et al, 1980; Hayashi et al, 1981; Hayashi, 1989; Iiyama et al, 1994).

1.3.2.2 Glucuronoarabinoxylan

GAXs comprise a family of polysaccharides consisting of a linear β-1,4-linked-D-xylopyranosyl backbone substituted by an array of mono- and oligosaccharide side chains and by *O*-acetyl groups and phenolic acids. The main substituents include single α-L-arabinofuranosyl and α-D-glucuronosyl (or its 4-*O*-methyl derivative) residues attached to the backbone xylopyranosyl residues at the C3 hydroxyl and C2 hydroxyl (Fry, 1988). In some GAXs, the single α-L-arabinofuranosyl residues can also be linked through the C2 hydroxyl as well as both the C2 and C3 hydroxyl of the backbone xylopyranosyl residues. The (1,4)-linked xylopyranosyl residues of the backbone may be acetylated at the C2 hydroxyl, C3 hydroxyl, or both (Aspinall, 1980; Gowda and Sarathy, 1987; Zablackis et al, 1995). The degree and pattern of substitution of the xylan backbone determines the shape of the molecule and its solution properties. Substitution of the xylan backbone by arabinosylation or acetylation, for instance, led to the xylan backbone taking a more extended conformation and increased the solubility (Waldron and Brett, 1983), presumably by preventing extensive intermolecular hydrogen bonding (reviewed by Gibeaut and Carpita, 1994). Typically GAXs make up roughly 5% of the primary cell wall in dicots, 20% of the primary walls of grasses, and 20% of the secondary walls in both dicots and grasses (Fry, 1988; Reiss et al, 1994).

1.3.2.3 (1,3), (1,4)-β-D-Glucans

The (1,3), (1,4)-β-D-glucans comprise a family of polymers of β-D-glucopyranosyl residues attached by both (1,3)- and (1,4)-glycosidic linkages.

They are heterogeneous with respect to molecular size and fine structure, varying with tissue, age and source (reviewed by Carpita and Gibeaut, 1993). Normally they consist of approximately 30% β-1,3- and 70% β-1,4-D-glucosidic linkages with more than 90% of the molecule containing β-1,3-D-linked cellotriosyl and cellotetraosyl residues. However, individual (1,3)- and (1,4)-linked residues, blocks of more than three contiguous (1,4)-linked residues, and regions of contiguous (1,3)-linked residues may be present (Buliga et al, 1986). The molecular weight of (1,3), (1,4)-β-D-glucans is generally estimated to range from 20,000 to 1,000,000 Da (Bacic et al, 1988). The (1,3), (1,4)-β-D-glucans are asymmetric molecules assuming an extended conformation in aqueous solution. They are not major components in dicots but are abundant neutral polysaccharides in the cell walls of grasses (Goldberg, 1985; Gibeaut and Carpita, 1994).

1.3.2.4 β-Mannans

β-Mannans possess a backbone of β-1,4-linked-D-mannopyranosyl residues, which in the case of (galacto)glucomannan is interrupted by a smaller proportion (25-35%) of β-D-glucopyranosyl residues. They are major components of endosperm walls of some seeds (Aspinall et al, 1953) and occur in moderate amounts in certain secondary cell walls and perhaps in small amounts in primary walls (Aspinall et al, 1953 and 1980; Bacic et al, 1988).

1.3.2.5 Galactomannans

Galactomannans comprise a β-1,4-linked D-mannan backbone substituted at C6 hydroxyl with single α-D-galactopyranosyl residues. Some members of the galactomannan family have β-1,3-D- and β-1,2-D-linkages in the mannan backbone, with substitution at the C2 hydroxyl of some β-1,3-D-linked mannopyranosyl residues with single α-D-galactopyranosyl residues. Galactomannans occur primarily in the endosperm walls of the seeds of Leguminosae and have a storage role. Unlike the β-1,4-D-mannans, they are

water-soluble and their solubility properties depend on the mannose: galactose ratio (Aspinall, 1959; McCleary, 1981).

1.3.3 Pectins

Pectins are considered the most complex class of wall polysaccharides and have been described as 'block' polymers (Aspinall, 1980; Jarvis, 1984; Bacic et al, 1988). They normally consist of a family of acidic polysaccharides (rhamno-galacturonans) and several neutral oligosaccharides and polysaccharides (arabinans, galactans, and arabinogalactans) which may be covalently attached to the rhamnogalacturonan backbone primarily through the rhamnopyranosyl residues. Pectic polysaccharides can be extracted from walls with water or aqueous solutions of chelating agents (Aspinall, 1982) or with purified polysaccharide hydrolases (Fry, 1988). Their molecular weights range from 30,000-300,000 Da (Northcote, 1972; Aspinall and Jiang, 1974; Jarvis, 1984; O'Neil et al, 1990; Kikuchi et al, 1996).

1.3.3.1 Homogalacturonan

Homogalacturonan is one of the major pectins in some plant cell walls, and consists mainly of contiguous unbranched α-1,4-linked D-galacturonic acid residues. More typically, the stretches of polygalacturonic acid are interrupted by rhamnose residues at about every 25 galacturonic acids units to give rise to rhamnogalacturonan, the major pectin backbone. The rhamnose residues may act as molecular 'punctuation marks' with the intervening homogalacturonan block being in some cases fully methylesterified and in others not esterified at all. They may also act as anchorage points for neutral side-chains such as arabinan, galactan or arabinogalactan (Fry, 1988).

1.3.3.2 Rhamnogalacturonan I (RG I)

The rhamnogalacturonan (RG I) backbone comprises α-1,4-linked D-galacturonosyl residues interspersed with α-1,2-linked-L-rhamnopyranosyl residues. Pectic preparations from some walls show uninterrupted homo-galacturonan regions with a DP of about 70, and a repeating sequence consisting

of galacturonosyl and rhamnopyranosyl residues. In some instances, the main neutral chains are attached to the backbone by the C4 hydroxyl of the 1,2-linked rhamnopyranosyl residues, and substitution of the galacturonosyl residues at either the C2 or C3 hydroxyl is found as well (deVries et al, 1983; Jarvis, 1984). Pectic polysaccharides isolated from different sources have varying degrees of methylesterification and hydroxyl acetylation of the 1,4-linked galacturonosyl residues (Aspinall, 1967 and 1974). Galacturonan chains assume a helical conformation in solution which enables chains to align alongside each other rather than becoming intertwined (Aspinall, 1982; Iiyama et al, 1994). The solution properties of the acidic backbone cause them to form gels (McNeill et al, 1980; Bacic et al, 1988).

1.3.3.3 Rhamnogalacturonan II (RG II)

Rhamnogalacturonan II (RG II) consists of approximately 60 glycosyl residues that include 2-*O*-methyl fucose, 2-*O*-methyl xylose, apiose, aceric acid (3-C-carboxyl-5-deoxy-L-xylose) and 3-deoxy-D-*manno*-octulosonic acid. Two heptasaccharide repeat units form about 60% of the molecule. RG II polysaccharides are isolated by hydrolysis with α-(1,4)-endopolygalacturonase and their presence in primary walls of other plants is inferred from the presence of unusual glycosyl residues determined with methylation analyses (Spellman et al, 1983; Stevenson et al, 1988).

Phenolic substitution of pectic polysaccharides has been observed by esterification to neutral sugars in polysaccharides from the pectic fraction of walls of suspension cultured spinach and sugar beet. Two oligosaccharide fragments contain ferulic acids which are characterised as 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose and 3(?)-*O*-(3-*O*-feruloyl-α-L-arabinofuranosyl)-L-arabinose (Fry, 1979 and 1983).

1.3.3.4 Arabinans

Arabinans as side-chain substituents, are polymers of α-1,5 linked-L-arabino-furanosyl residues branched through the C2 hydroxyl, the C3 hydroxyl or both. The arabinan may be also attached to the acidic rhamnogalacturonan through the C4 hydroxyl of the 1,2-linked rhamnopyranosyl residues via one to three β-1,4-linked D-galactopyranosyl residues. A linear α-1,5-linked L-arabinan can aggregate to form microcrystalline particles (McNeill et al, 1982, Bacic et al, 1988).

1.3.3.5 Galactans and Arabinogalactans

Galactans and pectic arabinogalactans are polymers of β-1,4-linked-D-galactopyranose residues that exist in the seed wall of particular species in small amounts (Bacic et al, 1988; Carpita and Gibeaut, 1993). Usually the galactan backbone is substituted with α-L-arabinofuranosyl side chains. They are constituents of extracellular spaces and possibly associated with proteins of the plasma membrane (Aspinall et al, 1967; Aspinall, 1980; Jarvis, 1984).

1.3.4 Callose

Callose is a polymer of β-1,3-linked D-glucopyranose units. It normally exists in higher plants as a component of special walls (e.g. pollen mother wall and cotton seed hairs) and appears at particular developmental stages. It is also formed in response to wounding by physical or chemical stimuli or to infection by microorganisms (Bishop and Ryan, 1987; Gibeaut and Carpita, 1994). In the plant wall, callose may be soluble in water, or acidic and alkaline solutions, and most preparations of callose are polydisperse (Stone and Clarke, 1992).

1.4 WALL POLYMER INTERACTIONS AND FRACTIONATION FOR POLYSACCHARIDE ANALYSIS

1.4.1 Polymer Interaction

Although wall polysaccharides are well characterised (Section 1.3), there are gaps in our knowledge about the molecular organisation and interaction of these polymers in the wall (Hoson, 1991). Models based on the physical (noncovalent) and chemical (covalent) associations among wall polysaccharides are fundamental to understanding wall structure (Levy et al, 1991; Carpita and Gibeaut, 1993). However, the full range of walls can not be encompassed by a single model due to differences in composition and function (Hearle, 1982; Selvendran, 1985; Iiyama et al, 1994).

In the primary walls of dicotyledons, pectic polysaccharides are the most important gel-forming polymers and are particularly rich in the cell plate at the earliest stage of wall formation (Jarvis, 1984). Cellulose microfibrils oriented periclinally and glycoproteins (e.g. extensin) anticlinally form the reinforcing frame in the wall (Lamport and Epstein, 1983; Bacic et al, 1988). Xyloglucans can coat cellulose microfibrils by hydrogen bonding and provide a key interaction between cellulose microfibrils and matrix polysaccharides (Bauer et al, 1973; Darvill et al, 1980). Covalent associations between gel-forming pectins and xyloglucan may also stabilise and strengthen the wall (Verma et al, 1982; Northcote, 1985; Selvendran, 1985; Morris, 1986; Wu et al, 1988; Roberts, 1989). Polysaccharide-protein, and polysaccharide-phenol-protein associations also exist in the wall (Fry, 1979, 1983 and 1988).

In the secondary wall, associations of cellulose microfibrils with the extended, relatively regular non-cellulosic polymers and with the lamellated lignin form a wall that is more rigid and has higher tensile strength than the primary wall (Northcote, 1972, Bacic et al, 1988).

1.4.2 Wall Fractionation

Approaches to plant cell wall fractionation have involved extraction of the cell wall to separate wall polysaccharides into different fractions (Selvendran, 1985; Brett and Waldron, 1990). Purification of wall polysaccharides requires information on the nature and character of each polysaccharide (Section 1.3) and about the interaction of wall polysaccharides as described previously (Section 1.4.1). Generally, procedures used for wall fractionation use chelating agents to remove divalent cations from pectic polysaccharides, alkali to disassociate hydrogen bonding between hemicelluloses and cellulose, and an acylating acid mixture to solubilise other materials. The final pellet insoluble in those solutions is expected to be crystalline cellulose (Scott, 1960 and 1965; Selvendran, 1985). Further extractions often use specific enzymes to release a particular polysaccharide from the wall, and other chemical reagents, such as 2% cetyltrimethylammonium bromide (CTAB; Fry, 1988) can be used to precipitate acidic polymers (e.g. pectins) from neutral polymers (e.g. XG). Care must be taken to minimise modification of the polysaccharides by endogenous enzymes or by the reagents used. However, there is no perfect procedure to extract wall polysaccharides in pure form (Aspinall, 1953, 1967 and 1982; Carpita, 1984; Carpita and Kanabus, 1988; Hoson, 1991). The extraction approach has also imposed certain limitations on views of wall polysaccharides. Extraction and purification of the wall components, for example, may cause only partial recovery of a particular type of molecule, and the recovered material may not represent all components. To overcome the potential loss, different extraction methods are compared especially chemical and enzymic extraction methods (Wada and Ray, 1978; Carpita and Kanabus, 1988; Fry, 1988; Heim et al, 1990b; Zablackis et al, 1995).

1.5 BIOSYNTHESIS OF WALL POLYSACCHARIDES

1.5.1 General Features of Polysaccharide Synthesis

The review by Delmer and Stone (1988) proposed that biosynthesis of plant cell wall polysaccharides involves three stages: prepolymerisation for precursor preparation in a subcellular compartment, polymerisation of chains on the membrane, and deposition of chains into the walls.

(1) Prepolymerisation is the process in which monomers (substrates, or precursors) are synthesised, activated and transported to polymerisation sites. The substrates are sugar nucleotides, or nucleoside diphosphate sugars which contain a monosaccharide attached through its glycosidic hydroxyl to the β-phosphate of a ribonucleoside (uridine, or guanidine) diphosphate (Ericson and Elbein, 1980; Reese, 1988; Gordon and Maclachlan, 1989). Most substrates are generated from the cellular pool of hexose phosphate by soluble nucleoside triphosphate pyrophosphorylases (Hall et al, 1982). They are formed by pathways in which the main flow of material is through UDP-glucose (UDP-Glc) derived from glucose-1-phosphate, or sucrose (Aloni et al, 1983; Kleczkowski, 1994). The enzyme associated with UDP-Glc provision for wall polysaccharide synthesis is considered to be a key enzyme of carbohydrate metabolism in sink organs, e.g. growing leaves (Hall et al, 1982; Claussen et al, 1985). Only small amounts of GDP-glucose are found in several plants and its pyrophosphorylase is present in many plants of mono- and dicots (Aloni et al, 1982; Barber, 1985). Sucrose or its phosphate may also be substrates for polysaccharide biosynthesis (Salvucci and Klein, 1993; Martin et al, 1993). In addition, UDP-Glc is the substrate for the formation of the UDP-derivatives of Gal*p*, Gal*p*U, Xyl*p*, Api*f*, Ara*p* and Rha*p* via epimerase, oxido-reductase and decarboxylase reactions (Duffus and Duffus, 1984; Gibeaut and Carpita, 1994).

Because it is unclear whether the enzymes generating precursors for polysaccharide biosynthesis are located in the lumen of the endoplasmic

reticulum, in the Golgi body, in the cytosol, or at the internal face of the plasma membrane, the control of the supply of precursors to the sites of polysaccharide synthesis is poorly understood (reviewed by Delmer and Stone, 1988). Experiments on glycosyl-transferases associated with membranes in plant and animal cells have suggested that UDP and GDP are effective inhibitors of UDP-Glc uptake and may act as allosteric inhibitors of glycosyltransferase reactions within the membrane (Delmer and Stone, 1988).

(2) The Golgi body is the site of synthesis of all wall matrix polysaccharides (Dhugga et al, 1991, Gibeaut and Carpita, 1993 and 1994), whereas cellulose (and perhaps callose; Section 1.5.2.2) is polymerised and deposited in the wall of higher plants by a plasma-membrane-localised cellulose synthase complex (Montezinos and Brown, 1976; Mueller and Brown, 1980; Mizuta and Harada, 1991; Mizuta and Brown, 1992b; Qi et al, 1995). The biosynthesis of all wall polysaccharides can be divided into three steps: chain initiation, chain extension, and chain termination (reviewed by Delmer and Stone, 1988).

Very little is known about the mechanism of chain initiation, even for well-studied polymers such as starch (James et al, 1985; Preiss, 1988; Delmer and Stone, 1988; Martin and Smith, 1995, Delmer and Amor, 1995). Limited evidence has suggested that there may be protein or polysaccharide primers for chain initiation, but it is not demonstrated that the primer remains attached to the final product. Based on understanding of chain elongation of bacterial wall polymers (James et al, 1985), lipid intermediates are expected to play a role in plant cell wall polysaccharide synthesis for chain initiation and extension (Hopp et al, 1978; Lezica et al, 1978; Helsper, 1979; Camirand et al, 1984). There is, however, no direct evidence to support lipid-linked intermediates in higher plant biosyntheses. From preliminary studies on β-glucan synthesis *in vitro* (Henry and Stone, 1982; Blaschek et al, 1983 and 1985; Pont-Lezica et al, 1986), it is presumed that the direction of chain extension is from the non-reducing end

(Delmer and Stone, 1988). No studies have given evidence regarding the mechanism of chain termination. Cellulose biosynthesis in cotton fibers is an interesting example of regulation of chain termination (Basra and Malk, 1984; Timpa and Triplett, 1993). The DP of cellulose may be a good indicator with which to examine the regulation of chain termination, because it is much increased in the secondary wall and temperature has little effect on the DP of cellulose, but large effects on the rate of deposition of polymers (Zhu et al, 1984; Ryser, 1985; Delmer and Stone, 1988).

(3) Because wall matrix polysaccharides are polymerised within the Golgi body (Dhugga et al, 1991, Gibeaut and Carpita, 1993), they are secreted into the cell wall via vesicles that leave the Golgi, move to the plasma membrane, and fuse with that membrane releasing their contents to the cell wall space. The enzymes catalysing the synthesis of cellulose and β-1,3-glucan may remain latent in the Golgi and secretory vesicles, and become functional only after inserting into the plasma membrane (Mueller and Brown, 1980; Delmer and Stone, 1988; Schindler et al, 1994).

1.5.2 Cellulose Biosynthesis

1.5.2.1 Mechanism of Biosynthesis

Relatively little is known of the mechanism of cellulose biosynthesis in higher plants (Delmer and Amor, 1995). The biosynthetic reaction involves the successive addition of glucosyl units from UDP-Glc to the non-reducing end of a β-1,4-linked glucan primer in a reaction catalysed by a cellulose synthase complex (Carpita and Delmer, 1981; Aloni et al, 1982 and 1983; Delmer and Amor, 1995). It is presumed that sugar nucleotides located in the cell cytoplasm donate the sugar residue via a plasma membrane bound synthase to the growing β-1,4-glucan chain (Montezinos and Brown, 1976; Blaschek et al, 1983; Delmer and Stone, 1988; Blanton and Northcote, 1990; Haigler, 1991; Amor et al, 1991;

Tarchevsky and Marchenko, 1991; Brown et al, 1994; Gibeaut and Carpita, 1995; Delmer and Amor, 1995; Brown et al, 1996).

During cellulose biosynthesis *in vivo*, β-1,4-glucan polymerisation and crystallisation are coupled (Delmer and Stone, 1988). Based on the form, pattern and crystallinity of cellulose in nature, however, the process leading from the polymerisation of single β-1,4-glucan chain to the crystallisation of microfibrils must be very complex and require many levels of organisation (Delmer and Amor, 1995) namely: (1) For β-1,4-linked glucan extension, each glucose added to the growing chain is rotated ~180 ° with respect to its neighbour so that the basic repeating unit of cellulose is cellobiose. In contrast, all other glucans [β-1,3-glucan, α-1,4-glucan and (1,3), (1,4)-β-D-glucans] chains are not perfectly extended and a disaccharide is not their repeating unit (Delmer and Amor, 1995). (2) From the extended nature of the β-1,4-glucan chain, cellulose in nature is believed not to exist as a single chain but to occur from the moment of synthesis as a composite of many chains, called microfibrils (Delmer and Amor, 1995; Brown et al, 1996). The precise association of β-1,4-glucan by hydrogen bonding suggests that the microfibrillar cellulose should have a perfect crystalline structure (Haigler, 1987). As indicated previously (Section 1.3.1), however, non-crystalline, or poorly crystalline microfibrils may exist in nature and further production can be induced by specific chemicals, leading to a controversy over whether the non-crystalline regions consist of single glucan chain. (3) The diversity of cellulose microfibril and crystallite sizes in nature is believed to reflect different arrangements of the cellulose synthase complex within the membrane (Haigler, 1987). (4) Microfibrils produced in nature are cellulose I containing parallel glucan chains (Section 1.3.1), but its biosynthesis is hard to explain with models which propose that glucan chain formation and crystallisation occur as completely separate processes (Leppard and Colvin, 1978; Burgess and Linstead, 1979). Thus, crystalline cellulose I is unlikely to be

synthesised *in vitro* unless the integrity of the transmembrane enzyme complex and the coupling of polymerisation and crystallisation can be preserved (Haigler, 1987). Moreover, there is little doubt that microtubules and hormones play some role in directing microfibril orientation and several hypotheses have been proposed, but the precise mechanism remains unclear (Robinson and Quader, 1981; Heath and Seagull, 1982; Mineyuki and Gunning, 1990; Seagull, 1990; Emons et al, 1992; Shibaoka, 1994).

1.5.2.2 Freeze-Fracture Observation

Because of technical difficulties, cellulose synthases in higher plants have not been purified and identified (Seitz and Emmerling, 1990). However, freeze-fracture techniques have indicated that multimeric complexes of intramembranous particles in the plasma membrane are cellulose synthases, often called terminal complexes (Rudolph et al, 1989; Mizuta and Harada, 1991; Mizuta and Brown, 1992b). Two main types of putative cellulose synthase complexes have been observed in different organisms: the linear type and the rosette type (Brown, 1985; Haigler, 1985; Emons, 1991). Linear complexes consisting of parallel rows of particles are mainly observed at the ends of microfibrils in freeze-fracture studies of the plasma membrane in algae (Brown and Montezinos, 1976; Willison and Brown, 1978; Itoh and Brown, 1984; Mizuta, 1985; Quader, 1991). The rosette type, containing six particles 8 nm in diameter with the whole rosette having a diameter of 22 nm, have been reported from algae (Giddings et al, 1980; Mueller and Brown, 1980; Staehelin and Giddings, 1982; Hogetsu, 1983; Herth, 1984 and 1985; Reiss et al, 1984; Brown, 1985), from lower plants (Wada and Staehelin, 1981; Emons, 1985; Brown, 1985) and from higher plants (Herth and Weber, 1984; Chapman and Staehelin, 1984; Herth, 1985). The numbers of rosettes in different regions of the plasma membrane is consistent with the relative activity of cellulose synthesis in those regions in developing xylem elements (Schneider and Herth, 1986). These

complexes are also reported to be labile (Herth and Weber, 1984), and they may be irreversibly disrupted during membrane isolation. When cell-free preparations are used for assaying cellulose synthase activity, most product is β-1,3-glucan with a low yield of cellulose (Li and Brown, 1993; Li et al, 1993; Okuda et al, 1993; Kudlicka et al, 1995). This raises the question of whether cellulose and callose are made by the same synthase (Pillonel and Meier, 1985; Delmer et al, 1991 and 1993; Slay et al, 1992). Research using photoaffinity labelling (Li et al, 1993) suggests that each glucan has a different catalytic subunit for synthesis. Brown and colleagues (Brown et al, 1996) have suggested that very low cellulose synthase activity *in vitro* could be caused by depletion of essential boundary lipid, by non-specific aggregation of hydrophobic polypeptides or by the disassociation of polypeptides which are required for callose synthase activity. Discussion of cellulose synthesis *in vitro* has focused on whether high molecular weight products of cellulose I can be harvested (Delmer et al, 1993; Brown et al, 1994).

1.5.2.3 Molecular and Genetic Analysis

Although cellulose synthase purification from higher plants has not recovered high enzyme activities (Delmer and Amor, 1995), high rates of *in vitro* cellulose synthesis in *Acetobacter xylinum* were first reported by Aloni et al (1982) when they included a soluble protein factor (diguanylate cyclase) and GTP. Subsequent studies indicated that cyclic diguanylic acid (c-di-GMP) generated from GTP by the protein factor, strongly activates cellulose synthesis *in vitro* (Ross et al, 1987; Amikam and Benziman, 1989). A cellulose synthase operon encoding four proteins was identified in *A. xylinum* by using genetic complementation and gene analysis with a mutant defective in cellulose production (Wong et al, 1990). Two techniques (product entrapment and photoaffinity labelling) were used to purify cellulose synthase from *A. xylinum* with three major polypeptides of 90, 67, and 54 kDa (Mayer et al, 1991). The

operon of four genes involved in cellulose biosynthesis in *A. xylinum* has been further characterised (Wong et al, 1990; Saxena et al; 1990, 1991, 1994 and 1995). The first gene (termed *BcsA*, or *AcsAB*) encodes an 83 KDa protein that binds the substrate UDP-Glc, and presumably catalyses the polymerisation of glucose residues to form β-1,4-glucan (Lin et al, 1990). The second gene may function as a regulatory subunit binding c-di-GMP (Mayer et al, 1991), and the other two genes are suggested to code for proteins controlling the secretion of polymer and the pattern of crystallisation (Saxena et al, 1994). Recently, the genes (*celA, B,* and *C*) involved in cellulose biosynthesis have also been cloned from *Agrobacterium tumefaciens* (Matthysse et al, 1995 a and b). The *celA* gene shows significant homology to the *BcsA/AcsAB* gene and its product transfers glucose from UDP-Glc to a lipid acceptor. The other genes may encode proteins including an endoglucanase which is thought to release an oligosaccharide which is finally polymerised to cellulose. Genes showing homologies to the *celA, B and C* have also been identified in *Escherichia coli*, but their functions are not clear (Ross et al, 1991).

In higher plants, attempts have been made to screen plant cDNA libraries by using the *A. xylinum* genes as probes. Experiments failed to identify similar plant genes, which suggests that plant do not contain genes that are similar to the bacterial genes (Delmer and Amor, 1995). A recent report has demonstrated, however, that two cotton cDNAs and one rice cDNA show weak homologies to the bacterial *celA* gene which encodes the catalytic subunit of cellulose synthase (Pear et al, 1996). These two cotton genes termed *celA1 and celA2* are highly expressed during active secondary wall cellulose synthesis but there is no definitive biochemical evidence that their products are directly functional in cellulose biosynthesis *in vivo*. It is also speculated that the products of such genes may interact with other factors, such as sucrose synthase, or a regulatory subunit for binding cyclic-di-GMP (Pear et al, 1996).

Although different models have been proposed to understand cellulose biosynthesis in higher plants (Amor et al, 1991; Emons, 1994; Gibeaut and Carpita, 1994; Delmer and Amor, 1995; Brown et al, 1996), they are mainly based on information from bacteria (Lin et al, 1985; Cannon and Anderson, 1991). It is believed that more genes will be involved in cellulose biosynthesis in higher plants than in bacteria because of the additional factors involved in cytoskeletal association and developmental regulation. The identification of genes in higher plants may allow genetic modifications to alter the structure or crystallinity of cellulose or to control the timing and extent of its deposition in specific tissues (Delmer et al, 1985; Delmer and Amor, 1995).

1.5.3 Biosynthesis of Non-cellulosic Polysaccharides

The enzyme systems associated with the synthesis of most other polysaccharides are located in the Golgi apparatus and the products are transported in vesicles to the plasma membrane (Fincher and Stone, 1982; Duffus and Duffus, 1984; Delmer and Stone, 1988; Brett and Waldron, 1990; White et al, 1993).

For the synthesis of hemicelluloses (XG and GAX), about half of the xylosyl residues of the main XG chain are acetylated and both UDP-D-xylose and UDP-L-arabinose act as sugar donors for the synthesis of arabinoxylan type polysaccharides. Little is known, however, of the details of the enzymic reactions involved. There are at least two types of enzymes involved in XG synthesis: glucosyltransferases and xylosyltransferases (Nishitani and Tominaga, 1992; White et al, 1993). With an enriched Golgi membrane preparation and millimolar concentrations of substrates (UDP-Glc and UDP-Xyl), the products synthesised *in vitro* contain unbranched β-1,4-glucan and XG (Campbell et al, 1988; Hayashi et al, 1988). With a reduced supply of UDP-Xyl, β-1,4-glucan is made in significant amounts (Campbell et al, 1988; Gordon and Maclachlan, 1989). Thus, glucosyltransferases and xylosyltransferases must be coordinated

during XG synthesis *in vivo* (Ray, 1980). GAXs are synthesised by a UDP-GlcA glucuronyl transferase with a UDP-Xyl dependent xylan synthase (Waldron and Brett, 1983). Addition of UDP-Ara and UDP-Xyl may cause more branched GAX formation *in vitro* (Baydoun et al, 1989). In addition, highly branched GAX accumulates in dividing and elongating cells, but in the change from elongation to differentiation, more and more unbranched GAX is formed (Gibeaut and Carpita, 1994).

In the case of pectin synthesis, several different nucleotide sugars are substrates for multiple synthase reactions in the Golgi (Duffus and Duffus, 1984; Delmer and Stone, 1988). The major glycosyl substrate is UDP-GalA. After polymerisation, the galacturonic acid residues may be methylated. The substrates for synthesis of the backbone may be provided from either the cytosolic or lumenal side of the Golgi (Driouich et al, 1993). There are many other substrates used for synthesis: UDP-galactose, -xylose, and -arabinose. These nucleotide-sugars are all derived from UDP-Glc using UDP-Glc dehydrogenase, C4-epimerase or other enzymes that may be membrane-bound. Moreover, GDP-rhamnose is also used for synthesis (Fincher and Stone, 1982; Feingold, 1982; Jarvis, 1984; Northcote, 1985; Gibeaut and Carpita, 1995).

Much more is known about callose synthesis in plants. When cells are wounded or attacked by pathogens, callose is synthesised at the plasma membrane (Delmer, 1987; Delmer et al, 1991 and 1993). As discussed previously (Section 1.5.2), the cell-free preparations made to study cellulose biosynthesis mainly produce callose, which suggests callose synthase may be formed by a dissociated or disorganised cellulose synthase (Gibeaut and Carpita, 1994; Schlüpmann et al, 1994; Brown et al, 1996). The enzymes for synthesis of (1,4)-linked and (1,3)-linked glucans have been purified from cellulose-producing fungi (Bulone et al, 1990). Unlike wound-induced callose synthase in

which Ca^{2+} greatly increases the rate of callose production, the constitutively active pollen tube synthase is not stimulated by Ca^{2+} *in vitro* (Schlüpmann et al, 1993). A 52-kDa polypeptide from cotton and 57 kDa polypeptide from carrot and beet have been identified using photoaffinitylabelling with 5-azido-UDP-Glc (Lawson et al, 1989; Frost et al, 1990; Delmer et al, 1991). UDP-pyridoxal and formaldehyde are inhibitors of callose synthase (Mason et al, 1990). Moreover, the lipid environment plays an important role in maintaining callose synthase activity (Wasserman and McCarthy, 1986) and a phospholipase inhibitor may prevent inactivitation of the synthase in some tissues (Ma et al, 1991).

(1,3), (1,4)-β-Glucan is also synthesised in the Golgi apparatus in grasses (Gibeaut and Carpita, 1993). UDP-Glc is the substrate for synthesis and Mg^{2+}, or Mn^{2+} are cofactors. Further, the synthase of (1,3), (1,4)-β-glucan may, like cellulose synthase (Section 1.5.2), revert to callose synthase when disrupted (Henry and Stone, 1982; Meikle et al, 1991).

1.6 CONTROL OF WALL POLYSACCHARIDE DEPOSITION

The polysaccharide composition of the cell wall varies considerably between different cell types and at different stages of cell division and growth. In particular, there are major differences between primary and secondary cell walls (Section 1.2). As introduced previously (Sections 1.5.2 and 1.5.3), cellulose and other wall polysaccharides (pectins and hemicelluloses) are synthesised in quite different locations in the cell. Thus, the mechanisms which coordinate deposition of these polysaccharides into the wall form an interesting topic. Whether cellulose levels determine matrix polysaccharide formation is not clear. But for different matrix polysaccharides, the main control appears to be at the level of the polysaccharide synthases. During cell division in dicotyledonous plants, for example, arabinosyltransferase activity is very high (Brett and Waldron, 1990). As secondary wall deposition begins in differentiating xylem, the activity of

enzymes involved in pectin synthesis becomes low, and the activity of xylosyltransferases rise. Changes in the activity of arabinosyl- and xylosyltransferases indicate that new mRNA and enzyme molecules may be synthesised (Brett and Hillman, 1985; Bolwell, 1993).

The enzymes catalysing sugar nucleotide formation may also control the composition of the matrix polysaccharides delivered to the wall. During secondary wall deposition, the activities of enzymes for UDP-Xyl and UDP-Glc formation greatly increase (Delmer and Stone, 1988). The secretory process for non-cellulosic polysaccharides may also be affected by the rate at which Golgi vesicles fuse with the plasma membrane (Waldron and Brett, 1985; Brett and Waldron, 1990; Gibeaut and Carpita, 1994).

1.7 OTHER CARBOHYDRATES IN PLANT CELLS

Starch is the major polysaccharide occurring in the cell cytoplast. It is produced from photosynthesis in green leaves of plants and stored in other organs (e.g. seeds, roots and stems) as an energy-storing carbohydrate. Like cellulose, starch is a homo-glucan polymer but differs from it in the nature of the glucan chain. There are two major type of starch: the linear polymer amylose composed of α-1,4 linked-D-glucopyranose residues with a DP of 1,000-2,000, and the branched chain polymer amylopectin containing 20-25 D-glucopyranose units linked α-1,4 and joined by α-1,6 linkages to form a branched structure. Normally, the former comprises 25% of total starch, and the latter 75% (Kainuma, 1988).

Starch is synthesised as a granule in leaf chloroplasts during the day and is subsequently degraded at night (Martin and Smith, 1995). In tissues storing starch, sucrose is considered to be the primary substrate for starch synthesis, and UDP- and ADP-glucose, fructose and some glucose are principal products involved in sucrose metabolism (Preiss, 1988). Because ADP-Glc is the

preferred nucleotide sugar for starch synthesis (Duffus and Duffus, 1984), the other three products are probably converted to ADP-Glc. Recent studies on starch synthase indicate that the nucleotide sugar precursors are derived from sucrose synthase, or from glucose-1-phosphate in a reaction catalysed by pyrophosphorylase (Martin and Smith, 1995). The origin of the primer required to begin synthesis is unknown, and the mechanisms controlling starch synthesis are still unclear (Preiss, 1988; Martin et al, 1993).

Glycolipids are lipids that contain monosaccharides, or oligosaccharides. In higher plants, all tissues examined have polar neutral lipids containing galactose that are called galactolipids (Elbein, 1980). These lipids are complex consisting mainly of mono- and digalactosyldiglycerides but with minor oligogalactosyldiglycerides (Elbein, 1980). The other lipid-linked monosaccharides may be mannose, glucose, or *N*-acetyl-glucosamine (Douce and Joyard, 1980). The monosaccharide lipids are precursors for the formation of the lipid-oligo-saccharides. These sugars may join to the lipid in a phosphoryl or pyrophosphoryl linkage (Douce and Joyard, 1980; Fry, 1988). The lipid-linked saccharides have been claimed to be involved in the glycosylation of complex carbohydrates and to be intermediates in cellulose biosynthesis (Hopp et al, 1978). In bacteria, the product of one gene (*celA*) has been shown to transfer the glucose from UDP-Glc to a lipid acceptor for cellulose synthesis (Matthysse et al, 1995 a and b), but in higher plants there is no report about lipid-saccharide involvement.

1.8 *ARABIDOPSIS* MUTANTS

A genetic approach to select mutants defective in various aspects of cellulose deposition provides many advantages for dissecting the process of cellulose synthesis (Kokubo et al, 1991). Mutants have contributed in important ways to understanding bacterial cellulose biosynthesis (Valla and Kjosbakken,

1982; Valla et al, 1989; Wong et al, 1990; Ross et al, 1991). Studies of β-glucan synthesis in the wall of fungi have also used various mutants (Diaz et al, 1993; Cameron et al, 1994; Enderlin and Selitrennikoff, 1994; Roemer et al, 1994). In plants, *Arabidopsis thaliana* is increasingly chosen as a good genetic "model" because of its short life cycle, small genome size, self-pollination etc. Various *Arabidopsis* mutants have increased understanding of many molecular processes (Koncz et al, 1992).

Since 2,6-dichlorobenzonitrile (DCB) was found to be a cellulose-synthesis inhibitor (Hogetsu et al, 1974), it has been extensively applied in studies of the plant wall (Venverloo et al, 1984; Gonzalez-Reyes et al, 1986; Delmer, 1985; Shedletzky et al, 1992; Mizuta and Brown, 1992a; Suzuki et al, 1992; Wells et al, 1994, Peng et al, 1998). The herbicide isoxaben also inhibits the synthesis of acid-insoluble cell wall materials, and *Arabidopsis* mutants resistant to it have been identified (Heim et al, 1989, 1990 a and b, 1991; Omura et al, 1990).

Using ethyl methanesulfonate mutagenesis, Williamson and colleagues have isolated several temperature-sensitive *Arabidopsis* mutants termed *rsw1, rsw2, rsw3* and *rsw5* thought from preliminary evidence to be defective in cellulose production (Arioli et al, 1995). Other mutants of *Arabidopsis* displaying altered patterns of cellulose deposition (Potikha and Delmer, 1995) and reduced cellulose deposition in secondary cell walls (Turner and Somerville, 1997) and showing fucose deficient cell walls (Reiter et al, 1993) have been identified in other laboratories.

Genetic analysis of mutants *rsw1, rsw2, rsw3* and *rsw5* has indicated that each mutant is controlled by a different gene. They show a radial swelling phenotype at the restrictive temperature (31 °C) when incubated on an agar medium with carbon supply (Baskin et al, 1992; Baskin and Williamson, 1992; Williamson unpublished). Unpublished, preliminary ^{14}C-glucose incorporation assays on seedlings grown for 5 days at 21 °C and 2 days at 31 °C showed that

mutants *rsw1, rsw2* and *rsw5* had reduced incorporation of ^{14}C-glucose into cellulose and much smaller alterations in incorporation into other wall polysaccharide fractions. The *rsw3* mutant appeared to have alterations in both cellulose and other polysaccharides.

1.9 AIMS OF THIS STUDY

This study provides the biochemical characterisation of the *rsw1, rsw2, rsw3* and *rsw5*. The ^{14}C-glucose incorporation experiments suggested that reduced cellulose synthesis is the major change that occurs when the mutants grow at the restrictive temperature. However, it has never been shown that the fractionation process used - which is similar to one that Heim et al (1991) used on *Arabidopsis* - effectively separates the major polysaccharides, nor has it been shown that there are no changes in any non-cellulosic polysaccharides in the mutants which go undetected when only ^{14}C-incorporation into the major fractions is measured. It will be shown that:

1. The fractionation procedure, when refined to separate charged and uncharged polysaccharides, resolves the major classes of polysaccharides in *Arabidopsis* and that these show compositions and structures typical of those seen for the carbohydrates of other dicots.

2. The major effects of the mutations in three of the four different genes (*rsw1*, *rsw2* and *rsw3*) are essentially similar. Cellulose production (acetic-nitric acid insoluble material) is reduced, production of a β-1,4-glucan that is more readily extracted and degraded than cellulose is increased, whereas changes in the production and structures of other wall polysaccharides are relatively minor. The mutant *rsw5* shows some reduction in cellulose synthesis without production of the glucan.

These findings with *rsw1* have already provided an essential part of the reasoning to conclude that the *RSW1* gene encodes a catalytic subunit of

cellulose synthase (Arioli et al, 1998 a and b) and the findings reported here for *rsw2*, *rsw3* and *rsw5* are likely to prove similarly important in determining the function(s) of those genes when they are cloned.

CHAPTER TWO

METHODS FOR CARBOHYDRATE ANALYSIS

2.1 INTRODUCTION

2.1.1 Carbohydrate Analysis

Carbohydrates in all organisms show enormous structural complexity compared with other classes of biopolymers. Their structural diversity results from numerous stereochemical centres, an anomeric group, multiple linkages between monosaccharides, and the potential for branching. A tetrasaccharide with any four different sugars, for example, could have any of more than 10, 000 different structures, but a tetranucleotide or a tetrapeptide containing any four different monomer subunits can each have only 24 different structures (Orlando and Yang, 1998). Such complexity makes analysis and structure determination particularly challenging.

Polysaccharides are the major constituents of plant cell walls. Analysis of wall fractions to determine the molecular structure of polysaccharides requires information on the nature and proportions of monosaccharide units, the position and configuration of the glycosidic linkages, the sequence of monosaccharide units, the types of branching present and the location and nature of substituents (Duffus and Duffus, 1984; Fry, 1988). Identification of the degradation products of polysaccharides, mainly derived from hydrolysis, and of the linkages present by methylation analysis, are required to obtain this information (Nevell, 1987).

2.1.2 Hydrolysis of Polysaccharides

The monosaccharide compounds are usually determined after acid hydrolysis. Treatment with 2 M TFA at 120 °C for one hour will completely hydrolyse most polysaccharides other than cellulose and pectins (Fry, 1988; Needs and Selvendran, 1993). Partial hydrolysis and isolation of the resultant

oligosaccharides can supply information about the arrangement of the component monosaccharides in the molecule.

The glycosidic linkage in cellulose is susceptible to acid-catalysed hydrolysis under suitable condition resulting in high yields of D-glucose (reviewed by Nevell, 1987). Dilute acids can be used for less extensive degradation, but very diluted acids may cause negligible degradation. Generally, cellulose due to its crystalline character, is only hydrolysed by 2 M TFA to a limited extent. Sulphuric acid has been commonly used to hydrolyse crystalline cellulose. α-D-Glucosidic linkages are more easily hydrolysed than β-D-linkages and 1, 6-linkages are generally more resistant to hydrolysis than others (Smidsrød et al, 1966; Ruiter et al, 1992). Moreover, uronic acid glycosyl linkages that are abundant in pectic polysaccharides are relatively resistant to acid-degradation (Fry, 1988). Hydrolysis of pectins by 2 M TFA at 120 °C for 1 h releases only a low yield of uronic acids, and more severe conditions cannot be used as the uronic acids are readily decarboxylated in hot acid (Ruiter et al, 1992).

Enzymic hydrolysis is an alternative to acid hydrolysis (York et al, 1985; Zablackis et al, 1995). If the specificity of the enzyme is known, much useful structural information can be obtained from understanding which bonds are attacked and which resist hydrolysis. The enzymic degradation of cellulose is described in Section 5.1.3.

2.1.3 Analysis of Monosaccharides

After the isolated and purified polysaccharides of the cell wall are hydrolysed by acids or enzymes, the resultant monosaccharides can be quantitatively determined by gas chromatography-mass spectrometry (GC/MS; Albersheim et al, 1967; Blakeney et al, 1983; Linskens and Jackson, 1986). A simple and rapid preparation of alditol acetates (AA) for monosaccharide

analysis by GC/MS was described by Blakeney et al (1983). This method can be performed in a single tube without transfers or evaporations. Sodium borohydride is used to reduce monosaccharides, and the resulting alditol is acetylated using 1-methylimidazole as the catalyst. Removal of borate from the reaction is not required and the acetylation is completed within 10 min at room temperature. This method has been applied to the quantitative analysis of monosaccharides in acid hydrolysed plant cell wall.

Generally, any carbohydrates (monosaccharides, oligosaccharides and polysaccharides) may be quantitatively analysed at the micro or semi-micro levels by means of colorimetric tests (Dubois et al, 1956; Fry, 1988). As this approach does not require degradation of polysaccharides, it can be used for determining the sugar content of samples which can not be efficiently hydrolysed by acids, such as cellulose or pectins rich in uronic acids (Fry, 1988; Ruiter et al, 1992). However, colorimetric tests can only determine classes of sugars; hexoses, pentoses, 6-deoxyhexoses and uronic acids rather than the individual sugars within each class.

2.1.4 Methylation Analysis

Methylation analysis is widely used to determine the position of linkages between monosaccharide residues in oligo- or polysaccharides. It involves four basic steps: complete methylation of all free hydroxyl groups in the carbohydrate; hydrolysis of the fully methylated structure to a mixture of partially methylated monosaccharides; reduction of the anomeric carbon and acetylation of the partially methylated monosaccharides to form volatile partially methylated alditol acetate (PMAA) derivatives; separation and identification of the mixture of PMAAs by GC/MS (Lindberg, 1972; Jones, 1972; Lindberg and Longren, 1978; O'Neil and Selvendran, 1980; Harris et al, 1984; Doares et al, 1991).

The Hakomori method for polysaccharide methylation was originally introduced in 1964 (Hakomori, 1964). His procedure used sodium hydride to generate the dimsyl anion which, when added to a polysaccharide in DMSO solution and treated with methyl iodide, resulted in high yields of the methylated saccharide. This represented a considerable advance over the methods previously used which had to be repeated a number of times to achieve acceptable levels of methylation.

The Hakomori method was further improved to give shorter reaction times, higher yields and cleaner gas chromatograms by Ciucanu and Kerek (1984) who introduced the use of solid sodium hydroxide. Needs and Selvendran (1993) further modified the method adding the base before the methyl iodide, rather than simultaneously, to improve the reliability and yields from the procedure.

The Hakomori method typically uses mg quantities of material, but it can be successfully performed on about 25 μg of saccharide (Waeghe et al, 1983). When only small amounts of material are available, however, the purity of the regents is a critical factor in reducing the chemical background in the final GC/MS analysis.

When complete methylation analysis is to be carried out on polysaccharides rich in uronic acids (such as pectins), it is necessary to reduce the carboxyl group with a deuterium labelled reagent to facilitate the identification, by MS, of the methylated sugars derived from the uronic acid residues. This can be carried out on the methylated polysaccharide using $NaBD_4$ on a carbodiimide-activated ester or $LiAlD_4$ in tetrahydrofuran (Lindberg, 1972; Lindberg and Longren, 1978). However, relatively large amounts of polysaccharide are required and yields are variable.

Once the polysaccharide has been successfully methylated, it is hydrolysed with TFA to its constituent, partially methylated monosaccharides. They are then

reduced and acetylated using the same procedures as for the monosaccharides (Section 2.1.3).

2.1.5 Separation and Identification of PMAAs by GC/MS

Identification of the PMAAs requires both good chromatographic resolution and their mass spectra (MS). With the advent of modern bonded-phase capillary chromatography, it is now possible to separate almost all PMAAs. This is important as the MS of, for example, the hexose PMAAs with the same pattern of methylation, are essentially identical. Thus, when dealing with a complex mixture of PMAAs, identification is based on both retention time, relative to an internal standard (IS) or the terminal PMAA, and the MS.

PMAA standards are usually prepared by deliberate undermethylation of 1-methyl glycosides. However, this has usually resulted in poor yields of the most undermethylated PMAAs which are poorly extracted from aqueous solution. Recently, Doares et al (1991) improved this procedure by adding an acetylation step after the undermethylation (Doares et al, 1991). The partially methylated and partially acetylated methyl glycoside was then readily extracted from the methylation solution prior to being hydrolysed, reduced and acetylated.

Electron impact mass spectrometry (EI-MS) uses a high-energy electron beam passing through the derivatives as they exit from the chromatography column to produce fragments from each derivative in a predictable manner (reviewed by Lindberg and Lonngren, 1978; Carpita and Shea, 1988). Primary fragments are produced by cleavage of carbon-carbon bonds of the alditol. Cleavage between contiguous alditol carbons with methoxyl groups is much easier than cleavage between methoxylated and acetoxylated carbons. Cleavage between contiguous acetoxylated carbons hardly occurs unless the derivatives are fully acetylated or contain a single methoxyl group. The secondary fragments form subsequently by loss of acetic acid (m/z 60) from acetoxyl groups and loss

of methanol (m/z 32) from methoxyl groups. Additional secondary fragments form by loss of formaldehyde (m/z 30) or ketene (m/z 42). Loss of the acetylium ion (m/z 43) is a common peak of all derivatives of neutral sugars and most methyl amino sugars. Each PMAA derivative exhibits characteristic fragments from which the original distribution of the methoxylated and acetoxylated carbons on the carbon skeleton can be deduced. An understanding of the rules of fragmentation allows the chemical structure of PMAAs to be identified from their MS. For the identification of otherwise symmetrical alditols generated by reduction, it is important to use $NaBD_4$ to introduce a deuterium at the C-1 position of the partially methylated sugars.

2.1.6 Aim of This Chapter

This chapter describes GC/MS methods for the quantitative determination of monosaccharides and for methylation analysis, demonstrates a simplified procedure to prepare PMAA standards for methylation analysis, and tabulates the relative retention times (RRT) and MS for the PMAA standards that were detected by GC/MS.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

The reagents used for chemical analyses were analytical grades obtained from Sigma Chemical Co (St Louis, MO, USA), Aldrich Chemical Co (Milwaukee, WI, USA) and Ajax Chemicals (Auburn, NSW, Australia). Chemicals for methylation analysis were of the highest grade available. DMSO was stored over approximately one quarter volume of 3 Å molecular sieves which had been activated by heating at 250 °C for 24 h.

2.2.2 GC/MS

A fused-silica capillary column (SGE: 12m x 0.22 mm internal diameter) coated with a 70% cyanopropyl polysilphenylene-siloxane bonded phase (BPX70, thickness: 0.25μm) was eluted with helium (inlet pressure 15 psi) directly into the ion source of a Fisons MD800 GC/MS (injection port 250 °C; interface 250 °C; source 200 °C). Three temperature programs were used for GC separation of the monosaccharide derivatives:

A, from 80 °C (held for 2 min) to 170 °C at 30 °C/min and then to 240 °C at 8 °C/min (held for 10 min) was used for the analysis of AAs in some experiments;

B, from 45 °C (5 min) to 220 °C at 15 °C/min (10 min) was used to analyse AAs in other experiments;

C, from 80 °C (2min) to 170 °C at 30 °C/min and then to 240 °C at 3 °C/min (4 min) for PMAAs.

The mass spectrometer was operated in the EI mode with an ionisation energy of 70 eV. Mass spectra were acquired with full scans based on the temperature programs:

A, from 50 to 250 u in 0.45s for alditol acetates;

B, from 40 to 450 u in 0.45s for PMAAS;

C, from 45 to 350 u in 0.45s for PMAAs.

2.2.3 Acid Hydrolysis

Non-cellulosic polysaccharides such as pectins, hemicelluloses and acid-soluble materials were hydrolysed using TFA (Ruiter et al, 1992). The polysaccharide sample (2-20 μg) was dissolved in 2 M TFA (100 μl) in a 3 ml glass test tube that was sealed by melting the neck in a flame and drawing it out slowly with forceps or in a 10ml teflon tube with screw cap. The tube was heated either at 120 °C for 1 h in an autoclave (15 psi) or in an oven at 120 °C for 1.5 h. After the tube was cooled, the sample was centrifuged at 2,100 *g* for 5 min, and the supernatant collected and dried under vacuum at 45 °C. The dried hydrolysate was stored at 4 °C.

For hydrolysis of crystalline cellulose, a dry sample (10-100 μg) was suspended in 72% (w/w) H_2SO_4 (100 μl) by shaking or stirring at room temperature for at least 1 h until completely dissolved. The sample was mixed well with 2 ml distilled water and heated at 120 °C as described in the TFA method above. The sample solution was partially neutralised by slow addition, with rapid stirring, of 0.18 M $Ba(OH)_2$ (5 ml). To complete the neutralisation, bromophenol blue (2 μl of 1%) was added as indicator, and the mixture was stirred with finely powdered $BaCO_3$ (0.2 g). The reaction with $BaCO_3$ was slow and stirring was continued until the indicator showed blue. After the solution was centrifuged at 2,100 *g* for 10 min, the supernatant was removed and stored at 4 °C until use.

Monosaccharide samples (2-20 μg) from acid-hydrolysis were mixed with a known amount of *myo*-inositol (20-100 nmol) as internal standard (IS).

This acid hydrolysis was also applied to the samples from complete methylation of polysaccharides and from partial methylation of monosaccharide standards. All samples were freeze dried or dried in a vacuum oven prior to reduction.

2.2.4 Reduction and Acetylation

A freshly prepared solution of $NaBD_4$ (100 µl; 10 µg/µl in 2 M NH_4OH) was added to each sample. Samples were capped, mixed well and incubated at 60 °C for 2 h. Excess $NaBD_4$ was decomposed by addition of acetone (50 µl). The sample was then dried under a stream of nitrogen at 40 °C. Acetic acid (20 µl), ethyl acetate (100 µl) and acetic anhydride (300 µl) were added to the dry reduced sample. After mixing well, perchloric acid (70%, 20 µl) was added and the solution mixed again. After 5 min cooling on ice, water (1 ml) was added, followed by 1-methylimidazole (20 µl). After mixing, the sample was allowed to stand for 5 min. Dichloromethane (200 µl) was added, mixed gently, and centrifuged (2,100 *g*; 10 seconds) for phase separation. The lower phase was removed with a glass pipette and stored at -20 °C in a screw-capped glass vial until analysed by GC/MS (Section 2.2.2).

2.2.5 Methylation Analysis

Polysaccharide samples (2-20 µg) in glass bottles were dried overnight at 60 °C in a vacuum oven and dissolved, under nitrogen, in DMSO (200 µl) with heating at 45 °C for the minimum time necessary. Finely powdered dry sodium hydroxide (10 mg) was added to the solution in DMSO under nitrogen at room temperature. Immediately, the sample mixture was sealed tightly with a cap, mixed well and heated at 30 °C for 1 h, then left for a further 1 h with intermittent vortexing. For DMSO-insoluble polysaccharides such as cellulose and starch, methyl iodide (10 µl) was added prior to heating at 30 °C. After intermittent vortexing for 1 h, methyl iodide (200 µl) was added under nitrogen dropwise over 30 s, and then the mixture was capped again, mixed well and heated at 30 °C for 1 h with intermittent vortexing. After methylation, the mixture was extracted with chloroform-methanol (1 ml; 2:1, v/v), and then

washed with water (1 ml). After centrifuging at 2,100 *g* for 10 s, the lower phase was collected by using a glass pipette, and evaporated in a 45 °C water bath under a stream of nitrogen (Needs and Selvendran, 1993).

The residue was hydrolysed with 2 M TFA (Section 2.2.3), reduced with $NaBD_4$ and acetylated with acetic anhydride (Section 2.2.4), prior to GC/MS assay (Section 2.2.2).

2.2.6 Preparation of PMAA Standards

Methylglycoside samples were weighed: 20 mg of each of methyl-α-D-glucopyranoside, methyl-β-D-galactopyranoside and methyl-α-D-mannopyranoside; 22.5 mg of methyl-α-D-fucopyranoside and methyl-α-D-rhammnopyranoside; 22.6 mg of methyl-β-D-xylopyranoside and methyl-β-D-arabinopyranoside. All samples were dried at 60 °C overnight under vacuum, and dissolved in DMSO (400 µl) under nitrogen. Finely powdered NaOH (20 mg) was added to each sample, and immediately vortexed for 10 min under nitrogen. After adding methyl iodide (100 µl), the reaction solution was mixed and heated at 30 °C for another 10 min. 1-Methylimidazole (200 µl) was added to catalyse acetylation, followed by acetic anhydride (2 ml) with further mixing. After 10 min at room temperature, water (5 ml) was added to decompose excess acetic anhydride. After cooling to room temperature, each sample was extracted with dichloromethane (2 × 1 ml) by briefly centrifuging for phase separation. The two dichloromethane extracts were combined in a single glass tube, and then washed with water (5 × 3 ml). The dichloromethane layer was evaporated in a stream of nitrogen at 40 °C, and the oily residue was then hydrolysed with 2 M TFA (Section 2.2.3), reduced and acetylated as described previously (Section 2.2.4) prior to GC/MS (Section 2.2.2).

2.3 RESULTS AND DISCUSSION

2.3.1 Standards for Monosaccharide Analysis

Equal amounts (500 nmol) of seven monosaccharides (L-rhamnose, L-fucose, D-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose) and IS (*myo*-inositol) were reduced, acetylated and resolved by GC/MS (Figure 2.1). RRTs of each monosaccharide against IS are presented in Tables 2.1 and 2.2. Statistical analysis showed that all RRTs had a very low standard derivation (±0.001, n=5). For quantitative calculations, the response factor for each monosaccharide was determined as described in Table 2.1.

2.3.2 PMAA Standards of Glucose

The preparation of PMAA standards was based on the method of Doares et al (1991) but, as described in Section 2.2.6, the method was modified by directly using NaOH as the base, instead of potassium hydride to generate the dimsyl anion *in situ.*.

The GC/MS chromatogram of the PMAAs of glucose is presented in Figure 2.2. The PMAAs corresponding to each of the 14 peaks were identified by observing the MS for each peak to deduce the identity of the PMAA(s) (Figure 2.3). The fourteen peaks labelled on the chromatogram were clearly separated by the column and only one peak (number 10) contained two derivatives (3,4-Me_2Glc[1] and 2,3-Me_2Glc). All PMAAs of glucose, except for 6-Me Glc, could be detected by GC/MS using the modified methylation procedures (Table 2.3). RRT values of the peaks for PMAAs are also given in Table 2.3.

The unlabelled peaks on the chromatogram (Figure 2.2) were not carbohydrates according to MS.

[1] PMAAs are referred to by abbreviated names. Full chemical names are provided in a table at the front of the thesis.

In conclusion, the method employed for partial methylation (Section 2.2.6) generates 15 of 16 PMAAs of glucose, and resolves them into 14 peaks on the GC/MS chromatogram.

2.3.3 PMAA Standards of Galactose and Mannose

GC/MS chromatograms of the PMAAs of galactose and mannose standards are shown in Figure 2.4. The PMAAs of galactose and mannose are identified in Figures 2.5 and 2.6 respectively. RRT values for PMAAs are given in Table 2.4.

Comparison of RRTs for glucose, galactose and mannose standards can distinguish most PMAAs with the same MS fragment patterns (Tables 2.3 and 2.4). The RRTs for 2,3,4,6-Me_4 Glc and 2,3,4,6-Me_4 Man are very similar as are those for 2,3,4-Me_3 Glc and 2,3,4-Me_3 Man.

Two PMAAs of galactose and five PMAAs of mannose were not detected as described in Table 2.4.

2.3.4 PMAA Standards of Pentoses and 6-Deoxyhexoses

Derivatives of most of the PMAAs of the standard pentoses (xylose and arabinose) and the 6-deoxyhexoses (fucose and rhamnose) were prepared from their methyl glycosides and identified by GC/MS analysis (Figure 2.7). MS are shown in Figures 2.8, 2.9, 2.10 and 2.11, respectively, and RRT values are given in Tables 2.5 and 2.6. Partial methylation of methyl D-xylopyranoside and methyl D-arabinopyranoside samples only gave PMAAs with the pyranose ring form. PMAAs of the pentoses with the furanose ring forms were generated from methylation (Section 2.2.5) of D-xylose and D-arabinose samples (Figures 2.12 and 2.13). Their RRTs are also given in Table 2.5.

It should be noted that 2,3-OMe2-Ara can be derived from either a 5-linked pentofuranose (eg 5-Ara*f*, common in pectic polysaccharides) or a 4-linked pentopyranose (eg 4-Ara*p* which is relatively uncommon in plant polysaccharides).

2.4 SUMMARY

Methods for the quantitative analysis of monosaccharides and for methylation analysis of polysaccharides by GC/MS are described. A simple and rapid method to prepare PMAA standards has been established by directly adding NaOH into the reaction sample to avoid an additional procedure in preparing potassium methylsulfinylmethanide. The modified procedures produce relatively good yields for most PMAAs. No corrections were made by determining response factors for the different PMAAs and quantitation of the methylation analyses was not attempted.

These methods form the basis for the study reported in Chapter Three where the value of a fractionation scheme is established by identifying and quantifying the major polysaccharides in different fractions prepared from wild type *Arabidopsis* seedlings.

Table 2.1 Relative retention times of AA to *myo*-inositol and their respective response factors [a]

Sugars	Retention Times (min)	RRT	Response [c] Factors
Rhamnitol	8.311±0.009 [b]	0.645±0.001	1.31±0.03
Fucitol	8.476±0.010	0.658±0.001	1.87±0.03
Arabinitol	9.523±0.009	0.739±0.001	1.30±0.01
Xylitol	10.452±0.009	0.812±0.001	1.10±0.01
Mannitol	11.654±0.009	0.905±0.001	0.77±0.01
Galactitol	11.988±0.010	0.931±0.001	0.85±0.01
Glucitol	12.394±0.009	0.962±0.001	0.70±0.01
Inositol	12.879±0.010	1.0	1.0

[a] The response factors were determined by GC/MS using temperature program A (Section 2.2.2).

[b] ± Standard deviation (SD), n=5.

[c] **Response factors =**

$$\frac{\text{total area of analyte}}{\text{total area of IS}} \times \frac{\text{moles of internal standard (IS)}}{\text{moles analyte}}$$

Moles of analyte in sample =

$$\frac{\text{peak (sample) area analyte}}{\text{peak area of IS}} \times \frac{\text{moles of IS}}{\text{response factor for analyte}}$$

Figure 2.1

GC/MS chromatogram of alditol acetates (AAs) of a standard mixture of monosaccharides and *myo*-inositol. RRT of each peak and its response factor are presented in Table 2.1

Peak 1, rhamnitol; **2**, fucitol; **3**, arabinitol; **4**, xylitol; **5**, mannitol; **6**, galactitol; **7**, glucitol; & **IS**, *myo*-inositol.

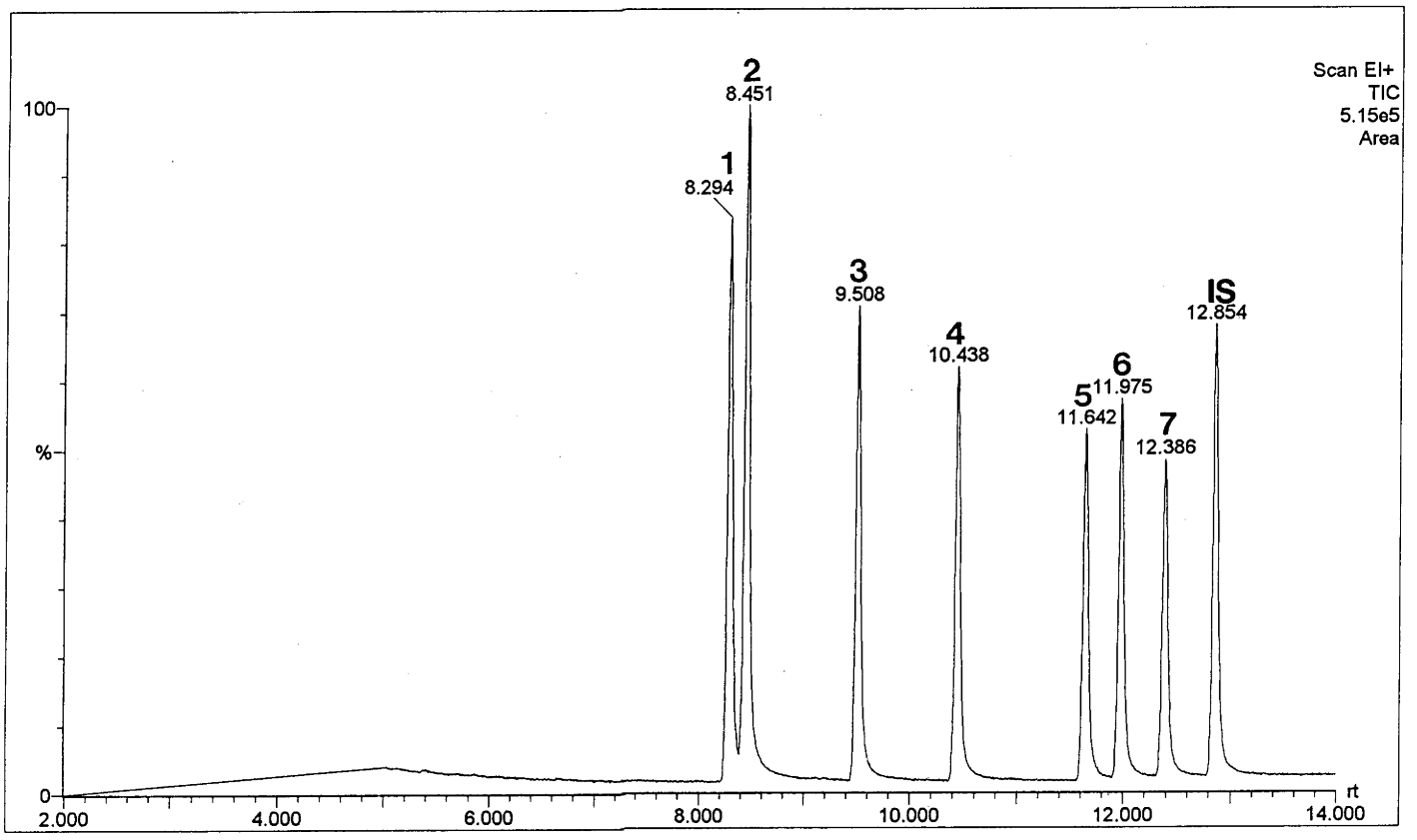

Scan EI+
TIC
5.15e5
Area
100
%
0
1
8.294
2
8.451
3
9.508
4
10.438
5
11.642
6
11.975
7
12.386
IS
12.854
rt
2.000
4.000
6.000
8.000
10.000
12.000
14.000

Figure 2.2

GC/MS chromatogram of PMAAs of glucose standard. RRT of each labelled peak is presented in Table 2.3. Mass spectra (MS) of each labelled peak and its deduced glycosyl linkage are presented in Figure 2.3 & Table 2.3, respectively. IS, Internal standard (*myo*-inositol).

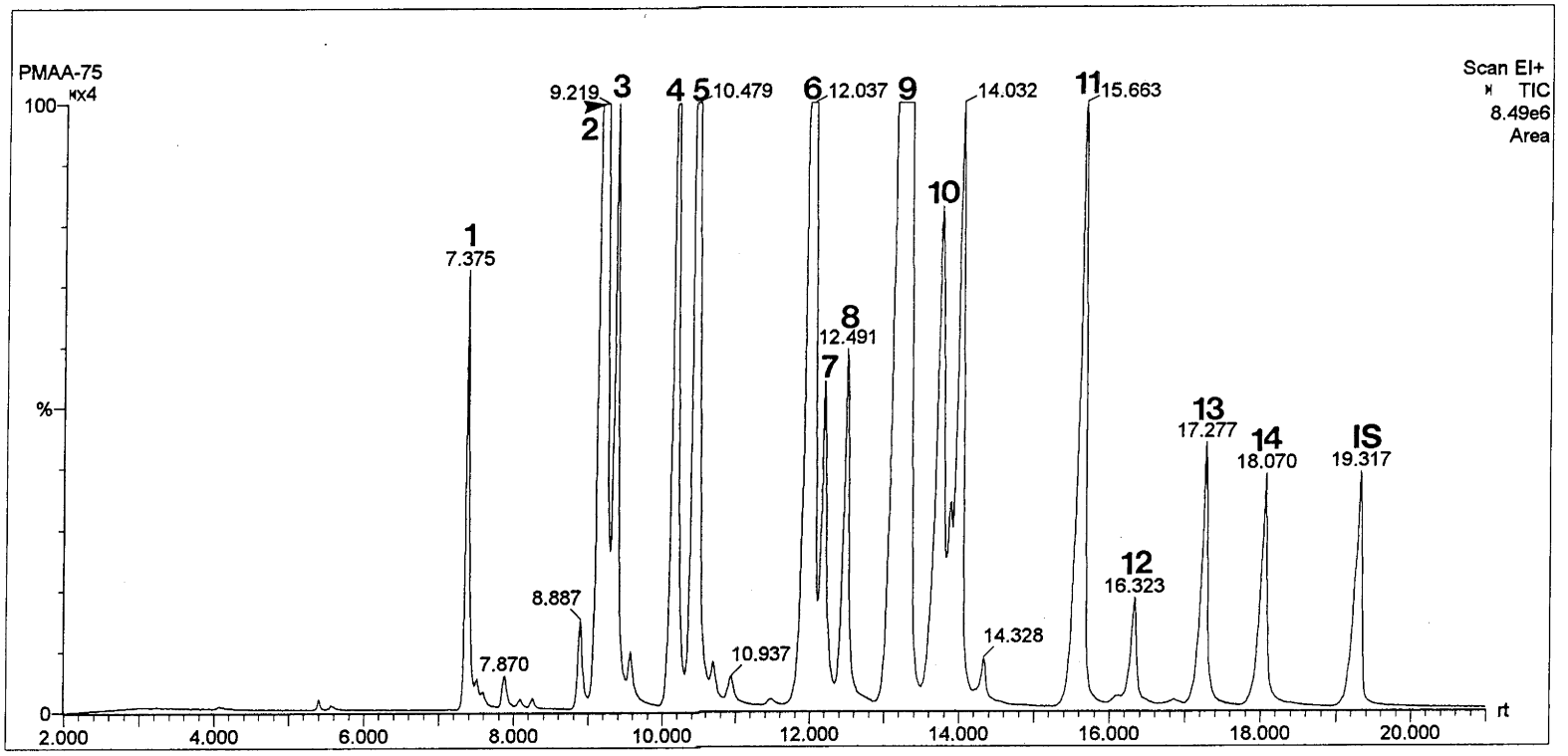
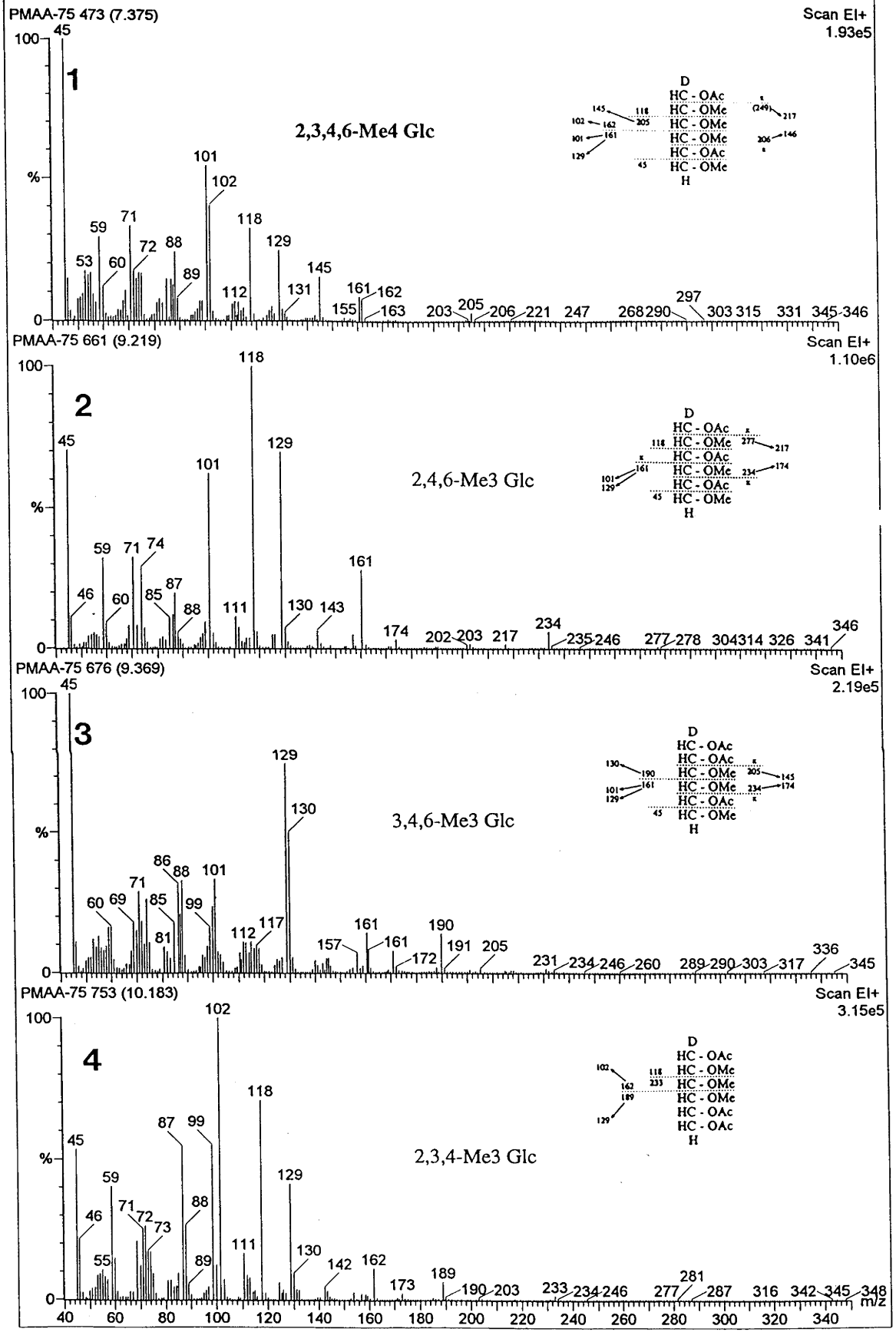
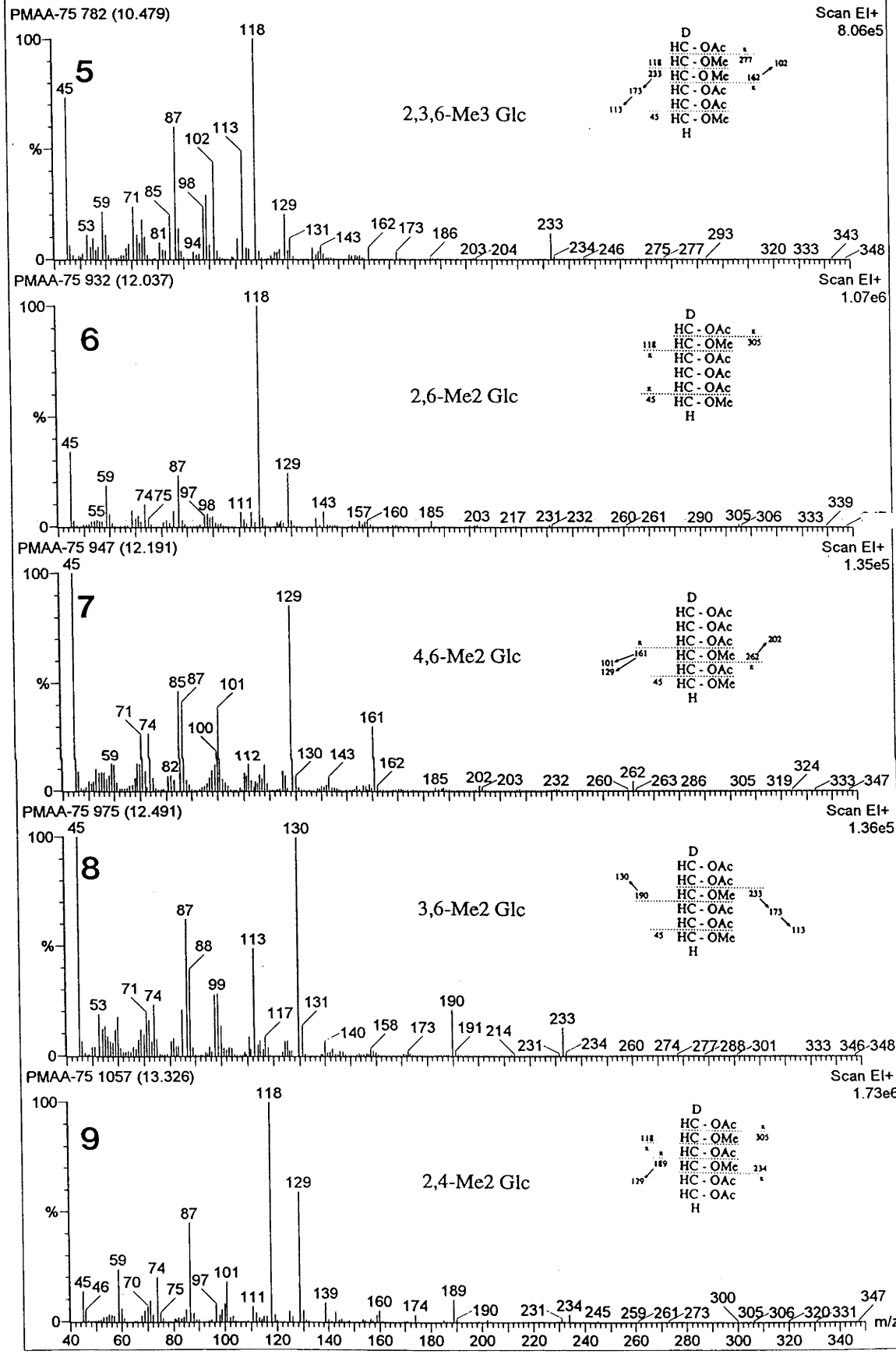

PMAA-75
Scan EI+
TIC
8.49e6
Area
100
%
0
rt
1
7.375
2
9.219
3
4
5
10.479
6
12.037
7
8
12.491
8.887
7.870
9
10
14.032
10.937
14.328
11
15.663
12
16.323
13
17.277
14
18.070
IS
19.317
2.000
4.000
6.000
8.000
10.000
12.000
14.000
16.000
18.000
20.000

Figure 2.3

MS of PMAAs of glucose standard from chromatogram in Figure 2.2.


PMAA-75 473 (7.375)
Scan EI+
1.93e5
1
2,3,4,6-Me4 Glc
PMAA-75 661 (9.219)
Scan EI+
1.10e6
2
2,4,6-Me3 Glc
PMAA-75 676 (9.369)
Scan EI+
2.19e5
3
3,4,6-Me3 Glc
PMAA-75 753 (10.183)
Scan EI+
3.15e5
4
2,3,4-Me3 Glc
m/z


PMAA-75 782 (10.479)
Scan EI+
8.06e5
5
2,3,6-Me3 Glc
PMAA-75 932 (12.037)
Scan EI+
1.07e6
6
2,6-Me2 Glc
PMAA-75 947 (12.191)
Scan EI+
1.35e5
7
4,6-Me2 Glc
PMAA-75 975 (12.491)
Scan EI+
1.36e5
8
3,6-Me2 Glc
PMAA-75 1057 (13.326)
Scan EI+
1.73e6
9
2,4-Me2 Glc
m/z

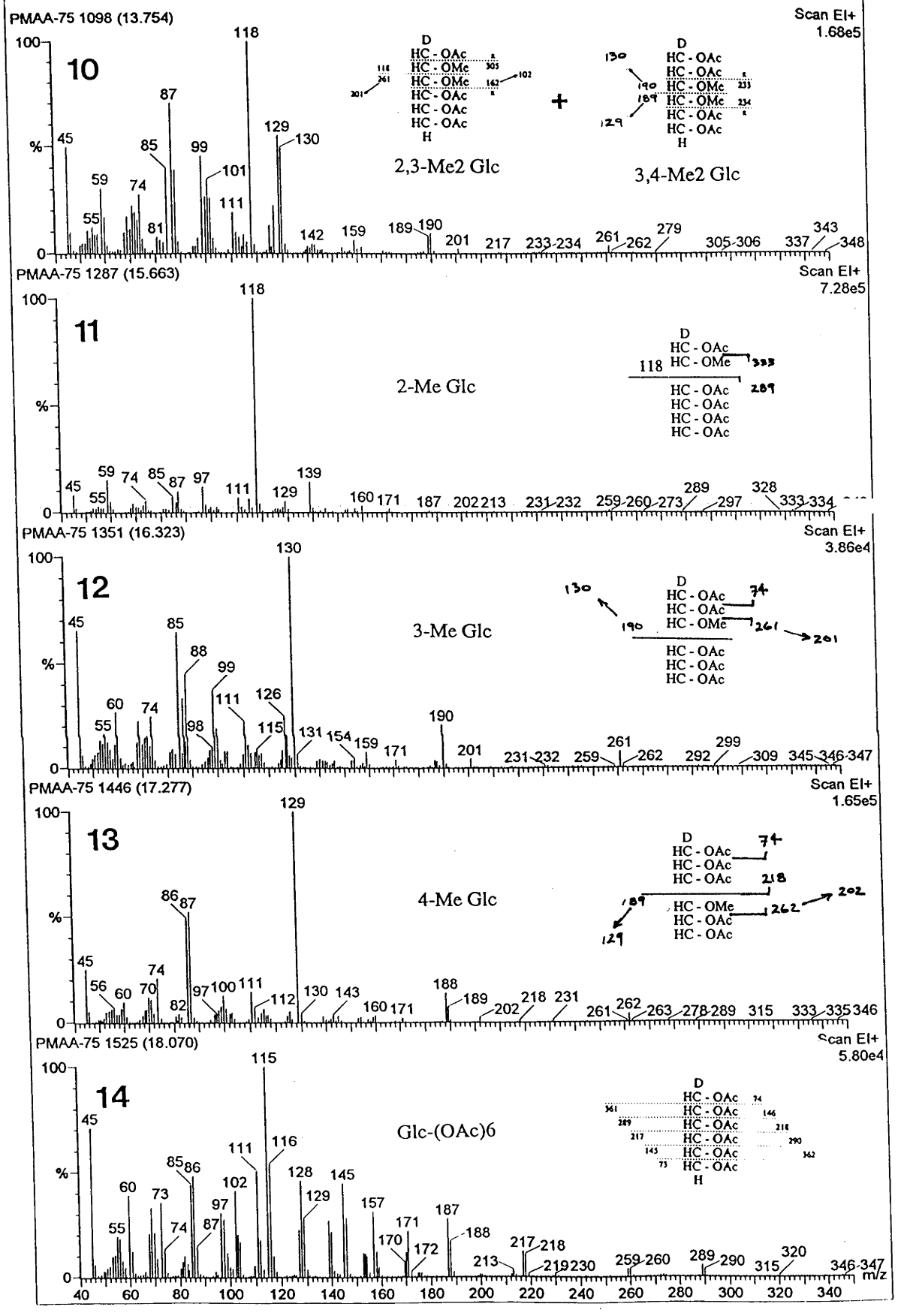

PMAA-75 1098 (13.754)
Scan EI+
1.68e5
10
2,3-Me2 Glc
3,4-Me2 Glc
PMAA-75 1287 (15.663)
Scan EI+
7.28e5
11
2-Me Glc
PMAA-75 1351 (16.323)
Scan EI+
3.86e4
12
3-Me Glc
PMAA-75 1446 (17.277)
Scan EI+
1.65e5
13
4-Me Glc
PMAA-75 1525 (18.070)
Scan EI+
5.80e4
14
Glc-(OAc)6
m/z

Table 2.3 Relative retention times of PMAAs of glucose standard to *myo*-inositol-hexaacetate and t-Glc*p*

Peak [a]	Derivative	Deduced Linkages	RRT-1	RRT-2	Ref-5 [b]	Ref-10 [c]
1	2,3,4,6-Me_4 Glc	t-Glc*p*	0.382	1	1	1
2	2,4,6-Me_3 Glc	3-Glc*p*	0.477	1.250	1.246	1.317
3	3,4,6-Me_3 Glc	2-Glc*p*	0.485	1.270	1.261	1.347
4	2,3,4-Me_3 Glc	6-Glc*p*	0.527	1.380	1.343	1.553
5	2,3,6-Me_3 Glc	4-Glc*p*	0.542	1.421	1.363	1.493
6	2,6-Me_2 Glc	3,4-Glc*p*	0.623	1.632	1.637	1.844
7	4,6-Me_2 Glc	2,3-Glc*p*	0.631	1.653	1.637	1.786
8	3,6-Me_2 Glc	2,4-Glc*p*	0.647	1.694	1.604	1.727
9	2,4-Me_2 Glc	3,6-Glc*p*	0.690	1.807	1.546	1.707
10	3,4-Me_2 Glc	2,6-Glc*p*	0.712	1.865	1.509	1.613
10	2,3-Me_2 Glc	4,6-Glc*p*	0.712	1.865	1.526	1.613
11	2-Me Glc	3,4,6-Glc*p*	0.811	2.124	1.784	-
12	3-Me Glc	2,4,6-Glc*p*	0.845	2.213	1.832	1.970
13	4-Me Glc	2,3,6-Glc*p*	0.894	2.343	1.899	1.970
	6-Me Glc	2,3,4-Glc*p*	-	-	1.633	-
14	Glc-$(OAc)_6$	-	0.935	2.445	1.973	2.191
IS	myo-inositol-Ac_6	-	1	2.619	-	-

[a] Peak number indicated in chromatogram (Figure 2.2).

[b] *and* [c] Data from Carpita and Shea (1989, 176-177).

Figure 2.4

GC/MS chromatograms of PMAAs of galactose (**A**) & mannose (**B**) standards. RRT of each labelled peak is presented in Table 2.4. MS of each labelled peak are presented in Figures 2.5 & 2.6, and its deduced glycosyl linkage is described in Table 2.4.

IS, Internal standard (*myo*-inositol).

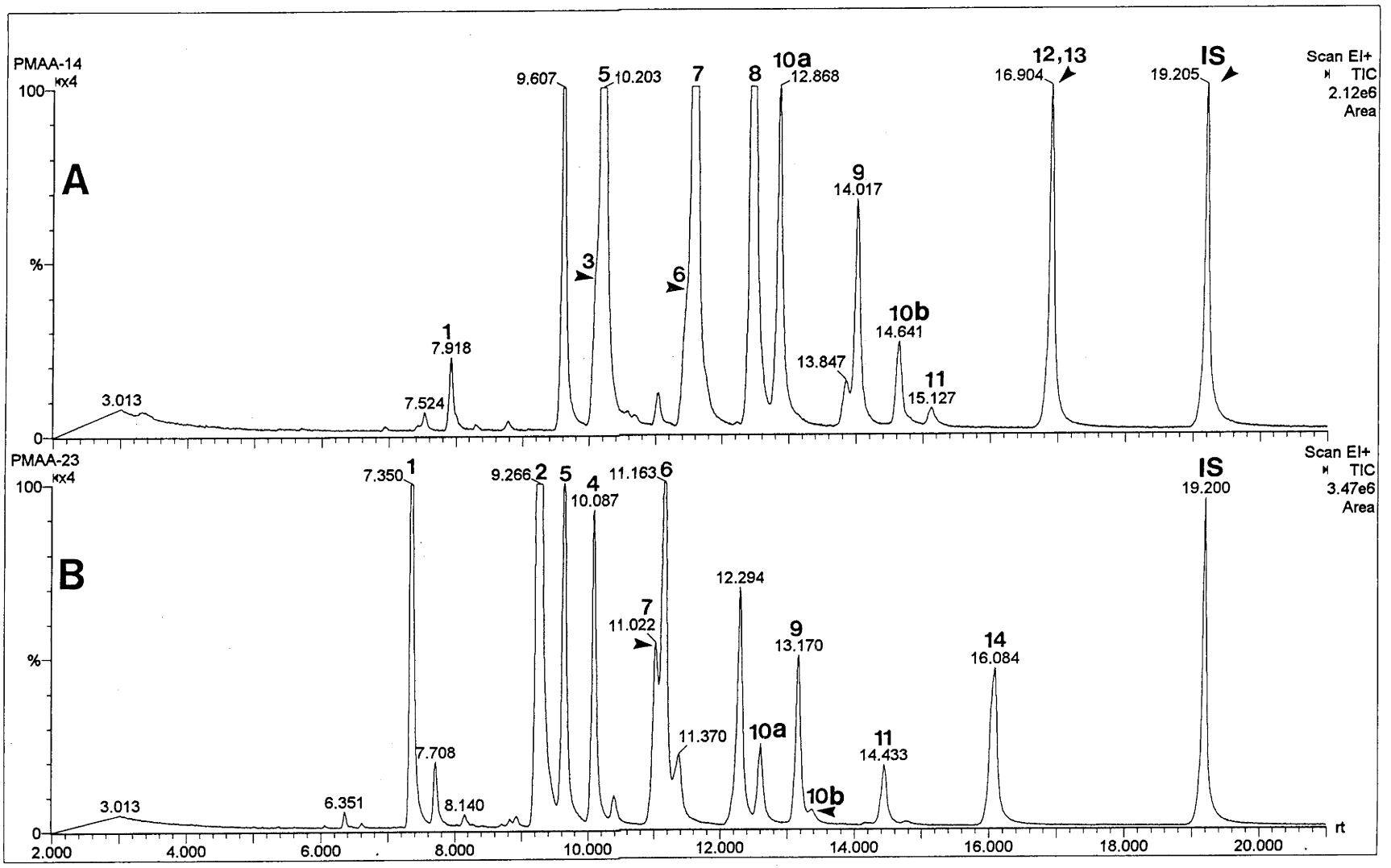
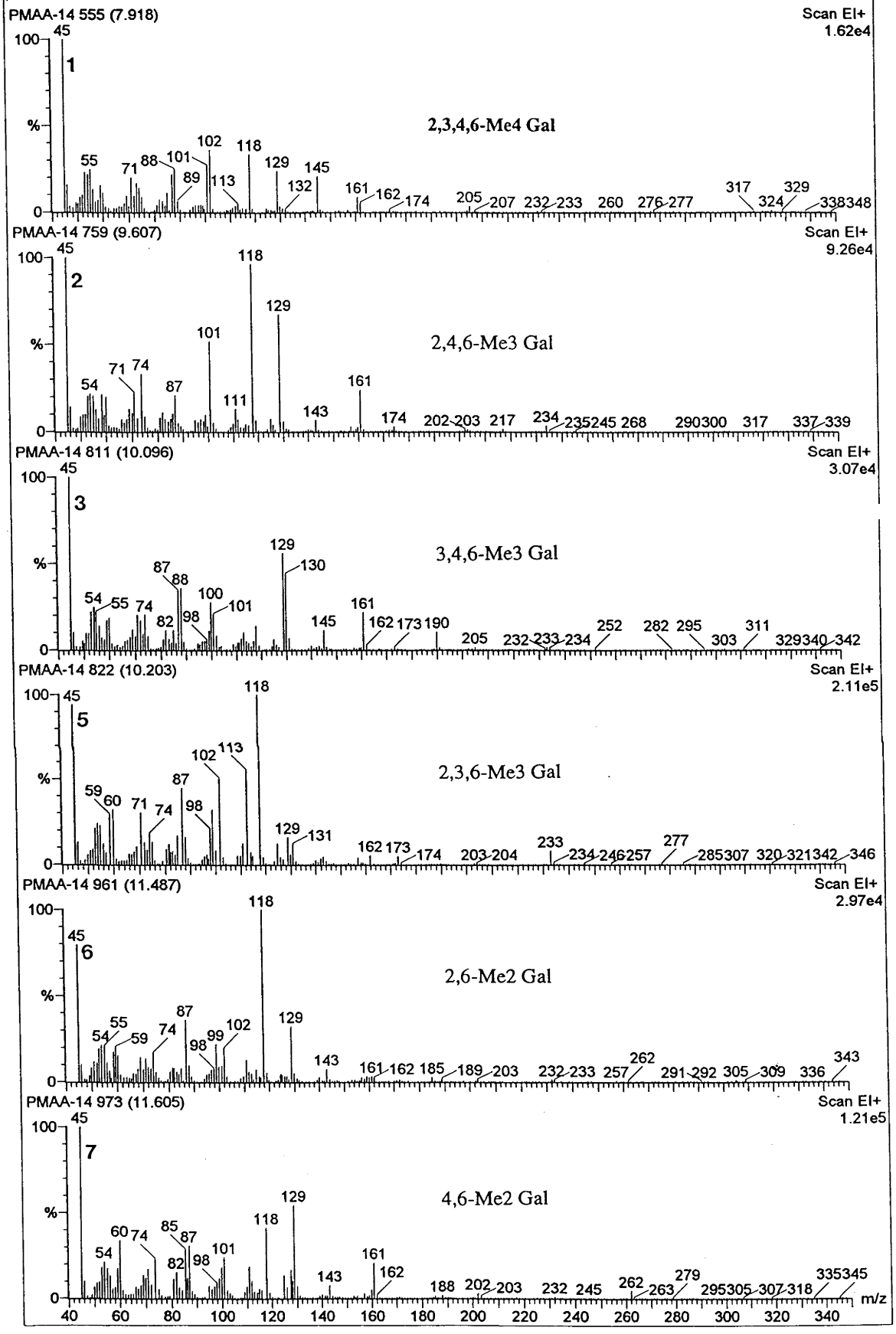

PMAA-14
Kx4
100
%
0
A
9.607
5
10.203
7
8
10a
12.868
12,13
16.904
IS
19.205
Scan EI+
TIC
2.12e6
Area
9
14.017
3
6
10b
14.641
1
7.918
13.847
11
15.127
3.013
7.524
PMAA-23
Kx4
100
%
0
B
7.350
1
9.266
2
5
4
10.087
11.163
6
IS
19.200
Scan EI+
TIC
3.47e6
Area
12.294
7
11.022
9
13.170
14
16.084
11.370
10a
11
14.433
10b
7.708
3.013
6.351
8.140
rt
2.000
4.000
6.000
8.000
10.000
12.000
14.000
16.000
18.000
20.000

Figure 2.5

MS of PMAAs of galactose standard from chromatogram in Figure 2.4, A.


PMAA-14 555 (7.918)
Scan EI+
1.62e4
1
2,3,4,6-Me4 Gal
PMAA-14 759 (9.607)
Scan EI+
9.26e4
2
2,4,6-Me3 Gal
PMAA-14 811 (10.096)
Scan EI+
3.07e4
3
3,4,6-Me3 Gal
PMAA-14 822 (10.203)
Scan EI+
2.11e5
5
2,3,6-Me3 Gal
PMAA-14 961 (11.487)
Scan EI+
2.97e4
6
2,6-Me2 Gal
PMAA-14 973 (11.605)
Scan EI+
1.21e5
7
4,6-Me2 Gal
m/z

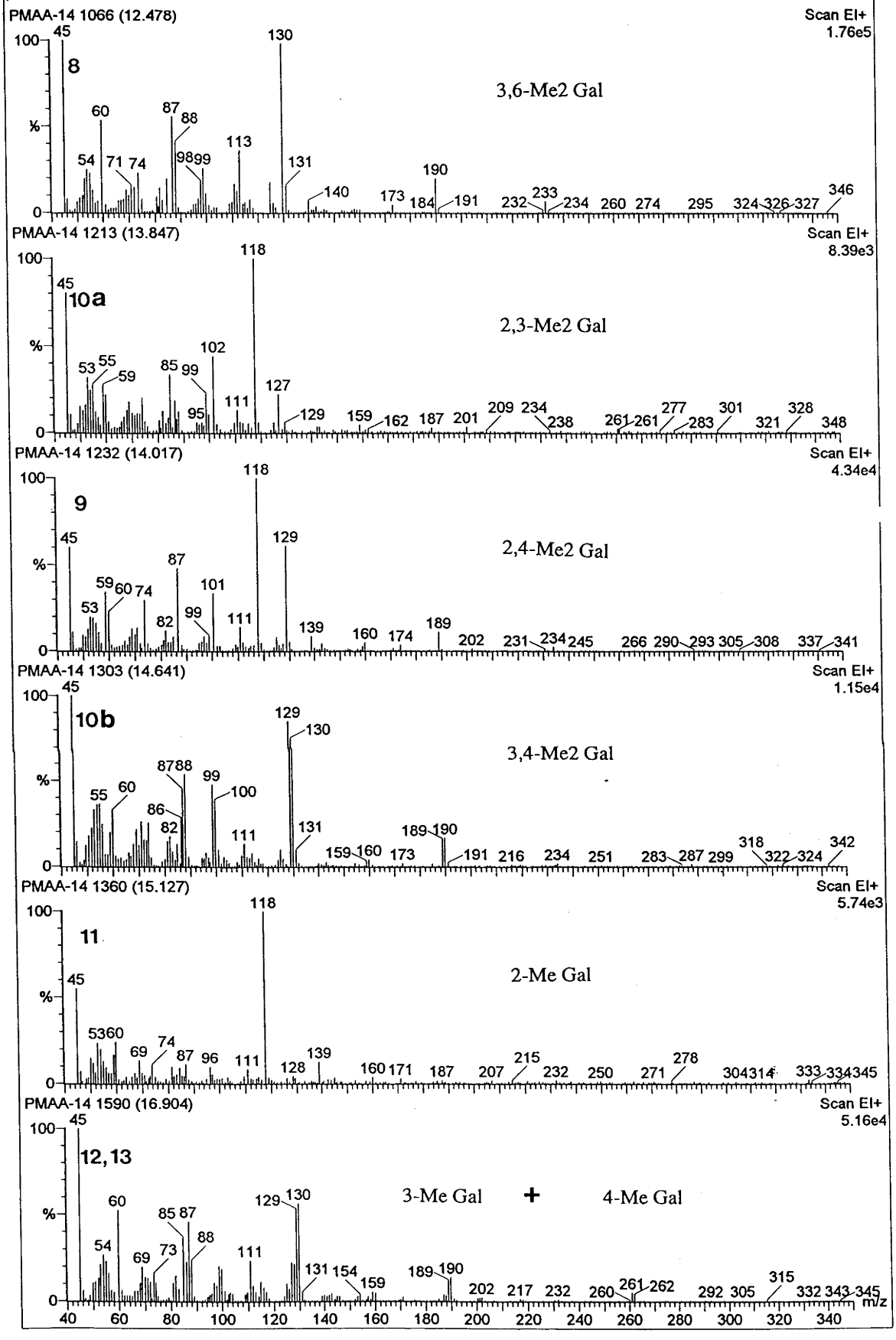
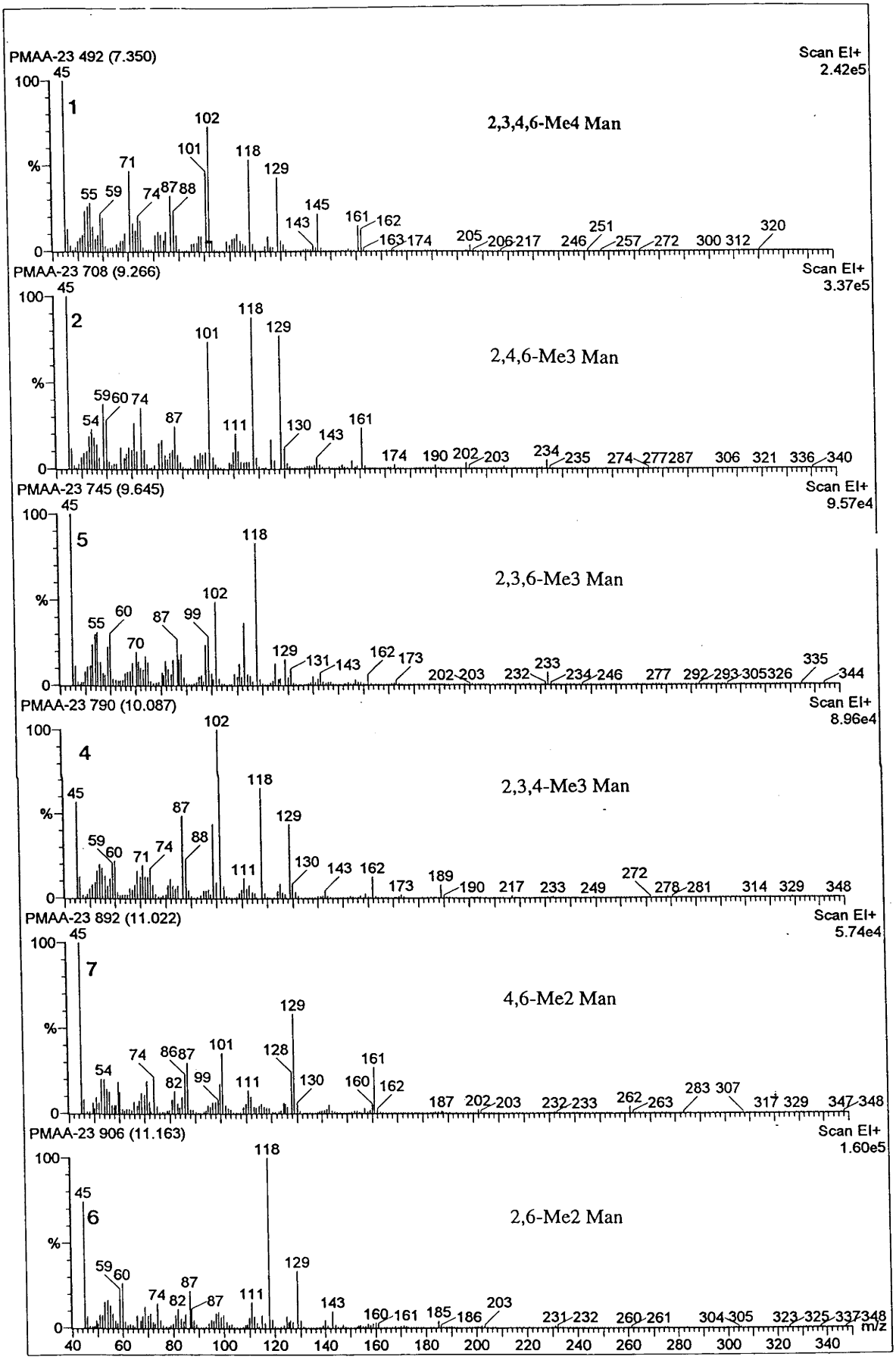

PMAA-14 1066 (12.478)
Scan EI+
1.76e5
8
3,6-Me2 Gal
PMAA-14 1213 (13.847)
Scan EI+
8.39e3
10a
2,3-Me2 Gal
PMAA-14 1232 (14.017)
Scan EI+
4.34e4
9
2,4-Me2 Gal
PMAA-14 1303 (14.641)
Scan EI+
1.15e4
10b
3,4-Me2 Gal
PMAA-14 1360 (15.127)
Scan EI+
5.74e3
11
2-Me Gal
PMAA-14 1590 (16.904)
Scan EI+
5.16e4
12,13
3-Me Gal + 4-Me Gal
m/z

Figure 2.6

MS of PMAAs of mannose standard from chromatogram in Figure 2.4, B.


PMAA-23 492 (7.350)
Scan EI+
2.42e5
1
2,3,4,6-Me4 Man
PMAA-23 708 (9.266)
Scan EI+
3.37e5
2
2,4,6-Me3 Man
PMAA-23 745 (9.645)
Scan EI+
9.57e4
5
2,3,6-Me3 Man
PMAA-23 790 (10.087)
Scan EI+
8.96e4
4
2,3,4-Me3 Man
PMAA-23 892 (11.022)
Scan EI+
5.74e4
7
4,6-Me2 Man
PMAA-23 906 (11.163)
Scan EI+
1.60e5
6
2,6-Me2 Man
m/z

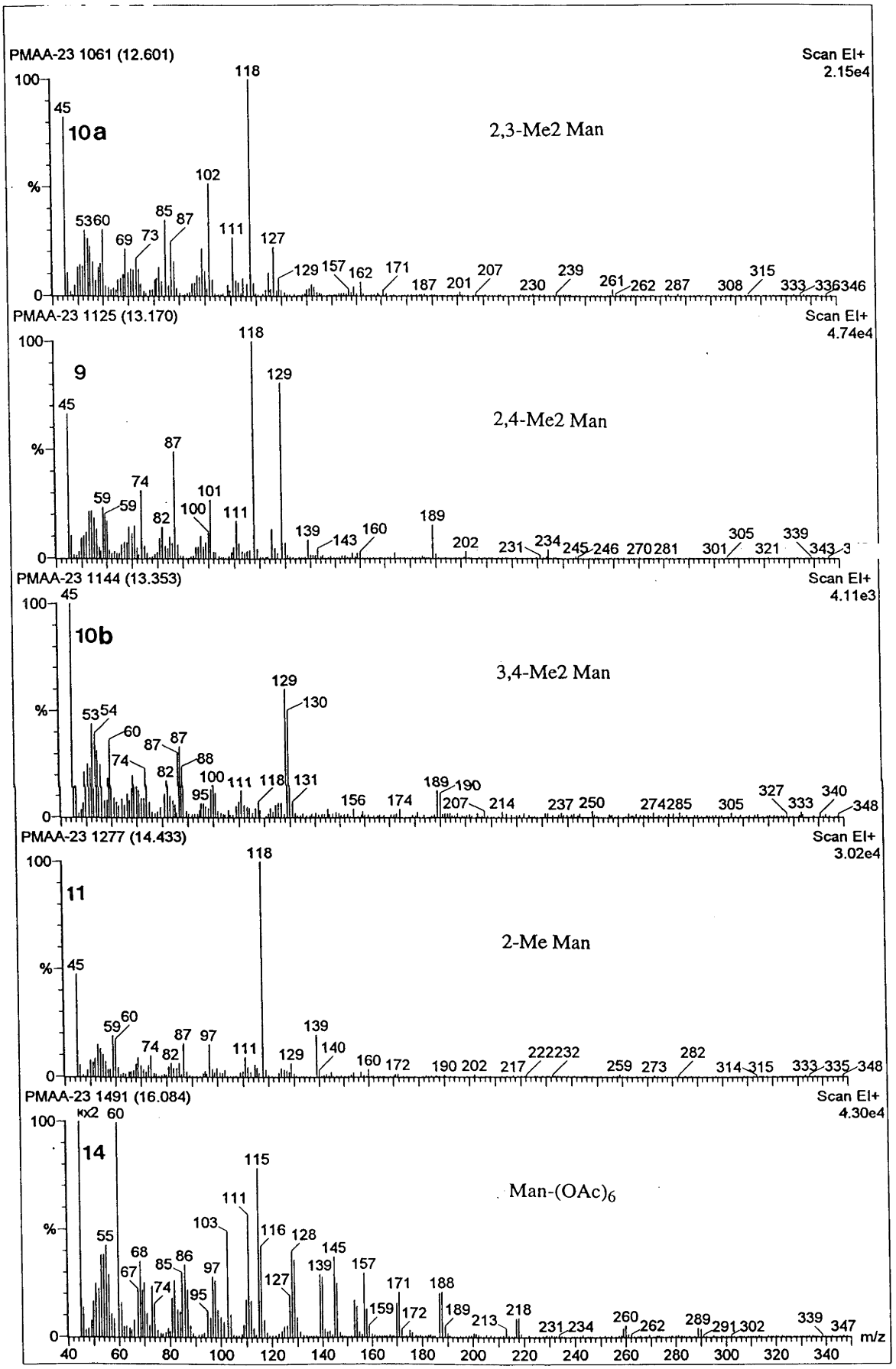

PMAA-23 1061 (12.601)
Scan EI+
2.15e4
10a
2,3-Me2 Man
PMAA-23 1125 (13.170)
Scan EI+
4.74e4
9
2,4-Me2 Man
PMAA-23 1144 (13.353)
Scan EI+
4.11e3
10b
3,4-Me2 Man
PMAA-23 1277 (14.433)
Scan EI+
3.02e4
11
2-Me Man
PMAA-23 1491 (16.084)
Scan EI+
4.30e4
14
Man-(OAc)6
m/z

Table 2.4 Relative retention times of PMAAs of galactose and mannose standard to *myo*-inositol-hexaacetate

Peak*	Derivative	Deduced Linkages	RRT	Peak	Derivative	Deduced Linkages	RRT
1	2,3,4,6-Me_4 Gal	t-Gal*p*	0.412	1	2,3,4,6-Me_4 Man	t-Man*p*	0.383
2	2,4,6-Me_3 Gal	3-Gal*p*	0.500	2	2,4,6-Me_3 Man	3-Man*p*	0.483
3	3,4,6-Me_3 Gal	2-Gal*p*	0.526	3	3,4,6-Me_3 Man	2-Man*p*	-
4	2,3,4-Me_3 Gal	6-Gal*p*	-	4	2,3,4-Me_3 Man	6-Man*p*	0.525
5	2,3,6-Me_3 Gal	4-Gal*p*	0.531	5	2,3,6-Me_3 Man	4-Man*p*	0.502
6	2,6-Me_2 Gal	3,4-Gal*p*	0.598	6	2,6-Me_2 Man	3,4-Man*p*	0.581
7	4,6-Me_2 Gal	2,3-Gal*p*	0.604	7	4,6-Me_2 Man	2,3-Man*p*	0.574
8	3,6-Me_2 Gal	2,4-Gal*p*	0.650	8	3,6-Me_2 Man	2,4-Man*p*	-
9	2,4-Me_2 Gal	3,6-Gal*p*	0.730	9	2,4-Me_2 Man	3,6-Man*p*	0.686
10b	3,4-Me_2 Gal	2,6-Gal*p*	0.762	10b	3,4-Me_2 Man	2,6-Man*p*	0.695
10a	2,3-Me_2 Gal	4,6-Gal*p*	0.721	10a	2,3-Me_2 Man	4,6-Man*p*	0.656
11	2-Me Gal	3,4,6-Gal*p*	0.788	11	2-Me Man	3,4,6-Man*p*	0.752
12	3-Me Gal	2,4,6-Gal*p*	0.880	12	3-Me Man	2,4,6-Man*p*	-
13	4-Me Gal	2,3,6-Gal*p*	0.880	13	4-Me Man	2,3,6-Man*p*	-
	6-Me Gal	2,3,4-Gal*p*	-		6-Me Man	2,3,4-Man*p*	-
14	Gal-$(OAc)_6$	-	-	14	Man-$(OAc)_6$	-	0.838
IS	myo-inositol-Ac_6		1	IS	myo-inositol-Ac_6		1

* Peak number indicated in chromatograms (Figure 2.4); The same MS for PMAA of each hexose (Glc, Gal and Man) labelled as the same peak number.

Figure 2.7

GC/MS chromatograms of PMAAs of xylose (**A**), arabinose (**B**), fucose (**C**), and rhamnose (**D**) standards. RRT of each labelled peak is presented in Tables 2.5 and 2.6. MS of each labelled peak presented in Figures 2.8, 2.9, 2.10, and 2.11, and its deduced glycosyl linkage is described in Tables 2.5 and 2.6. IS, Internal standard (*myo*-inositol).

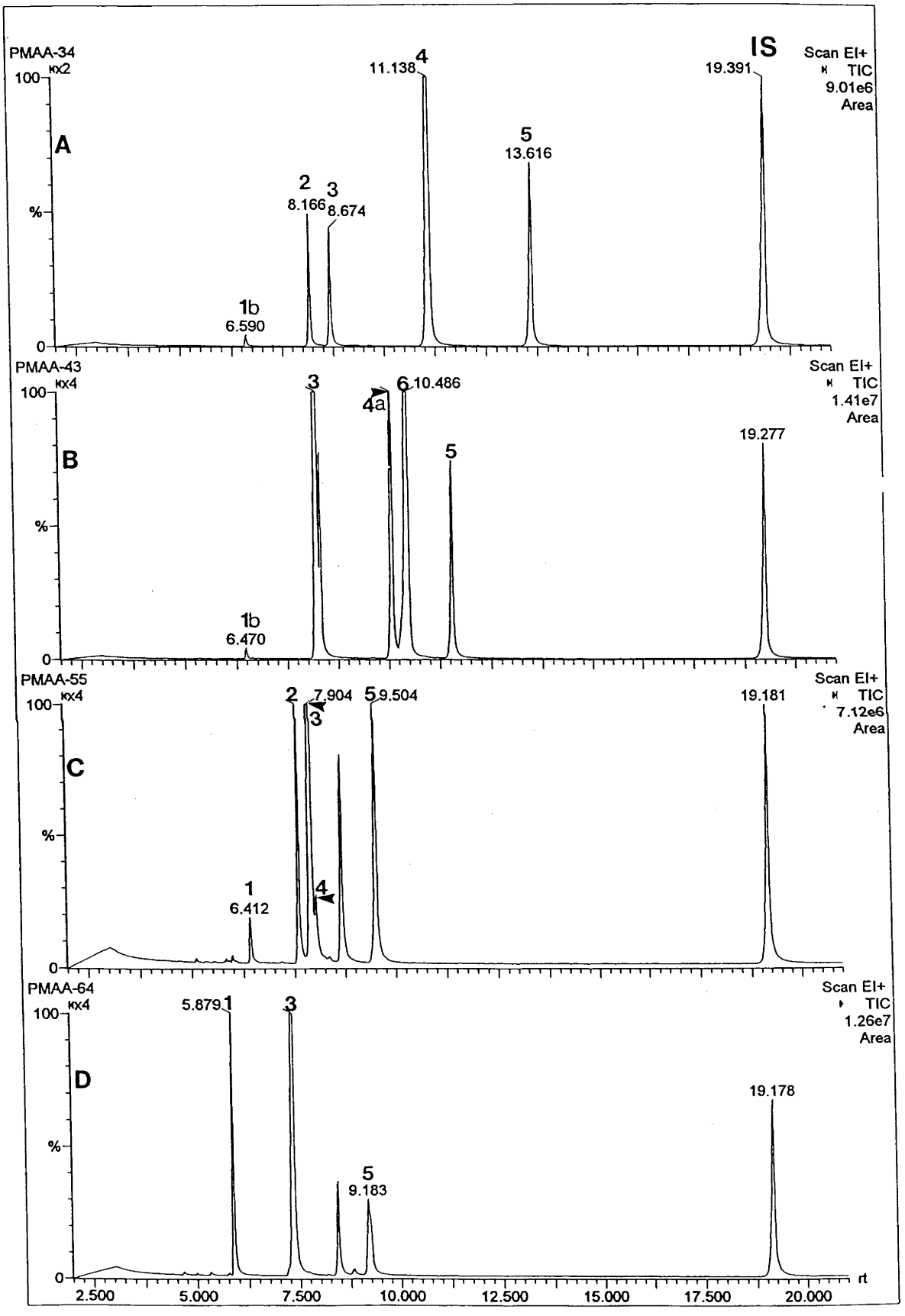
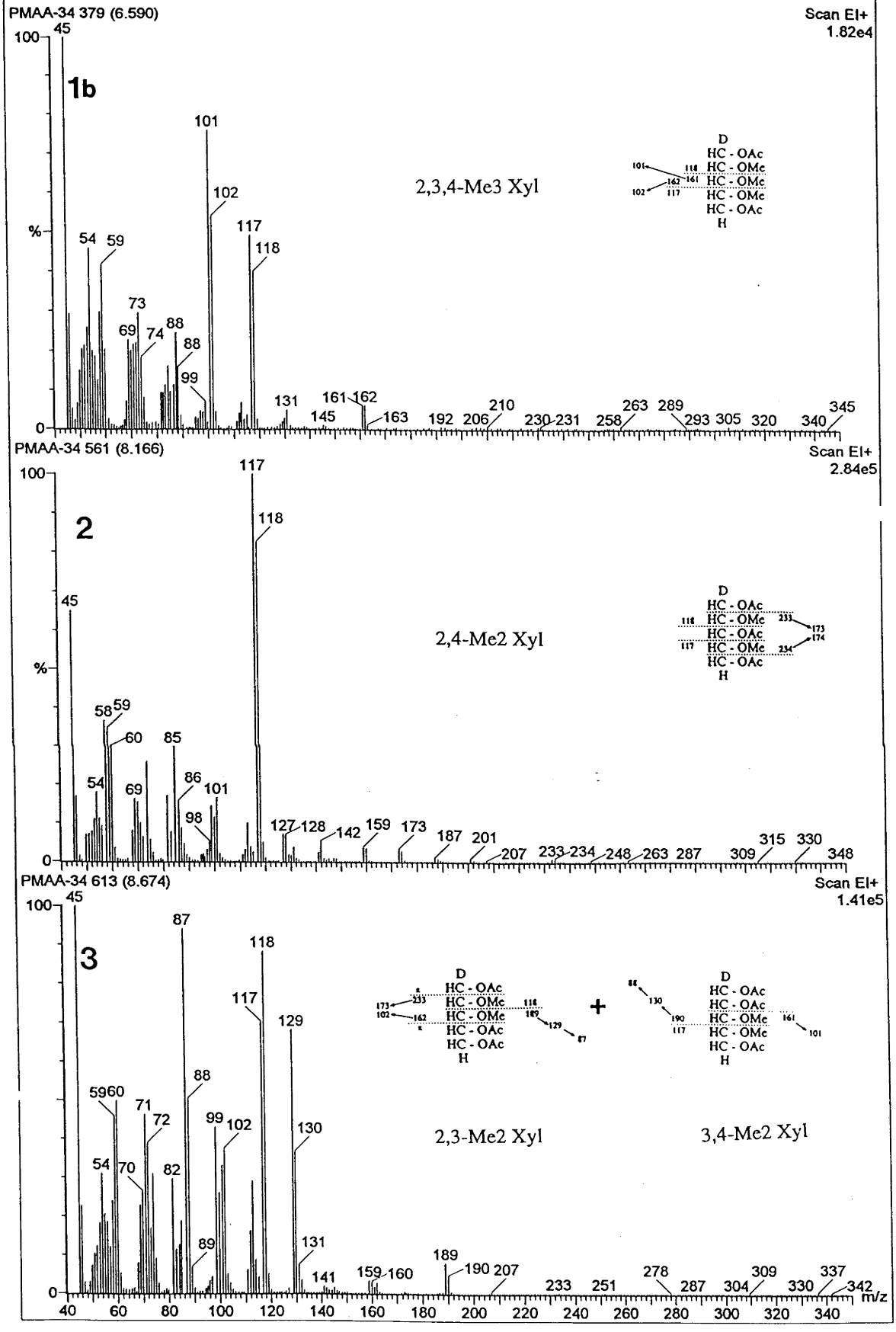

PMAA-34
Scan EI+
TIC
9.01e6
Area
A
1b
6.590
2
8.166
3
8.674
4
11.138
5
13.616
IS
19.391
PMAA-43
Scan EI+
TIC
1.41e7
Area
B
1b
6.470
3
4a
6
10.486
5
19.277
PMAA-55
Scan EI+
TIC
7.12e6
Area
C
1
6.412
2
7.904
3
4
5
9.504
19.181
PMAA-64
Scan EI+
TIC
1.26e7
Area
D
5.879
1
3
5
9.183
19.178
%
0
100
2.500
5.000
7.500
10.000
12.500
15.000
17.500
20.000
rt

Figure 2.8

MS of PMAAs of xylose standard from chromatogram in Figure 2.7, A.


PMAA-34 379 (6.590)
Scan EI+
1.82e4
1b
2,3,4-Me3 Xyl
PMAA-34 561 (8.166)
Scan EI+
2.84e5
2
2,4-Me2 Xyl
PMAA-34 613 (8.674)
Scan EI+
1.41e5
3
2,3-Me2 Xyl
3,4-Me2 Xyl
m/z

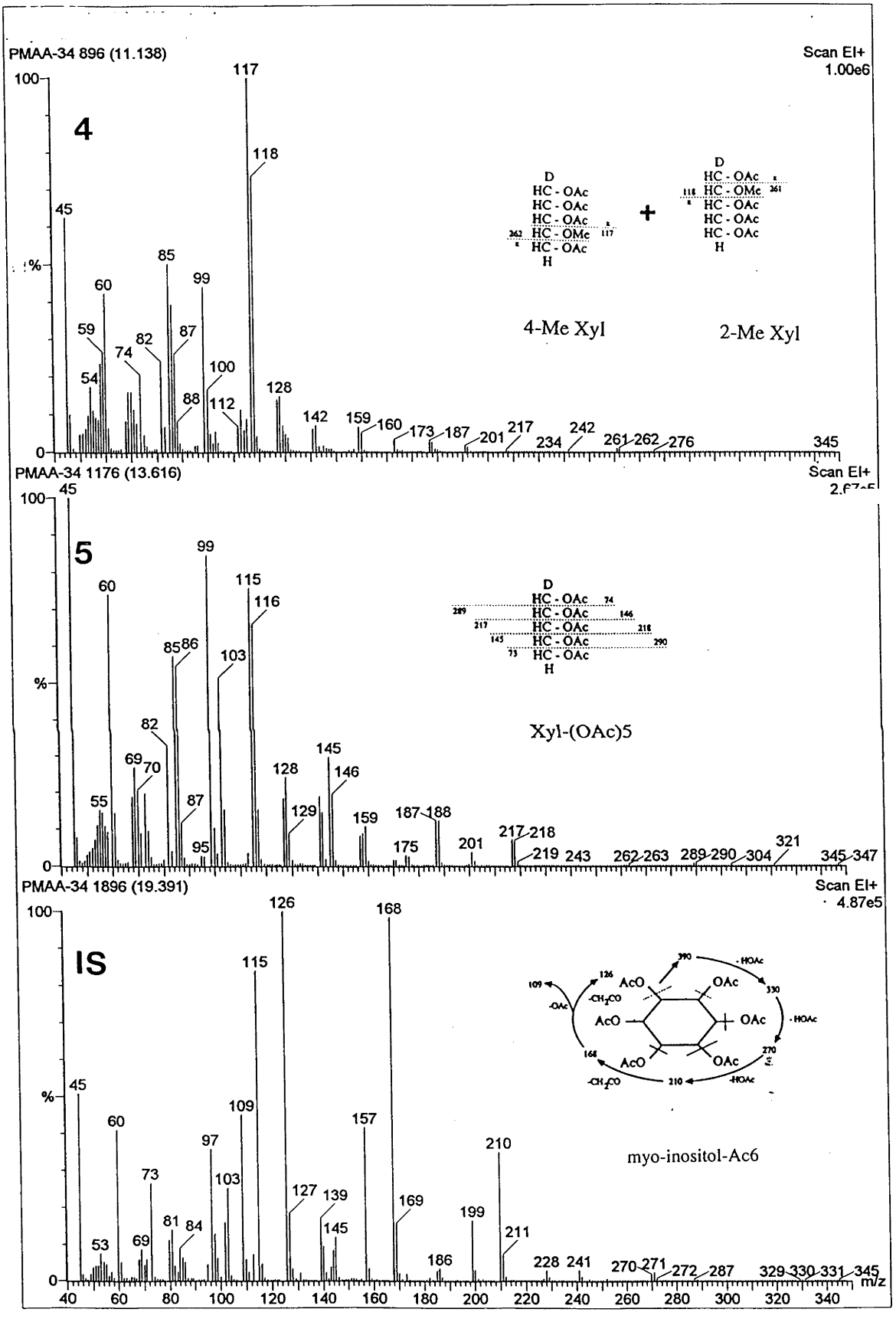

PMAA-34 896 (11.138)
Scan EI+
1.00e6
4
4-Me Xyl
2-Me Xyl
PMAA-34 1176 (13.616)
Scan EI+
5
Xyl-(OAc)5
PMAA-34 1896 (19.391)
Scan EI+
4.87e5
IS
myo-inositol-Ac6
m/z

Figure 2.9

MS of PMAAs of arabinose standard from chromatogram in Figure 2.7, B.

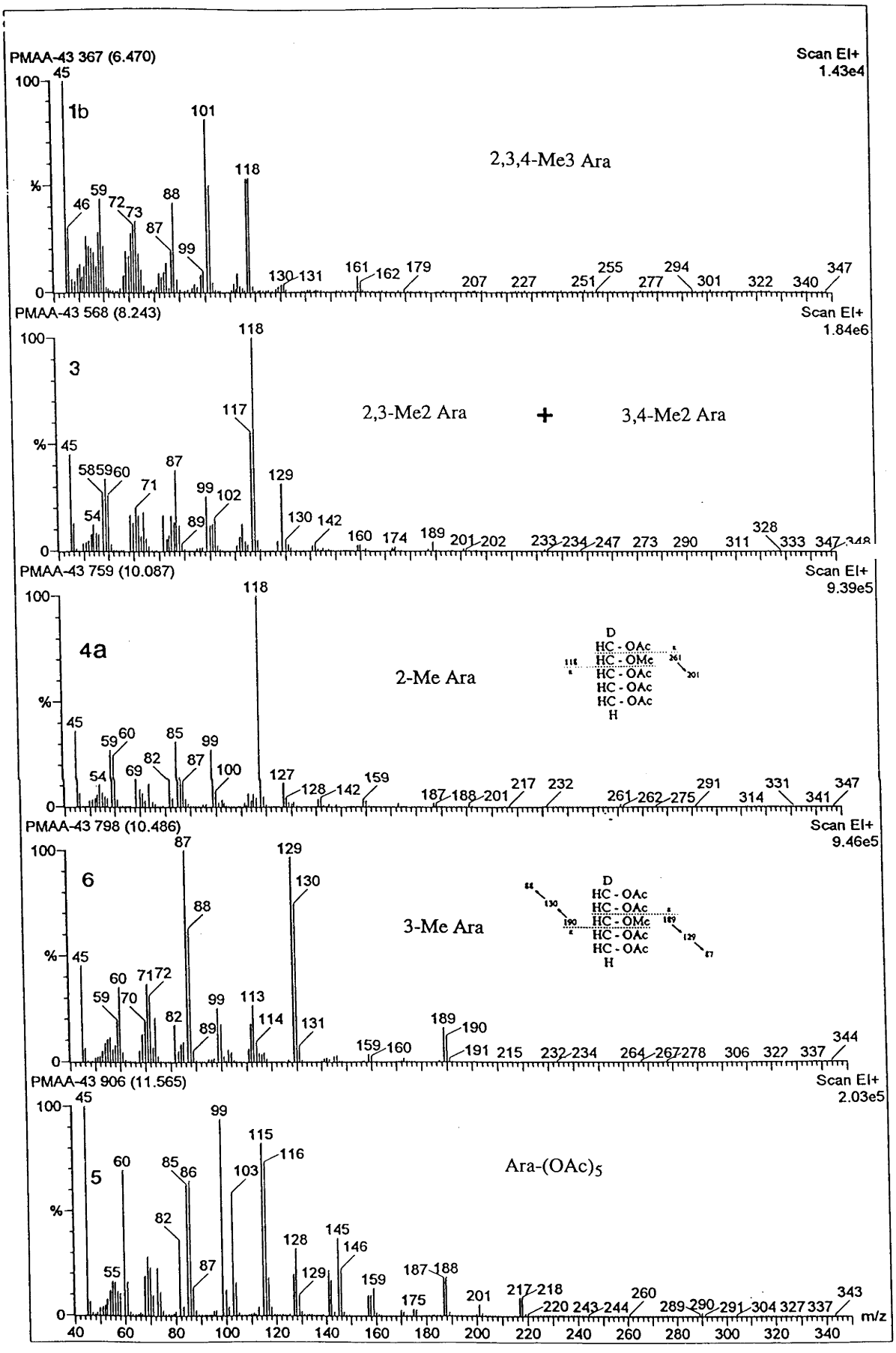

PMAA-43 367 (6.470)
Scan EI+
1.43e4
1b
2,3,4-Me3 Ara
PMAA-43 568 (8.243)
Scan EI+
1.84e6
3
2,3-Me2 Ara
+
3,4-Me2 Ara
PMAA-43 759 (10.087)
Scan EI+
9.39e5
4a
2-Me Ara
D
HC - OAc
HC - OMe
HC - OAc
HC - OAc
HC - OAc
H
PMAA-43 798 (10.486)
Scan EI+
9.46e5
6
3-Me Ara
D
HC - OAc
HC - OAc
HC - OMe
HC - OAc
HC - OAc
H
PMAA-43 906 (11.565)
Scan EI+
2.03e5
5
Ara-(OAc)5
m/z

Figure 2.10

MS of PMAAs of fucose standard from chromatogram in Figure 2.7, C.

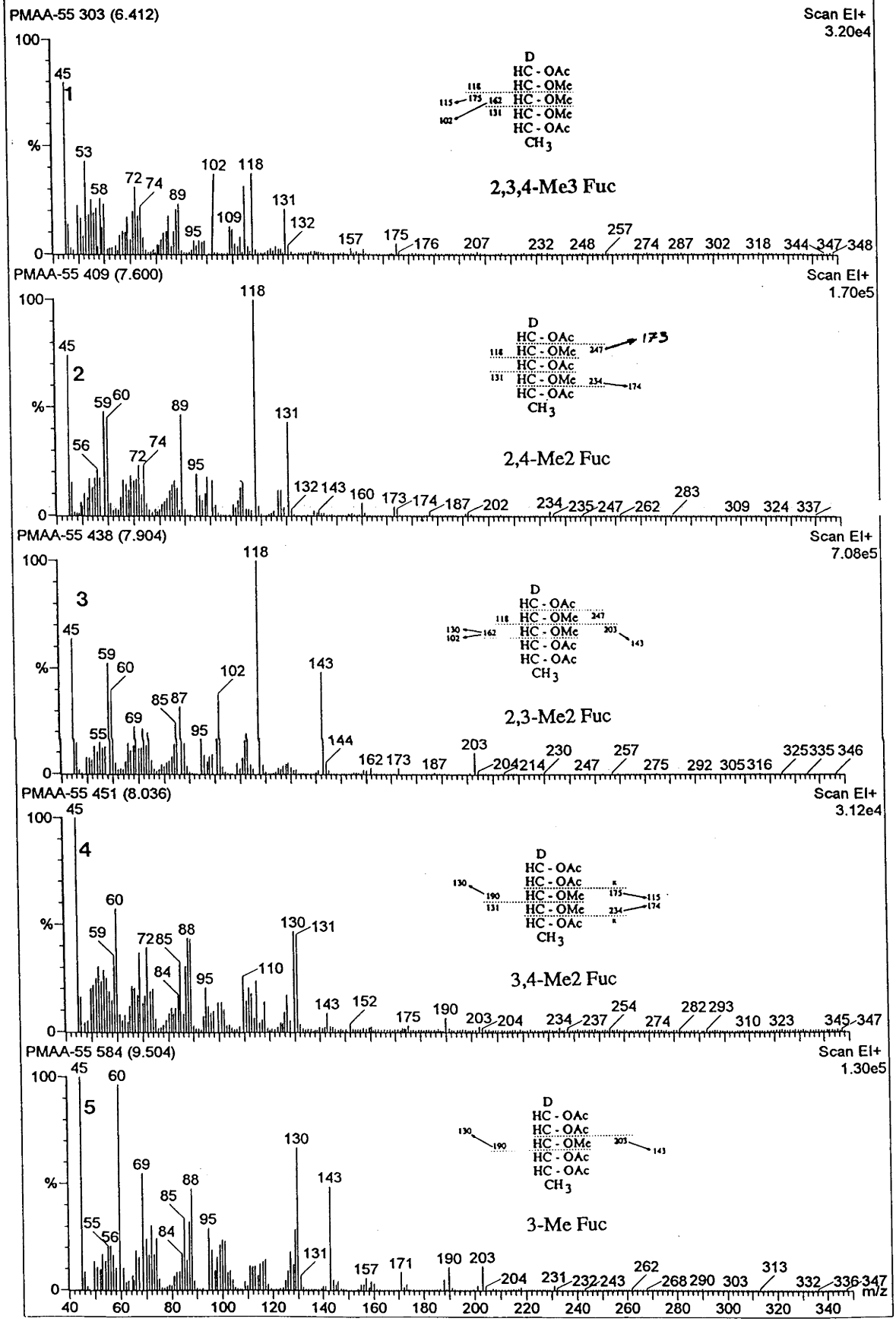

PMAA-55 303 (6.412)
Scan EI+
3.20e4
1
2,3,4-Me3 Fuc
PMAA-55 409 (7.600)
Scan EI+
1.70e5
2
2,4-Me2 Fuc
PMAA-55 438 (7.904)
Scan EI+
7.08e5
3
2,3-Me2 Fuc
PMAA-55 451 (8.036)
Scan EI+
3.12e4
4
3,4-Me2 Fuc
PMAA-55 584 (9.504)
Scan EI+
1.30e5
5
3-Me Fuc
HC - OAc
HC - OMe
CH3
m/z

Figure 2.11

MS of PMAAs of rhamnose standard from chromatogram in Figure 2.7, D.

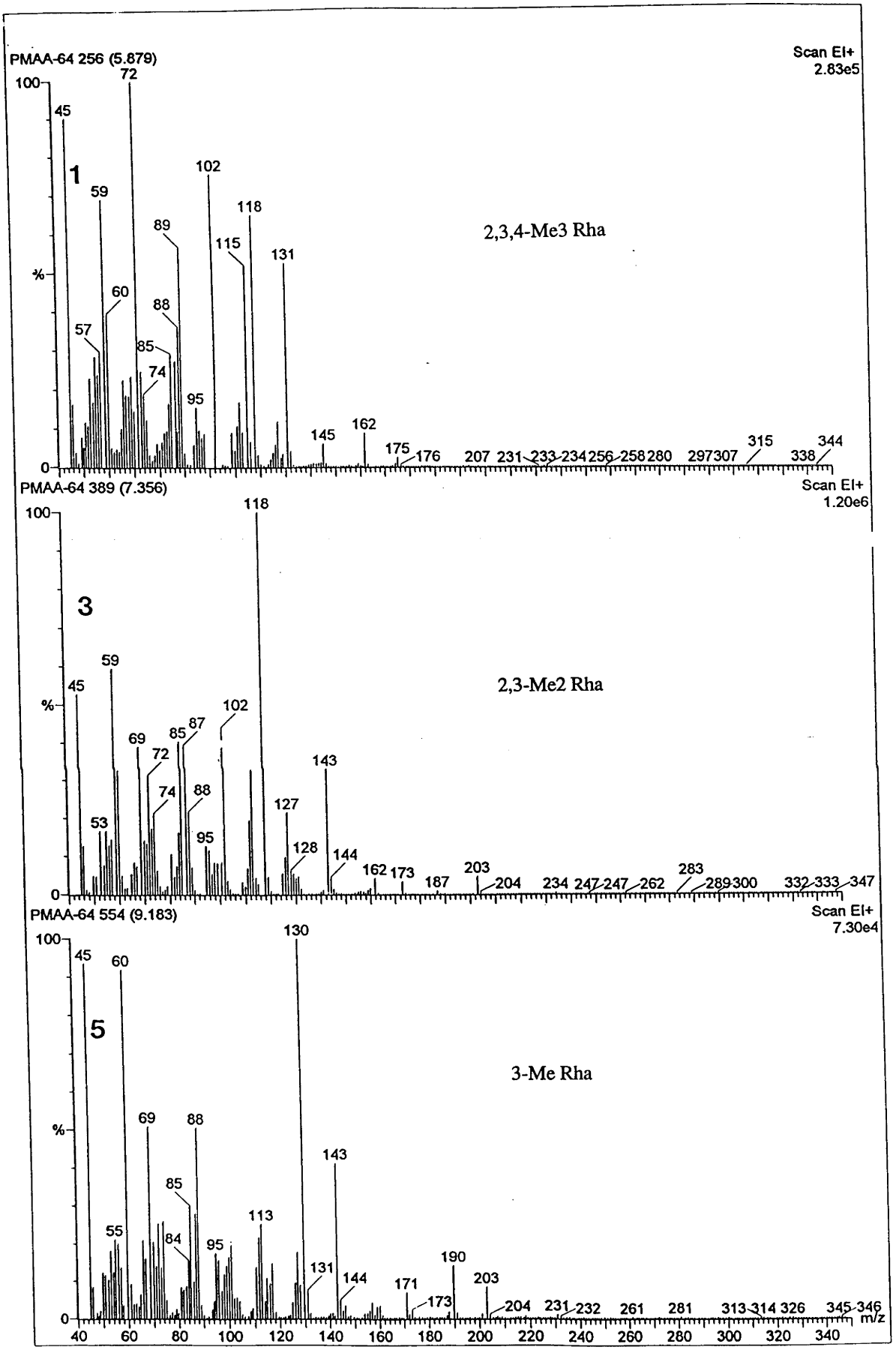

PMAA-64 256 (5.879)
Scan EI+
2.83e5
1
2,3,4-Me3 Rha
PMAA-64 389 (7.356)
Scan EI+
1.20e6
3
2,3-Me2 Rha
PMAA-64 554 (9.183)
Scan EI+
7.30e4
5
3-Me Rha
%
m/z

Figure 2.12

GC/MS Chromatograms of methylated alditol acetates of xylose (**A**) and arabinose (**B**) standards. RRT of each labelled peak is present in Table 2.5. MS of each labelled peak are presented in Figure 2.13, and its deduced glycosyl linkage is described in Table 2.5.

IS, Internal standard (*myo*-inositol).

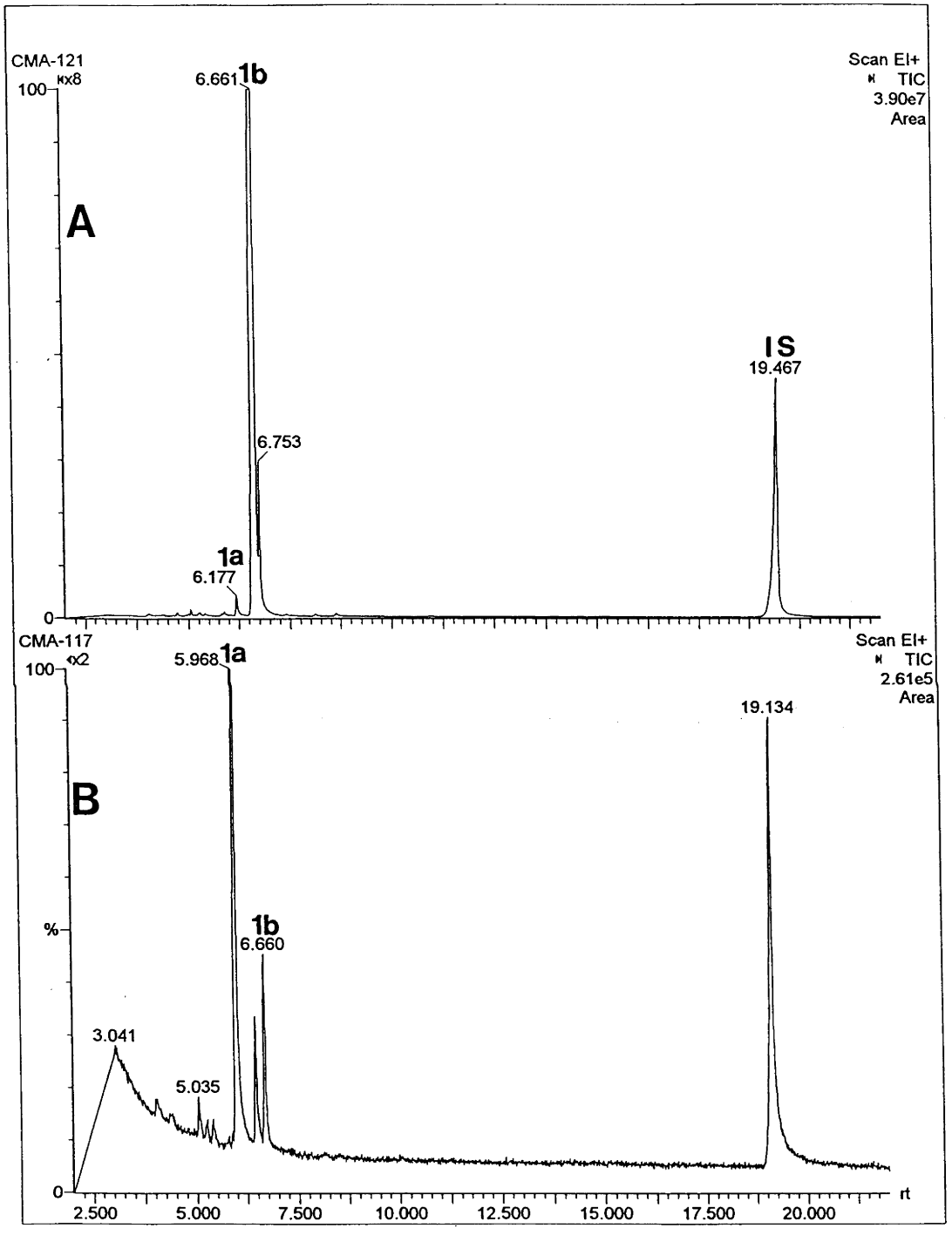

CMA-121
x8
100
0
A
6.661
1b
6.753
1a
6.177
IS
19.467
Scan EI+
TIC
3.90e7
Area
CMA-117
x2
100
%
0
B
5.968
1a
1b
6.660
3.041
5.035
19.134
Scan EI+
TIC
2.61e5
Area
2.500
5.000
7.500
10.000
12.500
15.000
17.500
20.000
rt

Figure 2.13

MS of PMAAs of xylose and arabinose from chromatograms in Figure 2.12.

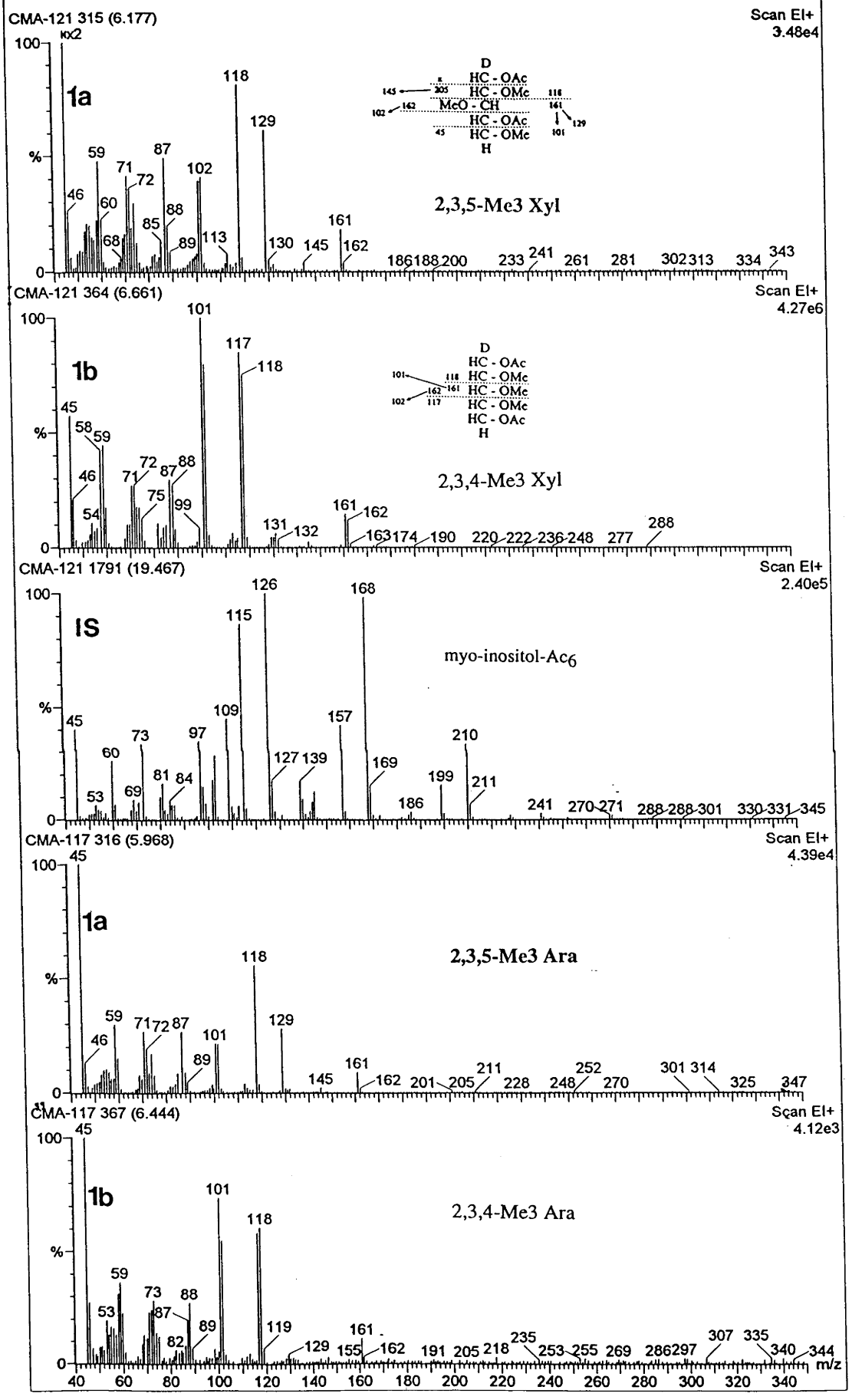

CMA-121 315 (6.177)
Scan EI+
3.48e4
1a
2,3,5-Me3 Xyl
CMA-121 364 (6.661)
Scan EI+
4.27e6
1b
2,3,4-Me3 Xyl
CMA-121 1791 (19.467)
Scan EI+
2.40e5
IS
myo-inositol-Ac6
CMA-117 316 (5.968)
Scan EI+
4.39e4
1a
2,3,5-Me3 Ara
CMA-117 367 (6.444)
Scan EI+
4.12e3
1b
2,3,4-Me3 Ara
m/z

Table 2.5 Relative retention times of PMAAs of xylose and arabinose standards relative to *myo*-inositol-hexaacetate

Peak*	Derivative	Deduced Linkage	RRT	Peak	Derivative	Deduced Linkage	RRT
1a	2,3,5-Me_3 Xyl	t-Xyl*f*	0.317	1a	2,3,5-Me_3 Ara	t-Ara*f*	0.312
1b	2,3,4-Me_3 Xyl	t-Xyl*p*	0.340	1b	2,3,4-Me_3 Ara	t-Ara*p*	0.336
2	2,4-Me_2 Xyl	3-Xyl*p*	0.421	2	2,4-Me_2 Ara	3-Ara*p*	-
3	2,3-Me_2 Xyl	4-Xyl*p*/5-Xyl*p*	0.447	3	2,3-Me_2 Ara	4-Ara*p*/5-Ara*p*	0.428
3	3,4-Me_2 Xyl	2-Xyl*p*	0.447	3	3,4-Me_2 Ara	2-Ara*p*	0.428
4a	2-Me Xyl	3,4-Xyl*p*	0.574	4a	2-Me Ara	3,4-Ara*p*	0.523
4b	4-Me Xyl	2,3-Xyl*p*	0.574	4b	4-Me Ara	2,3-Ara*p*	-
5	Xyl-$(OAc)_5$	-	0.702	5	Ara-$(OAc)_5$	-	0.600
6	3-Me Xyl	2,4-Xyl*p*	-	6	3-Me Ara	2,4-Ara*p*	0.544

* Peak number indicated in chromatograms (Figures 2.7 and 2.12); The same MS for PMAA of each pentose (xylose and arabinose) labelled as the same peak number.

Table 2.6 Relative retention times of PMAAs of fucose and rhamnose standards relative to *myo*-inositol-hexaacetate

Peak*	Derivative	Deduced Linkage	RRT	Peak	Derivative	Deduced Linkage	RRT
1	2,3,4-Me_3 Fuc	t-Fuc	0.334	1	2,3,4-Me_3 Rha	t-Rha	0.307
2	2,4-Me_2 Fuc	3-Fuc	0.396	2	2,4-Me_2 Rha	3-Rha	-
3	2,3-Me_2 Fuc	4-Fuc	0.412	3	2,3-Me_2 Rha	4-Rha	0.384
4	3,4-Me_2 Fuc	2-Fuc	0.419	4	3,4-Me_2 Rha	2-Rha	-
5	3-Me Fuc	2,4-Fuc	0.495	5	3-Me Rha	2,4-Rha	0.479
	2-Me Fuc	3,4-Fuc	-		2-Me Rha	3,4-Rha	-
	4-M Fuc	2,3-Fuc	-		4-Me Rha	2,3-Rha	-
	Fuc-$(OAc)_5$	-	-		Rha-$(OAc)_5$	-	-

* Peak number indicated in chromatograms (Figure 2.7); The same MS for PMAA of each 6-deoxyhexose (fucose and rhamnose) labelled as the same

CHAPTER THREE

A METHOD FOR THE FRACTIONATION OF THE POLYSACCHARIDES OF *ARABIDOPSIS* CELL WALLS

3.1 INTRODUCTION

3.1.1 The Plant Cell Wall

Plant cell walls are primarily composed of three major classes of polysaccharides: cellulose, hemicelluloses, and pectins. A number of different procedures which have been used for the fractionation of these polysaccharides (eg. Wada and Ray, 1978; Carpita and Kanabus, 1988; Heim et al, 1990 b; Zablackis et al, 1995) employ different methods for differential solubilisation of particular polysaccharides, such as the addition of chelating agents to remove divalent cations from pectic polysaccharides, alkali to weaken hydrogen bonding of hemicelluloses, and a mixture of acetic acid and nitric acid to solubilise non-crystalline cellulosic material (Fry, 1988).

3.1.2 Preparation of Plant Cell Walls

Plant cell walls can be prepared by homogenisation of tissue in a buffer (eg 20 mM HEPES or 0.5 M potassium phosphate, pH 7.0; Harris, 1983; Selvendran et al, 1985). The cells are physically disrupted and the cytoplasmic contents are washed out to leave walls which can be collected by centrifugation. Possible disadvantages of this approach include (Fry, 1988): (1) wall autolysis which can occur during homogenisation unless the preparation is kept at low temperature (0-4°C) to minimise wall enzyme activity; (2) loss of some water-soluble wall components, such as pectins; and (3) the requirement for very thorough mechanical treatment because the buffer itself has little disruptive effect on the tissue.

Several methods for cell disruption were evaluated (Goldberg, 1985; Fry, 1988) including: (1) grinding with a pestle and mortar in the presence of a little clean sand; (2) liquidising with a blender; (3) sonication; and (4) French pressure cell. For small amounts of materials, the grinding method is recommended. The procedure for the extraction of wall polysaccharides includes

use of organic chemicals (chloroform-methanol and acetone) to disrupt membranes and dissolve other lipid-compounds (Douce and Joyard, 1980) and reducing agents (eg 10 mM dithiothreitol or sodium metabisulphite) to minimise phenols becoming oxidised and cross-linked.

It is necessary to remove starch, which is abundant in some plant tissues, early in the extraction procedure. A generally satisfactory method for de-starching is to stir samples overnight at 25 °C with 90% aqueous DMSO (Selvendran et al, 1985; Heim et al, 1991). A disadvantage of this extraction is the removal of a small proportion of hemicellulose which may be recovered by washing several times with acetone (Fry, 1988). Carpita and Kanabus (1987) used sonication in DMSO followed by dilution of the sample to 20% DMSO, and treatment with glucoamylase to digest starch. Amylase is also be used to de-starch cell wall preparations (Zablackis et al, 1995), but commercial enzymes can be contaminated with other enzymes which digest wall polysaccharides (Fry, 1988), and long incubations required for complete digestion of starch may cause bacterial infection (Olaitan and Northcote, 1962).

3.1.3 Extraction and Fractionation of Cell Wall Polysaccharides

There are no perfect extractants for cell wall polysaccharides (Aspinall, 1982, Fry, 1988). Pectic polysaccharides are commonly isolated with aqueous chelating agents (such as ethylenediaminetetra acetate, EDTA), because most are rich in galacturonate residues so that adjacent polymer chains interact via Ca^{2+} ions. In the presence of EDTA to remove these ions, some pectic polysaccharides are gradually solubilised. There are few side effects if the wall treatment with chelating agents is kept at 25 °C. However, since chelating agents at 25 °C extract only a small proportion of pectin, and EDTA is difficult to remove by desalting, an alternative chelating agent (ammonium oxalate) is more commonly used (Carpita and Kanabus, 1988; O'Neil et al, 1990; Heim et al,

1991). The heating required for thorough extraction may cause polysaccharide degradation (Smidsrød et al, 1966).

After extraction of pectins in this way, alkali (either KOH or NaOH) is commonly used to solubilise hemicelluloses. It may also solubilise some residual pectic polysaccharides which have not been extracted by the chelating agent (Fry, 1988). The different hemicelluloses can be extracted with different concentrations of alkali (Carpita, 1984), and some hemicelluloses (such as mannans) are preferentially extracted in the presence of borate (Thornber and Northcote, 1962). Borohydride is added to the alkali to bring about reduction of the reducing termini of the polysaccharides, in order to avoid the alkali-induced depolymerisation process ("alkaline peeling") which might extract otherwise insoluble polysaccharides (Fry, 1988).

After the extraction of pectins and hemicelluloses, the remaining insoluble wall materials are digested in a mixture of acetic acid and nitric acid (Heim et al, 1990b). Very little is known about the chemical constitution of the acid-soluble materials (Updegraff, 1969, Fry 1988). The final insoluble wall material after this treatment is believed to be crystalline cellulose (Updegraff, 1969; Carpita, 1984; Heim et al, 1991).

Most of the polysaccharide fractions are contaminated with low molecular weight solutes, which must be removed before analysis. Desalting can be achieved by gel filtration (Fry, 1988), but there may be losses of small polymers which elute with the salt, or poor recoveries of polysaccharides because of precipitation on the column. Alternatively, polysaccharides may be precipitated with an organic solvent (Scott, 1960 and 1965), but there may be selective losses of the smaller polymers, and precipitated polysaccharide must be redissolved before analysis. On balance, the most practical method for desalting is dialysis. It removes DMSO, urea, inorganic salts, alkalis, and acids after neutralisation,

but not detergents, sodium hexametaphosphate and some chelating agents (Fry, 1988).

3.1.4 Aim of This Chapter

In this chapter, I adapt and validate a procedure which Heim et al (1991) used for the fractionation of *Arabidopsis* cell wall polysaccharides, but never validated by carbohydrate analyses. The method of Heim et al was taken from that used by Carpita (1984). I analyse each fraction for its monosaccharide composition, and use methylation analysis to determine linkage patterns. This information is used to assess the effectiveness of the fractionation procedure, and provides a background to assess changes in the wall polysaccharides in *Arabidopsis* mutants.

3.2 MATERIALS AND METHODS

3.2.1 Plant Growth

Seeds of wild type *Arabidopsis thaliana* (L.) Heynh (var. Columbia) were grown on agar media in growth chambers under constant light (80-90 μmol m^{-2} s^{-1}) and at 21°C for 7 days. Seeds were sterilised in 70% ethanol for 3 mins, followed by 5% (w/v) hypochlorite solution for 10 mins, rinsed well and plated on solidified agar medium. The medium comprised 2 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 90 μM iron-EDTA complex, 46 μM H_3BO_4, 9.1 μM $MnCl_2$, 0.77 μM $ZnSO_4$, 0.32 μM $CuSO_4$, 0.11 μM MoO_3, 1% (w/v) glucose, 1.2% (w/v) agar (Bacto-agar, Difco Laboratory, Detroit, Mich. USA), and 50 μM thiamine hydrochloride. Plates were sealed with laboratory film and placed vertically on racks.

Seven-day-seedlings were harvested, their roots and shoots collected separately, immediately frozen in liquid nitrogen and stored in sealed bottles at -70°C until use.

3.2.2 Plant Dry Weight Measurement

To measure plant dry weight, approximately 300 seedlings were rinsed well with cold distilled water and a sample checked under a microscope to ensure that all agar had been removed. The cleaned seedlings were dried for 16 h under vacuum in a freeze drier, then weighed. The seedlings' dry weight was determined in three replicates.

3.2.3 Spectrophotometric Assays

Total carbohydrates were analysed by the phenol/H_2SO_4 method (Dubois et al, 1956). An aliquot (0.4 ml) of aqueous sample (containing 2-15 μg carbohydrates) was added to 80% (w/w) phenol (10 μl), followed by conc H_2SO_4 (1 ml). The solution was shaken well and allowed to stand on the bench

until cool. Absorbance was read at 485 nm in a Shimadzu UV-265 spectrophotometer.

Total hexose contents of carbohydrates were determined by the anthrone/H_2SO_4 method (Dische, 1962; Fry, 1988). An aliquot (0.5 ml) of aqueous sample (containing 5-50 μg hexose) was added to 0.2 % anthrone (1 ml) in conc H_2SO_4, mixed well and incubated in a boiling water bath for 5 min. After the sample was cooled, its absorbance was read at 620 nm in the spectrophotometer. For the determinations of cellulose, the cellulose was dissolved in 67% (v/v) H_2SO_4 (1.0 ml) with shaking at 25 °C for 1 h (Fry, 1988), then a 10 μl aliquot used for the anthrone/H_2SO_4 method.

The orcinol/HCl method (Dische, 1962) was used to determine pentose contents. An aliquot (0.5 ml) of aqueous sample (containing 1-10 μg pentose) was added to 6% orcinol (67 μl) in ethanol, followed by 0.1% $FeCl_3.6H_2O$ (1.0 ml) in conc HCl, then mixed well and incubated in a boiling water bath for 20 min. After it was cooled, the sample was mixed again and its absorbance read at 665 nm.

Total uronic acids were measured by the *m*-hydroxybiphenyl/H_2SO_4 method (Blumenkrantz and Asboe-Hansen, 1973; Feingold, 1982). An aliquot (1 ml) of 0.5% borax ($Na_2B_4O_7.10H_2O$) in conc H_2SO_4 was added to an aqueous sample solution (0.2 ml, containing 1-20 μg uronic acid), mixed well and incubated in a boiling water bath for 5 min. After the sample was cool, absorbance was read at 520 nm. Then, 0.15% *m*-hydroxybiphenyl (20 μl) in 1 M NaOH was added with thorough mixing and the solution incubated at 25 °C for 5 min, and re-read at 520 nm. The increase in absorbance indicated the uronic acid content.

The cysteine/H_2SO_4 method was used to determine total 6-deoxyhexoses. An aliquot (1 ml) of 86% H_2SO_4 (v/v) was added to an aliquot of the sample solution (0.2 ml, containing 1-100 μg 6-deoxyhexoses), mixed well and

incubated in a boiling water bath for exactly 3 min. After the solution was cool, 3% L-cysteine-hydrochloride monohydrate (20 µl, in water) was added, mixed well and incubated at 25 °C for 2 h. The absorbance was read at 396 and 427 nm, and the difference (A_{396}-A_{427}) is proportional to 6-deoxyhexoses content.

Standard curves for all colorimetric methods were drawn using commercial samples: glucose for total carbohydrates or hexoses, xylose for pentoses, and galacturonic acid for uronic acids.

The methods described in Chapter Two, were also used here for monosaccharide composition determination and methylation analysis of each fraction.

3.2.4 Starch Detection with Iodine

A small amount of dry starch residue (100 µg-2 mg) was added to a drop of 0.33% iodine/0.67% potassium iodide. With starch a blue-black colour developed immediately.

3.2.5 Paper Chromatography

Paper chromatography was primarily used to identify whether a fraction contained GalA or GlcA (Hais and Macek, 1963). A sample (40-50 µg) of acid hydrolysate was gradually loaded onto the paper (Whatman 3 MM; 45 × 57 cm) to give a 0.5-1 cm diameter spot. Spots were spaced at 2-2.5 cm intervals. The chromatogram was developed in descending mode with butanol-acetic acid-water (12:3:5, v/v/v) for 18 h at room temperature, then immediately followed by ethyl acetate-pyridine-water (8:2:1, v/v/v) for 16 h. After the chromatographic paper was dried in the fume hood, spots were stained brown with silver nitrate/sodium hydroxide (Churms, 1982; Fry, 1988). For permanent storage, the stained paper was soaked in a 25% (v/v) solution of paper fixer (Ilford/Ilfospeed) for 30 seconds, then rinsed well and dried at room temperature. Standard R_f values were defined for each monosaccharide. Up to 1

mg sugar was applied in each spot, and as little as 0.1 μg of arabinose was detectable.

3.2.6 Fractionation of Cell Wall Polysaccharides

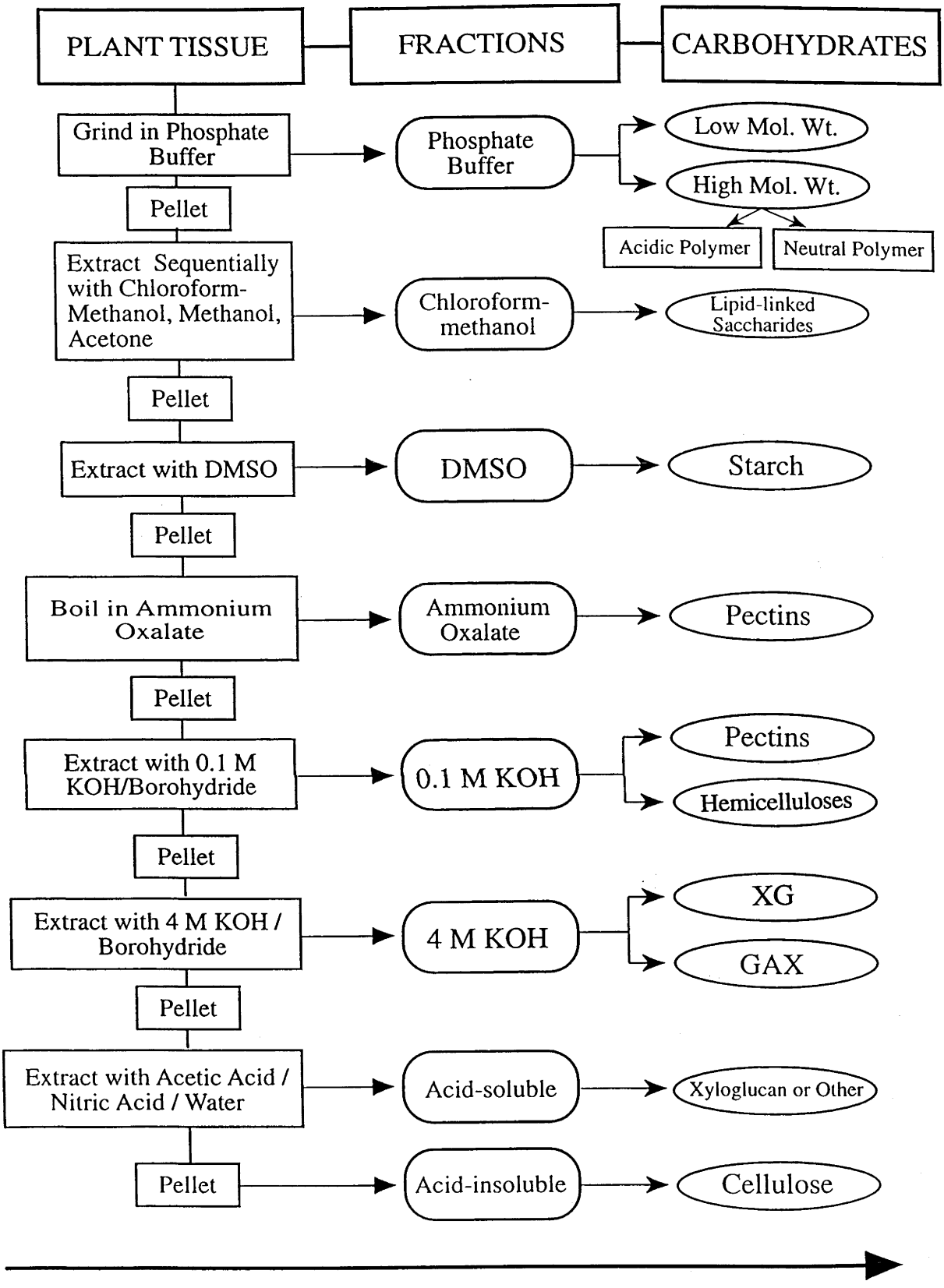
Cell walls were isolated and extracted by modifications of the procedures described by Carpita (1984) and Heim et al (1991) as summarised in Figure 3.1. Pellets were collected by centrifugation at 2,100 *g* for 10 min, unless mentioned separately. Three batches, each of about 100-150 seedlings were fractionated separately. For analyses using colorimetric tests, fractions from each batch of seedlings were analysed separately; for GC/MS analyses, fractions from all three extractions were pooled. Results from colorimetric tests are given with standard deviation. In addition, control experiments were conducted to check for agar medium contamination of fractions: (1), 200 seedlings were mixed with 200 mg agar medium and harvested; (2), 200 seedlings were harvested as normal; and (3), 200 mg agar only was taken. Fractionation was carried out on each of the three preparations.

3.2.6.1 Extraction with phosphate buffer

The dry seedlings were washed briefly with cold water several times and then ground thoroughly in a mortar with 3 ml cold 0.5 M potassium phosphate buffer (pH 7.0). The pestle and mortar were rinsed twice with 2 ml buffer and the combined homogenate centrifuged at 2,100 *g* for 10 min. The pellet was washed twice with 2 ml buffer and twice with 2 ml distilled water. The combined supernatants constituted the phosphate buffer fraction. This was dialysed (cellulose membrane; MWCO: 6-8, 000; Width: 40 mm; Diameter: 25.5 mm) extensively against distilled water and then lyophilised.

Figure 3.1

Procedure for Fractionation of *Arabidopsis* Cell Wall


PLANT TISSUE
FRACTIONS
CARBOHYDRATES
Grind in Phosphate Buffer
Phosphate Buffer
Low Mol. Wt.
High Mol. Wt.
Acidic Polymer
Neutral Polymer
Pellet
Extract Sequentially with Chloroform-Methanol, Methanol, Acetone
Chloroform-methanol
Lipid-linked Saccharides
Pellet
Extract with DMSO
DMSO
Starch
Pellet
Boil in Ammonium Oxalate
Ammonium Oxalate
Pectins
Pellet
Extract with 0.1 M KOH/Borohydride
0.1 M KOH
Pectins
Hemicelluloses
Pellet
Extract with 4 M KOH / Borohydride
4 M KOH
XG
GAX
Pellet
Extract with Acetic Acid / Nitric Acid / Water
Acid-soluble
Xyloglucan or Other
Pellet
Acid-insoluble
Cellulose

3.2.6.2 Extraction with chloroform–methanol

The cell wall pellet was stirred with 3 ml chloroform-methanol (1:1, v/v) for 1 h at 40 °C. After centrifugation, this step was repeated once for root tissue and twice for shoot tissue. The insoluble material was extracted at 40 °C with 2 ml methanol for 30 min, followed with 2 ml acetone. These steps were repeated once for root tissue, and twice for shoot tissue. After the pellet was washed twice with distilled water, all supernatants were combined and evaporated in a nitrogen stream to give the chloroform–methanol fraction.

3.2.6.3 Extraction with DMSO

A 3 ml aliquot of DMSO–water (9:1, v/v) was added to the cell wall pellet. The tube was purged with nitrogen, sealed, vortexed for 3 min and then rocked gently on a shaker. The root sample was extracted overnight, and the shoot sample for 24 h. After centrifugation, the pellet was washed twice with 2 ml DMSO–water, and then three times with 2 ml distilled water. The combined supernatants were dialysed and lyophilised to give the DMSO fraction.

3.2.6.4 Extraction with ammonium oxalate

The insoluble wall material was suspended in 0.5% ammonium oxalate (3 ml) and heated for 1 h in a boiling water bath. During this step, the sample was stirred vigorously every 10 min to prevent the accumulation of materials at the surface. After centrifugation and washing the pellet with distilled water (2 x 3 ml), the combined supernatants were dialysed and lyophilised to give the ammonium oxalate fraction.

3.2.6.5 Extraction with 0.1 M KOH

Under nitrogen, the cell wall pellet was suspended in 0.1 M KOH containing 1 mg/ml sodium borohydride (3 ml) and incubated for 1 h at 25 °C. This step was repeated once for root and twice for shoot tissue, and the pellets were washed once with 2 ml water. The combined supernatants were neutralised with acetic acid, dialysed and lyophilised to give the 0.1 M KOH fraction.

3.2.6.6 Extraction with 4 M KOH

The cell wall pellet was suspended in 4 M KOH containing 1 mg/ml sodium borohydride (3 ml) and incubated for 1 h at 25 °C. After centrifugation, this step was repeated once for root tissue and twice for shoot tissue. The combined supernatants were neutralised with acetic acid, dialysed and lyophilised to give the 4 M KOH fraction.

3.2.6.7 Extraction with acetic acid-nitric acid

The cell wall pellet was resuspended in 3 ml acetic acid–nitric acid–water (8:1:2, v/v/v) and heated for 1 h in a boiling water bath with stirring every 10 min. After centrifugation, the pellet was washed twice with water. The combined supernatants were diluted with water (5 ml), neutralised with 1 M KOH and acetic acid, dialysed and lyophilised to give the acid-soluble fraction. The remaining insoluble material was suspended in distilled water and lyophilised to give the acid-insoluble fraction.

3.2.6.8 Precipitation with CTAB

The phosphate buffer, ammonium oxalate, 0.1 M KOH and 4 M KOH fractions were further fractionated using cetyltrimethylammonium bromide (CTAB; Fry, 1988). Approximately 2 ml aliquots containing 1-2% carbohydrates were mixed with an equal volume of 1-2% CTAB containing 30 mM Na_2SO_4, and incubated at 37 °C overnight. After centrifugation at 2,100 *g* for 10 min, the supernatant containing the neutral polymers was collected. The pellet was re-dissolved in 3 ml of 5 M Na_2SO_4 by vortexing, freeze dried, mixed well with 80% ethanol, and centrifuged at 2,100 *g* for 10 min. The pellet contained acidic polysaccharides. To thoroughly wash out CTAB from the acidic polysaccharides, this 80% ethanol step was repeated. Finally, the pellet was de-salted by dialysis, and stored at 0-4°C until use.

The neutral polymers in the original CTAB supernatant were freeze dried, and re-suspended in 3 ml of 80% ethanol. After centrifugation, the pellet was resuspended in 2 ml distilled water, dialysed and stored frozen.

3.3 RESULTS AND DISCUSSION

Assessment of the fractionation is based on the monosaccharide composition of each fraction or sub-fraction and on the glycosidic linkage pattern identified by methylation analysis. Seedlings were grown on agar raising the possibility that agar adhering to the seedlings might contaminate the cell wall preparation. This possibility was eliminated as a significant problem by the control experiment described (Section 3.2.6). The 200 mg agar medium alone or mixed with the batch of seedlings was not dissolved by phosphate buffer or chloroform-methanol but was rapidly and completely dissolved by DMSO so that no pellet was obtained. The DMSO fraction showed, after dialysis and hydrolysis by TFA, a very high proportion of galactose and some glucose by GC/MS analysis (data not shown). From this experiment we cannot therefore exclude that some of the galactose found in the DMSO fraction of normally harvested plants (about 8% of DMSO fraction monosaccharides in the root) comes from agar carried over on the seedlings. The rapid and full solubilisation of agar in DMSO indicates that galactose found in subsequent fractions is very unlikely to represent agar contamination. Galactose in the DMSO fraction is less than 3% of total galactose recovered from all fractions making it a minor problem even for galactose.

3.3.1 Phosphate Buffer Fraction

The phosphate buffer fraction was expected to contain soluble proteins, metabolites and low molecular-weight carbohydrates (Fry, 1988). The monosaccharide composition (Tables 3.1, 3.2, 3.3, and 3.4), determined by GC/MS of alditol acetates after TFA hydrolysis, showed that approximately 80% of the monosaccharides in the phosphate buffer fraction were lost during dialysis prior to TFA hydrolysis. These are probably low molecular weight carbohydrates and metabolic intermediates such as nucleoside diphosphate-

Table 3.1 Monosaccharides from fractions of root tissue of *Arabidopsis* wild type (Columbia) grown at 21 °C [a]

Fractions	nmol/mg dry weight								
	UroA	Glc	Gal	Man	Xyl	Ara	Rha	Fuc	Total
Phosphate buffer LOW MOL. WT	83.94	92.23	50.78	12.44	20.21	83.94	48.19	50.78	442.51
HIGH MOL. WT	47.15	21.24	9.84	3.63	8.81	9.84	7.25	1.04	108.80
Chloroform/methanol	ND [b]	10.88	2.59	3.11	1.55	1.04	1.04	ND	20.21
DMSO	ND	21.76	2.59	ND	3.11	4.15	1.04	ND	32.65
Ammonium oxalate	144.04	18.13	17.62	2.59	16.06	33.68	11.92	4.15	248.19
0.1 M KOH	30.05	4.66	18.13	ND	9.84	12.95	4.66	3.11	83.40
4 M KOH	18.13	40.41	22.28	10.36	67.36	30.05	2.59	9.33	200.51
Acetic-nitric Acid-soluble	ND	7.77	1.04	2.07	3.11	2.07	3.11	1.04	20.21
Acetic-nitric Acid-insoluble	ND	515.03	ND	2.59	8.81	3.11	ND	ND	529.53
Total [c]	239.37	639.88	74.09	24.35	118.65	96.89	31.61	18.67	1243.51
%	19	52	6	2	10	8	2	1	100

a Uronic acids were determined by the *m*-hydroxybiphenyl/H_2SO_4 test, all others determined by GC/MS.

b ND, not detected.

c Total for each monosaccharide summed across all fractions, except the low molecular weight fraction.

Table 3.2 Monosaccharides from fractions of shoot tissue of *Arabidopsis* wild type (Columbia) grown at 21 °C [a]

Fractions	nmol/mg dry weight								
	UroA	Glc	Gal	Man	Xyl	Ara	Rha	Fuc	Total
Phosphate buffer LOW MOL. WT	65.35	105.80	3.14	9.21	43.62	154.31	140.78	8.92	531.13
HIGH MOL. WT	20.01	8.92	3.02	1.01	3.02	4.90	2.02	0.86	43.76
Chloroform/methanol	ND	4.75	25.19	1.58	1.58	0.72	0.43	ND	34.25
DMSO	ND	22.74	0.86	ND	1.15	1.01	0.29	ND	26.05
Ammonium oxalate	191.88	42.46	11.66	0.86	8.78	17.42	15.40	1.58	290.04
0.1 M KOH	16.70	1.58	5.18	ND	2.30	7.34	4.89	0.43	38.43
4 M KOH	9.79	28.65	11.95	8.64	26.34	13.10	0.86	3.17	102.50
Acetic-nitric Acid-soluble	ND	2.02	0.14	1.01	0.86	0.14	0.86	0.43	5.46
Acetic-nitric Acid-insoluble	ND	287.32	ND	2.16	4.17	2.02	ND	ND	295.67
Total [b]	238.38	398.44	58.00	15.26	48.20	46.65	24.75	6.47	836.15
%	28	48	7	2	6	5	3	1	100

a Uronic acids were determined by the *m*-hydroxybiphenyl/H_2SO_4 test, all others determined by GC/MS.

b Total for each monosaccharide summed across all fractions, except the low molecular weight fraction.

Table 3.3 Compositional analysis for fractions of root tissue of *Arabidopsis* wild type (Columbia) grown at 21 °C

Fractions	mol%								
	UroA	Glc	Gal	Man	Xyl	Ara	Rha	Fuc	Total
Phosphate buffer LOW MOL. WT	19	21	11	3	5	19	11	11	100
HIGH MOL. WT	43	20	9	3	8	9	7	1	100
Chloroform/methanol	ND	54	13	15	8	5	5	ND	100
DMSO	ND	67	8	ND	9	13	3	ND	100
Ammonium oxalate	58	7	7	1	6	14	5	2	100
0.1 M KOH	36	5	22	ND	12	16	5	4	100
4 M KOH	9	20	11	5	34	15	1	5	100
Acetic-nitric Acid-soluble	ND	39	5	10	16	10	15	5	100
Acetic-nitric Acid-insoluble	ND	97	ND	Tr *	2	1	ND	ND	100

* Tr, ≤ 0.5%.

Table 3.4 Compositional analysis for fractions of shoot tissue of *Arabidopsis* wild type (Columbia) grown at 21 °C

Fractions	mol%								
	UroA	Glc	Gal	Man	Xyl	Ara	Rha	Fuc	Total
Phosphate buffer LOW MOL. WT	11	19	7	2	8	27	25	1	100
HIGH MOL. WT	46	20	7	2	7	11	5	2	100
Chloroform/methanol	ND	14	74	5	4	2	1	ND	100
DMSO	ND	87	3	ND	5	4	1	ND	100
Ammonium oxalate	66	14	4	1	3	6	5	1	100
0.1 M KOH	43	4	14	ND	6	19	13	1	100
4 M KOH	10	28	12	8	26	13	1	3	100
Acetic-nitric Acid-soluble	ND	37	3	18	16	2	16	8	100
Acetic-nitric Acid-insoluble	ND	97	ND	Tr	2	1	ND	ND	100

Table 3.5 Compositional analysis of acidic and neutral polymers from phosphate buffer fraction

		UroA	Glc	Gal	Man	Xyl	Ara	Rha	Fuc	Total
ROOT [a]										
Pellet	**A** [b]	47.15	6.74	9.33	3.11	8.29	8.81	7.25	1.04	91.72
	B	52	7	10	3	9	10	8	1	100
Supernatant	**A**	ND	14.51	0.52	0.52	0.52	1.04	ND	ND	17.11
	B	ND	85	3	3	3	6	ND	ND	100
SHOOT										
Pellet	**A**	20.01	3.60	2.88	0.86	2.73	4.32	2.02	0.86	37.28
	B	54	10	8	2	7	12	5	2	100
Supernatant	**A**	ND	5.33	0.14	0.14	0.29	0.58	ND	ND	6.48
	B	ND	83	2	2	4	9	ND	ND	100

a Corrected for 80% (root) and 85% (shoot) recovery.

b A, nmol/mg dry weight; B, mol%.

sugars (Ericson and Elbein, 1980; Duffus and Duffus, 1984; Rees, 1988; Fry, 1988).

The carbohydrates in the dialysed phosphate buffer fraction were fractionationed into acidic and neutral polymers (Section 3.2.6.8) with CTAB (Table 3.5). Uronic acids were measured by the *m*-hydroxybiphenyl colour test, which does not distinguish between galacturonic acid and glucuronic acid. A hydrolysate of the acidic polysaccharides was therefore analysed by paper chromatography, which showed only galacturonic acid (data not shown). This is consistent with the presence of pectic polysaccharides (Zablackis et al (1995), and suggested that there was no contamination of this fraction with GAX.

The neutral polymers in the CTAB supernatant from the dialysed phosphate buffer fraction consisted almost exclusively of glucose (83-85%), with very small amounts of other monosaccharides (Tables 3.5). The pellet contained all the uronic acids detectable by the *m*-hydroxybiphenyl/H_2SO_4 method and 20% (root) and 15% (shoot) of the assayable uronic acids were lost when the pellet was processed with ethanol to obtain a clean GC/MS chromatogram. The appropriate correction factor was therefore applied to all monosaccharide determinations made on the pellet. Because less than 3% of total wall carbohydrates assayed by the phenol/H_2SO_4 colour test were lost during processing of the neutral polymer fraction, no correction was applied to those figures.

3.3.2 Chloroform–Methanol Fraction

It was expected that lipids that associated with the pellet would appear in this fraction (Carpita, 1984; Fry, 1988; Heim et al, 1991; Zablackis et al, 1995). These include the galactose-containing glycolipids (Douce and Joyard, 1980) and the lipid-linked saccharides which are intermediates in the formation of some complex carbohydrates and glycoconjugates (Hopp et al, 1978; Elbein, 1980; Douce and Joyard, 1980; Joyard and Douce, 1987).

In this fraction of *Arabidopsis* wild type seedlings (Tables 3.3 and 3.4), there were three major monosaccharide components (Gal, Glc and Man). The root tissue contained 54% glucose, 15% mannose and 13% galactose, but the shoot tissue had a very high proportion of galactose (74%), 14% glucose and 5% mannose. That is probably because chloroplasts in shoot tissue are rich in galacto-lipids (Douce and Joyard, 1980). It is unclear whether the small amounts of other monosaccharides (Xyl, Ara, and Rha) in the fraction (Tables 3.1 and 3.2) are from lipid-linked saccharides.

3.3.3 DMSO Fraction

The level of sugars in this fraction is low (Tables 3.1 and 3.2) relative to the other polysaccharide fractions and the main monosaccharide is glucose (Tables 3.3 and 3.4). Methylation analysis of this fraction yielded two major PMAAs which were identified by both RRT (Table 3.6) and MS (Figure 3.4, B; and Figure 3.5, B) as t-Glc*p* and 4-Glc*p*. The remaining minor peaks in the chromatogram were unidentifiable according to their MS (data not shown). The major polysaccharide therefore is 1,4-linked glucose residue consistent with starch being the main compound present (Heim et al, 1991). Moreover, about 84% of total glucose was released by α-amylase and α-glucosidase (method in Section 4.2.3.2) and a blue colour was given with the iodine–potassium iodide reagent. PMAAs indicating the branch points expected in amylopectin may have gone undetected with the low level of sample analysed.

The amounts of other monosaccharides in the DMSO fraction (Tables 3.1 and 3.2) were rather low and may be the result of a low level of polysaccharide degradation (Selvendran et al, 1985; Fry, 1988).

It is concluded that treatment with DMSO is mild and causes starch removal with co-extraction of limited amounts of other polysaccharides.

3.3.4 Ammonium Oxalate Fraction

The ammonium oxalate fraction (Tables 3.1 and 3.3) of *Arabidopsis* wild type root tissue contained 58% uronic acid and 14% arabinose. Glucose, galactose, xylose and rhamnose each comprised 5–7%. This fraction from shoot tissue had a similar composition (Tables 3.2 and 3.4), although the proportion of glucose was rather higher than in the root tissue. Methylation analysis for the CTAB pellet from the ammonium oxalate fraction of whole seedlings showed (Figures 3.6 A and 3.7; Table 3.7) PMAAs corresponding to most of the linked residues (t-Rha, 2-Rha, 2,4-Rha, t-Gal*p*, 4-Gal*p*, t-Ara*f*, t-Ara*p*, 5-Ara*f*, t-Xyl*p*) expected for pectic polysaccharides (Northcote, 1972; Jarvis, 1984; Carpita, 1984; Zablackis et al, 1995). The expected major residue of 1,4-linked galacturonic acid is not seen by this method, because the sample has not been treated to reduce an activated ester of the carboxyl prior to methylation. The identity of peak 6 could not be unambiguously assigned on the basis of RRT and MS. It may contain either or both of t-Man*p* and t-Glc*p*. Although the PMAA standards for 4-Ara*p*/5-Ara*f* and 2-Ara*p* co-chromatograph (Figure 2.9 and Table 2.5), the identity of peak 8 is deduced to be 5-Ara*f* as the lack of m/z 117 and 101 in the MS eliminates 2-Ara*p* as a possibility, and because 5-Ara*ff* rather than 4-Ara*p* is a common constitute of pectic polysaccharide. Although there is no PMAA standard for 2-Rha, peak 5 was assigned as 2-Rha because of the similarity of the MS to that of the stereo-isomer (2-Fuc) and because of the clear difference between the RRT for peak 5 (0.376) and 2-Fuc (0.419). A prominent peak (No, 11) on the chromatogram (Figure 3.6, A) corresponding to 1,4-linked Glc was observed, a finding made by others for the ammonium oxalate fraction (Carpita, 1984; Carpita and Kanabus, 1988; Shea et al, 1989; Kikuchi et al, 1996). Chapters Five and Six will show that a β-1,4-glucan may exist in the ammonium oxalate fraction of shoots, which probably explain why much more glucose was in this fraction prepared from shoots than when prepared from roots.

Figure 3.2

Paper chromatography of sugars released from TFA hydrolysed cell wal fractions hydrolysed by TFA or, in the case of the acid-insoluble fraction, by H_2SO_4.

Ammonium Oxalate Fraction: Lane **1**, root; Lane **2**, shoot

0.1 M KOH Fraction: Lane **3**, root; Lane **4**, shoot.

4 M KOH Fraction: Lane **5**, shoot.

Acid-soluble Fraction: Lane **6**, root; Lane **7**, shoot.

Acid-insoluble Fraction: Lane **8**, root; Lane **9**, shoot.

Standard Sample: Lane **10**, mixture of monosaccharides (GalA, GlcA, Gal, Glc, Ara, Xyl, Rha).

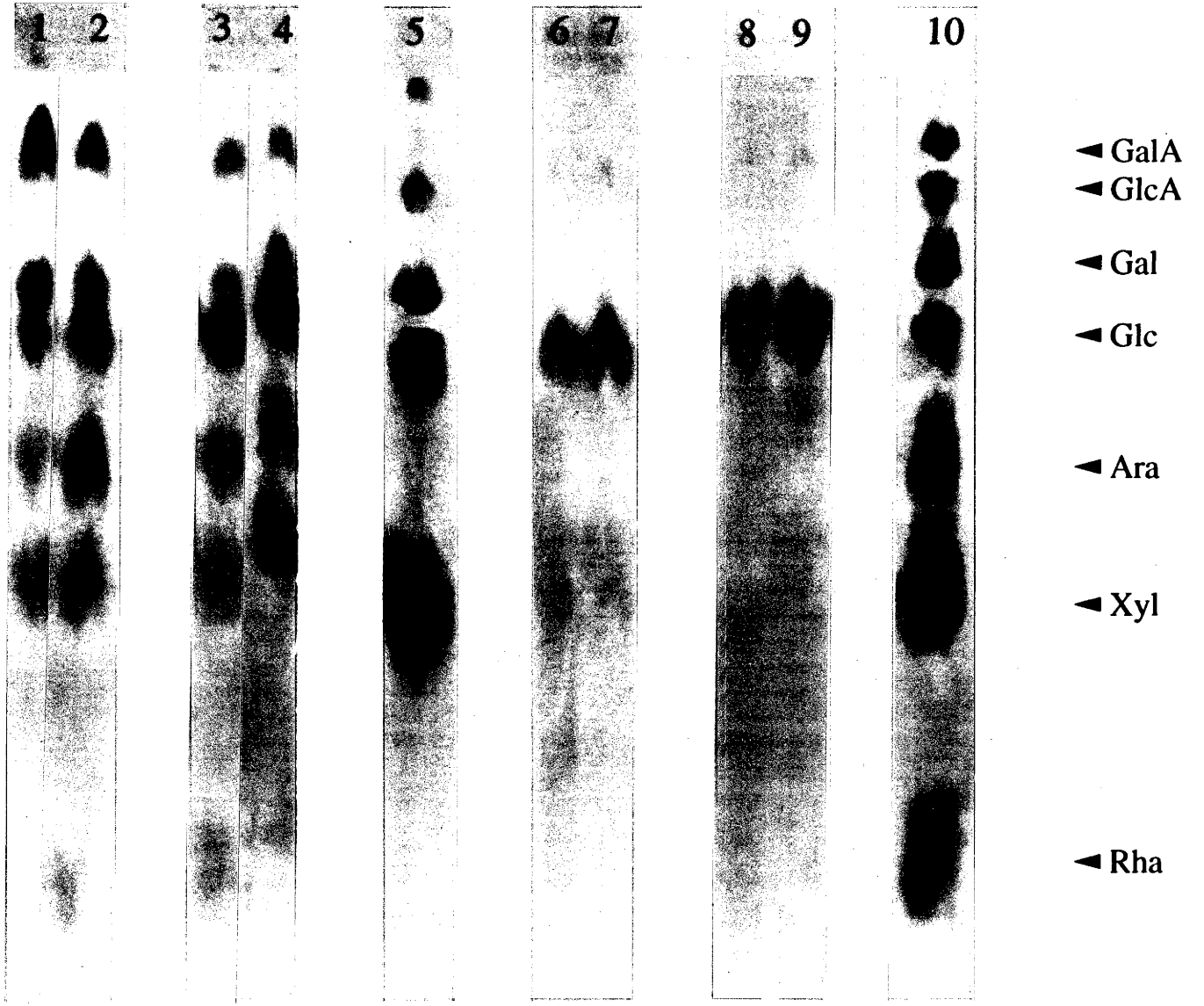

1
2
3
4
5
6
7
8
9
10
GalA
GlcA
Gal
Glc
Ara
Xyl
Rha

Figure 3.3

GC/MS chromatograms for AA of sugars:

A: From standard sample which was a mixture of monosaccharides:

Peaks: 1, Rha; 2, Fuc; 3, Ara; 4, Xyl; 5, Man; 6, Gal; 7, Glc;

IS, *myo*-inositol.

B, C, D, E: Released by TFA hydrolysis from fractions of the cell wall in the root tissue of wild type grown at 21 °C.

B, Ammonium oxalate fraction;

C, 0.1 M KOH fraction;

D, 4 M KOH fraction;

E, Acid-soluble fraction.

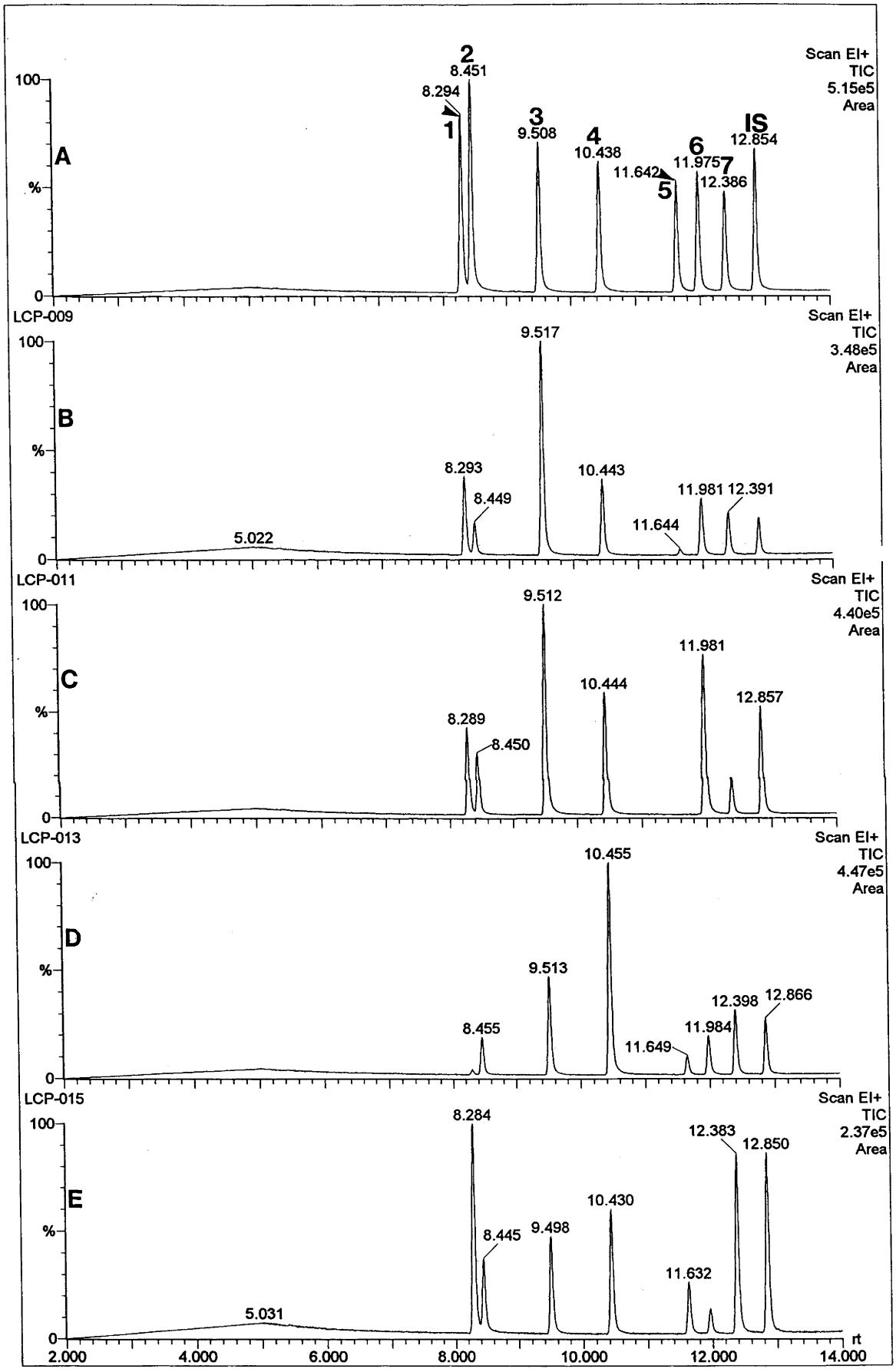

A
Scan EI+
TIC
5.15e5
Area
100
%
0
1
2
3
4
5
6
7
IS
8.294
8.451
9.508
10.438
11.642
11.975
12.386
12.854
LCP-009
B
Scan EI+
TIC
3.48e5
Area
9.517
8.293
8.449
10.443
11.644
11.981
12.391
5.022
LCP-011
C
Scan EI+
TIC
4.40e5
Area
9.512
11.981
10.444
12.857
8.289
8.450
LCP-013
D
Scan EI+
TIC
4.47e5
Area
10.455
9.513
12.398
12.866
8.455
11.984
11.649
LCP-015
E
Scan EI+
TIC
2.37e5
Area
8.284
12.383
12.850
10.430
8.445
9.498
11.632
5.031
2.000
4.000
6.000
8.000
10.000
12.000
14.000
rt

Figure 3.4

GC/MS chromatograms of PMAAs from:

A: Partial methylation of glucose standard (Figures 2.2 and 2.3).

B, C, D: Complete methylation analysis of :

B, DMSO fraction;

C, Acid-soluble fraction;

D, Acid-insoluble fraction.

E: Control, complete methylation analysis of blank sample.

RRT of each labelled peak is presented in Table 3.6. MS of each peak and its deduced glycosyl linkage are presented in Figure 3.5 and Table 3.6, respectively. IS, *myo*-inositol.

The regions (in B, C, D, and E) between the arrowheads were amplified two fold.

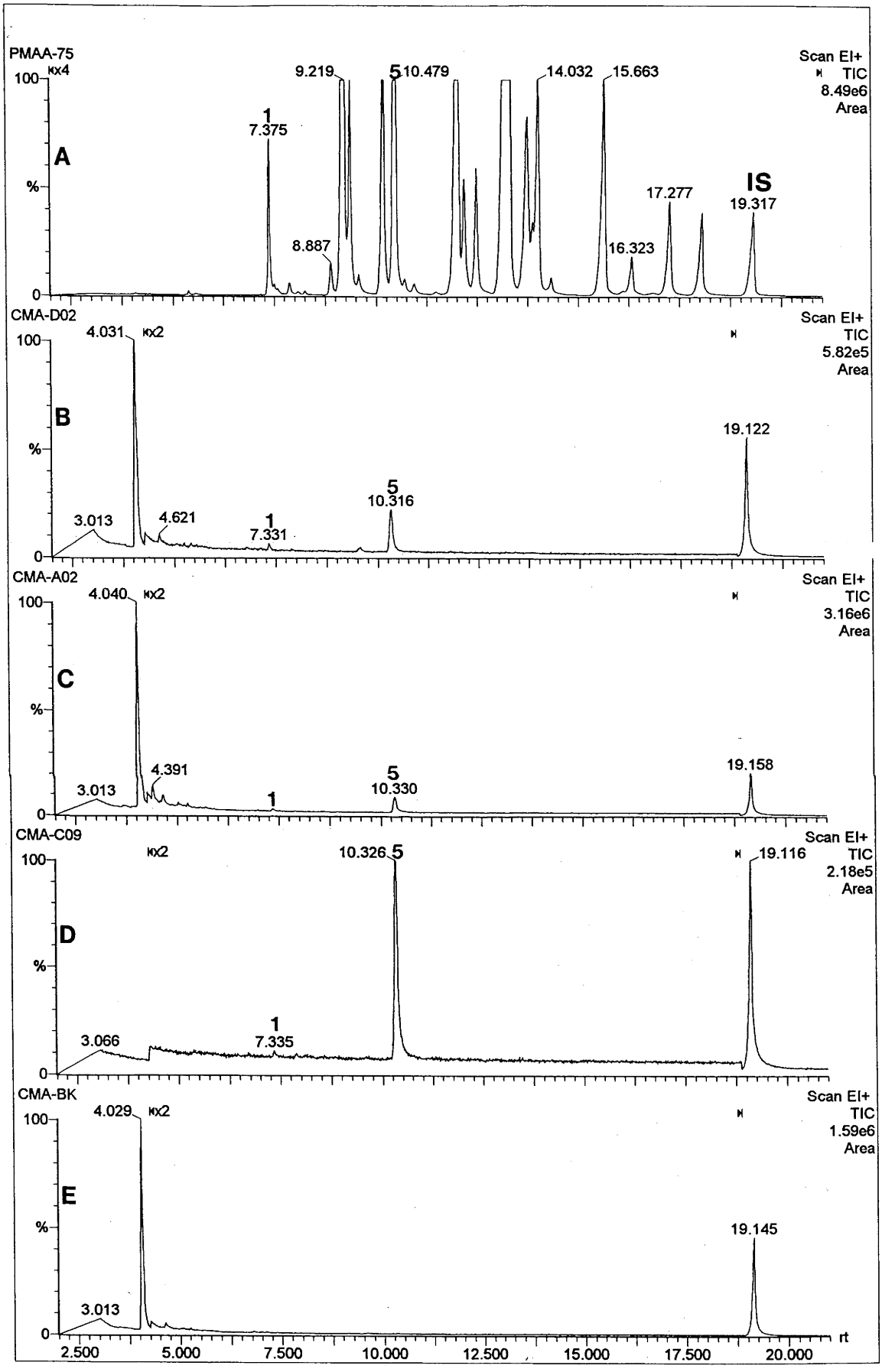
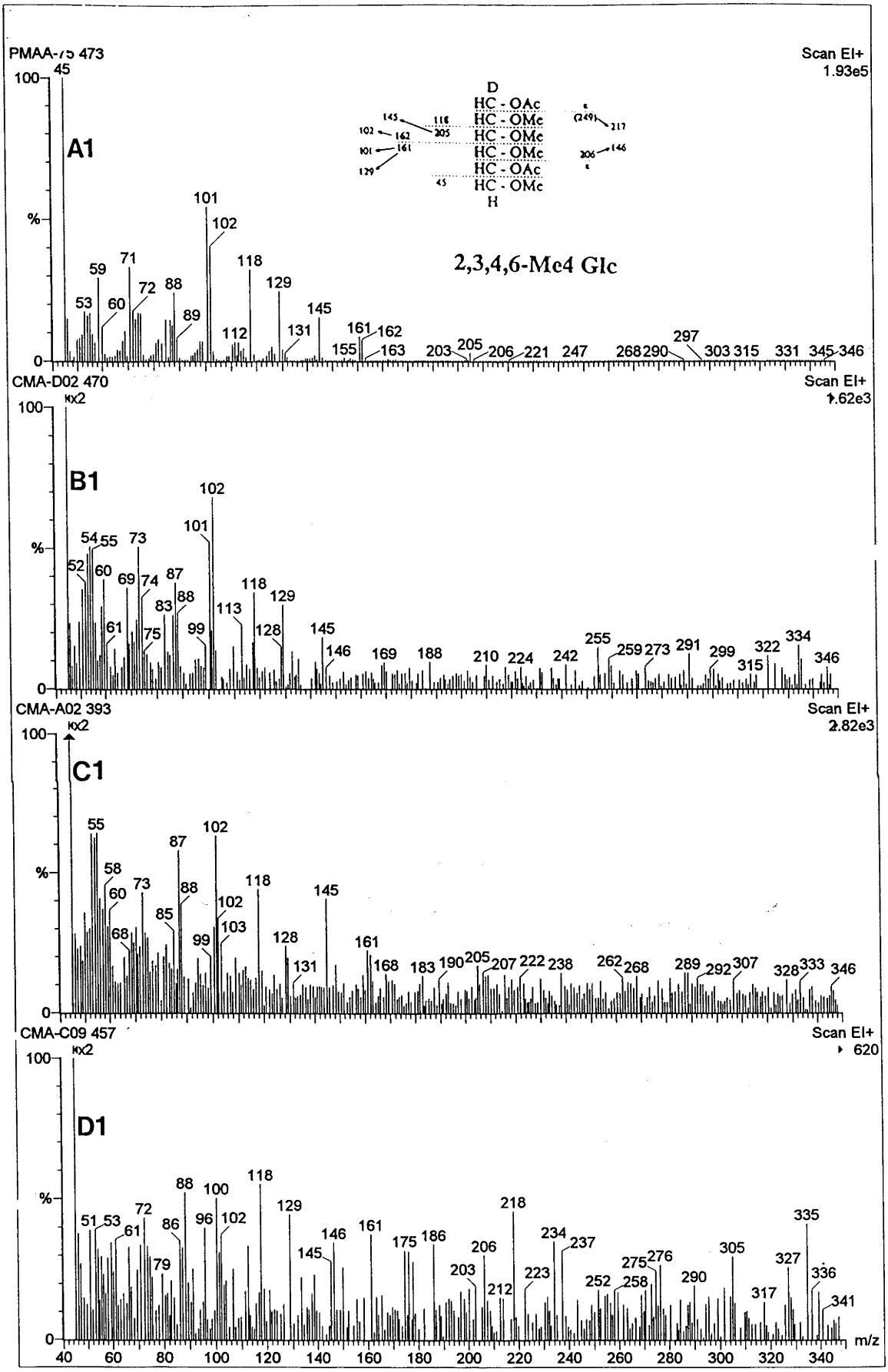

PMAA-75
Kx4
Scan EI+
TIC
8.49e6
Area
A
9.219
5
10.479
14.032
15.663
1
7.375
8.887
16.323
17.277
IS
19.317
CMA-D02
Scan EI+
TIC
5.82e5
Area
B
4.031
Kx2
19.122
5
10.316
3.013
4.621
1
7.331
CMA-A02
Scan EI+
TIC
3.16e6
Area
C
4.040
Kx2
4.391
19.158
3.013
5
10.330
1
CMA-C09
Scan EI+
TIC
2.18e5
Area
D
Kx2
10.326
5
19.116
3.066
1
7.335
CMA-BK
Scan EI+
TIC
1.59e6
Area
E
4.029
Kx2
19.145
3.013
100
%
0
2.500
5.000
7.500
10.000
12.500
15.000
17.500
20.000
rt

Figure 3.5

MS of each labelled peak in GC/MS chromatograms (Figure 3.4). The deduced glycosyl linkage of each labelled peak is given in Table 3.6.


PMAA-r5 473
Scan EI+
1.93e5
A1
2,3,4,6-Me4 Glc
CMA-D02 470
Scan EI+
1.62e3
B1
CMA-A02 393
Scan EI+
2.82e3
C1
CMA-C09 457
Scan EI+
620
D1
m/z

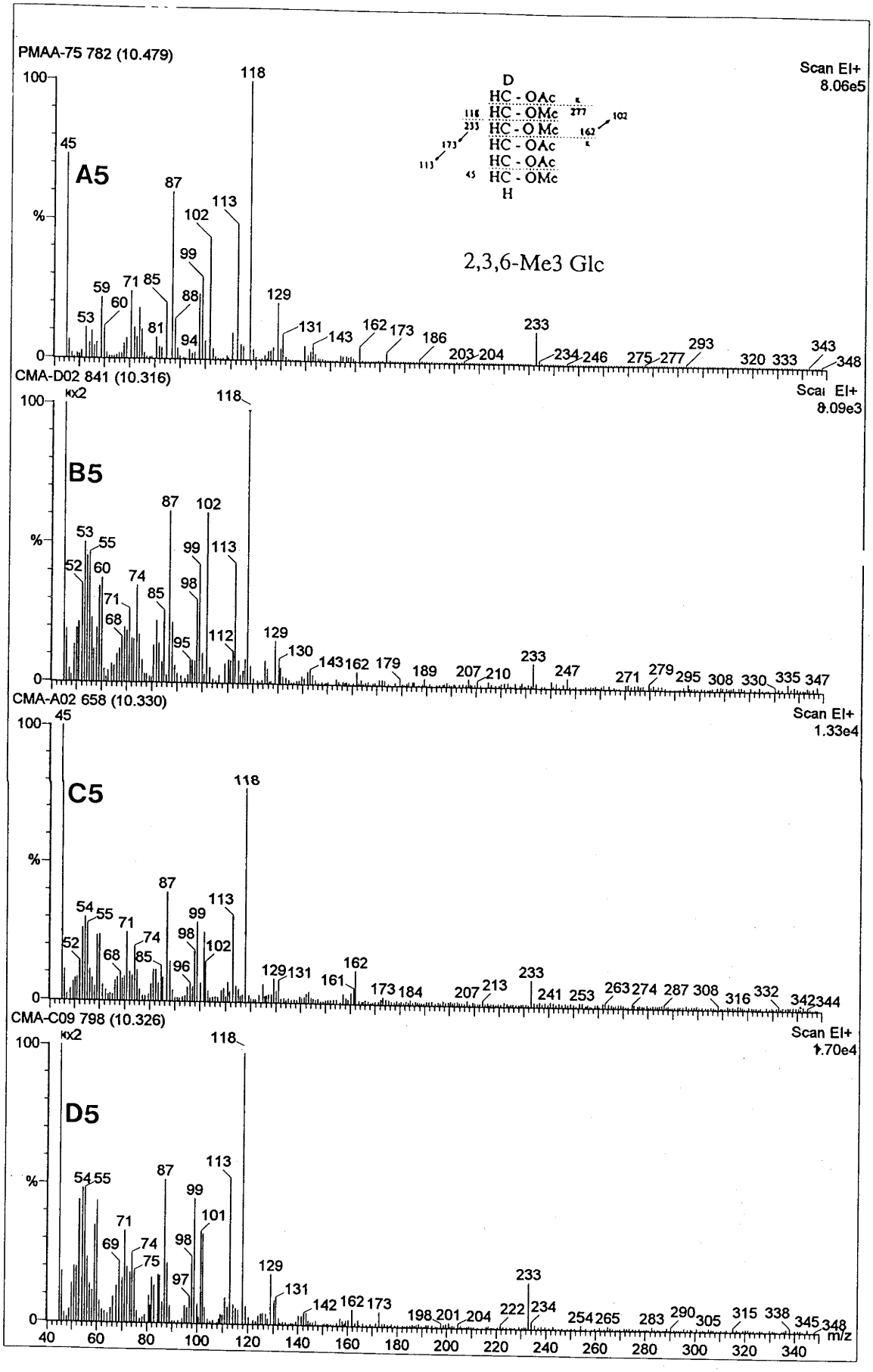

PMAA-75 782 (10.479)
Scan EI+
8.06e5
A5
2,3,6-Me3 Glc
D
HC - OAc
HC - OMe
HC - OMe
HC - OAc
HC - OAc
HC - OMe
H
CMA-D02 841 (10.316)
Scan EI+
8.09e3
B5
CMA-A02 658 (10.330)
Scan EI+
1.33e4
C5
CMA-C09 798 (10.326)
Scan EI+
1.70e4
D5
m/z

Table 3.6 Comparison of RRT of PMAAs derived from methylation analysis of DMSO (B), acid-soluble (C) and acid-insoluble (D) fractions of *Arabidopsis* wild type grown at 21 °C with glucose standard (A) (Chromatograms in Figure 3.4 and MS in Figure 3.5)

Peak *	Derivative	Deduced Linkage	RRT-1 *	RRT-2 *
A1	**2,3,4,6-Me_4 Glc**	**t-Glc*p***	**0.382**	**1**
B1	2,3,4,6-Me_4 Glc	t-Glc*p*	0.383	1
C1	2,3,4,6-Me_4 Glc	t-Glc*p*	0.384	1
D1	2,3,4,6-Me_4 Glc	t-Glc*p*	0.384	1
A5	**2,3,6-Me_3 Glc**	**4-Glc*p***	**0.542**	**1.42**
B5	2,3,6-Me_3 Glc	4-Glc*p*	0.539	1.41
C5	2,3,6-Me_3 Glc	4-Glc*p*	0.539	1.41
D5	2,3,6-Me_3 Glc	4-Glc*p*	0.540	1.41

*, RRT-1: Relative retention time to *myo*-inositol hexaacetate;

RRT-2: To t-Glc*p*.

Paper chromatography of a hydrolysate of this fraction (Figure 3.2, Lanes 1 and 2) showed galacturonic acid as well as arabinose, rhamnose, galactose and xylose. Glucuronic acid was not observed, indicating that this fraction was not significantly contaminated by GAX (Aspinall, 1980; Gibeaut and Carpita, 1994). This result is consistent with analyses of ammonium oxalate extracts of seven-day-old maize seedlings (Carpita and Kanabus, 1988) and of four-week-old seedlings of *Arabidopsis* (Zablackis et al, 1995), which likewise contained most of the pectic polysaccharides of the cell walls.

Although the three major types of pectic polysaccharides (homogalacturonan, RG I, and RG II) (McNeill et al, 1980 and 1982; Stevenson et al, 1988; Zablackis et al, 1995) expected to occur in the ammonium oxalate fraction were not separated and identified in this study, the higher proportion of arabinose, galactose and xylose in the fraction from root tissues (Table 3.3) suggests that roots might have a higher proportion of arabinans, galactans and arabinogalactans than shoots (Table 3.4). The higher percentage of uronic acid in the shoot tissue suggested a higher proportion of homogalacturonans, RG I, or RG II (Aspinall et al, 1967; McNeill et al, 1982; Jarvis, 1984).

To check whether extraction of pectic polysaccharides in boiling ammonium oxalate solution caused any depolymerisation, total hexoses, pentoses and uronic acids in the ammonium oxalate fraction were determined colorimetrically before and after dialysis (Table 3.8). Differences in uronic acids and pentoses resulting from dialysis were not significant by the t-test ($p \geq 0.1$; df, 10), but the difference in hexoses were significant ($p < 0.05$; df, 10) by 10%.

The ammonium oxalate fraction was then fractionated by CTAB precipitation to determine whether it contained neutral polymers with 1,4-linked glucose. The results will be described in Chapters Five and Six.

Figure 3.6

GC/MS chromatograms of PMAAs from methylation analysis of:

A: Pellet of ammonium oxalate fraction by CTAB precipitation;

B: Control, blank sample;

C: Supernatant of 4 M KOH fraction by a centrifugation (14, 000 *g*);

D: Control, blank sample.

RRT of each labelled peak is presented in Tables 3.7 and 3.8. MS of each peak is showed in Figures 3.7 and 3.8, and its deduced glycosyl linkages are presented in Tables 3.7 and 3.8 respectively. IS, *myo*-inositol.

The regions between the arrowheads were amplified four fold.

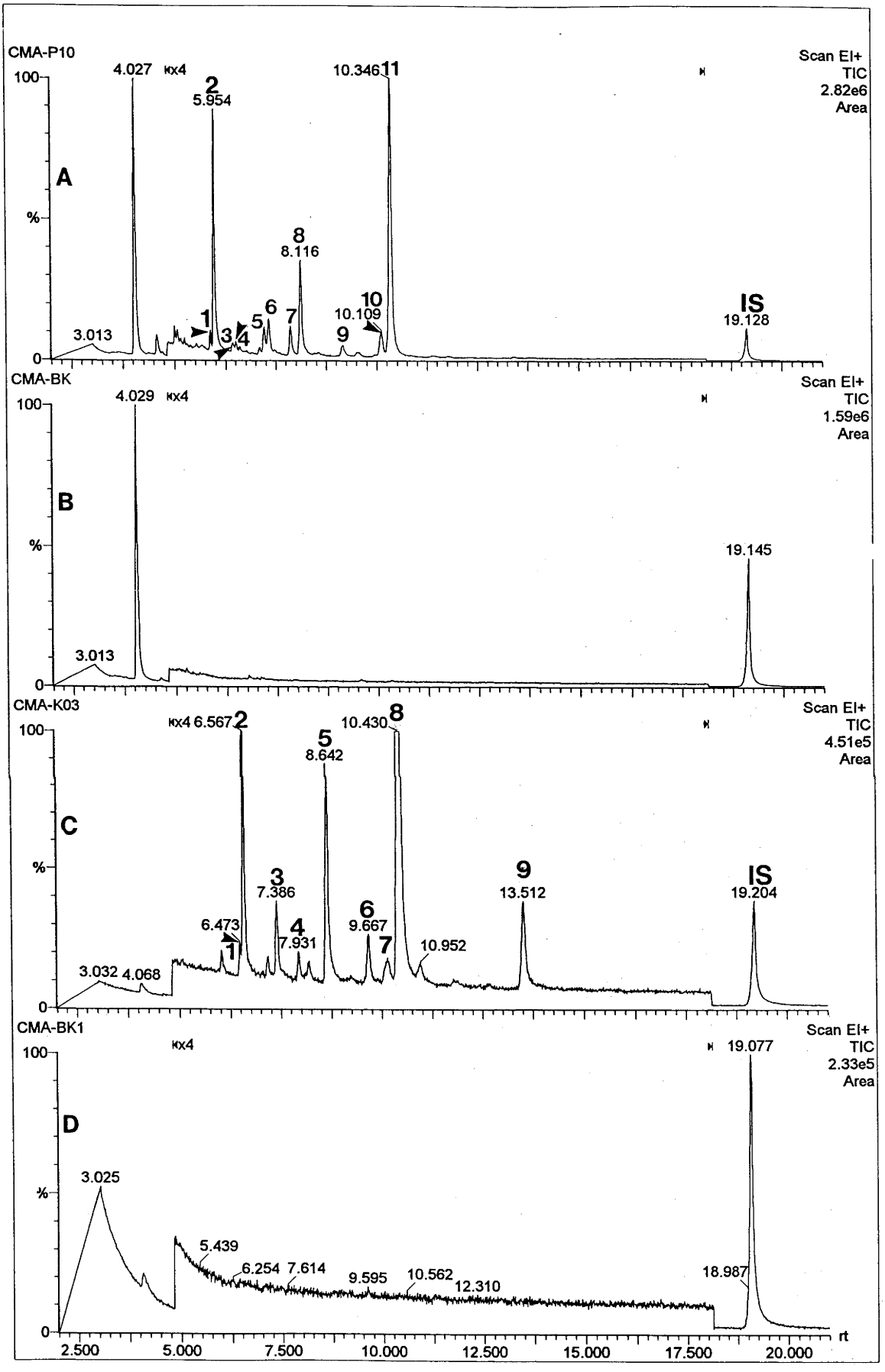
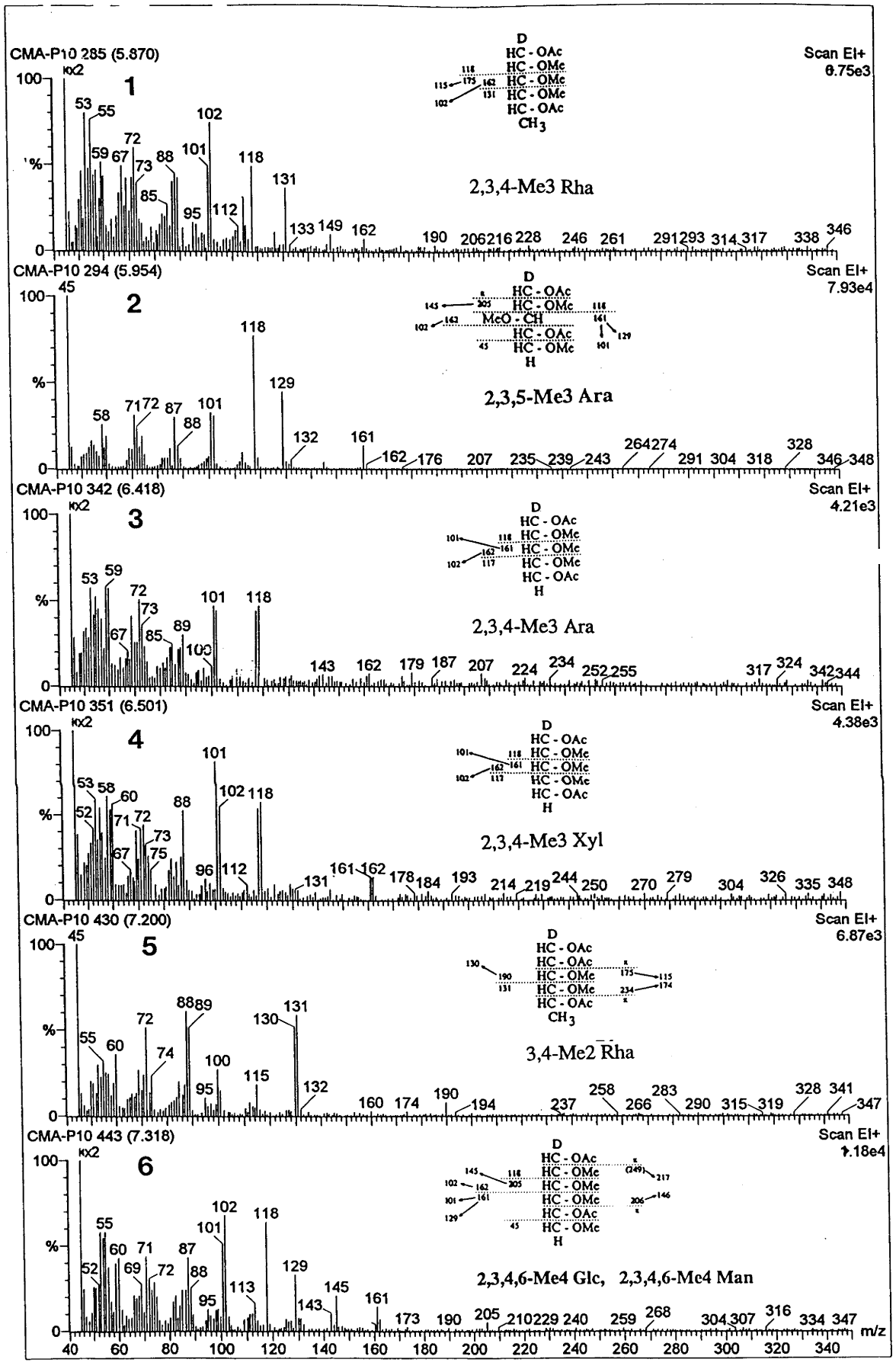

CMA-P10
Scan EI+
TIC
2.82e6
Area
A
4.027
5.954
10.346
8.116
10.109
19.128
3.013
IS
CMA-BK
Scan EI+
TIC
1.59e6
Area
B
4.029
19.145
3.013
CMA-K03
Scan EI+
TIC
4.51e5
Area
C
6.567
10.430
8.642
7.386
13.512
19.204
6.473
7.931
9.667
10.952
3.032
4.068
CMA-BK1
Scan EI+
TIC
2.33e5
Area
D
19.077
3.025
5.439
6.254
7.614
9.595
10.562
12.310
18.987
2.500
5.000
7.500
10.000
12.500
15.000
17.500
20.000
rt

Figure 3.7

MS of each labelled peak in GC/MS chromatogram A in Figure 3.6. The deduced glycosyl linkage of each labelled peak is summarised in Table 3.7.


CMA-P10 285 (5.870)
Scan EI+
6.75e3
1
2,3,4-Me3 Rha
CMA-P10 294 (5.954)
Scan EI+
7.93e4
2
2,3,5-Me3 Ara
CMA-P10 342 (6.418)
Scan EI+
4.21e3
3
2,3,4-Me3 Ara
CMA-P10 351 (6.501)
Scan EI+
4.38e3
4
2,3,4-Me3 Xyl
CMA-P10 430 (7.200)
Scan EI+
6.87e3
5
3,4-Me2 Rha
CMA-P10 443 (7.318)
Scan EI+
1.18e4
6
2,3,4,6-Me4 Glc, 2,3,4,6-Me4 Man
m/z

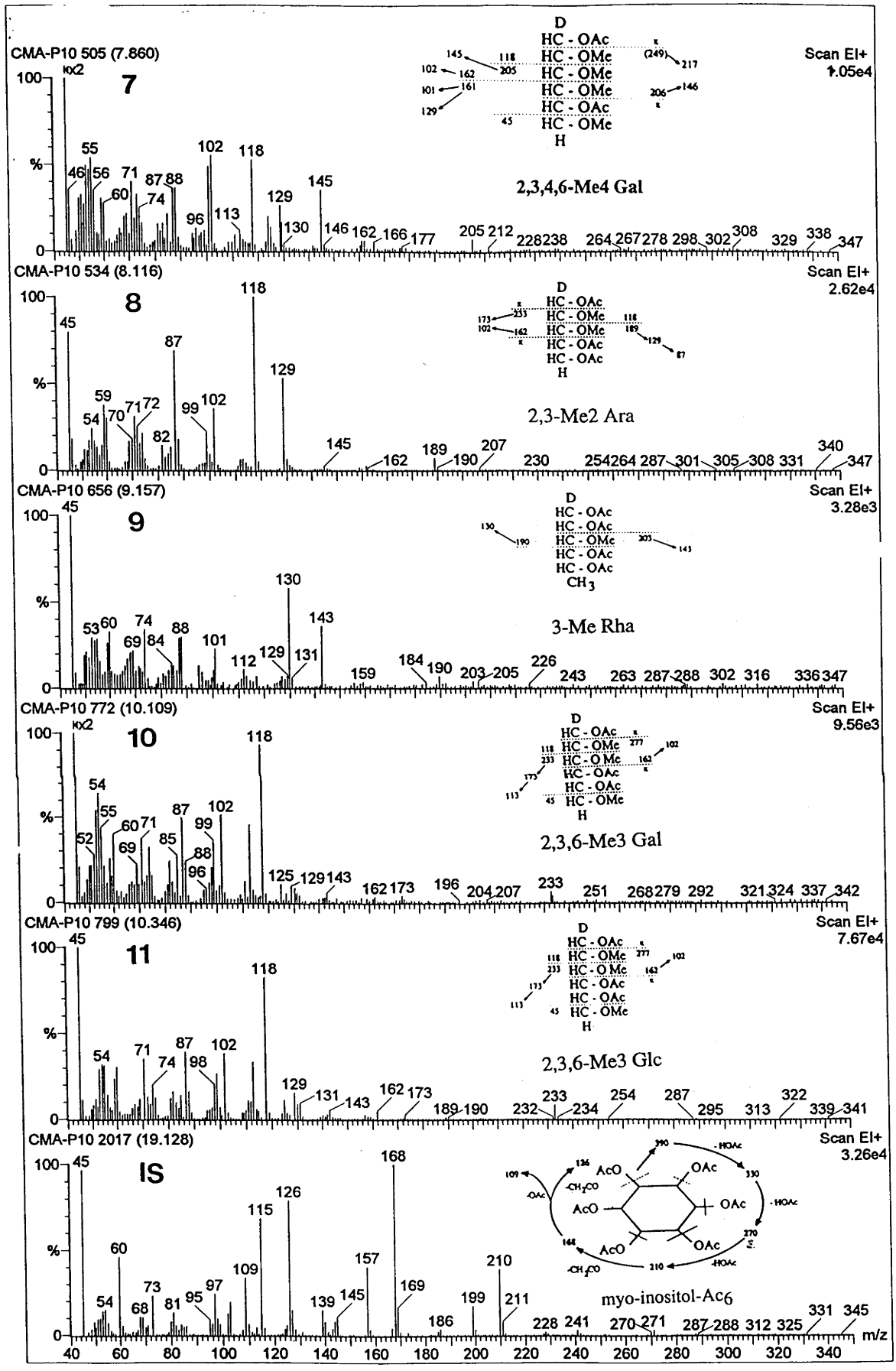

CMA-P10 505 (7.860)
Scan EI+
1.05e4
7
2,3,4,6-Me4 Gal
CMA-P10 534 (8.116)
Scan EI+
2.62e4
8
2,3-Me2 Ara
CMA-P10 656 (9.157)
Scan EI+
3.28e3
9
3-Me Rha
CMA-P10 772 (10.109)
Scan EI+
9.56e3
10
2,3,6-Me3 Gal
CMA-P10 799 (10.346)
Scan EI+
7.67e4
11
2,3,6-Me3 Glc
CMA-P10 2017 (19.128)
Scan EI+
3.26e4
IS
myo-inositol-Ac6
m/z

Table 3.7 Comparison of RRT of PMAAs derived from methylation analysis of the ammonium oxalate fraction of wild type grown at 21 °C with PMAA standards

Ammonium Oxalate Fraction				PMAA Standards			
Peak [a]	Derivative	Deduced Linkage	RRT	Table	Derivative	Deduced Linkage	RRT [b]
1	2,3,4-Me_3 Rha	t-Rha	0.307	2.5	2,3,4-Me_3 Rha	t-Rha	0.307
2	2,3,5-Me_3 Ara	t-Ara*f*	0.311	2.5	2,3,5-Me_3 Ara	t-Ara*f*	0.312
3	2,3,4-Me_3 Ara	t-Ara*p*	0.336	2.5	2,3,4-Me_3 Ara	t-Ara*p*	0.336
4	2,3,4-Me_3 Xyl	t-Xyl*p*	0.340	2.5	2,3,4-Me_3 Xyl	t-Xyl*p*	0.340
5	3,4-Me_2 Rha	2-Rha	0.376	2.5	3,4-Me_2 Fuc	2-Fuc	0.419
6	2,3,4,6-Me_4 Glc	t-Glc*p*	0.383	2.3	2,3,4,6-Me_4 Glc	t-Glc*p*	0.382
6	2,3,4,6-Me_4 Man	t-Man*p*	0.383	2.4	2,3,4,6-Me_4 Man	t-Man*p*	0.383
7	2,3,4,6-Me_4 Gal	t-Gal*p*	0.411	2.4	2,3,4,6-Me_4 Gal	t-Gal*p*	0.412
8	2,3-Me_2 Ara	5-Ara*f*	0.424	2.5	2,3-Me_2 Ara	4-Ara*p*/5-Ara*f*	0.428
9	3-Me Rha	2,4-Rha	0.479	2.5	3-Me Rha	2,4-Rha	0.479
10	2,3,6-Me_3 Gal	4-Gal*p*	0.528	2.4	2,3,6-Me_3 Gal	4-Gal*p*	0.531
11	2,3,6-Me_3 Glc	4-Glc*p*	0.541	2.3	2,3,6-Me_3 Glc	4-Glc*p*	0.542
IS	myo-inositol-Ac_6	-	1	2.3	myo-inositol-Ac_6	-	1

[a] Peak numbers for ammonium oxalate fraction indicated in Figures 3.6, A, MS presented in Figure 3.7.

[b] RRT of PMAA standards in Tables 2.3, 2.4 and 2.5.

Table 3.8 Effects of dialysis on monosaccharide levels in the ammonium oxalate fraction of wild type grown at 21 °C

Sugar	Root (nmol/mg dry weight)		Shoot (nmol/mg dry weight)	
	Undialysed	Dialysed	Undialysed	Dialysed
Uronic Acids	148±16 *	144±7	195±6	192±5
Pentoses	65±8	61±6	57±3	56±2
Hexoses	54±6	49±4	37±4	33±2
Total	267	254	289	281

* Standard deviation (n=6).

3.3.5 0.1 M KOH Fraction

The purpose of this mild extraction with alkali was to remove any remaining pectic polysaccharides which were bound strongly with the hemicelluloses and celluloses in the cell wall. Total sugars were much lower than in the ammonium oxalate fraction (Tables 3.1 and 3.2) and there was a high proportion of uronic acids although less than in the ammonium oxalate fraction (Tables 3.3 and 3.4).

Neutral and acidic sub-fractions were prepared by CTAB precipitation. The acidic and neutral sub-fractions from root tissue represented 70% and 30% respectively of the total carbohydrates measured by the phenol/H_2SO_4 test. In the shoot tissue, the acidic and neutral sub-fractions were respectively 80% and 20% of the total.

Paper chromatography (Figure 3.2; Lanes 3 and 4) showed a clear galacturonic acid spot without a glucuronic acid spot, which indicated that the acidic sub-fraction mainly contained pectic polysaccharides with no significant amounts of GAX. The neutral sub-fraction of the 0.1 M KOH fraction probably contained XG and, as will discussed in Chapters Five and Six, β-1,4-glucan.

3.3.6 4 M KOH Fraction

In the primary cell wall, XG and GAX are the major hemicelluloses of *Arabidopsis* (Zablackis et al, 1995). Hemicelluloses can be solubilised effectively with 4 M KOH solution containing 0.1% $NaBH_4$ (Carpita, 1984; Heim et al, 1991; Zablackis et al, 1995).

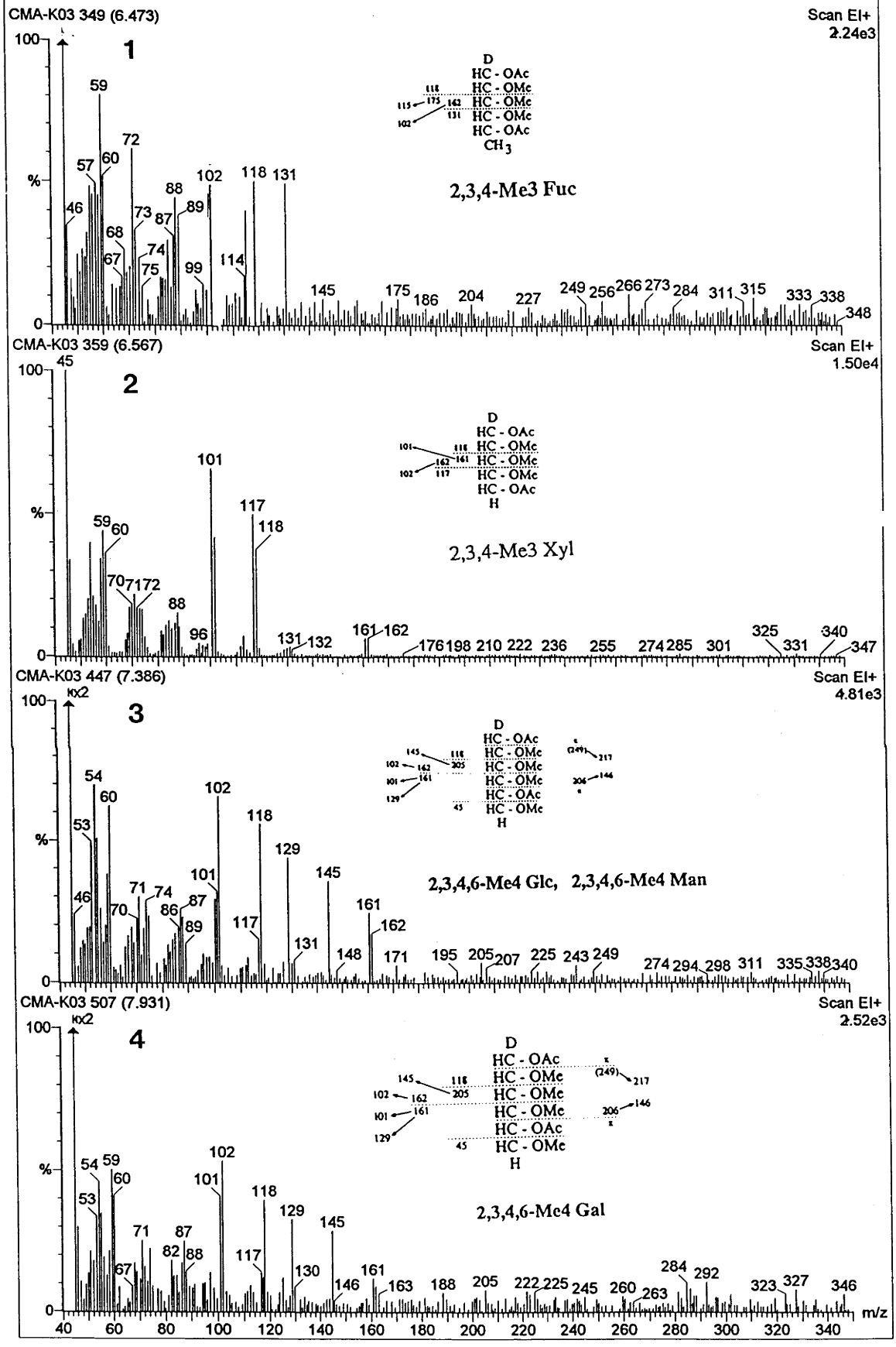
It was found that the 4 M KOH extracts from *Arabidopsis* root and shoot tissues (Tables 3.1 and 3.2), contained a high proportion of glucose (20% in root, 28% in shoot), xylose (34%, 26%) and arabinose (15%, 13%) with smaller amounts of galactose (11%, 12%) and uronic acids (9%, 10%) (Tables 3.3 and 3.4). The uronic acid in the fraction was shown to be glucuronic acid by paper chromatography (Figure 3.2, Lane 5). Galacturonic acid was not observed on the

Table 3.9 Compositional analysis for supernatant and pellet after CTAB fractionation of the 4 M KOH fraction of wild type grown at 21 °C

	mol%								
	UroA	Glc	Gal	Man	Xyl	Ara	Rha	Fuc	Total
ROOT									
Supernatant	ND	42	13	2	34	6	ND	3	100
Pellet	15	8	4	ND	42	30	1	ND	100
SHOOT									
Supernatant	ND	40	15	5	30	6	ND	4	100
Pellet	20	10	4	ND	38	27	1	ND	100

Figure 3.8

MS of each labelled peak in GC/MS chromatogram C in Figure 3.6. The deduced glycosyl linkage of each labelled peak is summarised in Table 3.10.


CMA-K03 349 (6.473)
Scan EI+
2.24e3
1
D
HC - OAc
HC - OMe
HC - OMe
HC - OMe
HC - OAc
CH3
2,3,4-Me3 Fuc
CMA-K03 359 (6.567)
Scan EI+
1.50e4
2
D
HC - OAc
HC - OMe
HC - OMe
HC - OMe
HC - OAc
H
2,3,4-Me3 Xyl
CMA-K03 447 (7.386)
Scan EI+
4.81e3
3
D
HC - OAc
HC - OMe
HC - OMe
HC - OMe
HC - OAc
HC - OMe
H
2,3,4,6-Me4 Glc, 2,3,4,6-Me4 Man
CMA-K03 507 (7.931)
Scan EI+
2.52e3
4
D
HC - OAc
HC - OMe
HC - OMe
HC - OMe
HC - OAc
HC - OMe
H
2,3,4,6-Me4 Gal
m/z

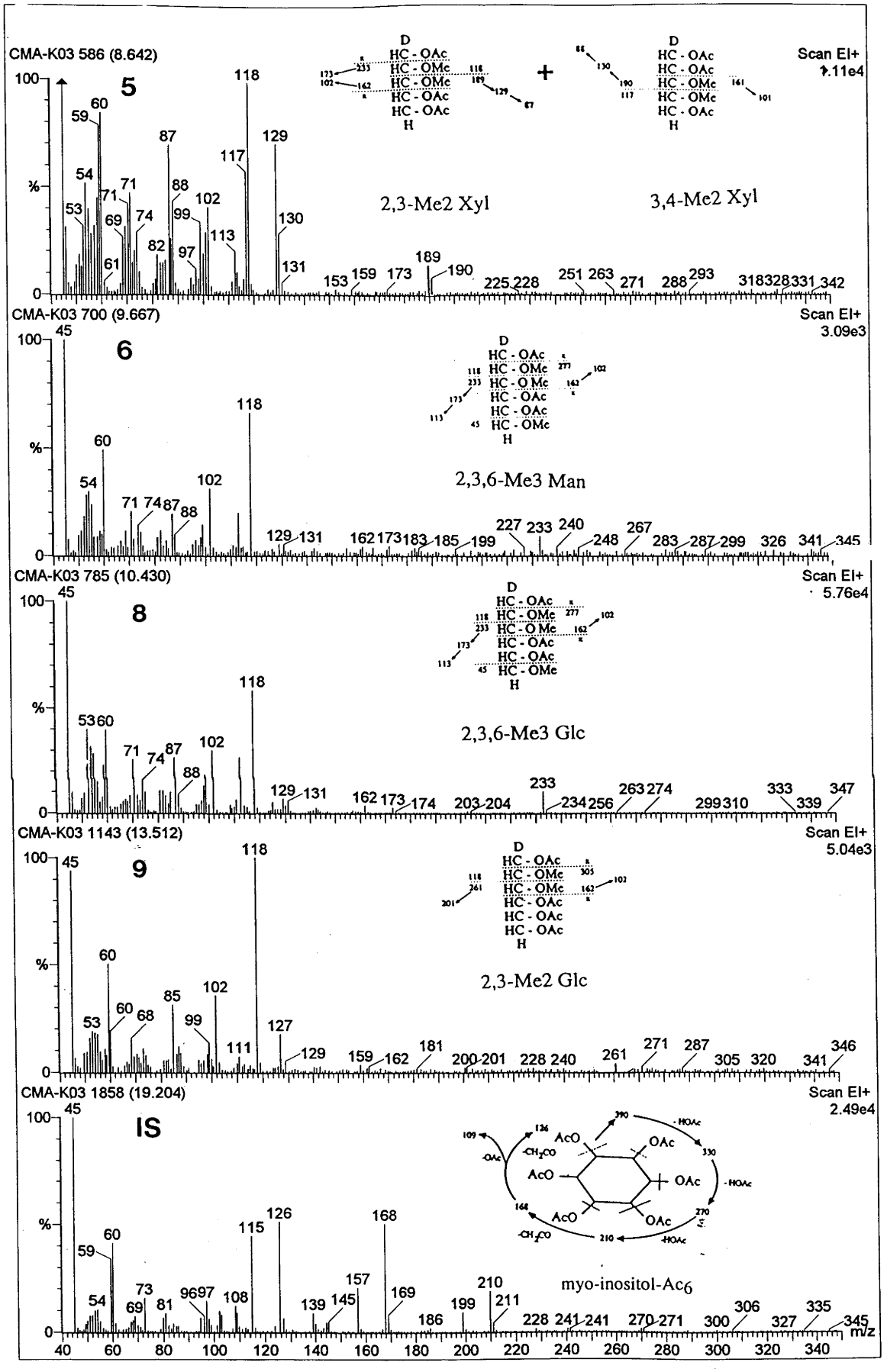

CMA-K03 586 (8.642)
Scan EI+
5
2,3-Me2 Xyl
3,4-Me2 Xyl
CMA-K03 700 (9.667)
Scan EI+
3.09e3
6
2,3,6-Me3 Man
CMA-K03 785 (10.430)
Scan EI+
5.76e4
8
2,3,6-Me3 Glc
CMA-K03 1143 (13.512)
Scan EI+
5.04e3
9
2,3-Me2 Glc
CMA-K03 1858 (19.204)
Scan EI+
2.49e4
IS
myo-inositol-Ac6
m/z

Table 3.10 Comparison of RRT of PMAAs derived from methylation analysis of the 4 M KOH fraction with PMAA standards

4 M KOH Fraction				PMAA Standards			
Peak [a]	Derivative	Deduced Linkage	RRT	Table	Derivative	Deduced Linkage	RRT [b]
1	2,3,4-Me_3 Fuc	t-Fuc	0.337	2.5	2,3,4-Me_3 Fuc	t-Fuc	0.334
2	2,3,4-Me_3 Xyl	t-Xyl*p*	0.342	2.5	2,3,4-Me_3 Xyl	t-Xyl*p*	0.340
3	2,3,4,6-Me_4 Glc	t-Glc*p*	0.385	2.3	2,3,4,6-Me_4 Glc	t-Glc*p*	0.382
3	2,3,4,6-Me_4 Man	t-Man*p*	0.385	2.4	2,3,4,6-Me_4 Man	t-Man*p*	0.383
4	2,3,4,6-Me_4 Gal	t-Gal*p*	0.413	2.4	2,3,4,6-Me_4 Gal	t-Gal*p*	0.412
5	2,3-Me_2 Xyl	4-Xyl*p*	0.450	2.5	2,3-Me_2 Xyl	4-Xyl*p*	0.447
5	3,4-Me_2 Xyl	2-Xyl*p*	0.450	2.5	3,4-Me_2 Xyl	2-Xyl*p*	0.447
6	2,3,6-Me_3 Man	4-Man*p*	0.503	2.4	2,3,6-Me_3 Man	4-Man*p*	0.502
7	3,4,6-Me_3 Gal	2-Gal*p*	0.527	2.4	3,4,6-Me_3 Gal	2-Gal*p*	0.526
8	2,3,6-Me_3 Glc	4-Glc*p*	0.543	2.3	2,3,6-Me_3 Glc	4-Glc*p*	0.542
9	2,3-Me_2 Glc	4,6-Glc*p*	0.704	2.3	2,3-Me_2 Glc	4,6-Glc*p*	0.712
IS	myo-inositol-Ac_6	-	1	2.3	myo-inositol-Ac_6	-	1

[a] Peak numbers from chromatogram in Figure 3.6, C and MS in Figure 3.8.

[b] RRT of PMAA standards from Tables 2.3, 2.4 and 2.5.

paper chromatography, and rhamnose comprised only 1% of the fraction (Tables 3.3 and 3.4), indicating that little or no pectic polysaccharides were being extracted.

Glucose and glucuronic acid are expected to be present only in XG and GAX, respectively (Carpita, 1984; Fry, 1988; Gibeaut and Carpita, 1994). Thus, these two monosaccharides can in principle be used to estimate the proportions of XG and GAX in the 4 M KOH fraction. However, as will be discussed in Chapters Five and Six, some of the glucose observed in the shoot tissue is due to a β-1,4-glucan.

The 4 M KOH fraction was separated by CTAB precipitation into neutral (supernatant) and acidic (pellet) fractions. Approximately 69% and 79% of the total hemicelluloses as measured by the phenol/H_2SO_4 test, were in the neutral fraction from root and shoot tissues respectively (data not shown). Compositional analysis by GC/MS (Table 3.9) showed that the supernatant (neutral polymer) had a broadly typical monosaccharide composition for XG which is widespread in dicotyledons (Xyl 38%, Glc 45%, Gal 8% and Fuc 7%; York et al, 1985) including *Arabidopsis* leaves (Zablackis et al, 1995). However, the occurrence of 2-5% mannose and higher than expected galactose suggest that the neutral polymers may contain small amount of β-1,4-D-mannans and galactomannans (Aspinall, 1980; McCleary, 1981; Bacic et al, 1988).

The high levels of xylose, arabinose and uronic acid in the pellet of acidic polymers (Table 3.9) are broadly typical of GAX (Fry, 1988: 57% Xyl, 36% Ara and 7% GlcA; Gibeaut and Carpita, 1994: 55% Xyl, 33% Ara and 11% GlcA). The relatively high proportion of uronic acids (15-20%) and the occurrence of other monosaccharides (8-10% Glc and 4% Gal) in the acidic polymer leaves open the possibility that other wall polysaccharides are present. There were no major differences between root and shoot tissues.

Methylation analysis (Figure 3.6 C and Figure 3.8; Table 3.10) was carried out on the supernatant after the 4 M KOH fraction from whole seedlings had been centrifuged at 14,000 *g* for 1 h (see Chapter Five). Peaks for t-Xyl*p*, 2-Xyl*p*, 4-Glc*p*, 4,6-Glc, t-Fuc, 2-Gal, and t-Gal*p* were detected, most of those expected for a typical XG structure (York et al, 1985; Shea et al, 1989; Zablackis et al, 1995). A small peak with appropriate RRT (0.527) to be the 2-Gal*p* also expected for XG occurs at peak 7. Chapter Five will discuss the form of the 4-Glc*p* detected in this fraction. Peak 6 corresponding to 4-Man*p* is consistent with the presence a β-1,4-D-mannan or a glucomannan (Section 1.3.2.4) in the fraction as discussed above. Peak 3 could contain both t-Man*p* and t-Glc*p*. MS for peak 5 show it contains both 2-Xyl*p* (present in XG) and 4-Xyl*p* (present in GAX). A small peak with an RRT (0.314) appropriate for t-Ara*f*, another GAX component, elutes before peak 1 but its identity could not be fully confirmed by MS. Any other unlabelled peaks are not PMAAs according to their mass spectra.

Less than 5% of total carbohydrates were lost from the 4 M KOH fraction during dialysis (data not shown), indicating that 4 M KOH extraction at 25 °C caused little depolymerisation.

3.3.7 Acid-soluble Fraction

Very little is known about the composition of the acid-soluble fraction (Carpita, 1984; Carpita and Kanabus, 1988; Heim et al, 1991). But, from the sequential fractionation of the cell wall, we expected it might contain non-crystalline cellulose and any residual non-cellulosic wall polysaccharides. The fraction contained very low monosaccharide levels (Tables 3.1 and 3.2) with a high proportion of glucose (41% in root and 42% in shoot) (Tables 3.3 and 3.4; Figure 3.2, Lanes 6 and 7). Relatively high proportions of xylose (17% root, 16% shoot), arabinose (11%, 3%), and rhamnose (15%, 16%) suggested that the fraction might still contain pectins and hemicelluloses. Levels of uronic acids

Table 3.11 Estimation by methylation analysis of the DP of the 1,4-glucans from the acid-soluble fraction and acid-insoluble fraction of whole seedlings of wild type grown at 21 °C *

Samples	Ratio of 4-Glc*p* to t-Glc*p* in Peak Area	DP	MW (Da)
Cellobiose Standard	0.5	2	342
1,4-Glucan from Acid-soluble Fraction	9.4	19	3096
1,4-Glucan from Acid-insoluble Fraction	58.7	117	18972

*GC/MS chromatograms for the PMAAs of fractions in Figure 3.4; and for PMAAs of cellobiose standard in Figure 5.4.

were too small to be determined by the *m*-hydroxybiphenyl/H_2SO_4 test, and the polymers containing mannose (10% root, 19% shoot) may be β-1,4-mannans and galactomannans as described previously (Section 3.3.6).

Methylation analysis of the acid-soluble fraction (Figures 3.4 and 3.5; Table 3.6, C) showed a peak of typical of a 1,4-linked glucan and approximately 60% of the glucose in the fraction was released by endo-cellulase (method in Section 5.2.3.1), consistent with the glucose being mainly in the form of non-crystalline cellulose. The DP of the 1,4-linked glucan was estimated as 19 from the ratio of 4-Glc*p* : t-Glc*p* on methylation analysis (Table 3.11). Other peaks on the chromatogram (Figure 3.4, C) were not carbohydrates, according to their MS (data not shown). Thus, the nature of other monosaccharides in the fraction was unclear.

3.3.8 Acid-insoluble Fraction

This final fraction of cell wall polysaccharides was expected to be crystalline cellulose which is insoluble in most extractants (Carpita, 1984; Fry, 1988; Heim et al, 1991). Glucose accounts for 97% of total sugar in the fraction by GC/MS analysis after hydrolysis by H_2SO_4 (Tables 3.1, 3.2, 3.3 and 3.4) and paper chromatography (Figure 3.2; Lanes 8 and 9) showed a single clear spot for glucose. Methylation analysis (Figures 3.4 and 3.5, D) gave only two peaks, corresponding to t-Glc*p* and 4-Glc*p* (Table 3.6, D) and indicating the presence of a 1,4-linked glucan. An estimate of the DP of the 1,4-linked glucan made from the ratio of terminal and 4-linked glucose (Table 3.11) shows a much higher value than that from the acid-soluble fraction.

The acetic-nitric acid-insoluble fraction was also hydrolysed with 2 M TFA (121 °C, 1 h). Crystalline cellulose is not hydrolysed under these conditions (Fry, 1988). The hydrolysate consisted of 60-67% glucose, 6-10% mannose, 20% xylose and 7-10% arabinose (Table 3.12), consistent with the presence of non-crystalline cellulose and small amounts of non-cellulosic polysaccharides.

Table 3.12 Fractionation of the acid-insoluble fraction into cellulose and TFA-soluble materials

Fractions	Sugars	nmol/mg dry weight	
		Root	Shoot
Acetic-nitric acid-insoluble [a]	Total hexoses	515±49	287±28
TFA-soluble [b]	Glc	29	12
	Man	3	2
	Xyl	9	4
	Ara	3	2
Crystalline cellulose [c]	Glc	483	273

[a] Total hexoses by the anthrone/H_2SO_4 test.

[b] Monosaccharides by GC/MS.

[c] Crystalline cellulose as acetic-nitric acid-insoluble hexoses minus TFA-soluble hexoses.

This is probably because those non-cellulosic polysaccharides are strongly associated with crystalline cellulose by either strong hydrogen bonding or cross-linking. Total TFA-soluble monosaccharides were less than 10% of the final TFA-insoluble residue in which only glucose was detected by GC/MS after hydrolysis with H_2SO_4.

3.3.9 Polysaccharides of the *Arabidopsis* Wild Type Cell Wall

The data showed that pectic polysaccharides were extracted with the ammonium oxalate, phosphate buffer and 0.1 M KOH fractions. The calculation (Tables 3.13) indicated that about 62% (root) and 80% (shoot) of total pectins were extracted with the ammonium oxalate fraction. The phosphate buffer and 0.1 M KOH fractions contained 23% and 15% of pectins respectively in root tissue and 11% and 9% in the shoot.

Extremely small amounts of hemicelluloses were extracted with the DMSO and acid-soluble fractions. Approximately 89% and 11% of total hemicelluloses (Table 3.14) occurred in the 4 M KOH and 0.1 M KOH fractions respectively of root tissue. In the shoot tissue, only 7% of hemicelluloses were extracted with the 0.1 M KOH fraction, and about 93% was recovered in the 4 M KOH fraction. XG was recovered in the 4 M KOH fraction and possibly in the 0.1 M KOH fraction (Sections 3.3.5 and 3.3.6). GAX was only found in the 4 M KOH (Table 3.14). XG accounted for 72% (root) and 79% (shoot) of total hemicelluloses, and GAX accounted for 28% and 21%.

After the calculation of total pectic polysaccharides, XG and GAX, the cell wall composition was summarised in Table 3.15. The results indicated that 7-day-old *Arabidopsis* seedlings have a broadly similar cell wall composition to those of other dicotyledons, which typically show a high proportion of pectic polysaccharides, smaller amounts of hemicelluloses and 9-40% cellulose (Fry, 1988; Bacic et al, 1988; Hayashi, 1989). The level of cellulose is therefore at the

Table 3.13 Estimation of pectic polysaccharides in the cell wall of *Arabidopsis* wild type *

Fractions	Root		Shoot	
	nmol/mg DW	%	nmol/mg DW	%
Phosphate Buffer	92	23	37	11
Ammonium Oxalate	248	62	269	80
0.1 M KOH	59	15	30	9
Total	399	100	336	100

* Phosphate buffer pectins as total monosaccharides in the CTAB pellet; Ammonium oxalate pectins as total monosaccharides in that fraction (Tables 3.1 and 3.2) minus 21 nmol glucan in the case of the shoot (Chapter Five); 0.1 M KOH pectins as total monosaccharides in that fraction (Tables 3.1 and 3.2) x 0.7 for root and x 0.8 for shoot (Section 3.3.5).

Table 3.14 Estimation of XG and GAX and their distribution between fractions from the cell wall of *Arabidopsis* wild type*

Hemicelluloses	Fractions	Root	Shoot
		nmol/mg dry weight	nmol/mg dry weight
XG	0.1 M KOH	25	8
	4 M KOH	138	80
GAX	4 M KOH	62	23
Total		225	111

* XG in the 0.1 M KOH fraction as total fraction monosaccharides (Tables 3.1 and 3.2) x 0.3 (root) and 0.2 (shoot) to give the neutral fraction (Section 3.3.5); XG in the 4 M KOH fraction as total fraction monosaccharides x 0.69 (root) and 0.78 (shoot) to give neutral fraction (Section 3.3.6). GAX similar except x 0.31 (root) and 0.22 (shoot) for acidic fraction (Section 3.3.6).

Table 3.15 Cell wall composition of *Arabidopsis* wild type (Columbia) grown at 21 °C *

Polysaccharides	Root		Shoot	
	nmol/mg dry weight	%	nmol/mg dry weight	%
Crystalline cellulose	483	41	273	37
Pectins	399	34	336	45
XG	163	14	88	12
GAX	62	5	23	3
Acid-soluble carbohydrates	64	6	26	3
Total	1171	100	746	100

* Crystalline cellulose from Table 3.12; Pectins Table 3.13; XG + GAX Table 3.14; Acid-soluble carbohydrates from acetic-nitric acids soluble (Tables 3.1 and 3.2) plus TFA soluble (Table 3.12).

upper end of the reported range and substantially higher than estimated by Zablackis et al (1995) for *Arabidopsis* leaves.

3.3.10 Monosaccharide Distribution in Plant Cell Polysaccharides

The distribution of particular monosaccharide in different polysaccharides is shown in Tables 3.16 and 3.17. The majority of glucose (78% in the root and 67% in the shoot) is present in crystalline cellulose, and more than 85% of total uronic acids and rhamnose are present in pectic polysaccharides. Galactose, xylose, and arabinose occurred largely in both pectins and hemicelluloses. Because chloroplast membranes are rich in galactolipids (Section 3.3.2), about 43% of galactose is found in lipids from shoot tissue, and much less in root lipids with most in pectins and hemicelloses. Rhamnose mainly occurred in pectins, fucose, mannose and xylose mainly in hemicelluloses, but with substantial amount in pectins.

In Tables 3.1 and 3.2, glucose represents about 48-52% of all monosaccharides present in wall polysaccharides plus starch and lipid-linked saccharides. The uronic acids accounted for 19-28%, galactose, xylose, and arabinose for 6-10%, mannose, rhamnose and fucose accounting for about 3% each.

Table 3.16 Monosaccharide distribution in polysaccharides from root tissue of *Arabidopsis* wild type *

Polysaccharides	mol %							
	UroA	Glc	Gal	Man	Xyl	Ara	Rha	Fuc
Lipid-Linked Saccharides	ND	2	4	13	1	1	4	ND
Starch	0	4	0	0	0	0	0	0
Pectins	89	4	55	24	26	54	86	35
Hemicelluloses	11	7	40	43	62	40	10	50
Cellulose	0	78	0	0	0	0	0	0
Others	ND	5	1	20	10	5	0	15
Total	100	100	100	100	100	100	100	100

* **Lipid-linked saccharides** were from chloroform-methanol fraction (Section 3.3.2); **starch** from DMSO fraction digested by α-amylase and α-1,4 glucosidase; **pectins** from the phosphate buffer, ammonium oxalate and 0.1 M KOH fractions (Sections 3.3.1, 3.3.4 and 3.3.5); **hemicelluloses** from 0.1 M KOH and 4 M KOH fractions (Sections 3.3.5 and 3.3.6); **cellulose** from acid-insoluble fraction (Section 3.3.8); **others** from acid-soluble fraction (Section 3.3.7), acid-insoluble fraction hydrolysed by 2 M TFA, and supernatant of the phosphate buffer and ammonium oxalate fractions separated by CTAB, and other monosaccharides of DMSO fraction after subtraction of starch.

Table 3.17 Monosaccharide distribution in polysaccharides from shoot tissue of *Arabidopsis* wild type (Columbia)

Polysaccharides	mol %							
	UroA	Glc	Gal	Man	Xyl	Ara	Rha	Fuc
Lipid-Linked Saccharides	ND	1	43	10	3	2	2	ND
Starch	0	6	0	0	0	0	0	0
Pectins	95	7	32	11	28	59	92	39
Hemicelluloses	5	7	24	57	58	33	6	48
Cellulose	0	67	0	0	0	0	0	0
Others	ND	12	1	22	11	6	ND	13
Total	100	100	100	100	100	100	100	100

3.4 SUMMARY

A published method (Heim et al, 1991) has been assessed for the fractionation of cell wall polysaccharides from small amounts (3-10 mg dry weight) of *Arabidopsis* tissue. The method is shown to provide a rapid, simple and effective procedure to separate the different polysaccharides classes. The effectiveness of the separation of the different classes is demonstrated by determination of the monosaccharide composition and methylation analysis of the different fractions. The results show that the cell walls of *Arabidopsis* wild type (Columbia) seedlings are broadly similar to those of many other dicotyledons.

In the next chapters, I will investigate the production of cellulose in the mutants, whether some of the 4-linked glucose detected in the ammonium oxalate and alkali fractions is from a β-1,4-glucan, and then analyse the quantity, composition and structure of the major non-cellulosic polysaccharides in all mutants and wild type grown at 21 °C and 31 °C.

CHAPTER FOUR

REDUCED PRODUCTION OF CELLULOSE IN RADIAL SWELLING MUTANTS

Some of the work in this chapter has been published in abstracts:

Williamson, R., Peng, L., Rolfe, B. and Redmond, J. (1995). Radial Swelling Mutants of *Arabidopsis* Which are Deficient in Cellulose Synthesis (abstr). Immunology and Cell Biology, 73: A9.

Arioli, T., Betzer, A., Peng, L., ..., and Williamson, R. (1995). Radial Swelling Mutants Deficient in Cellulose Biosynthesis (abstr, J 5-013). J. Cell Biochem., 440: 21A.

4.1 INTRODUCTION

Cellulose occupies 9-40 % of the dry weight of the primary cell wall in plants (Dahlgren et al, 1985; Bacic et al, 1988). In the cell wall of *Arabidopsis* leaves, it has been reported to represent only 14% (Zablackis et al, 1995), but the measurements in Chapter Three show much higher levels in seedlings. The width, DP, and crystallinity of the cellulose microfibrils are highly variable according to the source and age of the plant tissue (Fry, 1988; Section 1.3.1).

As described in Chapter One, understanding of cellulose biosynthesis in higher plants has been limited by the failure to isolate cellulose synthase (Gibeaut and Carpita, 1994; Delmer and Amor, 1995).

To elucidate the biosynthesis of cellulose, chemical mutagenesis and genetic screening have been used to select several temperature-sensitive *Arabidopsis thaliana* mutants *rsw1, rsw2, rsw3* (Baskin et al, 1992) and *rsw5* (Williamson et al unpublished results). These showed a radial swelling phenotype when transferred from 21 °C to 31 °C (Baskin et al, 1992). Radial swelling might result from insufficient cellulose to create a wall of adequate strength and preliminary data from a ^{14}C-glucose incorporation assay indicated that mutants incubated at 31 °C for 2 days showed reduced ^{14}C-glucose incorporation into cellulose (Williamson et al, unpublished).

In this chapter, some morphological observations of seven-day-old *Arabidopsis* seedlings grown at 21 and 31 °C will be presented, a reduction in cellulose synthesis demonstrated in the mutants together with the accumulation of greatly increased levels of glucose in the ammonium oxalate, 0.1 M KOH and 4 M KOH fractions.

4.2 MATERIALS AND METHODS

Three replicate samples comprising 100-150 seeds of wild type (var Columbia), *rsw1*, *rsw2*, *rsw3* (Baskin et al, 1992) and *rsw5* (Williamson et al unpublished) were grown at 21 °C for 7 days (Section 3.2.1) and three replicates of each were grown at 21 °C for 2 days followed by 5 days at 31 °C. All mutants had been backcrossed several times. The other growth conditions and the harvesting of roots and shoots were as described in Section 3.2.1. Dry weight determinations (Section 3.2.2) and the basic fractionation methods (Section 3.2.6) have been described.

4.3 RESULTS

4.3.1 Morphogenesis and Dry Weight

The phenotypes of three mutants *rsw1, rsw2* and *rsw3* showing radial swelling when incubated at 31 °C have been observed (Figures 4.1 and 4.2). These mutants, which were originally characterised as showing temperature-sensitive phenotypes (Baskin et al, 1992), maintained healthy growth, although incubated for five days at the restrictive temperature (31 °C) after two days for germination at normal temperature (21 °C). Measurements of root and hypocotyl lengths (Tables 4.1 and 4.2) indicated that all mutant seedlings grown at 21 °C had a similar appearance to the wild type and statistical analysis by *t*-test showed that there were no significant differences in root and shoot lengths between the wild type and all mutants ($p > 0.05$).

At 31 °C, however, mutants *rsw1, rsw2* and *rsw3* showed radially swollen roots (Baskin et al, 1992) that were much shorter than those of the wild type grown under the same conditions (Table 4.1, $p < 0.05$), and their hypocotyl (Table 4.2) and leaves showed a slower growth than the wild type. Although the wild type seedlings at 31 °C for five days also had shorter roots than at 21 °C, the roots retained a normal unswollen appearance and the hypocotyls were stimulated to grow longer. As a further comparison, when initially grown at 21 °C for three days and then transferred to 31 °C for another two days (Figure 4.2; Tables 4.1 and 4.2), the wild type seedlings were stimulated to grow longer than those grown at 21 °C for all five days, but elongation in the mutant seedlings, after transfer to 31 °C for two days, was quickly restrained due to a radial swelling of the roots.

Although all mutants showed radial swelling after growth at 31 °C for five days, they also had small differences in the extent of swelling and in growth as reported by Baskin et al (1992). When maintained at 31 °C for five days, *rsw1*

Table 4.1 Root Lengths (*cm*) of Seedlings of *Arabidopsis* Wild Type and Mutants *

Materials	3 Days at 21 °C	5 Days at 21 °C	3 Days at 21 °C + 2 Days at 31 °C	3 Days at 31 °C	5 Days at 31 °C
Wild Type	1.08±0.16	2.33±0.26	2.65±0.51	0.85±0.05	1.33±0.19
rsw1	1.19±0.14	2.33±0.17	1.53±0.09	0.30±0.06	0.42±0.12
rsw2	0.98±0.11	2.03±0.26	1.35±0.24	0.40±0.04	0.70±0.12
rsw3	1.22 ±0.12	2.53±0.27	1.73±0.20	0.40±0.05	0.42±0.06
rsw5	1.16±0.18	2.44±0.30	1.96±0.24	0.95±0.05	1.34±0.14

* All seedlings germinated for two days at 21 °C, before growth in the conditions shown; SD, n=50.

Table 4.2 Hypocotyl Lengths (*cm*) of Seedlings of *Arabidopsis* Wild Type and Mutants *

Materials	3 Days at 21 °C	5 Days at 21 °C	3 Days at 21 °C + 2 Days at 31 °C	3 Days at 31 °C	5 Days at 31 °C
Wild Type	0.10±0.01	0.20±0.02	0.25±0.03	0.50±0.05	0.55±0.04
rsw1	0.10±0.01	0.20±0.01	0.20±0.02	0.10±0.01	0.20±0.02
rsw2	0.10±0.01	0.20±0.01	0.20±0.01	0.10±0.01	0.25±0.03
rsw3	0.10±0.01	0.20±0.02	0.20±0.02	0.20±0.01	0.25±0.02
rsw5	0.10±0.01	0.20±0.02	0.25±0.02	0.30±0.02	0.35±0.04

* All seedlings germinated for two days at 21 °C, before growth in the conditions shown; SD, n=50.

Figure 4.1

Wild type and three radially swollen mutants (*rsw1, rsw2* and *rsw3*) of *Arabidopsis thaliana*. After 2 days at 21 °C for germination, plants were grown for 5 days at 21 °C (*A-D,* left panel), or for 5 days at 31 °C (*A-D,* central and right panels).

A, wild type; B, *rsw1*; C, *rsw2*; D, *rsw3*.

Bar (in B central panel) = 0.62 mm for right panel, = 5.0 mm for left and central panels.

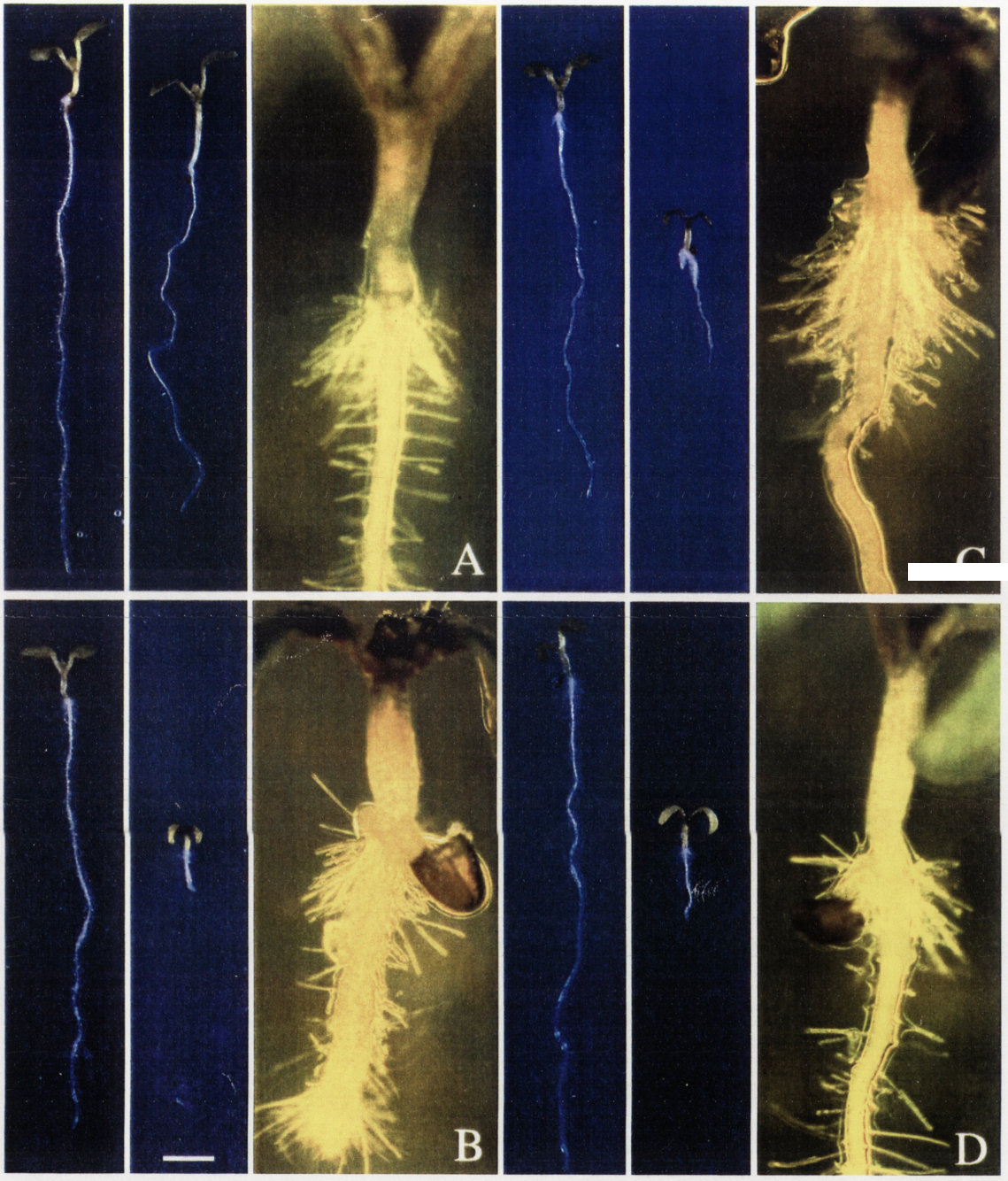

A
B
C
D

Figure 4.2

Wild type and three radially swollen mutants (*rsw1, rsw2* and *rsw3*) of *Arabidopsis thaliana*. After 2 days at 21 °C for germination, plants were grown for 3 days at 31 °C (*A-D*, left panel), or for 5 days at 31 °C (*A-D*, central panel), or for 3 days at 21 °C followed by 2 days at 31 °C (*A-D*, right panel).

A, wild type; B, *rsw1*; C, *rsw2*; D, *rsw3*.

Bar (in D left panel) = 0.14 cm for left and 0.16 cm for central panels;

Bar (in D right panel) = 0.13 cm for right panel.

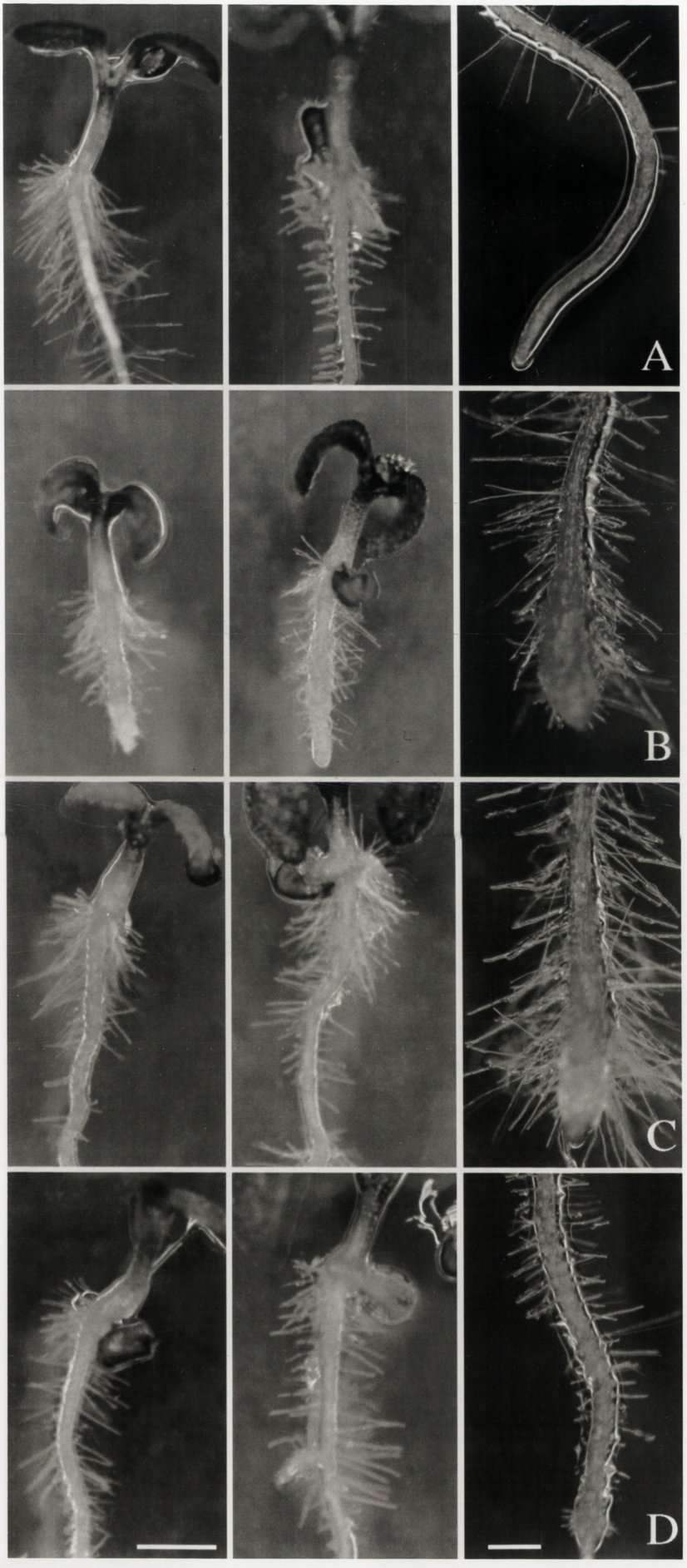
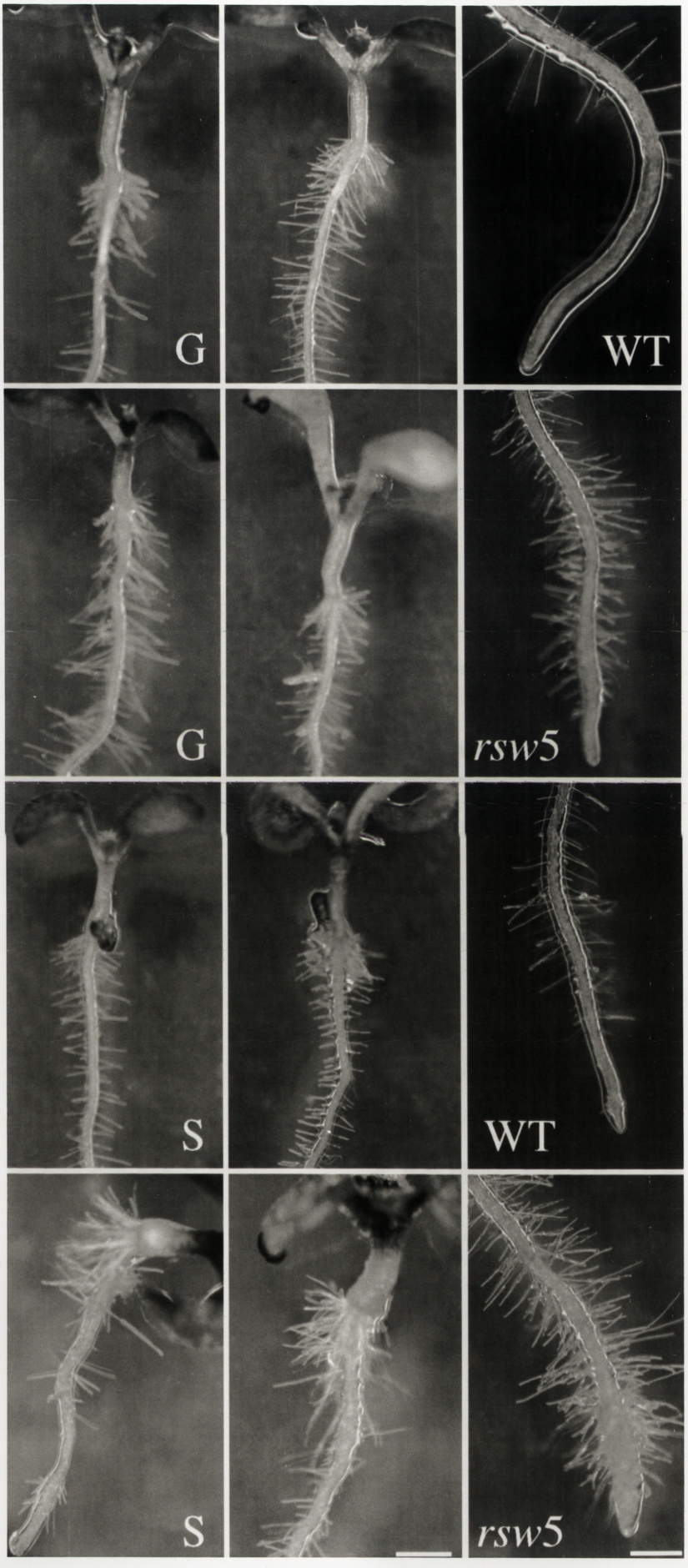

A
B
C
D

Figure 4.3

Wild type (**WT**) and mutant (***rsw5***) of *Arabidopsis thaliana*. After 2 days at 21 °C for germination, plants were grown on glucose-containing agar medium (**G**), or sucrose-containing agar medium (**S**) for 5 days at 21 °C (left panel), or for 5 days at 31 °C (central panel), or 3 days at 21 °C followed by 2 days at 31 °C (right panel).

Bar (in central panel) = 2.8 mm for left and central panels;

Bar (in right panel) = 1.2 mm for right panel.


G
WT
G
rsw5
S
WT
S
rsw5

was the most affected with the whole root showing strong swelling. However, the root of *rsw2*, after three days' incubation at 31 °C, started to grow faster than *rsw1* (Table 4.1) and to become less swollen in the meristem. By contrast, *rsw3* showed least swelling and its root curled up from the surface of the agar.

One mutant, *rsw5*, did not show clear radial swelling at 31 °C, when grown on the standard agar medium (Section 3.2.1) with glucose. It was found later that if glucose was replaced by sucrose, *rsw5*, like the other three mutants, showed radial swelling at 31 °C. The wild type still retained a normal shape as observed on the glucose medium (Figure 4.3). The *rsw5* seedlings used for biochemical analyses in this study were grown on glucose-containing medium and so did not show strong radial swelling.

The dry weights of seedlings grown for 2 days at 21 °C and 5 days at 31 °C or 21 °C (Section 3.2.2) are presented in Table 4.3. At 21 °C, the dry weights of the roots of all mutants were significantly higher than that of the wild type (p <0.05), but at 31 °C only *rsw2* and *rsw5* were significantly higher than wild type at the same temperature. Although the shoots of all mutants at 21 °C also had much greater dry weights than the wild type did, at 31 °C *rsw1* and *rsw5* showed significantly lower dry weights (p <0.05) whereas *rsw2* and *rsw3* had greater dry weights.

The ratio (31 : 21 °C) of dry weights (Table 4.3) indicated that growth at 31 °C stimulated shoot growth in seedlings of all *Arabidopsis* mutants (1.08-1.37) and wild type (1.67), but inhibited root growth (0.56-0.94). Associated with their root radial swelling at 31 °C, however, *rsw1*, *rsw2* and *rsw3* seedlings had greater decreases in dry weights (23-44% of their weight at 21 °C) than did the non-swelling wild type and *rsw5* (6-18%). In the shoots, all mutants showed lower increases in dry weights (8% -37%) than did wild type (67%).

Table 4.3 Dry Weight of Seedlings of *Arabidopsis* Wild Type and Mutants *

Materials	μg/plant				31 °C : 21 °C	
	Root		Shoot			
	21 °C	31 °C	21 °C	31 °C	Root	Shoot
Wild Type	19.3±0.56	15.88±0.44	69.47±0.28	116.15±0.8	0.82	1.67
rsw1	27.03±0.84	15.99±1.16	86.43±0.8	93.75±0.6	0.59	1.08
rsw2	32.46±0.68	25.12±1.2	96.52±0.82	124.52±0.8	0.77	1.29
rsw3	25.81±1.37	14.47±0.89	96.56±0.13	131.82±2.3	0.56	1.37
rsw5	24.16±0.85	22.74±0.42	78.42±0.22	107.81±2	0.94	1.37

* ± SD, n=3.

4.3.2 Reduction of Cellulose Biosynthesis

The cellulose of the *Arabidopsis* cell wall is recovered in the acid-insoluble fraction (Section 3.2.6.7). Methylation analysis for the acid-insoluble fraction in the root and shoot tissue from all mutants only showed two peaks for t-Glc*p* and 4-Glc*p*, a typical 1,4-linkage pattern (chromatogram not shown). Wild type and mutants grown at 31 °C, still had the same peaks indicating 1,4-linkages.

The glucose contents of the cellulose in mutants and wild type was determined by the anthrone/H_2SO_4 method and expressed as nmol/mg dry weight seedlings (Table 4.4). The mutants grown at 21 °C for 5 days (Figure 4.4, Table 4.4) showed no significant differences from the wild type in cellulose production in either roots or shoots ($p > 0.05$), except in the root of *rsw2* and in the shoot of *rsw5* which had respectively 70% and 73% of the cellulose of the wild type. At 31 °C, however, all mutants had much less cellulose than the wild type ($p < 0.05$). The roots of mutants *rsw1, rsw2, rsw3* and *rsw5* had only 60%, 44%, 51% and 65% respectively of the cellulose of the wild type grown at 31 °C, and the shoots had only 44, 38, 50 and 50% of the cellulose of the wild type (Figure 4.4, Table 4.4).

The Rw value gives the ratio of cellulose (nmol/mg dry weight) in a 31 °C plants to the cellulose in a 21 °C plant. It measures the impact of high temperature (31 °C) on cellulose biosynthesis. The results (Figure 4.5, Table 4.4) showed that wild type had Rw values >1 in root and shoot tissue, suggesting that cellulose biosynthesis was stimulated at 31 °C relative to other activities which contribute to dry weight. In contrast, the Rw values of mutants *rsw1, rsw2* and *rsw3* were much less than 1, varying from 0.60 to 0.79. Mutant *rsw5* also showed Rw values in root (0.82) and shoot (0.90) tissue that were lower than those in wild type. The data show that all mutants were strongly inhibited in cellulose biosynthesis when grown at 31°C whereas the wild type was stimulated.

Table 4.4 Glucose Content of Cellulose from Acid-insoluble Fraction in *Arabidopsis* Wild Type and Mutants *

Materials	nmol glucose/mg dry weight				Rw	
	Root		Shoot		31 : 21 °C	
	21 °C	31 °C	21 °C	31 °C	Root	Shoot
Wild Type	483±25	526±25	273±28	363±18	1.1	1.3
rsw1	401±15	315±20	218±20	159±19	0.79	0.73
rsw2	336±22	234±28	230±16	139±12	0.70	0.60
rsw3	435±36	270±29	285±19	181±18	0.62	0.63
rsw5	416±18	343±22	200±14	180±15	0.82	0.90

* The glucose of cellulose determined by the anthrone/H_2SO_4 test; SD, n=3.

Figure 4.4

The cellulose content (nmol glucose/mg dry weight) from the acid-insoluble fractions of four mutants expressed as a % of the cellulose from the wild type grown at the same temperature.

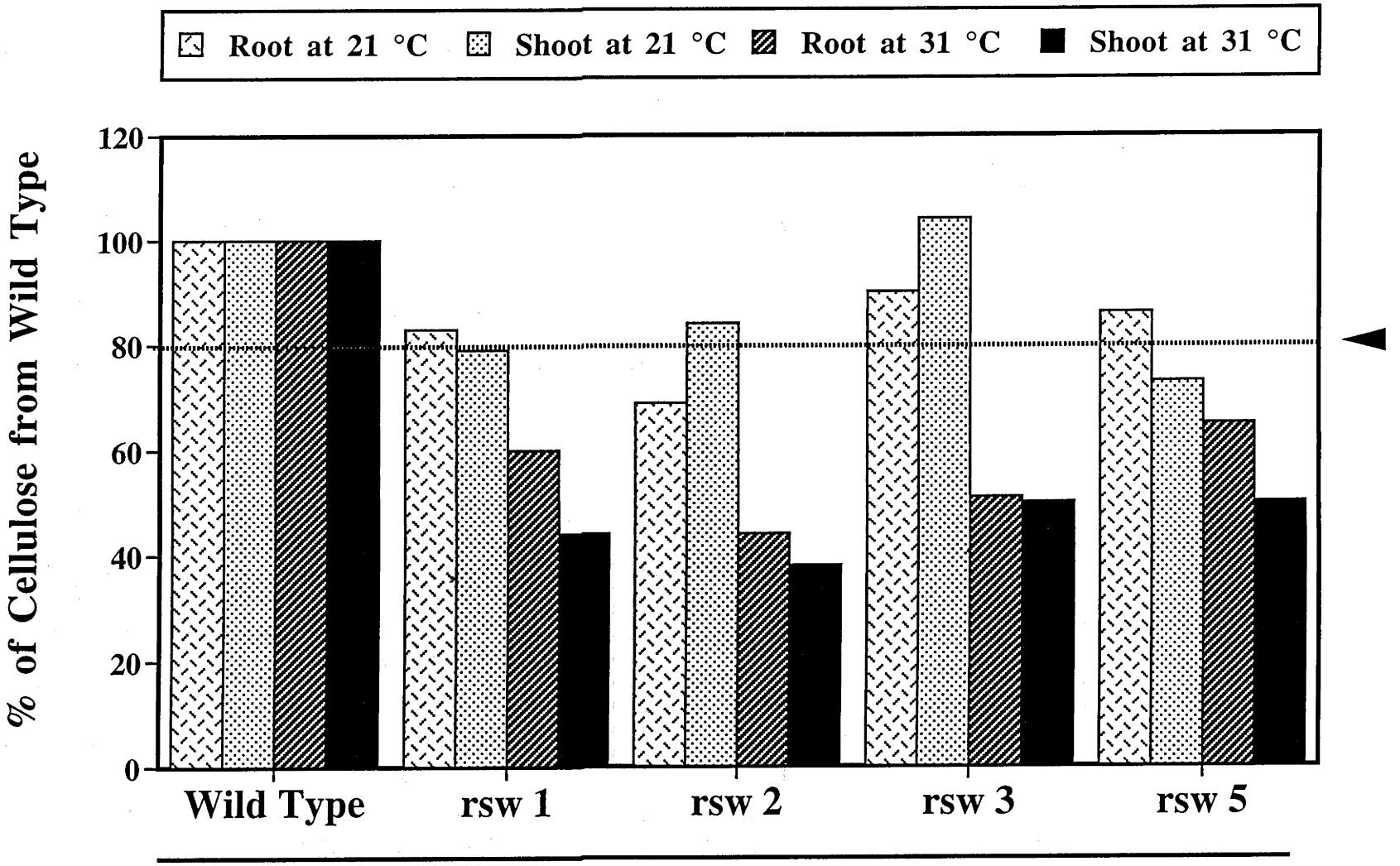
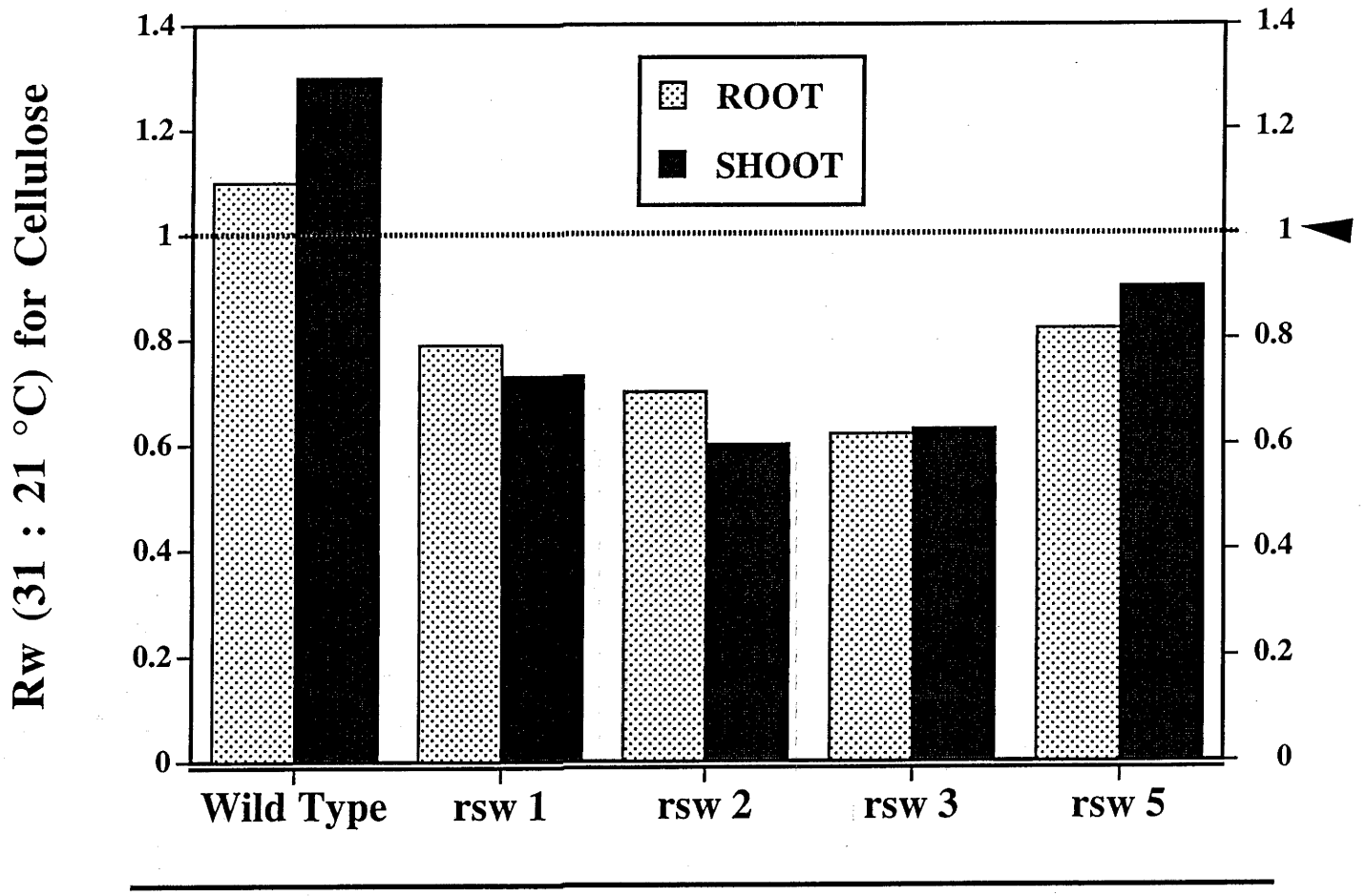

Root at 21 °C
Shoot at 21 °C
Root at 31 °C
Shoot at 31 °C
% of Cellulose from Wild Type
120
100
80
60
40
20
0
Wild Type
rsw 1
rsw 2
rsw 3
rsw 5

Figure 4.5

Rw (31 °C : 21 °C) for cellulose (nmol/mg dry weight) in the acid-insoluble fractions of *Arabidopsis* wild type and mutants grown at 21 and 31 °C.


Rw (31 : 21 °C) for Cellulose
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ROOT
SHOOT
Wild Type
rsw 1
rsw 2
rsw 3
rsw 5

Although the quantity of cellulose was much reduced in mutants at 31 °C, its DP estimated by methylation analysis was similar in all mutants and wild type, whether grown at 21 °C or 31 °C (data not shown).

4.3.3 Increased Glucose Levels in Fractions Expected to Contain Non-Cellulose Polysaccharides

Arabidopsis mutants grown at 31 °C were, as just described, greatly inhibited in cellulose production. In apparent compensation for the cellulose deficiency, a lot of glucose was unexpectedly found in the ammonium oxalate, 0.1 M KOH and 4 M KOH fractions of shoot tissue of *rsw1*, *rsw2* and *rsw3* grown at 31 °C (Figure 4.6, Table 4.5). The *rsw5* mutant, however, did not contain more glucose than wild type grown at the same temperature. The increase in glucose occurred only in the shoots of the three mutants and was not seen in the roots (Table 4.6).

Figure 4.6

Paper chromatography of sugars released by TFA from the ammonium oxalate fraction of the cell wall of shoots in *Arabidopsis* wild type and mutants grown at 21 °C and 31 °C:

At 21 °C:

Lane 1 Wild Type;

Lane 2, 3, 4, 5 *rsw5, rsw1, rsw2* and *rsw3.*

At 31 °C:

Lane 6 Wild Type;

Lane 7, 8, 9, 10 *rsw5, rsw1, rsw2* and *rsw3.*

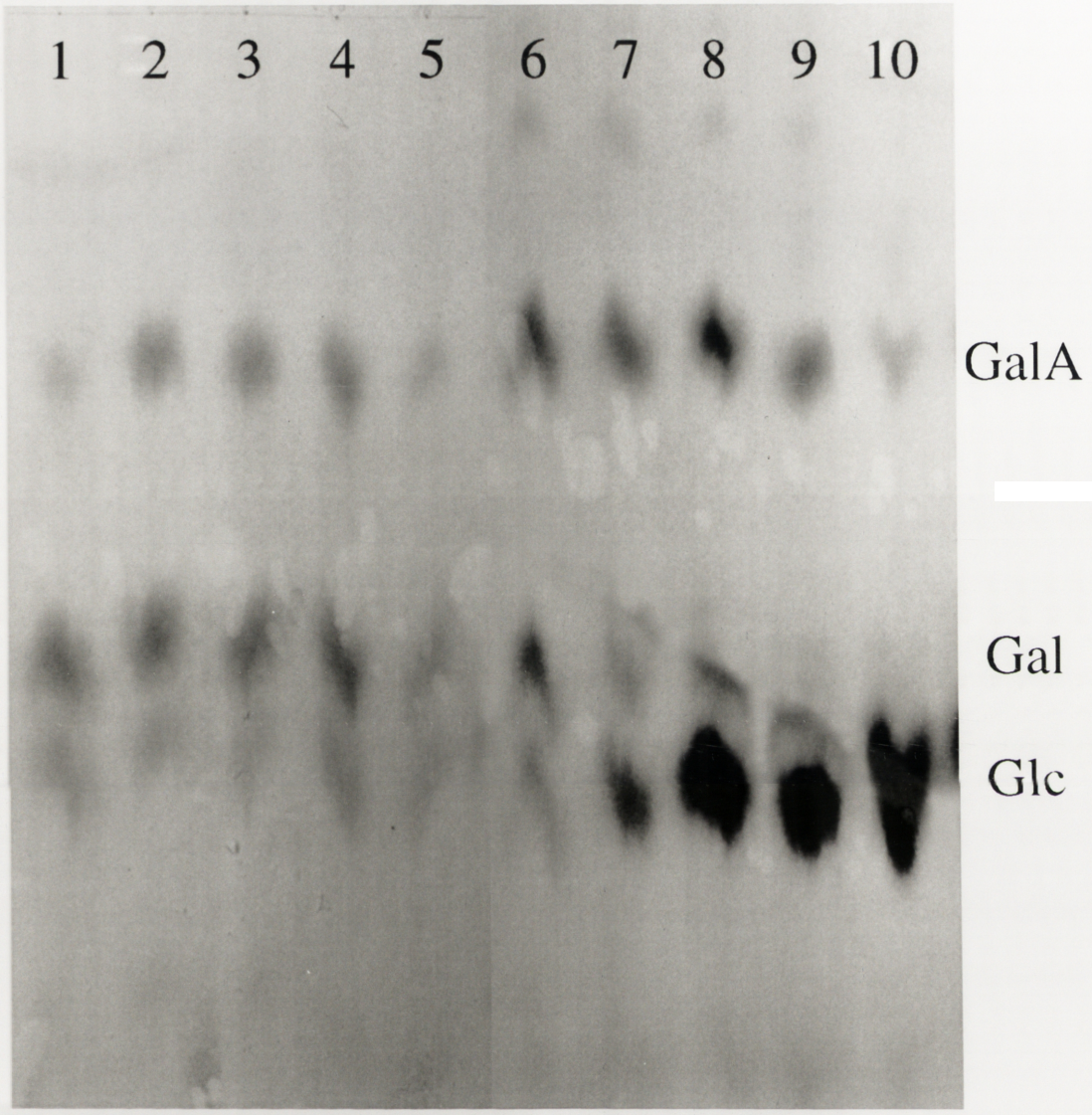

1 2 3 4 5 6 7 8 9 10
GalA
Gal
Glc

Table 4.5 Glucose (nmol/mg dry weight of seedlings) in Three Fractions from Shoots of *Arabidopsis* Wild Type and Mutants Grown at 21 °C and 31 °C

Fractions	Wild Type		*rsw1*		*rsw2*		*rsw3*		*rsw5*	
	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C
Ammonium Oxalate	42	80	47	221	20	184	29	155	33	54
0.1 M KOH	2	6	5	21	5	28	6	52	5	16
4 M KOH	29	52	26	93	38	122	42	130	30	59
Total	73	138	78	335	63	334	77	337	68	129

Table 4.6 Glucose (nmol/mg dry weight of seedlings) in Three Fractions from Roots of *Arabidopsis* Wild Type and Mutants Grown at 21 °C and 31 °C

Fractions	Wild Type		*rsw1*		*rsw2*		*rsw3*		*rsw5*	
	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C
Ammonium Oxalate	18	16	13	26	14	19	14	25	10	12
0.1 M KOH	5	5	4	6	4	4	3	7	3	3
4 M KOH	40	44	38	37	31	37	39	43	32	31
Total	63	65	55	69	49	60	56	75	45	46

4.4 DISCUSSION

The production of cellulose (expressed as nmol glucose/mg plant dry weight) is similar in mutants and wild type at 21 °C except in the case of the root of *rsw2* and the shoot of *rsw5* but is greatly reduced at 31 °C in all mutants. This is seen both by expressing the cellulose content of the mutants as a percentage of wild type and by comparing the Rw values which are the ratio of cellulose content at 31 °C to the cellulose content of the same genotype at 21 °C. This shows that the *RSW1, RSW2, RSW3* and *RSW5* genes are involved in cellulose production although it does not yet show that they are involved specifically in cellulose production rather than being involved in general cell wall production. An unexpected finding is that the shoots of *rsw1, rsw2* and *rsw3* grown at 31 °C show much enhanced levels of glucose in their ammonium oxalate, 0.1 M KOH and 4 M KOH fractions. In the next chapter it will be shown that the excess glucose in those fractions is in the form of β-1,4-glucan.

CHAPTER FIVE

PURIFICATION OF β-1,4-GLUCAN FROM *rsw1*, *rsw2* AND *rsw3* BUT NOT FROM WILD TYPE OR *rsw5*

Some of the work in this chapter has been published:

Arioli, T. et al (1998). Molecular Analysis of Cellulose Biosynthesis in *Arabidopsis*. Science, 279: 717-720.

5.1 INTRODUCTION

In the previous chapter it was shown that mutants *rsw1*, *rsw2*, *rsw3* and *rsw5* are defective in cellulose production when grown at their restrictive temperature of 31 °C. It was noted that three fractions containing non-cellulosic polysaccharides in wild type (Chapter Three) contained elevated levels of glucose when prepared from the shoots of *rsw1*, *rsw2* and *rsw3* seedlings growing at 31 °C but not when prepared from the roots. In this chapter, it will be shown that most of that glucose is in the form of a β-1,4-glucan.

5.1.1 β-1,4-Glucan

There are four major homo-glucose polymers (glucans) in higher plants: cellulose (β-1,4-glucan), starch (α-1,4-glucan and α-1,4-glucan branched with 1,6-glucose units), callose (β-1,3-glucan) and β-(1,4; 1,3)-glucan (Duffus and Duffus, 1984; Fry, 1988; Stone and Clarke, 1992; Gibeaut and Carpita, 1994). These glucans occur in the cell wall except for starch which occurs in the cytoplasm (Bacic et al, 1988; Martin et al, 1993).

Cellulose has a compact, tightly bonded structure that is fibrous, insoluble in aqueous solvents, and provides high strength in the wall (Woods, 1959; Kennedy et al, 1989). Cello-oligosaccharides (β-1,4-glucan) are known to become insoluble when DP ≥8 (Tonnesen and Ellefsen, 1971). When a single hydroxyl of one in ten anhydroglucoses is substituted, however, the resultant β-1,4-glucan becomes soluble in neutral aqueous solution (Atalla, 1998). A soluble β-1,4-glucan was isolated from *Acetobacter xylinum*, and methylation analysis showed a low level of β-1,2-linked glucose units along this β-1,4-glucan chain (Colvin et al, 1977). In higher plants, Satoh et al (1976) also recognised a possible soluble β-1,4-glucan in homogenates of *Phaseolus aureus* seedlings by its easy hydrolysis with cellulase. The authors also proposed that this β-1,4-

glucan might be an intermediate in cellulose biosynthesis *in vivo*. On the other hand, native cellulose consists of crystalline and amorphous regions (Shafizadeh and McGinnis, 1971; Brett and Waldron, 1990) and several chemicals (dyes and fluorescent brighteners) interfere with the crystallisation of newly polymerised glucan chains into cellulose fibrils (Haigler et al, 1980; Herth, 1980; Haigler, 1991; Okuda et al, 1994). However, it remains unknown whether cellulose that is amorphous or altered in crystallisation by chemicals is soluble in neutral aqueous media.

5.1.2 β-1,4-Glucan Identification

Identification of the molecular structure of any polysaccharide basically requires information on the composition and sequence of monosaccharides, the position and configuration of the glycosidic linkages, the types of branching present and the location of substituents (Duffus and Duffus, 1984). The monosaccharide composition and methylation analysis of the main wall polysaccharides (cellulose, pectins and hemicelluloses) of *Arabidopsis* wild type provides basic information on composition and linkages. Those methods cannot, however, distinguish β-1,4-glucan (cellulose) from α-1,4-glucan (one type of starch). On the other hand, the high resistance to extraction under mild conditions and to hydrolysis by bases or acids (except concentrated H_2SO_4; Section 2.1), allows cellulose to be extracted separately from other wall polysaccharides (pectins and hemicelluloses) and starch (see Chapter Three). The contrasting response of cellulose and α-1,4-glucan to hydrolysis by 2 M TFA, for example, can be used to identify them. If, however, the β-1,4-glucan is soluble for some reason and so susceptible to TFA hydrolysis, then this property would not help in their identification.

5.1.3 β-1,4-Glucan Digestion by Enzymes

Digestion by enzymes specific for either β-1,4- or α-1,4- linkages provides a powerful way to identify the configuration of glucans. There are different types of enzymes which can hydrolyse β-1,4-glucan in micro-organisms (Reese, 1977) such as endo-β-1,4-glucanase (EC 3.2.1.4), and exo-cellobiohydrolase (EC 3.2.1.91; Wood and McCrae, 1986; Wood and Bhat, 1988). The hydrolysis of cellulose by enzymes is extremely complex, due to the dual nature of the insoluble substrate (crystalline and amorphous) and the complex enzymatic system (reviewed by Rapp and Beermann, 1991). Although native celluloses are very appropriate for studying complete cellulase preparations, swollen or regenerated celluloses are commonly used as substrates due to their increased susceptibility to enzymic degradation (reviewed by Fincher and Roberts, 1987; Shoemaker et al, 1998). Endo-β-1,4-glucanase activity is determined in extracts of many plant tissues (particularly growing tissue) by measuring the rate of loss of viscosity of carboxymethylcellulose solution. Although carboxymethylcellulose is the most commonly used substrate, neutral derivatives such as hydroxyethylcellulose are preferable due to their much lower sensitivity to pH and other ionic effects (Child et al, 1973). There are few reports of plant enzymes which hydrolyse crystalline cellulose microfibrils in the way that enzymes from some micro-organisms do (Maclachlan and Carrington, 1991).

Some indirect approaches have been employed to test the degradation by plant β-1,4-glucanases *in vivo* (Verma et al, 1982). Green et al (1977) have devised a continuous, polarographic assay to detect cellulase activity by coupling the liberation of glucose from microcrystalline cellulose with oxygen consumption via glucose oxidase. Observing short fiber production from the native substrate is an optical way to measure cellulolytic activity (Shoemaker and Brown, 1978) with reduction either in turbidity of suspensions of

microcrystalline cellulose (Palva and Nevalainen, 1976) or in binding of Congo Red dye (Wood, 1981).

Cellulases preferentially attack interior linkages of cellulose and their pH optima are between 4.5 and 7.0. Various other polysaccharide hydrolases, in addition to those mentioned above, have been identified in plant tissues, such as α-amylase, and β-1,3-glucanase. Their pH optima are usually in the acidic range (Duffus and Duffus, 1984; Preiss, 1988; Brett and Waldron, 1990; Stone and Clarke, 1992).

Starch degradation in plants involves two major enzymes: firstly starch is broken down to maltose by α-amylase. Secondly, maltose is digested into glucose by α-glucosidase (Preiss, 1988; Martin and Smith, 1995).

5.1.4 Aim of the Chapter

In Chapter Three, I documented the main polysaccharides in the fractions of the cell wall of *Arabidopsis* wild type grown at 21 °C. A relatively high proportion of glucose was found in the ammonium oxalate fraction of shoots and methylation analysis showed that most of it was 4-linked. In Chapter Four, *Arabidopsis* mutants and wild type grown at 31 °C were shown to have an even higher glucose content in their ammonium oxalate, 0.1 KOH and 4 M KOH fractions. This chapter will use those fractions prepared mainly from mutant seedlings grown at 31 °C to show that most of that glucose occurs as a β-1,4-glucan (Figure 5.1).

5.2 MATERIALS AND METHODS

5.2.1 Plant Material

Large scale preparations of glucan for characterisation were made by harvesting about 5000 whole seedlings of wild type and mutants (*rsw1, rsw2, rsw3* and *rsw5*) that had been grown for two days at 21 °C, followed by five days at 31 °C. Because of the large number of seedlings involved, the harvested tissue was not separated into roots and shoots. The preparation of neutral and acidic subfractions from the ammonium oxalate fraction using 2% CTAB was described in Chapter Three. The β-1,4-glucan from the neutralised and dialysed 0.1 and 4 M KOH fractions was precipitated by centrifugation at 14, 000 *g* for 1 h (Figure 5.1). Methods of carbohydrate analysis were described in Chapters Two and Three.

Purifiable glucan and enzymically releasable glucose were quantified using the three replicate batches of wild type and mutant seedlings grown at 21 °C and 31 °C that were described in Chapter Four.

5.2.2 Enzymes and Substrates

Endo-cellulase (EC 3.2.1.4; from *Trichoderma sp.*), exo-1,3-β-D-glucanase (EC 3.2.1.58) and XG (from tamarind seed) were purchased from Megazyme (Ireland) Pty Ltd. The following products were obtained from Sigma Chemical Co. (St. Louis MO): α-amylase (EC 3.2.1.1; from *Porcine pancreas*), α-glucosidase (EC 3.2.1.20; from rice), β-glucosidase (EC 3.2.1.21; from almonds), D-(+)-cellobiose, D-(+)-cellopentaose, D-(+)-cello-oligosaccharides, laminarin (from *Laminaria digitata*, L-9634), and starch (soluble ACS reagent, S-9765). Cellulose was a powder (for chromatography, CF11, Whatman Co).

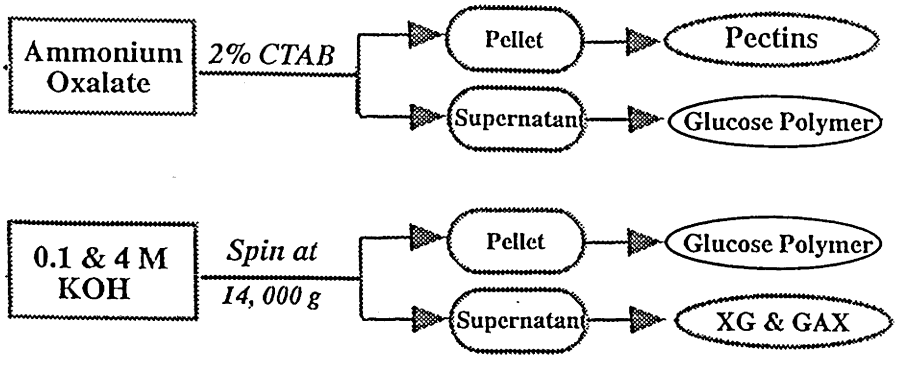


Figure 5.1

Procedure For Purification of β-1,4-Glucan

5.2.3 Enzyme-Catalysed Hydrolysis of Carbohydrates

For each enzyme reaction described below, two reaction controls were run: free glucose was determined after incubation with enzyme only (ie no substrate) and after incubation with substrate only (ie no enzymes). Any glucose determined from those was subtracted for quantitative calculation of glucose released.

5.2.3.1 Endo-cellulase and β-glucosidase

A dry sample of carbohydrate (2-20 μg) was dissolved in 50 mM sodium acetate (400 μl; pH 4.7, adjusted with acetic acid). Endo-cellulase only, or a mixture of endo-cellulase and β-glucosidase was added to make the final activity of each enzyme 0.125 U/ml. The solution was mixed well, capped tightly, and incubated at 37 °C for 48 h. The solution was then centrifuged at 14, 000 *g* for 20 min and the supernatant collected, freeze-dried and stored at 4 °C until analysed by GC/MS.

5.2.3.2 α-Amylase and α-glucosidase

A dry sample of carbohydrate (20-100 μg) was dissolved in 50 mM phosphate buffer (400 μl, pH 7), and α-amylase (final level, 0.125 U/ml) was added, mixed well, capped and incubated at 37 °C for 48 h. After the enzyme reaction, the solution was freeze-dried, and dissolved in 50 mM sodium acetate solution (400 μl) with α-glucosidase (0.06 U/ml; pH 4.7, adjusted by acetic acid). After incubation at 37 °C for 48 h, the solution was centrifuged at 14, 000 *g* for 20 min, and the supernatant was collected, freeze-dried and analysed by GC/MS.

5.2.3.3 Exo-1,3-β-D-glucanase

The dry substrate sample (2-20 μg) was dissolved in 50 mM sodium acetate (400 μl, pH 4.7, adjusted with acetic acid), and exo-1,3-β-D-glucanase (0.15 U/ml) was added. The solution was mixed well, capped tightly, and incubated at 37 °C for 48 h. Then, the solution was centrifuged at 14, 000 *g* for 20 min and the supernatant collected, freeze dried and stored at 4 °C until analysed by GC/MS.

5.3 RESULTS

5.3.1 The Glucan from *rsw1* Shoots

5.3.1.1 Purification

The various monosaccharides seen when the ammonium oxalate fraction prepared from 5000 whole *rsw1* seedlings grown at 31 °C is hydrolysed with TFA (Figure 5.2, B) were all found in the pellet after CTAB precipitation (Figure 5.2, D). Only glucose, however, was detected in the supernatant (Figure 5.2, C). No further peaks were detected when the chromatogram was amplified eight-fold. Furthermore, uronic acids, that were the most abundant constituent of the ammonium oxalate fraction, were not detected in the supernatant by the *m*-hydroxybiphenyl/H_2SO_4 test (data not shown). The method will detect uronic acids down to about 10 nmol/ml (see also Fry, 1988) so, given that samples containing 3500 nmol/ml glucose were analysed, this places uronic acids at <0.35 residues per 100 glucose residues. The supernatant purified from the ammonium oxalate fraction was therefore judged to be a glucan (or homo glucose polymer) without other monosaccharide constituents.

After dialysis (MWCO: 6-8, 000) of the supernatant for 3 days to remove any small molecules including any remaining CTAB, the glucan formed a visible pellet when centrifuged at 14, 000 *g* for 1 h. Almost all glucose was in the pellet according to GC/MS analysis of TFA hydrolysates (data not shown). The same amount of crude ammonium oxalate fraction prior to CTAB purification was shown in preliminary experiments to form only a smaller pellet when centrifuged for 1 h at 14, 000 *g*. The pellet only contained 37% of the glucose in the fraction (96 nmol/mg dry weight seedlings) with small amounts of all other monosaccharides making up about 15% of the total monosaccharides in the pellet.

Figure 5.2

GC/MS chromatograms of AAs from:

A, Standard mixture of monosaccharides: Peaks 1, rhamnose; 2, fucose; 3, arabinose; 4, xylose; 5, mannose; 6, galactose; 7, glucose; IS, *myo*-inositol.

B, Sugars released by TFA hydrolysis of ammonium oxalate fraction of whole seedlings of *rsw1* grown at 31 °C.

C, Sugars released by TFA hydrolysis of supernatant purified by 2% CTAB from the ammonium oxalate fraction of whole seedlings of *rsw1* grown at 31 °C.

D, Sugars released by TFA hydrolysis of pellet purified by 2% CTAB from the ammonium oxalate fraction of whole seedlings of *rsw1* grown at 31 °C.

E, Control: Sugars of supernatant purified by 2% CTAB from the ammonium oxalate fraction without TFA hydrolysis.

The scale of the chromatograms (in C and E) were amplified by eight in the region between the arrowheads.

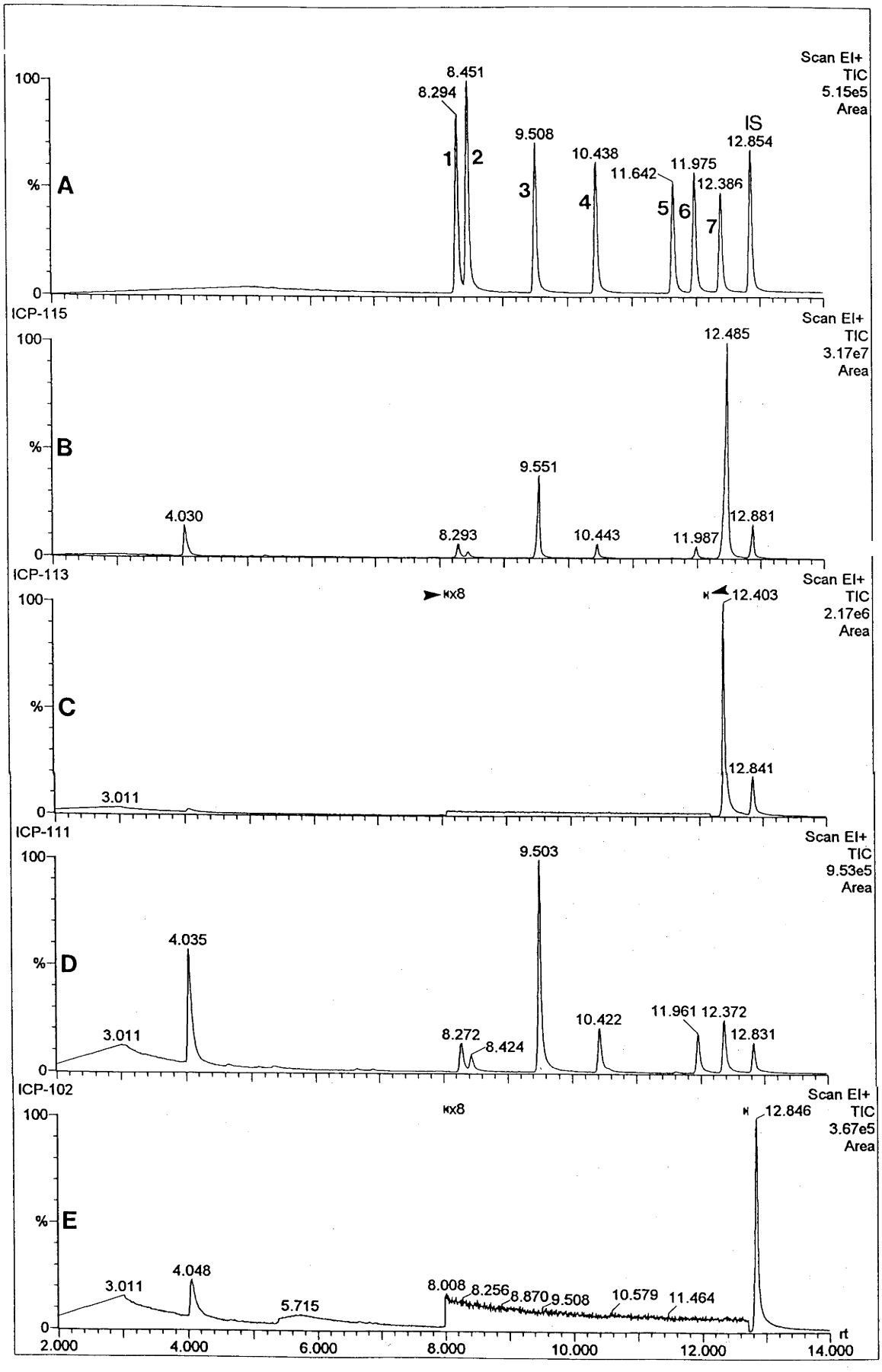

Scan EI+
TIC
5.15e5
Area
100
%
A
0
8.451
8.294
1
2
9.508
3
10.438
4
11.642
5
11.975
6
12.386
7
IS
12.854
ICP-115
Scan EI+
TIC
3.17e7
Area
12.485
B
9.551
4.030
12.881
8.293
10.443
11.987
ICP-113
Scan EI+
TIC
2.17e6
Area
x8
12.403
C
12.841
3.011
ICP-111
Scan EI+
TIC
9.53e5
Area
9.503
4.035
D
3.011
8.272
8.424
10.422
11.961
12.372
12.831
ICP-102
Scan EI+
TIC
3.67e5
Area
x8
12.846
E
4.048
3.011
8.008
8.256
8.870
9.508
10.579
11.464
5.715
rt
2.000
4.000
6.000
8.000
10.000
12.000
14.000

Figure 5.3

GC/MS chromatograms of AAs from:

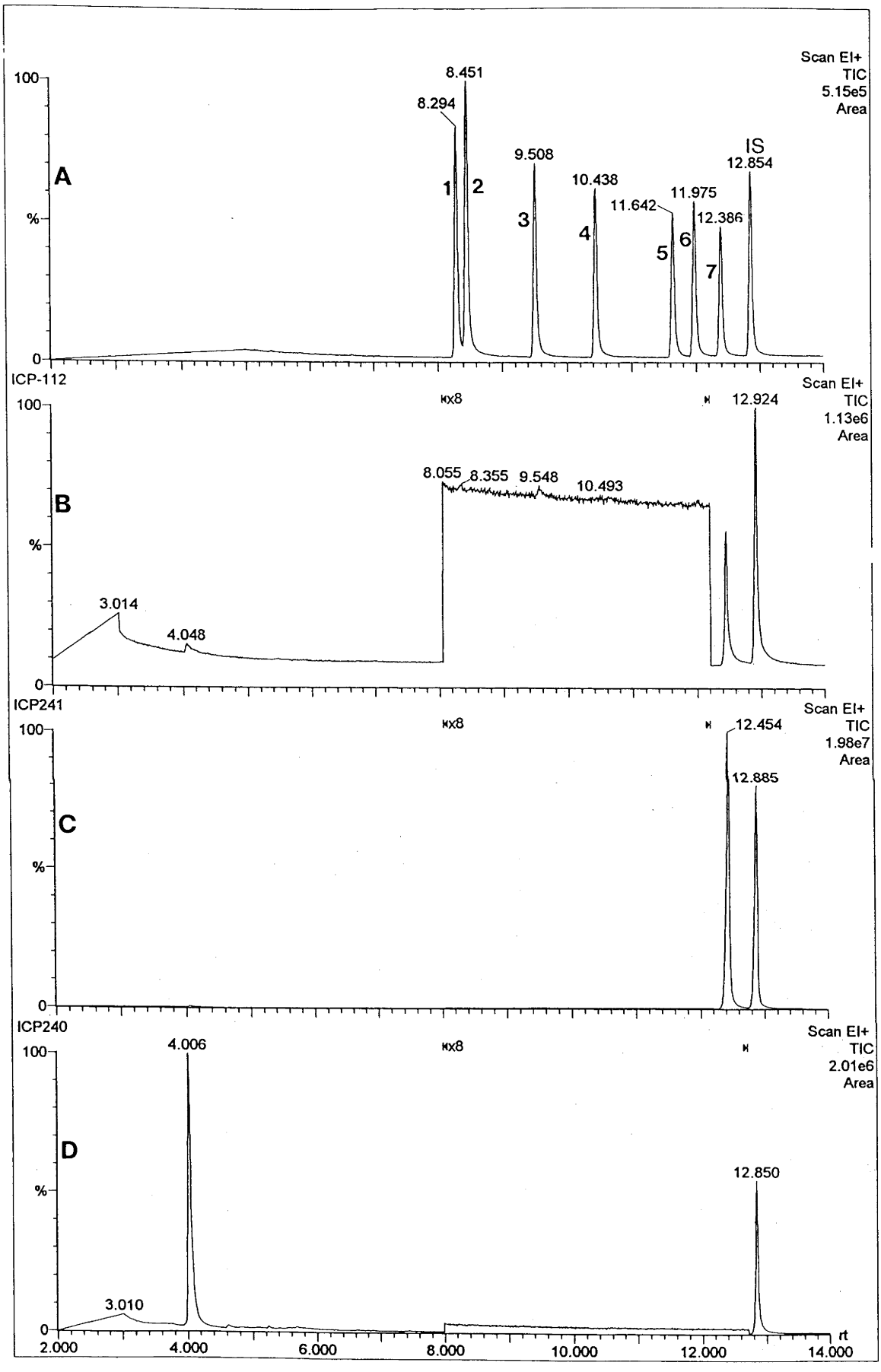
A, Standard mixture of monosaccharides: Peaks 1, rhamnose; 2, fucose; 3, arabinose; 4, xylose; 5, mannose; 6, galactose; 7, glucose; IS, *myo*-inositol.

B, Sugars released by TFA hydrolysis of pellet purified from 0.1 M KOH fraction of whole seedlings of *rsw1* grown at 31 °C.

C, Sugars released by TFA hydrolysis of pellet purified from 4 M KOH fraction of whole seedlings of *rsw1* grown at 31 °C.

D, Control: Sugars from pellet purified from the 4 M KOH fraction without TFA hydrolysis.

The regions (in B, C, D) between the arrowheads were amplified by eight.


A
Scan EI+
TIC
5.15e5
Area
100
%
0
8.451
8.294
9.508
10.438
11.642
11.975
12.386
IS
12.854
1
2
3
4
5
6
7
ICP-112
B
Scan EI+
TIC
1.13e6
Area
12.924
8.055
8.355
9.548
10.493
3.014
4.048
ICP241
C
Scan EI+
TIC
1.98e7
Area
12.454
12.885
ICP240
D
Scan EI+
TIC
2.01e6
Area
4.006
12.850
3.010
2.000
4.000
6.000
8.000
10.000
12.000
14.000
rt

Because the 0.1 and 4 M KOH fractions mainly contained the neutral polysaccharide XG that would not precipitate with CTAB (Sections 3.3.5 and 3.3.6), neutralised and dialysed fractions were centrifuged at 14, 000 *g* for 1 hour to see if a glucan could be isolated (Figure 5.1). Chromatograms of the TFA hydrolysis products of the 4 M KOH pellet showed only a clear peak for glucose even when the remainder of the chromatogram was amplified by a factor of eight (Figure 5.3, C). Chromatograms of the pellet from the 0.1 M KOH fraction showed a major glucose peak with, when amplified by a factor of eight, only small traces of arabinose and possibly xylose and rhamnose. The colorimetric test did not detect any uronic acids in samples containing 3500 nmol/ml glucose. The data show that a glucan also occurred in the 0.1 M and 4 M KOH fractions and could be pelleted from the neutralised and dialysed fractions under the conditions used.

5.3.1.2 Linkage analysis

Methylation analysis of the glucan purified by CTAB fractionation of the ammonium oxalate fraction and by centrifugation of the 4 M KOH fraction of *rsw1* grown at 31 °C showed only two peaks on GC/MS chromatograms (Figure 5.4, Peaks, B1 and B5; C1 and C5). PMAAs in these peaks were identified by comparing MS fragmental patterns and RRTs with those for PMAAs of glucose, cellobiose and laminarin standards (Figure 5.4, A, D and E; Figure 5.5; Table 5.1). It was judged that the PMAAs were 1,5-di-*O*-acetyl-(1-deuterio)-2,3,4,6-tetra-*O*-methyl-D-glucitol (Peaks B1 and C1) and 1,4,5-tri-*O*-acetyl-(1-deuterio)-2,3,6-tri-*O*-methyl-D-glucitol (Peaks B5 and C5), respectively. The very minor peaks visible when other regions of the chromatogram were amplified by a factor of four (Figure 5.4, B) did not give mass spectra consistent with their being PMAAs. In particular there were no peaks showing RRT equal either to 1,3,5-tri-*O*-acetyl-(1-deuterio)-2,4,6-tri-*O*-methyl-D-glucitol from the laminarin standard (Figures 5.4 and 5.5; Peak E2) which would have indicated

Figure 5.4

GC/MS chromatograms of PMAAs from:

A, Glucose standard.

B, Methylation analysis of the supernatant purified by 2% CTAB from the ammonium oxalate fraction of whole seedlings of *rsw1* grown at 31 °C.

C, Methylation analysis of the pellet purified from 4 M KOH fraction of whole seedlings of *rsw1* grown at 31 °C.

D, Methylation analysis of cellobiose.

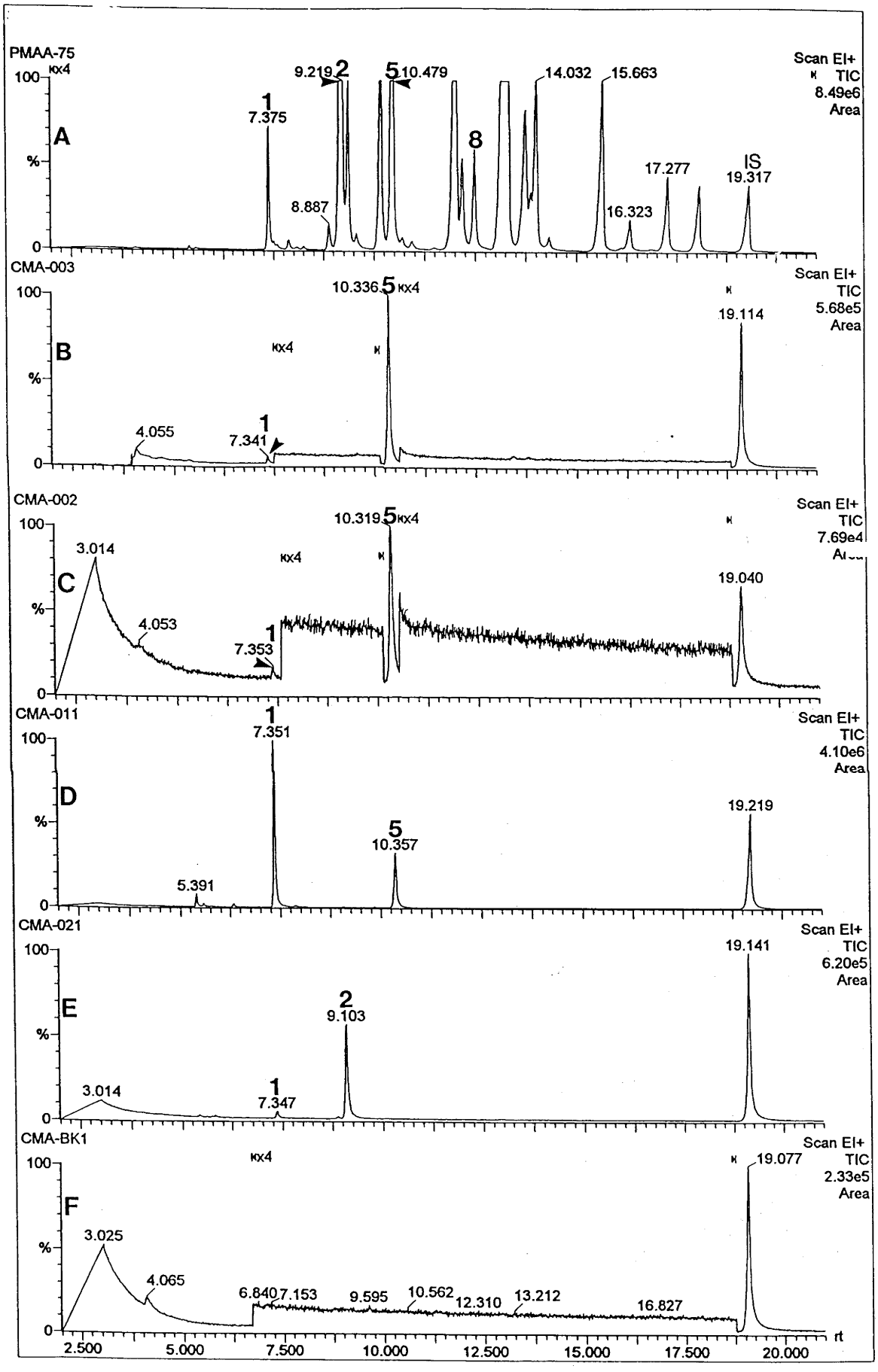
E, Methylation analysis of laminarin.

F, Control: Methylation analysis of blank.

Peak **1**: 2,3,4,6-Me_4 Glc; Peak **2**: 2,4,6-Me_3 Glc; Peak **5**: 2,3,6-Me_3 Glc; Peak **8**: 3,6-Me_2 Glc; **IS**: Internal Standard (*myo*-inositol).

Peaks were identified on the basis of RRT (Table 5.1) and mass spectra (Figure 5.5).

The regions (in B, Cand F) between the arrowheads were amplified by four.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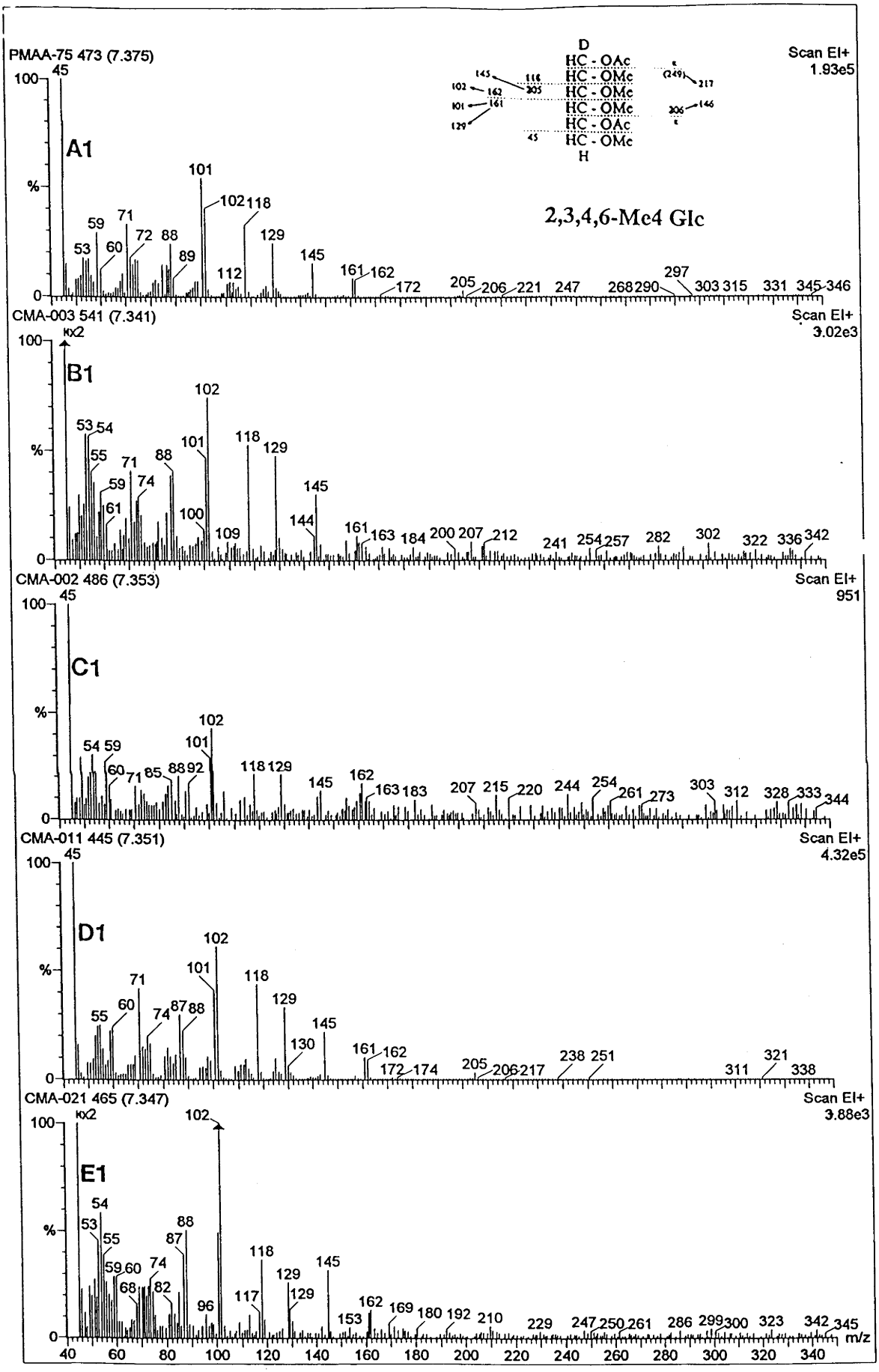
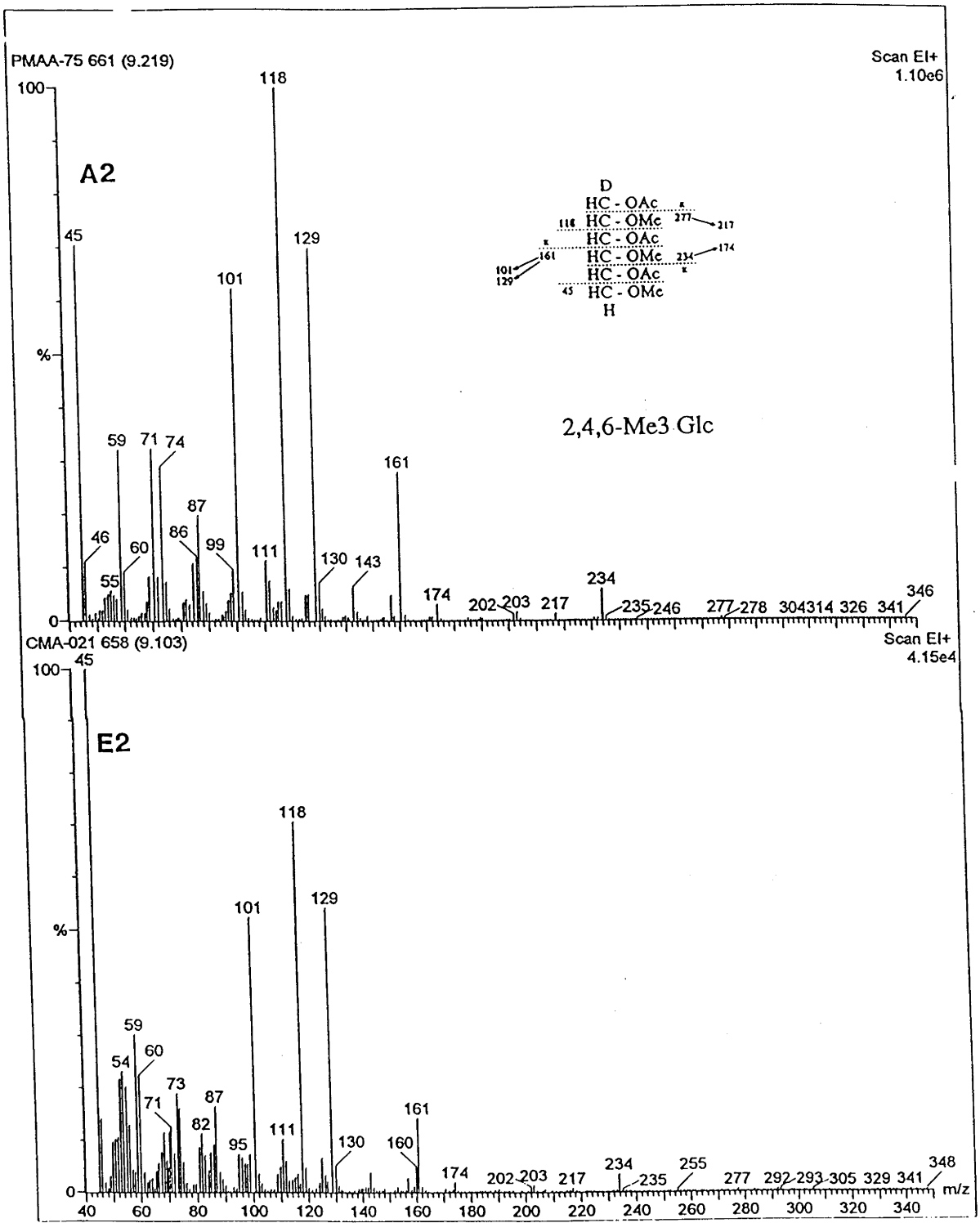
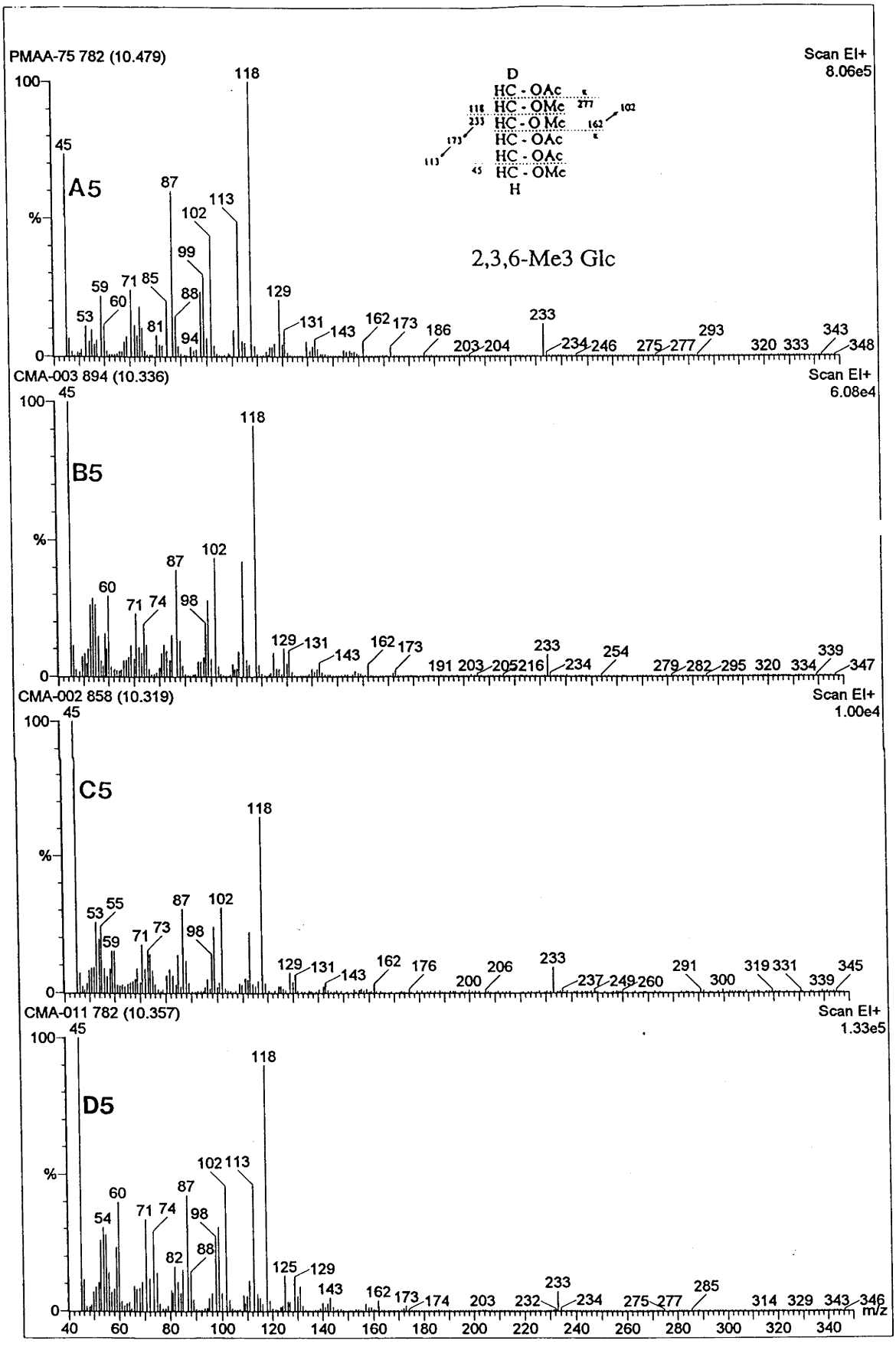
Scan EI+
TIC
8.49e6
Area
A
7.375
9.219
10.479
14.032
15.663
8.887
16.323
17.277
IS
19.317
CMA-003
Scan EI+
TIC
5.68e5
Area
B
10.336
19.114
4.055
7.341
CMA-002
Scan EI+
TIC
7.69e4
C
3.014
10.319
19.040
4.053
7.353
CMA-011
Scan EI+
TIC
4.10e6
Area
D
7.351
19.219
10.357
5.391
CMA-021
Scan EI+
TIC
6.20e5
Area
E
19.141
9.103
3.014
7.347
CMA-BK1
Scan EI+
TIC
2.33e5
Area
F
19.077
3.025
4.065
6.840
7.153
9.595
10.562
12.310
13.212
16.827
2.500
5.000
7.500
10.000
12.500
15.000
17.500
20.000
rt

Figure 5.5

Mass spectra of each labelled peak in GC/MS chromatograms (Figure 5.4). The deduced glycosyl linkage of each labelled peak are summarised in Table 5.1.


PMAA-75 473 (7.375)
Scan EI+
1.93e5
A1
2,3,4,6-Me4 Glc
D
HC - OAc
HC - OMe
HC - OMe
HC - OMe
HC - OAc
HC - OMe
H
CMA-003 541 (7.341)
Scan EI+
3.02e3
B1
CMA-002 486 (7.353)
Scan EI+
951
C1
CMA-011 445 (7.351)
Scan EI+
4.32e5
D1
CMA-021 465 (7.347)
Scan EI+
3.88e3
E1
m/z


PMAA-75 661 (9.219)
Scan EI+
1.10e6
A2
D
HC - OAc
HC - OMe
HC - OAc
HC - OMe
HC - OAc
HC - OMe
H
2,4,6-Me3 Glc
CMA-021 658 (9.103)
Scan EI+
4.15e4
E2
m/z


PMAA-75 782 (10.479)
Scan EI+
8.06e5
A5
2,3,6-Me3 Glc
D
HC - OAc
HC - OMe
HC - O Me
HC - OAc
HC - OAc
HC - OMe
H
CMA-003 894 (10.336)
Scan EI+
6.08e4
B5
CMA-002 858 (10.319)
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1.00e4
C5
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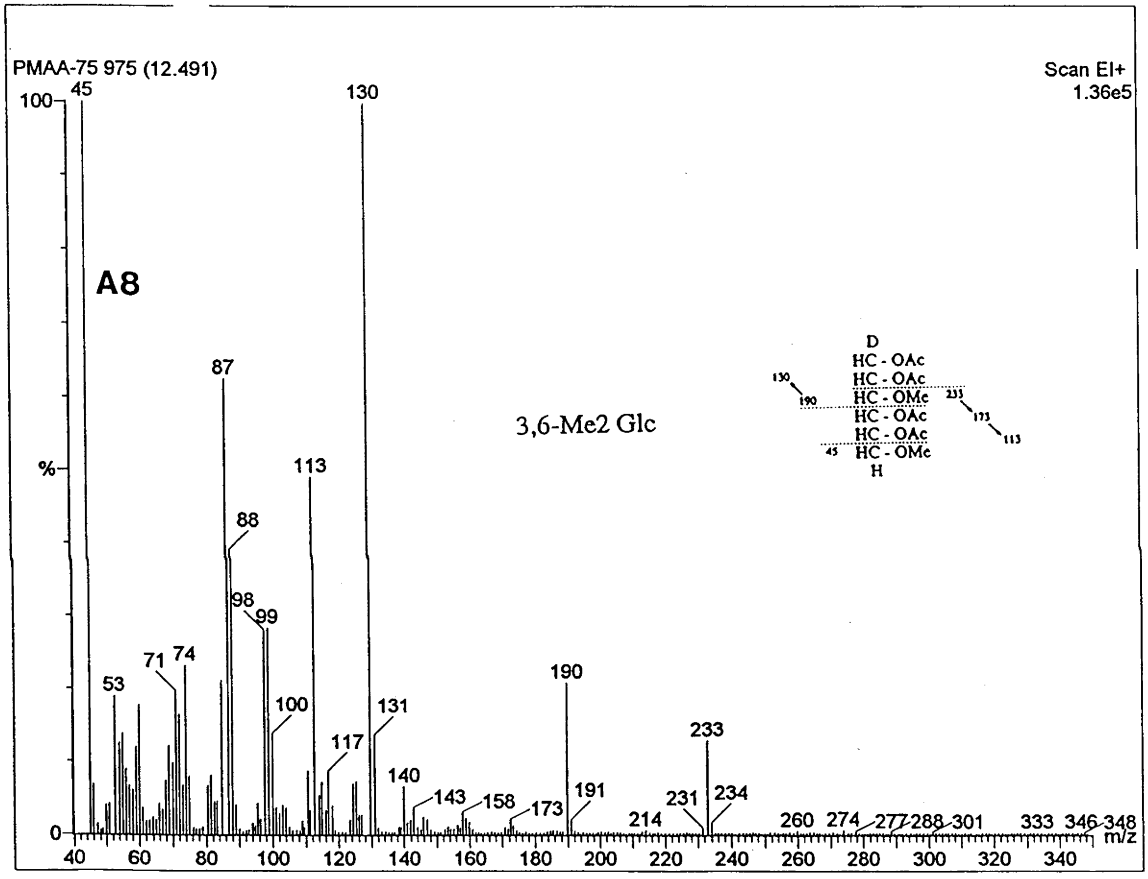
Scan EI+
1.33e5
D5
m/z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3,6-Me2 Glc
D
HC - OAc
HC - OAc
HC - OMe
HC - OAc
HC - OAc
HC - OMe
H
%
m/z

Table 5.1 Comparison of RRT of PMAAs derived from methylation analysis of the CTAB supernatant (B) purified from the ammonium oxalate fraction and the pellet (C) purified from the 4 M KOH fraction of whole seedlings of *rsw1* grown at 31 °C, glucose standard (A), cellobiose (D) and laminarin (E)

(Chromatograms in Figure 5.4 and MS in Figure 5.5)

Peak	Derivative	Deduced Linkage	RRT-1 *	RRT-2
A1	**2,3,4,6-Me_4 Glc**	**t-Glc*p***	**0.382**	**1**
B1	2,3,4,6-Me_4 Glc	t-Glc*p*	0.384	1
C1	2,3,4,6-Me_4 Glc	t-Glc*p*	0.385	1
D1	2,3,4,6-Me_4 Glc	t-Glc*p*	0.382	1
E1	2,3,4,6-Me_4 Glc	t-Glc*p*	0.384	1
A2	**2,4,6-Me_3 Glc**	**3-Glc*p***	**0.477**	**1.25**
E2	2,4,6-Me_3 Glc	3-Glc*p*	0.476	1.24
A5	**2,3,6-Me_3 Glc**	**4-Glc*p***	**0.542**	**1.42**
B5	2,3,6-Me_3 Glc	4-Glc*p*	0.541	1.41
C5	2,3,6-Me_3 Glc	4-Glc*p*	0.542	1.40
D5	2,3,6-Me_3 Glc	4-Glc*p*	0.539	1.41
A8	**3,6-Me_2 Glc**	**2,4-Glc*p***	**0.647**	**1.694**

*, RRT-1: Relative retention time to *myo*-inositol hexaacetate;

RRT-2: To t-Glc*p*.

Table 5.2 Estimation by methylation analysis of the DP of the 1,4-glucan purified from the ammonium oxalate and 4 M KOH fractions of whole seedlings of *rsw1* grown at 31 °C *

Samples	Ratio of 4-Glc*p* to t-Glc*p* Peak Areas	DP	MW (Da)
Cellobiose Standard	0.5	2	342
1,4-Glucan from Ammonium Oxalate Fraction	32.5	65	10548
1,4-Glucan from 4 M KOH Fraction	17.4	35	5688

* GC/MS chromatograms for the PMAAs in Figure 5.4.

1,3-linkages (Table 5.1, E2), or to peaks from any other potential linkages such as peak A8 (1,2,4,5-tri-*O*-acetyl-(1-deuterio)-3,6-di-*O*-methyl-D-glucitol) indicating a 2,4-linked glucose as expected from Atalla's speculative structure of a substituted 1,4-glucan (Atalla, 1998; Figures 2.2 and and 2.3 provide details of all PMAAs of the glucose standard). It is concluded that only 1,4-linkages can be detected in the glucans purified in the supernatant from the CTAB-fractionated ammonium oxalate fraction and in the pellet from the 4 M KOH fraction.

The DP of those glucans was estimated by comparing the ratio of 4-Glc*p* to t-Glc*p* using a cellobiose standard to give relative response factors (Table 5.2). The glucan purified from the ammonium oxalate fraction had a DP of 65, almost double the DP of 35 for the glucan from the 4 M KOH fraction.

5.3.1.3 Configuration of the 1,4-glucan

The 1,4-glucan purified as described from the three fractions could be either a β-1,4-glucan (cellulose), an α-1,4-glucan (amylose), or a mixture of the two. A sample of the dried 1,4-glucan (150-200 μg) gave no blue colour with iodine (0.33%) and KI (0.67%), whereas starch samples (50, 100 and 200 μg) gave a clear blue reaction (method in Section 3.2.3). The starch showed a clear positive reaction with the iodine solution even if mixed with other carbohydrates (starch/cellulose, or /pectin, or /XG: total 100 μg, 1:1, w/w).

To test the nature of the 1,4-linkage, an endo-cellulase was used to digest the 1,4-glucan (glucose concentration: 3500 nmol/ml) purified by CTAB from the ammonium oxalate fraction (Table 5.3). After incubation with the enzyme at 37 °C for 48 h, the 1,4-glucan sample released about 92% of its total glucose as free glucose as judged by GC/MS (Figure 5.6, B). On the chromatogram, the 1,4-glucan samples whether purified from the ammonium oxalate fraction (Figure 5.6, B), or from the 0.1 and 4 M KOH fractions (Figure 5.7, B and C) showed only one peak corresponding to glucose. In comparison, only about 1%

Figure 5.6

GC/MS chromatograms of AAs from:

A, Standard mixture of monosaccharides: Peaks 1, rhamnose; 2, fucose; 3, arabinose; 4, xylose; 5, mannose; 6, galactose; 7, glucose; IS, *myo*-inositol.

B, Sugars released by endo-cellulase at 37 °C for 48 h from supernatant purified by 2% CTAB from the ammonium oxalate fraction of whole seedlings of *rsw1* grown at 31 °C.

C, Control: Sugars of supernatant purified by 2% CTAB from the ammonium oxalate fraction of whole seedlings of *rsw1* grown at 31 °C without endo-cellulase incubation.

D, Control: Sugars of endo-cellulase only (ie no sample).

The regions (in B, C and D) between the arrowheads were amplified by four.

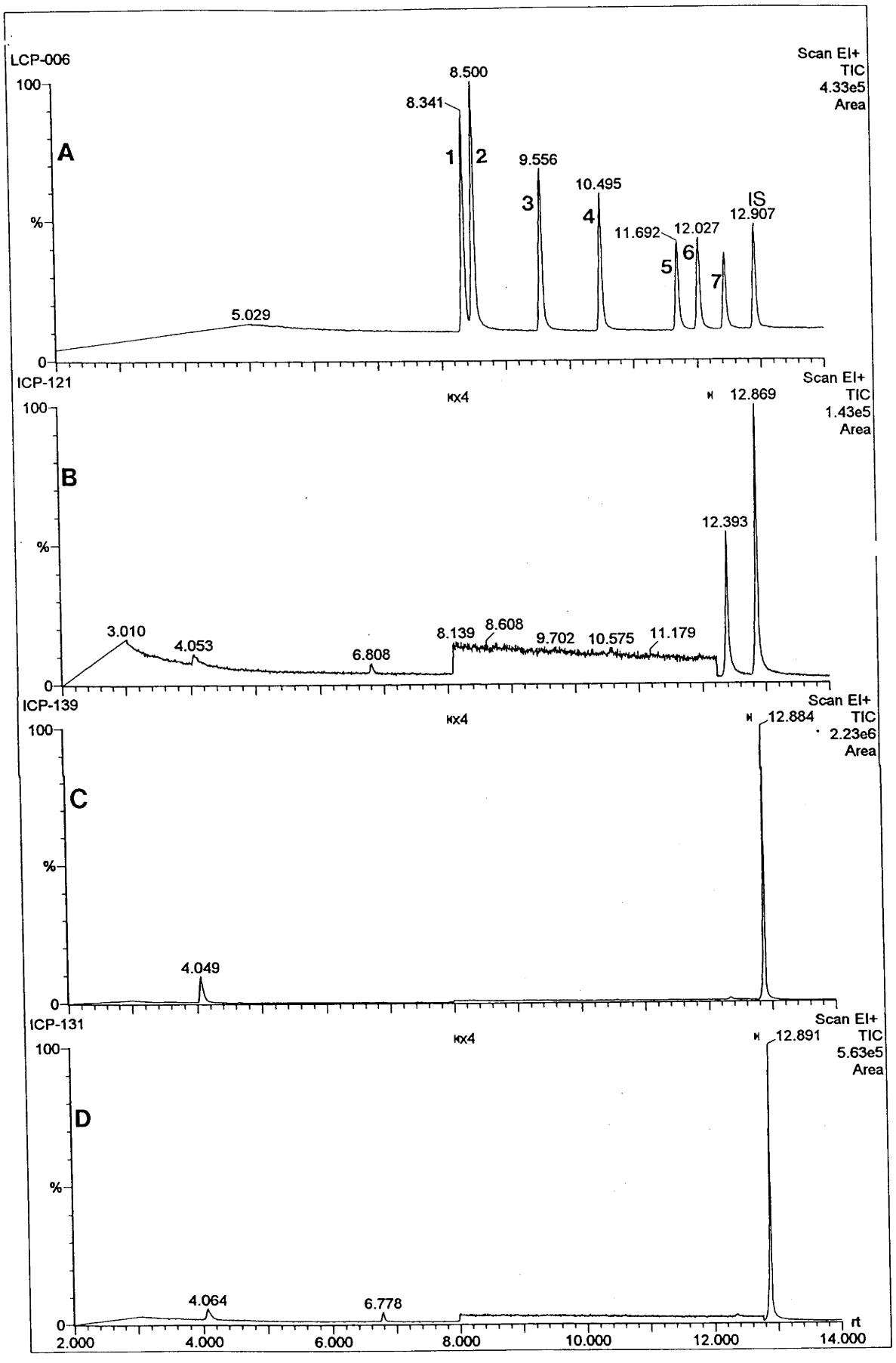

LCP-006
Scan EI+
TIC
4.33e5
Area
A
100
%
0
8.500
8.341
1
2
9.556
3
10.495
4
IS
12.907
11.692
12.027
5
6
7
5.029
ICP-121
Scan EI+
TIC
1.43e5
Area
Kx4
12.869
B
12.393
3.010
4.053
6.808
8.139
8.608
9.702
10.575
11.179
ICP-139
Scan EI+
TIC
2.23e6
Area
Kx4
12.884
C
4.049
ICP-131
Scan EI+
TIC
5.63e5
Area
Kx4
12.891
D
4.064
6.778
rt
2.000
4.000
6.000
8.000
10.000
12.000
14.000

Figure 5.7

GC/MS chromatograms of AAs from:

A, Standard mixture of monosaccharides: Peaks 1, rhamnose; 2, fucose; 3, arabinose; 4, xylose; 5, mannose; 6, galactose; 7, glucose; IS, *myo*-inositol.

B and C, Sugars released by endo-cellulase at 37 °C for 48 h from:

B, pellet centrifuged (14, 000 *g*, 1 h) from the 0.1 M KOH fraction of whole seedlings of *rsw1* grown at 31 °C;

C, pellet centrifuged (14, 000 *g*, 1 h) from the 4 M KOH fraction of whole seedlings of *rsw1* grown at 31 °C.

D, Control: Sugars of pellet centrifuged (14, 000 *g*, 1 h) from the 4 M KOH fraction of *rsw1* grown at 31 °C incubated in absence of the endo-cellulase.

The regions (in B, C and D) between the arrowheads were amplified by four.

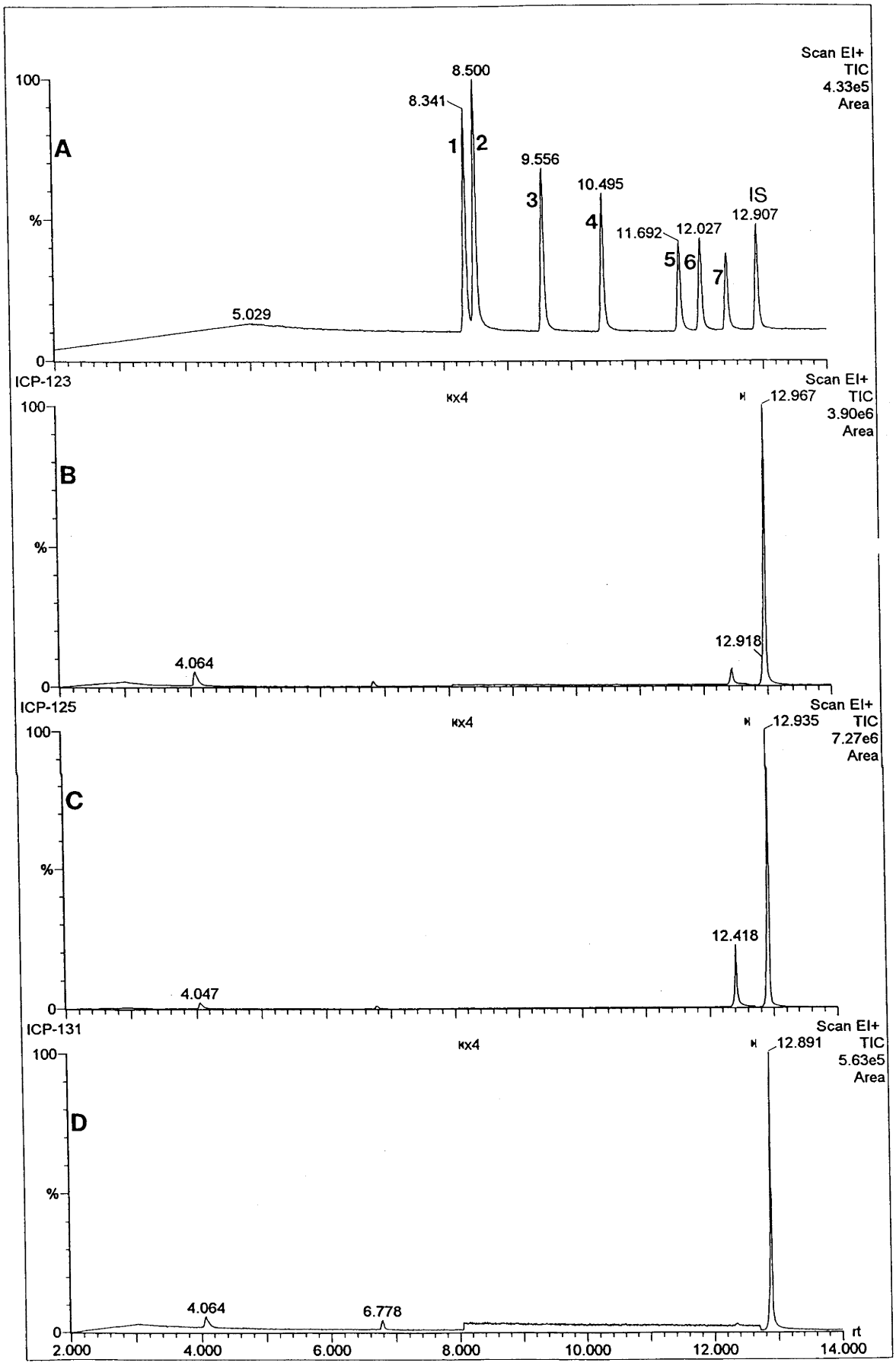

Scan EI+
TIC
4.33e5
Area
A
100
%
0
8.500
8.341
1
2
9.556
3
10.495
4
11.692
5
12.027
6
7
IS
12.907
5.029
ICP-123
Scan EI+
TIC
3.90e6
Area
B
12.967
12.918
4.064
ICP-125
Scan EI+
TIC
7.27e6
Area
C
12.935
12.418
4.047
ICP-131
Scan EI+
TIC
5.63e5
Area
D
12.891
4.064
6.778
rt
2.000
4.000
6.000
8.000
10.000
12.000
14.000

Figure 5.8

GC/MS chromatograms of AAs from:

A, Standard mixture of known monosaccharides: Peaks 1, rhamnose; 2, fucose; 3, arabinose; 4, xylose; 5, mannose; 6, galactose; 7, glucose; IS, *myo*-inositol.

B and C, Sugars released by α-amylase and α-glucosidase at 37 °C for 48 h from:

B, supernatant purified by CTAB from ammonium oxalate fraction of whole seedlings of *rsw1* grown at 31 °C;

C, pellet centrifuged (14, 000 *g*, 1 h) from the 4 M KOH fraction of whole seedlings of *rsw1* grown at 31 °C.

D, Control: sugars of α-amylase and α-glucosidase only.

The regions (in B, C and D) between the arrowheads were amplified by four.

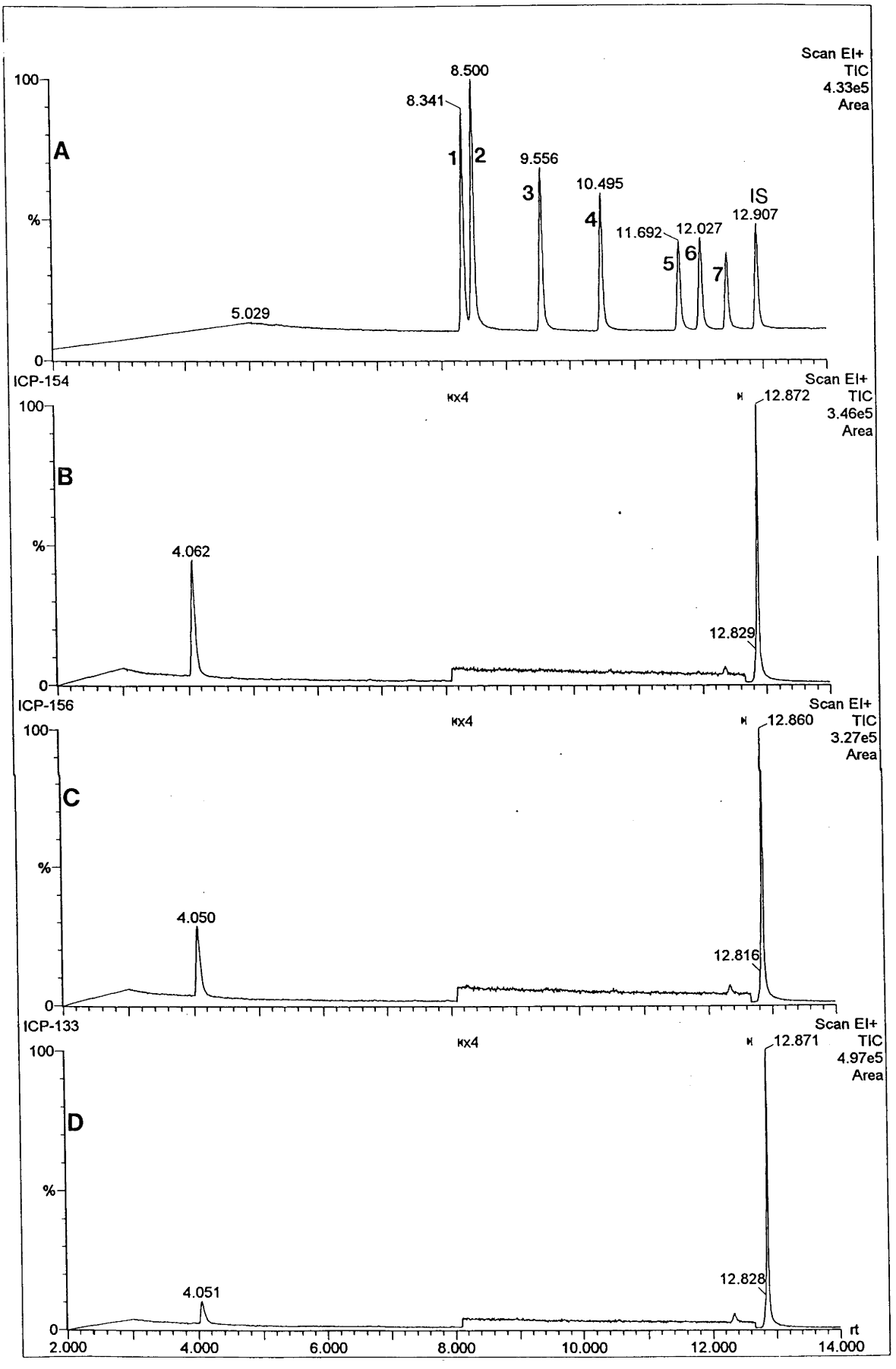

A
Scan EI+
TIC
4.33e5
Area
100
%
0
8.500
8.341
1
2
9.556
3
10.495
4
11.692
5
12.027
6
7
IS
12.907
5.029
ICP-154
Scan EI+
TIC
3.46e5
Area
12.872
B
4.062
12.829
ICP-156
Scan EI+
TIC
3.27e5
Area
12.860
C
4.050
12.816
ICP-133
Scan EI+
TIC
4.97e5
Area
12.871
D
4.051
12.828
rt
2.000
4.000
6.000
8.000
10.000
12.000
14.000

Table 5.3 Glucose released by endo-cellulase from different concentrations (nmol/ml) of β-1,4-glucan & starch *

	β-1,4-Glucan concentration						Starch concentration			
	1000	2000	2500	3000	3500	4500	2000	4000	6000	8000
1st experiment	779	1688	2090	2875	3433	3785	ND	ND	47	119
2st experiment	761	1712	2312	2545	3009	4045	ND	ND	73	95
Mean	770	1700	2201	2710	3221	3915	ND	ND	60	107
% of total glucose released	77	85	88	90	92	87	ND	ND	1	1.3

*Glucose content determined by GC/MS; β-1,4-glucan from the supernatant purified by 2% CTAB from the ammonium oxalate fraction of whole seedlings of *rsw1*; β-1,4-glucan and starch were diluted from a stock solution whose concentration was determined by GC/MS after TFA hydrolysis; enzyme digestion was at 37 °C for 48 h.

of total glucose was released from starch (6000 nmol/ml) by endo-cellulase digestion (Table 5.3). Moreover, incubating the 1,4-glucan samples with α-amylase and α-glucosidase at 37 °C for 48 h released only about 3% of total glucose (Figure 5.8, B and C; Table 5.4). A starch sample, however, was converted completely to glucose by those enzymes (Table 5.4).

To demonstrate the specificity of those enzymes, further carbohydrate standards (cellobiose, cello-pentaose, cello-oligosaccharides, cellulose, XG and laminarin) were used as substrates (Table 5.5). Interestingly, endo-cellulase, like β-glucosidase, was able to digest cellobiose completely, whereas the percentages of total glucose released from cello-pentaose and cello-oligosaccharides were less than the 92% released from the β-1,4-glucan digestion (Table 5.5). As expected, none of the standard substrates was significantly digested by α-amylase, α-glucosidase, or exo-β-1,3-glucanase. Laminarin was degraded into glucose only by exo-β-1,3-glucanase whereas no glucose was released from cellulose (completely insoluble in enzymic reaction solution) or from XG by any of the enzymes. Clearly, the endo-cellulase was specific for β-1,4-glucan digestion but could not degrade the crystalline cellulose standard.

In conclusion, the 1,4-glucan samples from all three fractions contain largely or entirely β-1,4-linkages.

5.3.1.4 Characteristics of β-1,4-glucan digestion

A further series of experiments were carried out to characterise the digestion of the β-1,4-glucan by endo-cellulase and other enzymes.

The percentage of glucose released from the β-1,4-glucan by endo-cellulase (0.125 U/ml) rose as the substrate concentration was increased (Table 5.3). On the other hand, different quantities of endo-cellulase (0.100, 0.125, 0.50, 1.00 U/ml) had little effect on the extent of β-1,4-glucan digestion (from 88% to 92%), when the optimum β-1,4-glucan concentration (3500 nmol/ml) was used (data not shown). In Table 5.4, the optimum incubation time for β-1,4-glucan

Table 5.4 The effects of time and enzyme specificity on the release of glucose from β-1,4-glucan & starch *

Enzyme	Glucose Released	β-1,4-Glucan						Starch			
		4h	8h	16h	32h	48h	72h	4h	16h	32h	48h
Endo-cellulase	nmol/ml	ND	ND	1577	2975	3221	3235	ND	ND	ND	53
	% of total	ND	ND	45	85	92	92	ND	ND	ND	1
α-Amylase & α-Glucosidase	nmol/ml	ND	ND	ND	ND	107	118	2251	4750	5071	5015
	% of total	ND	ND	ND	ND	3	3	45	95	101	100

* Glucose determined by GC/MS; β-1,4-glucan, 3500 nmol/ml; Starch, 5000 nmol/ml.

Table 5.5 Percentage of glucose released by different enzymes from various standard substrates *

	Cello-biose	Cello-pentaose	Cello-oligosaccharides	Cellulose	XG	Laminarin
Endo-cellulase	99	88	73	ND	<2	ND
β-Glucosidase	101	15	5	ND	<1	ND
α-Amylase	<1	ND	ND	ND	ND	ND
α-Glucosidase	<1	ND	ND	ND	ND	ND
Exo-β-1,3-Glucanase	ND	ND	ND	ND	ND	53

* Glucose determined by GC/MS; Enzymes at 37 °C for 48 h; Initial concentration of glucose in all substrate samples was adjusted to 3500 nmol/ml on the basis of GC/MS determination after TFA hydrolysis, or in the case of cellulose, on the basis of anthrone/H_2SO_4 determination.

Table 5.6 Comparison of the ability of different enzymes to digest β-1,4-glucan *

Enzymes	Glucose Released	
	nmol/ml	As % of Total Glucose
Endo-cellulase	3221	92
Endo-cellulase + β-Glucosidase	3225	92
β-Glucosidase	95	3
α-Amylase	16	<1
α-Amylase+ α-Glucosidase	20	<1
α-Glucosidase	12	<1
Exo-β-1,3-Glucanase	0	ND

* β-1,4-glucan concentration, 3500 nmol/ml; Enzyme digestion at 37 °C for 48 h;

digestion is investigated. The β-1,4-glucan was not completely hydrolysed by endo-cellulase even if incubated for 72 h. Supplementing the endo-cellulase with β-glucosidase did not increase glucose release, and β-glucosidase alone released only about 3% of the glucose (Table 5.6). A variety of other enzymes (α-amylase, α-amylase with α-glucosidase, α-glucosidase, exo-β-1,3-glucanase) were ineffective in releasing glucose (Table 5.6).

5.3.2 Glucan from *rsw2* and *rsw3* Shoots

The preceding results show that a β-1,4-glucan can be purified from the ammonium oxalate, 0.1 M KOH and 4 M KOH fractions prepared from the shoot of *rsw1* seedlings grown at 31°C. A less complete analysis was carried out on glucans purified from the shoots of *rsw2* and *rsw3* grown at 31°C. A mixture of the CTAB supernatant (50% glucose in the mixture based on the anthrone/H_2SO_4 test) and pellets prepared from the 0.1 M KOH (10% glucose) and 4 M KOH (40% glucose) fractions showed only glucose when hydrolysed by TFA and resolved by GC/MS (Figure 5.9), 1,4-linkages for the glucose when subject to methylation analysis (Figures 5.10 and 5.11; Table 5.7) and a β-configuration for the linkages based on a high yield of glucose released (more than 90%) by endo-cellulase digestion (data not shown; substrate concentration, 3500 nmol glucose/ml). Estimates of the DP of a mixture of the β-1,4-glucan from the three fractions made from the ratio of terminal and 4-linked glucose (Table 5.8) showed slightly higher values to those obtained from the *rsw1* glucan.

5.3.3 Attempted Purification of Glucan from *rsw5* and Wild Type

Levels of glucose in the ammonium oxalate and alkali fractions from wild type and *rsw5* grown at 31 °C were lower than in those from *rsw1*, *rsw2* and *rsw3* (Table 4.5). Purification of β-1,4-glucan using those fractions of whole

Figure 5.9

GC/MS chromatograms of AAs from:

A, Standard mixture of monosaccharides: Peaks 1, rhamnose; 2, fucose; 3, arabinose; 4, xylose; 5, mannose; 6, galactose; 7, glucose; IS, *myo*-inositol.

B and C, Sugars released by TFA hydrolysis of mixture of supernatant (50% of total glucose) purified by 2% CTAB from the ammonium oxalate fraction, and pellets purified by a centrifugation from the 0.1 M KOH fraction (10% of glucose) and 4 M KOH fraction (40% of glucose) of whole seedlings of *rsw2* (**B**) and *rsw3* (**C**) grown at 31 °C.

D, Control: Sugars of the mixture of supernatant and pellets of *rsw2* without TFA hydrolysis.

The regions (in B, C, D) between the arrowheads were amplified by four.

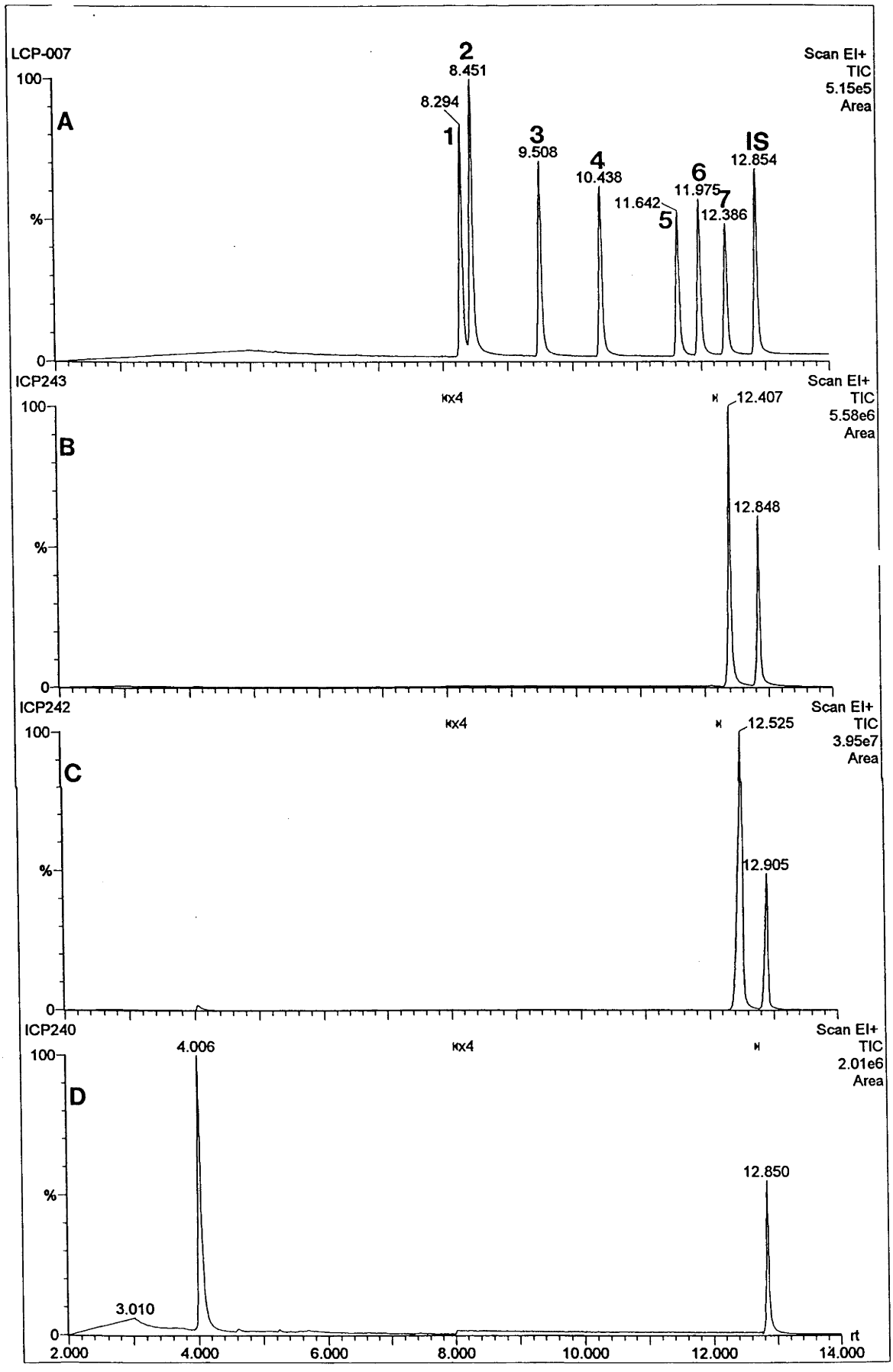

LCP-007
Scan EI+
TIC
5.15e5
Area
A
100
%
0
2
8.451
8.294
1
3
9.508
4
10.438
11.642
5
6
11.975
7
12.386
IS
12.854
ICP243
Scan EI+
TIC
5.58e6
Area
B
x4
12.407
12.848
ICP242
Scan EI+
TIC
3.95e7
Area
C
x4
12.525
12.905
ICP240
Scan EI+
TIC
2.01e6
Area
D
x4
4.006
12.850
3.010
rt
2.000
4.000
6.000
8.000
10.000
12.000
14.000

Figure 5.10

GC/MS chromatograms of PMAAs from:

A, Glucose standard.

B and **C**, Methylation analysis of the mixture of glucans purified from the ammonium oxalate fraction (50% of total glucose), 0.1 M KOH fraction (10% of glucose) and 4 M KOH fraction (40% of glucose) of whole seedlings of *rsw2* (**B**) and *rsw3* (**C**) grown at 31 °C.

D, Control: Methylation analysis of blank.

Peak **1**: 2,3,4,6-Me_4 Glc; Peak **5**: 2,3,6-Me_3 Glc;

Peaks **a** and **b**: non-carbohydrates; **IS**: Internal Standard (*myo*-inositol).

Peaks were identified on the basis of RRT (Table 5.7) and mass spectra (Figure 5.11).

The regions (in B, C and D) between the arrowheads were amplified by four.

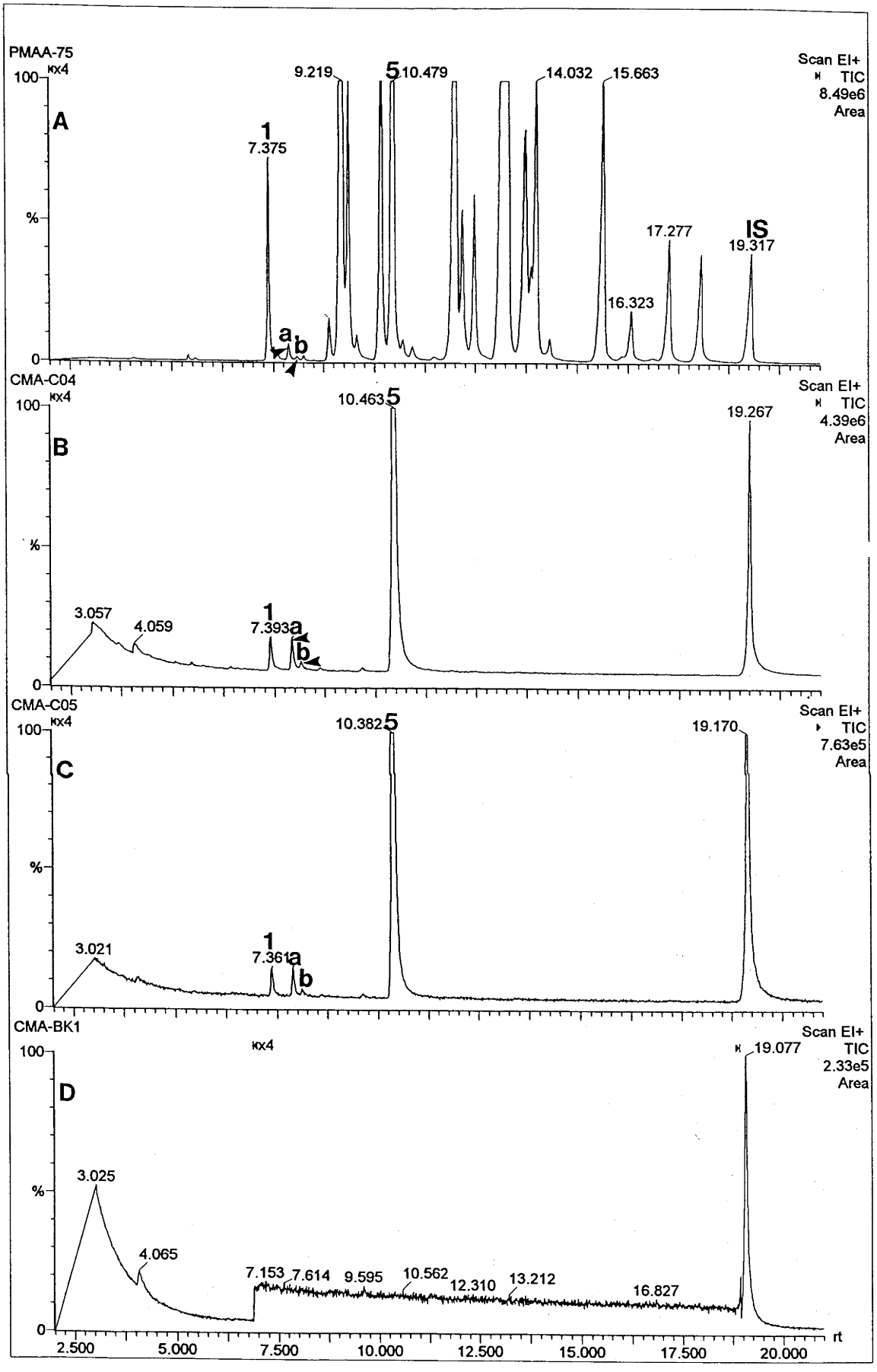
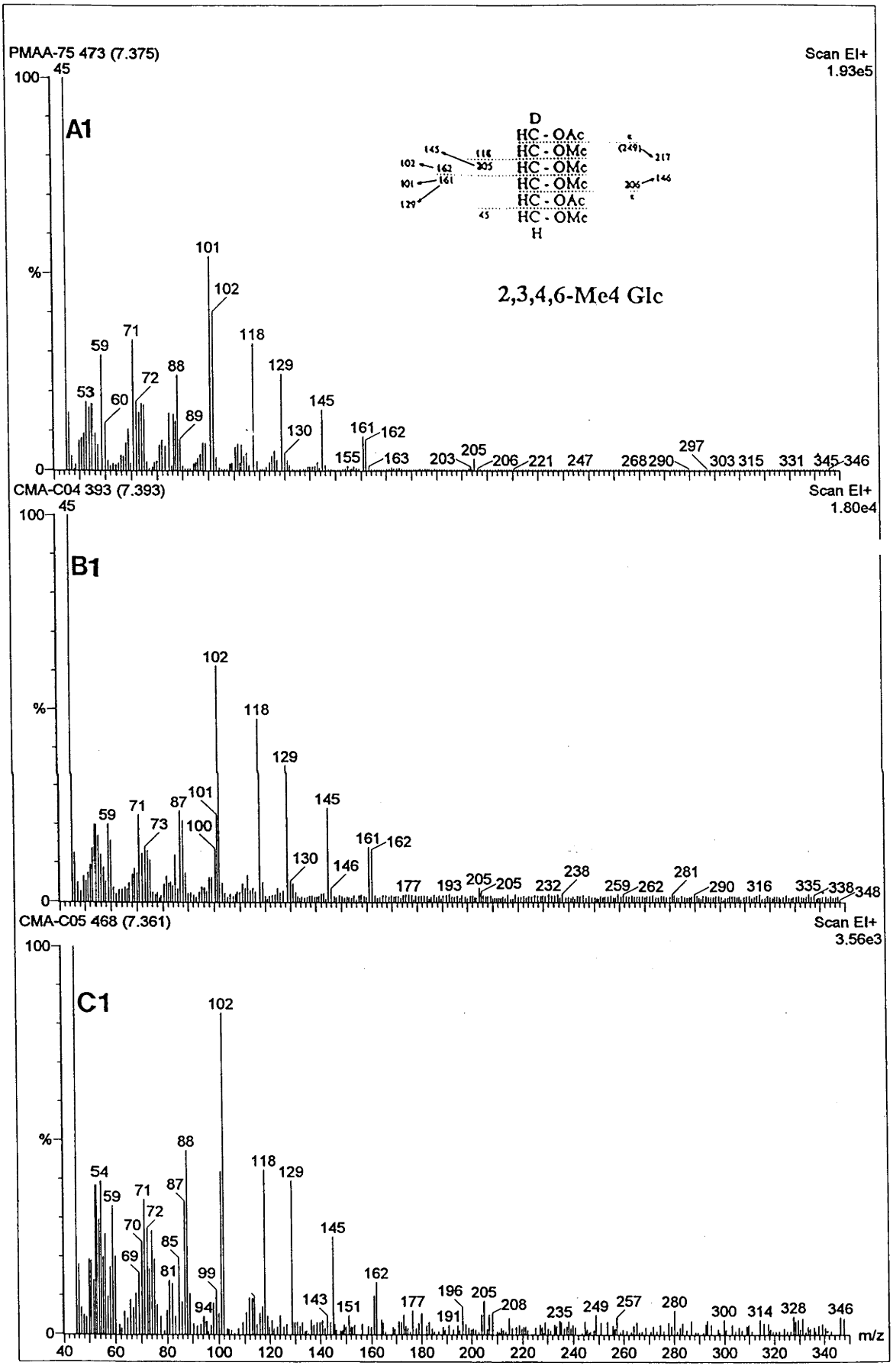
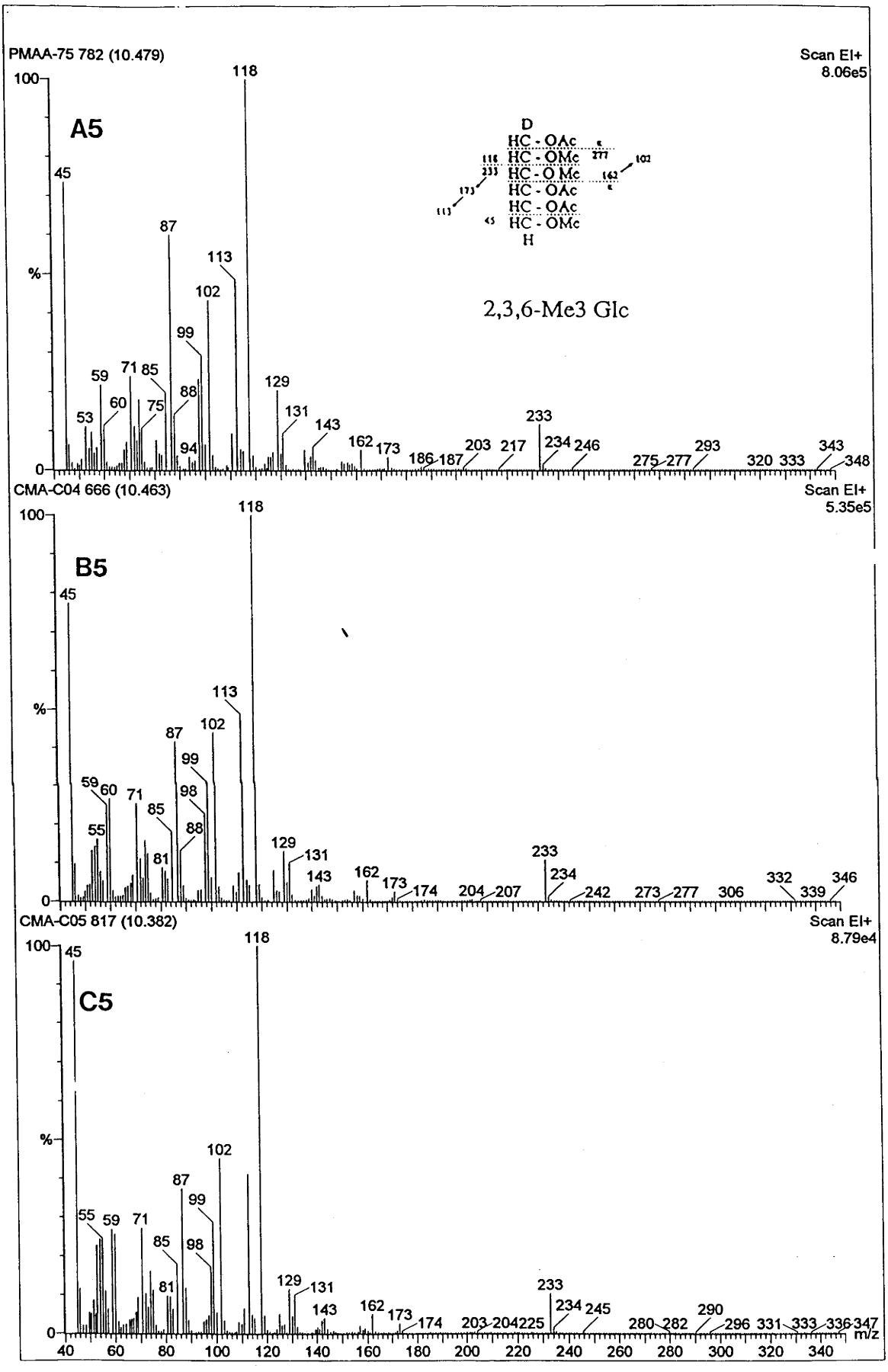
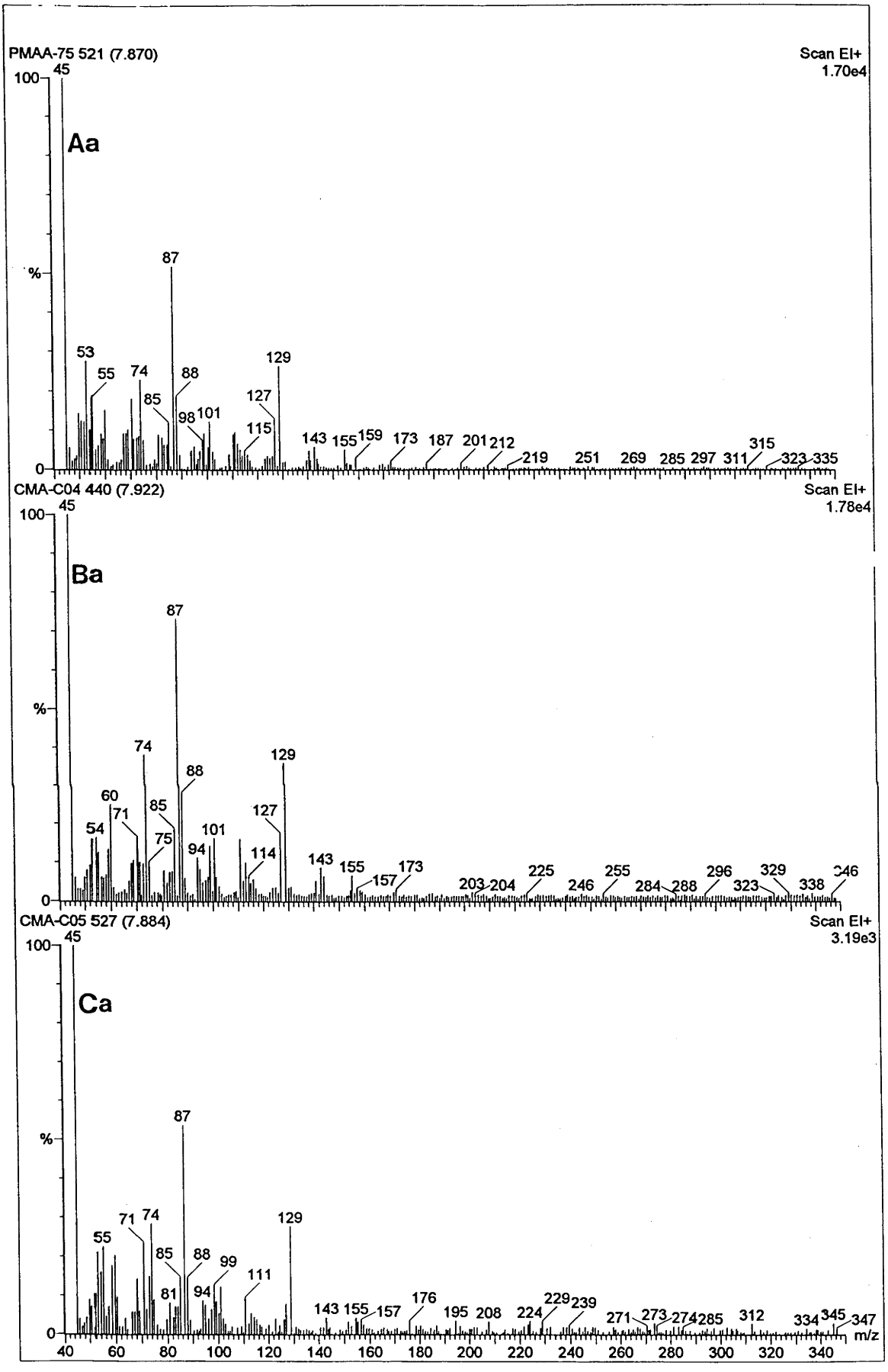

PMAA-75
100
kx4
A
9.219
5
10.479
14.032
15.663
Scan EI+
TIC
8.49e6
Area
1
7.375
a
b
17.277
IS
19.317
16.323
%
0
CMA-C04
100
kx4
B
10.463
5
19.267
Scan EI+
TIC
4.39e6
Area
3.057
4.059
1
7.393
a
b
%
0
CMA-C05
100
kx4
C
10.382
5
19.170
Scan EI+
TIC
7.63e5
Area
3.021
1
7.361
a
b
%
0
CMA-BK1
100
kx4
D
19.077
Scan EI+
TIC
2.33e5
Area
3.025
4.065
7.153
7.614
9.595
10.562
12.310
13.212
16.827
%
0
2.500
5.000
7.500
10.000
12.500
15.000
17.500
20.000
rt

Figure 5.11

Mass spectra of each labelled peak in GC/MS chromatograms (Figure 5.10). The deduced glycosyl linkage of each labelled peak are summarised in Table 5.7.


PMAA-75 473 (7.375)
Scan EI+
1.93e5
A1
D
HC - OAc
HC - OMe
HC - OMe
HC - OMe
HC - OAc
HC - OMe
H
2,3,4,6-Me4 Glc
CMA-C04 393 (7.393)
Scan EI+
1.80e4
B1
CMA-C05 468 (7.361)
Scan EI+
3.56e3
C1
%
m/z


PMAA-75 782 (10.479)
Scan EI+
8.06e5
A5
2,3,6-Me3 Glc
CMA-C04 666 (10.463)
Scan EI+
5.35e5
B5
CMA-C05 817 (10.382)
Scan EI+
8.79e4
C5
m/z


PMAA-75 521 (7.870)
Scan EI+
1.70e4
Aa
CMA-C04 440 (7.922)
Scan EI+
1.78e4
Ba
CMA-C05 527 (7.884)
Scan EI+
3.19e3
Ca
%
m/z

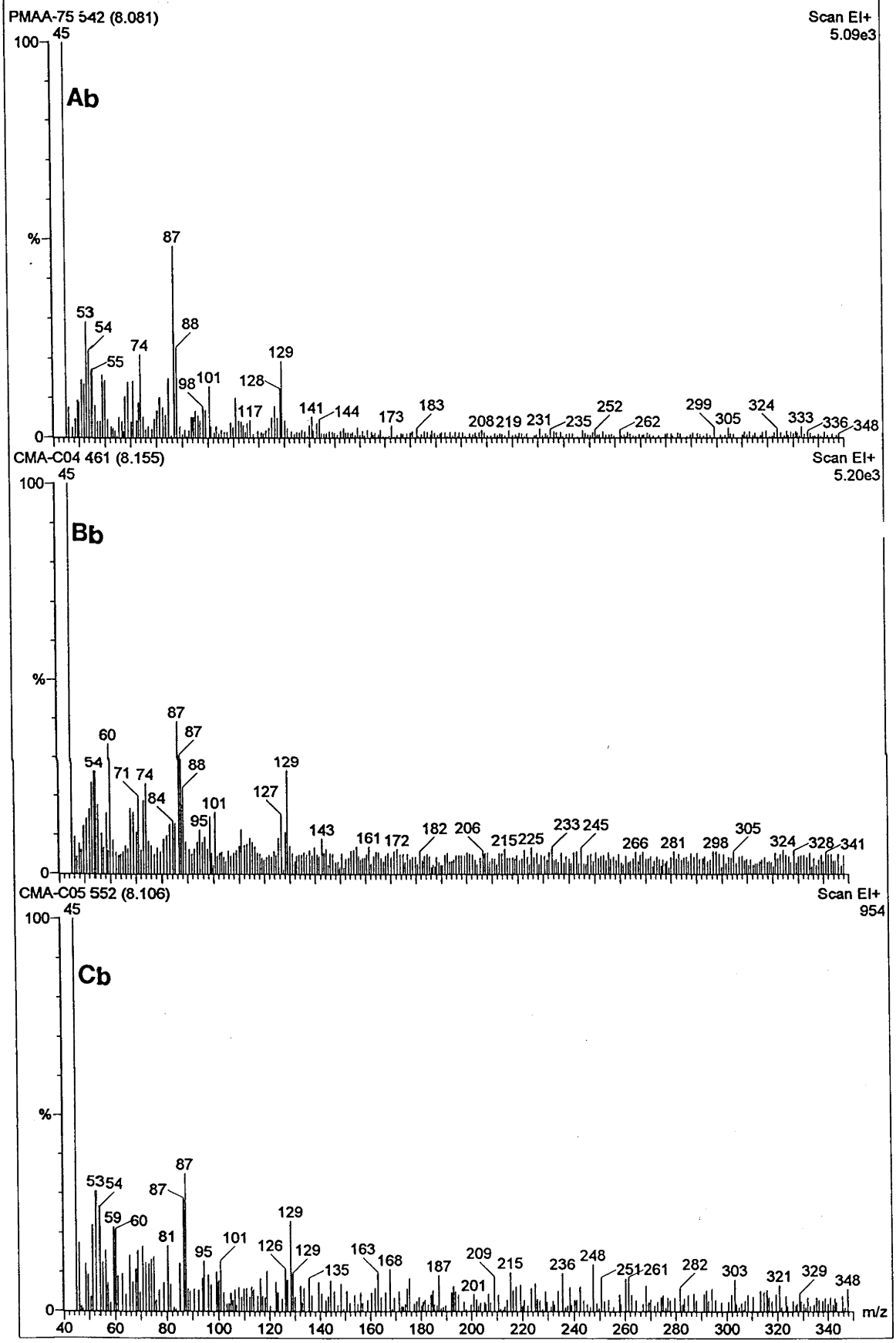

PMAA-75 542 (8.081)
Scan EI+
5.09e3
Ab
CMA-C04 461 (8.155)
Scan EI+
5.20e3
Bb
CMA-C05 552 (8.106)
Scan EI+
954
Cb
100
%
0
m/z
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340

Table 5.7 Comparison of RRT of PMAAs derived from methylation analysis of the mixture of glucans purified from the ammonium oxalate fractions, the 0.1 M KOH andthe 4 M KOH fractions of whole seedlings of *rsw2* (B) and *rsw3* (C) grown at 31 °C with the glucose standard (A)

(Chromatograms in Figure 5.10 and MS in Figure 5.11)

Peak	Derivative	Deduced Linkage	RRT-1 *	RRT-2
A1	**2,3,4,6-Me_4 Glc**	**t-Glc*p***	**0.382**	**1**
B1	2,3,4,6-Me_4 Glc	t-Glc*p*	0.384	1
C1	2,3,4,6-Me_4 Glc	t-Glc*p*	0.384	1
A5	**2,3,6-Me_3 Glc**	**4-Glc*p***	**0.542**	**1.42**
B5	2,3,6-Me_3 Glc	4-Glc*p*	0.543	1.42
C5	2,3,6-Me_3 Glc	4-Glc*p*	0.542	1.41
Aa	**Non-carbohydrates**	**-**	**0.407**	**1.07**
Ba	Non-carbohydrates	-	0.411	1.07
Ca	Non-carbohydrates	-	0.413	1.07
Ab	**Non-carbohydrates**	**-**	0.418	1.10
Bb	Non-carbohydrates	-	0.423	1.10
Cb	Non-carbohydrates	-	0.423	1.10

*, RRT-1: Relative retention time to *myo*-inositol hexaacetate;

RRT-2: To t-Glc*p*.

Table 5.8 Estimation by methylation analysis of the DP of the mixture of the 1,4-glucan purified from the ammonium oxalate fraction (50%), 0.1 M KOH (10%) and 4 M KOH fraction (40%) of whole seedlings of *rsw2* and *rsw3* grown at 31 °C *

Samples	Ratio of 4-Glc*p* to t-Glc*p* in Peak Area	DP	MW (Da)
rsw2	37.5	75	12168
rsw3	32.3	65	10548

*GC/MS chromatograms for the PMAAs in Figure 5.10.

seedlings from wild type and *rsw5* was attempted using similar amounts (glucose concentration, 3500 nmol/ml) of samples to those used for *rsw1*, *rsw2* and *rsw3*. No glucose (or other monosaccharide) was detected in the supernatant purified by CTAB precipitation of the pectins from the ammonium oxalate fraction, and no pellets formed on centrifugation (14, 000 *g*, for 1 h) of dialysed alkali fractions. The glucose present in those fractions is therefore not in the form of a glucan that can be recovered by the methods that were successful with *rsw1*, *rsw2* and *rsw3*.

5.3.4 Release of Glucose by Endocellulase

A glucan could not be purified from the ammonium oxalate and alkali fractions of *rsw5* and wild type but the methylation analysis reported in Sections 3.3.4 and 3.3.6 for wild type and to be reported in Chapter 6 for the mutants showed 4-linked glucose was the dominant form of glucose in the ammonium oxalate fraction and present in the alkali fractions. Therefore, the release of glucose by endocellulase digestion of the ammonium oxalate and alkali fractions was investigated to learn more about the form of that glucose.

Significant quantities of glucose can be released by endocellulase action on the whole fractions from wild type, *rsw1* and *rsw5* (Table 5.9). All therefore contain β-1,4 linkages that can be hydrolysed by this specific enzyme (Section 5.3.1.3). (The individual fractions from *rsw2* and *rsw3* were not tested.) However, wild type and *rsw5* differ from *rsw1* in an important way. The amounts of glucose released by endocellulase digestion of the ammonium oxalate and alkali fractions of *rsw1* are comparable to the yields of glucan that can be purified from those fractions and no further glucose is released from those *rsw1* fractions after glucan has been removed (Table 5.9; ammonium oxalate pellet and KOH supernatant). In contrast, whereas a glucan cannot be purified from wild type and *rsw5*, endocellulase will release glucose from their

Table 5.9 Glucose released by TFA hydrolysis and endo-cellulase digestion from the ammonium oxalate fraction and its supernatant and pellet subfractions and from the 4 M KOH fractions and its supernatant and pellet subfractions in wild type, *rsw5* and *rsw1* grown at 31 °C *

Fractions / Subfractions	Glucose (nmol/mg dry weight)					
	Wild Type		*rsw5*		*rsw1*	
	TFA	Enzyme	TFA	Enzyme	TFA	Enzyme
Ammonium Oxalate Fraction	**80**	**58**	**54**	**42**	**221**	**192**
Supernatant	ND	ND	ND	ND	195	180
Pellet	78	56	51	40	26	ND
4 M KOH Fraction	**52**	**20**	**59**	**19**	**93**	**54**
Supernatant	51	19	57	19	37	ND
Pellet	ND	ND	ND	ND	56	51

* Substrate concentration, 3500 nmol glucose/ml

CTAB pellets and from their alkali supernatants. They therefore also contain β-1,4-linked glucose but the methods that purify a glucan from *rsw1, rsw2* and *rsw3* do not separate it from the pectins and hemicelluloses in the two fractions respectively.

5.3.5 Glucose in the Acid-Soluble Fraction

Analysis of the acid-soluble material in wild type (Section 3.3.7) demonstrated that 4-linked glucose could be detected but the identities of the polymer(s) containing it were not determined. The quantities of glucose found in the acid soluble fraction prepared from mutants and wild type are shown in Table 5.10. In contrast to the increased glucose seen in the ammonium oxalate and alkali fractions from *rsw1*, *rsw2* and *rsw3*, the amount of glucose recovered in the acid-soluble fraction did not increase to high levels in any of the mutants. Endo-cellulase digestion was able to release about 50% of total glucose in this fraction.

5.3.6 Glucose in the Phosphate Buffer Fraction

Although significant amounts of pectic polysaccharides were also extracted with the phosphate buffer fraction in wild type and all mutants, no β-1,4-glucan was found in this fraction by the cellulase digestion test (Section 3.3.1 for wild type, data for *rsw1, rsw2, rsw3* and *rsw5* not shown).

5.3.7 Quantitation of Glucan and β-1,4-Linked Glucose in Shoots of Mutants and Wild Type

Table 5.10 summarises the data on the quantities of β-1,4-glucan that can be purified from *rsw1*, *rsw2* and *rsw3* together with the quantities of glucose that can be released from the various fractions by endocellulase digestion. The distribution of the glucan between the ammonium oxalate and alkali fractions is expressed on a percentage basis for *rsw1*, *rsw2* and *rsw3* in Table 5.11.

Table 5.10 Glucose (nmol/mg dry weight) released by TFA (**T**) and endo-cellulase (**E**) from fractions of shoots in wild type and *rsw5* and glucose released from fractions and β-1,4-glucan of shoots in *rsw1, rsw2* and *rsw3* grown at 31 °C *

Fractions	Wild Type				*rsw5*				*rsw1*				*rsw2*				*rsw3*			
	Whole Fraction		β-1,4-Glucan		Whole Fraction		β-1,4-Glucan		Whole Fraction		β-1,4-Glucan		Whole Fraction		β-1,4-Glucan		Whole Fraction		β-1,4-Glucan	
	T	**E**	**T**	**E**	**T**	**E**	**T**	**E**	**T**	**E**	**T**	**E**	**T**	**E**	**T**	**E**	**T**	**E**	**T**	**E**
Ammonium Oxalate	80	58	ND	ND	54	42	ND	ND	221	192	195	180	184	NA	147	NA	155	NA	132	NA
0.1 M KOH	7	4	ND	ND	16	11	ND	ND	21	12	14	12	28	NA	18	NA	52	NA	26	NA
4 M KOH	52	20	ND	ND	59	19	ND	ND	93	54	56	51	122	NA	61	NA	130	NA	73	NA
Acid-soluble	2	1	ND	ND	2	1	ND	ND	2	1	ND	ND	4	NA	ND	ND	4	ND	ND	ND
Total	141	83	ND	ND	131	73	ND	ND	337	259	265	243	338	NA	226	NA	341	NA	231	NA

* The glucose determined by GC/MS after TFA (T) hydrolysis or endo-cellulase (E) digestion. The β-1,4-glucan was purified in the supernatant subfraction by CTAB from the ammonium oxalate fraction of mutants (*rsw1, rsw2* and *rsw3*), and recovered in the pellet of 0.1 and 4 M KOH fractions by centrifugation; ND, not detected; NA, data not available (experiments were not done); Substrate concentration, 3500 nmol glucose/ml

Table 5.11 Percentage of β-1,4-Glucan in various fractions prepared from shoots of *rsw1*, *rsw2* and *rsw3* grown at 31 °C *

Fractions	*rsw1*	*rsw2*	*rsw3*
Ammonium Oxalate	74	65	57
0.1 M KOH	5	8	11
4 M KOH	21	27	32
Total	100	100	100

* The β-1,4-glucan determined by GC/MS after TFA hydrolysis, was purified in the neutral polymer fraction by CTAB from the ammonium oxalate fraction of mutants (*rsw1*, *rsw2* and *rsw3*), and recovered in the pellet of the 0.1 and 4 M KOH fractions by centrifugation. The glucose content of β-1,4-glucan from Table 5.10.

Table 5.12 Comparison of total production of β-1,4-glucan (crystalline and non-crystalline celluloses) from shoots of *rsw1, rsw2* and *rsw 3* with the crystalline cellulose alone from shoots of wild type and *rsw5* *

Materials	nmol glucose/mg dry weight		Rw
	21 °C	31 °C	31 : 21 °C
Wild Type	273	363	1.3
rsw5	200	180	0.9
rsw1	242	424	1.7
rsw2	250	365	1.5
rsw3	290	412	1.4

* The total glucose was the sum of glucose from cellulose in the acid-insoluble fraction (Table 4.4) and from the β-1,4-glucan recovered from four fractions (ammonium oxalate, 0.1 M KOH, 4 M KOH and acid-soluble; Table 5.10); **Cellulose** determined by anthrone/H_2SO_4 test; **β-1,4-Glucan** (non-crystalline cellulose) determined by GC/MS; Rw, total β-1,4-glucan in 31 °C plants : total in 21 °C plants.

The production of β-1,4-glucan (crystalline and non-crystalline cellulose) is compared for wild type and the different mutants in Table 5.12.

5.3.8 β-1,4-Glucan and 4-Linked Glucose in the Root

Substantial amounts of β-1,4-glucan were therefore found in the ammonium oxalate and alkali fractions from shoots of *rsw1*, *rsw2* and *rsw3* and endocellulase-releasable glucose was found in the shoots of wild type and mutant. It was shown in the previous chapter (Table 4.6) that the amounts of glucose in those fractions from mutant and wild type roots were much lower than the amounts in the corresponding fractions from shoots (Table 4.5). No glucose was released by endocellulase digestion of ammonium oxalate, 0.1 M KOH, and 4 M KOH fractions prepared from the roots of all mutants and wild type, whether grown at 21 °C or 31 °C. The only glucose released by endocellulase from fractions prepared from roots was extremely small amounts in the acid-soluble fraction (see Section 3.3.7 for wild type).

5.4 DISCUSSION

The increased glucose present in the ammonium oxalate, 0.1 M KOH and 4 M KOH fractions from shoots of *rsw1, rsw2* and *rsw3* has been shown to result from the presence of a β-1,4-glucan that can be purified. The evidence for its identification will be assessed, its properties *in vitro* and in the wall considered and its biosynthesis discussed. The state of glucose in the comparable fractions from *rsw5* and wild type will then be discussed in the light of the presence of glucose that could be released by endocellulase digestion but where it proved impossible to purify a glucan using the methods that were successful with *rsw1*, *rsw2* and *rsw3*.

5.4.1 β-1,4-Glucan from the Shoots of *rsw1*, *rsw2* and *rsw3*

5.4.1.1 Purification

It has been shown that a β-1,4-glucan can be purified from the CTAB supernatant of the ammonium oxalate fraction and as pellets from the 0.1 and 4 M KOH fractions of the cell wall of *rsw1, rsw2* and *rsw3* seedlings grown at 31 °C (Section 5.3). Identification as a β-1,4-glucan rests on three main findings: monosaccharide composition indicating a homo-glucose polymer without any other monosaccharides; methylation analysis demonstrating only 1,4-linkages; digestion by various specific enzymes, particularly endo-cellulase, confirming a predominant, perhaps exclusive β-configuration for the 1,4-linkages. Those analyses used for the β-1,4-glucan identification may not, however, identify its complete molecular structure. Only monosaccharides have been analysed leaving open the issue of whether the β-1,4-glucan is associated with other molecules such as proteins or lipids.

5.4.1.2 Physical and Chemical Characteristics of β-1,4-Glucan

Because of its crystalline structure, cellulose is hardly hydrolysed to glucose by 2 M TFA at 120 °C for 1 h (Fry, 1987). Although crystalline cellulose can be attacked by some cellulases (Wood, 1991, Shoemaker et al, 1998), it was not detectably hydrolysed into glucose by the endo-cellulase used in this study. The β-1,4-glucan purified from the ammonium oxalate and alkali fractions was, however, efficiently converted to glucose both by TFA and by endo-cellulase, suggesting it is likely to be non-crystalline, or poorly-crystallised cellulose. However, the β-1,4-glucan was purified by CTAB from the ammonium oxalate fraction and the β-1,4-glucan in the KOH fractions could be precipitated by centrifugation (14, 000 *g* for 1 h; Section 4.3.1) showing that the β-1,4-glucan probably does undergo self-association once extracted.

5.4.1.3 β-1,4-Glucan May Interact with Wall Polysaccharides

The β-1,4-glucan in the ammonium oxalate fraction extracts with pectins but does not associate tightly with them (Section 1.4.1) because it is readily separated from them by CTAB. It can then be collected as a pellet by centrifugation. According to preliminary results, however, the β-1,4-glucan could not be completely precipitated from the crude ammonium oxalate fraction by a similar centrifugation and the pellet formed contained small amounts of pectins. This may indicate that the pectins associate to some extent with the β-1,4-glucan and interrupt its self-crystallisation and therefore sedimentation. Many alternatives are possible, however, including that some other component is purified away from the glucan by the CTAB and/or ethanol used in separating the pectins.

The 4 M KOH used in fractionation dissociates hydrogen bonds (Fry, 1988). The β-1,4-glucan that remained in the wall after ammonium oxalate extraction, and which extracted into the 4 M KOH fraction, may therefore have been bound by hydrogen bonds. Binding could be to XG, to cellulose, or to itself

in the wall (Levy et al, 1991; Section 1.4.1). Dialysis to remove KOH might then cause self-association of the β-1,4-glucan which would form the pellet after centrifugation. Separation of the β-1,4-glucan from XG by centrifugation points to the glucan having different physical and chemical properties from the XG which is still soluble in neutral media.

Association of the β-1,4-glucan with pectins and XG may therefore play an important role in maintaining the β-1,4-glucan as non-crystalline, or poor-crystalline structure in the cell wall but, if those associations exist, they are readily overcome *in vitro*.

5.4.1.4 β-1,4-Glucan May be Linked with Other Compounds

The β-1,4-glucan purified from wall fractions could be linked to other compounds such as proteins, lipids or a range of small molecules. Such complexes have been suggested to play an important roles in initiating β-1,4-glucan formation in bacterial cellulose biosynthesis (review by Atalla, 1998) or in arranging β-1,4-glucan to form crystalline cellulose (Brett and Waldron, 1990). Such linkages might account for incomplete digestion of the β-1,4-glucan by endo-cellulase, although other explanations, such as its partial self-crystallisation inhibiting the digestion, are possible. Compounds linked to the β-1,4-glucan may hinder the complete crystallisation of the β-1,4-glucan even if it is fully purified. Furthermore, if the linked compounds were soluble in neutral media, this may explain why the β-1,4-glucan could be left in the supernatant when pectins precipitated by CTAB were collected from the ammonium oxalate fraction by low speed centrifugation. That the β-1,4-glucan could be precipitated by higher speed centrifugation might be due to partial self-crystallisation of the β-1,4-glucan once purified (Section 4.4.2). This might perhaps be helped by the denaturing of linked compounds by CTAB or ethanol during purification.

The estimates of DP are lower than those for crystalline cellulose obtained by the same methods (Section 3.3.8; Table 3.11) but still high enough to cause

self-association (Tonnesen and Ellefsen, 1971). Any linkages at the non-reducing terminus would lead to overestimation of the DP.

5.4.2 Formation of the β-1,4-Glucan in *rsw1*, *rsw2* and *rsw3*

One or more glucosyltransferases in the Golgi apparatus are involved in synthesising the β-1,4-glucan backbone of XG. A relatively unbranched β-1,4-glucan and XG products can be produced *in vitro* by a Golgi membrane preparation with addition of UDP-Glc and UDP-Xyl (Campbell and Hillman, 1988; Hayashi et al, 1988; Section 1.5.3). There is, however, no direct evidence that β-1,4-glucan (non-crystalline cellulose) can be formed *in vivo* by the Golgi apparatus. Because the β-1,4-glucan is abundant in mutants which are defective in cellulose production, it seems more likely that the non-crystalline β-1,4-glucan is made at the cell surface by cellulose synthase and fails to crystallise into microfibrils for some reason.

Cellulose I is produced in plants by cellulose synthase complexes *in vivo* (reviewed by Haigler, 1987). The formation of non-crystalline cellulose in mutants showing reduced cellulose suggests that the coupling of β-1,4-glucan polymerisation and crystallisation is disrupted in mutants at high temperature. This is different from the behaviour seen in preparations for cellulose biosynthesis *in vitro* where the integrity of the transmembrane enzyme complex is apparently damaged so that callose is the major product with limited yields of cellulose (Li et al, 1993; Delmer et al, 1993; Brown et al, 1994).

5.4.3 A β-1,4-Glucan Can not be Purified from Wild Type and *rsw5*

Substantial amounts of glucose occur in the ammonium oxalate and alkali fractions from wild type and *rsw5*. This is predominantly 4-linked in the ammonium oxalate fraction and a significant proportion of the glucose in all fractions is released by endocellulase digestion. In *rsw1*, all the endocellulase-releasable glucose was recovered as glucan from the ammonium oxalate and

alkali fractions. Significant amounts of glucose remained in the CTAB pellet and the alkali fraction supernatants but none was released by the enzyme. In contrast, none of the endocellulase-releasable glucose was purified as a glucan from *rsw5* or wild type and endocellulase still released part of the glucose from the CTAB pellet and alkali supernatants. The reasons for these differences are completely unclear but they imply that the endocellulase releasable glucose in wild type and *rsw5* is much more strongly associated, perhaps by covalent linkages, with the pectins and hemicellulases than is the case in *rsw1, rsw2* and *rsw3*. The next chapter will contain further discussion of the possible form of this glucose.

5.4.4 Root and Shoot Differences

Glucose makes up a smaller proportion of the monosaccharides found in the ammonium oxalate and alkali fractions prepared from roots than it does in the fractions from shoots. Moreover, none of that glucose is released by endocellulase, In this it resembles that proportion of the total glucose in the corresponding fractions from shoots which cannot be released by endocellulase. This will be explored further in the next chapter.

5.5 SUMMARY

A glucan has been purified in the supernatant from the CTAB-precipitated ammonium oxalate fraction, and in pellets from the 0.1 M KOH and 4 M KOH fractions of the cell wall in shoots of *rsw1*, *rsw2* and *rsw3* grown at 31 °C. Methylation analysis and specific enzymic digestion demonstrated that the glucan had only β-1,4-linkages. Moreover, it appears to be a non-crystalline (or poorly crystalline) cellulose with relatively high DP and an ability to self-associate in vitro that is probably not fully seen in vivo. Many questions regarding its possible linkage to other compounds and its solubility properties remain to be resolved in future studies. Its accumulation in cellulose-defective mutants suggests that it is probably made by cellulose synthase rather than by a Golgi enzyme but direct evidence is not available. Judged by enzyme digestion, substantial amounts of β-1,4-linked glucose may also exist in the shoots of wild type and *rsw5* grown at 31 °C but a glucan cannot be purified by the methods that work with *rsw1*, *rsw2* and *rsw3*. The exact form of the glucose and the reason why it cannot be purified remain to be determined.

CHAPTER SIX

ANALYSIS OF NON-CELLULOSIC POLYSACCHARIDES

6.1 INTRODUCTION

Pectins and hemicelluloses are the main non-cellulosic polysaccharides in the cell wall (Section 1.1). These two major polysaccharide classes differ greatly in monosaccharide composition, which determines their behaviour in the cell wall (Duffus and Duffus, 1984; Bacic et al, 1988; Fry, 1988).

The nature, properties and biosynthesis of the main matrix polysaccharides were described in Chapter One. It was also pointed out that cellulose and non-cellulosic polysaccharides (pectins and hemicelluloses) are synthesised in quite different locations in the cell and very little is known about how these polysaccharides combine to form the cell wall. Several radial swelling mutants have been shown (Chapter Four) to have much less cellulose when grown at 31 °C and, in the case of three mutants, to produce excess of a β-1,4-glucan that extracts readily from the walls of shoot tissue (Chapter Five). The β-1,4-glucan, however, was not found in root tissue. Those results show that the *RSW1*, *RSW2*, *RSW3* and *RSW5* genes are required for normal cellulose production but they leave open the possibility that they also affect the production of other polysaccharides either directly or indirectly as a consequence of less cellulose being produced. The mutations are in different genes each of which may, at one extreme, have completely different functions in cellulose synthesis or, at the other extreme, each of which may be members of a multigene family encoding catalytic subunits related to the already cloned *RSW1* (Arioli et al, 1998a). It cannot therefore be assumed that all mutants show identical carbohydrate compositions. In this Chapter, I investigate the composition of pectins and hemicelluloses and the impact of changes in cellulose biosynthesis on their deposition in the wall. Outside the wall, the question of whether reduced cellulose production affects starch biosynthesis is briefly analysed.

6.2 MATERIALS AND METHODS

The growth and extraction of plant material was described in Chapters Three, Four and Five. Monosaccharide analyses were conducted on the batches of about 150 seedlings that were separated into roots and shoots (Chapter Four) while methylation analysis was conducted on fractions prepared from about 5000 whole seedlings as described in Chapter Five. The experimental methods used for determination of monosaccharide composition and methylation analysis by GC/MS were described in Chapters Two and Three. Methods of enzyme digestion and β-1,4-glucan purification were described in Chapter Five.

6.3 RESULTS

6.3.1 Pectic Polysaccharides

As described in Chapter Three for wild type grown at 21°C (Section 3.3.4), most pectic polysaccharides were extracted with ammonium oxalate. The monosaccharide composition of the ammonium oxalate fraction derived from the wild type and mutants grown at 21 °C or 31 °C was determined (Table 6.1). The composition is broadly similar for all genotypes and temperatures except for the wide variations in glucose content noted in Section 4.3.3. The variations were shown to result in the most extreme cases from the presence of a β-1,4-glucan in the fractions from shoots of *rsw1*, *rsw2* and *rsw3* grown at 31 °C (Chapter Five). A glucan could not be purified by comparable methods from the shoots of wild type and *rsw5* grown at 31 °C but part of the glucose they contained was released by endo-cellulase digestion (Section 5.3.3) as was part of the glucose in the shoots of all genotypes grown at 21 °C. No glucose was released by endocellulase from ammonium oxalate extracts prepared from roots (Section 5.3.8 for 31 °C; data not shown for 21 °C). The second of the columns under glucose in Table 6.1 ("non-releasable glucose") shows the quantities of glucose that could not be released by endo-cellulase digestion or purified as glucan in the case of *rsw1*, *rsw2* and *rsw3* shoots. When the proportions of different monosaccharides are calculated using non-releasable glucose rather than total glucose (Figures 6.1 and 6.2), there is only limited compositional variation between genotypes and temperatures. Uronic acids are the dominant component and comigrate with galacturonic acid on paper chromatography (data not shown). The proportion of uronic acids was slightly higher in shoots than in roots whereas the percentage of arabinose was consistently higher in roots.

Methylation analysis of the CTAB pellet from whole wild type seedlings grown at 21 °C revealed a glycosidic linkage pattern typical of pectins from

Table 6.1 Monosaccharides (nmol/mg dry weight seedlings) from the Ammonium Oxalate Fraction [a]

COMPONENT		Uronic acid	Glucose		Galactose	Mannose	Xylose	Arabinose	Rhamnose	Fucose	Total [d]
WT roots	21°[e]	144	18[b]	18[c]	18	3	16	34	12	4	249
	31°	139	16	16	20	3	16	42	14	4	254
WT shoots	21°	192	42	21	12	1	9	17	15	2	269
	31°	241	80	22	16	1	8	31	17	2	338
rsw1 roots	21°	145	13	13	17	1	13	21	9	4	223
	31°	177	26	26	23	3	24	44	18	5	320
rsw1 shoots	21°	179	47	23	14	1	9	19	14	1	260
	31°	242	221	26	18	2	12	27	14	1	342
rsw2 roots	21°	147	14	14	16	2	12	30	11	3	235
	31°	107	19	19	15	2	11	29	11	3	197
rsw2 shoots	21°	204	20	10	10	1	6	15	11	1	258
	31°	239	184	26	25	2	13	38	22	2	367
rsw3 roots	21°	146	14	14	18	2	14	38	15	4	251
	31°	153	25	25	17	3	11	30	11	3	253
rsw3 shoots	21°	184	29	14	13	1	7	18	13	1	251
	31°	196	155	24	17	2	7	17	11	1	275
rsw5 roots	21°	142	10	10	17	3	17	40	14	5	248
	31°	126	12	12	18	2	16	44	18	4	240
rsw5 shoots	21°	230	33	14	10	1	10	23	19	2	309
	31°	204	54	23	6	1	6	21	12	1	274

[a] Uronic acids were determined by the *m*-hydroxybiphenyl-H_2SO_4 test; other monosaccharides by GC/M[illegible] after TFA hydrolysis; [b] Total glucose from Table 4.5;
[c] Non-releasable glucose, comprising glucose not released by endo-cellulase or, in the case of shoots of *rsw1, rsw2* and *rsw3,* glucose not purifiable as β-1,4-glucan;
[d] Only the non-releasable glucose value used for total; [e] Data for wild type at 21 °C reproduced from Tab[illegible] 3.1 and 3.2 which used total glucose for total monosaccharides.

Figure 6.1

Monosaccharide composition of the ammonium oxalate fractions from the root tissue of *Arabidopsis* wild type and mutants grown at 21 and 31 °C.

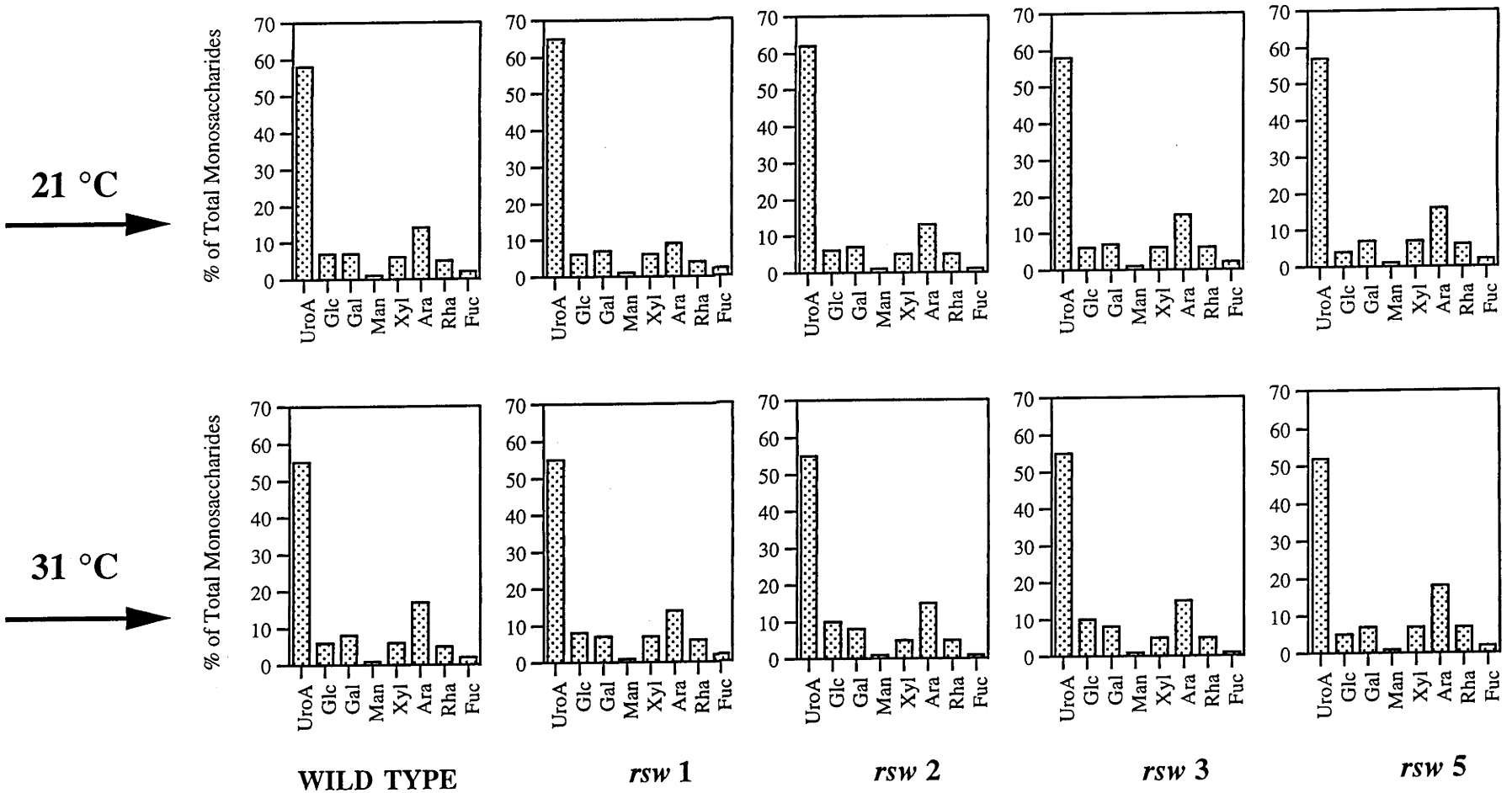

21 °C
31 °C
% of Total Monosaccharides
0
10
20
30
40
50
60
70
UroA
Glc
Gal
Man
Xyl
Ara
Rha
Fuc
WILD TYPE
rsw 1
rsw 2
rsw 3
rsw 5
ROOT TISSUE

Figure 6.2

Monosaccharide composition of the ammonium oxalate fractions from the shoot tissue of *Arabidopsis* wild type and mutants grown at 21 and 31 °C using corrected glucose values from Table 6.1.

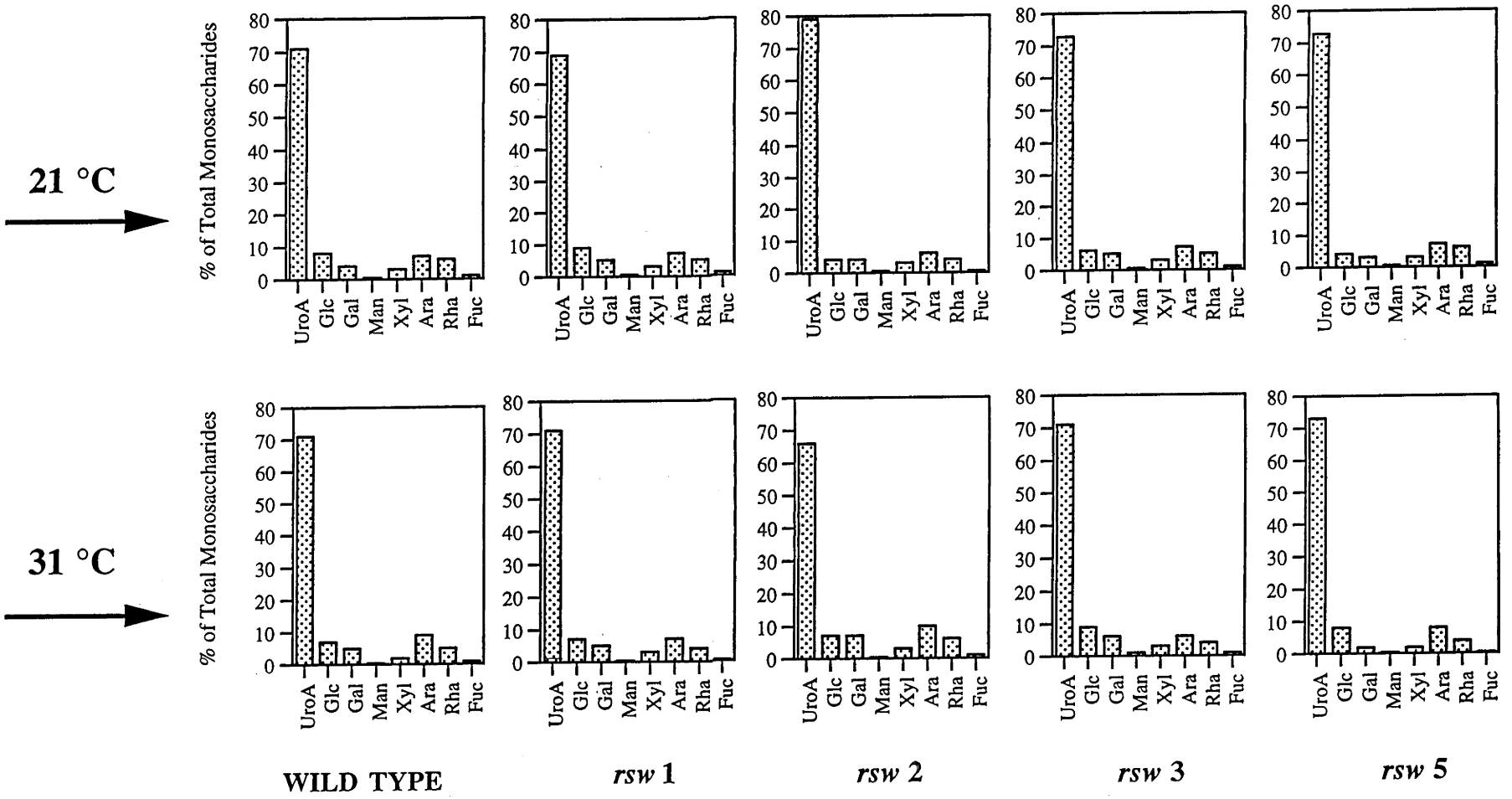

21 °C
31 °C
% of Total Monosaccharides
80
70
60
50
40
30
20
10
0
UroA
Glc
Gal
Man
Xyl
Ara
Rha
Fuc
WILD TYPE
rsw 1
rsw 2
rsw 3
rsw 5
SHOOT TISSUE

Figure 6.3

GC/MS chromatograms of PMAAs from methylation analysis of the pectins precipitated with CTAB from the ammonium oxalate fractions from whole seedlings of:

A, wild type grown at 21 °C;

B, wild type at 31 °C;

C, *rsw1* at 31 °C;

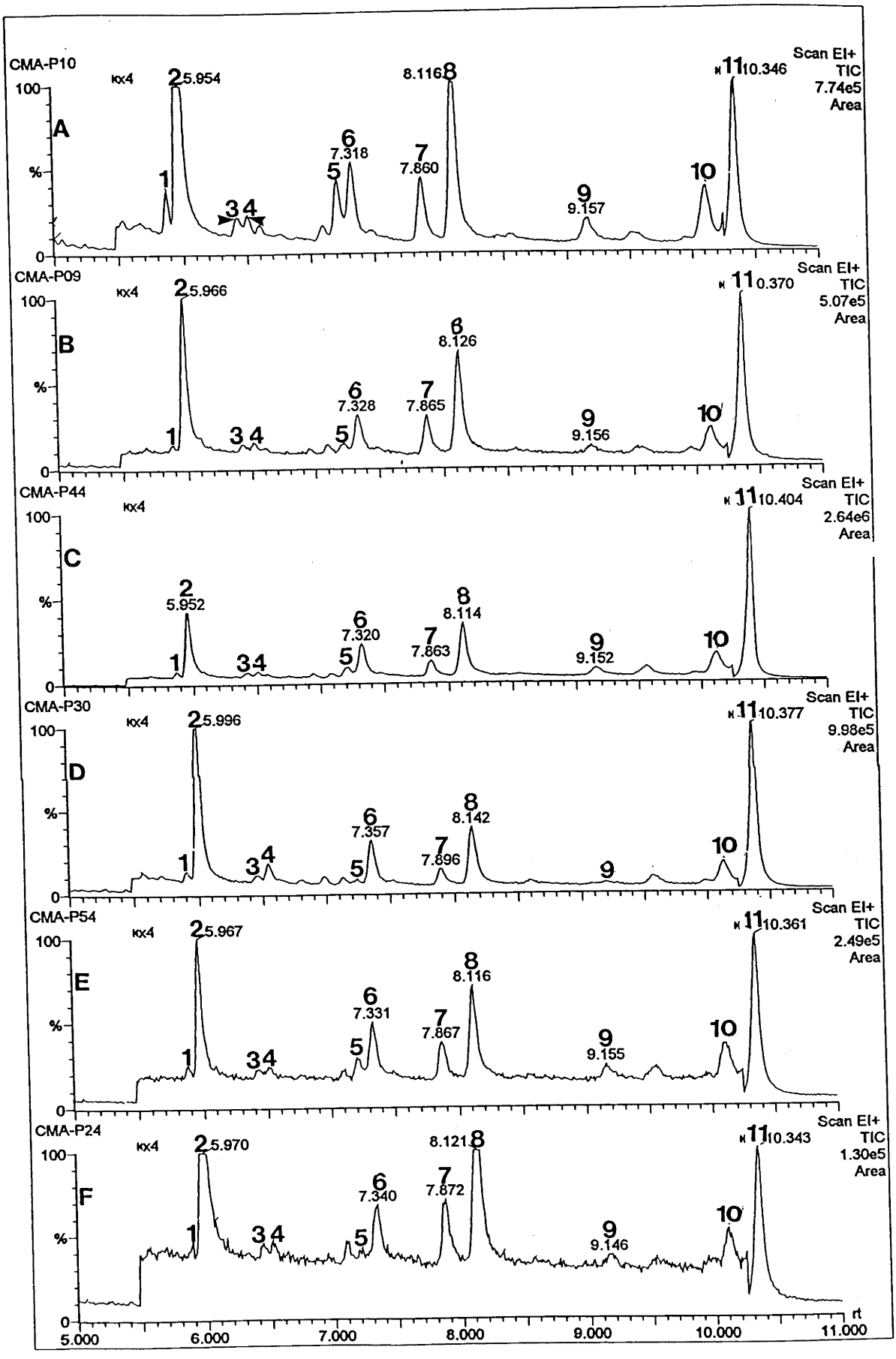
D, *rsw2* at 31 °C;

E, *rsw3* at 31 °C;

F, *rsw5* at 31 °C.

The full length scan of chromatogram **A** is presented in Figure 3.6. The RRT of each labelled peak in **A** is given in Table 3.7. MS of each peak is shown in Figure 3.7, and its deduced glycosyl linkages are presented in Table 3.7.

Peaks assigned the same number have similar MS patterns and RRT values (data not shown); All chromatograms were amplified by four over the indicated rang to clarify small peaks.


CMA-P10
Scan EI+
TIC
7.74e5
Area
A
5.954
8.116
10.346
7.318
7.860
9.157
CMA-P09
Scan EI+
TIC
5.07e5
Area
B
5.966
10.370
8.126
7.328
7.865
9.156
CMA-P44
Scan EI+
TIC
2.64e6
Area
C
10.404
5.952
8.114
7.320
7.863
9.152
CMA-P30
Scan EI+
TIC
9.98e5
Area
D
5.996
10.377
8.142
7.357
7.896
CMA-P54
Scan EI+
TIC
2.49e5
Area
E
5.967
10.361
8.116
7.331
7.867
9.155
CMA-P24
Scan EI+
TIC
1.30e5
Area
F
5.970
10.343
8.121
7.340
7.872
9.146
100
%
0
5.000
6.000
7.000
8.000
9.000
10.000
11.000
rt

many dicots (Section 3.3.4). A very similar linkage pattern was observed in pellets from the wild type and from all mutants grown at 31 °C (Figure 6.3). It included peaks for t-Rha, 2-Rha, 2,4-Rha, t-Gal*p*, 4-Gal*p*, t-Ara*f*, t-Ara*p*, 4-Ara*p*, t-Xyl*p*, most of the linkages expected for dicot pectins. Peak 11 (not amplified in Figure 6.3) indicates 4-linked glucose that remained in the CTAB pellet. The number of terminal glucose residues cannot be estimated since they are not resolved from terminal mannose in peak 6. The unlabelled small peaks in the chromatograms did not give MS that clearly identified them as particular PMAAs (data not shown).

Pectic polysaccharides extracted not only into the ammonium oxalate fraction, but also into the phosphate buffer and 0.1 M KOH fractions from the walls of wild type at 21 °C (Chapter Three). Table 6.2 shows the monosaccharide composition of the 0.1 M KOH fraction with glucose again presented as total and non-releasable (ie not purifiable as a glucan in the case of *rsw1*, *rsw2* and *rsw3* shoots or releasable by endocellulase elsewhere). Fractionation with CTAB was not carried out but the general consistency of composition suggests that the mutants did not show major changes in the mixture of pectins and XG deduced for the wild type at 21 °C. CTAB pellets from the phosphate buffer fractions of wild type and mutant were analysed by spectrophotometric assays (Table 6.3) and again show only relatively minor changes in the mutants. Estimates of the quantity of pectins in each fraction show that roots (Table 6.4) released more pectins into the phosphate buffer fraction than did shoots (Table 6.5) and the proportion released slightly decreased when *rsw1*, *rsw3* and *rsw5* were grown at 31 °C.

In summary, the major variation in the composition of the ammonium oxalate fractions comes from the increased quantities of glucose found in fractions prepared from shoots of *rsw1*, *rsw2* and *rsw3* grown at 31 °C. Compositional variations are much smaller if glucose that cannot be released by

Table 6.2 Monosaccharides (nmol/mg dry weight seedlings) from the 0.1 M KOH Fraction [a]

COMPONENT		Uronic acid	Glucose		Galactose	Mannose	Xylose	Arabinose	Rhamnose	Fucose	Total [d]
WT roots	21°[e]	30	5[b]	5[c]	18	ND	10	13	5	3	83
	31°	26	5	5	10	ND	8	13	5	3	69
WT shoots	21°	17	2	1	5	ND	2	7	5	ND	37
	31°	17	6	3	6	ND	2	6	4	ND	38
rsw1 roots	21°	20	4	4	12	ND	8	12	4	2	61
	31°	39	6	6	13	ND	12	16	6	3	94
rsw1 shoots	21°	22	5	2	5	ND	2	6	4	ND	42
	31°	22	21	7	5	ND	2	5	2	ND	43
rsw2 roots	21°	19	4	4	10	ND	8	11	4	2	59
	31°	25	4	4	10	ND	8	12	5	2	66
rsw2 shoots	21°	14	5	2	7	ND	2	8	5	ND	38
	31°	15	28	10	5	ND	2	6	3	ND	40
rsw3 roots	21°	24	3	3	8	ND	5	10	3	2	54
	31°	37	7	7	12	ND	7	11	4	2	79
rsw3 shoots	21°	12	6	3	5	ND	1	5	3	ND	30
	31°	8	52	26	3	ND	1	3	1	ND	43
rsw5 roots	21°	29	2	2	10	ND	9	13	5	2	71
	31°	27	3	3	10	ND	9	14	6	3	71
rsw5 shoots	21°	13	5	2	6	ND	4	2	4	ND	33
	31°	11	16	5	3	ND	1	4	2	ND	27

[a] Uronic acids were determined by the *m*-hydroxybiphenyl-H_2SO_4 test; other monosaccharides by GC/M after TFA hydrolysis; [b] Total glucose from Table 4.5;
[c] Non-releasable glucose, comprising glucose not released by endo-cellulase or, in the case of shoots of *r* *v1*, *rsw2* and *rsw3*, glucose not purifiable as β-1,4-glucan;
[d] Only the non-releasable glucose value used for total; [e] Data for wild type at 21 °C reproduced from Tabl 3.1 and 3.2 which used total glucose for total monosaccharides.

Table 6.3 Carbohydrates (nmol/mg dry weight seedlings) from Pellet by CTAB precipitation with Dialysed Phosphate Buffer Fraction *

COMPONENT	Uronic acids	Hexoses	Pentoses	6-Deoxy-Hexoses	Total
WT roots 21°	34	24	13	5	76
31°	47	31	19	7	104
WT shoots 21°	14	10	6	3	33
31°	18	14	6	3	41
rsw1 roots 21°	40	28	15	6	89
31°	30	23	10	4	67
rsw1 shoots 21°	11	8	4	2	25
31°	10	24	5	2	41
rsw2 roots 21°	40	26	16	5	87
31°	31	21	11	5	68
rsw2 shoots 21°	10	7	4	1	22
31°	12	6	5	2	25
rsw3 roots 21°	61	42	15	9	127
31°	32	20	12	5	69
rsw3 shoots 21°	6	3	2	1	12
31°	9	8	4	2	23
rsw5 roots 21°	43	36	18	8	105
31°	40	21	12	5	68
rsw5 shoots 21°	8	6	3	1	18
31°	8	24	6	3	41

* Method for carbohydrate analysis described in Section 3.2.3.

Table 6.4 Distribution of Pectic Polysaccharides in Fractions from Roots*

Fraction	%				
	Wild Type	*rsw1*	*rsw2*	*rsw3*	*rsw5*
21 °C					
Phosphate Buffer	20	25	24	31	26
Ammonium Oxalate	65	63	65	60	62
0.1 M KOH	15	12	11	9	12
Total	100	100	100	100	100
31 °C					
Phosphate Buffer	26	14	22	18	19
Ammonium Oxalate	63	71	64	67	67
0.1 M KOH	11	15	15	15	14
Total	100	100	100	100	100

* Pectic polysaccharides from total monosaccharides of ammonium oxalate fraction (Table 6.1), total monosaccharides of 0.1 M KOH fraction (Table 6.2) x 0.7 for root, and total carbohydrates of pellet from phosphate buffer fraction (Table 6.3).

Table 6.5 Distribution of Pectic Polysaccharides in Fractions from Shoots *

Fraction	%				
	Wild Type	*rsw1*	*rsw2*	*rsw3*	*rsw5*
21 °C					
Phosphate Buffer	10	7	7	5	6
Ammonium Oxalate	81	82	82	87	87
0.1 M KOH	9	11	10	8	7
Total	100	100	100	100	100
31 °C					
Phosphate Buffer	10	9	6	7	12
Ammonium Oxalate	83	83	86	83	82
0.1 M KOH	7	8	8	10	6
Total	100	100	100	100	100

* Pectic polysaccharides from total monosaccharides of ammonium oxalate fraction (Table 6.1), total monosaccharides of 0.1 M KOH fraction (Table 6.2) x 0.8 for shoot, and total carbohydrates of pellet from phosphate buffer fraction (Table 6.3).

endocellulase or purified as a β-1,4-glucan is considered. There are likewise no major changes in pectin distribution and, together with a lack of major changes seen by methylation analysis, this leads to the conclusion that none of the mutants show major changes in the biosynthesis of pectins.

6.3.2 Hemicelluloses

Hemicelluloses (XG and GAX with some β-1,4-D-mannans or galactomannans) mainly extracted with the 4 M KOH fraction from the cell walls of wild type grown at 21 °C (Section 3.3.6). The monosaccharide composition of the 4 M KOH fraction derived from the wild type and mutants grown at 21 °C or 31 °C was determined (Table 6.6). In all cases, root fractions have roughly twice the total monosaccharides as shoots when compared on a dry weight basis. As occurred with the ammonium oxalate fraction, the quantities of glucose in the shoots of *rsw1*, *rsw2* and *rsw3* varied widely and a figure for non-releasable glucose is again provided after subtracting glucose that can be released by endo-cellulase digestion or, in the case of shoots of *rsw1*, *rsw2* and *rsw3* grown at 31 °C, purified as β-1,4- glucan. The *rsw2* shoot at 31 °C appeared to have an unusually low galactose level and the differences in arabinose levels in the *rsw1* shoot grown at the two temperatures were unusually large. When expressed in terms of percentage composition, the mutants, whether grown at 21°C or 31 °C, had a generally similar monosaccharide composition to the wild type in both roots (Figure 6.4) and shoots (Figure 6.5).

Differences in monosaccharide composition between roots and shoots were more consistent and more prominent than differences due to genotype or temperature. The higher total monosaccharides in the 4 M KOH extracts of roots compared to shoots came mainly from xylose, to a lesser degree from arabinose, uronic acids and galactose with smaller excesses of fucose and little difference in mannose or rhamnose (Table 6.6). CTAB fractionation of the 4 M KOH fraction

Table 6.6 Monosaccharides (nmol/mg dry weight seedlings) from the 4 M KOH Fraction[a]

COMPONENT		Uronic acid	Glucose		Galactose	Mannose	Xylose	Arabinose	Rhamnose	Fucose	Total[d]
WT roots	21°[e]	18	40[b]	40[c]	22	10	67	30	3	9	200
	31°	25	44	44	27	9	64	32	3	7	211
WT shoots	21°	10	29	26	12	9	26	13	1	3	100
	31°	6	52	36	16	10	22	10	tr	4	104
rsw1 roots	21°	18	38	38	20	9	60	29	2	8	184
	31°	31	37	37	21	8	54	33	3	6	193
rsw1 shoots	21°	9	26	19	10	6	16	8	1	2	71
	31°	13	93	37	14	5	21	19	1	2	112
rsw2 roots	21°	17	31	31	20	8	54	30	2	7	169
	31°	20	37	37	24	8	53	33	2	7	184
rsw2 shoots	21°	12	38	27	16	7	23	16	1	3	105
	31°	9	122	43	6	6	23	19	tr	2	108
rsw3 roots	21°	22	39	39	23	11	65	30	2	9	201
	31°	20	43	43	23	10	52	29	3	7	187
rsw3 shoots	21°	7	42	30	11	7	22	11	1	3	92
	31°	4	130	40	11	6	22	14	tr	2	99
rsw5 roots	21°	14	32	32	17	9	61	29	2	6	170
	31°	15	31	31	15	8	51	24	2	7	153
rsw5 shoots	21°	6	30	21	9	6	18	11	1	2	74
	31°	4	59	28	14	5	16	8	tr	2	77

[a] Uronic acids were determined by the *m*-hydroxybiphenyl-H_2SO_4 test; other monosaccharides by GC/MS after TFA hydrolysis, tr: <0.5 nmol/mg dry weight); [b] Total glucose from Table 4.5; [c] Non-releasable glucose, comprising glucose not released by endo-cellulase or in the case of shoots of *rsw1*, *rsw2* and *rsw3*, glucose not purifiable as β-1,4-glucan; [d] Only the non-releasable glucose value used for total; [e] Data for wild type at 21 °C reproduced from Table 3.1 and 3.2 which used total glucose for total monosaccharides.

Figure 6.4

Monosaccharide composition of the 4 M KOH fractions from the root tissue of *Arabidopsis* wild type and mutants grown at 21 and 31 °C.

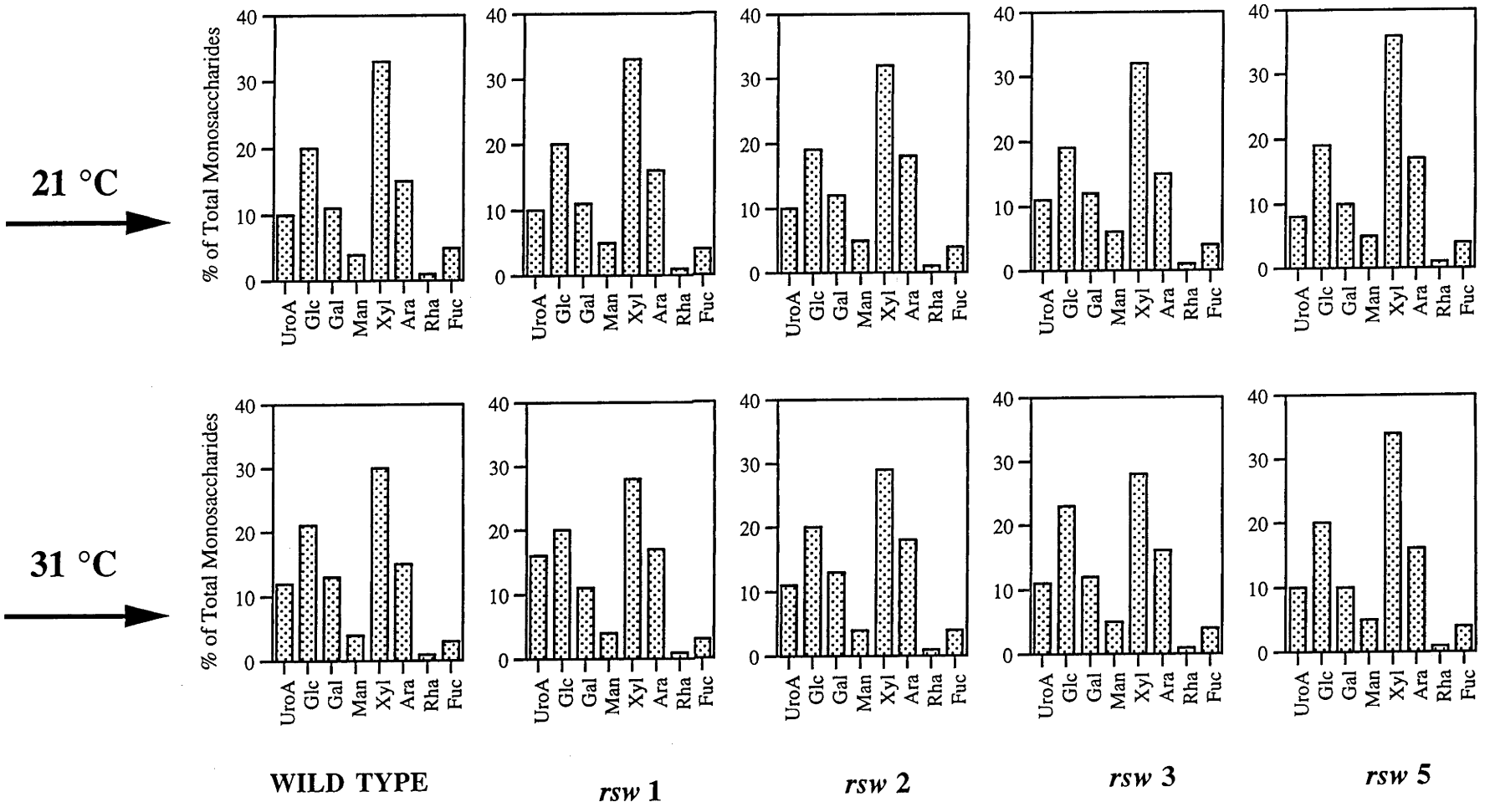
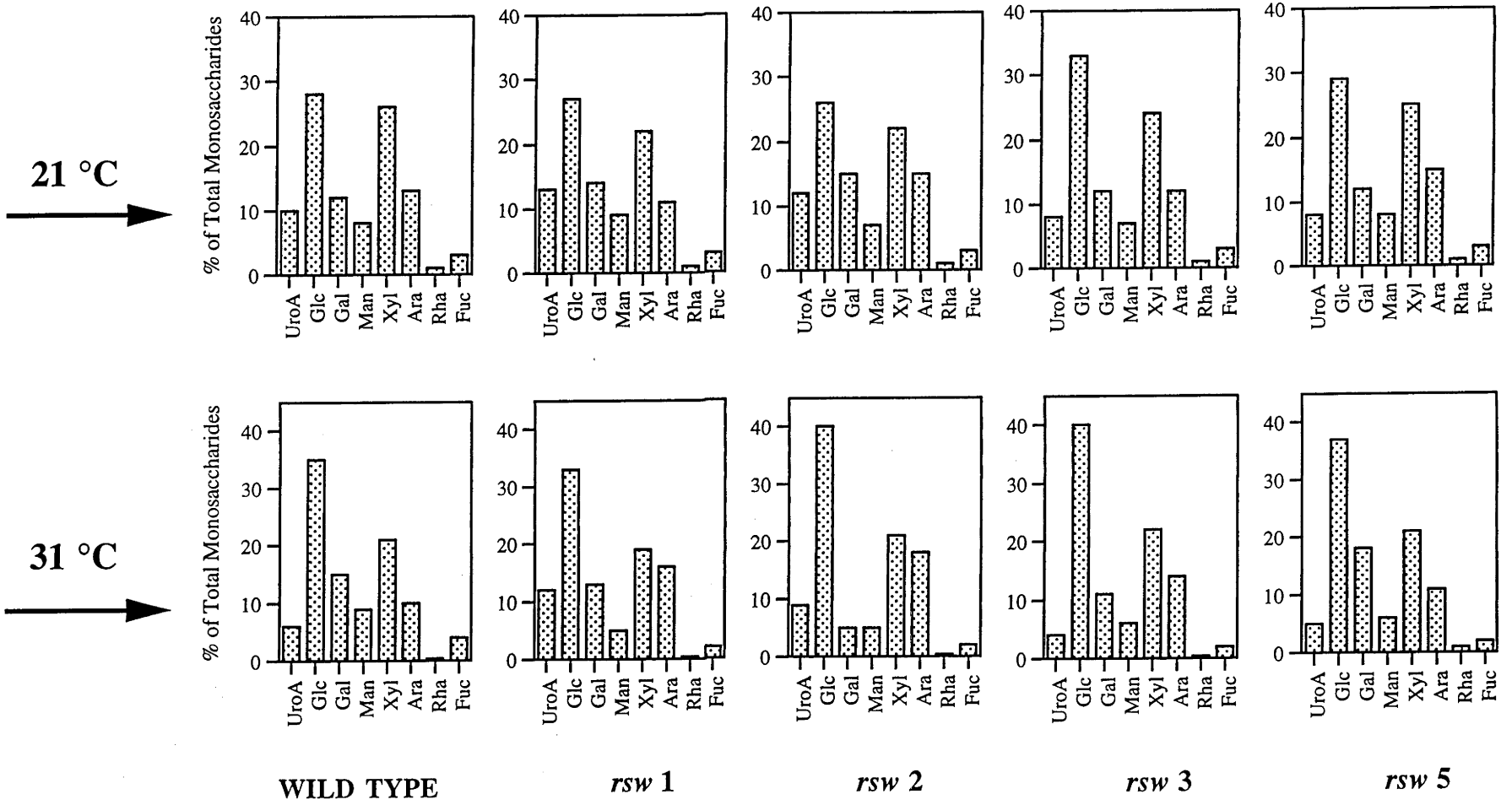

21 °C
31 °C
% of Total Monosaccharides
0
10
20
30
40
UroA
Glc
Gal
Man
Xyl
Ara
Rha
Fuc
WILD TYPE
rsw 1
rsw 2
rsw 3
rsw 5
ROOT TISSUE

Figure 6.5

Monosaccharide composition of the 4 M KOH fractions from the shoot tissue of *Arabidopsis* wild type and mutants grown at 21 and 31 °C using corrected glucose values from Table 6.6.


21 °C
31 °C
% of Total Monosaccharides
0
10
20
30
40
UroA
Glc
Gal
Man
Xyl
Ara
Rha
Fuc
WILD TYPE
rsw 1
rsw 2
rsw 3
rsw 5
SHOOT TISSUE

from wild type grown at 21 °C showed that all glucuronic acid and most of the arabinose occurred in the pellet (Table 3.9), probably in the form of GAX (Section 3.3.6). As a result it was estimated that both XG and GAX were more abundant in roots than shoots of wild type but GAX was present in greater excess in roots (Table 3.13). That increase in the ratio of GAX:XG is likely to hold for all mutants at both temperatures given the consistently higher levels of uronic acids and arabinose in the roots of all genotypes at both temperatures (Table 6.6).

Methylation analysis was carried out on hemicelluloses in neutralised and dialysed 4 M KOH fractions prepared from whole seedlings and centrifuged to remove β-1,4-glucan (Figure 6.6). The PMAA products from all mutants grown at 31 °C resembled those from the wild type at 21 and 31 °C. As discussed for the wild type (Section 3.3.6), most of the PMAAs expected from XG are found (peak 1, t-Fuc, peak 2, t-Xyl*p*; peak 4, t-Gal*p*; peak 5, 2-Xyl*p*; peak 7, 4-Glc*p*; peak 8, 4,6-Glc*p*), together with at least one for GAX (Peak 5, 4-Xyl*p* and possibly t-Ara*f*) and two for β-1,4-D-mannans or galactomannan (peak 3, t-Man*p*; peak 6, 4-Man*p*). Other unlabelled peaks in the chromatograms did not give MS from which PMAAs could be identified (data not shown).

The monosaccharide composition of the 0.1 M KOH fractions was presented in Table 6.2. By applying the methods described in Section 3.3.10 to distinguish pectins from hemicelluloses in the 0.1 M KOH fractions, it was estimated that about 10% of total hemicelluloses were extracted into the 0.1 M KOH fraction in all mutants and wild type (Tables 6.7 and 6.8).

In summary, the hemicelluloses produced by the mutants in both roots and shoots resembled wild type in monosaccharide composition and deduced linkages. Hemicelluloses were more abundant in roots than in shoots on a nmol monosaccharide/mg dry weight basis and the ratio of GAX: XG was probably higher in roots than in shoots.

Figure 6.6

GC/MS chromatograms of PMAAs from methylation analysis of the supernatant (hemicelluloses) after spinning down β-1,4-glucan from the 4 M KOH fractions from whole seedlings of:

A, wild type grown at 21 °C;

B, wild type at 31 °C;

C, *rsw1* at 31 °C;

D, *rsw2* at 31 °C;

E, *rsw3* at 31 °C;

F, *rsw5* at 31 °C.

The full length scan of chromatogram **A** is presented in Figure 3.6C. The RRT of each labelled peak in the chromatogram is given in Table 3.10. MS of each peak is shown in Figure 3.8, and its deduced glycosyl linkages are presented in Table 3.10.

Peak assigned the same number have similar MS patterns and RRT values (data not shown). All chromatograms were amplified by two.

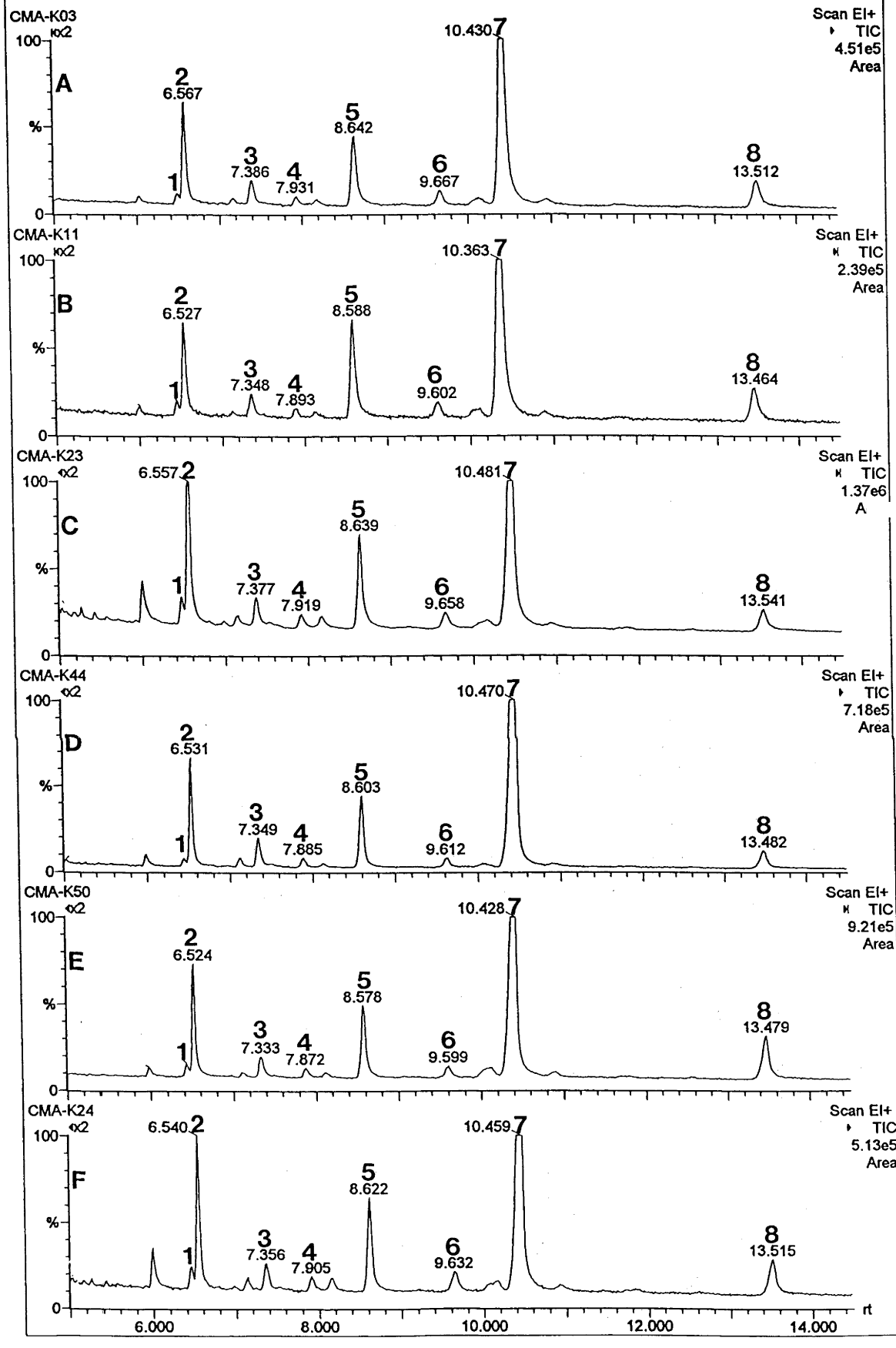

CMA-K03
Scan EI+
TIC
4.51e5
Area
A
1 6.567
2 7.386
3 7.931
4 8.642
5 9.667
6 10.430
7 13.512
CMA-K11
Scan EI+
TIC
2.39e5
Area
B
1 6.527
2 7.348
3 7.893
4 8.588
5 9.602
6 10.363
7 13.464
CMA-K23
Scan EI+
TIC
1.37e6
A
C
1 6.557
2 7.377
3 7.919
4 8.639
5 9.658
6 10.481
7 13.541
CMA-K44
Scan EI+
TIC
7.18e5
Area
D
1 6.531
2 7.349
3 7.885
4 8.603
5 9.612
6 10.470
7 13.482
CMA-K50
Scan EI+
TIC
9.21e5
Area
E
1 6.524
2 7.333
3 7.872
4 8.578
5 9.599
6 10.428
7 13.479
CMA-K24
Scan EI+
TIC
5.13e5
Area
F
1 6.540
2 7.356
3 7.905
4 8.622
5 9.632
6 10.459
7 13.515
100
%
0
6.000
8.000
10.000
12.000
14.000
rt

Table 6.7 Distribution of Hemicelluloses in Fractions from Roots *

Fraction	%				
	Wild Type	*rsw1*	*rsw2*	*rsw3*	*rsw5*
21 °C					
0.1 M KOH	11	9	10	7	11
4 M KOH	89	91	90	93	89
Total	100	100	100	100	100
31 °C					
0.1 M KOH	9	12	10	11	12
4 M KOH	91	88	90	89	88
Total	100	100	100	100	100

* Hemicelluloses from total monosaccharides of 4 M KOH fraction (Table 6.6), and total monosaccharides of 0.1 M KOH (Table 6.2) x 0.3 for root.

Table 6.8 Distribution of Hemicelluloses in Fractions from Shoots *

Fraction	%				
	Wild Type	*rsw1*	*rsw2*	*rsw3*	*rsw5*
21 °C					
0.1 M KOH	7	10	7	6	9
4 M KOH	93	90	93	94	91
Total	100	100	100	100	100
31 °C					
0.1 M KOH	7	7	7	8	6
4 M KOH	93	93	93	92	94
Total	100	100	100	100	100

* Hemicelluloses from total monosaccharides of 4 M KOH fraction (Table 6.6), and total monosaccharides of 0.1 M KOH (Table 6.2) x 0.2 for shoot.

6.3.3 Levels of Total Pectins and Hemicelluloses

The total levels of pectins and hemicelluloses were estimated for the wild type and mutants and are shown with the previously reported levels of cellulose and β-1,4-glucan (Table 6.9). When total hemicelluloses, pectins and crystalline cellulose levels in the mutants are compared to the levels in wild type at the same temperature, the underproduction of crystalline cellulose in the shoots of all mutants at 31 °C is large compared to any effects on pectins and hemicelluloses. In roots, cellulose underproduction is large in *rsw1*, *rsw2* and *rsw3*, but not much larger than that for pectins and hemicelluloses in the case of *rsw5* (Figures 6.7 and 6.8). The deduced percentage compositions of the walls are presented in Figures 6.9 and 6.10.

6.3.4 Percentage Composition of Cell Walls

The percentages of the major polysaccharide types in cell walls of wild type and mutants are given in Figures 6.9 and 6.10 which are derived from Table 6.9. All mutants grown at 21 °C for 5 days appear to have similar cell wall composition to wild type. Walls of roots have higher percentages of cellulose and hemicelluloses and less pectins than do walls of shoots. Moreover, mutants and the wild type have 3% or 4% of β-1,4-glucose in the walls of shoots.

When grown at 31 °C for 5 days, the wild type shows little change in wall composition. The shoots of all mutants (Figure 6.9), however, show a lower percentage of cellulose, a reduction that is substantially compensated by increased accumulation of non-crystalline cellulose with modest changes in pectins and hemicelluloses. In the root (Figure 6.10), the decreased percentage of cellulose is offset by an increase in the percentages of hemicelluloses and/or pectins. Mutant *rsw1* shows an increase in the percentage of pectins, *rsw2* an increase in the percentage of hemicelluloses, whereas *rsw3 and 5* have increases in the percentage of both polysaccharides.

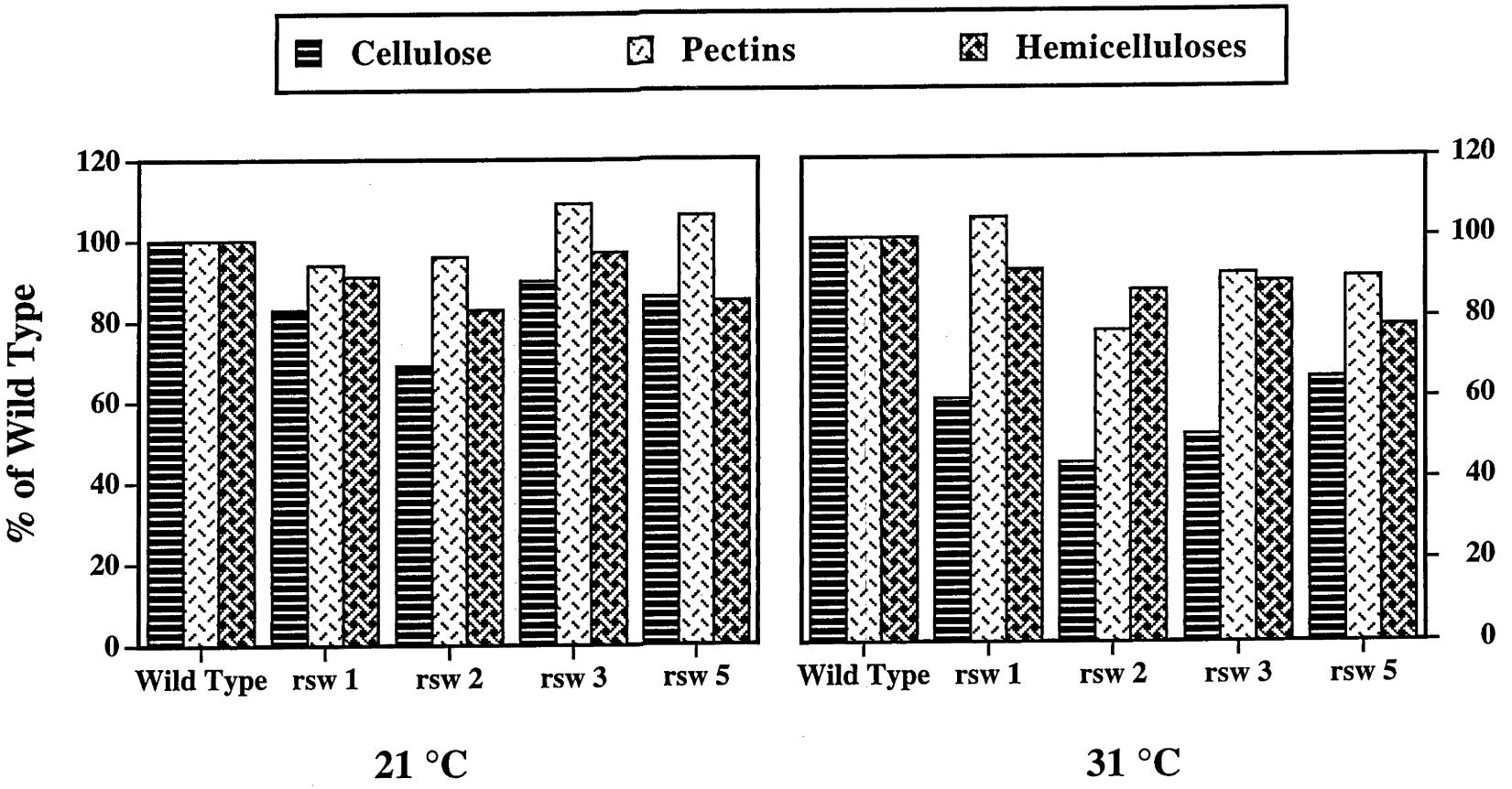
Table 6.9 Total Wall Polysaccharides of *Arabidopsis* Wild Type and Mutants Grown at 21 and 31 °C *

Carbohydrates	nmol/mg dry weight									
	Wild Type		*rsw1*		*rsw2*		*rsw3*		*rsw5*	
	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C
ROOT										
Cellulose	483	526	401	315	336	234	435	270	416	343
Pectins	383	406	355	453	363	311	416	377	403	358
Hemicelluloses	225	232	202	221	187	204	217	211	191	174
Total	1091	1164	958	989	886	749	1068	858	1010	875
SHOOT										
Cellulose	273	363	218	159	230	139	285	181	200	180
β-1,4-Glucan				266		228		233		
β-1,4-Glucose	22	83	24		20		24		21	73
Pectin	332	409	319	417	310	424	287	332	353	337
Hemicellulose	107	112	79	121	113	116	98	108	81	82
Total	734	967	640	963	673	907	694	854	655	672

* **Cellulose** data from Table 4.4; **Pectins** from total monosaccharides of ammonium oxalate fraction (Tables 6.1), total monosaccharides of 0.1 M KOH fraction (Table 6.2) x 0.7 for root and x 0.8 for shoot, and total carbohydrates of pellet from phosphate buffer fraction (Table 6.3); **Hemicelluloses** from total monosaccharides of 4 M KOH fraction (Tables 6.6) and total monosaccharides of 0.1 M KOH fraction (Table 6.2) x 0.3 for root and x 0.2 for shoot; **β-1,4-Glucan** purified from ammonium oxalate, 0.1 M KOH and 4 M KOH fractions of *rsw1, rsw2* and *rsw 3* at 31 °C; **β-1,4-Glucose** from endo-cellulase digestion of ammonium oxalate, 0.1 M KOH, 4 M KOH and acid-soluble fractions of wild type and *rsw5* at 31 °C, and digestion of all fractions of mutants and wild type at 21 °C (Table 5.10).

Figure 6.7

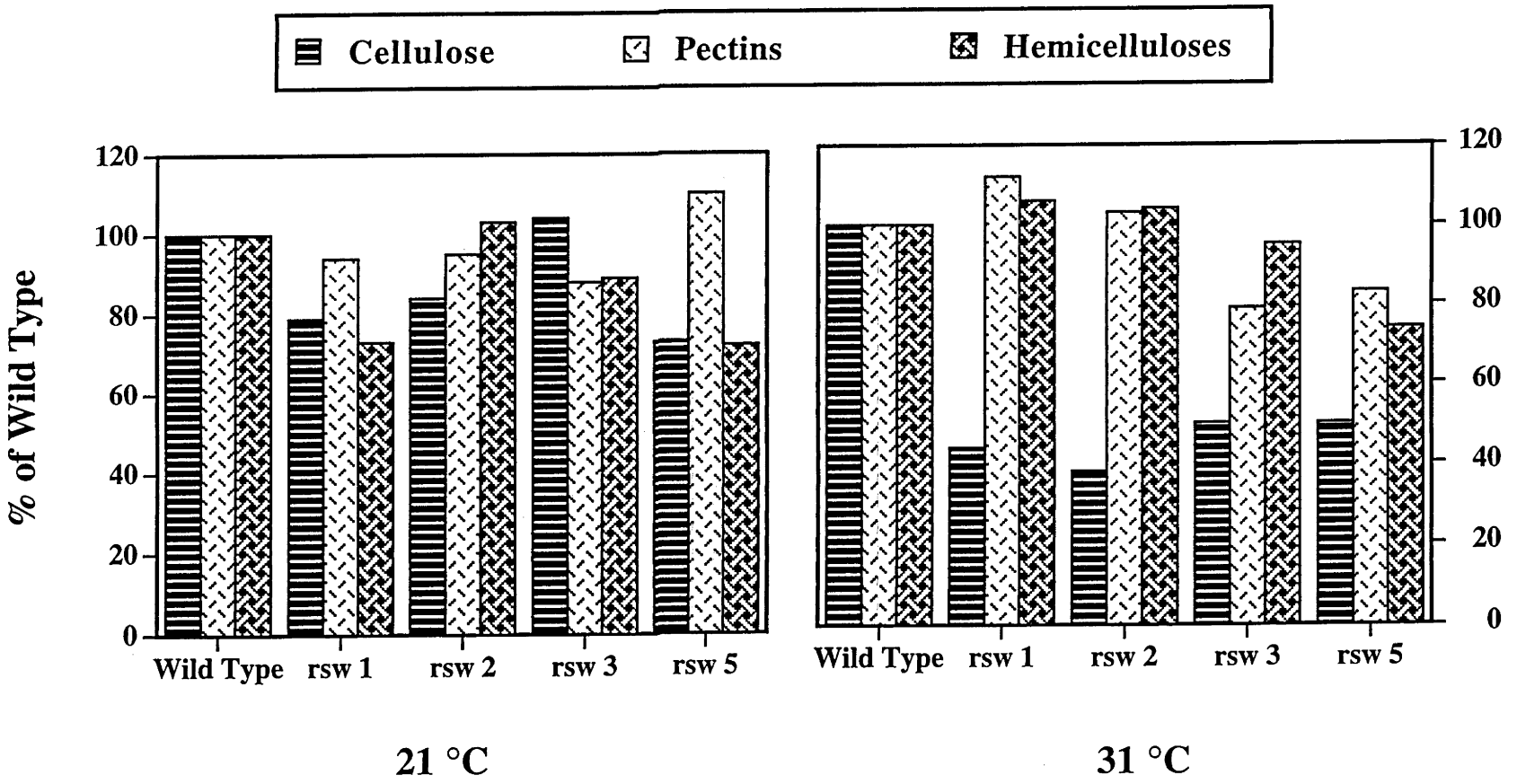
Contents (nmol/mg dry weight) of cellulose, total pectins and hemicelluloses of roots from four mutants expressed as a % of those from wild type grown at 21 °C and 31 °C; Data from Table 6.9.



Total Wall Polysaccharides in Roots of *Arabidopsis*

Figure 6.8

Contents (nmol/mg dry weight) of cellulose, total pectins and hemicelluloses of shoots from four mutants expressed as a % of those from wild type grown at 21 °C and 31 °C; Data from Table 6.9.



Total Wall Polysaccharides in Shoots of *Arabidopsis*

Figure 6.9

The wall composition in the shoot tissue of *Arabidopsis* wild type and mutants grown at 21 °C and 31 °C; β-1,4-Glucan only for *rsw1, rsw2* and *rsw3* grown at 31 °C and β-1,4-Glucose for any other cases; All data from Table 6.9.

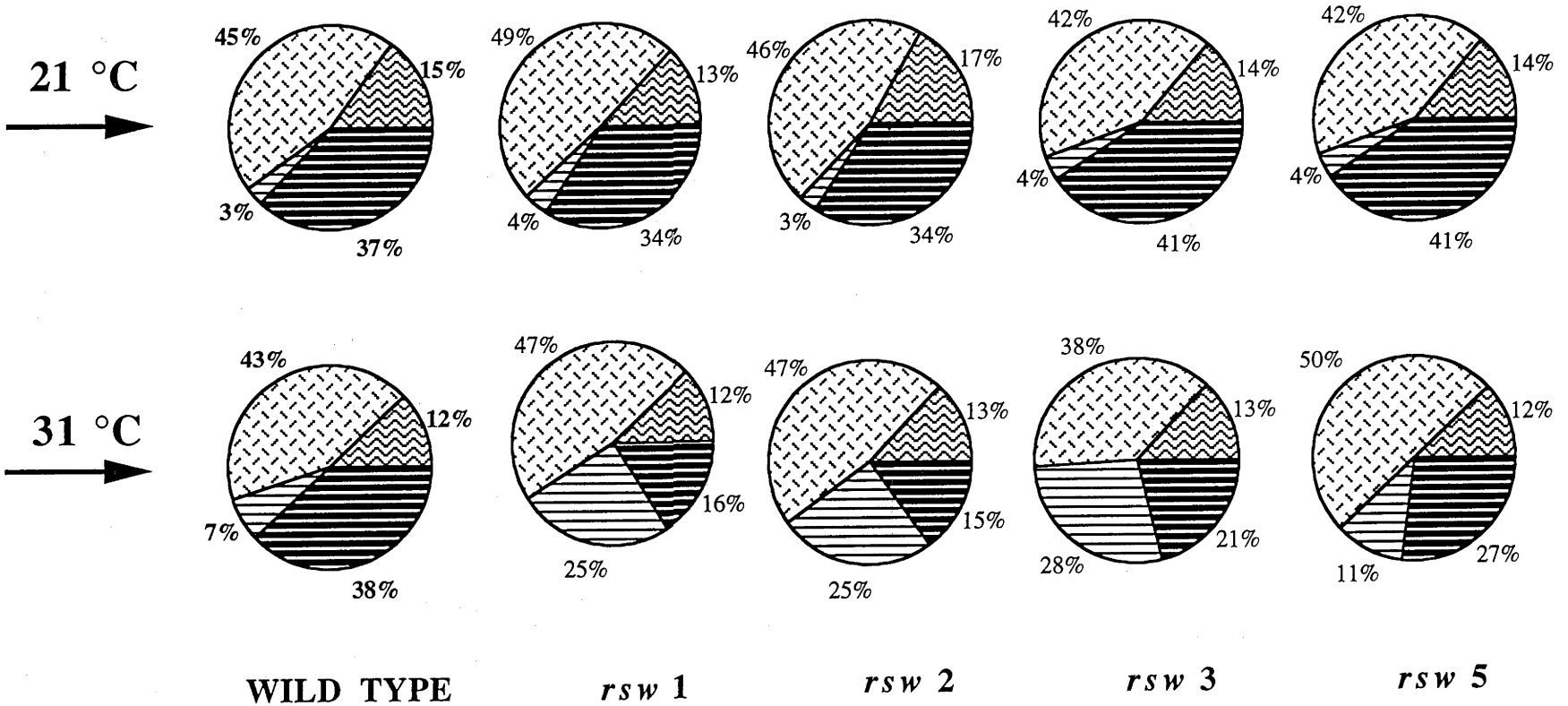
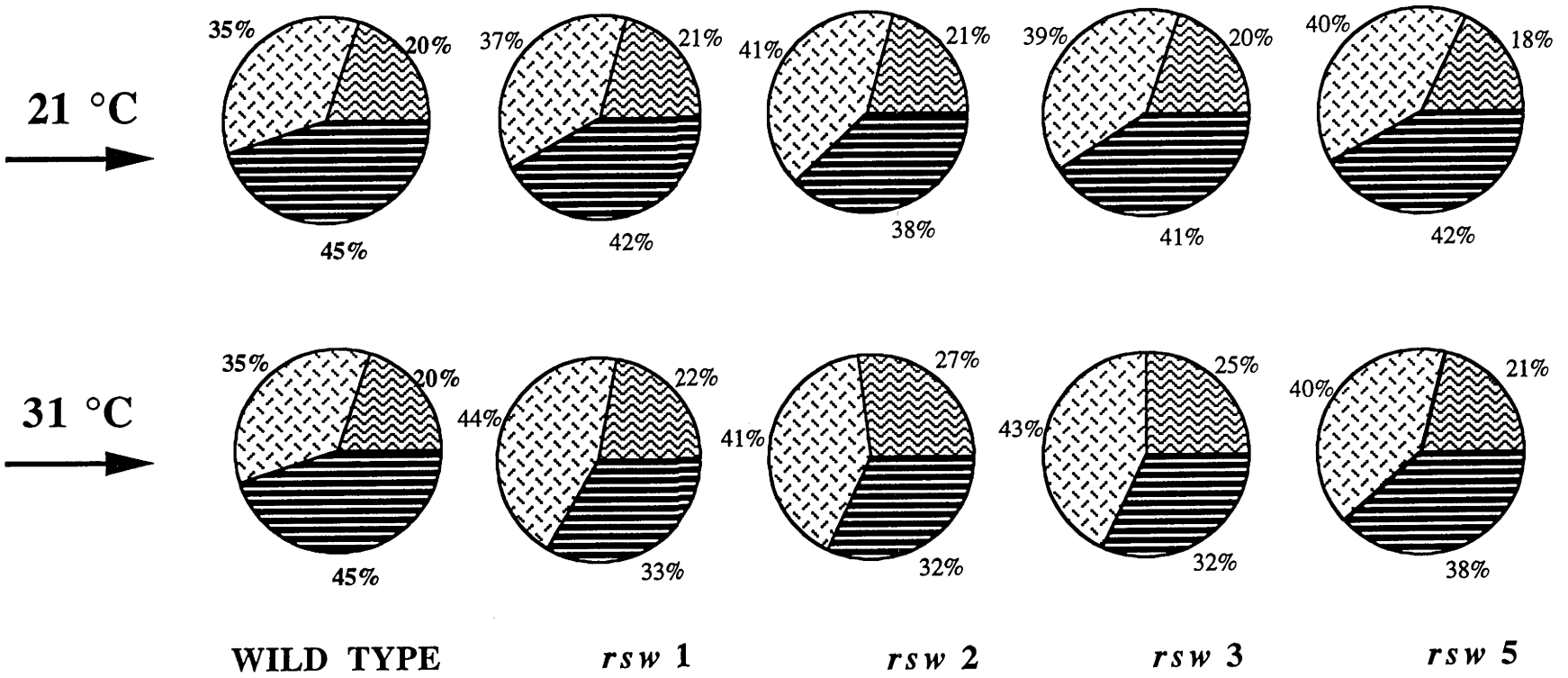

21 °C
45%
15%
3%
37%
49%
13%
4%
34%
46%
17%
3%
34%
42%
14%
4%
41%
42%
14%
4%
41%
31 °C
43%
12%
7%
38%
47%
12%
16%
25%
47%
13%
15%
25%
38%
13%
21%
28%
50%
12%
27%
11%
WILD TYPE
rsw 1
rsw 2
rsw 3
rsw 5
SHOOT TISSUE
Cellulose
Pectins
b-1,4 Glucan
Hemicelluloses

Figure 6.10

The wall composition in the root tissue of *Arabidopsis* wild type and mutants grown at 21 °C and 31 °C; All data from Table 6.9.


21 °C
35%
20%
45%
37%
21%
42%
41%
21%
38%
39%
20%
41%
40%
18%
42%
31 °C
35%
20%
45%
44%
22%
33%
41%
27%
32%
43%
25%
32%
40%
21%
38%
WILD TYPE
rsw 1
rsw 2
rsw 3
rsw 5
ROOT TISSUE
Cellulose
Pectins
Hemicelluloses

6.3.5 Starch

It has been shown that starch in *Arabidopsis* seedlings is extracted in the DMSO fraction (Section 3.3.3). The glucose content of the DMSO fraction (Table 6.10) indicated that mutants and wild type at 31 °C both accumulated much more starch than at 21 °C. In the shoots, mutants *rsw1, rsw2* and *rsw3* had slightly higher Rw values (2.1-2.6) for starch production than did the wild type (1.6) and *rsw5* (1.3). In roots, however, the Rw values (4.1-7.0) in mutants *rsw1, rsw2* and *rsw3* were very much higher than those in wild type (1.7) and *rsw5* (1.4). The glucose contents of starch in the roots of all mutants except *rsw2* were not significantly different from wild type when grown at 21 °C ($p > 0.05$), but much more glucose occurred as starch in *rsw1, rsw2* and *rsw3* than in the wild type or *rsw5* at 31 °C ($p < 0.05$).

It appeared that part of the glucose not used for cellulose biosynthesis in the roots of *rsw1, rsw2* and *rsw3* at 31°C was stored as starch.

6.3.6 Glucose Distribution

The distribution of glucose in all cell polysaccharides is presented in Tables 6.11 and 6.12.

All mutants showed only small differences from the wild type for glucose distribution in shoots at 21 °C (Table 6.11). Mutants *rsw1* and perhaps *rsw5* had slightly lower percentages of glucose as cellulose than the wild type did, and a little more glucose as starch. The inhibition of cellulose biosynthesis in mutants at 31 °C, was accompanied by major alterations in glucose distribution in shoots (Table 6.11). The wild type at 31 °C had only 5% less glucose in cellulose than at 21 °C, whereas *rsw1, rsw2* and *rsw3* had about 40% less glucose as cellulose than at 21 °C, and *rsw5* had 17% less glucose. Mutants *rsw1, rsw2* and *rsw3* had about 30% more glucose in non-crystalline cellulose (β-1,4-glucans) and about

5% more in starch than at 21 °C. Mutant *rsw5* at 31 °C had 14% more glucose in the β-1,4-glucose. The percentage of total glucose in other polysaccharides (pectins and hemicelluloses) showed only small differences between 21 °C and 31 °C.

Thus, the shoots of mutants at 31 °C a lower percentage of glucose as cellulose and a higher percentage as non-crystalline cellulose (β-1,4-glucan) and some as starch.

In roots of plants grown at 31 °C, mutants *rsw1, rsw2* and *rsw3* had about 20% less of their total glucose as cellulose than at 21 °C, whereas wild type and *rsw5* were essentially unaltered (Table 6.12). There were large increases in the percentage of glucose as starch in *rsw1, rsw2* and *rsw3* with little change in other polysaccharides at 31 °C.

Therefore, the reduction of glucose as cellulose in mutants at 31 °C mainly diverts glucose into either non-crystalline cellulose (shoots) or starch (roots) without clear changes in pectins and hemicelluloses.

Table 6.10 Glucose Content of Starch from DMSO Fraction of *Arabidopsis* Wild Type and Mutants *

Materials	Glucose as Starch (nmol/mg dry weight)				Rw	
	Root		Shoot		31 : 21 °C	
	21 °C	31 °C	21 °C	31 °C	Root	Shoot
Wild Type	22±1	37±2	23±1	37±4	1.7	1.6
rsw1	18±1	126±6	56±2	144±3	7.0	2.6
rsw2	11±1	66±4	25±3	65±8	6.2	2.6
rsw3	19±1	77±4	41±3	86±8	4.1	2.1
rsw5	18±1	24±3	33±1	42±3	1.4	1.3

* The glucose of DMSO fraction was determined by anthrone/H_2SO_4 test; SD, n=3;

Rw, Glucose of starch ratio in 31 °C plants : 21 °C plants.

Table 6.11 Percentage of Total Glucose Present in Different Carbohydrates in Shoots *

Carbohydrates	Wild Type		*rsw1*		*rsw2*		*rsw3*		*rsw5*	
	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C
Lipid-Linked Saccharides	1	1	1	1	1	1	1	1	2	1
Starch	6	7	16	22	8	13	10	15	11	12
Pectins	6	4	6	10	3	5	4	4	5	6
Hemicelluloses	8	7	7	7	10	10	9	9	8	10
Cellulose	73	68	61	24	72	27	70	32	67	50
β-1,4-Glucan	-	-	-	36	-	44	-	39	-	-
β-1,4-Glucose	6	13	9	-	6	-	6	-	7	21
Total	100	100	100	100	100	100	100	100	100	100

* **Lipid-linked saccharides** were from chloroform-methanol fraction (Section 3.3.2); **starch** from DMSO fraction (Section 3.3.3); **Pectins** from the phosphate buffer, ammonium oxalate and 0.1 M KOH fractions (Sections 3.3.1, 3.3.4 and 3.3.5); **Hemicelluloses** from 0.1 M KOH and 4 M KOH fractions (Sections 3.3.5 and 3.3.6); Only non-releasable glucose used for pectins and hemicelluloses from those fractions; **Cellulose** from acid-insoluble fraction (Section 3.3.8); **β-1,4-glucan** from ammonium oxalate, 0.1 M KOH and 4 M KOH fractions purified from *rsw1, rsw2* and *rsw3* grown at 31 °C , or **β-1,4-Glucose** from endo-cellulase digestion of ammonium oxalate, 0.1 M KOH, 4 M KOH and acid-soluble fractions of wild type and *rsw5* at 31 °C, and digestion of all fractions of mutants and wild type at 21 °C (data from Table 5.10).

Glucose of cellulose and starch determined by anthrone/H_2SO_4 test, all other determined by GC/MS.

Table 6.12 Percentage of Total Glucose Present in Different Carbohydrates in Roots *

Carbohydrates	Wild Type		*rsw1*		*rsw2*		*rsw3*		*rsw5*	
	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C	21 °C	31 °C
Lipid-Linked Saccharides	2	1	3	2	2	2	1	2	2	1
Starch	4	6	3	24	3	18	4	18	4	6
Pectins	3	2	3	5	3	5	3	6	2	3
Hemicelluloses	8	8	8	8	9	11	8	11	7	8
Cellulose	82	82	82	60	82	63	83	62	84	81
β-1,4-Glucose	1	1	1	1	1	1	1	1	1	1
Total	100	100	100	100	100	100	100	100	100	100

* **Lipid-linked saccharides** were from chloroform-methanol fraction (Section 3.3.2); **starch** from DMSO fraction (Section 3.3.3); **Pectins** from the phosphate buffer, ammonium oxalate and 0.1 M KOH fractions (Sections 3.3.1, 3.3.4 and 3.3.5); **Hemicelluloses** from 0.1 M and 4 M KOH fractions (Sections 3.3.5 and 3.3.6); **Cellulose** from acid-insoluble fraction (Section 3.3.8); **β-1,4-Glucose** from endo-cellulase digestion of acid-soluble fractions of wild type and all mutants grown at 21 °C and 31 °C.

Glucose of cellulose and starch determined by anthrone/H_2SO_4 test, all other determined by GC/MS.

6.4 DISCUSSION

6.4.1 Overall Picture of Pectins and Hemicelluloses

Changes in the monosaccharide composition and glycosidic linkage patterns of the non-cellulosic polysaccharides in the mutants were relatively small as were the differences in their estimated quantities when compared to changes in cellulose amounts (*rsw5* was a partial exception to this picture in that its root showed changes in cellulose content that were little bigger than those in non-cellulosic polysaccharides). This suggests, therefore, that the reduced cellulose biosynthesis in the mutants did not affect the synthesis of pectins and hemicelluloses in any major way suggesting that the genes concerned are involved specifically in cellulose synthesis. This is reasonable because cellulose and non-cellulosic polysaccharides are synthesed at quite different locations in the cell (Delmer and Stone, 1988; Section 1.5.1) but shows that the rate of synthesis of matrix polysaccharides is not kept closely in step with the rate of cellulose production. Decreased use of glucose for cellulose synthesis appears to result in glucose being diverted into a non-crystalline cellulose in the shoot and into starch in the root with little change in pectins and hemicelluloses. Brett and Waldron (1990) suggested that the main controls for polysaccharide production were at the level of the polysaccharide synthase. If correct, the reduced cellulose synthase activities of the mutants at high temperature therefore did not have a direct effect on the activities of synthases making non-cellulosic polysaccharides.

6.4.2 1,4-Linked Glucose

Methylation analysis shows 4-Glc*p* in the ammonium oxalate, 0.1 M KOH and 4 M KOH fractions of all mutants and wild type, whether grown at 21 °C or 31 °C. There is also 4,6-Glc*p* in the KOH fractions as expected for XG. The glucose could be further divided into two types: endo-cellulase releasable and non-releasable. Almost all of the endo-cellulase releasable glucose in shoots of *rsw1, rsw2* and *rsw3* at 31 °C is recovered as the purified β-1,4-glucan. Although such β-1,4-glucan was not successfully purified in other cases, there is endo-cellulase releasable glucose in wild type and *rsw5* shoots grown at 31 °C and in shoots of all genotypes grown at 21 °C. The demonstrated specificity of the endo-cellulase makes it highly probable that the glucose released was β-1,4-linked. Glucose release precludes a highly substituted structure of the sort seen in XG where, although endocellulase can release oligosaccharides (Zablackis et al, 1995), the linkages of xylose residues to the β-1,4-glucan backbone prevent endocellulase from releasing glucose. I therefore favour the view that a β-1,4-glucan is present even where it cannot be purified but that the glucan is strongly associated or perhaps covalently linked to non-cellulosic polysaccharides or other compounds in the fraction. This would prevent glucan purification by the method used in this study.

The glucose in the 0.1 M and 4 M KOH fractions that is not released by endocellulase could come from (1) incomplete digestion of the glucan since only 92 % of total glucose was released under optimal conditions from the glucan purified from *rsw1*, *rsw2* and *rsw3*; (2) the glucose of XG which is not released by endocellulase as a result of the frequent substitutions along the β-1,4-glucan backbone. The nature of the non-released glucose that is present in the ammonium oxalate fraction is unknown although 4-Glc*p* has been found by

several authors pectin fractions (Carpita, 1984; Carpita and Kanabus, 1988; Shea et al, 1989; Kikuchi et al, 1996).

If this hypothesis is correct, the glucan for some reason does not exist in roots where no endocellulase releasable glucose is detected in any genotype or growth temperature.

6.5 SUMMARY

The monosaccharide composition and glycosidic linkage patterns of the pectins and hemicelluloses in the roots and shoots of all mutants resembled those of the wild type irrespective of whether the plants were grown at 21 or 31 °C. Changes in the levels of total pectins and hemicelluloses were small relative to the changes in cellulose biosynthesis, and each mutant probably showed only small differences in the proportions of different types of pectins and hemicelluloses. Moreover, with reduced cellulose production, glucose was redistributed into non-crystalline cellulose (in shoots) and into starch (in roots) and slightly into wall matrix polysaccharides (pectins and hemicelluloses). The evidence supports the view that mutations are in genes quite directly involved in cellulose biosynthesis and shows that the rate of production of non-cellulosic polysaccharides is not closely coupled to the rate at which cellulose is being synthesised.

CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE STUDIES

7.1 SUMMARY

This study has shown mutants *rsw1, rsw2, rsw3* and *rsw5* have defects in genes that are involved in cellulose biosynthesis. The reduced cellulose biosynthesis in the shoots of mutants grown at 31 °C is almost compensated by production of non-crystalline cellulose (Sections 5.3.3 and 5.4.5). It suggests that the coupling of β-1,4-glucan polymerisation and crystallisation is affected in the mutants at their restrictive temperature. Freeze fracture observations have shown that the putative cellulose synthase complexes (rosettes) of *rsw1* disappear from the plasma membrane at 31 °C and its gene has been cloned (Arioli et al, 1998). It encodes a catalytic subunit of cellulose synthase.

The formation of non-crystalline cellulose accompanying the reduced cellulose synthesis *in vivo* is different from the defect seen with cell-free preparations for cellulose biosynthesis *in vitro* where callose is the major product when the yield of cellulose is limited.

Roots of mutants do not accumulate non-crystalline cellulose along with their reduction of cellulose synthesis. The reason for this is not understood.

7.2 FUTURE STUDIES

Many possible directions for future studies exist:

1) Biochemistry of cellulose biosynthesis: Measurements of ^{14}C-sucrose and glucose incorporation into the wall polysaccharides of *rsw5* and wild type grown at 21 and 31 °C could show whether *rsw5* has a reduced incorporation into cellulose and perhaps other wall polysaccharides when it shows more radial swelling on sucrose than on glucose.

The chemical and physical properties (DP, crystallinity and interaction with matrix polysaccharides) of non-crystalline cellulose and cellulose could be determined for the wild type and all mutants.

Non-crystalline cellulose could be used as a probe to search for factors (protein or other carbohydrates) which may either activate β-1,4-glucan polymerisation or control cellulose crystallisation. Techniques such as HPLC and two dimensional electrophoresis can be applied to analyse any associated molecules during the extraction of non-crystalline cellulose.

2) Cloning of the *RSW2*, *RSW3* and *RSW5* genes would make a major contribution to understanding their roles in cellulose production.

3) Cellulose biosynthesis *in vitro* cell-free preparations for cellulose biosynthesis could be observed to see whether the major product is non-crystalline cellulose in mutants or callose as in the wild type.

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