The Biochemistry and Molecular Biology of Intercellular Adhesion in Plant Tissue Culture

by

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Abstract

The adhesion between neighbouring plant cells is established as cells are formed during cytokinesis through the middle lamella that is made principally of pectins and proteins. Pectins are secreted into the cell wall in a highly methylesterified form and subsequently de-esterified *in muro* by pectin methyl esterase (PME, E.C. 3.1.11). The present study reports on the biochemical characterization and immunochemical analyses of phosphate buffer/EDTA pectic extracts associated with cell-cell adhesion in suspension cultures of wild type (WT), salt tolerant (HHS) cell lines and synchronized Arabidopsis suspension cultures. Using the synchronized cultures, The PME-mediated configuration of pectins at the onset of adhesion during cytokinesis, was assessed through the analysis of the expression patterns of the PME isoforms annotated to be expressed throughout the cell cycle

The wild type Arabidopsis seemed to maintain the intercellular adhesion through the gelling of the highly methylated JIM7 recognized homogalacturonans that were shown to be abundant in the primary cell walls, middle lamellae and cellular junctions, possibly due to the hydrophobic interactions between the methoxy groups. The rhamnogalacturonan-I fraction was rich in arabinan side chains reflecting the proliferative state of the cells. The increase in arabinan content was accompanied by a reduction in the galactan content 4 days after subculturing. The cell walls of salt tolerant Arabidopsis contained the JIM7 and LM7recognized epitopes along with a high degree of branching of rhamnogalacturonan-I carrying galactans and arabinans as side chains. The change in the detected epitopes is thought to play a role in the ability of the cells to withstand the high osmotic pressure and increase the in the level of adhesion between cells. The JIM5 low methylesterified HGs were less abundant in both cultures, and the absence of the 2F4 antibody recognizing the Ca^{2+} egg boxes could be attributed to the scarce amounts of Ca^{2+} present in the culturing medium

The immunochemical studies of the pectin extracted from the synchronized Arabidopsis suspension cultures after washing out aphidicolin indicated that the recognition of both of JIM7 and JIM5 varied in parallel during the cell cycle, whereas, the recognition of arabinan increased during the cell division. The sequence and phylogenetic analysis of ten PME isoforms that were annotated to be expressed at one or more phases of the cell cycle of synchronized *Arabidopsis thaliana* suspension cultures (Menges and Murray, 2002 and 2003), revealed that only five of these genes could be PMEs. The genes At4g02330, At1g02810, At2g26440, and At2g47550 were thought to be of type II PMEs which have a pre-pro-catalytic domains and At5g47500 is a type I PME that lack the pro-region. The amino acid sequence of At4g12390 showed similarities with the N-terminal pro-peptides of plant PME and invertase inhibitors.

The expression of several PME genes was studied in suspension cultures of *Arabidopsis thaliana* synchronised using aphidicolin. Semi-quantitative PCR experiments showed that the expression of At5g47500 transcript was always detected during M phase of the cell cycle. The rest of the genes failed to show consistent patterns of expression. Northern blots revealed that mRNA coding for At5g47500 decreases during S and G2 phases and accumulates during the M phase of the cell cycle. Our results suggest that this PME isoform is involved in the modulations of the cell walls as the cells are going through division and cytokinesis.

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List of abbreviations

AIM	Alcohol insoluble material
AIR	Alcohol insoluble residue
AGPs	Arabinogalactan proteins
Api	Apiose
Ara	Arabinose
biotin-UTP	Biotin linked uridine triphosphate
bp	Base pair
BSA	Bovine serum albumin
BSA /PBST	Bovine serum albumin in phosphate buffer saline containing Tween 20
CalS	callose synthase
CDKs	Cyclin dependent kinases
CDKB2;2	Cyclin dependent kinase B2;2
Cnr	Colourless non-ripening tomato
CWP	Cell wall proteins
CYC	cyclins
cycD3	cyclin
DAPI	4'-6-Diamidino-2-phenylindole
DCEP	Nuclease free water
DM	Degree of methylesterification
DNA	Deoxy ribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EGTA	ethylene glycol bis (ß-aminoethylether)-N, N, N', N'- tetraacetic acid
Fig	Figure
FITC	Fluorescein isothiocyanate
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
δ	Gamma
GDP	Guanidine diphosphate

Glu	Glucose
GluA	Glucuronic acid
GRP	glycine rich protein
GT	Glycosyltransferase
h	Hour
HHS	Habituated to High Salt
HG	Homogalacturonans
HG-galacturonosyl transferase	Homogalacturonan-galacturonosyl transferase
HG-PT	homogalacturonan methyl transferase
HPAE-PAD	High performance anion exchange chromatography coupled with pulsed amperometric detection
HRGP	hydroxyproline-rich glycoprotein
M	Molar
mM	Millimolar
Man	Mannose
M/A index	Metaphase/anaphase index
µg	Microgram
µl	Microliter
MPBS	Fat free milk in phosphate buffer saline
nm	Nanometer
NMP	nucleotide monophosphate
NDP	nucleoside diphosphate
oPME	Pectin methylesterase extracted from orange peel
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline containing Tween 20
PC	Paper chromatography
PCW	Primary cell wall
PG	Polygalacturonase
PGA-GalA transferase	polygalacturonic acid-GalA transferase
PME	Pectin methyl esterase
PMEG buffer	Buffer made of 50 mM PIPES, 2 mM MgSO4, 5

	mM EGTA, 2% glycerol, pH 6.8
PMT	pectin methyltransferase
PRP	proline rich protein
RT	Reverse transcription
RT-PCR	Reverse transcription-Polymerase chain reaction
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan II
Rha	Rhamnose
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT–PCR	Reverse transcription–PCR.
sec	Second
SAM	S-adenosyl-methionine
SDS	sodium dodecyl sulfate
T/Ca/S	Buffer made of 20 mM Tris-HCl pH 8.2, 0.5 mM $CaCl_2$ and 150 NaCl mM
TFA	Triflouroacetic acid
UA	Uronic acid
UDP	Uridine diphosphate
UTP	Uridine 5´ -triphosphate
UV	Ultra violet irradiation
v/cm	Volume to centimeter
v/v	Volume to volume ratio
w/v	Weight to volume ratio
Xyl	Xylose
XG	Xyloglucan
XGA	Xylogalacturonan
XET	Xyloglucan endotransglycosylase

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I recognize that this research would not have been possible without the financial assistance of Philadelphia University/ Jordan, and express my gratitude to the administration of the University.

Declaration

I hereby confirm that the work presented in this thesis is my own and expressed in my own words. It has not previously been submitted for a degree at any other institution. Any use made within it of works of other authors in any form is properly acknowledged at the point of use. A list of the references employed is included.

Signature ……..

Date …….

Chapter 1

General Introduction

1.1 Plant cell wall: Structure and function

The presence of a cell wall is one of the major features that distinguish the plants from animals. The plant extracellular matrix (cell wall) is an intricate structure that constitutes the raw material that is used to manufacture textiles, paper, lumber and other products. It is involved in maintaining the size, shape, growth and development of plant cells, and their protection from the adverse changes in the surrounding environment including the invasion of pathogens and predators, dehydration and mechanical abrasion (Gaffe *et al.*, 1997; Willats *et al*., 2006). The cell wall material is also of great importance for human and animal nutrition. As the whole world is in a rush for the production of biofuels, cellulosic alcohol is increasingly becoming an option for liquid transportation fuels (Chapple *et al*., 2007). The cell wall is a characteristic, semi rigid, dynamic interface that surrounds the plant cell and defines the distinctive morphology of the entire plant.

The plant cell deposits its wall through secretary pathways during cytokinesis. The mitotic spindle remnants give rise to a plant-specific polarized microtubular array known as a phragmoplast which is laid down in the center of the dividing cell, and serves as a framework for the assembly of the Golgi derived exocytic vesicles. More evidence is emerging supporting the turnover of parental cell wall components through plasma membrane and cell wall derived endocytic vesicles carrying the cargo to the developing cell plate (Baluška *et al*., 2002; Dhonukshe *et al*., 2006). The cell plate expands from the middle out through the addition of secretory vesicles at the periphery. It fuses with the parental cell plasma membrane/ primary cell wall at specific cortical sites previously defined by the preprophase band of microtubules (Verma, 2001). Once the attachment has taken place, the cell plate undergoes a process of maturation during which callose is replaced by cellulose and pectin (Assaad, 2001). The daughter cells proceed to deposit the primary cell wall on both sides of the cell plate, which becomes the middle lamella and the region of intercellular attachment. The internal turgor pressure developing in rapidly growing soft tissues keeps the primary cell walls in permanent tension. It tends to force the plant cells towards a spherical shape.

The cell junctions are subjected to forces tending towards cell separation (Jarvis, 1998). The tensile force initiates the development of an intercellular space at a predetermined controllably degrading localized region of the older parental primary cell wall, thus separating the cell from its neighbours at the tricellular junctions (Jarvis, 1998; Jarvis, 2003; Willats *et al.,* 2001c). This space may be filled with pectins glueing the older middle lamella of the parent cell with the newly forming middle lamella (Parker *et al.*, 2001) or open up to form an intercellular air space (Jarvis, 2003). The middle lamella is made principally of pectins and proteins (Swords and Staehelin, 1993; Smallwood *et al*., 1994 and 1995), and the primary cell wall consists of a rigid skeleton of cellulose microfibrils embedded in a gel-like matrix composed of pectic compounds, hemicellulose, enzymes and structural proteins (Carpita and Gibeaut, 1993; Willats *et al*., 2001b).

Certain specialized cells lay down a further, thicker, more rigid and often multilayered secondary cell wall, that is deposited to the inside of the primary wall after the cell has stopped growing. It is mainly made of cellulose and hemicellulose impregnated with lignin (Brett and Waldron, 1996), however, the secondary cell walls of the macrosclereids and osteosclereids of pea testa and flax fibers are non-lignified with cellulose, hemicellulose and pectins constituting the major components (McCartney and Knox, 2002, His *et al*., 2001). In Arabidopsis anthers, the pollen mother cell develops a specialized secondary cell wall composed almost entirely of callose (Francis et al., 2006). Secondary cell walls are frequently a feature of cells specialized in providing mechanical support and structural reinforcement to the plant body. The presence of a secondary cell wall lining the water and solute conducting cell, allows the cell to resist forces of gravity and/or tensional forces associated with the transpirational pull of a column of water.

Conduits called plasmodesmata penetrate cell walls, providing pathways for transporting cytoplasmic molecules from one cell to another (Brett and Waldron, 1996; Orfila and Knox, 2000). The plasmodesmata are laid down at cytokinesis during the formation of the new cell plate. Secondary

plasmodesmata are inserted into the cell wall during phases of rapid cell expansion.

1.2 Architecture of mature cell walls

The current model of the plant cell wall visualizes the wall structure as containing a number of structurally independent networks which, when superimposed upon one another, interact further to give rise to the whole complex structure as shown in fig.1.1.

A cellulose-hemicellulose network is a strong load bearing fibrillar network that gives each cell its stable shape (Cosgrove, 2001; Micheli, 2001). This network is composed of cellulose microfibrils that consist of many parallel chains of unbranched (1→4)-ß-D-glucose polymers. Hemicelluloses typically are branched polysaccharides characterized by a strong tendency to bind to the surface of cellulose, and may link two microfibrils together (Cosgrove, 1997). Hemicelluloses are thought to form tethers that can hold the microfibrils in place and/or as a lubricating coating to prevent direct microfibril-microfibril contact. During growth, the molecular tethers are assumed to relax to allow separation of the microfibrils (Brett and Waldron, 1996; Cosgrove, 2001).

A structurally independent and functionally interacting pectin network and protein (extensin) network are believed to occupy the space within the cellulose-hemicellulose network. The functional interaction between the pectin network and other networks seems to play a role in controlling the pore size in the wall, and accordingly the movements of molecules through the wall (Fleischer *et al*., 1999). The extensin network is made of a hydroxyproline rich glycoprotein. It is laid down in a pattern that is perpendicular to the cellulose-hemicelluloses network, making the walls more rigid. The lignin network is the last to be formed, transforming the wall into a rigid impermeable structure specialized to provide extra strength. Cutins and suberins may also be present in certain cell types to decrease the permeability of the cell and to

provide an extra protective barrier. Once assembled, the cell wall architecture of growing cells has to be extensible. Plant growth and development rely on discrete and coordinated changes in the original cell wall structure with the deposition of newly synthesized wall material (Carpita *et al*., 1996; Carpita *et al*., 2001).

The architecture and function of the cell wall of germinating pollen grain extending through the style seems to differ from the cell walls of other vegetative plant cells. The initial wall formed at the growing tip is made of pectin, while another mature secondary wall made of callose, proteins, arabinogalactan proteins and pollen extensin like proteins is deposited behind the growing tip. In some plant species, cellulose was found to be associated with the callose wall though no significant amounts of xyloglucan were detected in pollen tube cell walls (Lord, 2000; Abreu and Oliveira, 2004; Geitmann and Steer, 2006).

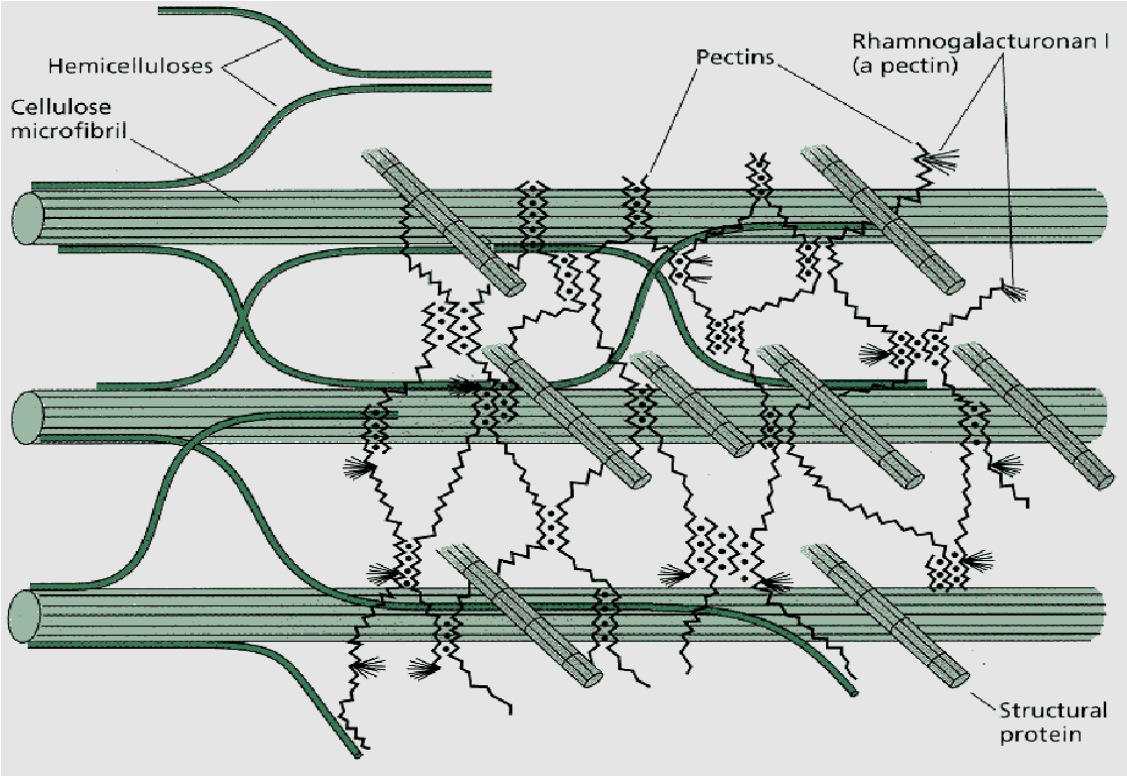


Fig. 1.1: Diagrammatic representation of the architecture of the current model of the plant primary cell wall (Brett and Waldron, 1996).

1.3 Intercellular Adhesion

Adhesion between plant cells is a fundamental feature of plant growth and development and is an essential part of the strategy by which growing plants achieve and maintain mechanical strength. The adhesion between neighbouring plant cells is established as cells are formed during cytokinesis through the middle lamella in order to create an apoplastic continuum. The plant cell remains in contact with the same neighbouring cells throughout its growth and development, except during the phases of intrusive growth, like the penetration of pollen tube through stylar tissue (Jauh and Lord, 1996; Bosch *et al*., 2005; Mollet *et al*., 2000), the differentiation of tracheids (Kalev and Aloni, 1998), elongation and increase in the diameter of vessel elements, xylem fibers, laticifers, and flax fiber cells (Ronald *et al.*, 1995; Serpe *et al*., 2001; Willats *et al*., 2001b; Jarvis, 2003; Siedlecka *et al*., 2008). The development of intercellular spaces in some tissues seems to occur as a response to the tensile forces created by turgor-imposed stresses that tend to make cells adopt a spherical shape, and then as a result of the controlled splitting at the middle lamella, the cells tear away from their neighbours at each corner (Jarvis, 1998). The tricellular junctions and their reinforcing zones act as the first line of defense against cell separation (Willats *et al.*, 2001b and 2001c; Jarvis *et al.*, 2003). Several studies have shown the importance, degree of coordination, modification and localized targeted extracellular metabolism between a network of intercellular spaces, middle lamellae and the involved cells. The increase in the shelf life and crispness of fruits and vegetables is dependent on the modifications of pectin and maintenance of cell-cell adhesion (Orfila, et al., 2001; Guillon *et al*., 2008). The fruit of the *Colourless non-ripening* (*Cnr*) tomato mutant was characterized with a mealy phenotype and a much reduced cell-to-cell adhesion in the pericarp of the ripe fruit, as a result of the reduction in the activities of a variety of pectin degrading enzymes (Thompson *et al*., 1999; Eriksson *et al*., 2004). The occurrence and role of pectic polysaccharides at the outer face of unadhered cell walls at plant surfaces is less well documented. In many cases, a layer of

pectin is thought to occur between the cell wall and the cuticle in aerial parts of land plants (Willats *et al*., 2001b).

1.4 Chemical structure of major wall polysaccharides

Eukaryotic cell walls differ significantly in composition and physical structure from prokaryotic cell walls. The cell walls of fungi are made of chitin and beta glucans, while mannan is a major constituent of cell walls in yeast. Plants comprise ≈ 35-40 cell types, each of which is distinctive in its position, size, shape, and wall characteristics. All the plant cells possess a primary cell wall which mainly consists of cellulose, hemicellulose, pectin and protein. Cellulose is the most abundant polysaccharide in plants followed by hemicelluloses and pectin. The primary cell walls (type I) of dicots and non-commelinoid monocots contain mainly cellulose, xyloglucan and pectin, with minor amounts of arabinoxylans, glucomannans and galactoglucomannans (Yong *et al*., 2005). The primary walls of grasses (type II) are composed of cellulose microfibrils, glucurono-arabinoxylans, and mixed-linkage (1→3),(1→4)-β-D-glucans, together with smaller amounts of glucomannans, xyloglucans, pectins, and a network of polyphenolic substances (McCann *et al*., 2007).

1.4.1 Cellulose and callose

Cellulose is a tightly packed aggregate of linear polymers of (1→ 4)-β-linked glucose residues, ranging in size from 2000 to 25000 glucose residue, forming long parallel chains of fibers. The structure of cellulose microfibrils is remarkably uniform throughout the higher plants (Brett and Waldron, 1996). They tend to have a very high tensile strength equivalent to steel. Cellulose is important industrially as it is the major constituent of cotton fibers, wood and paper. Despite the fact that humans are incapable of digesting cellulose, it is an important ingredient in human diet, it is presence in the human diet aids in

the smooth working of the intestinal tract. Some animals can digest cellulose through the activity of cellulose hydrolyzing enzymes secreted by symbiotic bacteria living in their guts.

Cellulose synthesis occurs at rosette like synthase complexes that consist of six hexagonally arranged subunits that are embedded in the plasma membrane. Each rosette subunit contains six CesA proteins, providing a total of thirty-six CesA proteins per rosette. Cellulose microfibrils are formed by the crystallization of 36 β-glucan parallel chains of intermediate length (20-40nm) held together by hydrogen bonds and van der Waals forces (fig. 1.2). *CESA* genes encoding cellulose synthases are regarded as the major sink for atmospheric carbon, and are known to occur in many isoforms, however, the various isoforms in each species are differentially expressed (Galway, 2006). Arabidopsis contains 10 *CESA* genes named *AtCESA1- AtCESA*10 forming a subfamily of the cellulose synthase like genes. *CESA1*, *CESA3*, and *CESA6* are required for cellulose biosynthesis in primary cell walls, whereas *CESA4*, *CESA7,* and *CESA8* are required for cellulose biosynthesis during secondary wall deposition (Lerouxel *et al*., 2006). The orientation of cellulose microfibrils correlates well with the orientation of cortical microtubules, suggesting that microtubules somehow control the orientation of microfibril deposition (Lerouxel *et al*., 2006; Paredez *et al*., 2006). Using an Arabidopsis *CESA6* mutant (*prc1-1*) with a yellow fluorescent-tagged version of *CESA6*, the CesA protein within a rosette, was shown to move within the plasma membrane in a linear track that aligned with the cortical microtubule. A significant pool of the labelled CesA6 protein was located in the Golgi (Paredez *et al*., 2006).

In plasmolysed *Tradescantia virginiana* leaf epidermal cells, the plasma membrane was pinned to the cell walls through cellulose microfibrils produced by the cellulose synthase complexes. Cellulase treatment resulted in the loss of some of the connecting fibres (Lang *et al*., 2004). The biochemical analyses of the cell wall components indicated that the plant compensates for the disruption in the synthesis of any of the components like cellulose, and depending on the plant species, through the construction of walls that are richer in pectin (Encina et al., 2002 and Manfield et al., 2004), and in some

cases structural proteins (Sabba et al., 1999). The reduction in cellulose formation due to the application of herbicide like isoxaben and dichlobenil (2,6-dichlorobenzonitrile) that interfere with cellulose synthesis to the suspension cultures of *Phaseolus vulgaris* and *Arabidopsis thaliana* respectively is partly compensated for by an increased production of pectic domains like homogalacturonan (HG). The calcium mediated cross-linking of HG is thought to compensate to some extent for the diminished load bearing capacity of the disrupted cellulose network (Encina *et al*., 2002; Manfield *et al*., 2004; García-Angulo, et al., 2006).

Callose is a linear (1→3)-β-glucan with some (1→6)- branches that has been localised at cell plate of dividing cells, plasmodesmata, root hair, spiral thickenings in tracheids, around pollen mother cells, sieve plates and as a response to wounding, pathogen infection and physiological stress (Hong *et al*., 2001; Verma, 2001; Francis *et al*., 2006). In higher plants, the activity of callose synthase (*CALS*) is found to be associated with the cellulase synthase fraction of the plasma membrane. Callose synthesis occurs within the cell plate. The developing immature cell plates are fluid and wrinkled, whereas mature cell plates are more stiff and flat as a result of the removal of callose and deposition of cellulose and pectin (Assaad, 2001). The presence of callose layer in pollen tubes is thought to help in the extensin and mechanical stabilization of the growing pollen tube (Mollet *et al*., 2000; Bosch *et al*., 2005; Geitmann and Steer, 2006).

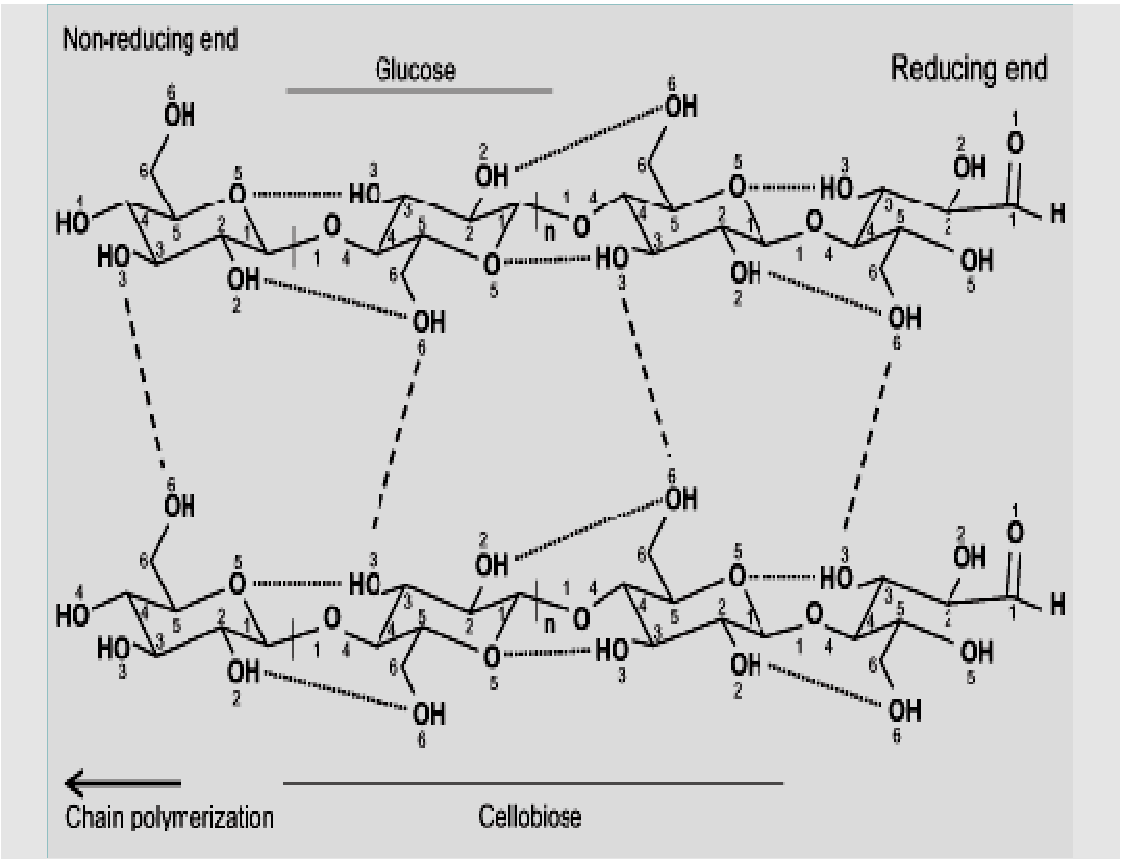


Fig. 1.2 Structure of cellulose polymers (Festucci-Buselli, *et al*., 2007)

1.4.2 Hemicellulose

The non-cellulosic (hemicellulose and pectic) polysaccharides are assumed to be constructed in the Golgi apparatus, packaged in secretory vesicles, and exported to the cell plate forming zone, where they are integrated with cellulose microfibrils afterwards (Zhang and Staehelin, 1992; Ridley *et al*.,2001; Lord and Mollet, 2002; Reiter, 2002). Hemicelluloses can be grouped into four main classes according to the main type of sugar residues present: xylans, xyloglucans, mannans and mixed linkage β-glucans. Within each of these classes a number of sub-groups can be divided due to the chemical nature of their side chains (Lerouxel, *et al*., 2006; Hoch, 2007). It is thought that cellulose synthase like (*CSL*) genes might encode Golgi-localized glycan synthases that are involved in the biosynthesis of hemicelluloses. The *Csls* are a family of genes that have sequence similarity to the *CESA* genes

and appear to be present in all plant genomes. The cellulose synthase-like (*CSL*) genes are subdivided into groups *CSLA* to *CSLH.* Certain *CSL* groups are common to all plants, whereas others are present only in specific species of plants (Lerouxel *et al*., 2006).

The dicots and monocots differ substantially in their hemicellulose composition. Xyloglucan (XG) is the major hemicellulose in type I primary walls, and glucoronoarabinoxylans (GAX) are characteristic of type II cell walls (Cordenunsi et al., 2008). Xyloglucan is composed of ß-(1→4)-D-glucan backbone substituted with (1→6)-α-D-xylose or xylose-galactose-fucose moieties (Reiter, 2002). Glucuronoarabinoxylans have a linear β-(1→4)-linked D-xylose backbone with both neutral and acidic side chains attached at intervals along its length. The acidic side chains are terminated with glucuronosyl or 4-*O*-methyl glucuronosyl residues, whereas the neutral side chains are composed of arabinose and/or xylose residues. Xyloglucans are considered as the most important partners of cellulose microfibrils in rendering strong mechanical properties of type I cell walls.

Glucuronoarabinoxylans are thought to have a similar role in type II cell walls (Cosgrove, 2000). Xyloglucan can either bind to the surface of cellulose microfibrils or cross link the adjacent microfibrils. Besides to their structural function, xyloglucans are also known to serve as reserves in seeds (Hoch, 2007). The interaction between xyloglucan and cellulose was claimed to occur when the xyloglucan molecules assembled in the Golgi apparatus, are secreted into the cell wall in a soluble form and integrated with the newly synthesized cellulose microfibrils. The linkage between xyloglucan and pectin is thought to be formed through NDP-sugar-dependent chain elongation of xyloglucans upon an RG-I primer present within the endo-membrane system, most likely the Golgi bodies (Popper and Fry, 2008). In rose suspension culture cells, about one third of the xyloglucan is covalently linked to rhamnogalacturonan I (Thompson and Fry, 2000).The immuno-labelling of the *mur*1 Mutant of Arabidopsis using the CCRC-M1 monoclonal antibody, showed that the fucose containing xyloglucans were restricted to the apices of the primary and lateral roots, root hairs and the walls of the epidermis and

pericycle layers of the mature roots (Freshour *et al*., 2003). The *mur1* mutant of Arabidopsis is deficient in fucose in the shoot system of the plant. The gene associated with *mur1* was shown to be blocked at the first nucleotide sugar inter-conversion step, in which a GDP-mannose-4,6-dehydratase is used to biosynthesize GDP-fucose, which is the sugar nucleotide substrate required by the fucosyltransferase responsible for the incorporation of fucose.

Xyloglucan metabolism plays a key role in the control of cell elongation and enlargement by serving as a substrate for xyloglucan endotransglucosylase (XET), which is capable of cutting and rejoining the intermicrofibrillar xyloglucan chains. Newly synthesized XG polymers are incorporated into the wall by the grafting activity of XET (Pilling and Hőfte, 2003; Chanliaud *et al*., 2004; Popper and Fry, 2005). The exogenous application of auxin to intact plants is known to induce rapid elongation in the plant tissues. The cell wall acidification is an essential component of auxin-induced cell expansion. The reduction in apoplastic pH has been suggested to activate cell wall-modifying enzymes including expansins, which catalyze the breakage of bonds between cellulose and hemicellulose and rearrange cell wall polymers (Cosgrove, 1997).

Several varieties of the mannan polysaccharides have been characterized, including pure mannans, galactomannans, glucomannans, and galactoglucomannans. The backbones of mannan consist either of β-(1→4) linked D-mannose units (mannans), or of a combination of β-(1→4) linked D-mannose and β-(1→4) linked D-glucose residues (glucomannans). Both of galactomannans and galactoglucomannans have D- galactose side chains in varying abundance (Hoch, 2007). Mannan polysaccharides are widespread among land plants, bryophytes and charophytes and are also present in many algal species (Popper and Fry, 2003). They appear to have a structural reinforcing role and act as a major storage polysaccharides in the endosperms of some plant species (Liepman *et al*., 2007). In Arabidopsis, mannans were immunolocalized in the thickened secondary cell walls of xylem elements (Handford *et al*., 2003). Very little mannan could be detected in cells lacking secondary thickenings, such as leaves (Zablackis *et al*., 1995).

In wood cell walls of Sitka spruce, mannans were abundant in early and late wood and scarce at the resin channel forming cells (Altaner *et al*., 2007). In dicotyledonous plants, such as peas, Arabidopsis and tobacco, the occurrence of xylans and arabinoxylans was restricted to the secondary cell walls as in xylem and sclerified parenchyma (McCartney *et al.*, 2005).

1.4.3 Proteins

There may be as much as several hundred proteins, whether structural proteins or enzymes, in the cell wall. Several proteomic studies performed on Arabidopsis revealed the presence of at least 404 cell wall proteins (CWPs, Pont-Lezica, 2008). About 87% of these CWPs were described as functional proteins. The remaining 13% are proteins of unknown functions. Proteins acting on polysaccharides are the most abundant. The apoplasmic enzymes are classified into three different categories, soluble, ionically bound and covalently bound- depending on the treatment necessary for their release from cell walls (Carpin *et al*., 2001; Jamet *et al.,* 2008). Examples of proteins with enzymatic activities include peroxidases, pectinases, pectin methylesterases, cellulases, invertases and expansins.

Proteins are involved in the regulation of several important events related to plant growth and development, including cell expansion and proliferation, formation and differentiation of vegetative and floral organs, and sexual reproduction. According to Showalter (2001), CWP could be classified according to their most abundant amino acid components into four major classes including: hydroxyproline-rich glycoproteins (HRGP), proline-rich glycoproteins (PRP) and glycine-rich glycoproteins (GRP).

Arabinogalactan proteins (AGPs) are a widely distributed hydroxyproline-containing class of proteoglycans. The AGP consists of a hydroxyproline-rich core protein which is decorated by arabinose and galactose-rich polysaccharide units. Several AGPs are characterized by a C-terminal glycosylphosphatidylinositol (GPI) anchor that allows for their attachment to

the plasma membrane. They are present in the majority of plants. In bryophytes, AGPs were detected at the plasmalemma/wall interface of water conducting cells (Ligrone *et al*., 2002). The AGPs are Golgi synthesized and are among the first components laid down in the developing cell plates (Freshour *et al*., 1996). In higher plants, these molecules are involved in the development and differentiation of cells and tissues (Smallwood *et al*., 1994; Freshour *et al*, 1996; Wiśniewska and Majewska-Sawka, 2007), in pollen-pistil adhesion, pollen tube germination and guidance through the style (Pilling and Hőfte, 2003; Abreu and Oliveira, 2004), in addition to their role in signalling for programmed cell death (Showalter, 2001; Ligrone *et al*., 2002). AGP-rich extracts isolated from the media of embryogenic and non-embryogenic suspension cultures of sugar beet (*Beta vulgaris* L.) are able to enhance the organogenesis of guard protoplast-derived calli and to increase the number of shoots formed (Wiśniewska and Majewska-Sawka, 2007). Much of the evidence relating to AGP function has been based on the use of monoclonal antibodies directed against their carbohydrate moieties (Knox, 1997; Ligrone *et al*., 2002).

Extensin is a well-studied HRGP. It is described as a rodlike firmly bound highly insoluble wall protein that is reported to play a role in the cessation of growth; however, new evidence is emerging emphasizing its essential role in the initiation of growth during cytokinesis. The positively charged extensin scaffold reacts with acidic pectin to form extensin pectate, which further templates the cell plate components in the new cross wall and enable its fusion with the mother cell wall (Cannon *et al*., 2008).
Expansins are cellulose bound enzymes responsible for acid induced loosening and extension of cell walls (Cosgrove, 1997). They are categorized into two families called α-expansins and β-expansins. Grass pollen allergens have significant sequence similarities to β-expansins (Cosgrove, 2000). Both families have similar rheological effects though acting on different substrates. The expansins can disrupt the attachment sites between cellulose and xyloglucans promoting the slippage of the microfibrils followed by the growth and extension of cells. Higher levels of α-expansins activities were found in

type I cell walls, whereas, β-expansins have stronger selectivity for type II walls (Valdivia *et al*., 2007).

Yieldins and xyloglucan endotransglycolsylases (XET) are other apoplasmic proteins that appear to cause alterations to the tensile load bearing cellulose microfibrils- hemicellulose network.

1.4.4 Pectin

Pectins, one of the main components of the extracellular matrix, are complex polysaccharides containing homogalacturonans (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) domains. Xylogalacturonan (XGA) and apiogalacturonan can also be found in particular species. These polysaccharides appear to be present in all cells but their relative abundance and structural details differ between cell types and species. It was reported that one third (~35%) of the primary cell wall molecules of dicotyledonous and non-graminaceous monocotyledonous plants are made of pectin, and about 5 -10% of the walls of grasses are made of pectins (Jarvis, 1984; Carpita & Gibeaut, 1993; Willats *et al*., 2001b; Lord and Mollet, 2002; Guillemin *et al*., 2005). Pectin content is greatly reduced or absent in non-extendable secondary cell walls, however. Trees like *Pinus sylvestris* are reported to have lignified pectic middle lamellae to withstand the compressive force developing as the tree grows (Hafren *et al*., 2000). Pectin is the main charged component of plant cell walls. It consists partially of negatively charged polygalacturonic acid chains capable of interacting with positively charged molecules such as polyamines or cations. It can bind to proteins carrying positive charges and thus influence the cell wall properties during assembly and different developmental stages (Carpin *et al*., 2001).

1.4.4.1 Biosynthesis of pectin

It is estimated that pectins are synthesized from nucleotide sugars by at least 53 different enzymatic activities (Ridely 2001). The biosynthetic enzymes required are glycosyltransferases and decorating enzymes including methyltransferases, acetyltransferases and feruloyltransferases. Glycosyltransferases (GTs) constitute the largest portion of the pectin biosynthetic enzymes; they specialize in the transfer of one glycosyl residue from a nucleotide sugar donor to the non-reducing end of an oligo- or polysaccharide acceptor. Galacturonosyl transferases are present in the cell walls of all land plants. A HG-galacturonosyl transferase isoform (*GAUT1*) was purified from Arabidopsis and was shown to be involved in HG biosynthesis (Sterling *et al*. 2006).

Pectins are synthesized in the *cis*-Golgi apparatus, methylesterified at the C-6 carboxyl in the medial Golgi by pectin methyltransferase (PMT) and modified by the addition of side chains to the backbones in the trans Golgi apparatus, giving rise to RGI , RGII and xylogalacturonan, before exocytosis to the apoplasmic space as highly methylesterified polymer. (Carpita and Gibeaut, 1993; Ridley *et al*., 2001; Willats *et al*., 2001b; Mouille et al, 2007). Nucleotide sugars are the immediate substrates for the synthesis of pectin. They are synthesized on the cytosolic side of the Golgi and transported into the Golgi lumen by specific nucleotide-sugar: nucleoside monophosphate antiporters. The released nucleoside diphosphate (NDP) is hydrolyzed by Golgi-localized nucleoside diphosphatase into nucleotide monophosphate (NMP) and inorganic phosphate which are transported out of the Golgi through the antiporters (Ridely *et al*., 2001). The activity of homogalacturonan methyl transferase (HG-PT) and polygalacturonic acid-GalA (PGA-GalA) transferase were detected in the microsomes of tobacco suspension cultures (Doong *et al*., 1995; Goubet *et al*, 1998). The (HG-PT) was capable of transferring the methyl group from S-adenosyl-methionine (SAM) to the C6 carboxyl of HG, and PGA-GalA transferase catalyses the transfer of GalA from UDP-GalA to a growing GalA- containing HG, RG-I or RG-II.

1.4.4.2 Functions of pectins

Pectins are a family of structurally heterogeneous polymers. The fine structure of the pectic matrix is modified extensively *in muro* during development and in response to local functional requirements by an array of modifying enzymes, many of which have been identified. They play a distinct role in cell wall porosity, expansion and cell-cell adhesion that in turn determines the mechanical properties of the cell wall (Knox, 1992; Cosgrove, 2000; Jarvis *et al*., 2003; Jones *et al*., 2003; Orfila and Knox, 2000; McCartney and Knox, 2002). Pectins have a wide spectrum of applications in several biotechnological, agricultural, nutritional and economical fields. They are used as gelling and stabilizing agents in the production of jams and jellies, fruit juices, confectionary products and bakery fillings. Pectin is also used for the stabilisation of acidified milk drinks and yogurts (Thakur *et al*., 1997; Willats *et al*., 2006). Pectin has beneficial effects on human health. It is thought to lower cholesterol levels, serum glucose levels and may have anticancer activities inducing apoptosis in human colonic adenocarcenoma cells (Willats *et al*., 2006). Oligosaccharide fragments of pectins are thought to have roles in signalling, development and growth during the life cycle of the plant (Dumville and Fry, 2000; Ridely *et al*., 2001). Pectins are primary targets of attack by invading microbes. The pathogen or endogenous secreted enzymes release oligo-GalA (2-20) residues that would function as potent elicitors of plant defense response (Cosgrove, 2005, Pilling and Höfte, 2003, Bédouet, *et al*., 2005). The pectin network was reported to be a target for special developmental modifications during the life cycle of a plant, such as cell wall swelling and softening during fruit ripening (Steele *et al*., 1997; Dumville and Fry, 2000; Barnavon *et al*, 2001; Brummell *et al*., 2004; Almeida *et al*., 2008), cell separation during abscission of fruits, leaves, floral parts, pods, and seeds, pollen grains dehiscence and root cap cell differentiation (Wen *et al*., 1999; Roberts *et al*., 2000).

1.4.4.3 Chemical structure of Pectic polysaccharide

Three major classes of pectic polysaccharides are HG, RG-I and RG-II. The presence of D-galacturonic acid (GaIA) is common to all these classes. Pectin can be extracted from cell walls by a variety of methods, including hot acid, mild alkaline treatment and chelating compounds.

1.4.4.3.1 Rhamnogalacturonan-I (RG-I)

It is an acidic domain consisting of as many as 100 repeats of (1→2)-α–L-rhamnose- and (1→ 4)–α-D-galacturonic acid in which 20-80% of the rhamnose residues are substituted at C-4 with branched side chains of neutral sugars. The neutral sugar side chains include linear chains of (1→ 4)-β-linked D-galactose residues, chains of (1→5)-α-linked arabinose residues that are frequently branched at O-3 and sometimes at O-2 and arabinogalactans I or arabinogalactan II. The arabinogalactan I side chains are generally made of β-(1→ 4) galactose chains with arabinose branches, while arabinogalactan II are highly branched made of (1→ 3)-β- and (1→ 6)- β-linked galactose residues which are more common in proteoglycans but may also be part of RGI (Brett and Waldron, 1996; Ros *et al*., 1996). In some species, for example sugar beet, the arabinose and galactose residues in RG-I side chains can be substituted with ferulic acid esters (Lévigne *et al*., 2004; Guillemin *et al.*, 2005).

RG-I side chains occur in distinct regions of cell walls and stages of cell development (Rhiouey, 1995 a and b; Jones *et al*., 1997; Willats *et al*. 2001a; McCartney *et al*., 2000; McCartney and Knox 2002; Guillemin *et al*. 2005). The presence of arabinan and galactan side chains was associated with cell proliferation and differentiation respectively (Jones *et al*., 1997; Willats *et al*., 1998 and1999b; McCartney *et al*., 2000; Orfila and Knox 2000). Actively proliferating cells usually lack detectable amounts of galactan side chains but instead display large amounts of arabinose rich domains as shown in suspensions and callus cells of carrot (Kikuchi *et al*., 1996). In the

Amaranthaceae, of which spinach and sugar beet are members, arabinan and galactan side chains are esterified by ferulic acid. The RG-I associated galactans were found to occur in a restricted manner at the transition zone of Arabidopsis root marking the onset of rapid cell elongation. The occurrence of the galactan side chains appeared to be at the zone in which xyloglucan endotransglycolsylases activity and its donor substrate have been identified to be co-localized (McCartney *et al*., 2003). The sporogenous cells of sugar beet anthers were marked with the presence of arabinan that disappeared after the completion of the gametophytic phase, the entry in meiosis correlated with the appearance of galactans in the walls of meiocytes (Majewska-Sawka *et al*. 2004). A study on guard cells indicated that arabinans are required to maintain cell wall flexibility by preventing homogalacturonan polymers from forming tight associations (Jones *et al*., 2003 and 2005). The cell walls of Sitka spruce contained the arabinans, lining the intercellular spaces between tracheids in severe compression wood and parenchymatic ray cells, possibly indicating the importance of this polymer in cell adhesion (Altaner *et al*., 2007).

1.4.4.3.2 Rhamnogalacturonan II (RG-II)

Rhamnogalacturonan II (RG-II) is a pectic polymer that is known to occur in all the primary cell walls studied to date , but is thought to be absent from the middle lamellae region (Matoh *et al*., 1998; Ridley *et al*., 2001; Vincken *et al*., 2003). It is thought to be covalently linked to HG (Ishii and Matsunaga, 2001; O'Neill *et al*., 2004). It is a branched pectic domain containing a HG backbone of 9 GalA residues that are (1→4)–α-linked, and is substituted by 4 heteropolymeric oligosaccharide side chains. RG-II consists of at least 12 different monosaccharides in more than 20 different linkages (Scheller *et al*., 2007). The Rha residues are much less abundant than in RG-I, and are present at the side chains of RG-II instead of in the backbone. Apiose residues in the side chain of separate RGII polymers can form a covalent cross-link in the form of a borate diester (Ishii *et al*, 1999; O'Neil *et al*., 1996). The cross linking of borate-RG-II is expected to control pore size in the wall

and plays a role in establishing a scaffold of pectic polymers to which other molecules in the wall can bind (Ridley *et al*., 2001). The RG-II dimer is thought to enhance intercellular attachment. The failure of the cells to form such dimers due to boron deficiency or a mutation in the plant genetic make up causes drastic morphological abnormalities. Boron is essential for pollen tube growth, it is thought to be involved in the binding of pollen tubes to the stylar matrix by means of RG-II borate cross links (Lord and Mollet, 2002). The growth of the pollen tube through pistil tissues was impaired with the absence of boron (Matoh *et al*., 1998). The loosely-attached callus cells of *nolac-H18,* a mutant of *Nicotiana plumbaginifolia*, failed to undergo organogenesis and shoot development due to a mutation in the glucuronosyltransferase gene (NpGUT1) that synthesizes the β-D-GlcA-(1→4)-α-L-Fuc-(1→), which is a part of one of the side chains of RGII. The lack of glucuronic acid moiety on RG-II side chain prevented the formation of borate dimers. Around 50% of RG-II in the mutant was present as monomers, whereas more than 95% of RG-II in the wild type tobacco was present as dimers (Iwai *et al*., 2002). Arabidopsis plants carrying the *mur1* mutation are dwarfed and have brittle stems. The dwarf phenotype of *mur1* plants was a result of reduced RG-II cross linking as a result of replacing fucose with galactose residues (Glushka *et al*., 2003)

1.4.4.3.3 Homogalacturonan (HG)

This is a linear homopolymer of (1→4)-α-linked D- galacturonic acid (GalA). It is thought to contain 100-200 GalA residues of which 70-80% are methyl esterified in the Golgi at C-6 carboxyl before secretion into the wall (Fig. 1.3, Thibault *et al*., 1993; Relat *et al*., 2001; Le Goff *et al*., 2001). HG may, depending on the plant source, also be partially O-acetylated at C-3 or C-2. HG can be further substituted by apiose or xylose and is then referred to as apiogalacturonan or xylogalacturonan respectively (Kikuchi *et al*., 1996; Renard *et al*., 1997; Ishii, 1997; Le Goff *et al*., 2001). HG can be acted upon by several cell wall based enzymes like pectin methyl esterases (PMEs), polygalacturonases (PG) and pectate lyases (PL). The end product of the

action of these enzymes could influence and determine the properties of the pectic network (MacKinnon *et al*., 2002). The PME deesterifies the HG facilitating the accessibility of polygalacturonases and pectin lyases that would hydrolyse the HG chain. On the other hand, the presence of contiguous de-esterified galacturonic acid residues allows cross-linking of HG chains by ionic interaction with calcium ions forming a network of calcium pectinate gel (Fig. 1.4). Calcium bound matrices are important in strengthening the cell wall. The combined action of PME and PG releases oligogalacturonides which are known to serve as signals in a range of plant processes. The difference in the levels of firmness between 12 varieties of strawberry was attributed to the activity of PME and PG; the softest varieties had the highest PME and PG activities (Lefever *et al,* 2004). The degree of methylesterification of the walls of xylem fibers appears to play an important factor in wood biotechnology and the determination of wood quality. One of the PME isoforms in aspen (PtPME1) was found to negatively affect wall plasticity of wood fibers, thus impairing its ability to intrusively grow and penetrate the middle lamellae of the adjacent cells, which ultimately led to the reduction in the length and width of xylem cells (Siedlecka *et al*., 2008).

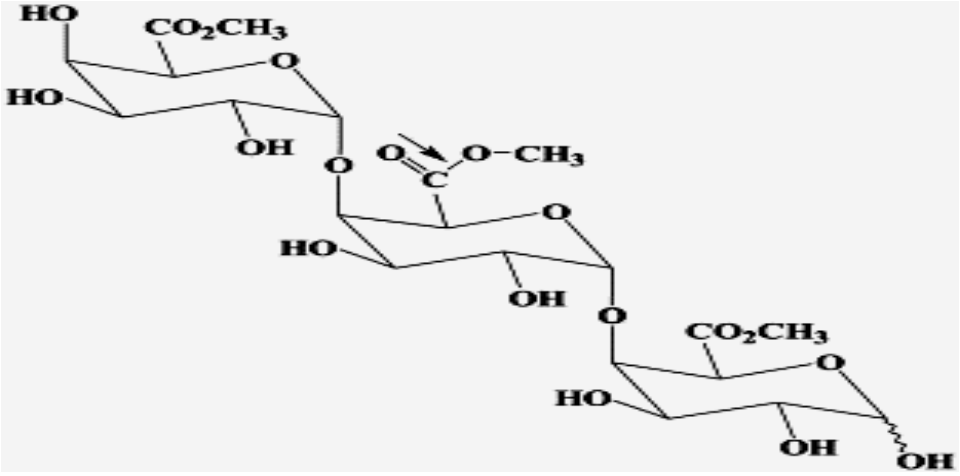


Fig.1.3 Chemical structure of HG carrying methyl ester groups (Jenkins *et al*., 2001).

Homogalacturonan is known to be involved in a range of cell wall activities that influence cell adhesion, cell expansion, wall porosity and defense

(Minorsky, 2002; Derbyshire *et al*., 2007). They are abundant in the middle lamellae, and especially in reinforcing zones at the tricellular junctions and the corners of intercellular spaces, which are key load bearing locations (Parker *et al*., 2001; Willats *et al*., 1999b and 2001c). Pectins of middle lamella are characterized with low or zero level of RGI (Jones *et al*., 1997; McCartney *et al*., 2000) and RGII (Matoh *et al*, 1998). The presence of acetylated homogalacturonan throughout cell walls of suspension-cultured sugar beet cells was associated with decreased levels of intercellular adhesion (Liners *et al*., 1994). It was reported by Ralet *et al* (2003) that a degree of acetyl esterification of HG above 15% hinders pectin gel formation.

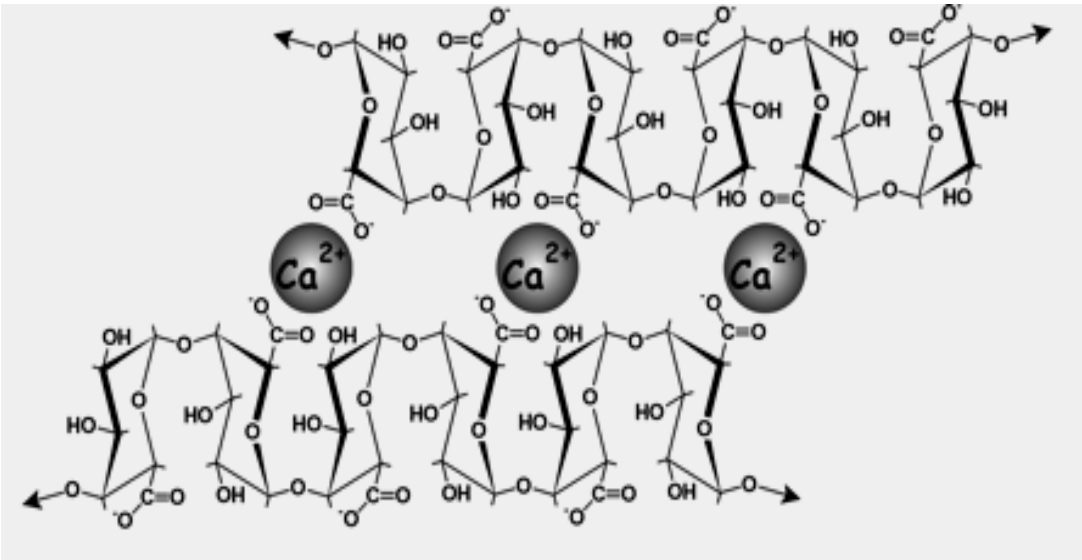


Fig. 1.4 The interaction between Ca^{2+} and unesterified carboxyl groups of GA residues of two HG chains (Vincken *et al*, 2003).

Xylogalacturonan (XGA) has a HG backbone with 25–75% of the galacturonic acid (GalA) units being substituted with xylose. The GalA sugar residues of the XGA backbone can be methyl-esterified (Schols *et al*., 1995; Le Goff *et al*., 2001). The presence of Xylogalacturonan (XGA) has been detected in the cell walls of tissues and organs of several plants such as peas, carrot suspension cultures and Arabidopsis seedlings (Kikuchi et. al, 1996; Le Goff *et al*., 2001; Willats *et al*., 2004; Zandleven *et al*., 2007). The presence of

XGA was restricted to the loosely attached inner parenchyma cells of pea testa, and root cap cells of carrot, maize and Arabidopsis root apices (Willats *et al*., 2004).

1.5 Supramolecular architecture of pectins

Two types of models for pectin structure have been proposed; earlier, it was thought that extended chains of covalently linked alternating RG-I, RG-II and HG domains constituted the backbone of pectic polymers with RG-I and RGII side chain residues interspersing the chains (Fig. 1.5). Another alternative model of pectin structure was proposed by Vincken *et al* (2003) states that RG-I could serve as the backbone of the network on which HG, RG-II, arabinans, galactans, arabinogalactans (AGs) and all other pectic domains are attached (Fig.1.7). Recently, the covalent linkage of a HG or a XGA domain to RGI was demonstrated at the oligomer level using controlled acid hydrolysis of apple MHR (modified pectic hairy regions) mixture of oligomers (Fig. 1.6). The presented results partly correspond with the first model in which the backbone consisted of consecutive HG and RGI structural elements (Coenen *et al.*, 2007). The pectic network is thought to bind to cellulose through the neutral sugar side chains, and the extent of binding varies with respect to the nature and structure of side chains (Iwai *et al*., 2001; Zykwinska, *et al*., 2005). The ability of pectins in tethering of cellulose microfibrils could be of great significance, especially in the cell walls that are poor in xyloglucan like in the case of sugar beet, potato, celery, onion and carrot (Zykwinska *et al.*, 2007). Some evidence for the occurrence of a covalent linkage between xyloglucan and pectin was reported by Femenia, *et al* (1999), Abdel-Massih *et al*. (2003), Cumming *et al*. (2005) and Popper and Fry (2008). The pectin hemicellulose association was reported to exist in a wide range of suspension cultures of angiosperms possessing type I and type II cell walls (Popper and Fry, 2005). Incubating pea Golgi membranes with UDP-[^{14}C] galactose led to the synthesis of a large molecular weight compound that could bind to paper and could be digested by cellulase, the obtained result indicated that the radioactivity was incorporated into galactan

side-chains of RG-I, and that pectin-xyloglucan bond was formed during the biosynthesis of the two polymers in Golgi apparatus (Abdel-Massih *et al*., 2003). The possible presence of covalent cross links between xyloglucan and RG-I was thought to occur via arabinan side chains of pectin (Brett *et al*, 2005). Abdel Massih *et al* (2007) proposed that the attachment between the xyloglucan and nascent pectin occurs after the formation of homo-galacturonans, but before the pectin leaves the Golgi apparatus. The pectin backbone was thought to elongate while attached to a protein, which may act as a primer. When the galactan side-chains are added, the protein becomes detached from the pectin, and xyloglucan becomes linked to some of the galactan side-chains. Their proposed model in Fig. 1.8 agrees with the new publications suggesting the linear arrangement of RG-I and polygalacturonan in the backbone of pectin. In a recent paper, Popper and Fry (2008) reported that in Arabidopsis suspension cultures xyloglucan–pectin bonds are shown to be formed intra-protoplasmically and the xyloglucan chains are thought to be built up de novo, using NDP-sugars as donor substrates, on an RG-I side-chain. The anionic xyloglucan–pectin complex formed is believed to remain stable in the primary cell wall, and to allow the efficient binding, assembly and correct deposition of cell wall polymers and maintenance of intercellular adhesion.

Although according to the listed references, the constituents of the pectic network are linked to one another through glycosyl links, borate diesters, calcium ion cross-links and/or the oxidative coupling of arabinan and galactan side chains containing ferulic acid, the arrangements, patterns and frequencies of specific domains within multi-domain chains have not been determined and thus the above proposed models remains to be elucidated and proven (Willats *et al*., 2001b; Ridley *et al*., 2001; Vincken *et al*., 2003; Clausen *et al*. 2003; Cosgrove, 2005; Abdel Massih *et al*., 2007; Coenen *et al*., 2007; Verhertbruggen and Knox 2007).

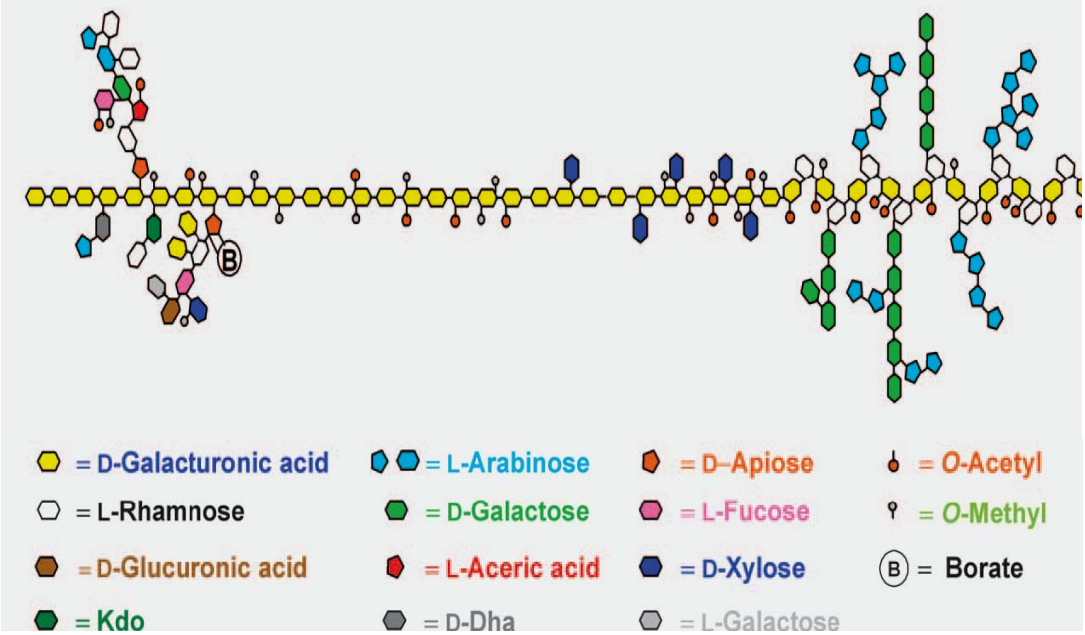


Fig. 1.5 diagrammatic representation of more possible recent macromolecular structure of pectin (Vincken *et al*, 2003; Scheller *et al*., 2007)

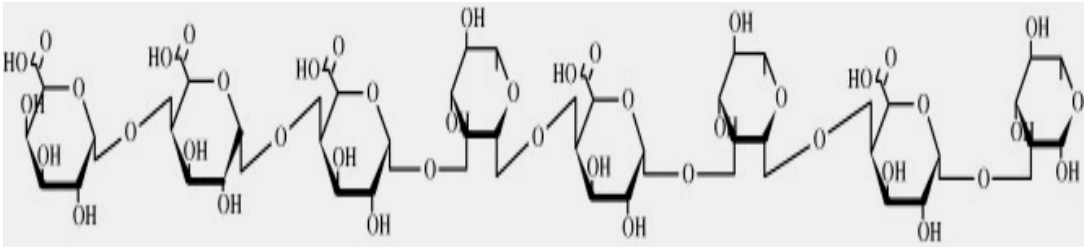


Fig 1.6 Representation of a connection between rhamnogalacturonan type I and homogalacturonan (Coenen *et al*., 2007).

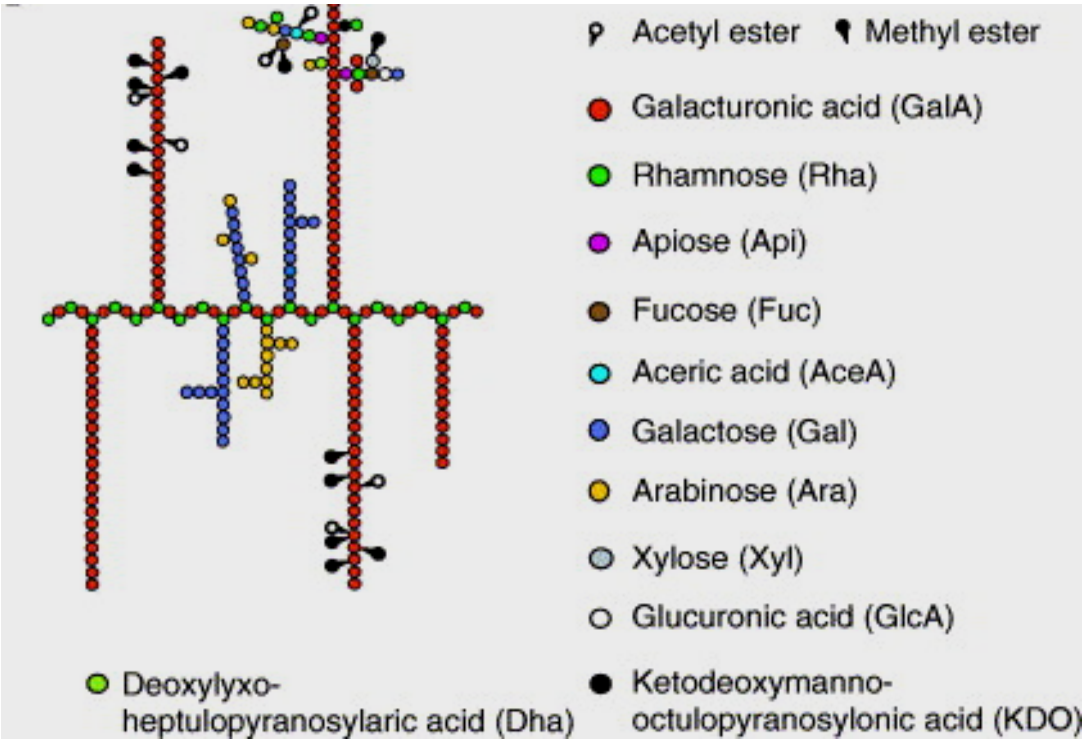


Fig. 1.7 diagrammatic representation of macromolecular structure of pectin according to the model proposed by Vincken *et al.* (2003) and Willats *et al*. (2006)

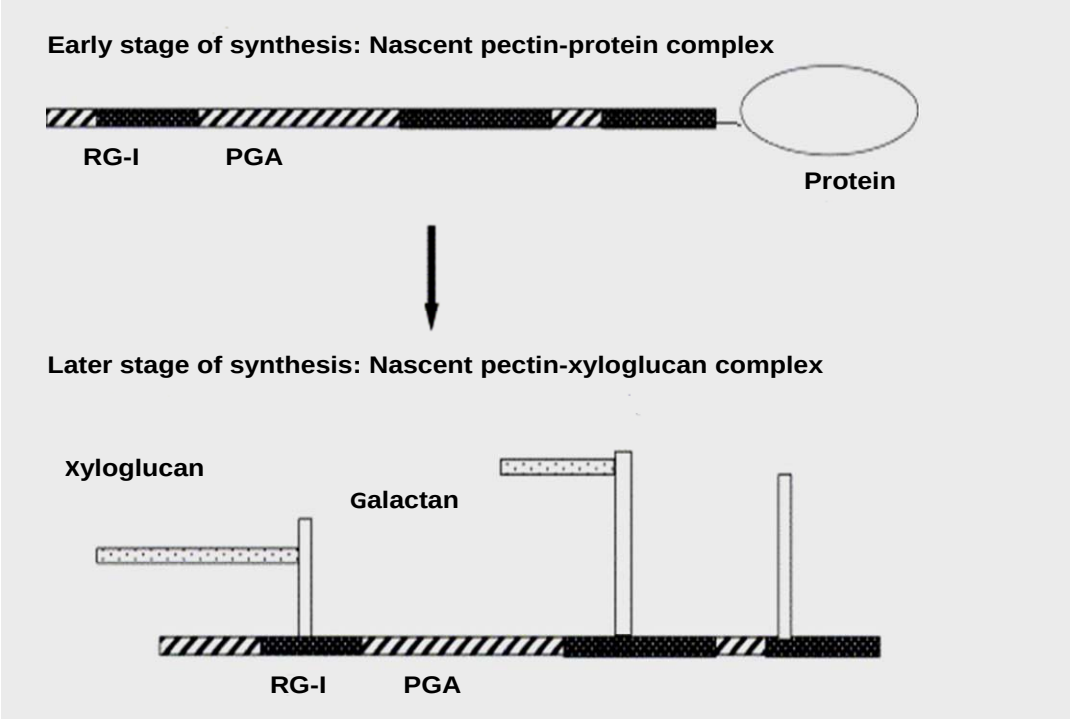


Fig. 1.8 Possible model of sequential stages of pectin biosynthesis (Abdel-Massih *et al.*, 2007)

1.6 Arabidopsis as a model to study Cell walls

In recent years, Arabidopsis has become a key model species in plant biotechnology and agricultural research. *Arabidopsis thaliana* is a little flowering weed that is a member of the mustard (Brassicaceae) family, and is currently used as a model plant for the study of cellular and molecular sides of plant cell wall biogenesis, assembly and modification. Arabidopsis has a small completely sequenced genome with high transformation efficiency, and powerful reverse and forward genetics (Arabidopsis Genome Initiative, 2000; Chen *et al*., 2004). It is small in size and has a short generation cycle. The amount of its DNA per cell ranks among the least of all known plants. Arabidopsis roots offer several advantages for the study of cell wall dynamics. The epidermis, cortex, endodermis and pericycle, each is made of a single layer of cells except area closest to hypocotyle where two layers of cortical cells are present The root cap is made of 1 to 3 layers of cells (Freshour *et al*, 1996). Studying the polysaccharide components of the Arabidopsis roots could give an insight into the structure of the cell walls of many roots systems of other higher plants.

Recent functional genomics and mutant studies have allowed the identification and characterization of many Arabidopsis mutants with altered cell wall composition. Several Arabidopsis mutants were characterized with phenotypic defects in cell adhesion, such as, *qua1, qua2, quartet, mur1 and emb30* (Freshour *et al*., 2003). The phenotype of Arabidopsis *quasimodo1* mutant showed a deficiency in cell adhesion and stunted growth. The analysis of the quasimodo1 (*qua1-1)* and (qua1-2) mutants showed several cell wall defects with a particularly large decrease in the activity of HG-galacturonosyl transferase in Arabidopsis seedlings and root calli suspension cultures (Bouton *et al*. 2002; Leboeuf *et al*., 2005). The *quasimodo2* (*qua2*) mutant was related to a defect in a putative S-adenosyl methionine dependent methyltransferase gene (Mouille *et al*. 2003). Arabidopsis *emb30* showed abnormal localization and accumulation of pectin in intercellular/interstitial spaces rather than in the corners (Capodicasa *et al*., 2004) The Arabidopsis

quartet mutant has microspores that fail to separate during pollen development as a result of the persistence of pectin in pollen mother cell wall (Francis *et al*., 2006).

1.7 Aims of the Project

In this project, taking into consideration that pectin methylesterase is one of the wall based enzymes modulating pectin structure that is implicated in cell-cell adhesion; we propose that the establishment of intercellular adhesion through the action of PMEs starts at the time of cytokinesis when the cell is laying down the cell plate which becomes the middle lamella. The degrees and patterns of deesterification of homogalacturonans will determine the onset, level and strength of intercellular adhesion. The project was designed to

1. Characterize and assess the role of pectic polysaccharides in cell-cell adhesion of Arabidopsis suspension culture with respect to their biochemistry and epitope distribution within the middle lamellae, especially at tricellular junctions.

2. Assess the role of pectin methyl esterases in generating epitopes important in intercellular adhesion, by determining which PME genes are expressed in culture and then down regulating these genes.

3. Use synchronized suspension cultures to study the pectins laid down at cell division, especially during the formation of tricellular junctions, and PME genes expressed during cell division.

4. Assess the possible modulations in the middle lamella and adhesion as a response to adaptation to salinity using salt tolerant Arabidopsis cell line.

Chapter 2

Materials and method

All chemicals were purchased from Sigma (UK), unless stated otherwise.

2.1 Plant material and growth conditions

Wild type *Arabidopsis thaliana L*. ecotype Columbia cultures were originally initiated from stem explants by May and Leaver (1993). The suspension cultures were established by transferring approximately 2 g of healthy callus cells into 250 ml Erlenmeyer flasks containing 100 ml of the growth medium. The cells were grown in MSMO medium (Sigma, Murashige and Skoog basal salts with minimal organics containing macro, micronutrients and vitamins as described by Lensmaier and Skoog (1965)) supplemented with 3% (w/v) sucrose, 0.5 mg/l NAA (α-naphthalene acetic acid) and 0.05 mg/l kinetin. The pH of the culture media was adjusted to 5.8 using 2M KOH before autoclaving at 121^{o}C for 20 min. The cultures were kept at 26°C under continuous light of PPFD (photon flux density) of 20 µmol m^{-2} s^{-1} on a rotary shaker at 150 rpm. The suspensions were maintained routinely by transferring 10 ml of the culture every 7 days at early stationary phase to a 250 ml flask containing 90 ml fresh media. The suspension cells were used in the experiments after three months of regular subculturing

The salt tolerant Arabidopsis HHS (Habituated to High Salt) cell line was established by Dr. Peter Dominy (University of Glasgow). Successive subculturing of the wild type Arabidopsis over a period of two years into a medium of higher salinity resulted in the establishment of a suspension that can tolerate up to 300 mM NaCl. The HHS cell culture was maintained in a medium made of the same Sigma MSMO, supplemented with 3% sucrose (w/v), 0.5 mg/l NAA, and 0.05 mg/l Kinetin and 300 mM NaCl (17.5 g/l), at PH 5.8. The cultures (45 ml) were grown in 150 ml flasks at 20°C under continuous light of PPFD (photon flux density) of 20 µmol m^{-2} s^{-1} on a rotary shaker at 150 rpm.

The growth curve of the culture was followed by determining the packed cell volume (PCV) every day over a growth period of 10 days. Aliquots (5 ml) of

suspension cultured cells were sampled, centrifuged at 300 xg for 3 min and PCV was calculated as the volume of the cell pellet divided by the total volume. The recorded data represent the average of three replicate samples taken every day during the culture cycle from stocks each containing 180 ml media and 20 ml cells in 500 ml conical flasks.

2.2 Preparation of alcohol insoluble material (AIM)

Arabidopsis cell cultures (100 ml) were allowed to settle in a measuring cylinder. The volume of settled cells was recorded and excess medium poured off into a 250 ml conical flask. Ethanol was added to the precipitated cells to give a final concentration of 70% (v/v) ethanol (Fry, 1988). After heating at 70^{o}c for 30 min to inactivate the endogenous enzymes, the mixture was cooled and centrifuged at 4000 rpm for 10 min. The supernatant was collected and the alcohol insoluble residue (AIR) was resuspended and washed twice in 70% ethanol to remove low molecular weight sugars and other metabolites while retaining macromolecules like proteins, starch and wall polymers.

The culture medium was adjusted to 70% (v/v) ethanol and incubated overnight at 4^{o}C. The precipitated sugars were collected by centrifugation at 4000 rpm for 10 min and the pellet was washed three times with 70% ethanol and stored at -20^{o}C for further analysis.

2.3 Extraction of cell wall pectin

To solubilise the ionically-bound homogalacturonans (HGs), the alcohol insoluble material was extracted using 50 mM ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA) in sodium phosphate buffer pH 6.8 at 70^{o}C for 30 min (Abdel-Massih et al., 2003 and 2007). The mixture was cooled and centrifuged at 4000 rpm for 10 min. The supernatant was collected and the

pellet was washed twice with EDTA/phosphate buffer, each time collecting the supernatant. The extracted pectins were dialyzed overnight at 4^{o}C against distilled water. The volume of the dialysed pectin was recorded and the pectin stored at -20^{o}C.

2.4 Analysis of the extracted pectin

2.4.1 Determination of the total sugar content of the extracted pectin

The amount of total carbohydrate in hydrolyzed and non-hydrolyzed EDTA/phosphate extracts was determined by the phenol-sulphuric acid method (Dubois, 1956). Galactose was used as a reference to construct a standard curve. Phenol (5%, 0.4 ml) was added to 0.4 ml of the sugar containing sample, then using a glass pipette, 2 ml of concentrated sulphuric acid was added rapidly making sure to direct the stream of acid against the liquid surface rather than the sides of the test tubes. Blanks were prepared by substituting distilled water for the sugar solution. The absorbance of the characteristic yellow orange color was measured at 490 nm.

2.4.2 Determination of total galacturonic acid content of the extracted pectin

The total uronic acid content was assayed using the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973; Wicker and Leiting, 1995) with galacturonic acid as a standard. Using 50mM sodium phosphate buffer pH 6.8, different dilutions of 1mg/ml D- galacturonic acid were distributed into screw capped test tubes. Concentrated sulphuric acid containing 0.0125 M sodium tetraborate (1.2 ml) was added taking care that the tetraborate solution hit the center of the liquid so the heat of dilution of sulphuric acid would drive the reaction. The test tubes were left to cool down to room temperature after which 20 µl of 0.15% (w/v) m-hydroxydiphenyl in 0.5% (w/v)

NaOH were added to the tubes except the blank. To the blank 20 µl of 0.5% NaOH were added. The absorbance was measured at 520 nm and the uronic acid content was interpolated from the corresponding reference curve.

2.4.3 Estimation of the degree of esterification of the extracted homogalacturonan

2.4.3.1 Base catalysed deesterification

Aliquots of the extracted pectin (1 ml) were treated with a mild alkali (saponified) by mixing the pectins with 40 µl of 1M NaOH in order to reach a pH of 12 as was detected by pH paper. The mixture was incubated at 4^{o}C for 1 h. Then the pH was adjusted to pH 7 using 60 µl of 0.49 M phosphoric acid. The alkaline deesterified solution was used to quantify the amounts of the released methyl esters.

2.4.3.2 Determination of the released methyl groups

The amount of methanol formed after saponification of the pectins was analysed by using a colorimetric method according to Klavons and Bennett (1986). To construct a methanol calibration curve, serial dilutions of stock methanol were prepared containing 0.0, 0.1, 0.2, 0.4, 0.5 and 0.6 µmol, to a final volume of 1 ml using 50mM sodium phosphate buffer pH 6.8. Alcohol oxidase from *Pichia pastoris* (EC 1.1.3.13, Sigma) 1unit/ml was added to all the test tubes and incubated at 25^{o}C for 20 min. The enzyme oxidises methanol to formaldehyde. A 2 ml solution containing 2 M ammonium acetate, 50 mM acetic acid and 20 mM 2,4-pentanedione was added to all the test tubes and the reaction mixture was incubated at 60^{o}C for 15 min. A colored product called 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine is formed as a result of the condensation of the formaldehyde with 2,4-pentanedione. The samples were left to cool down to room temperature and the absorbance was determined with a spectrophotometer at 412 nm.

The amount of released methyl groups was estimated from the standard calibration curve. The ratio of methanol to galacturonic acid was used to calculate degree of methylesterification (DM %). Control reactions were done to detect a possible interference of background free methyl groups or residual ethanol in the extracted pectin. Ethanol will compete with the released methyl groups for the alcohol oxidase forming acetaldehyde.

2.4.4 Determination of sugar composition

2.4.4.1 Preparation of the samples

A portion (10 ml) of the phosphate/EDTA extracted dialysed pectins was added to 40 ml of ethanol and kept at 4^{o}C for 48h. The precipitated pectin was withdrawn and concentrated to approximately 1ml with a rotating evaporator at 40^{o}C and hydrolysed in 2M trifluoroacetic acid (TFA) for 1h at 121^{o}C. TFA was evaporated under a stream of N_2. The hydrolysates were re-dissolved in 250 µl distilled water prior to the detection of the neutral fraction of TFA hydrolysates by paper chromatography (PC) and high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAE-PAD).

2.4.4.2 Paper chromatography

Before placing any sugars on the paper, four horizontal straight lines were drawn at 6, 11, 13, 15 cm from top of Whatman no. 1 paper. Sample spots of the acid hydrolysates were loaded on the paper along the 15 cm line. The neutral sugars were separated by descending paper chromatography using a solvent system composed of pyridine: ethyl acetate: acetic acid: water (3:18:1:1). The chromatograms were allowed to run for 36 hr. Sugars were visualised on the paper chromatograms by staining with aniline hydrogen phthalate or silver nitrate (Fry, 1988). The standard sugars were divided into two batches A and B and TFA treated under the same conditions. Batch A

contained a mixture of 5mg/ml of each of fucose (Fuc), galacturonic acid (GalA), mannose (Man), xylose (Xyl) and galactose (Gal). Batch B included 5 mg/ml of each of glucose (Glu), glucuronic acid (GluA), arabinose (Ara), rhamnose (Rha) and 1,2:3,5-di-o-isopropyl-idene-α-D apiose (Api).

2.4.4.3 High performance anion exchange chromatography with pulsed amperometric detection (HPAEC- PAD)

The composition of the neutral sugar content of the acid hydrolysates was detected using high performance anion exchange chromatography coupled with pulsed amperometric detection (HPEA-PAD, Sullivan and Douek, 1994). The samples were mixed with an anion exchange resin (Dulite) and filtered through sterile millipore filters prior to injection on a Dionex analytical Carbopac PA-10 column. The eluents included deionized water, 100mM NaOH and 350mM NaOH prepared from a carbonate free NaOH stock. Carbonate, being a divalent anion at pH $\geq$ 12, binds strongly to the columns and interferes with carbohydrate binding, causing a drastic decrease in column sensitivity and a loss of resolution and efficiency. The eluent flow rate was 1ml/min at room temperature. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold detector. The surface of the electrode was cleaned between measurements to remove any residues from previous runs. A post run flush of the column with 350 mM NaOH was done to wash out the strongly retained species.
The standards included arabinose, rhamnose, galactose, glucose, mannose and xylose. Standard curves were constructed relating the concentration of each of the standard sugars to the obtained peak area. The resulting linear equations were used to quantity the amounts of the detected sugars in the tested samples. The initial runs showed that fucose was present in low stable quantities so it was used as an internal standard. The components were compared to standard monosaccharides by their retention times. All the peak areas were normalized to the area of fucose before the calculations of the detected sugars. The detected fraction was expressed as % mol of the total detected neutral sugars.

2.5 Immunochemical studies

The anti-pectin antibodies used in this study, including the rat monoclonal antibodies JIM5, JIM7, LM7, LM5 and LM6 and the mouse monoclonal antibody 2F4, were all purchased from PlantProbes – Leeds/ UK.

2.5.1 Immuno-dot assay

Equal amounts (10 µl) of the extracted pectic polysaccharides containing (1, 0.5 or 0.2 µg sugars) were spotted as 1 µl aliquots onto nitrocellulose membranes and air dried at room temperature for at least 1h. All subsequent treatments were done at room temperature. Membranes were blocked with 1M phosphate buffered saline (PBS, 0.14M NaCl, 2.7mM KCl, 7.8mM $Na_2HPO_4.12\ H_2O$, 1.5mM KH_2PO_4) pH 7.2 containing 5% Marvel fat free milk powder (MPBS, Marvel, Premier Beverages, UK) for 1 h. The membranes were then incubated with the following primary monoclonal antibodies: JIM5, JIM7, LM5, LM6 and LM7 according to Willats *et al* (2001c) and Clausen *et al*. (2003). All primary antibodies were diluted 1/10 (w/v) in MPBS for 2 hours. After washing extensively under running tap water, membranes were left for 10 minutes rocking in PBS containing 0.1% (v/v) tween 20 prior to incubation for 2 h in secondary antibody (goat anti-rat-IgG, whole molecule, coupled to horseradish peroxidase) diluted 1/1000 in MPBS. Membranes were washed again before development in substrate solution made of 25 ml deionized water, 5 ml methanol containing 10 mg/ml 4-chloro-1-naphthol and 30 µl 6% (v/v) H_2O_2. The reaction was stopped by replacing the substrate solution with tap water. Citrus fruit pectins with various degrees of esterification 89, 65 and 34% were used as control standards.

2.5.2 Immunolocalization of cell wall pectic epitopes

2.5.2.1 Preparation of plant material for microscopy

Representative samples of Arabidopsis cells during the different phases of the culture cycle were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M PBS, pH 7.2, for 1 h at room temperature and then washed extensively in PBS. The fixed cells were further processed and embedded in LR white resin as described by Willats *et al*. (1999b and 2001c).

The cells were extra fixed in a 0.1M sodium cacodylate buffer containing 1% glutaraldehyde and 2% formaldehyde pH 7.0 for 1 h at room temperature. After 3 washes with the buffer, the cells were resuspended in buffer containing 3% agar at 40^{o}C and vortexed. The blocks were dehydrated in a series of graded ethanol concentrations consisting of 10%, 20% at room temperature for 20 min and 30% at 40^{o}C for 20 min, and infiltrated with LRWhite resin (London Resin, Reading, UK) containing 0.5% benzoin methyl ether with regular changes of resin over 2 days. Resin was polymerized in gelatine capsules using UV light for 12 h at -20^{o}C and 12 h at room temperature. One micrometer (1µm) thick sections were used in the immunofluorescence analysis and 50 nm thick sections were used in the immunoelectron microscopy studies.

2.5.2.2 Immunofluorescence localization of pectic epitopes

The resin embedded plant material (1µm thick) was blocked in 5% fat free milk in phosphate buffered saline (MPBS) according to Willats *et al*. (1999 and 2001c) for at least 2 h at room temperature. Gentle rocking was maintained during the immunolabelling steps. Sections were then incubated overnight in a solution containing one of the monoclonal antibodies; JIM5, JIM7, LM5, LM6 and LM7 diluted 1:10 v/v in MPBS at 4^{o}C. After washing the sections several times in an excess of PBS, they were incubated overnight at 4^{o}C in darkness with the goat-anti rat fluorescein isothiocyanate (FITC) conjugated secondary antibody diluted 1:100 v/v in MPBS. The same labelling steps were followed

with the monoclonal antibody 2F4 except that the buffer used (T/Ca/S) consisted of 20 mM Tris-HCl pH 8.2, 0.5 mM $CaCl_2$ and 150 mM NaCl, and the goat-anti mouse FTIC conjugated secondary antibody was diluted 1:100 v/v in free fat milk T/Ca/S. Sections were washed again in buffer before being mounted in anti fade solution (Citiflour AF3, Agar scientific, Stansted, UK) and examined with epifluorescence microscope. Control sections were treated in parallel but with the omission of the primary antibodies.

In some cases, the embedded sections were submitted to enzymatic (Pectin methylesterase from orange peel (oPME)) and/or chemical (alkaline deesterification) treatment, before immunolabelling as described by Guillemin *et al*. (2005) and Leboeuf *et al*. (2005). The *in situ* deesterification of pectins was carried out by incubating the resin embedded sections with 100 µl of oPME (105 U/ml) in Tris-HCl pH 7.5 at 30^{o}C for 30min, or using 100 µl of 0.05M NaOH for 30 min at 4^{o}C. Sections were rinsed thoroughly with deionized water before labelling with JIM5 or JIM7 as described above.

2.5.2.3 Immunoelectron microscopy of pectic epitopes

The resin embedded sections (~ 50 nm thick) were blocked using 1% (w/v) bovine serum albumin (BSA) in PBS containing 2% Tween 20 (PBST). The monoclonal antibodies JIM5, JIM7, and LM7 were diluted 1/5 in 1% BSA/PBST overnight at 4^{o}C. After washing with PBST, sections were incubated overnight at 4^{o}C in goat anti-rat secondary antibody conjugated to 10 nm colloidal gold diluted 1/20 in BSA /PBST. Sections were washed with PBST and post-stained with 2% uranyl acetate. The same labelling procedure were followed with the monoclonal antibody 2F4 except that it was diluted in 1% BSA in T/Ca/S buffer, and the use of goat anti mouse secondary antibody conjugated to10nm colloidal gold diluted in the same buffer. Sections were observed with an electron transmission microscope.

2.6 Synchronization of Arabidopsis of cell suspension

Aphidicolin, which is a fungal toxin extracted from *Cephalosporium aphidicolia*, was used to reversibly block the cell cycle progression of the Arabidopsis cultures as described by Menges and Murray (2002). All glassware was thoroughly washed and autoclave sterilized. A 40 ml aliquot of 7 days old culture was subcultured in 200ml fresh medium containing 4 µg/ml aphidicolin and incubated at 26°C under continuous light of PPFD (photon flux density) of 20 µmol m^{-2} s^{-} on a rotary shaker at 150 rpm for 22 h. Cells were washed with 1 liter MSMO through filter paper under mild suction making sure not to dry the cells. The cells were quickly resuspended in 250 ml MSMO and incubated under the same cultivation conditions as above. Representative samples were collected at regular intervals after washing out the aphidicolin, and were used to determine the mitotic index and for RNA extraction. RT-PCR was then used to analyze the expression of cell cycle marker genes and pectin methylesterase genes.

2.6.1 Cell number and mitotic index

A 0.5 ml aliquot of cell suspension was collected every hour after washing out aphidicolin. After removing the supernatant, the cells were fixed with 3.7% formaldehyde in PMEG buffer (50 mM PIPES, 2 mM MgSO4, 5 mM EGTA, 2% glycerol, pH 6.8) according to Menges and Murray (2002). Cells were washed with PBS buffer and the cells were separated by digestion for 2hr with 1% cellulase and 1% pectinase (extracted from *Trihoderma* sp, Duchefa) and 0.4 M mannitol at room temperature. The DNA of cells was stained with 1µg/ml DAPI (4', 6-diamidino-2-phenyl-indole) for ½ hr. After washing the cells several times with PBS, the number of dividing cells was estimated by UV light microscopy. The mitotic index represented the percentage of cells with DAPI stained metaphase/anaphase figures.

2.6.2 RNA Extraction

Arabidopsis cells were collected by the vacuum assisted filtration of 5 ml of suspension cells through a glass sintered funnel every 2 hours. The cells were quickly collected in a pre-weighed sterile eppendorf tube, weighed again, frozen in liquid nitrogen and stored at -80^{o}C until further use for RNA extraction. RNA was extracted using the Gentra kit (Qiagen) according to manufacturer's instructions. The RNA precipitate was resuspended in 20 µl DEPC treated water. To eliminate residual genomic DNA present in the preparation, the samples were treated with DNA-free kit (Ambion). The total RNA concentration and purity were estimated by the measurement of absorbance at 230, 260 and 280. The quality of RNA was assessed by loading 5 µg of RNA denatured by glyoxal load buffer dye (Ambion) on a 1.5% agarose gel electrophoresis. RNA was stored at –80°C until further use

Total RNA (5 µg) were used as templates for reverse transcription (RT) reactions to synthesize the first strand cDNA, using the Superscript first strand cDNA synthesis kit according to manufacturer's instruction (Invitrogen). An aliquot (1µl) of the cDNA mix was used as a template in a 25 µl volume for subsequent Reverse transcription-Polymerase chain reaction (RT-PCR) amplification using the primer combinations listed in tables 2.1 and 2.2. The PCR conditions were 95^{o}C for 15 min and 30 cycles of 92^{o}C for 45 sec, 58^{o}C for 45 sec, 72^{o}C for 45 sec with a final extension of 10 min at 72 ^{o}C. The resulting PCR products were separated by gel electrophoresis on 1.5 % agarose gel containing ethidium bromide, and photographed. Samples were treated identically but without reverse transcriptase as a negative control in order to exclude contamination with genomic DNA.

2.6.3 Primers of cell cycle marker genes

Progression into the different cell cycle stages (G1, S, G2 and M) was estimated by the expression data of four cell cycle marker genes (cyclin dependent kinase CDKB2, cyclin D3 (cycD3) and Histone (H4)). Using the

TAIR website (www.arabidopsis.org) we designed exon-exon primers. The primer sequences are given in table 2.1. As a control, primers specific to the actin7 gene were used. Gel images were analyzed quantitatively with image analysis software and the relative expression levels were normalised with respect to actin7.

Locus	Forward primer	Reverse primer	Expected size of Amplified Product (bp)
At1g20930 (CDKB2;2)	CTCACATCGTTAGGTTG ATGGAT	AGGCTTAAGATCCCT GTGCAAA	228
At4g34160 (cycD3)	CGATTCGGAAGGAGGA AGAAAGTAG	TCGAGACAGCTGAGT CCTTGTTCTT	232
At1g76160 (Histone, H4)	TGGGAAAGGGAGGAGC GAAGA	GACAACATCCATGGC GGTCA	232
At5g09810 (actin 7)	CGTACAACCGGTATTGT GCT	TTTTCTCTCTGGCGG TGCAA	570

Table 2.1 summarizes the accession number, amplified fragment size of each gene, and the specific primer pairs used of cell cycle marker genes

2.6.4 Cell cycle pectin methylesterase genes

The nucleotide sequences of six cell cycle expressed PMEs were retrieved from the TAIR website (www.arabidopsis.org), and used to design exon-exon primers. Primers were designed to obtain an amplified product in a range of 200-650 bp. The primer sequences are given in table 2.2. The actin7 gene was used as a control. Gel images were analyzed quantitatively with image analysis software and the relative expression levels were normalised with respect to actin7.

Locus	Forward primer	Reverse primer	Expected size of Amplified Product (bp)
At4g02330	TCTTGGGCCCATCCAAAGAA	AATTCCCCGTTCCGTTTTGG	205
At1g02810	CGCCACATTTGCTGTGACAGC	TGGAATTAATCACGTGGTAACCAGG	612
At2g26440	TTAGAAGATTCTAGCGACGGGTATG	AAAGTGTGTCCTGGTAACCATCAAT	439
At4g12390	TTATGGACGGTGTGGTAAAATCAGC	AGCATAAACATCCCAAGCCTACACA	243
At2g47550	TCAGCCACATTTATTCTATCAGGTCC	GCCGTAACCTCGTTCGATTGAC	255
At5g47500	CGGATTTTACAGAGAGAAAGTG	GCGCAGTATTCGTGAAGCTAA	212
At5g09810 (Actin 7)	CGTACAACCGGTATTGTGCT	TTTTCTCTCTGGCGGTGCAA	570

Table 2.2 summarizes the accession number, amplified fragment size of each gene, and the specific primer pairs used of PME isoform genes annotated to be expressed during the cell cycle.

2.7 Phylogenetic analysis of cell cycle expressed PMEs

A phylogenetic tree was constructed to show the possible structural and consequent relationship between the sequences of the cell cycle putative PME isoforms listed in Table (2.2). The sequences were retrieved from the TAIR website (www.arabidopsis.org), and the phylogenetic tree was established with the neighbour-joining method using the MEGA program version 3.1 (Kumar *et al*., 2004).

2.8 Northern hybridisation analysis

All the glassware used and working area were treated with Ambion's RNase Zap, followed by rinsing with high quality RNase-free water to make sure that

all were clean and RNase-free. RNA probes were generated by *in vitro* transcription from DNA templates. The RT-PCR product of At5g47500 (212 bp) was extracted from the agarose gel using the gel extraction kit (Qiagen) and used to synthesize the template. The extracted pure DNA was amplified with the primers linked to the bacteriophage promoter T3 (At5g47500-T3-r, AATTAACCCTCACTAAAGCAGTATTCGTG) and (At5g47500-f, AAGATTGCTCCCGGATTTTACAGAGAG) using the following PCR conditions: 95^{o}C for 15 min, 30 cycles of 92^{o}C for 45 sec, 58^{o}C for 45sec and 72^{o}C for 45 sec with a final extension of 10 min at 72 ^{o}C. The RNA probe was synthesized through the transcription of the template using Maxiscript *in vitro* transcription kit (Ambion) in the presence of biotin-substituted nucleotides (biotin-UTP). After transcription the reaction was run on a denaturing polyacrylamide gel to separate the probe. The gel was either stained or UV shadowed to identify and cut the full-length probe from the gel. The probe was eluted in an overnight incubation at 30^{o}C in elution buffer made of 0.5M NH_4-acetate/ 1mM EDTA/ 0.2% SDS. The amount and quality of the synthesized probe was estimated spectrophotometrically by measuring the absorbance at 230, 260 and 280 nm.

Equal amounts (15 µg) of total RNA were denatured with glyoxal dye and loaded on 1.2% (w/v) denaturing agarose formaldehyde gel electrophoresis. The gel was run at 5 v/cm according to the recommendations of the NorthernMax-Gly Kit (Ambion). The RNA on the gel was transferred onto a BrightStar-Plus Positively Charged Nylon Membrane (Ambion) and later crosslinked to the membrane in a non-covalent interaction via exposure to short wave ultraviolet light. The RNA sequences of At5g47500 were detected on the blot by overnight hybridization to the biotin labelled probe at 65^{o}C. A marker mixture made of 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, and 9 kilobase nucleotide sequences was included and hybridized with the marker Probe provided by the manufacturer (BrightStar Biotinylated RNA Millennium™ Markers, Ambion).

After probe hybridization and washing to remove non-specific labelling, the hybridization signal was detected by BrightStar BioDetect kit (Ambion). The

chemiluminescent detection of probed mRNA involved blotting the membrane with a conjugate of the enzyme alkaline phosphatase and a ligand (Streptavidin) with a high binding affinity for biotin. The alkaline phosphatase caused the degradation of the added substrate CDP-Star which resulted in light emission. The blots were exposed to X-ray film overnight. The resulting band identified by the probe indicated the size of the mRNA, and the intensity of the band corresponded to the relative abundance.

Chapter 3

Biochemical and immunochemical analysis of pectins of Arabidopsis cell walls

3.1 Introduction

Plant suspension cultures have been the favourite subjects for studies of cell growth and wall biochemistry, in part because their walls are relatively homogenous and can be conveniently studied in the absence of developmental processes. As in intact seedlings, the cell wall dynamics in cells growing in a suspension are achieved via two distinct steps: the biosynthesis of cell wall components by the actions of membrane-bound enzymes followed by the assembly and rearrangement of cell wall structures *in muro* via the actions of extracellular proteins. Several studies reported about the biochemical macromolecular constituents of the cell walls of actively proliferating cells in suspension. The callus and suspension cultures of carrot and sugar beet, Sycamore, Tobacco BY-2 and *Arabidopsis thaliana* were investigated, all the tested species showed the presence of the regular components of celluloses, hemicelluloses and pectins (Kikuchi, 1996; Willats *et al*., 2000b; Majewska-Sawka and Münster 2004; Leboeuf *et al*., 2004 and 2005).

Pectin rich cell wall interfaces, mainly middle lamella and cellular junctions, are reported to mediate intercellular adhesion and provide mechanical support. Unesterified homogalacturonan cross linked by calcium in the middle lamella is thought to play a major role in cell adhesion. The strength of the interaction between Ca^{2+} and pectin increases with decreasing average degree of pectin methylesterification and increased length of the unsubstituted galacturonan back bone (Knox, 1992; Jarvis *et al*., 2003). The adhesion between the neighbouring cells living in a suspension culture resulted in the presence of cell aggregates forming clusters of varying sizes. It was reported by Leboeuf *et al*. (2004) that Arabidopsis suspension cells living in the stationary phase were less adherent than proliferating cells during the division and expansion phases, and they suggested that homogalacturonans, pectic side chains and arabinogalactan proteins were involved in the intercellular attachment. Earlier, in suspension cultures of Paul's Scarlet Rose, galactose containing wall domains were thought to be involved in the maintenance

intercellular adhesion (Wallner and Nevins, 1974). In recent years, the use of monoclonal antibody probes directed against a range of polysaccharides and proteoglycan epitopes in pectic domains gave a more rapid and reliable insight into the occurrence, distribution and possible functions of pectins throughout intact plant tissues and cells living in cultures (Knox, 1997; Jones *et al*., 1997; Willats *et al*, 1998, 1999b and 2000a).

Aims of this chapter:

This chapter reports on the biochemical characterization and the immunochemical analyses of EDTA/phosphate pectic extracts and the *in situ* distribution of homogalacturonan and RG-I domains *in muro* in sections of resin embedded cells, in an attempt to study the role of pectins in the cell-cell adhesion of *Arabidopsis thaliana* ecotype Columbia suspension culture cells

3.2 Growth kinetics of *Arabidopsis thaliana* suspension cultures

The cultures used were derived from an original line initiated by May and Leaver, 1993. The big cellular clumps were removed by sieving the culture under aseptic conditions in an attempt to produce a relatively finer and more homogeneous cell suspension of cell clusters less than 1mm in diameter. Microscopically, the green Arabidopsis cultures consisted of small cell aggregates and single cells. The cell aggregates were made of cells varying in size and shape (Fig. 3.1). The stationary phase was characterised by a high degree of aggregation.

The growth curve of the suspension cultured *Arabidopsis thaliana* is illustrated in Fig. 3.2. The packed cell volume (PCV) expressed as millilitre (ml) of cells per ml suspension culture was used to follow the growth of the cell suspensions. The growth of the culture progressed into two reproducible phases, an exponential phase between days 2 and 6 followed by the stationary phase. During the exponential phase, the PCV increased steadily

up to day 6 showing a 4-fold increase in PCV. After day 7 the PCV stayed at the same level as a characteristic of cells in the stationary phase. The doubling time of the culture was 3.1 days. Samples representing the lag phase between days 0-2 were not recorded as it was noticed that taking aliquots at these time intervals disrupted the growth curve possibly because of the reduction in the amount of initial inoculum transferred during subculturing.

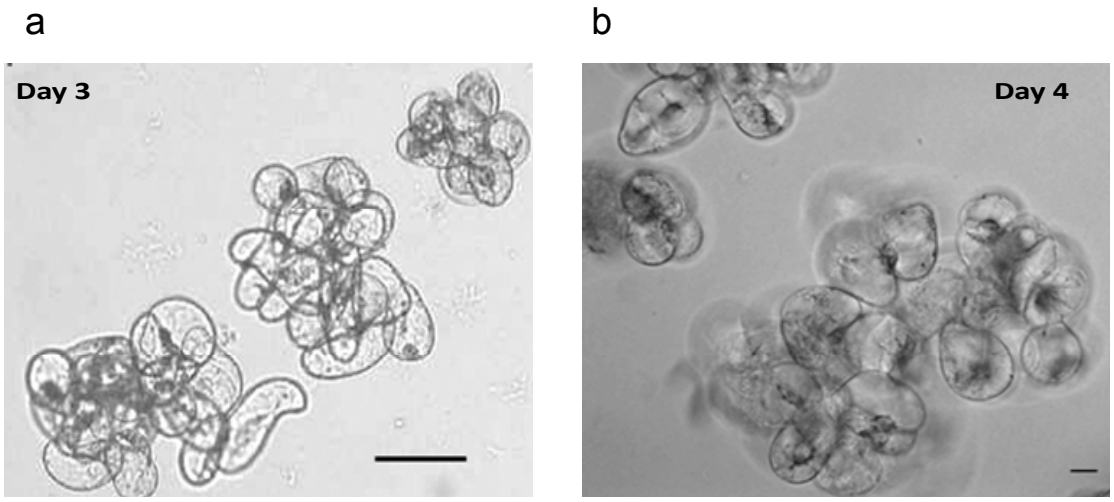


Fig. 3.1 Light microscopic view of cell morphology and clump sizes of Arabidopsis suspension cultures, samples representing cells in 3 days (a) and 4 days (b) after subculturing. Scale bar representing 10 µm.

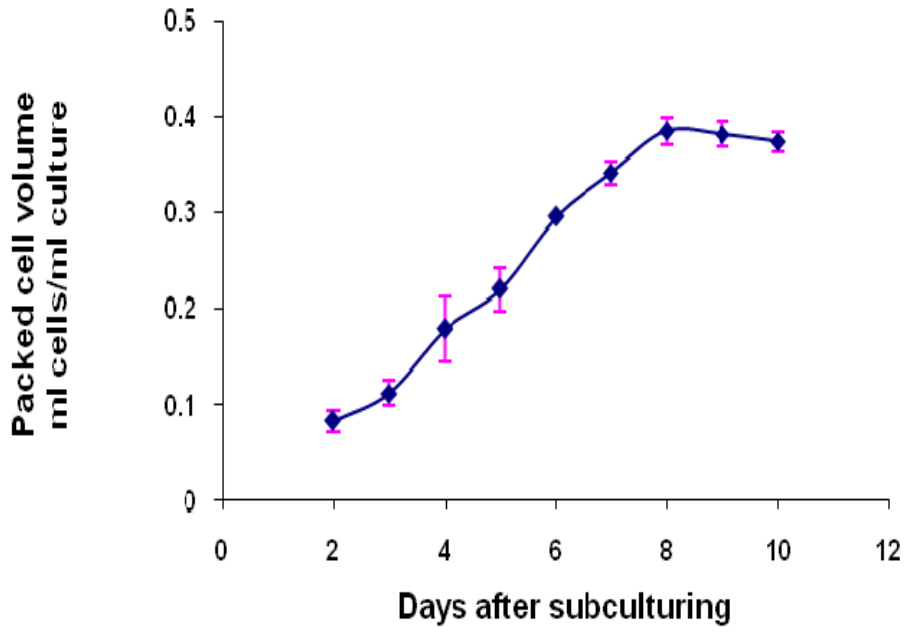


Fig. 3.2 Growth curve of Arabidopsis suspension culture. The graph shows mean ± SD for three different cultures.

3.3 Chemical analysis of extracted pectin

3.3.1 Sugar content of the extracted pectin

The total extracted sugar and uronic acid (UA) contents of the phosphate/EDTA buffer extracts of the alcohol insoluble residue are illustrated in Fig.3.3. The alcohol insoluble residue includes the Arabidopsis cells with the intracellular proteins, RNA, starch and wall polymers. It is free from chlorophyll, low molecular weight sugars, amino acids, organic acids and inorganic salts (Fry, 1988). The extraction of dicot cell wall pectin with chelating agents brings Ca^{2+} crosslinked HG rich pectins into solution accompanied by some cell separation (Parker *et al*., 2001; McCartney and Knox, 2002; Jarvis, 2003; Vincken *et al*., 2003; Hepler, 2005). Ca^{2+} chelating agents alone were reported to be sufficient to extract HG anchored within the wall by calcium bridges, and covalently crosslinked RG-I, inducing separation of onion parenchyma cells (Ng *et al*., 2000). However, in cereals and related monocots the abundance of pectins in the primary cell wall is relatively low and cell separation by chelating agents is not generally possible.
The ratio of UA to the total extracted carbohydrate ranged from 59% to 64% of the extracted pectin suggesting that significant quantities of homogalacturonans were present in the extracts.

The total carbohydrate and uronic acid contents of the extracted pectin dialysed against water and expressed as µg sugar /ml cells, decreased significantly (at 5% level) after the first day of subculturing by 22 and 16% respectively. For the rest of the sampling period, the change in the total extract content of cell wall sugars was insignificant and in parallel with the changes of UA content. A slight increase of 11 and 19% in the total sugar and UA content respectively was observed at day 4. However, there were no significant changes in the proportions of UA along the culture cycle. This result is in agreement with the observation of Leboeuf *et al*. (2004) in the cell walls of Arabidopsis suspension cultures.

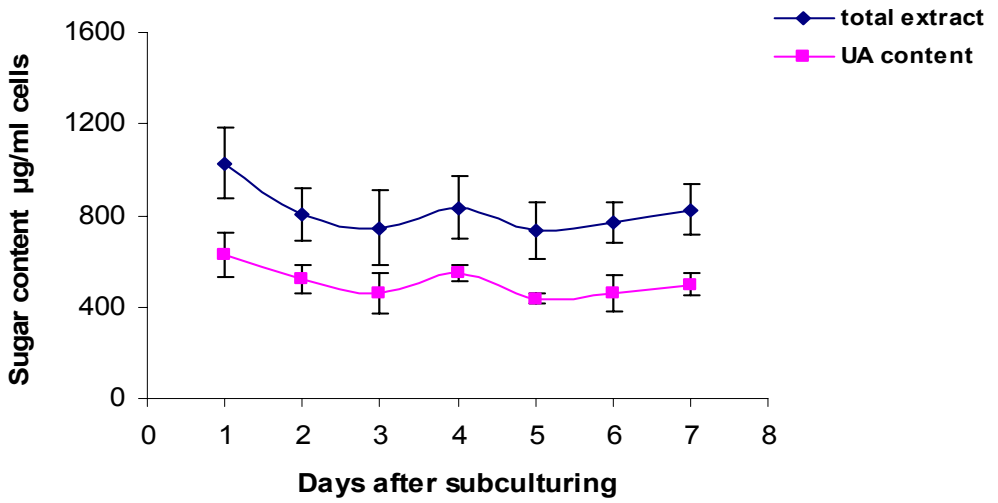


Fig. 3.3 Changes in the amounts of total extracted carbohydrates (●) and uronic acid (■) in the EDTA/phosphate buffer extracts during the culture cycle. The graph shows mean ± SD for three different cultures.

3.3.2 Degree of methylesterification of extracted pectin

The degree of methylesterification of HG fraction of the extracted pectin was determined by saponification for 1 h at pH 12 at 4^{o}C, followed by the enzymic oxidation of the released methanol by alcohol oxidase (Klavons and Bennett 1986; Guillemin *et al*., 2005). The ratio of methanol to uronic acid (UA) content was used to estimate the degree of methylesterification (%DM). Samples of the extracted pectin were tested for the presence of background free methyl groups before the alkali demethylation of the extracted HG. The results did not show any methyl contaminants that might interfere with estimates of the released methyl groups after the dilute alkali treatment. The % DM during the culture cycle varied from 36% to 60% (Fig. 3.4). The %DM of buffer chelator soluble pectin increased during the linear and elongation stages of growth to 60% at day 6. However, its level decreased to 40% at day 7 as the cells were proceeding into the stationary phase.

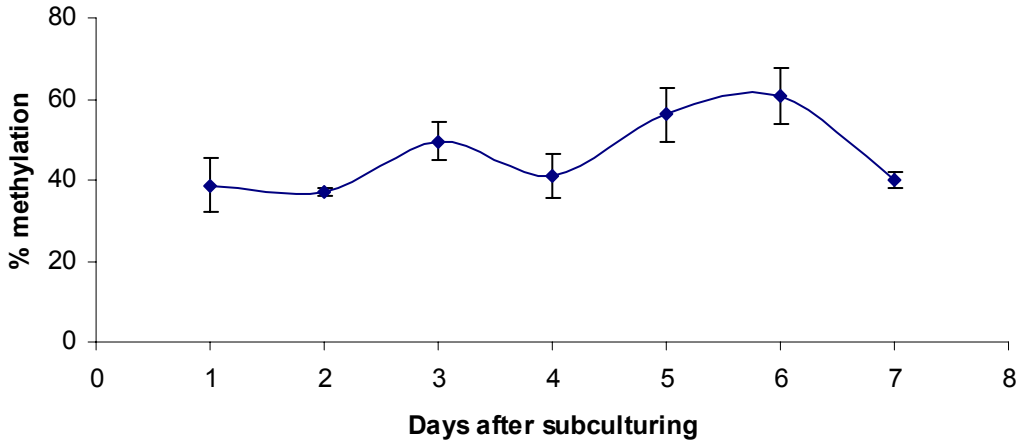


Fig. 3.4 Degree of methylation of EDTA/ phosphate buffer extracts during the culture cycle. The graph shows mean ± SD for three different cultures.

3.3.3 The neural sugar composition of the extracted pectin

The neutral fraction of trifluoroacetic acid (TFA) hydrolysates of the extracted pectin and ethanol precipitated was analysed by paper chromatography (PC) and high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The acid hydrolysis (2M TFA, 120^{o}C, 1h) was used to essentially release the neutral non-cellulosic sugar residues present in the extracted pectin with little or no decomposition of the free monosaccharides (Fry, 1988; Popper and Fry, 2004).

3.3.3.1 Precipitation of the extracted pectin

The EDTA/phosphate extracted sugars were precipitated from the aqueous solution (10 ml) at 4^{o}C for 48 h by the addition of ethanol (40 ml) to a final concentration of 80%. Some of sugars formed insoluble droplets that remained suspended in the solution and others formed a gel like layer at the bottom of the graduated cylinder. The sugar content of the samples during preparation was listed in Table 3.1. Most of the pectin was lost through the preparation whether during the precipitation, concentration and/or acid

hydrolysis, thus the chromatographic analysis of the released neutral sugars gave only a qualitative insight onto the component sugars.

Days after sub-culturing	**Sugar content (µg/ 10 ml pectic extracts)**	***Sugar content after TFA hydrolysis (µg/ml cells)***	***% recovery of extracted pectin***
1	1282.43 ± 85.49	207.56 ± 9.73	16 %
2	1143.59 ± 171.25	276.89 ± 32.64	24 %
3	1137.42 ± 167.13	341.89 ± 66.57	29 %
4	1218.35 ± 193.19	348 ± 23.46	28 %
5	1212.08 ± 135.36	222.08 ± 27.87	18 %
6	1321.03 ± 126.46	370.52 ± 42.27	28 %
7	1607.2 ± 128.0	312.1 ± 33.22	19 %

Table 3.1 Sugar content of the pectic extracts and TFA hydrolysed samples. The table shows mean ± SD for three different EDTA/phosphate extracts from three different cultures.

3.3.3.2 Paper chromatography

The pattern of separation of the standard sugars was studied before the analysis of the extracted pectin (Fig. 3.5). The standard sugars were divided into two batches A and B and developed in a system composed of ethyl acetate: pyridine: acetic acid: water (18:3:1:2). Batch A contained a mixture of fucose (Fuc), galacturonic acid (GalA), mannose (Man), xylose (Xyl) and galactose (Gal). Batch B included glucose (Glu), glucuronic acid (GluA), arabinose (Ara), rhamnose (Rha) and 1, 2:3, 5-di-o-isopropyl-idene-α-D apiose (Api). Apiose was not detected on the chromatogram whether stained with aniline phthalate or sliver nitrate, possibly because the TFA hydrolysis of 1, 2:3, 5-Di-O-isopropyl-idene-α-D-apiose did not release the requested

apiose or it has a high rate of migration and leaked into the developing solution. A large faint spot of Rha was located on the chromatogram, the rest of the included sugars showed different mobilities. In order to study the component neutral sugars of the extracted pectin, the optimum separation was obtained after running the chromatogram for 36 h using the same solvent system (Fig. 3.6)

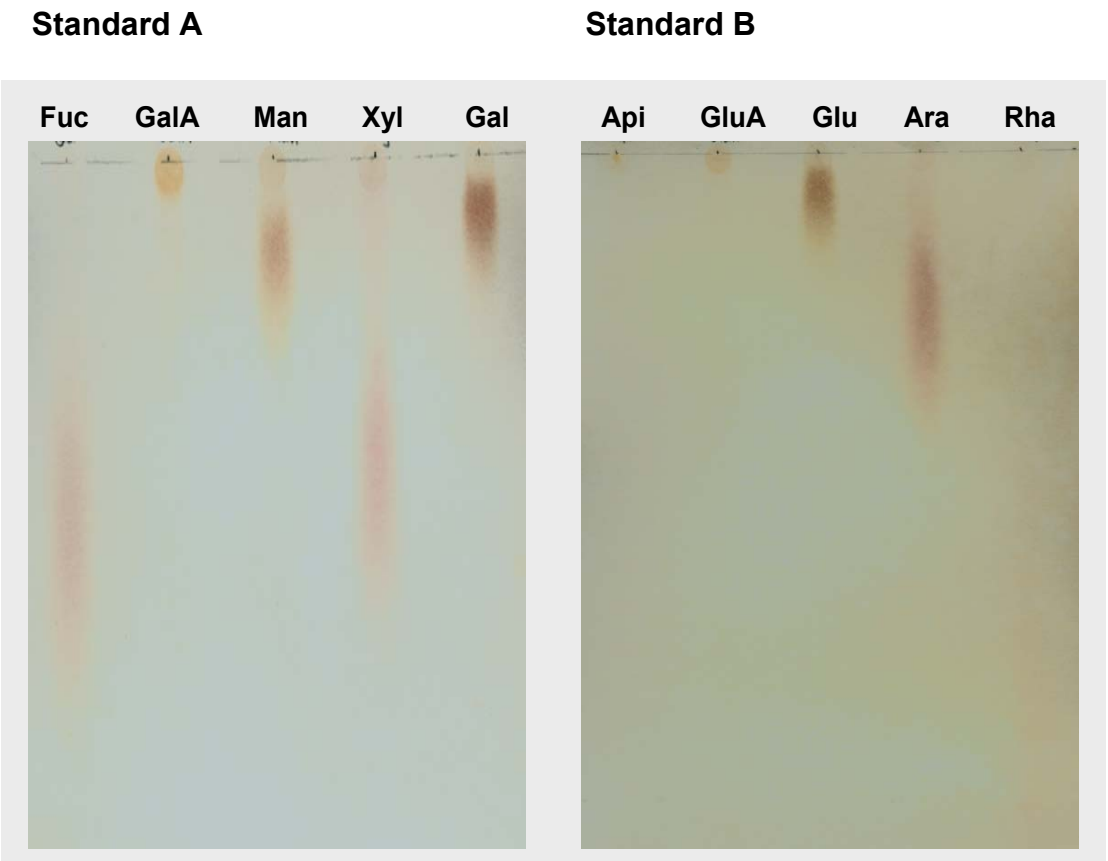


Fig. 3.5 Paper chromatography of standard sugars. Standard A contains 5 mg/ml of fucose (Fuc), galacturonic acid (GalA), mannose (Man), xylose (Xyl) and galactose (Gal). Standard B contains 5 mg/ml of apiose (Api), glucuronic acid (GluA), glucose (Glu), arabinose (Ara) and rhamnose (Rha).

The chromatogram obtained revealed the presence of galacturonic (GalA) and glucuronic acids (GluA), galactose (Gal), some glucose (Glu), arabinose (Ara), and a small portion of xylose (Xyl). Mannose (Man) was not detected in the chromatogram. Fucose (Fuc) was included in standard A but was not detected on the chromatogram; possibly it has leaked out of the Whatmann paper. The chromatogram did not detect the presence of Rha residues as part

of the neutral sugars, though it is an essential residue in the backbone of rhamnogalacturonan I (RG-I). There is an unidentified spot with mobility just less than the Rha standard. It could be a modified form of Rha or of another sugar. But it seems that most or all of the Rha was lost during the hydrolysis. Previous studies reported the EDTA/phosphate co-extraction of Rha and HG (Leboeuf *et al*., 2004). The same carbohydrates are components of the pectins obtained from other plant raw materials (Asamizu *et al*., 1984; Jones *et al*., 2003).

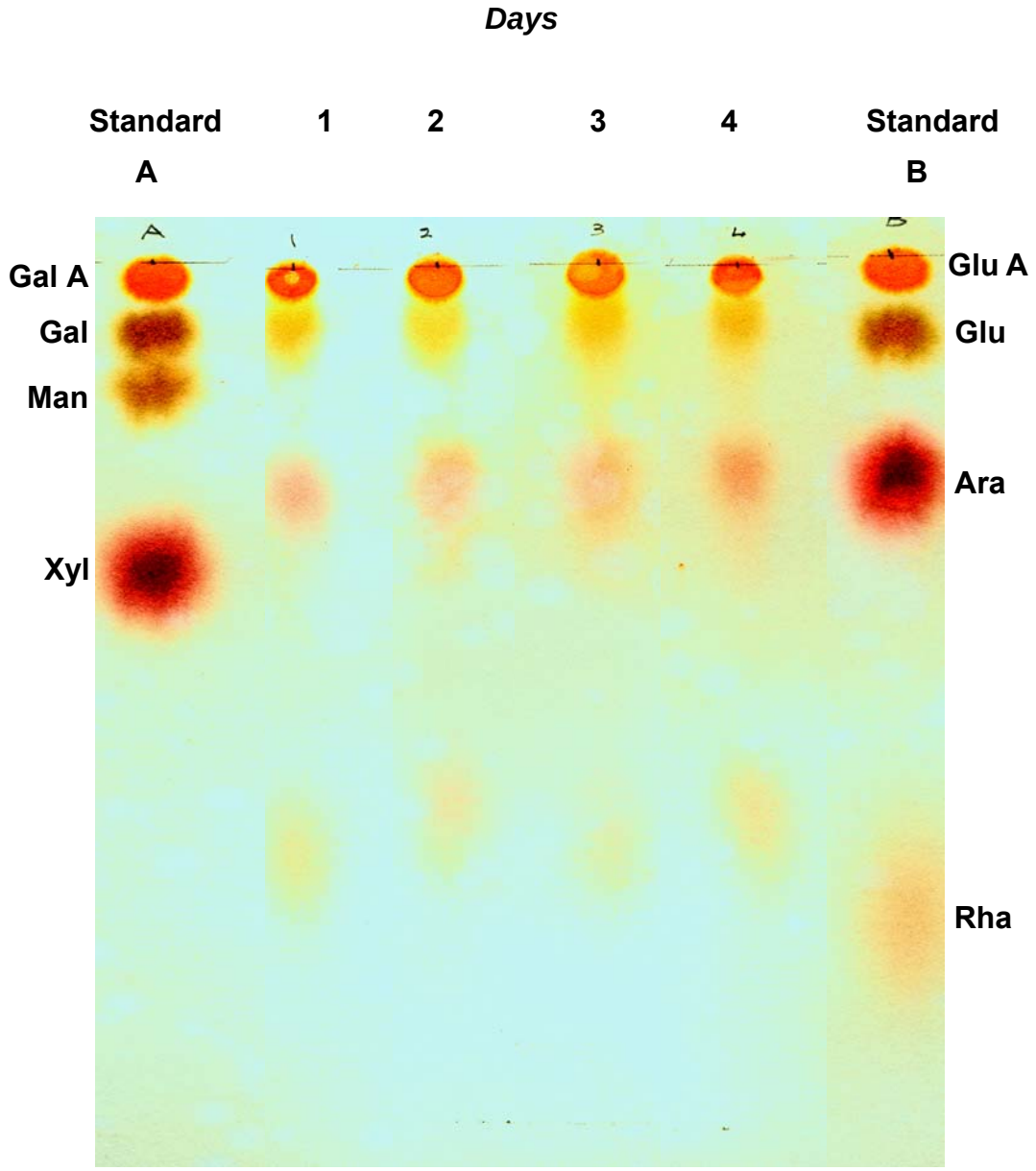


Fig. 3.6a Paper chromatography of the extracted pectins during day 1, 2, 3 and 4 after subculturing. The results were reproducible in three different EDTA/phosphate pectic extracts of three different cultures.

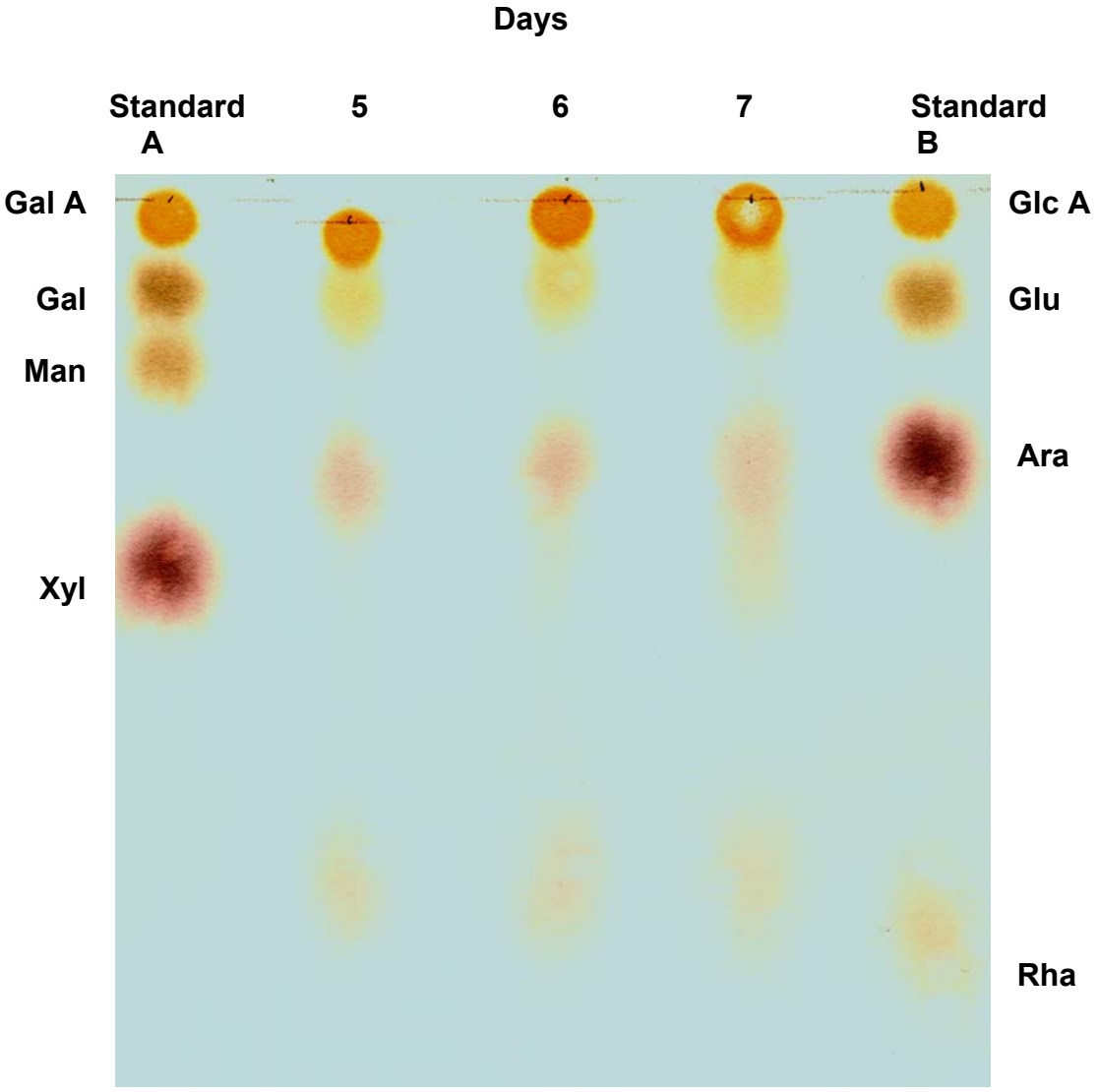


Fig. 3.6b Paper chromatography of the extracted pectins during day 5, 6 and 7 after subculturing. The results were reproducible in three different EDTA/phosphate pectic extracts of three different cultures.

3.3.3.3 High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).

The HPAEC-PAD chromatogram of sugar hydrolysates of the phosphate buffer extracted pectic material showed that the major neutral sugars detected were characteristic of pectins and included Gal, Ara, Glu, and Rha, whereas Xyl, Man and Fuc represented the minor sugars (Fig. 3.7 and 3.8). Arabinose and galactose were not reliably resolved and the mannose peak tended to tail.

A comparison of the component sugar residues of pectic fractions extracted from *Arabidopsis thaliana* suspension cultures (Bouton *et al*., 2002), sycamore suspension cells (Ishii *et al*., 1989), *Poplus alba* suspension culture (Kakegawa *et al*., 2000), and our suspension cultured Arabidopsis showed the presence of Ara, Rha, Xyl, Gal, Glu, Man and Fuc as the main neutral component residues of the cell wall pectin matrix.

The results in Fig 3.8 suggest that both Gal and Ara showed opposing changes between days 3 to 7. Arabinose content increased by 24% while Gal content decreased by 15%. The same period showed an increase in the detected Rha while there was no detectable change in the proportions of Glu, Xyl and Man. The obtained results suggest an increase in Rha residues commonly associated with rhamnogalacturonan-I (RG-I) decorated with arabinose side chains. Some type-I arabinogalactan could be synthesized during this period. Actively proliferating cells were reported to display large amounts of arabinose rich domains as shown in suspensions or callus cells of carrot and sugar beet (Kikuchi, 1996 and Majewska-Sawka and Münster 2004).However in our culture we can not confirm these observations as we could not define which of the monosaccharides was/were lost during the sample preparation, and thus the results were just interpreted qualitatively.

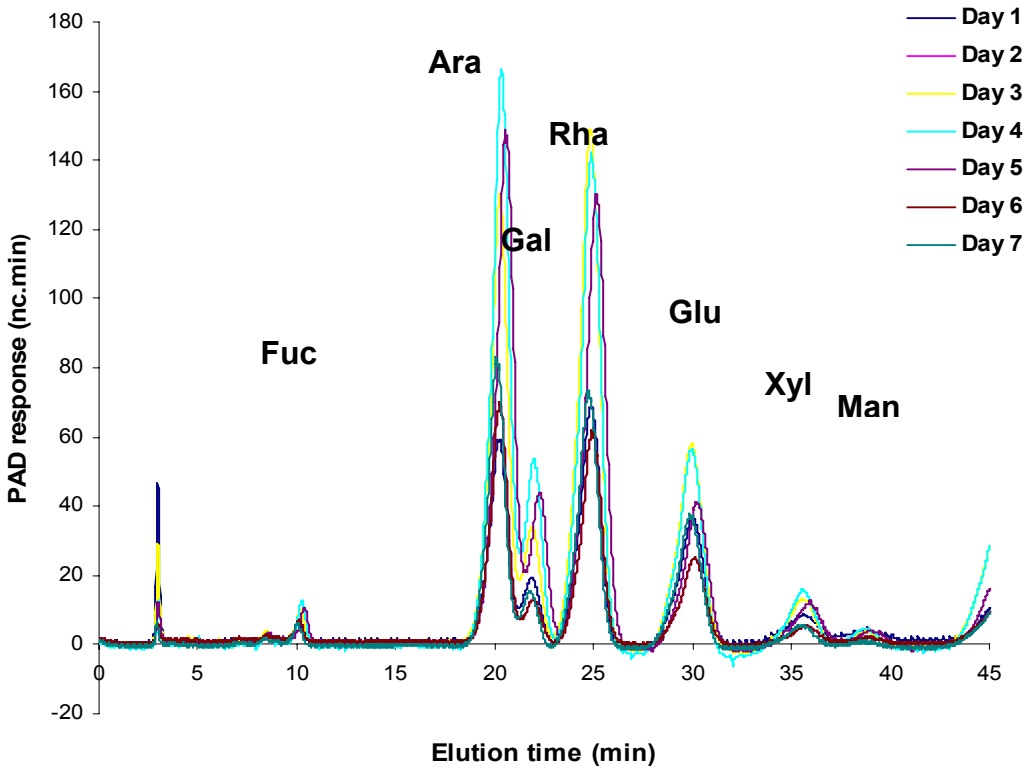


Fig 3.7 The HPAEC-PAD Chromatograms showing the elution of the component neutral sugars during the culture cycle.

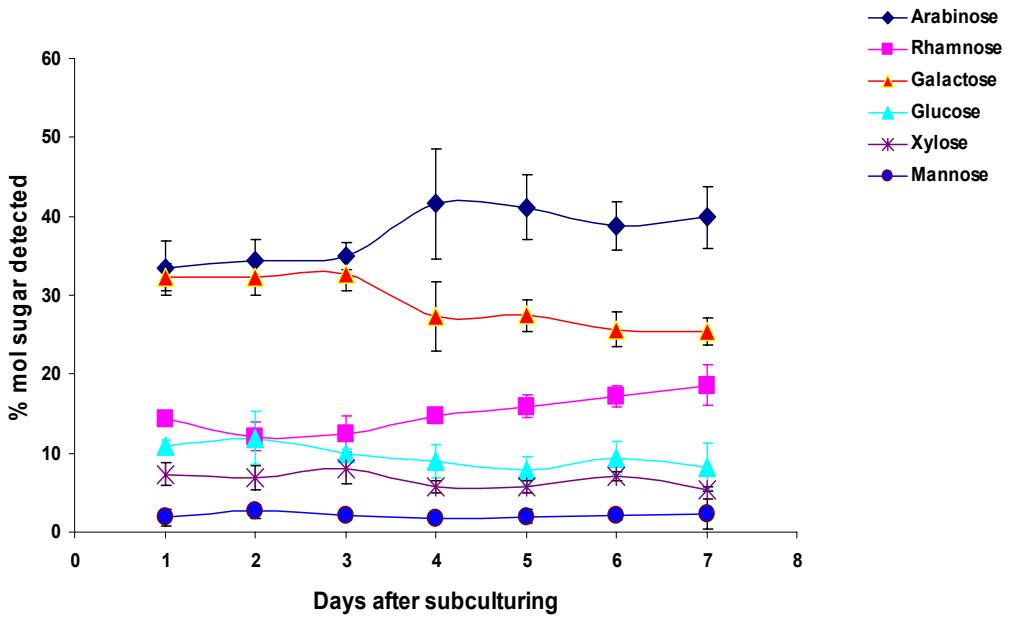


Fig. 3.8 Molar composition of neutral sugars detected on HPAEC-PAD of EDTA/ phosphate pectic extracts during culture cycle. The graph shows mean ± SD for three different cultures.

3.4 Detection of de-esterified and methylesterified homogalacturonans during culture cycle

Binding of the different anti-HG and anti RG-I side chains monoclonal antibodies to the Arabidopsis extracted pectic polysaccharides, as seen in the obtained immuno-profiles, and immuno-labelling of the resin embedded sections using immuno-fluorescence and immuno-electron microscopy, were used to investigate the structural relationship between the patterns and extents of methylesterification of HG epitopes, RG-I side chains and the adhesion between cells through the culture cycle.

3.4.1 Binding of monoclonal antibodies JIM7 and JIM5 to the extracted HG

The appearance and the diffusion of the 50mM EDTA/ 50mM sodium phosphate solubilised polysaccharides, forming two rings away from the point of application could be attributed to the relative mobilities of the different pectic components within the sample. Smaller and/or less branched HG components migrated further from the point of application while larger and/or branched components produced a dot or ring before attaching to the nitrocellulose during drying (Willats *et al*., 1999a and b). The same pattern of migration of pectin when applied on nitrocellulose membrane was observed using sugar beet extracted pectins (Guillemin *et al*., 2005) and lime pectins (Willats *et al*., 2000b and 2001c).

The monoclonal antibody JIM7 recognizes highly methylesterified residues with adjacent or flanking unesterified GalA (Clausen *et al*., 2003). It bound strongly to the extracted pectins from Arabidopsis cells during the whole culture cycle (days 1-7). The signal appeared to decrease slightly between days 1 to 4. It increased again during late division and stationary phase. This might reflect the continuous production and investment of the cell in the already established and newly deposited and formed cell walls (Fig. 3.9).

Samples of the same extracts were also probed with JIM5 (Fig 3.9) that bound strongly to methylesterified GalA residues up to a level of about 40%

degree of esterification (Willats, 2001c). The EDTA extracted pectins containing the epitope appeared to be present through the culture cycle except on day 4 during the exponential phase of culture growth. The results showed that the amount of chelator extracted HG with a low degree of methylation was much less than the highly methylesterified HG detected by JIM7. The JIM5 reactive epitope was recognized in both the unbranched and highly branched fractions of pectin.

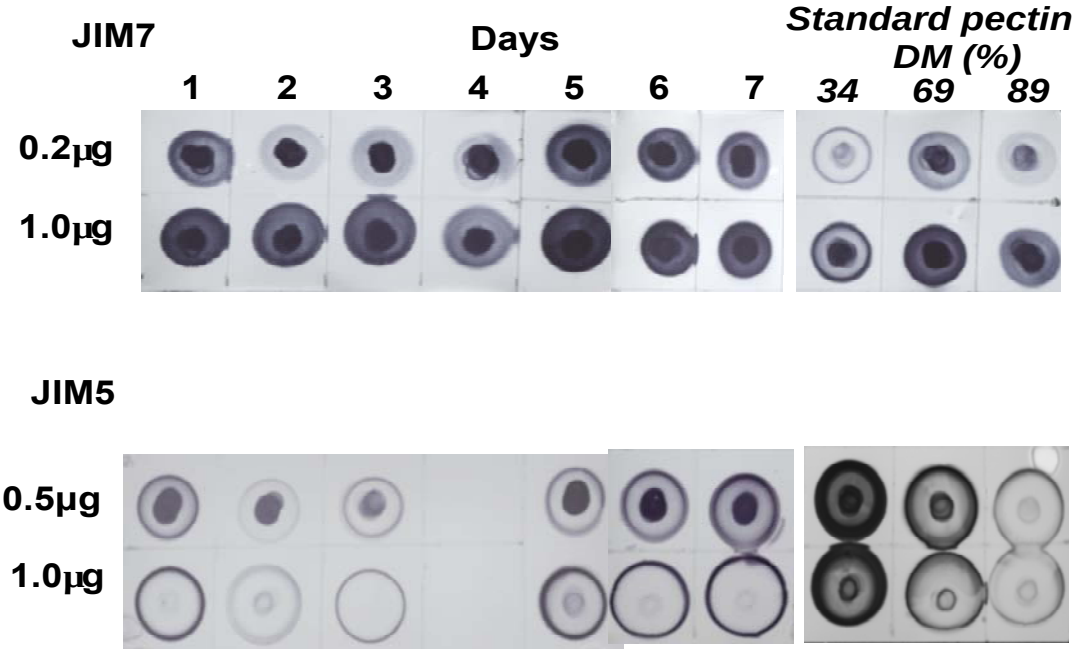
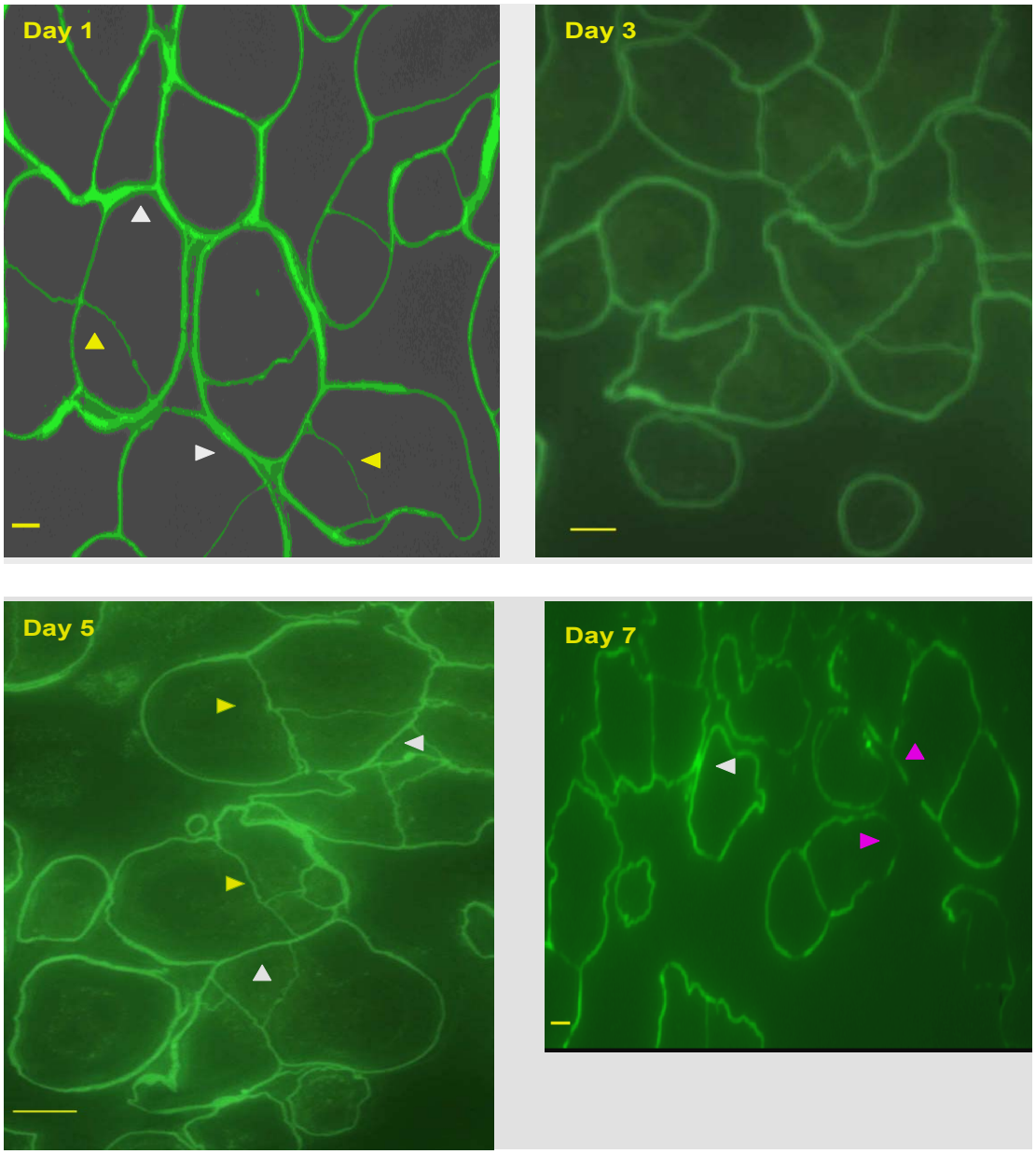


Fig. 3.9 Immuno-dot assay of monoclonal antibody JIM7 and JIM5 binding to the EDTA solubilised pectins during the culture cycle. The results were reproducible in two phosphate/EDTA pectic extracts of two different cultures.

3.4.2 Immuno-labelling of Arabidopsis cell walls with monoclonal antibody JIM7

The JIM7 epitope was abundantly distributed throughout the primary cell walls and middle lamellae of resin embedded Arabidopsis cells during the culture cycle (Fig. 3.10). Images representing the different phases of the culture cycle (day 1- lag/early exponential phase, day 3- mid exponential, day 5-late exponential and day 7- stationary phase) are shown to indicate the changes

of the recognized epitope. The older mature cell walls (white arrow) exhibited a stronger labelling compared to newly formed cell walls (yellow arrow). In day 7, the samples showed some patchy separated signals (pink arrow), maybe reflecting changes in the cell walls as the cells were entering the stationary phase. All control sections remained unlabelled when the primary antibodies were omitted.



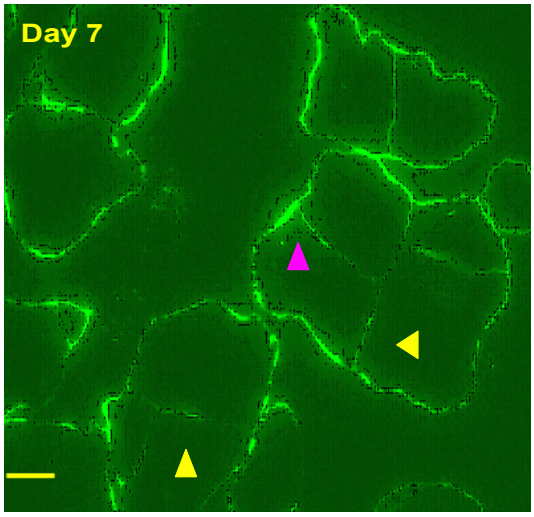


Fig. 3.10 Immunolocalization of JIM7 epitopes in resin embedded sections of wild type *Arabidopsis* cells in days 1, 3, 5, and 7. Colored arrows were used to indicate strength of the signal; yellow to label newly formed walls, white to label mature walls and pink to indicate the patchy pattern of labelling. Black dots appearing in day 7 image are artefacts due to gain in software. Scale bar represents 10 µm.

The labelling with gold-conjugated secondary antibody demonstrated the presence of the epitope in the primary cell walls mainly facing the plasma membrane of day 1 sections. It was absent from the middle lamellae except at cell junctions (Fig. 3.11). The internally located gold dots are possibly indicating the active biosynthesis of highly methylesterified pectins transported in vesicles. As the culture proceeded through the exponential phase, the gold particles were distributed throughout the different wall zones including primary walls, middle lamellae and cellular junctions (Fig 3.12). In day 7, during the stationary phase, the distribution of JIM7 highly esterified HG was uneven and patchy around the same cell, possibly due to the loss of active epitope as a result of pectin methylesterase activity (fig 3.13). This result is in agreement with the reduction in the degree of methylesters released upon saponification of the EDTA/phosphate extracted pectin.

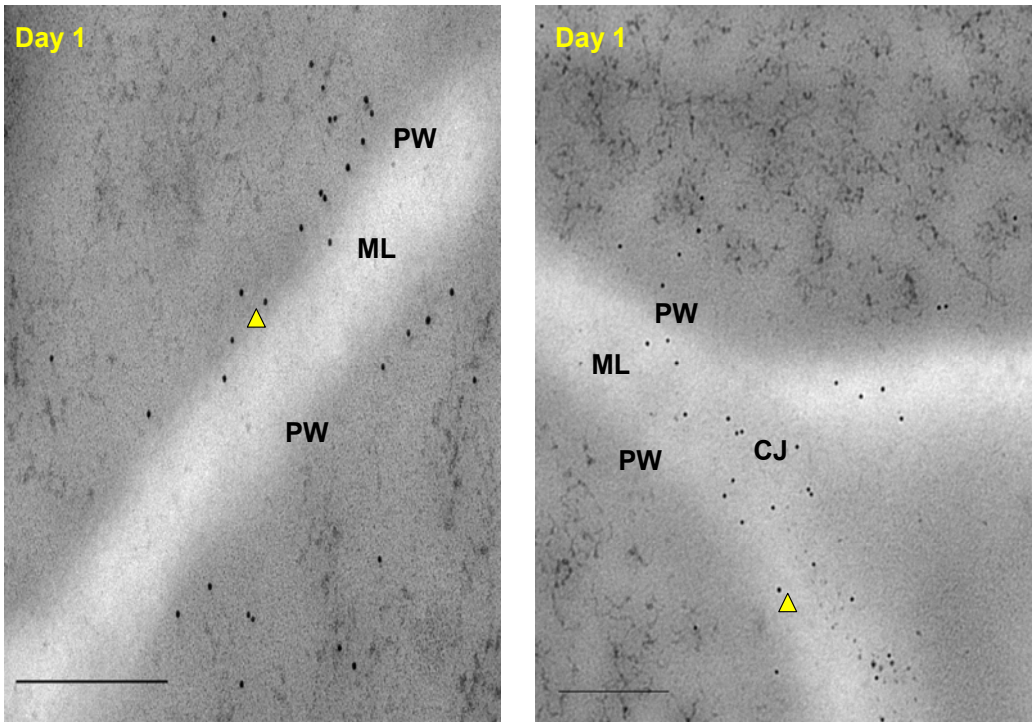


Fig. 3.11 TEM images showing immunogold labelling of resin embedded sections of day 1 using monoclonal antibody JIM7. Yellow arrows show the JIM7 recognized epitopes. Abbreviations; Primary cell wall (PW), middle lamella (ML) and cellular junction (CJ). Scale bar represents 10 µm.

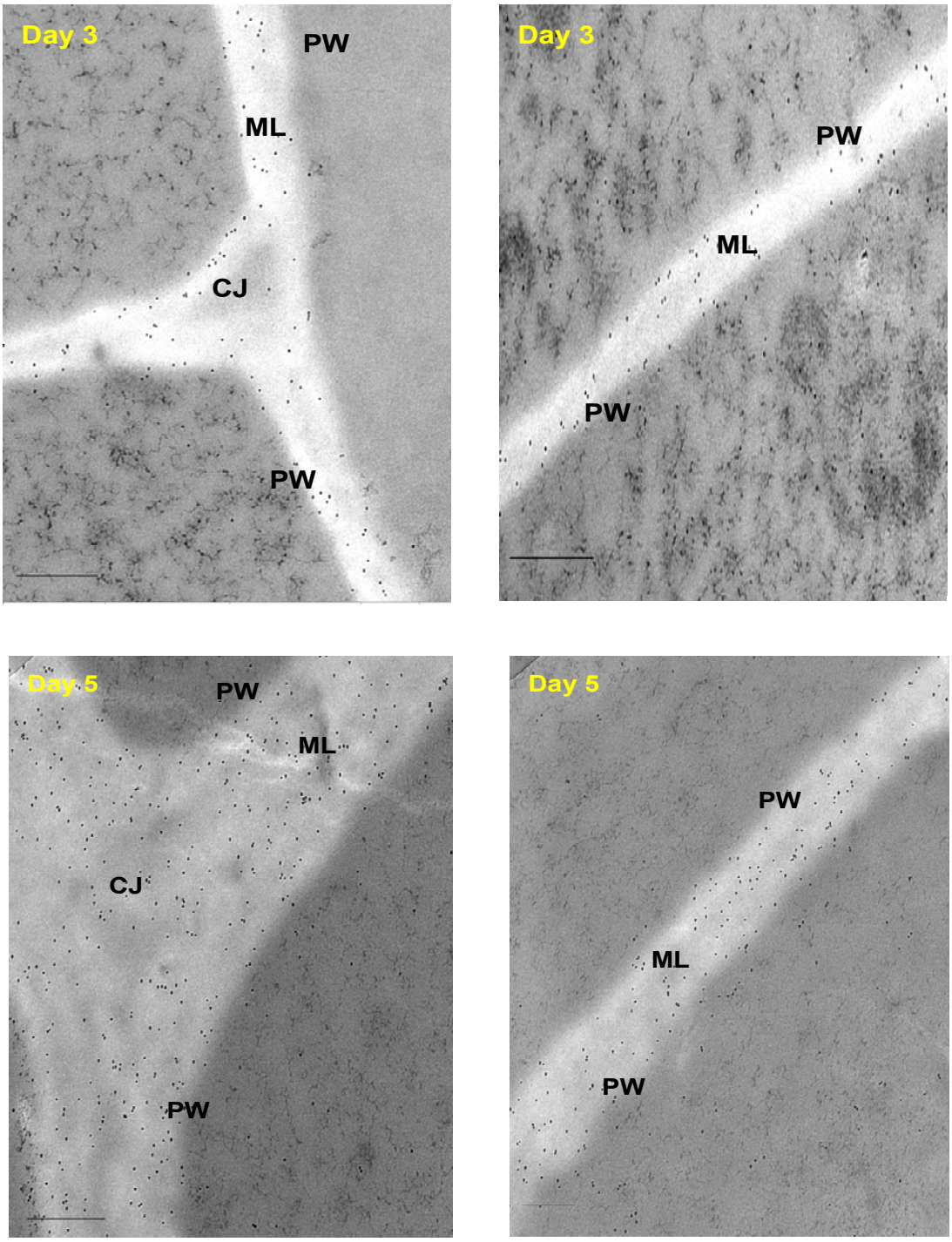


Fig. 3.12 TEM images showing immunogold labelling of resin embedded sections of days 3 and 5 using monoclonal antibody JIM7. Abbreviations; Primary cell wall (PW), middle lamella (ML) and cellular junction (CJ). Scale bar represents 10 µm.

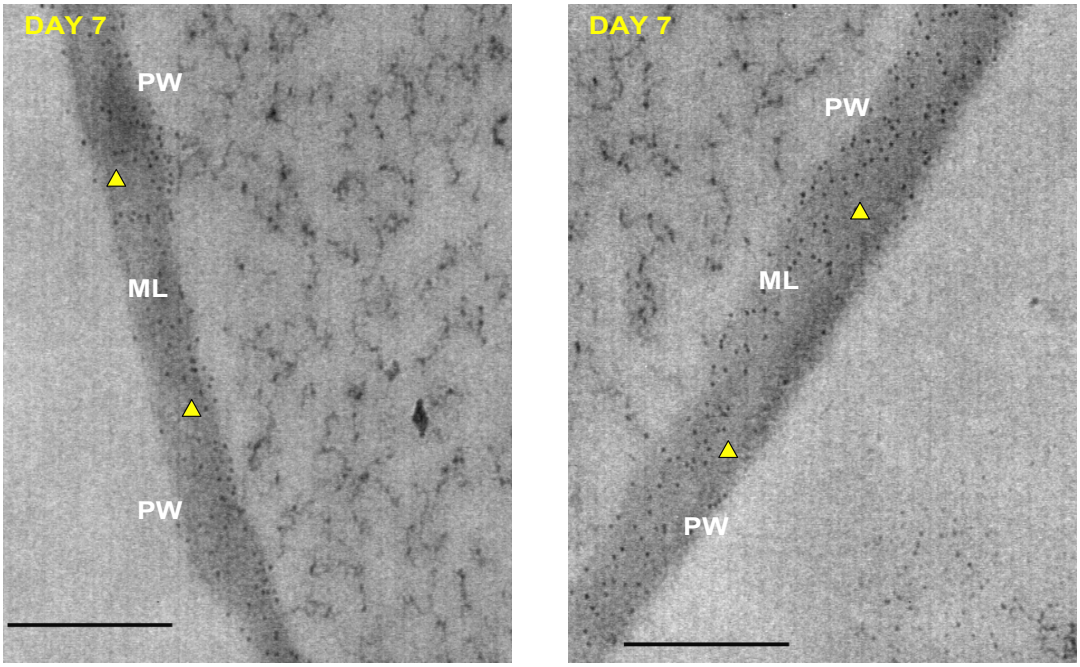


Fig. 3.13 TEM images showing immunogold labelling of resin embedded sections of day 7 using monoclonal antibody JIM7. Yellow arrows show the JIM7 recognized epitopes. Abbreviations; Primary cell wall (PW), middle lamella (ML) and cellular junction (CJ). Scale bar represents 10 µm.

4.4.3 Immuno-Labelling of Arabidopsis cell walls with monoclonal antibody JIM5

The JIM5 antibody binding to the resin embedded sections was visualized with FTIC and gold conjugated secondary antibodies (Fig3.14, 3.15). The immuno-fluorescent labelling with JIM5 indicated that HG with a low DM was less abundant in the cells of Arabidopsis cells (Fig 3.14). JIM5 HG epitope seemed to be restricted to some cells and cell wall domains, it occurred at lower levels with a weaker signal compared to JIM7 on the surfaces of cells during the different stages of culture cycle. The JIM5 epitope was unequally distributed between the different cells. A stronger signal existed at the mature cell wall especially between the neighbouring cells (Fig 3.14, white arrowhead). A weaker signal was obtained on the newly formed cells (Fig 3.14, yellow arrowhead).

The immunogold labelling of low esterified pectins with JIM5 antibody showed few gold particles confined to the middle lamellae and the cell junctions during the different phases of growth (Fig. 3.15 yellow arrowhead). No JIM5 labelling was demonstrated in the primary cell walls at any of the studied phases. This observation fits with the immuno-profiles and immunofluorescent labelling that indicated that JIM5 epitope seemed to be less abundant than JIM7 in the walls of Arabidopsis.

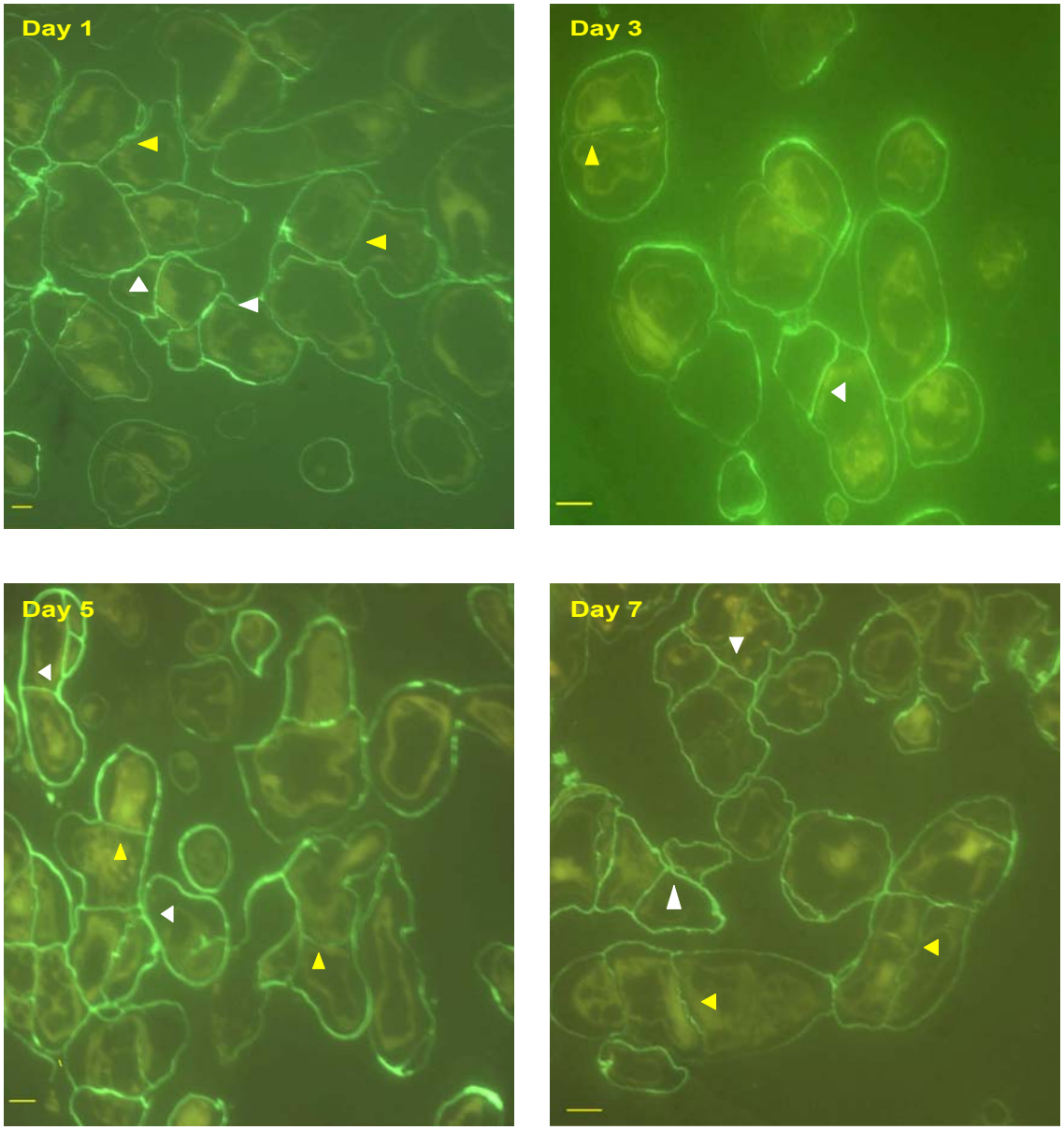


Fig. 3.14 Immunolocalization of JIM5 epitopes in resin embedded sections of wild type Arabidopsis cells in days 1, 3, 5 and 7. Colored arrows were used to indicate strength of the signal; yellow to label newly formed walls and white to label mature walls. Scale bar represents 10 µm.

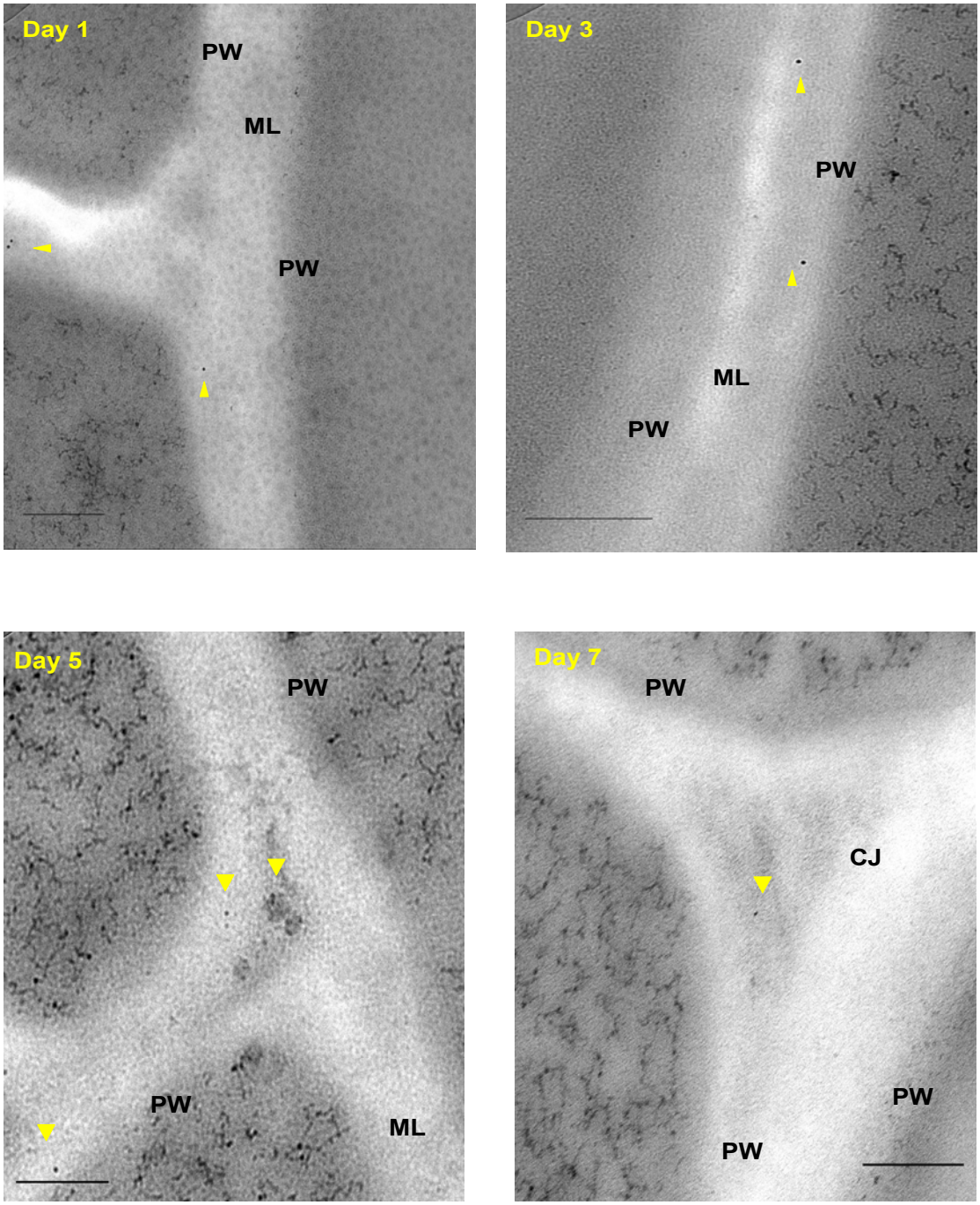


Fig. 3.15 Immunogold labelling of JIM5 recognized HG in wild type Arabidopsis resin embedded cells in days 1, 3, 5 and 7 after subculturing. Yellow arrows are used to label the JIM5 recognized epitopes. Abbreviations: primary wall (PW), middle lamella (ML) and cellular junction (CJ). Scale bar represents 10 µm.

3.4.4 Immuno-labelling of Arabidopsis cell walls with monoclonal antibodies LM7 and 2F4

LM7 is a monoclonal antibody that recognizes randomly methylated HG with a non blockwise distribution. The epitopes recognized by LM7 were not detected in the immunoprofiles of the EDTA/phosphate solubilised pectin samples nor in the resin embedded sections labelled with the antibody. The absence of the LM7 epitope could be attributed to the instability of the epitope, which was reported to be extremely labile and tends to be lost during sample freeze/thawing and processing (Willats *et al*., 2001c). Labelling of the glutaraldehyde fixed un-embedded cells was not easy due to the autofluorescence of the chlorophyll inside the cells and the clumpy nature of the culture (results not shown).

The monoclonal 2F4 antibody recognizes the calcium induced egg box dimers of nine acidic HG residues (Liners and Van Cutsem 1992; Willats *et al*., 2000b). The 2F4 labelling pattern of the embedded cells was examined by immunofluorescence and immuno-electron microscopy in middle lamellae and junction zones. The 2F4 was not detected in the embedded cells possibly reflecting the absence of the Ca^{2+} bridges which apparently are not playing a strong role in the adhesion between cells in clusters (data not shown).

3.4.5 De-esterification of resin embedded cells

The methylesters localized on the HG of the cell walls of resin embedded cells were removed through the treatment of sections with pectin methylesterase (oPME) or 1M NaOH. Pectin methylesterase isolated from orange peel (oPME) has been used in previous research works to modify to some extent the degree and patterns of HG esterification (Willats *et al*., 2001b; Leboeuf *et al*., 2005; Guillemin *et al*., 2005; Sobry *et al*., 2005). During the culture cycle, the JIM7 epitope was abundant and distributed in all the primary cell walls of Arabidopsis cells (fig. 3.10, 3.11 and 3.12). After the alkali treatment, no labelling was found in the cell walls using JIM7 or JIM5, while labelling with

JIM7 after the hydrolysis of methylesters using oPME was reduced suggesting that the epitope recognized by JIM7 was no longer present in the treated sections (Fig 3.16). In contrast, JIM5 epitope was less abundant and not evenly distributed through the primary cell walls of wild type Arabidopsis (Fig.3.14 and 3.15), and yet labelling the cells with JIM5 did not show an increase in the level of unmethylated GalA after treating the sections with oPME (results not shown). The alkali seemed to have a stronger deesterification effect than oPME, possibly because NaOH can have a better access to pectin deep within the resin and oPMEs are large protein molecules that can only access the surface of the sections. These results indicate that the differences in labelling observed in the immersion labelling and electron microscopy experiments were not due merely to differences in the permeability to antibody probes but reflected the abundance of the epitope throughout the cell walls of the suspension cultured cells.

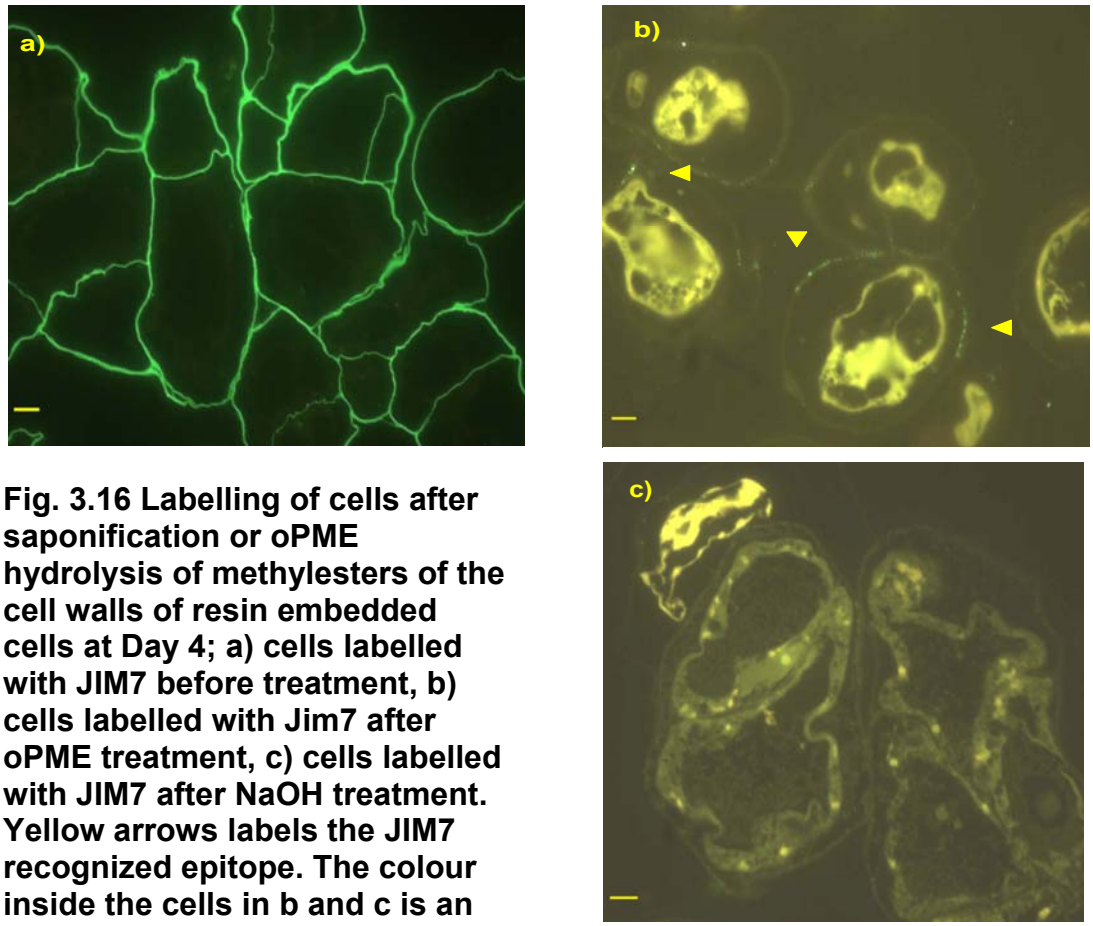


Fig. 3.16 Labelling of cells after saponification or oPME hydrolysis of methylesters of the cell walls of resin embedded cells at Day 4; a) cells labelled with JIM7 before treatment, b) cells labelled with Jim7 after oPME treatment, c) cells labelled with JIM7 after NaOH treatment. Yellow arrows labels the JIM7 recognized epitope. The colour inside the cells in b and c is an artefact due to gain in software. Scale bar represent 10 µm

3.5 Distribution of rhamnogalacturonan I-associated epitopes

Anti galactan and anti arabinan probes, LM5 and LM6 respectively, were used to characterize the occurrence of RG-I in the extracted pectin and *in situ* localization of these epitopes in the suspension cultured Arabidopsis cells. The RG-I associated (1→4)-ß-D-galactan and (1→5)-α-Arabinan epitopes are common features of the side chains attached to rhamnose residues in the backbone of RG-I. The citrus pectic polysaccharides with different degrees of esterification were used as standards as they were reported to bind to these two epitopes (Jones *et al*., 1997; Willats *et al*., 1998). All cell walls of embedded cells remained unlabelled when the primary monoclonal antibodies were omitted.

The LM5 antibody was used to probe the presence of galactan side chains. It bound to the extracted pectin during all the days of the culture cycle indicating that these pectins contain at least four consecutive units of (1→4)-ß-linked Gal (Jones *et al*, 1997) (Fig. 3.15). The epitope was less abundant than the JIM7 epitope, and was more concentrated in the central dots as part of the large highly branched pectins. The LM5 detected epitopes could not be detected in the resin embedded sections using light microscopy. In previous studies, the recognition of LM5 binding (1→4)-ß- Gal was related to the differentiation and elongation of cells (Willats *et al*., 1999b; McCartney, *et al*., 2003). The restricted occurrence of (1→4)-ß-D-galactan epitope at the surface of wild type Arabidopsis roots appears to be a molecular marker for rapid cell elongation. (McCartney *et al*., 2003

The monoclonal antibody LM6 recognises five residues of (1→5)- α-linked arabinose residues (Willats *et al*., 1998). LM6 bound strongly to the two forms of extracted pectins; the highly branched immobile part showing at the central spot in comparison with the small less branched mobile fraction, during all the days of the culture cycle (Fig. 3.17). This result indicates that (1→5)-α-linked Ara sequences are more abundant than the LM5 recognized epitope in the EDTA/phosphate extracted pectin fraction of the Arabidopsis suspension

cells. This epitope is frequently associated with the primary cell walls of young proliferating cells (Willats *et al*., 1999b; McCartney *et al*., 2000). Large amounts of arabinose rich domains were detected in carrot meristems (Willats *et al*., 1999b) and suspension and callus cells of carrot and sugar beet (Kikuchi *et al*., 1996; Majewska-Sawka and Műnster, 2003).

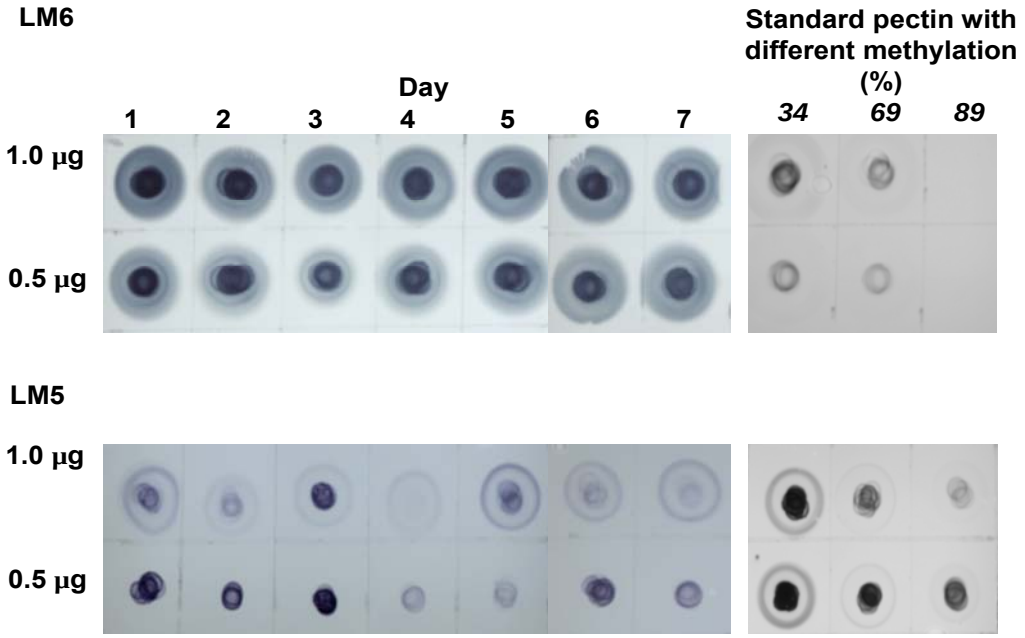


Fig. 3.17 Immuno-dot assay of monoclonal antibody LM6 and LM5 binding to the extracted pectins during culture cycle. The results were reproducible in two phosphate/EDTA pectic extracts of two different cultures.

During the culture cycle, the arabinan recognized by LM6, like the highly methylesterified HG, was abundant throughout the primary cell walls of LR White resin embedded Arabidopsis cells. No differences were observed in the abundance of LM6 between the different days. However, newly formed cell walls contained a lower level of the epitope (Fig. 3.18).

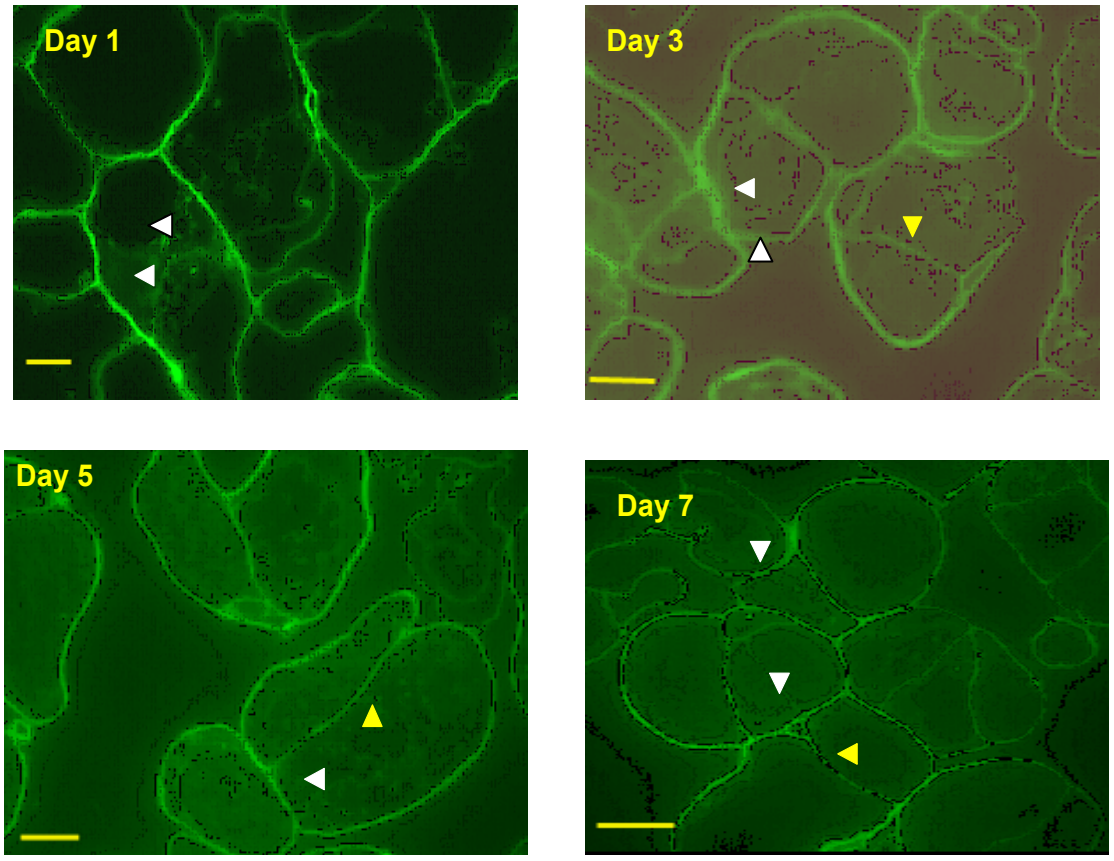


Fig. 3.18 Immunolocalization of LM6 epitopes in resin embedded sections of wild type Arabidopsis cells in days 1, 3, 5, and 7. Colored arrows were used to indicate strength of the signal; yellow to label newly formed walls and white to label mature walls. Scale bar represents 10 µm.

3.6 Discussion

The growth kinetics of *Arabidopsis thaliana* cell suspensions following their subculture into fresh medium could be divided into two reproducible phases, namely an exponential cell proliferation phase in days 2 to 7 and a stationary phase after day 7.There may have been a lag phase during days 0-2. The increase in packed cell volume (PCV) during the period of exponential phase could be associated with a high rate of cell division, whereas the stationary phase was marked with a constant PCV, as a result of a reduction in the rate of cell division, as a consequence of the depletion of nutrient media and accumulation of metabolic by-products. The cell suspension culture used was composed of single cells and aggregates of less than 1mm in diameter. Very

few intercellular spaces located at the tricellular junctions of cells could be seen microscopically. This could be related to the lack of the turgor generated tensile forces that tend to separate cells at the tricellular junctions (Jarvis *et al*., 2003).

During the successive phases of culture cycle, as a result of cell division and growth, numerous new primary cell wall components are expected to synthesized, assembled, modified, and turned over, The primary cell wall is composed of cellulose, hemicellulose, pectin, and structural protein (Brett and Waldron, 1996). Pectic polysaccharides, especially polymers of HG, are enriched in intercellular matrices (middle lamellae) and are thought to play a key role in cell-cell adhesion (Willats *et al*. 2001a and b; Jarvis *et al*., 2003; Vincken *et al*. 2003). The presence of these cell wall components was reported in the suspension cultured cells *of* Arabidopsis (Leboeuf, *et al*., 2005) and sycamore (Keegstra *et al*., 1973).

The total amounts of the extracted sugars using a system of phosphate/chelator buffer did not show a marked change during the different days of our cultures. The sugar composition of the extracts was analysed using paper chromatography, high performance anion exchange chromatography with pulsed pad amperometric detection (HPEAC-PAD) and colorimetric assays. The pectic extract was precipitated by adding ethanol to a ratio of 80%, concentrated in vacuum and acid hydrolysed at 120^{o}C for 1h. The percentage recovery of sugar was less than 10% of the initial sugar content extracted from the alcohol insoluble cellular residues, and thus the obtained results were analysed to provide a qualitative view of the changes of the composing monosaccharides during the culture cycle.

The chelator/phosphate soluble fraction of Paul's scarlet rose (Wallner and Nevins, 1974), sycamore (Ishii *et al*., 1989), soybean (Ishikawa *et al*., 2000), apple pectins (Zsivanovits, *et al*., 2004), *Myrothamnus flabellifolius* (Moore *et al*., 2006) and Arabidopsis suspension cultured cells (Manfield *et al*., 2004; Leboeuf *et al*., 2005) possessed a pectic character as shown by high GalA content with additional sugars as galactose, arabinose, rhamnose, fucose,

mannose, xylose and glucuronic acid. The chelator phosphate buffer solubilises the calcium associated and highly methylesterified homogalacturonans (HG) with some neutral sugars attached to rhamnogalacturonan (Willats, *et a*l., 2001a; Garcia-Angulo, 2006). The primary cell walls of Arabidopsis leaves were reported to be remarkably rich in phosphate buffer soluble pectic polymers. This observation suggests that much of the pectic components of Arabidopsis leaves are relatively weakly held in the wall network (Zablackis *et al*., 1995).

The UA assay used responds to both glucuronic and galacturonic acids (Blumenkrantz and Asboe-Hansen, 1973). The major uronic acid present in many of the tested land plants was GalA (Wallner and Nevins, 1974; Ishikawa *et al*., 2000; Popper and Fry, 2004; Abdel-Massih *et al.*, 2007), and thus the UA assay is used to reflect the relative content of GalA; a major component of HG. The relative overall amount of the EDTA/phosphate extracted sugars expressed as µg sugars/ ml cells did not change significantly during the culture cycle. The GalA fraction constituted over half of the pectic extract and its quantity did not change prominently during the 7 days following subculturing. According to the observed results, the pectic matrix could be undergoing some structural modifications although there was no considerable change in the number of uronic acid residues.

The presence of GalA, Rha, Gal and Ara in the EDTA/phosphate solubilised sugars suggested the release of pectins with HG and RG-1. The uronic acid content, as determined by the Blumenkrantz and Asboe-Hansen (1973) method, arabinose and galactose, as shown in PC and HPAEC –PAD chromatograms, were the major components of the EDTA/phosphate soluble extracts, while rhamnose, glucose, xylose and fucose are present in minor quantities. The presence of arabinose and galactose could be attributed to the presence of arabinans, galactans and arabinogalactans that are ubiquitous constituents of the side chains of RG-I in the primary walls of dicotyledonous species**.** The detection of glucose and xylose could be caused by the co-extraction of some xyloglucan and/or xylogalacturonan covalently linked to pectin. Femenia *et al*. (1999) and Thompson and Fry (2000) provided

evidence for covalent linkages between pectin and xyloglucan in cell walls of cauliflower stems and rose suspension cultures, respectively. The covalent linkage between xyloglucan and pectin was proposed to be formed via arabinan side chains during the biosynthesis of the hemicellulosic and pectic matrices in the Golgi apparatus (Brett *et al*., 2005). In Arabidopsis roots and carrot suspension cultures, the presence of xylogalacturonan was restricted to the loosely attached cells of root caps and free surfaces of cell clusters, respectively (Willats *et al*., 2004). In another study, small quantities of xylogalacturonan were reported to be part of the pectic polysaccharides of Arabidopsis stems, young and mature leaves (Zandleven *et al*., 2007). Some of the glucose fraction could be coming from callose, a major component of the forming cell plates during cytokinesis (Minorsky, 2002). The small quantities of mannose (Man) detected in the EDTA/phosphate extracts could be related to the hemicellulosic galactoglucomannans, which have been shown to be synthesized by mannan synthase in the Golgi apparatus of Arabidopsis callus cells (Hanford *et al*., 2003). Fucose is detected in small quantities and possibly is a xyloglucan linked residue (Zablackis *et al*, 1995; Kakegawa *et al*., 2000). The system used could not detect the presence of apiose residues in tested pectic extracts

The degree of methylesterification of the EDTA/phosphate extracts ranged from 40 to 60%. Most of the increase was recorded towards the end of the exponential phase at day 5 and 6. The increase in the degree of methylation between days 1 and 6, even though the total sugars and GA content didn't change markedly during the days 1 to 6, reflects the continuous investment of cells in the primary cell walls with newly synthesized highly methylated HG. This observation is in agreement with previous studies of different suspension cultured cells. The degree of methylesterification of suspension cultured cells of tobacco, flax and maize increased during the phase of cell division and elongation, and decreased during the stationary phase (Kim and Carpita, 1992; Schaumann *et al*., 1993; McCann *et al*., 1994).

The increase in hypocotyl length of Arabidopsis was accompanied by an increase in the degree of methylation (Derbyshire *et a*l, 2007). During the

stationary phase at day 7, the percentage of methylester groups detected on the extracted HG was less than 40%, possibly reflecting an increase in the activity of pectin methylesterase. Leboeuf *et al*., 2005 reported a reduction in the cluster size and cell-cell adhesion of wild type Arabidopsis as the culture entered the stationary phase. In order to demonstrate and emphasize the effect of pectin methylesterase hydrolysis and saponification on the HG of Arabidopsis cells, day 4 harvested LR White resin embedded cells were subjected to the two treatments prior to immunolabelling with JIM7, which recognizes the highly esterified GalA backbone of pectin (Clausen *et al*., 2003). The dilute alkali treatment at 4^{o}C for 1 h removed the JIM7 epitopes, while the orange pectin methylesterase (oPME) resulted in a weaker dot like pattern of labelling. The JIM5 labelling of low-methyl HG disappeared after the NaOH treatment and did not increase significantly after the treatment with oPME. The results obtained strongly suggest that methylesters are removed more completely by saponification and the action of oPME seems to be more selective and recognizes specific patterns of methylesterification.

The immunolabelling results supported the biochemical data and indicated the high methylester content of pectin in the Arabidopsis suspension cells. The use of the currently available monoclonal antibody probes directed against a range of polysaccharides has proven to be a valuable, rapid and reliable tool for studies of the dynamics of cell wall architecture and cell development (Knox, 1997; Willats, 2000a). JIM5 and JIM7 are rat monoclonal antibodies that recognize different patterns of methylesterification on HG; JIM7 binds to a HG with a high degree of methylesterified residues ((Knox, 1997; Willats, 2000a, Clausen *et al*., 2003), whereas JIM5 preferentially recognizes a HG backbone with a low degree of methylesterified residues (Willats *et al*., 2001b). The abundance of JIM7 detected highly methylesterified GalA in Arabidopsis suspension cultured cells has been reported earlier by Leboeuf *et al* (2005) and Manfield *et al*. (2004). In general, the pectic polysaccharides present in the walls of young or actively growing plant cells were found to be highly methylesterified whereas walls of mature cells contain strongly acidic pectin (Satoh, 1998). The JIM7 epitope was detected in the primary cell walls and cellular junctions of day 1 cells; however, it was not seen in the middle

lamellae. During the exponential phase, the epitope was uniformly distributed over the primary cell walls, middle lamellae and cellular junctions. Mature cell walls exhibited a stronger labelling signal compared with newly formed cell walls. This observation could be attributed to the biosynthesis and deposition of highly methylesterified homogalacturonans on the sides of the cell walls of daughter cells, after the completion of cytokinesis and separation between dividing cells (Vincken *et al*., 2003). In a study of Golgi apparati of suspension cultured sycamore maple (*Acer pseudoplantus* L.) cells, JIM7 antibodies strongly labelled the medial and trans Golgi cisternae suggesting that in plant cells the methylesterification of the carboxyl groups of the GA residues occurs in the medial and trans cisternae of Golgi bodies (Zhang and Staehlin, 1992). Immunocytolabelling of pea stem cortical cells indicated that low methylesterified or deesterified HG is often localized to middle lamellae, cell corners and around air spaces whereas more highly esterified pectins were distributed throughout the cell wall. At day 7, as the cells were entering the stationary phase, the JIM7 labelling was uneven around the cells, possibly reflecting modifications in the degree of methylesterification as a result of the action of pectin methylesterase. This observation coincides with the reduction in the degree of methylesterification of the EDTA/phosphate HG extracts.

During the culture cycle the JIM5 HG epitope was restricted to some cells and cell wall domains as illustrated in Fig. 3.13. No considerable changes could be detected with light microscopy. Few immunogold labelled JIM5 epitopes were recognized in the middle lamellae of the cells during the different phases of culture cycle. No labelling was seen in the primary cell walls. In carrot cultured cells, highly acidic pectin and highly methylesterified pectin were reported to be present in the middle lamella and primary cell walls, respectively (Liners and Cutsem, 1992). A homogalacturonan epitope recognized by JIM5 was detected in the cell walls of carrot suspension culture cells (Willats *et al*., 1999b), pea root apex (Willats *et al*., 2004), tomato fruit particularly in the locular jelly-like cavity surrounding the seeds (Jones *et al*. 1997, Steele *et al*., 1997), middle lamella and cell junctions of young and mature flax fibers (His *et al*., 2001) and in the endocytic vesicles involved in the turnover of plasma membrane proteins and cell wall pectins involved in

the initiation and formation of the cell plates of dividing cells of Arabidopsis and maize root cells and in tobacco BY-2 suspension cells (Dhonukshe *et al*., 2006) .

The homogalacturonans reactive to the 2F4 antibody that binds specifically to the calcium dimerized sequence of at least nine consecutive HG residues (Liners *et al*., 1992; Willats *et al*., 2001a), and LM7 antibody that recognizes non-blockwise partially methylesterified HG (Clausen, *et al*., 2003), were not detected in the resin embedded cell sections. The absence of Ca^{2+} bound matrices detected by the antibody 2F4 could be attributed to the scarcity of available calcium ions in the culturing medium. In pea stem cortical parenchyma, the LM7 epitope was located and restricted to the corners of the cell wall linings of the intercellular spaces at the point of cell to cell contact. In outer thickened cell walls of epidermal cells, the LM7 epitope occurred in discrete regions that were associated with cell junctions and maybe involved in maintaining the integrity of the outer cell layer (Willats *et al*, 2001b).

A comparison between the immunoprofiles suggests the presence of the HG epitopes recognized by JIM5 and JIM7 in both of the two forms of pectins; that is the large branched and smaller un-branched pectins. A large proportion of the extracted HG is methylesterified. A slight increase in its levels was noticed during the exponential and stationary phase. The binding of JIM5 was weaker than JIM7 reflecting that much of the chelator extracted pectins was highly methylesterified HG. The recognition level of JIM5 epitope reduced during the 3 days after subculturing, it disappeared during day 4 and picked up again towards the end of the exponential phase and during the stationary phase. The observed changes are possibly reflecting some modifications in the degrees of methylation of the HG backbone during those phases.

The RG-I associated LM5 and LM6 monoclonal antibodies recognise four residues of (1→4)-ß-D galactan and five residues of (1→5)-α-L arabinan, respectively (Jones *et al*., 1997 and Willats, *et al*. 1998). The immunodot assay (IDA) revealed that the LM6 (1→5)-α-arabinan epitope was abundant in the EDTA/phosphate extracts during the culture cycle. The immunolabelling of

embedded sections showed that the LM6 epitope was more abundant in the mature primary cell walls. Differences in the labelling intensity between the old and newly deposited primary cell walls may reflect differences in the degree of RG-I branching. Presence of arabinan was reported to contribute to the elasticity and flexibility of the cell wall (Renard and Jarvis, 1999). Arabinan in sugar beet can participate in pectin cross link via oxidative coupling of ferulic acid esterifying the arabinose and thus playing a direct role in the control of cell wall extensibility and mechanical properties (Guillemin *et al*., 2005). Ferulic acid dimers are thought to be involved in cell adhesion (Waldron *et al*., 1997).

The immunodot assay (IDA) of chelator/phosphate buffer extract probed with monoclonal antibody LM5 indicated the presence of (1→4)-β-galactan in small quantities during the different stages of the culture cycle. The failure of labelling of the embedded sections using LM5 could be attributed to the loss of the epitope during the processing of the sample Actively proliferating cells usually have low detectable amounts of LM5 recognizable epitopes, but instead display large amounts of LM6 reactive arabinan rich domains, as shown in both of the carrot (Willats *et al*., 1999b) and Arabidopsis (McCartney *et al*., 2003) root meristems, and suspension and callus cells of carrot, *Poplus* and sugar beet, (Kikuchi *et al*., 1996; Satoh, 1998; Willats *et al*., 1999b; Kakegawa *et al*., 2000; Majewska-Sawka and Munster, 2003).

The results obtained indicate that although the Ca^{2+} bound matrices seem to be an important factor in cell adhesion in many plant systems, the abundance of epitopes recognized by JIM7, along with the limited labelling of JIM5 and the absence of 2F4 reactive epitopes, rules out any significant contribution of calcium-pectate gels to the adhesion between the Arabidopsis suspension cells, and suggests that pectins with a level of esterification higher than 50% are probably making up the HG fraction of the cell wall.

The highly methylesterified HG rich pectic network abundant in middle lamellae and cellular junctions could be playing a role in the adhesion

between cells possibly by forming hydrogen bridges and hydrophobic forces between methoxy groups. Highly methylesterified pectins are thought to be involved in cell-cell adhesion in many plant species. For instance they seemed to be responsible for the tight cell adhesion between *Sinapis alba* shoot meristematic cells (Sobry *et al*., 2005). The high levels of arabinan residues as side chains of RG1 and wall arabinogalactan proteins (AGP) have been proposed to have a role in cell adhesion (Johnson *et al*., 2003; Leboeuf *et al*., 2004). This observation is in agreement with the reported results using tobacco wild type and nolac H14 (non-organogenic callus) mutant in which the loose intercellular attachment between the cells was related to low levels of arabinan residues in the side chains of pectic extracts (Iwai *et al*., 2001).

As a conclusion, the analysis of the buffer/chelator extracted pectin indicated the abundance of highly methylated HGs that are possibly through hydrophobic interactions, playing a role in the gelling of the pectin and the maintenance of intercellular adhesion between the cells. The pectic network of Arabidopsis cell walls was rich in Ara reflecting the proliferative state of the cultures. The other neutral sugars mainly Rha, Xyl, Gal, GluA, and Man are typical components of the pectic network of dicot plants. For future work, the use of a sequential extraction of all the pectin in the cell wall will give a better insight on the changes of the pectic network during the culture cycle.

Chapter 4

Abundance of pectin esterase transcripts in synchronized Arabidopsis cultures across the cell cycle

4.1 Introduction

Most of the photosynthetically fixed carbon is incorporated into cell wall assembly, making plant cell walls the most abundant source of terrestrial biomass and renewable energy (Coutinho *et al*., 2003; Reiter, 2002). The ability of plants to detect and respond to changes in their cell walls, and the cross talk between wall synthesizing and modifying mechanisms is organized through an array of enzymes, many of which have been identified. Based on genome sequencing, plants contain many more carbohydrate active enzymes than other types of organisms. Approximately 10% of the cell wall mass is made of proteins that are encoded by large gene families (Kwon *et al*., 2005). Pectin methylesterases (PMEs, EC 3.1.1.11) are enzymes belonging to family CE8 which is one out of 14 families classified within the Carbohydrate esterase category of the CAZY database (http://www.cazy.org/fam/CE8.html). Within a given sequence family, three dimensional (3D) structure fold and catalytic mechanisms are conserved. PMEs are involved in the demethyl-esterification of pectin, producing methanol and a carboxylic acid moiety on the pectin polymer (Wojciechowski and Fall, 1996).

The deesterification of pectins catalysed by PMEs leads to a complete reorganization of the cell wall in several ways. It contributes to the stiffening and rigidity of the cell wall by producing blocks of unesterified carboxyl groups that can associate with other HGA chains by calcium cross links, forming gel like matrices (Jarvis, 1984; Rihouey *et al*., 1995b; Willats *et al*., 2001a, b and c), The mechanical properties and porosity of these gels depend strongly on whether the esterification pattern is random (non-block) or linear (block wise) giving rise to blocks of free carboxyl groups (Willats *et al*., 2001c, Micheli, 2001). The strength of the interaction between Ca^{2+} and pectin increases with decreasing average degree of pectin methylestrification and increased length of the unsubstituted galacturonan back bone. The PME demethylesterfication of homogalacturonan releases protons that reduce the apoplasmic pH and promote the action of expansins (Carpita *et al*., 1996) and hydrolases, such as polygalacturonases and pectin lyases (Michei *et al*., 2001; Lefever *et al*.,

2004; Arancibia and Motsenbocker, 2006) that will degrade the pectic matrix, contributing to the loosening and expansion of the cell wall. The treatment of epidermal strips of *Vicia faba* possessing a type I cell wall, and those of *Commelina communis* and *Zea mays* possessing a type II wall, with PME and endopolygalacturonase caused an increase in the stomatal aperture on opening despite the fact that the three species are representatives of different cell wall types, reflecting a conserved functional role of pectins in guard cells (Jones *et al*., 2005).

PMEs are found in all species of higher plants, phytopathogenic bacteria and fungi and in symbiotic microorganisms (Raiola *et al*, 2004; Lievens *et al*., 2002). PMEs were reported to be involved in important developmental processes such as Arabidopsis silique development (Louvet, *et al*., 2006), fruit maturation in tomato (Eriksson *et a*l., 2004), *Phaseolus* (Stolle-Smits *et al*., 1999), Strawberries (Castillejo *et al*., 2004), grape berries (Barnavon *et al*., 2001), and banana (Nguyen *et al*., 2002)., microsporogenesis (Francis *et al*., 2006), pollen tube growth (Jiang *et al*., 2005; Tian *et al*., 2006), breaking seed dormancy and germination (Ren and Kermode, 2000), hypocotyl elongation (Al-Qsous *et al*., 2004), root development (Wen *et al*., 1999) and defence mechanisms against pathogens (Ridely *et al*., 2001). Recently, it has been proposed that the structural plant component pectin could contribute to up to 40% of the total global greenhouse gas methane (CH_4) emissions as a result the action of PMEs on the methyl groups (Keppler *et al*., 2006; Schiermeier, 2006), followed by reduction of methanol to methane.

Pectin methylesterase (PME) genes occur in multigene families and encode isoforms differing in molecular weight, isoelectric point (pI) and biochemical properties (Carbonell *et al*., 2006). It is often mentioned that plant PMEs with basic pI , which represent most isoforms, act in block wise fashion, while plant PMEs with an acidic pI act in a non block wise more random fashion (Micheli, 2001; Bosch *et al*., 2005), some PME isoforms are either constitutively expressed or differentially regulated in response to specific developmental or environmental cues (Manfield *et al*., 2004). In tomato plants, multiple isoforms of PME were reported to be either tissue specific or ubiquitously expressed

(Gaffé *et al*., 1994 and 1997). *In muro*, the demethylation of pectins is spatially and temporally regulated according to the cell type and the developmental stage of organs.

The sequencing of the *Arabidopsi*s genome has emphasized the massive genetic investment that underpins pectin biosynthesis and modification within the primary cell wall matrix (Arabidopsis Genome Initiative 2000). This plant is known to possess around 25498 genes of which > 3800 genes, ~15% of its genome, are likely to participate in cell wall biogenesis, modification, assembly and disassembly during cell development (Carpita *et al*., 2001; Yokoyama and Nishitani, 2004). It is been reported that 66 PME-related genes occur and are fairly scattered within the Arabidopsis genome (TAIR, http://www.arabidopsis.org).

PME genes can be divided into type I and type II genes depending on the expression of pre and pro proteins encoded by the PME genes and considered to be signatures of PME (Micheli, 2001). Type I genes contain five or six introns and a short or nonexistent pro region, and type II genes contain only two or three introns and a long pro region, The pre N-terminal region or signal peptide is required for protein targeting to the endoplasmic reticulum-Golgi endomembrane system for processing and secretion (Dorokhov *et al*., 2006). The pro region consists of a large peptide made of around 250 amino acids and is cleaved off at some point in the secretory pathway. The pro region could be acting as an inhibitor or intramolecular chaperone which could either prevent the correct folding of PME or directly inhibit the enzyme activity to prevent the premature demethylation of pectins before their insertion in the cell wall (Micheli *et al*., 2001; Di Matteo *et al.*, 2005; Bosch *et al*., 2005). About 65% the PME isoforms present in the Arabidopsis genome encode a pro-region with a predicted molecular mass of 15-25 kDa and acidic to alkaline pIs (Pelloux *et al*., 2007). Numerous studies have shown that the pro region shows similarities with PME inhibitors (Bosch *et al*., 2005; Louvet, *et al*., 2006). PME proteins of bacteria and fungi do not contain any pro region (Markovic and Janecek 2004)

To date, the structures of only two PMEs, one from carrot (Johansson *et al*., 2002) and one from the bacterium *Erwinia chrysanthemi* (the agent of the soft rot of plants (Jenkins *et al*., 2001)) and one PMEI from Arabidopsis (At-PMEI, Hothorn *et al*., 2004b) have been resolved. AtPMEI-1 and AtPMEI-2 are two genes encoding functional inhibitors of pectin methylesterase in wild type Arabidopsis that showed similarities to PME pro regions (Richard *et al*., 1994; Wolf *et al*., 2003; Giovane *et al*., 2004 and Raiola *et a*l., 2004). Previous studies reported the isolation and characterization of AtPME1 (At1g53840), AtPME2 (At1g53830) and AtPME3 (At3g14310) (Richard *et al*., 1994 and 1996; Micheli *et al*., 1998). The exogenous foliar application of methanol to the growing Arabidopsis seedlings induced the expression of AtPME1 isoform and promoted the growth of the leaves (Ramírez *et al*., 2006). AtPME1 was identified as an Arabidopsis pollen specific PME isoform (Tian *et al*., 2006). Recently, it was demonstrated by Röckel *et al*. (2008) that the PME isoform (AtPPME1, At1g69940) could interact *in vitro* with AtPMEI2 (At3g17220), leading to the inactivation of AtPPME1. The PMEI2 were shown to be localized to Brefeldin A-induced aggregates accumulating at the flanks of the growing tip of the tobacco pollen tube. Changes in the expression patterns of At4g12390, At4g02330, At2g26440 and At2g47550 in isoxaben-habituated Arabidopsis suspension cells were attributed to cell wall modifications to compensate for the disruption of cellulose synthase (Manfield, *et al*., 2004). At4g02330 (AtPMEpcrB) was detected and reported to be expressed in Arabidopsis rosette leaves and floral branches (Micheli *et al*., 1998).

Aims of the chapter

The aim of this chapter was to use immunochemical analysis and transcript profiling of the cell cycle expressed annotated PME isoforms using fast-growing synchronized cultures of wild type *Arabidopsis thaliana*, in an attempt to define the biological role and expression timing of these isoforms. This information could then be compared with the biosynthesis and deposition of HG epitopes in parental cell walls during the different phases of mitotic cell division, the development of the cell plate in daughter cells and the onset of adhesion between parental and daughter newly formed cells. The purpose of

using synchronized cell culture is to have a high proportion of cells proceeding to the same event of the cell cycle at the same time in the absence of developmental processes. The first part of the work involved checking that the cultures progressed through the cell cycle in a detectable and reproducible fashion. The synchronous progression of cells into the cell cycle was followed through the mitotic index and expression pattern of some cell cycle marker genes including cyclin dependent kinase (CDKB2), cyclin (cycD3) and histone (H4). The synchronized cultures were used to assess the expression patterns of the PMEs annotated to be expressed during the cell cycle.

4.2 Synchronization of Arabidopsis cultures

The cell cycle consists of two major events, DNA replication (S phase) and mitosis (M phase) interrupted by two gap phases, G1 (between M and S phase) and G2 (between S and M phases). The G1 phase is assumed to be the gate through which most cells resume cell cycle progression.
The progression of cells through the cell cycle is controlled by a conserved mechanism based on sequential transient formation and activation of complexes between cyclin-dependent kinases (CDKs) and their activating subunits, the cyclins (CYC). Aphidicolin is a fungal toxin that was reported to reversibly block cell cycle progression by inhibiting the action of DNA polymerase α and δ at the late G1/early S phase boundary. Washing out aphidicolin leads to the synchronous resumption of S phase with subsequent transition of cells through S and G2 phases until mitosis (Planchais *et al*., 2000; Menges and Murray 2002).

4.2.1 Cell cycle progression after washing out aphidicolin

The efficiency of cell synchronization was monitored by recording the mitotic index and analysis of the transcriptional patterns of some cell cycle marker genes

4.2.1.1 Mitotic index after aphidicolin-induced synchronization

The mitotic index was determined by UV light microscopic analysis of at least 800 cells stained with DAPI (0.1 µg/ml 4',6-diamino-2-phenylindole) during cell cycle progression (Fig. 4.1). The maximal mitotic index of ~ 12% reflects the percentage of cells in pro-metaphase, metaphase and anaphase and was recorded 13-14 h after washing out aphidicolin. Examples of the DAPI stained nuclei are illustrated in Fig 4.2. During different synchronizations, the pattern of the mitotic index curve was always similar to that in Fig. 4.1. The maximum index reported was in the range of 8-12%. The increase in the number of dividing cells was first detectable some 6 h after the release of aphidicolin to reach a maximum at 13-14 h (M phase). The timing of the maximum mitotic index was reproducible in four separate synchronizations. According to the obtained curve the relative duration of each of the cell cycle phases was nominated as; G1 and S phases at t=0 to t=4, S and G2 phase at t=4 to t=8, G2 and M phase at t=8 to t=18, and the subsequent G1 phase at t=20 to the end of sampling period.

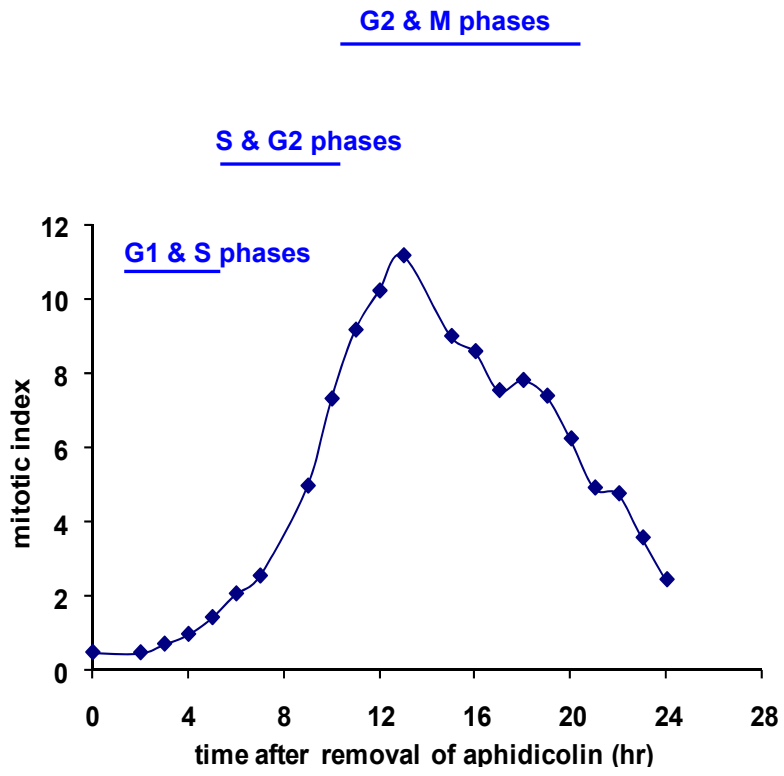
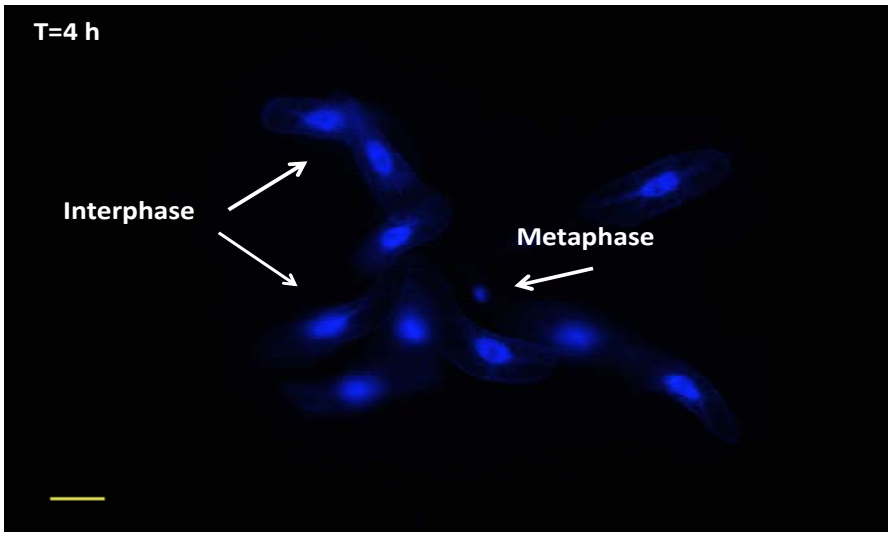


Fig. 4.1 Mitotic index as the culture progressed through the culture cycle after washing out aphidicolin.

(A)



(B)

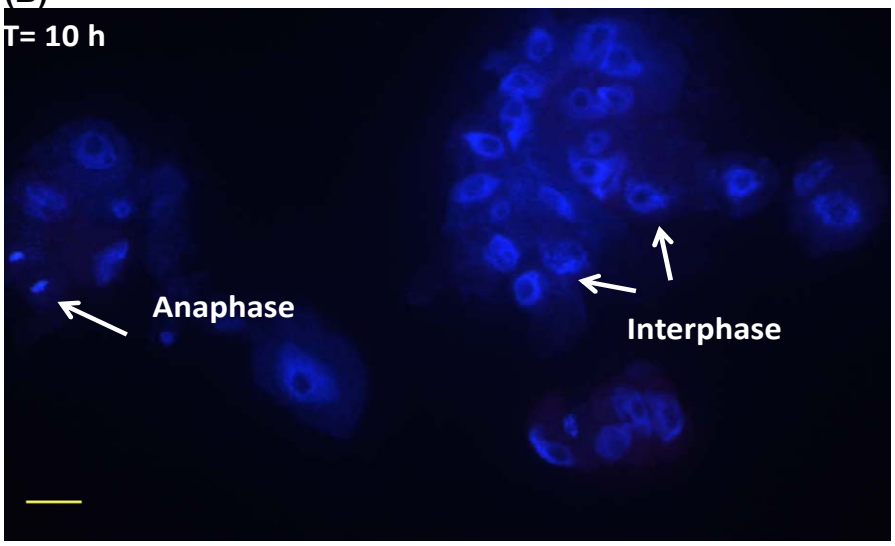


Fig. 4.2 Mitotic activity of synchronized Arabidopsis cells stained with Dapi after 4 and 10 hours of washing out aphidicolin. (A) A cell in metaphase and other cells in interphase. (B) A cell in anaphase and other cells in interphase. Scale bar represents 10 µm.

4.2.1.2 Analysis of cell cycle marker gene transcript profiles using reverse transcription-PCR

Using cDNA synthesized from RNA isolated from samples collected every 2 hours after the removal of aphidicolin, transcript profiles of three cell cycle marker genes including cyclin dependent kinase (CDKB2;2), cyclin (cycD3) and histone (H4) were analyzed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) to illustrate the progression of the cell cycle (Fig. 4.3 A, B). The constitutively expressed gene actin7 was used as a control that was not regulated by the cell cycle.

The activity of Cyclin dependent kinase B2;2 (CDKB2;2, At1g20930) was associated with cells of high mitotic activity in Arabidopsis flowers and suspension cultures (Boudolf, *et al*., 2001; Menges *et al*., 2002 and 2003; Pina *et al*., 2005), suggesting that it might be playing a role at the onset of/or progression through mitosis. The expression levels of CDKB2;2 are higher during the G2 and M phase as illustrated in Fig. 4.3. The results showed a reduction in its levels as the cells were preparing for division during S phase between t=2 to t=6, however, it increased again and remained at high levels during the division phase. In the subsequent G1, its levels dropped again. During the different experiments, the expression levels of CDKB2 were higher from G2- to M-phase. This result is consistent with earlier reports suggesting a role of B-type CDKs in the control of mitosis (Dewitte and Murray, 2003; Lee *et al*., 2004; Menges *et al*. 2005).

The microarray data showed that CYCD3;1 (At4g34160), a member of the D-type cyclins whose expression was mainly correlated with proliferating cells, was the highest cyclin expressed in the synchronized Arabidopsis suspension cultures (Menges *et al*., 2005). The analysis of CYCD3;1 expression profile (Fig. 4.3), showed a slight reduction in its levels progressively as the cells proceeded through S phase at t=0 to t= 4. Its levels were higher during the mitotic phase and peaked at t=18. During the different replicate experiments, the CYCD3;1 levels were higher during the M phase regardless of the time at which it showed the peak activity.

The mRNA of histone H4 (At5g07660) gene is known to accumulate during the DNA synthesis at the G1/S transition. The highest levels of H4 existed at S phase 2 h after removing the block (fig. 4.4). Lower levels of the transcript were expressed to the end of the sampling period. Similar patterns of H4 expression were recorded by Zhang *et al*., 2005 using Arabidopsis suspension cultures.

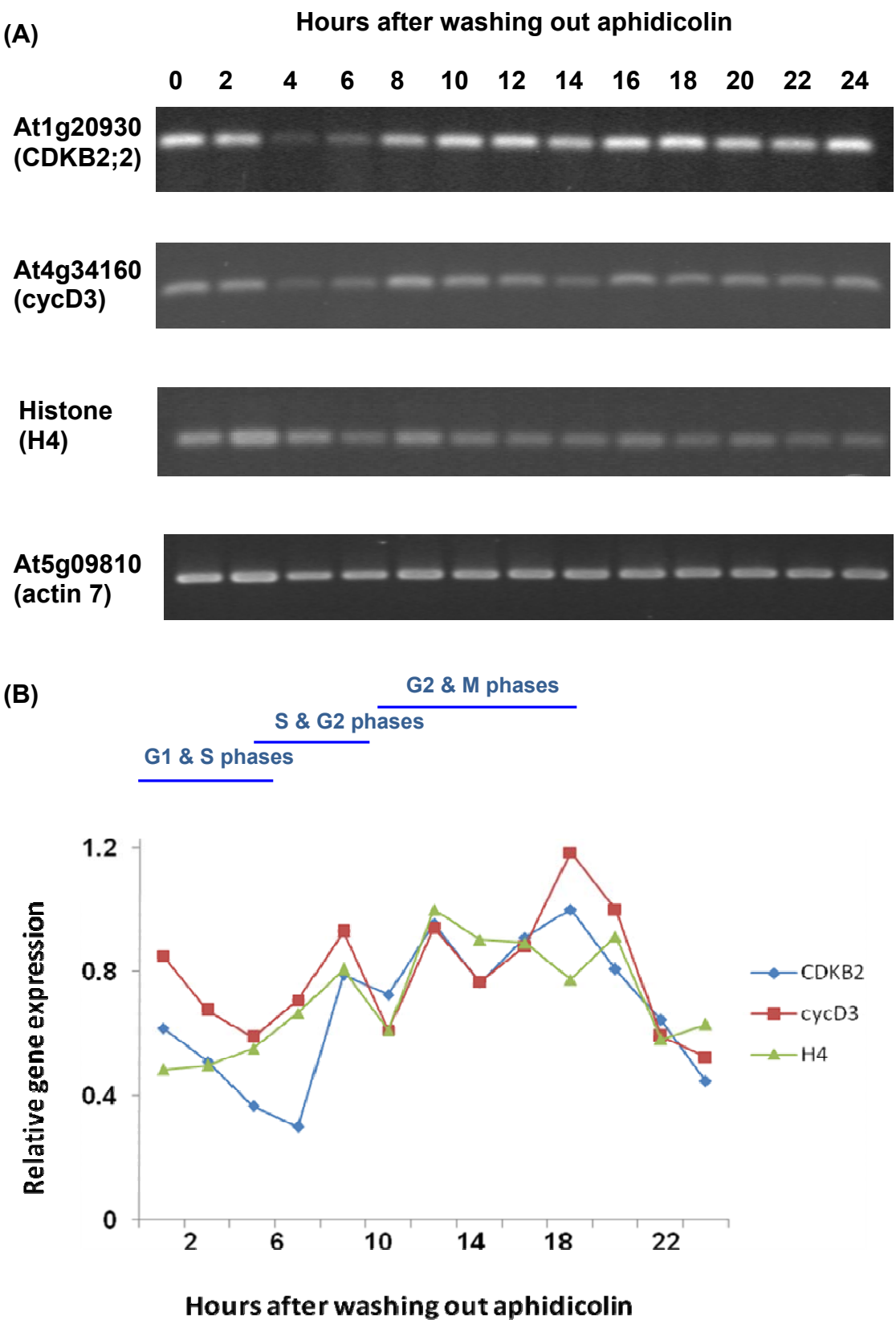


Fig. 4.3 Expression patterns (A) and Quantitative relative expression (B) of CDKB2;1 , cycD3;1, and H4 genes during the Cell cycle after washing out aphidicolin.

4.2.2 Characteristics of phosphate/EDTA extracted pectin during cell cycle

4.2.2.1 Changes in the total sugar and GA content of extracted pectin.

The total sugar content of EDTA/phosphate extracted pectin from cells over a period of 24 h (from t=0 to t=24 h) after the release from aphidicolin ranged between 800 and 1250 µg/ml cells reflecting an increase of 55% in the initial amount of pectic extracts from the cells (table 1, Fig 4.5). Most of this increase was recorded within the period of t=6 to t=18h after washing out aphidicolin at the time when the cells were entering the G2 phase at t= 6h and all through the M phase. As the cells were exiting the cell cycle and preparing for the second division the amount of extracted sugar was less by 14% and increased afterwards to the end of the sampling period at t=24h.
The total extracted sugar refers to the sum of the solubilised pectin from the living cells preparing for division, and a portion of dead cells transferred into the culture during subculturing.

Time after washing out Aphidicolin (Hours)	Total EDTA / phosphate extracted pectin (µg pectin/ ml packed cell volume)	Total GA in extracted pectin (µg pectin/ ml packed cell volume)	% GA content in extracted pectin	% methyl esterified GA residues
0	**808**	**361**	**45**	**28**
2	**767**	**408**	**53**	**25**
4	**756**	**451**	**59**	**25**
6	**823**	**488**	**58**	**26**
8	**984**	**508**	**51**	**26**
10	**1011**	**537**	**53**	**31**
12	**937**	**577**	**61**	**34**
14	**944**	**573**	**60**	**36**
16	**1108**	**732**	**66**	**33**
18	**1205**	**740**	**61**	**36**
20	**1026**	**543**	**52**	**33**
22	**1166**	**576**	**49**	**33**
24	**1255**	**536**	**42**	**38**

Table 4.1 Total sugar, GA and degree of methylesterification of 50mM EDTA/ 50 mM phosphate buffer extracted pectin from alcohol insoluble cell wall materials

The water and chelator solublized pectin extracted after the release of the block showed that the uronic acid fraction of the extracted pectin increased progressively through the cell cycle (t=0 to t=18 h). However, the UA content at t=16 was higher than at the other sampling periods, and it remained at the same level at t=18. At t=20, UA content was 27% less than its content at t=18 h and remained at a constant level to the end of the sampling period. An earlier study of *Arabidopsis thaliana* wild type suspension cultures originated from root calli reported glucuronic and galacturonic acids as the major components of uronic acids (Leboeuf *et al*., 2005). (Table 4.1, Fig 4.4).

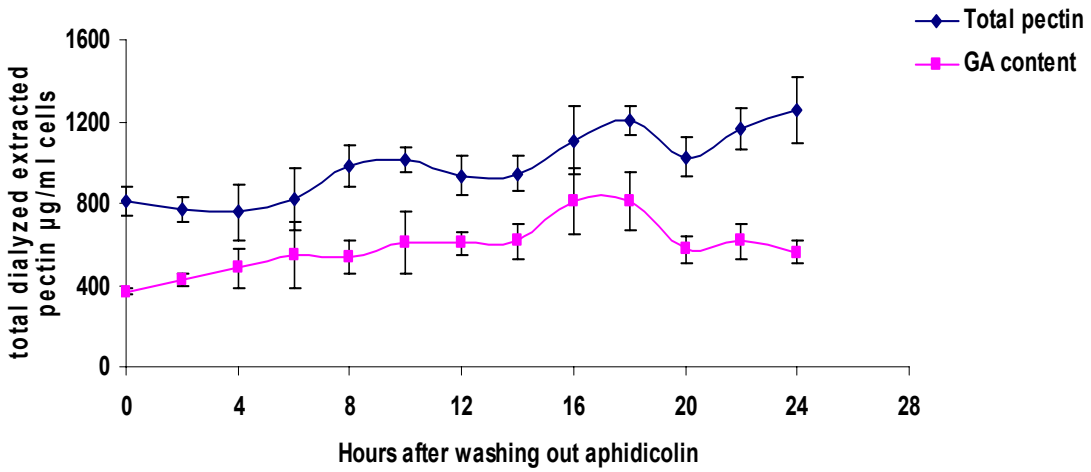


Fig. 4.4 Changes in the total extracted pectin and galacturonic acid after washing out aphidicolin. The graph shows the mean ± SD for three separate synchronization experiments.

4.2.2.2 Degree of methylesterification of extracted homogalacturonan

The amount of methylesterification of HGs was estimated through the saponification of the pectic extracts using 1M NaOH at 4^{o}C. The released methanol was oxidized by alcohol oxidase, and the produced formaldehyde was determined colorimetrically with acetylacetone (pentane-2,4-dione) and ammonia according to Klavons and Bennett (1986). Alcohol oxidase activity is not specific for methanol; other short-chain alcohols such as ethanol can also act as substrates. Samples of the extracted pectin were tested for the presence of free methyl groups and/or remains of ethanol in which the Arabidopsis cells were originally boiled. Our results did not show any contaminants that might interfere with estimates of the released methyl groups after the dilute alkali treatment.

The degree (percentage) of methylation (DM %) is calculated as the ratio of methyl groups released upon NaOH saponification to the UA fraction of EDTA/phosphate extracted pectin. During the cell cycle, the DM% of the extracted homogalacturonans increased through the sampling period from 28% at t=0 to 38% at t=24 h after washing out the block (Fig. 4.5).The increase in DM% was recorded as the cells were in G2 phase and increased

further as the cells were actively dividing and depositing newly synthesized highly esterified pectins (t=6 to t=18).

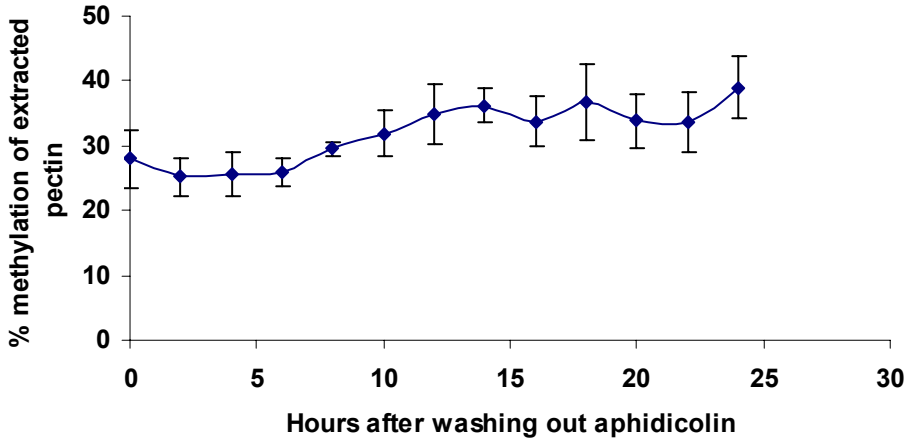


Fig. 4.5 Degree of methylation of buffer/chelator soluble pectin from alcohol insoluble material. The graph shows mean ± SD for three separate synchronization experiments.

4.2.3 Immuno-analysis of the extracted pectin

The monoclonal antibodies directed against HG and RG-I were used to assess the dynamic deposition and assembly of methylesterified/deesterified epitopes, through the analysis of EDTA/phosphate solubilised pectin during cytokinesis and subsequent formation of the cell plate.

4.2.3.1 Binding of monoclonal antibody JIM7 and JIM5 to the extracted homogalacturonan during the cell cycle

Monoclonal antibodies JIM5 and JIM7 recognize partially methylesterified epitopes of HG with low and high degree of methylation (DM %) respectively (Clausen *et al*., 2003; Willats *et al*., 2000a and b). Immunodot assay (IDA) analysis of the extracted pectin indicated the presence of the two pectic components, each containing fractions of methylated HG. The highly branched polymer with a lower rate of mobility is localized in the central dot

while the unbranched fraction formed an outer ring away from the point of application on the nitrocellulose membrane.

The immunoprofile of pectins reactive to JIM7 supported the biochemical data and indicated an increase in highly esterified pectin in the highly branched pectin localized in the central dot and the mobile linear pectin forming the outer ring during the G2 and M phases (Fig. 4.6). The JIM 5 binding to the outer ring and central dot indicated that both pectic components contained low-ester HG (Fig.4.7). The darker stronger reaction at the central dots indicates that the higher level of low-ester HG is located in the highly branched fraction of the extracted pectin. Similar to the JIM7 reaction, the signal seemed to become weaker during the S phase and increase again through G2, M, and subsequent G1 phases. The amounts of the two epitopes recognized by JIM5 and JIM7 seemed to vary in parallel during the cell cycle.

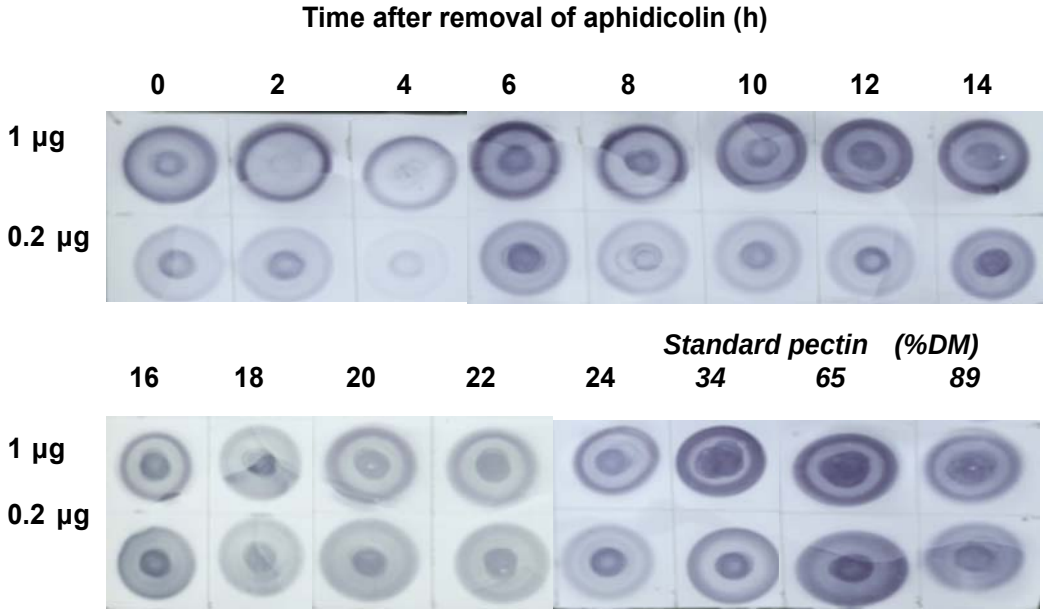


Fig. 4.6 Immuno-dot assay of monoclonal antibody JIM7 binding to the extracted pectins during the cell cycle. The results were reproducible in two separate EDTA/phosphate pectic extracts solubilised from two different cultures

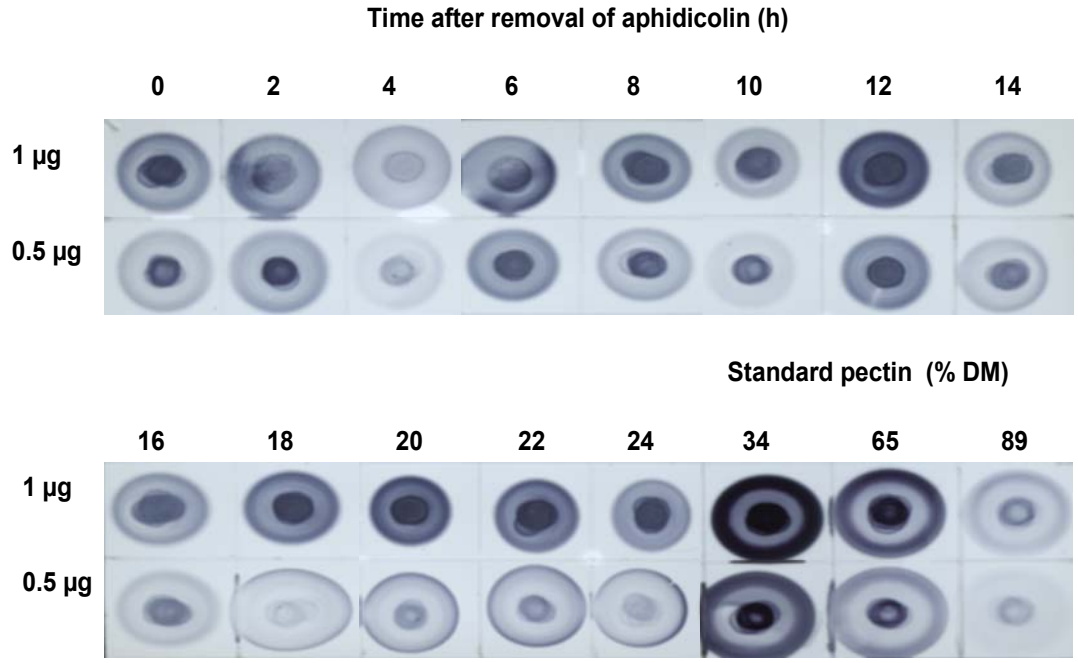


Fig. 4.7 Immuno-dot assay of monoclonal antibody JIM5 binding to the extracted pectins during the cell cycle. The results were reproducible in two separate EDTA/phosphate pectic extracts solubilised from two different cultures

The use of the monoclonal antibody LM7 failed to show any reaction regardless of the amount of applied pectin possibly due to the fact that the LM7 epitope is extremely labile as demonstrated by Willats *et al*. (2001c).

4.2.3.2 Binding of monoclonal antibody LM5 and LM6 to the extracted pectin during the cell cycle

The neutral side chains of RG-I domain of pectic polysaccharides were probed with monoclonal antibodies LM5 and LM6 directed against defined epitopes of (1→4)-ß-galactan (Jones *et al*., 1997 and Willats, *et al*., 1998) and (1→5)-α- arabinan (Willats *et al*., 1998). The tested immuno profiles indicated that the (1→5)-α- arabinan epitope was abundantly present in the EDTA/phosphate solubilised fraction of cell wall pectins. The recognized epitope was present in the distinct pectic components of differing mobilities

(Fig. 4.8). The binding of LM6 to the extracted pectin increased after t=6 as the cells progressed from G2 into M phase, which suggests the occurrence of branching or further substitution of the side chains as the cells are progressing through the cell cycle. The LM6 reactive epitope increased again to the end of the sampling period as the cells are going into the next G1 phase. The (1→4)-ß-galactan epitope was essentially absent or present at low levels in these extracts (data not shown).

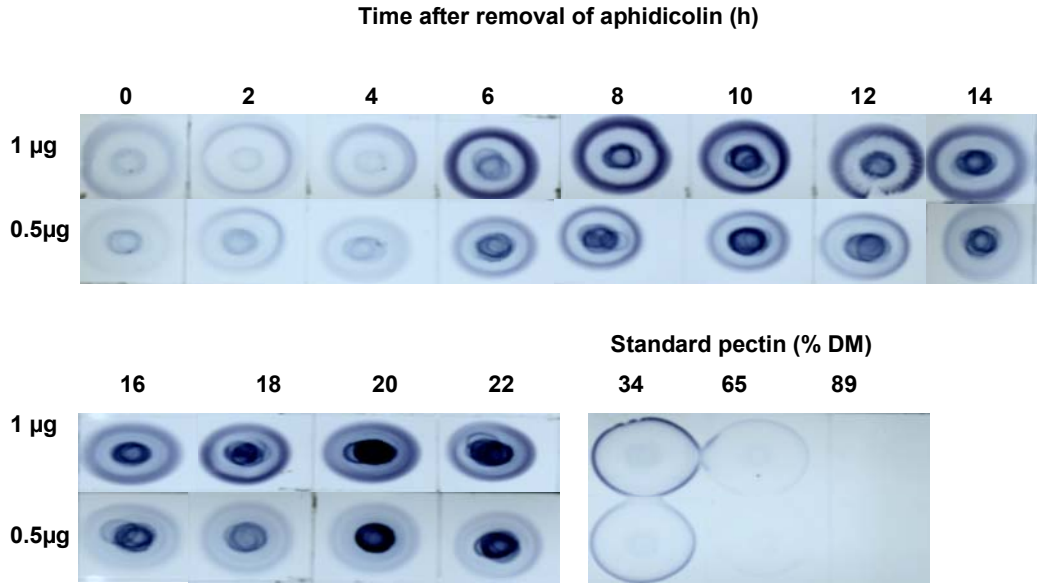


Fig. 4.8 Immuno-dot assay of monoclonal antibody LM6 binding to the extracted pectins during the cell cycle. The results were reproducible in two separate EDTA/phosphate pectic extracts solubilised from two different cultures

4.2.4 Abundance of pectin methylesterases during the cell cycle.

4.2.4.1 Phylogenetic analysis of cell cycle expressed PMEs.

The phylogenetic tree (Fig. 4.9) of the protein sequences of the synchronized Arabidopsis cell cycle related putative PMEs isoforms listed in table (4.1) allows the distinction of three major groups named group A to C, possibly with

different putative functions. Group A is composed of five members, At4g02330, At1g02810, At2g47550, At2g26440 and At5g47500. Group B contains only one member, At4g12390, whereas At1g76160, At4g12420, At4g22010 and At4g25240 are members of Group C and are now annotated in databanks as multi-copper oxidases.

Gene name	LENGTH (aa)	molecular weight	isoelectric point	Domains (# of domains)	Cell cycle expression phase
At2g26440	548	60417.0	4.9298	PMEI & PME	M
At4g02330	574	63944.0	8.6243	PMEI & PME	G1
At1g02810	580	63953.0	8.9025	PMEI & PME	G1
At4g12390	207	23078.0	10.2591	PMEI	G1
At5g47500	363	40042.0	8.8961	PME	M
At2g47550	561	61498.0	9.0312	PMEI & PME	S
At1g76160	542	60041.0	8.5742	Multi-copper oxidase	G1
At4g12420	588	65638.0	9.4644	Multi-cupper oxidase	G1
At4g22010	542	60456.0	10.2469	Multi-copper oxidase	G1
At4g25240	590	65877.0	7.3968	Multi-copper oxidase	M

Table 4.2 Cell cycle expressed annotated PME isoforms listed in Menges *et al*. (2003).

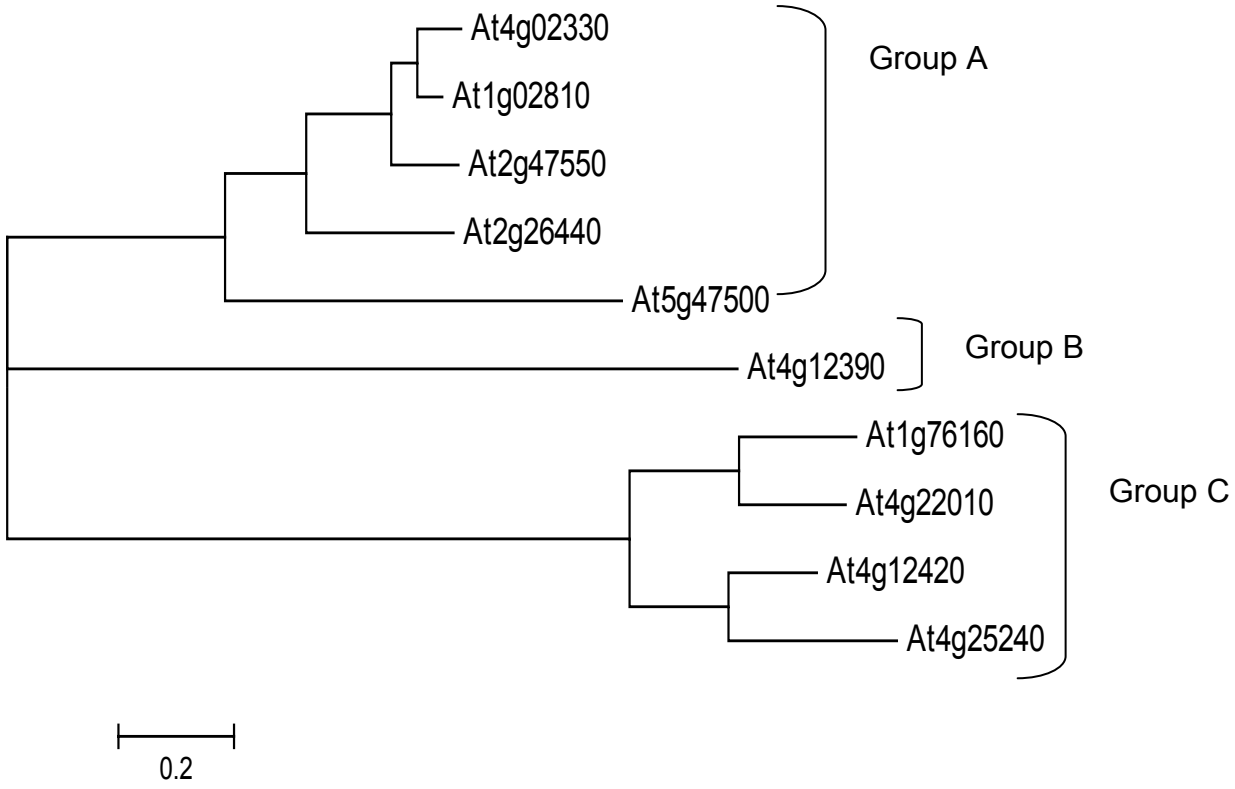


Fig 4.9 Phylogenetic tree analysis of the cell cycle expressed PME sequences using MEGA version 3.1 based on Neighbour-Joining method (Kumar *et al*., 2004).

A further alignment using ClustalW (www.ebi.ac.uk) of amino acid sequences of groups A, B and C against the functionally characterized type II AtPME3 (At3g14310: Micheli *et al*., 1998) AtPMEI-1 and AtPMEI-2 (At3g17220 At1g48020 respectively: Raiola *et al*., 2004), AcPMEI that have been detected in kiwi fruit and Nt-INH expressing an invertase inhibitor isoform identified in *Nicotiana tabaccum* (Scognamiglio *et al*., 2003) (Fig. 4.10), revealed that the genes At2g26440, At4g02330, At1g02810, At2g47550 and At4g12390 shared the presence of the four conserved cysteine residues in the N terminal region (Fig. 4.10 yellow shading). These residues were reported to be involved in the formation of two intramolecular disulfide bridges critical for protein folding in PMEI and invertase inhibitors. However, AtPME1-1, AtPMEI1-2, AcPMEI and AtPME3 at the pro region shared the presence of the fifth Cysteine residue which was absent in all invertase inhibitor sequences identified so far (Scognamiglio *et al*., 2003). The cleavage site yielding mature PME as

reported by Giovane *et al*. (2003), occurs close to the sequence RRKLLM and is conserved in At1g02810, At4g02330 and At 2g47550. At4g12390 is of lower similarity to *Arabidopsis thaliana* PMEIs and in the TAIR website is annotated as a putative invertase/ PMEI inhibitor. Invertase inhibitors share some structural similarities with PMEIs though they have a completely different target enzyme. Earlier reports stated that individual members of the invertase inhibitor/ PMEI protein family are inhibitors of either PME or invertase but never both (Wolf *et al*., 2003).

```
At4g02330       --------------MLSLKLFLVTLFLS--LQTLFIAS----------QTLLP--SNSSS
At1g02810       --------------MESPIFILITLSFF--LQSVLASS----------QTLS-----NSS
At2g47550       --------------MSQKLMFLFTLACLSSLPSPFISA----------QIPAIGNATSPS
At2g26440       --------------MALSSFNLSSLLFLLFFTPSVFSYSY--------QPSLNPHETSAT
At3g14310       MAPSMKEIFSKDNFKKNKKLVLLSAAVALLFVAAVAGISAGASKANEKRTLSPSSHAVLR
At1g48020       -----------MAANLRNNAFLSSLMFLLLIGSSYAITSS-----------------EMS
At3g17220       -----------MAAYLTNRVLMSSLMFFVMTGSLNAQVA-----------------DIK
AcPMEI          -----------------ENHLIS-----------------------------------
At5g47500       ------------------------------------------------------------
Nt-INH          --------------MKNLIFLTMFLTILLQTNANN---------------------LVE
At4g12390       -----------MEPKLTHLCYCLLLFLPLLCQSTIAKPSS--------SPNPSSSINFIV

At4g02330       TICKTTPDPKFCKSVFPQTS----QGDVREYGRFSLRKSLTQSRKFTRTIDRYLKRNNAL
At1g02810       TICKTTPDPKYCKSVFPHS-----QGNVQQYGCFSIRKSLSQSRKFIRTVDRYIKRNAHL
At2g47550       NICRFAPDPSYCRSVLPNQ-----PGDIYSYGRLSLRRSLSRARRFISMIDAELDRKGKV
At2g26440       SFCKNTPYPDACFTSLKLSISINISPNILSFLLQTLQTALSEAGKLTDLLSGAGVSNN--
At3g14310       SSCSSTRYPELCISAVVTAGG-VELTSQKDVIEASVNLTITAVEHNYFTVKKLIKKRKG-
At1g48020       TICDKTLNPSFCLKFL--NTKFA-SPNLQALAKTTLDSTQARATQTLKKLQSIIDGGVD-
At3g17220       AICGKAKNQSFCTSYMKSNPKTS-GADLQTLANITFGSAQTSASEGFRKIQSLVKTATN-
AcPMEI          EICPKTRNPSLCLQALESDPRSA-SKDLKGLGQFSIDIAQASAKQTSKIIASLTNQATD-
At5g47500       ------------------------------------------------------------
Nt-INH          TTCKNTPNYQLCLKTLLSDKRSA-TGDITTLALIMVDAIKAKANQAAVTISKLRHSNPP-
At4g12390       SSCRVTRYQTLCVKCLAAFADKIRRNENQ-LAQTALAVTLVRVQSTTIYVGKLTKARRIK

At4g02330       LSQSAVGALQDCRYLASLTTDY-LITSFETVNIT---TSSKTLSFSKADEIQTLLSAALT
At1g02810       SQPAVIRALQDCRFLAGLTMDY-LLTSFETVNDTSAKTSFKPLSFPKADDIQTLLSAALT
At2g47550       AAKSTVGALEDCKFLASLTMDY-LLSSSQTADST------KTLSLSRAEDVHTFLSAAIT
At2g26440       LVEGQRGSLQDCKDLHHITSSF-LKRSISKIQDG-------VNDSRKLADARAYLSAALT
At3g14310       LTPREKTALHDCLETIDETLDE-LHETVEDLHLYP----TKKTLREHAGDLKTLISSAIT
At1g48020       --PRSKLAYRSCVDEYESAIGN-LEEAFEHLASG-----------DGMGMNMKVSAALD
At3g17220       --PTMKKAYTSCVQHYKSAISS-LNDAKQSLASG-----------DGKGLNIKVSAAME
AcPMEI          --PKLKGRYETCSENYADAIDS-LGQAKQFLTSG-----------DYNSLNIYASAAFD
At5g47500       -----MAQLTNSLNYLFSVSLL-LFVSFHCLCFR----------------FSLVAACS
Nt-INH          --AAWKGPLKNCAFSYKVILTASLPEAIEALTKG-----------DPKFAEDGMVGSSG
At4g12390       --RREYLAVKDCVENLGDGLEM-LAQSMRELKQVGRSGRDRDEFLWRLSNVETWVSAALT
                              .           *  :                        .:

At4g02330       NEQTCLDGINTAAS--SSWTIRNGVALPLINDTKLFSVSLALFTKGWVPKKKKQVASYSW
At1g02810       NEQTCLEGLTTAASYSATWTVRTGVALPLVNDTKLLGVSLALFTKGWVPKKKKRAG-FAW
At2g47550       NEQTCLEGLKSTAS-------ENGLSGDLFNDTKLYGVSLALFSKGWVPRRQ-------R
At2g26440       NKITCLEGLESASG-----PLKPKLVTSFTTTYKHISNSLSALPK---QRRT--------
At3g14310       NQETCLDGFSHDDAD---KQVRKALLKGQIHVEHMCSNALAMIKNMTDTDIAN-------
At1g48020       GADTCLDDVKRLRS------VDSSVVNNSKTIKNLCGIALVISNMLPRN-----------
At3g17220       GPSTCEQDMADFK-------VDPSAVKNSGDFQNICGIVLVISNMM--------------
AcPMEI          GAGTCEDSFEGPPN------IPTQLHQADLKLEDLCDIVLVISNLLPGS-----------
At5g47500       NS------------------TDDQQIQHHHHRKWVGPSG--------------------
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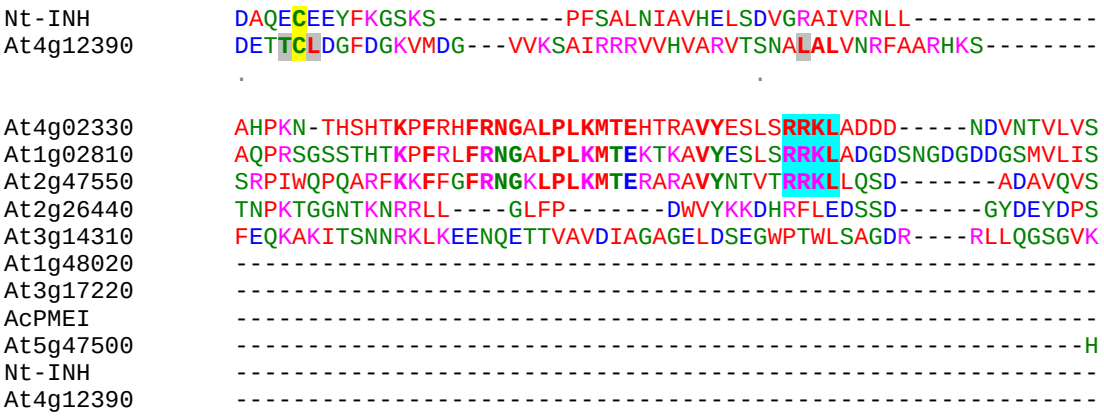

Fig 4.10 Clustal W alignment at the N-terminal of peptide sequences of At4g02330, At1g02810, At2g47550, At2g26440, At4g12390 and At5g47500 against AtPME3 (At3g14310) and PMEI-1 and PMEI-2 (At1g48020 and At3g17220 respectively), AcPMEI and Nt-INH.

Since the identity level in the deduced amino acid sequences of the N-terminal pre (signal) region is not very high, it may suggest that the secretory pathways of these putative PME isoforms are different, and that this region may contribute to specify a biological function to each isoform. Both Type I and Type II showed the presence of short consensus motifs, motif I (GxYxE) with a conserved tyrosine residue, which has been proposed to play a role in the catalytic mechanism, motif II (QAVAL), motif III (QDTL), motif IV (GTXDFIFG) and motif V (YLGRPWK) within the carboxy terminal regions which correspond mainly to the catalytic domain of PME precursors (Fig. 4.11, yellow shading). These motifs have been recognized as a signature for PMEs in Arabidopsis shoots and other plant species (Micheli, *et al* 1998; Lievens *et al*., 2002, Pelloux *et al*., 2007). These conserved motifs were not detected in the alignment of the copper oxidase encoding genes; At4g25240, At4g22010, At4g12420 and At1g76160.

The two aspartic acid residues, two glutamines and one arginine residue (Fig. 4.11, yellow shading), characteristic of the active site of the enzyme, are conserved in the cell-cycle-related type I and II PMEs (Jenkins *et al*., 2001, Johansson *et al*., 2002, Dorokhov *et al*., 2006, Pelloux, *et al*., 2007). The At5g47500 showed a similarity to At3g14310 at the catalytic domain without a pro region and thus it could be a type I PME.

```
                                                        GxYxE
At4g02330       DIVTVNQNGTGNFTTITEAVNSAPNKTDGTAGYFVIYVTSGVYEENVVIAKNKRYLMMIG
At1g02810       DIVTVSQDGTGNFTNITAAVAAAPNNTDGSAGFFLIYVTAGIYEEYISIAKNKRYMMMIG
At2g47550       DIVTVIQNGTGNFTTINAAIAAAPNKTDGSNGYFLIYVTAGLYEEYVEVPKNKRYVMMIG
At2g26440       ESLVVAADGTGNFSTINEAISFAPNMSNDR---VLIYVKEGVYDENIDIPIYKTNIVLIG
At3g14310       ADATVAADGSGTFKTVAAAVAAAPENSNKR---YVIHIKAGVYRENVEVAKKKKNIMFMG
At1g48020       ------------------------------------------------------------
At3g17220       ------------------------------------------------------------
AcPMEI          ------------------------------------------------------------
At5g47500       KVITVSLNGHAQFRSVQDAVDSIPKNNNKS---ITIKIAPGFYREKVVVPATKPYITFKG
Nt-INH          ------------------------------------------------------------
At4g12390       ------------------------------------------------------------

At4g02330       DGINRTVVTGNRNVVDG------WTTFNSATFAVTSPNFVAVNMTFRNTA-----GPEKH
At1g02810       DGINQTVVTGNRSVVDG------WTTFNSATFAVTAPNFVAVNITFRNTA-----GPEKH
At2g47550       DGINQTVITGNRSVVDG------WTTFNSATFILSGPNFIGVNITIRNTA-----GPTKG
At2g26440       DGSDVTFITGNRSVGDG------WTTFRSATLAVSGEGFLARDIMITNTA-----GPEKH
At3g14310       DGRTRTIITGSRNVVDG------STTFHSATVAAVGERFLARDITFQNTA-----GPSKH
At1g48020       ------------------------------------------------------------
At3g17220       ------------------------------------------------------------
AcPMEI          ------------------------------------------------------------
At5g47500       AGRDVTAIEWHDRASDLGANGQQLRTYQTASVTVYANYFTARNISFTNTAPAPLPGMQGW
Nt-INH          ------------------------------------------------------------
At4g12390       ------------------------------------------------------------

                QAVAL                 QDTL                GTxDFIFG
At4g02330       QAVAMRSSADLSIFYSCSFEAYQDTLYTHSLRQFYRECDIYGTVDFIFGNAAVVFQDCNL
At1g02810       QAVALRSGADFSIFYSCSFEAYQDTLYTHSLRQFYRECDVYGTVDFIFGNAAVVFQNCNL
At2g47550       QAVALRSGGDLSVFYSCSFEAYQDTLYTHSLRQFYRECDVYGTVDFIFGNAAVVLQNCNL
At2g26440       QAVALRVNADFVALYRCVIDGYQDTLYTHSFRQFYRECDIYGTIDYIFGNAAVVFQGCNI
At3g14310       QAVALRVGSDFSAFYNCDMLAYQDTLYVHSNRQFFVKCLIAGTVDFIFGNAAVVLQDCDI
At1g48020       ------------------------------------------------------------
At3g17220       ------------------------------------------------------------
AcPMEI          ------------------------------------------------------------
At5g47500       QAVAFRISGDKAFFSGCGFYGAQDTLCDDAGRHYFKECYIEGSIDFIFGNGRSMYKDCEL
Nt-INH          ------------------------------------------------------------
At4g12390       ------------------------------------------------------------

                                                                 YLGRPWK
At4g02330       YPRQPMQNQFNAITAQGRTDPNQNTGISIHNCTIKPADDLVSSNYTVKTYLGRPWKEYSR
At1g02810       YPRKPMPNQFNAITAQGRSDPNQNTGTSIQNCTIKPADDLVSSNYTVKTYLGRPWKEYSR
At2g47550       YPRQPRKGQSNEVTAQGRTDPNQNTGTAIHGCTIRPADDLATSNYTVKTYLGRPWKEYSR
At2g26440       VSKLPMPGQFTVITAQSRDTQDEDTGISMQNCSILASEDLFNSSNKVKSYLGRPWREFSR
At3g14310       HARRPNSGQKNMVTAQGRTDPNQNTGIVIQKCRIGATSDLQSVKGSFPTYLGRPWKEYSQ
At1g48020       ------------------------------------------------------------
At3g17220       ------------------------------------------------------------
AcPMEI          ------------------------------------------------------------
At5g47500       HS---IASRFGSIAAHGRTCPEEKTGFAFVGCRVTGTG---------PLYVGRAMGQYSR
Nt-INH          ------------------------------------------------------------
At4g12390       ------------------------------------------------------------

At4g02330       TVFMQSYIDEVVEPVGWREWNGDFALS-TLYYAEYNNTGSGSSTTDRVVWPGYHVIN-ST
At1g02810       TVYMQSYIDGFVEPVGWREWNGDFALS-TLYYAEYNNTGPGSNTTNRVTWPGYHVIN-ST
At2g47550       TVVMQTYIDGFLEPSGWNAWSGDFALS-TLYYAEYNNTGPGSDTTNRVTWPGYHVIN-AT
At2g26440       TVVMESYIDEFIDGSGWSKWNGGEALD-TLYYGEYNNNGPGSETVKRVNWPGFHIMG-YE
At3g14310       TVIMQSAISDVIRPEGWSEWTGTFALN-TLTYREYSNTGAGAGTANRVKWRGFKVITAAA
At1g48020       ------------------------------------------------------------
At3g17220       ------------------------------------------------------------
AcPMEI          ------------------------------------------------------------
At5g47500       IVYAYTYFDALVAHGGWDDWDHKSNKSKTAFFGVYNCYGPGAAATRGVSWAR---ALDYE
Nt-INH          ------------------------------------------------------------
At4g12390       ------------------------------------------------------------
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At4g02330       DANNFTVENFLLGDGWMVQSGVPYISGLLS 573
At1g02810       DAANFTVTGLFIEADWIWKTGVPYTSGLIS 579
At2g47550       DASNFTVTNFLVGEGWIGQTGVPFVGGLIA 560
At2g26440       DAFNFTATEFITGDGWLGSTSFPYDNGI-- 547
At3g14310       EAQKYTAGQFIGGGGWLSSTGFPFSLGL-- 592
At1g48020       -------------------------------
At3g17220       -------------------------------
AcPMEI          -------------------------------
At5g47500       SAHPFIAKSFVNGRHWIAPRDA-------- 362
Nt-INH          -------------------------------
At4g12390       -------------------------------
```

Fig. 4.11 Clustal W Alignment at the catalytic domain of the amino acid sequences of At4g02330, At1g02810, At2g47550, At2g26440, At4g12390 and At5g47500 against AtPME3 (At3g14310), PMEI-1, PMEI-2, AcPMEI and Nt-INH.

4.2.4.2 Patterns of PME transcripts during cell cycle

To ensure specificity and efficiency of PME isoforms during PCR amplification, the total RNA was always treated with DNase prior to first strand cDNA synthesis, and the primers were designed to cover an exon-exon junction. All primer pairs produced a single DNA product of the expected size. Menges *et al*. (2002) suggested that At4g02330 and At1g02810 genes were expressed during G1 phase, At2g47550 in S phase and At2g26440 genes in M phase. However, the data obtained in this project were not consistent between the experiments and so are not shown.

The At5g47500 (Fig.4.12) isoform displayed a more consistent expression during the M phase. However its expression during the G1 varied between the different synchronizations.

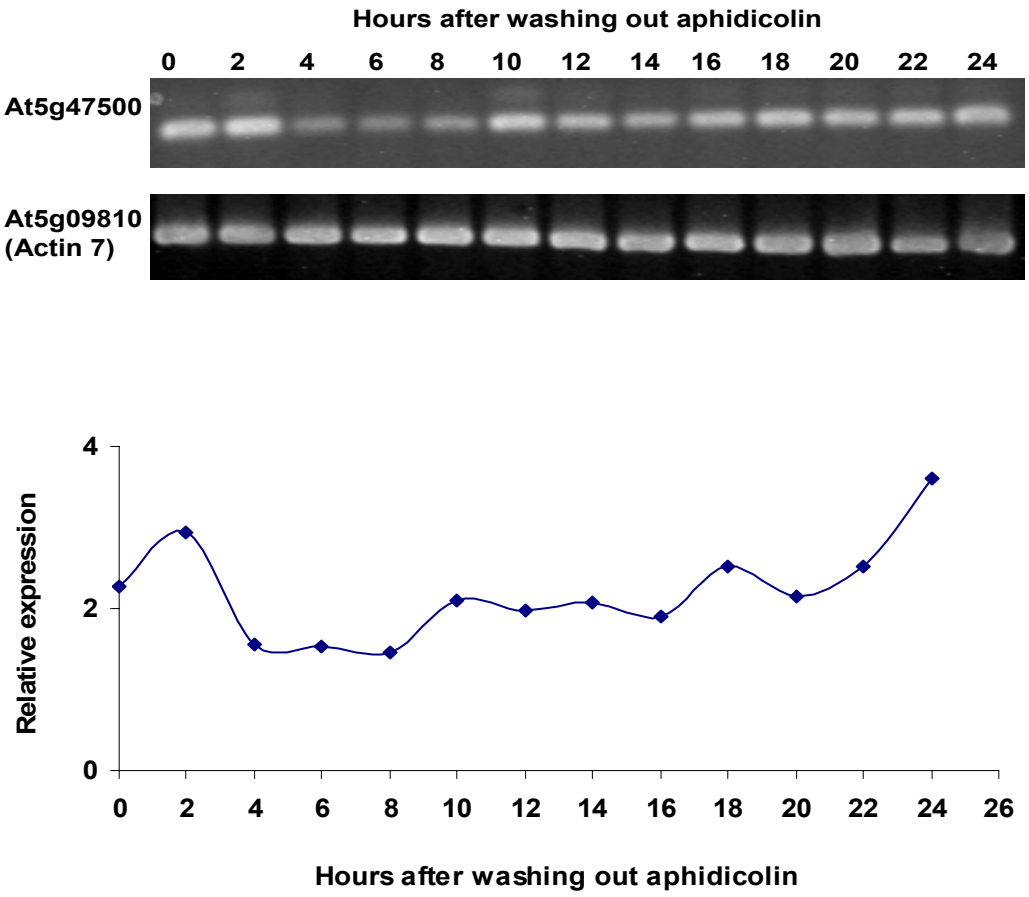

Fig 4.12. Quantitative relative expression of At5g47500 through the cell cycle after washing out aphidicolin, presented as relative expression after normalization with At5g09810 (Actin 7)

4.2.4.3 Northern blot analysis of At5g47500 expression.

The PME isoform At5g47500 PCR product of 212 bp was used to generate templates for T3 labelled antisense probe (238 bp) with (At5g47500-T3-r **AATTAACCCTCACTAAAGCAGTATTCGTG** and At5g47500-f **AAGATTGCTCCCGGATTTTACAGAGAG**) (Fig. 4.13). A MAXIscript Ambion kit was used to prepare a biotinlytaed RNA probe that was hybridized with 15 µg of total RNA to detect the expression levels of At5g47500 mRNA during the different phases of cell cycle (Fig. 4.13). As a general control for the experiment, 18S and 25S rRNA profiles were checked after electrophoresis.

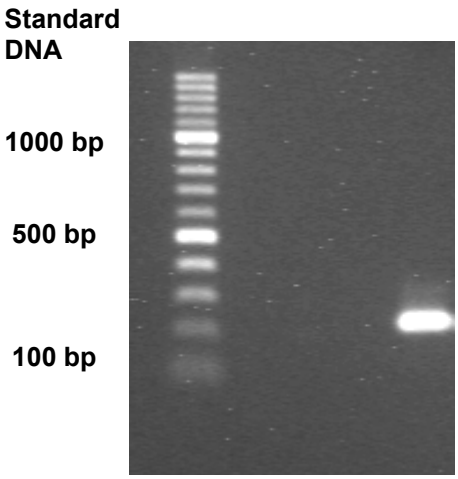

Fig. 4.13 PCR product 212bp amplified with a set of primers of T-3 reverse linked and a forward

The Northern gel-blot analysis of gene expression pattern of At5g47500 demonstrated a transcript of approximately 1.75 Kb of At5g47500 mRNA during the cell cycle (Fig 4.14). The transcript levels decreased after t=0 to as the cells were exiting G1 into S phase. No transcripts could be detected between t=4 to t=8 through the S and G2 of the cell cycle. The At5g47500 mRNAs accumulated from t= 10 to t=24 h. The levels of expression increased progressively during M phase between t=10 and t= 18, slightly decreased at t=20 and remained steady up to t=24. Our results using the northern hybridization were consistent with the analyses using RT-PCR, indicating that the At5g47500 gene may play a role in pectin modulation during the cell cycle, especially the M phase.

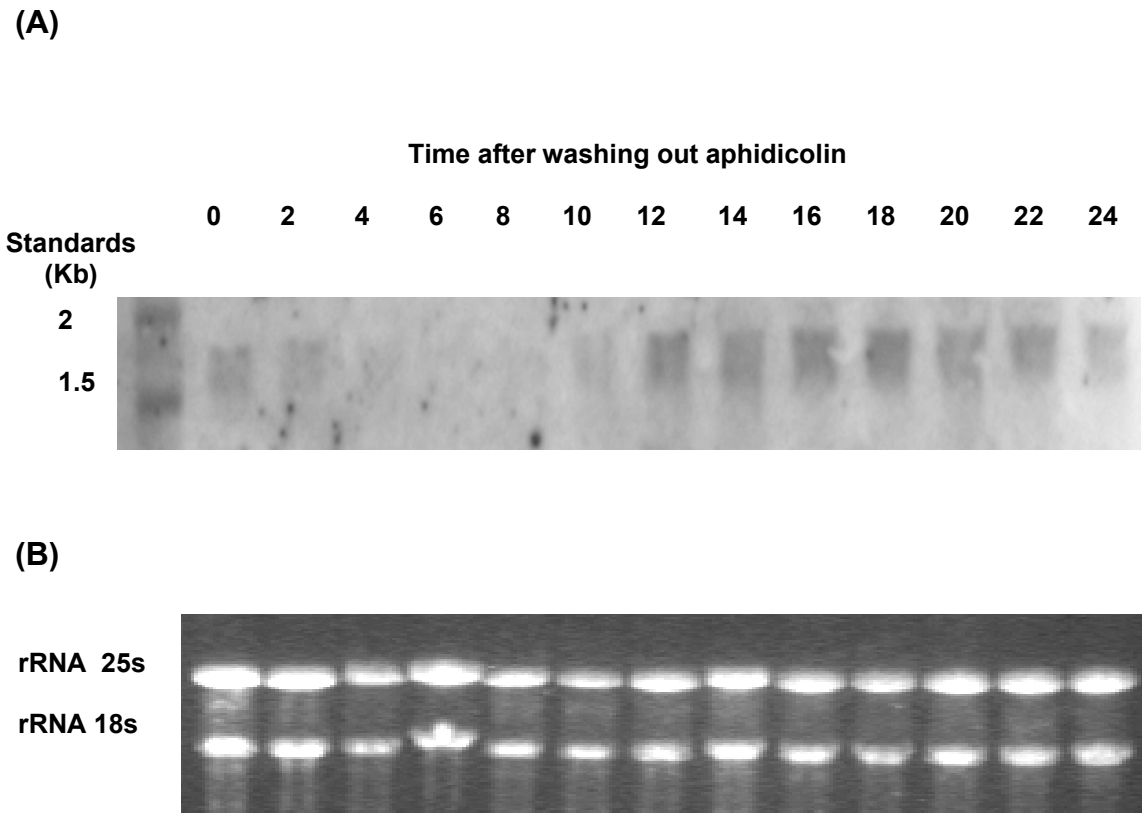

Fig. 4.14 Northern blot analysis of At5g47500 gene expression in Arabidopsis suspension during cell cycle. (A) Northern blot of total RNA hybridized with biotin labelled probes. (B) Equal total RNA (15 µg) stained with ethidium bromide as a loading control.

4.3 Discussion

The fungal toxin aphidicolin has been found to be an effective method of reversibly blocking cell cycle progression in a number of plant systems like tobacco BY-2 (Nagata *et al*.,1992; Sorrell, *et al*., 1999) and *Arabidopsis* thaliana (Menges and Murray, 2002). The use of aphidicolin leads to cessation of cell cycle progression and accumulation of cells primarily in late G1/early S phase boundary (Planchais *et al*., 2000). After the release of the block, the synchronous resumption of the cell cycle was followed by monitoring changes in the mitotic index, and using RT-PCR to analyze the expression profiles of the cell cycle marker genes including CDKB2;2, CYCD3;1 and H4.

Traditionally, the cell cycle is divided into four phases: G1, S, G2, and M phase of which G1 is considered as the most significant restriction point. During this phase the cells must integrate a variety of nutritional, hormonal and developmental signals necessary to irreversibly proceed through the cell cycle (Menges *et al*., 2006). The removal of aphidicolin resulted in the progression of the cell cycle, with a peak in the mitotic index after 13-14 h of washing out the inhibitor. The percentage of cells in mitosis (mitotic index) varied between 8 and 12% between the different synchronizations. It could have been underestimated due to the small genome size of Arabidopsis cells and as only the stages with highly condensed visible chromosomes in metaphase and anaphase were scored. However, the peaks of the mitotic indices reported in some published data are close to our value (Menges *et al*., 2002; Zhang *et al*., 2005)

D-type cyclin (CycD3;1) RNA was detected during the cell cycle as indicated by the expression profile of the cDNA amplified with exon-exon designed primers. Higher levels of expression were recorded during the late G2 and M phases between t=10 to t=18 h. The expression of CycD3;1 is regulated by external signals such as the availability of sucrose and plant hormones particularly cytokinin (Menges *et al*., 2006). The data presented in this work is consistent with the abundance of tobacco (Nicta;CycD3;1) showing higher levels of the transcript in mitotic cells during the exponential phase of the culture cycle and M phase of the cell cycle of aphidicolin synchronized tobacco BY-2 suspensions (Sorrell, *et al*., 1999). Similar results were observed in *Arabidopsis thaliana* suspension cultures originated from root calli and synchronized by a combination of sucrose starvation and cyclohexmide (Zhang *et al*., 2005), and in aphidicolin synchronized MM2d Arabidopsis cell line established by Menges and Murray (2002).

B-type CDKs (CDKB) make up a class of plant-specific CDKs that display a peak of activity at the G2 and M phases (Joubés *et a*l., 2000; Inzé and De-Veylder, 2006). In Arabidopsis, CDKB2;2 as a core cell cycle regulator gene, was upregulated in the actively dividing cells of young leaves and floral organs, whereas the microarray data of another study failed to detect any

expression of this gene in the mature organs (Wang and Yang, 2007). The increase in the expression of this gene was used as a marker to determine the G2 and the mitotic phase (M) in the synchronized suspension cultures of MM1d and MM2d Arabidopsis cell lines. (Menges *et al*., 2003 and 2005). In our culture, a higher level of the transcript of CDKB2;2 was present in the blocked cells and during the G2 and M phase between t= 8 to t=18 h after the release of aphidicolin.

Histones have been related to the condensation of the chromatin, which is one of the important morphological changes reported during the cell cycle (Kodama *et al*., 1994). The peak of Histone (H4) expression was recorded during the S phase at t= 2–4 h following the release of the cells from aphidicolin. This observation is in agreement with previous observations (Menges and Murray, 2002; Zhang *et al*., 2005). Thus the correlation between the mitotic index and the transcription profiles of CycD3;1, CDKB2;2,and H4 indicated that the cells were reasonably arrested in the G_1/S phase of the cell cycle. The synchronized cells entered the S phase at approximately 2 h after the release of aphidicolin, passed through G2 at t=6 to t=10, and then into the M phase with a peak M/A index at t=14h. The cells proceeded into the subsequent G1 of the next cell cycle at t=20 h after washing out the block.

In the last stage of the cell cycle during cytokinesis, Golgi-derived membranous vesicles carrying cell plate materials are transported to the equatorial zone of the phragmoplast. The arriving vesicles fuse forming a continuous, interwoven, tubulo-vesicular membranous network which is composed of various carbohydrate polymers, with callose, xyloglucan, pectin and arabinogalactans as the major lumenal components of the forming cell plates (Staehelin and Moore, 1995; Robertson *et al*., 1995; Verma, 2001; Yokoyama and Nishitani, 2001; Segui-Simarro *et al*., 2004). The cell plate undergoes a complex process of maturation during which callose is replaced by cellulose (Verma, 2001).

The EDTA/phosphate solubilised the pectin that was loosely attached to the cell wall through ionic calcium bonds (Brummell *et al*., 2004). The sugar

content of the extract was quantified using the phenol sulphuric method (Dubois, 1956). The amount of extracted sugar increased during G2 and M phases and peaked at t=18 h. It was significantly reduced (at 5% level) at t=20. It was 14% less than the extracted pectin at t=18. For the rest of the sampling period, the levels of extracted sugar increased again (t=22 to t=24 h) and returned to a level similar to that at t=18 (Fig. 4.4). The increase during G2 and M phase could be attributed to the cell wall soluble sugars stored in the Golgi stacks that have been reported to accumulate at the spindle poles, Golgi belt zone and around the phragmoplast where the cell plate will be formed, during the pro-metaphase, metaphase and cytokinesis respectively (Nebenführ *et al*., 2000). Golgi stacks are distributed roughly equally among the daughter cells to ensure optimal cell viability. In dividing somatic cells, the Golgi derived vesicles that give rise to cell plate and cell wall contains pectin, callose, xyloglucan and arabinogalactan (Matar and Catesson, 1988; Freshour *et al*., 1996; Staehelin and Moore, 1995; Sonobe *et al*., 2000; Verma; 2001). Highly esterified pectins in these vesicles form gels through hydrophobic interactions facilitated by the removal of water and presenting a stable elongated cell plate building blocks that direct the fusion of the new vesicles to what is called the dumbbell ends of the fusing vesicles (Segui-Simarro, *et al*., 2004). The reduction at t=20 possibly reflects the cell wall resistance to hot buffer-EDTA extraction when the developing cell plate fuse with the parent cell plasmalemma, agreeing with previous observation in dividing meristematic cells of *Phaseolus vulgaris* root (Matar and Catesson, 1988), or it could be attributed to the loss of small pectin molecules from extracts during the dialysis and sample preparation. The primary walls are then deposited on each side of the middle lamella which might explain the increase in pectic extracts during the subsequent G1. The increase in the number of secretory vesicles associated with the active synthesis of the cell wall was observed in the second G1 phase of the synchronized *Catharanthus roseus* cultures (Amino *et al*. 1984; Hirose and Komamine, 1989). The final stage in the completion of the cell plate is the maturation stage, a process that is accompanied by the replacement of callose by cellulose transforming the fluid wrinkled cell plate to a stiff, flat, cellulose rich cell wall. (Nebenführ *et al*., 2000).

The uronic acid content increased during the cell cycle after the release of aphidicolin and peaked at t=16 and t=18 h constituting 66% and 61% of the extracted pectin respectively. However, the UA level decreased in the subsequent G1 (Fig 4.4). The progressive increase in the UA acid content during S, G2 (t=2 to t=10) possibly could be related to the modulations in the parental cell walls as the cells are preparing for cytokinesis. The increase in methylated UA content during M phase could be related to the exocytotic Golgi derived vesicles (Freshour *et al*., 1996), and the endocytotic vesicles as a result of the turn over of the parental cell wall components to the phragmoplast where these vesicles will unload their cargo (Baluśka *et al*., 2005). The methylesterification of HG is believed to take place in the Golgi bodies through the activities of pectin methyl transferase (PMT) (Vannier *et al*., 1992) and deesterified in muro by pectin methylesterase (PME) (Gaffe *et al*., 1994). The reduction in the extracted UA at t=20 although the total solubilised pectin increased possibly reflects the resistance of chelator extractable HG, due to the modulation in the newly developing cell wall, and the deposition of other sugar components like weakly bound water soluble xyloglucans.

The amount and pattern of HGA methylation is important for wall function in growth and development. Homogalacturonan (HG) in the walls of young cells is highly methylesterified, whereas a lower degree of esterification characterises the cell walls of older cells. (Schaumann *et al*., 1993).The immuno-dot assay (IDA) of equal quantities of uronic acid for 24 h after washing out aphidicolin, using JIM7 and JIM5 detecting highly and low methylated HG respectively, showed the presence of the two epitopes in the cell walls of the treated cells at G1 phase (t=0). Both epitopes existed in the two extracted fractions of pectin; the branched, large in size, immobilized extracts and the less branched, smaller in size, highly mobile fraction of the extract.

The reaction between JIM7 and its epitope during the cell cycle fits with the curve illustrating the changes in the degree of methylesterification of the

extracted HG, Higher levels of this epitope were detected during G2, M and subsequent G1 phase of the cell cycle, more pronounced in the larger highly branched fraction of extracted pectin during the M phase between t= 10 to t=18. The IDA and the % of methylesterification seemed to stay at the same level during the following G1 phase despite the changes in the amount of extracted pectin and its UA fraction at t=20 and 24 h of the sampling period. The highly methylated HG detected during the M phase could be attributed to the Glogi derived vesicles transporting highly methylated HG that tend to accumulate in the phragmoplast and used in the assembly of the plate.

The JIM5 recognized epitopes were detected during the cell cycle and the subsequent G1 phase. JIM5 binding to the outer ring and central dot indicated that both pectic components contained low-ester HG. The more intense binding of JIM5 at the central dot reflect the high level of deesterification of the highly branched fractions. The fraction of JIM5 reactive epitope seemed to decrease during late S and early G2 phases possibly reflecting changes in the parental cell walls Ca^{2+} bound matrices allowing the extension and increase in the cell size preparing for the cytokinesis. The intensity of the signal seemed to increase during the late G2 and M phase possibly due to the turn over of the parental cell wall components during cell division. Brefeldin-A induced compartments of growing maize (*Zea mays*) root apices demonstrated the presence of endosoms carrying xyloglucan, rhamnogalacturonan II dimers cross-linked by borate diol diester, partially esterified (up to 40%) homogalacturonan pectins, rhamnogalacturonan I decorated with galactan and arabinan side chains (Baluśka *et al*., 2002 and 2005).

LM6 is a monoclonal antibody that recognizes five residues in (1→5)-α-arabinan (Jones *et al*., 1997; Willats *et al*., 1998). Our results showed the presence of the epitope in the EDTA/buffer solubilised pectin during all the phases of the cell cycle, however, a significant intense labelling was noticed in G2 and M phases. This suggests the biosynthesis and secretion of linear stretches of arabinan of at least five residues associated with RG-I at the developing cell plate. In carrot suspension cultures, LM6 labelling was abundant in the cell walls of cells in a proliferative state (Willats *et al*., 1999b).

The failure to detect galactan side chains of RGI of the extracted pectins using LM5 is possibly due to the proliferative state of culture cells. The presence of LM5 reactive (1→4)-ß-galactan epitope in carrot suspension cultures has been related to the developmentally regulated changes as the cells switch from the proliferation to elongation state (Willats *et al*., 1999b).

The Phylogenetic analysis using MEGA version 3.1 based on Neighbour-Joining method (Kumar *et al*., 2004) and clustalW alignment (www.ebi.ac.uk), of the cell cycle putative PME amino acid sequences, categorized these genes into three distinct groups of which Group A showed significant similarities to the characterized, AtPMEI1-1, AtPMEI1-2, AcPMEI, Nt-NIH and AtPME3 at the pro region. The members of group A could be either of type I PME which has a pre-catalytic domain represented by At5g47500, and type II which has a pre-pro-catalytic domains represented by At4g02330, At1g02810, At2g26440, and At2g47550.

The comparative analysis of amino acid sequences of type II PMEs with AtPME3, AtPME1-1, AtPME1-2, AcPMEI and Nt-NIH have shown that they all shared the presence of four conserved Cysteine residues at the pro-region of At4g02330, At1g02810, At2g47550 and At2g26440. The pro region is thought to have an inhibitory function toward is own PME. The amino acid sequence of At4g12390 showed similarity with the N-terminal pro-peptides of plant PME and invertase inhibitors, in particular, the four cysteine residues involved in disulfide bridges. The invertase inhibitor shared similarities with PMEI though PMEI had extra fifth cysteine residues that have not been detected in all invertase inhibitor isoforms (NIHs) studied so far. The comparison at the C-terminal region showed the presence of five characteristic sequence fragments (GxYxE, QAVAL, QDTL, DFIFG, YLGRPWK) that have been recognized as signatures of the PME structures and six amino acids residues strictly located at the active site; two aspartic acids (D), arginine (R) and two glutamines (Q) and many of aromatic residues lining the cleft where the substrate is likely to bind (Jenkins *et al*., 2001).

In all the repeated synchronizations, the expression levels of At5g47500 were always detected during M phase of the cell cycle. The rest of the genes failed to show consistent patterns of expression possibly. The expression of At5g47500 was detected in the treated cells and decreased during S and G2 phase at t=2 to t= 8 after which it increased during the division of the parent cells. Using biotin labelled probes, a positive signals of a single transcript of approximately 1.75 Kb were recognized during G1 at t=0, M phase at t=12 to t=18 h, and subsequent G1 at t=20 to t=24 h. The signal was stronger during the M phase. The obtained signals agree with the cell-cycle-expressed pattern reported by RT-PCR. The size of At5g47500 full genomic DNA is around 1,925 Kb and the full cDNA size is 1,430kb as predicted by Tair website (www.arabidopsis.org). The At5g47500 cDNA annotated in Tair website could be starting at a point upstream to the predicted initiation code explaining the difference in the obtained and predicted size of mRNA. At5g47500 is one of the PME isoforms with a basic isoelectric point (pI) which is likely to act in a linear block wise fashion as proposed by Micheli *et al*. (2001). It is possibly involved in the formation of a small fragment of deesterified stretches of homogalacturonan forming Ca^{2+} bound matrices. The differences in the expression profiles of cell cycle putative PMEs obtained in this study compared to those reported by Menges and Murray (2003) may be partly due to cultures being independently generated. The studied suspension culture was quite recently generated and some genetic changes are thought to occur in cultures grown for many generations.

As a conclusion, the obtained data are reflecting the importance of the methylated HG in the onset of intercellular adhesion. Out of the five putative cell cycle expressed PMEs, only At5g47500 encoding a type I PME showed a systemic consistency in its expression during M phase, possibly attributing to the deesterification of the pectins as they are laid down in the site of developing cell plate. The exact mode and pattern of demethylation as a result of the activity of PME encoded by At5g4700 remains to be elucidated in future work.

Chapter 5

Biochemistry of adhesion in suspension cultures of salt tolerant Arabidopsis

5.1 Introduction

Plants have a remarkable ability to cope with variable biotic and abiotic environmental stresses like salinity, drought, wounding, heavy metal toxicity, nutrient deficiency and pathogen invasion. Soil salinity is one of the major environmental factors limiting agricultural productivity in many arid and semi-arid regions of the world. Based on their ability to grow on high salt medium, plants are traditionally classified as glycophytes or halophytes. Glycophytes are sensitive and can not tolerate high salinity. Halophytes are more tolerant to high concentrations of NaCl and can grow in habitats excessively rich in salts, such as salt marshes, sea coasts, and saline deserts. For glycophytes, salinity imposes a major constraint on plant growth, development and survival. High levels of salts could lead to ionic stress especially sodium toxicity, physiological drought, osmotic stress due to the increase in osmotic potential, and secondary stresses such as nutritional disorders and oxidative stress (Zhu, 2001; Xiong and Zhu, 2002; Zhou *et al*., 2007). High salinity causes pleiotropic effects in plant growth such as reduced cell expansion, decreased protein synthesis, and accelerated cell death. A high level of Na^+ is toxic to plants because it disturbs the cytoplasmic $K^{+/}Na^+$ homeostasis (Taji *et al*., 2004). Tolerance of some plant species to soil salinity is achieved by either extruding Na^+ out of cells, or compartmentalizing sodium ions into the vacuole away from the cytosol and excretion of Na^+ via plasma membrane Na^+/H^+ antiporters. The Na^+/H^+ antiporters in the plasma membrane and tonoplast play a vital role in pumping Na^+ either out of cells (plasma membrane antiporter) or into vacuole (vacuolar antiporter) in exchange of H^+ (Chen *et al*., 2007). A fundamental difference between true halophytes and salt-tolerant glycophytes is that in general halophytes are accumulators of salt whereas salt tolerant glycophytes or salt-tolerant ecotypes of normally salt-sensitive glycophytes are salt excluders (Chaudhary *et al*., 1996).

The cell wall, as the site of interactions with abiotic and biotic environments, undergoes several physical and biochemical alterations as a response to living in a stressed environment (Degenhardt and Gimmler, 2000). Some data

are available describing the effect of salinity on the mass of cell walls and the structural modifications associated with the processes of withstanding the increased concentrations of salt. Some plant species had thicker cell wall with higher masses like *Citrus limon* calli (Piqueras *et al*., 1994), while others showed a reduction in the relative mass of their cell walls such as suspension cultured tobacco cells (Iraki *et al*., 1989b). The exposure of *Aster tripolium* leaves, *Gossypium hirsutum* roots, and tobacco suspension cultures to high NaCl concentrations imposed a reduction in their cellulose content and increased proportions of non-cellulosic components compared to the walls of unadapted cells (Binet, 1985; Zhang and Lauchli, 1993; Iraki *et al*., 1989b,). The leaves of *Chloris gayana* exhibited a reduced rate of expansion after exposure to NaCl (Ortega and Taleisnik, 2003; Ortega *et al*., 2006).

Arabidopsis is a typical glycophyte and not particularly salt tolerant. However, a number of studies suggested that it may contain most, if not all, of the salt tolerance genes that one might expect to find in halophytes (Taji *et al.*, 2004). These findings have led to the hypothesis that many halophytes may use the same mechanisms of salt tolerance found in glycophytes and that subtle differences in regulation may account for the large variations in tolerance or sensitivity between glycophytes and halophytes. A recent study of the transcript profiling data of Arabidopsis seedlings treated with 150mM NaCl for 3h and 24h showed that the changes in the level of expression of 1500 genes were strongly regulated as a salt stress response. Fewer than 25% of these genes were salt stress specific, of which many are induced only in roots. Two cell wall modifying genes, xyloglucan endotransglycosylase and pectin methylesterase (PME; At1g21850), were reported to be exclusively up-regulated by salt stress in roots (Ma *et al*., 2006). Normal tobacco cells cannot grow in the presence of 100 mM NaCl. After salt adaptation, some tobacco cells are able to tolerate five times that much salt (Hasegawa *et al*., 2000). Similarly, alfalfa cells as well as intact plants have been adapted to tolerate very high levels of NaCl (Winicov, 1991).

Aim of this chapter

The aim of this chapter is to use salt tolerant Arabidopsis suspension cultures adapted to 300mM NaCl (HHS cell line) to study the pectin epitopes and their roles in mediating cell adhesion through *in situ* immunocytochemical analyses, along with the biochemical characterization of the pectic extracts during the proliferation of the culture.

5.2 Growth kinetics of *Arabidopsis thaliana* suspension cultures

The *Arabidopsis thaliana* HHS (Habituated to High Salt) cell line tolerant to 300 mM NaCl was generated by Dr. Peter Dominy - University of Glasgow. Non-treated wild type cell suspension cultures were accommodated to growth in high salt concentrations through successive subculturing with gradual increments of NaCl concentration over a period of 2 years. The HHS culture was green in color and contained a mixture of spherical and some more elongated cells. The cells formed larger clusters that seemed to be difficult to disaggregate by shaking.

The pattern of growth of the salt tolerant Arabidopsis suspension cultured cells was similar to that of the previously described wild type. However, the maximal packed cell volume (PCV) achieved by the cells was much lower. The exponential phase lasted between days 2 to 6 followed by a marked reduction in the rate of increase in PCV. Seven days after the previous subculturing, the PCV increased by 150 % compared to the PCV at day 2 during the early division phase. Days 0 to 2 could have represented the lag period after which the packed cell volume increased markedly due to increase in the division rate.

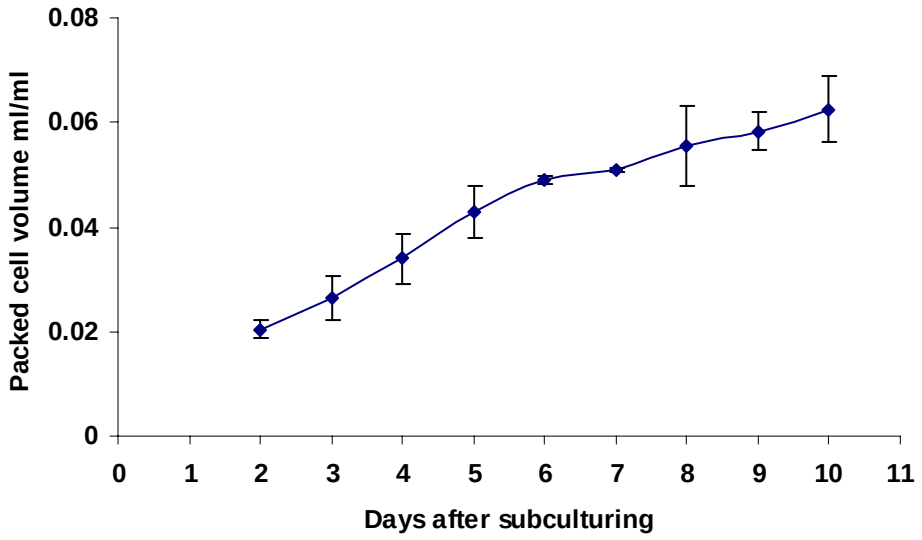

Fig. 5.1 Growth curve of suspension cultures of HHS cell line of *Arabidopsis thaliana.* The graph shows mean ± SD for three different cultures.

5.3 Chemical analysis of extracted pectin

5.3.1 Sugar content of the extracted pectin

Pectins are known to be solubilised using EDTA/phosphate buffer by the breakage of egg box calcium cross-bridges between adjacent pectate sequences. The total extracted sugar increased between days 1 to 5. A sharp increase was recorded at day 1 after subculturing, followed by a reduced rate of increase between days 2 to 5, after which it remained essentially unchanged for the rest of the culture cycle (Fig. 5.2). At day 5, the amount of extracted sugar had increased by 79% compared to day 1.

Galacturonic acid (GalA) is the most abundant plant cell wall (PCW) uronic acid (UA) (Popper and Fry, 2004; Abdel-Massih *et al.*, 2007). The UA fraction of the EDTA pectic extracts increased during the exponential phase. It constituted 35% on day 2 and 68% on day 6 of the solubilised pectin (Fig 5.2), while this fraction constituted an almost constant proportion ranging between 59 to 64% of the chelator extract of the wild type Arabidopsis line (Fig 3.3).

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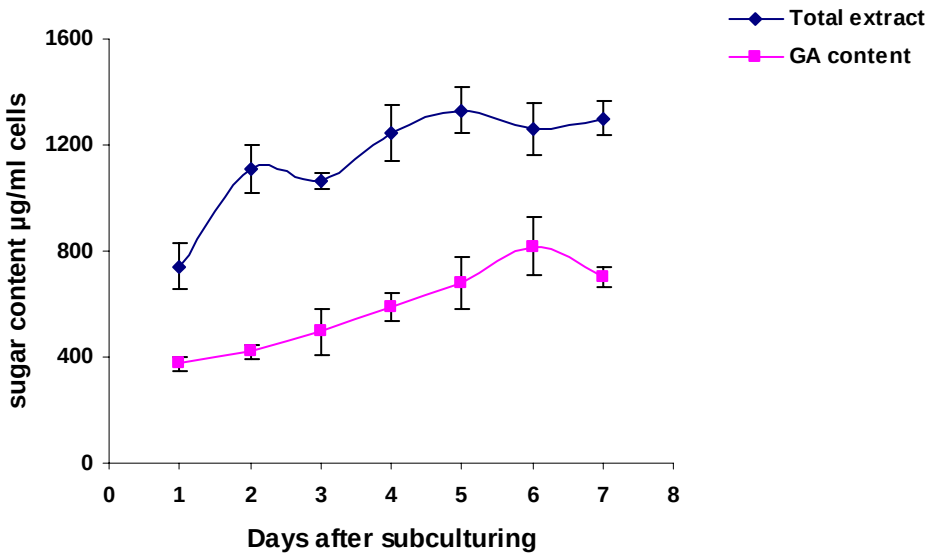

Fig. 5.2 Changes in the contents of phosphate/EDTA buffer extracted pectic sugars and GA fraction during the culture cycle of the HHS *Arabidopsis thaliana* cell lines. The graph shows mean ± SD for three separate experiments.

5.3.2 Determination of the level of methylesterification of extracted homogalacturonan.

The chelator/phosphate buffer extract contained both esterified and non esterified HG. The degree of esterification of the GalA fraction was deduced from the measurements of methanol released by saponification using a dilute alkali at 4C^{o}.The extracted HG showed a reduction by about 30% in the degree of acid esterification compared with the wild type (wt), indicating possibly higher levels of Ca^{2+} bound matrices during mid/late exponential and early stationary phase. The degree of methylesterification (DM) of pectic extracts of wild type Arabidopsis increased (40 to 60%) during the exponential phase and was reduced at the onset of the stationary phase on day 7 (Fig. 3.4)

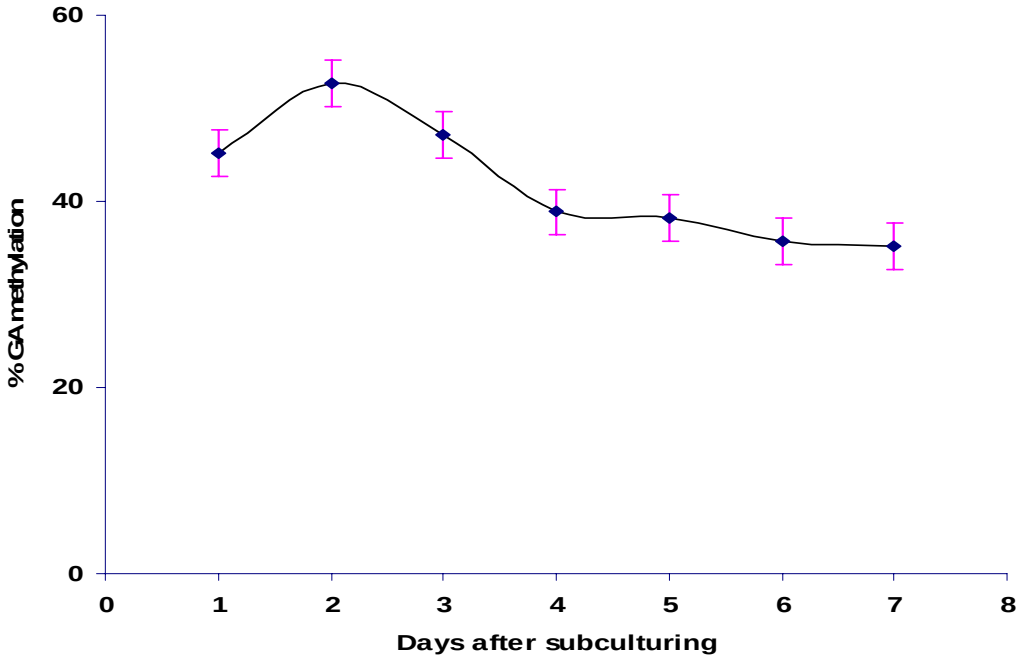

Fig. 5.3 Degree of methylesterification of the EDTA/phosphate extracts of HHS *Arabidopsis thaliana* cell line during the culture cycle. The graph shows mean ± SD for three separate experiments.

5.4 Detection and localization of homogalacturonans with low and high levels of methylesterification during culture cycle

Anti-HG and RG-I side chains monoclonal antibodies JIM5, JIM7, LM7, LM5 and LM6 were used to characterize the occurrence and methylesterification of HG in the cell walls of salt tolerant HHS *Arabidopsis thaliana* cell line cultured cells. Both the degree and the pattern of de-esterification influence the binding capacity of these antibodies to HG domains whether *in muro* or in the EDTA/phosphate released pectin. The monoclonal JIM 7 antibody detects relatively high level of HG methylesterification with flanking unesterified GalA residues, and JIM5 binds to unesterified GalA residues with adjacent or flanking methylesterified units. LM7 preferentially recognizes HG with a random non-blockwise pattern of esterification (Willats *et al*., 2000b; Clausen *et al*., 2003), and 2F4 recognizes low methylesterified homogalacturonan associated with calcium (Liners *et al.*, 1992).

5.4.1 Binding of monoclonal antibody JIM7 and JIM5 to the extracted pectins

The immunodot analysis (IDA) revealed the presence of the JIM7 recognized epitope in all the EDTA/phosphate fractions of the HHS cell line, suggesting the abundance of highly esterified pectin across the cell walls during culture cycle (Fig. 5.4). The intensity of labelling was stronger in the central dots representing the larger highly branched fraction of the extracted pectin. The JIM7 obtained immunoprofiles did not differ much from the blots of the wild type Arabidopsis extracts using the same antibody.

The immuno dot blots using JIM5 demonstrated the presence of short stretches of contiguous GalA residues in the chelator/phosphate extracts of Arabidopsis salt tolerant cells which possibly were calcium-complexed. The profiles obtained showed stronger labelling during the exponential phase between days 2 to 5 (Fig. 5.4). The abundance of the JIM5 reactive epitope was more pronounced in the large highly branched fraction of the extracted pectin. This result fits with the reduction in the degree of methylesterification during the exponential phase, possibly through the action of PMEs.

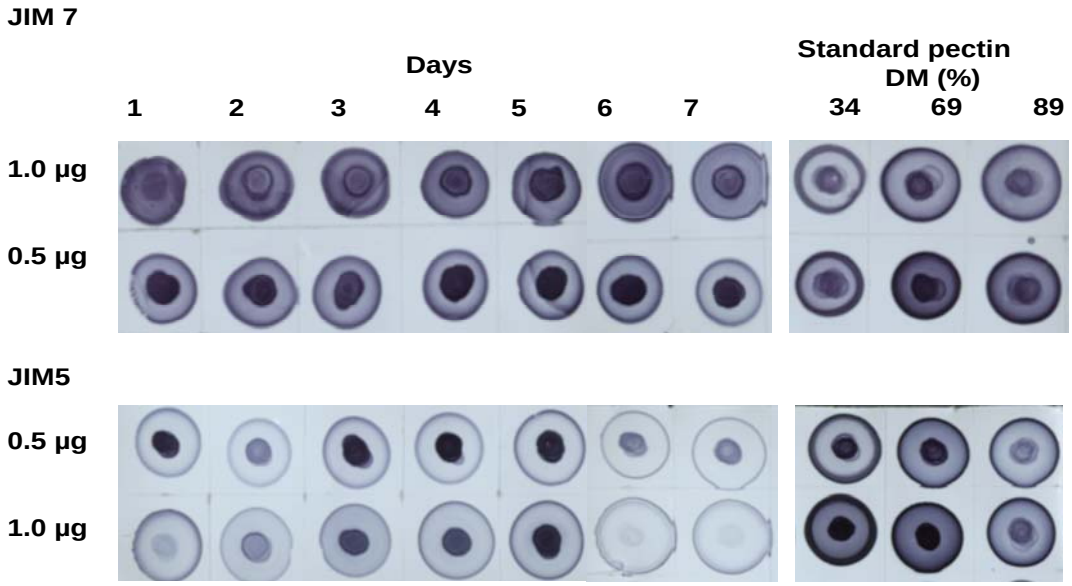

Fig. 5.4 Immuno-dot assay of monoclonal antibody JIM7 and JIM5 binding to the EDTA/phosphate buffer solubilised pectins from HHS Arabidopsis cells during the culture cycle. The results were reproducible in the extracts of two different cultures.

5.4.2 Immuno-Labelling of Arabidopsis cell walls with monoclonal antibodies JIM7 and JIM5

The signals resulting from JIM7 binding were found both in young thin walls formed just after cell division (white arrows), and in older thicker walls. Within one aggregate composed of several cells, a higher level of the signal was detected between neighbouring cells where a stronger adhesion seemed to prevail (yellow arrows), and in cell walls that surrounded aggregates and remained in direct contact with the environment (Fig. 5.5). The epitope is deposited in tricellular junctions developing between adjacent cells.

The low ester HGs detected by the JIM5 antibody were deposited in the walls of salt tolerant Arabidopsis resin embedded cells. It seemed to exist in sporadic dot-like spots (Fig 5.6). The labelling pattern indicated unevenness of distribution within cell walls of a cluster of cells. The JIM5 binding polymers appeared as irregular aggregates in some cells and in some wall areas. The level of labelling of the newly formed cells (white arrows) was less compared to the matured established primary cell walls. The label was distributed less homogeneously throughout a wall of a cell and the walls adjoining neighbour cells showed stronger signals (yellow arrow) compared to unadhered surfaces at the boundaries of cell clusters. The presence of JIM5 epitopes is thought to contribute to cell adhesion through Ca^{2+} cross-linking (Carpita & McCann 2000).

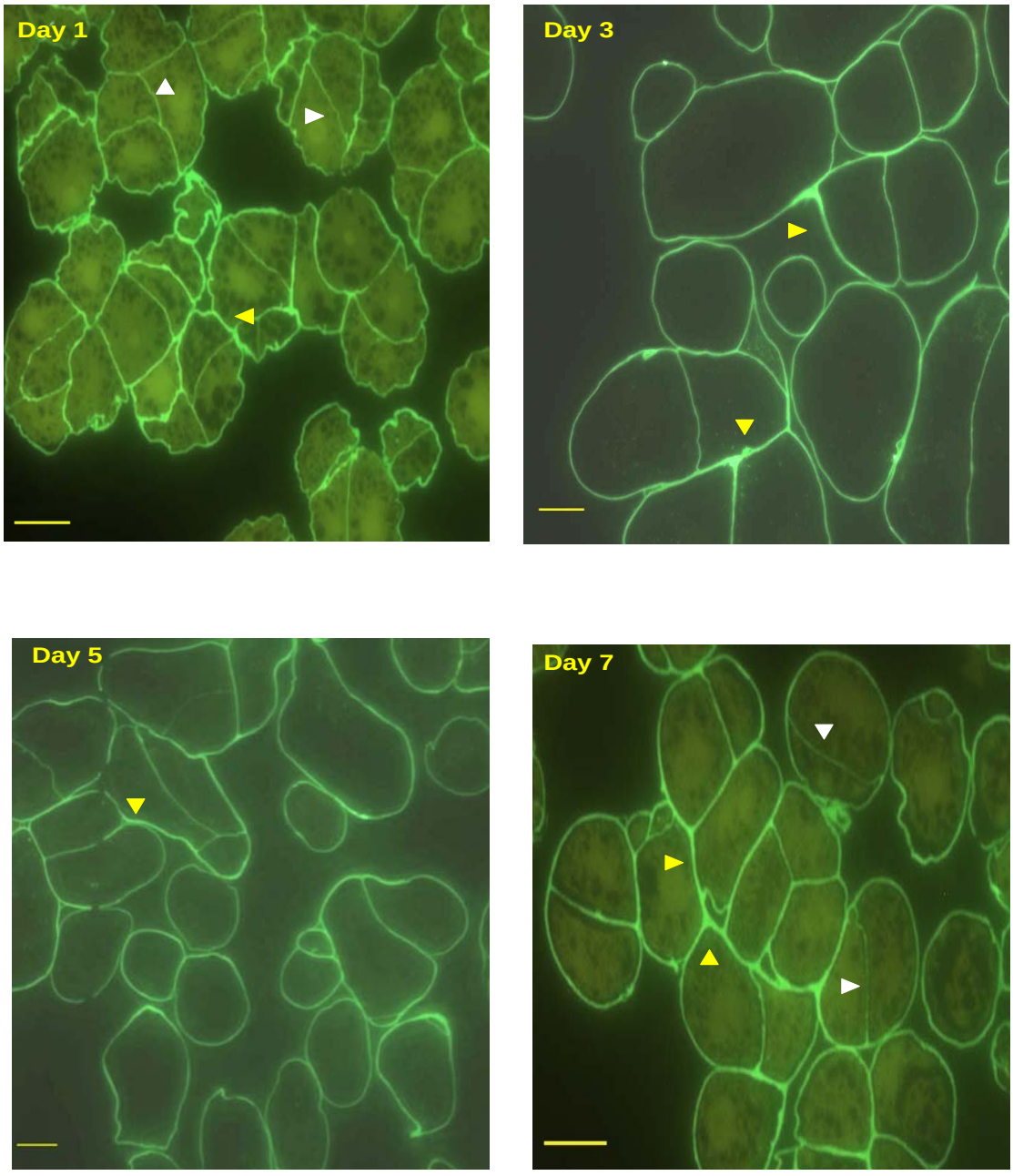

Fig. 5.5 Immunolocalization of JIM7 recognized epitopes in resin embedded sections of HHS Arabidopsis cells in days 1,3,5, and 7. Colored arrows were used to indicate strength of the signal; yellow to label adhesion between neighbouring cells and white to label newly formed young cell walls. Scale bar representing 10 µm.

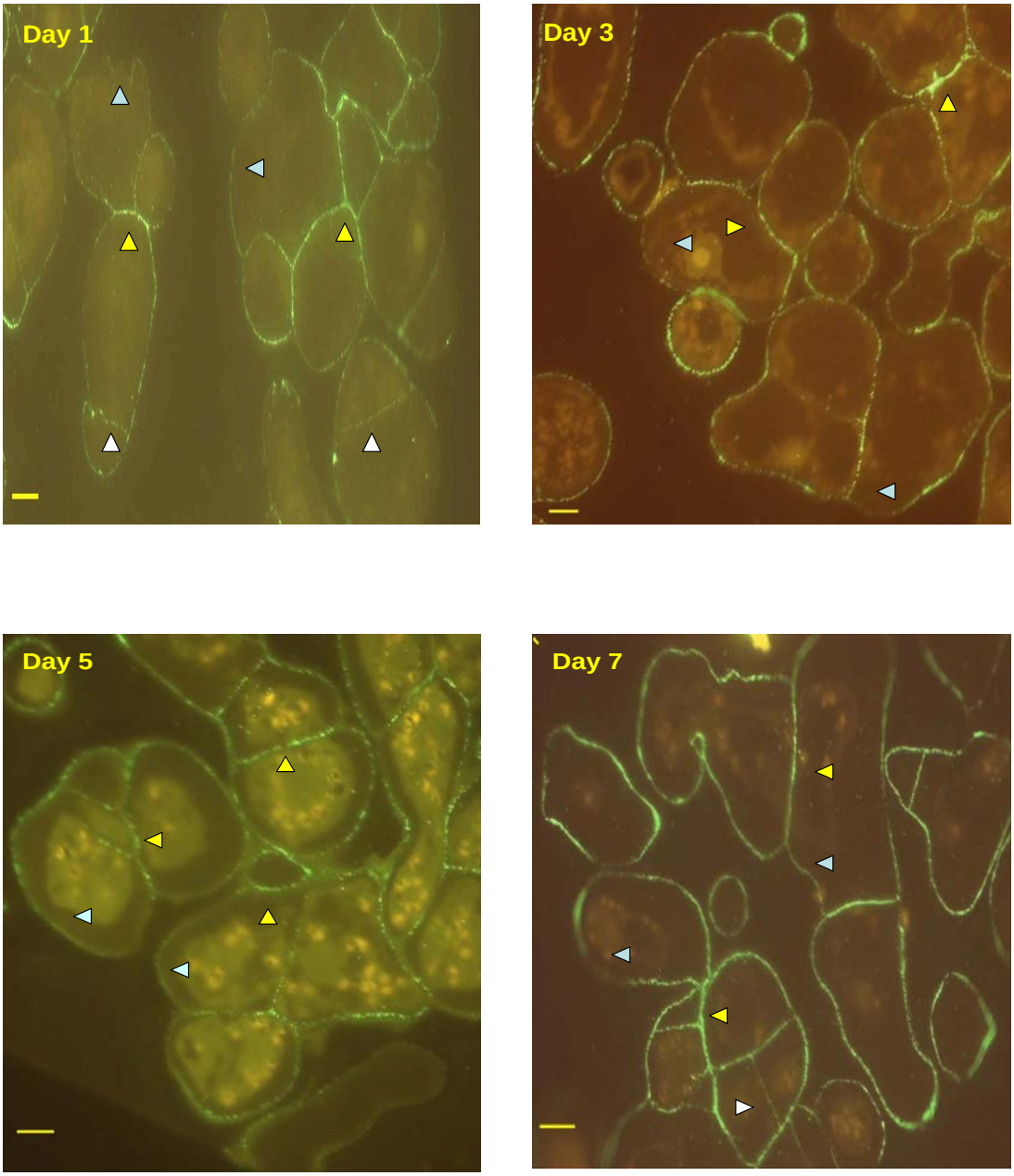

Fig. 5.6 Immunolocalization of JIM5 recognized epitopes in resin embedded sections of HHS Arabidopsis cells in days 1,3,5, and 7. Colored arrows were used to indicate strength of the signal; yellow to label adhesion between neighbouring cells, white to label newly formed young cell walls and blue to label the unadhered surfaces of cells in clusters. Scale bar representing 10 µm.

5.4.3 Immuno-Labelling of Arabidopsis cell walls with monoclonal antibody LM7 and 2F4

The epitope recognized by LM7 was observed in the immunodot labelling of EDTA/ phosphate extracts from salt tolerant Arabidopsis cells (fig 5.7). A slight increase in the strength of the reaction was noticed as the cells proceeded through the culture cycle. Again, the epitope seemed to prevail in the two pectic extracts though was more pronounced in the central dot of more branched large fraction of the extract. The trials of the immuno *in muro* localization of the LM7 reactive epitope in the sections of the embedded cells of HHS cell line failed to detect the presence of the epitope. This observation is in agreement with previous studies referring to the instability, lability and loss of this epitope during the preparation of the tested plant material (Willats *et al*., 2001c). The HG microdomain detected by the monoclonal antibody LM7 was reported to occur specifically at the junction zones between separated and adhered cell walls during intercellular space formation in parenchyma systems (Willats *et al*. 2001c).

The 2F4 monoclonal antibody recognizes only pectic polysaccharides of low ester content (DM < 30%) that possess oligomers of at least 9 GalA able to form calcium dimers inducing intermolecular associations (Liners *et al*. 1992). This epitope was not detected in the tested resin embedded cells possibly due to the absence of the epitope and/or conformational change in the GalA chains during the processing of the sample.

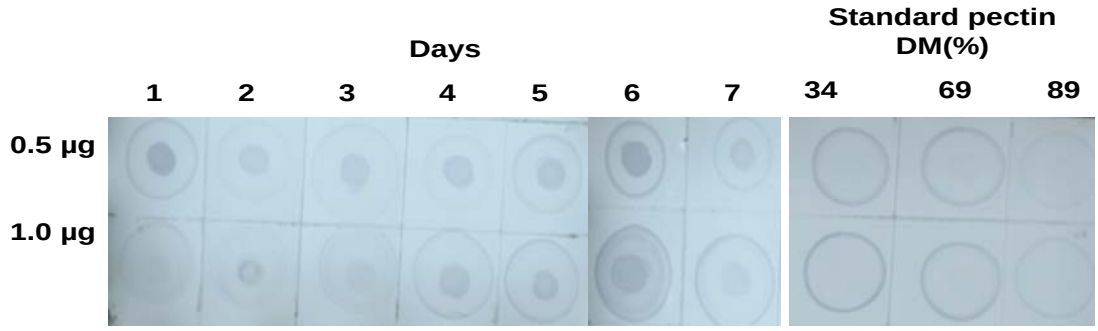

Fig. 5.7 Immuno-dot assay of monoclonal antibody LM7 binding to the EDTA/phosphate buffer solubilised pectins from HHS Arabidopsis cells during the culture cycle. The results were reproducible in the extracts of two different cultures.

5.5 Distribution of Rhamnogalacturonan I-associated Epitopes.

The location of RG-I associated epitopes were investigated using anti-arabinan and anti-galactan monoclonal antibodies. LM5 recognizes an epitope of four residues of (1→4)-ß-D-galactan (Jones *et al*., 1997), and LM6 recognizes five residues of (1→5)-α -L-arabinan (Willats *et al*., 1998). Control samples showed a complete lack of staining and no fluorescence signal

5.5.1 Binding of monoclonal antibody LM5 and LM6 to the extracted pectins

The immunoprofiles shown in Fig. 5.8 suggests that the EDTA/phosphate extracts were rich in epitopes that bind the two anti-pectin antibodies, LM5 and LM6. The (1→4)-ß-D-galactan and (1→5)-α -L-arabinan mainly belonging to RGI side chains were mostly detected in the large highly branched fraction of the extracted pectin. No significant differences in the levels of the LM5 and LM6 epitopes were detected during the culture cycle, although a slight reduction in the strength of the signal for LM6 epitope was observed on days 6 and 7. In wild type Arabidopsis, LM6 epitopes were detected and appeared to be more abundant in the pectic extracts. The

results obtained may indicate the presence of a RG-I of a higher degree of branching with more galactan side chains, which possibly is playing a role in the adaptation of the cell walls to 300 mM NaCl.

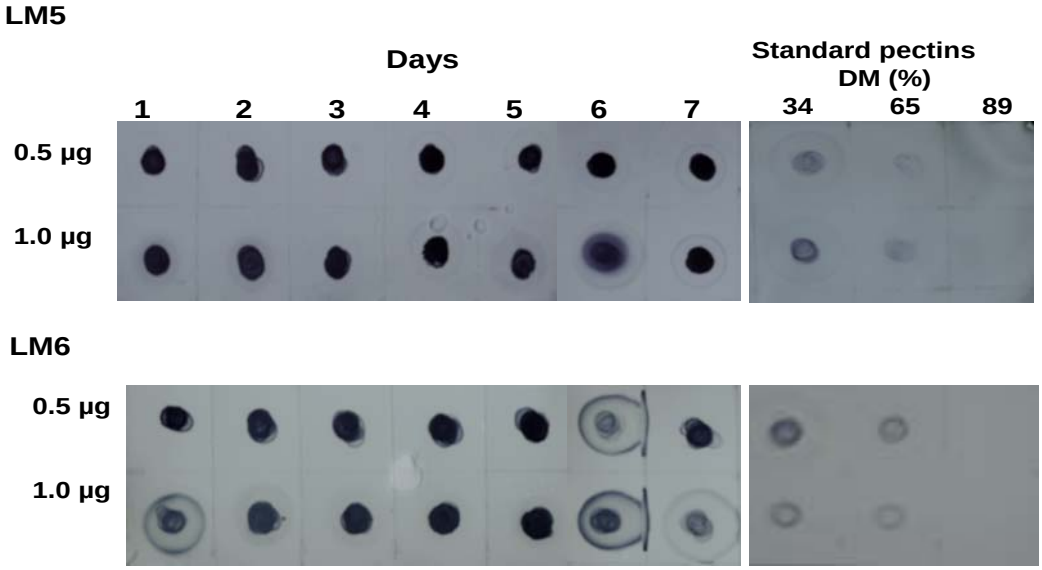

Fig. 5.8 Immuno-dot assay of monoclonal antibody LM5 and LM6 binding to the EDTA/phosphate buffer solubilised pectins from HHS Arabidopsis cells during the culture cycle. The results were reproducible in the extracts of two different cultures.

5.5.2 Immuno-Labelling of Arabidopsis cell walls with monoclonal antibody LM5

Surprisingly, the salt tolerant embedded cells displayed detectable amounts of the LM5 reactive epitope mainly at the external surfaces of cell clusters facing the culture media, and through out the primary cell walls of single cells and small aggregates (Fig. 5.9). Mainly, the galactan epitopes were scarce and less abundant in the middle lamellae; however, the epitope could be located in few cell junctions as in Day 3.

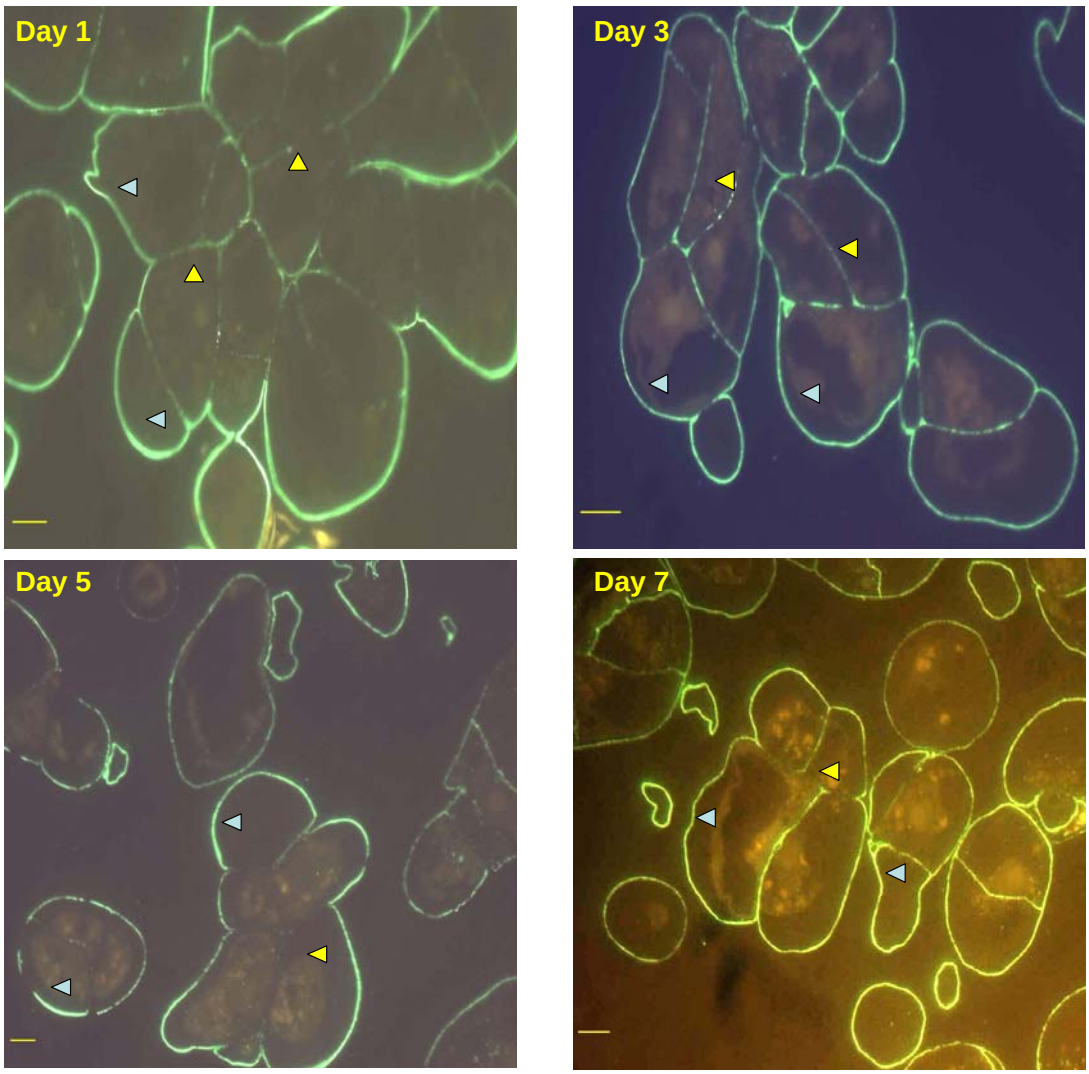

Fig. 5.9 Immunolocalization of LM5 recognized epitopes in resin embedded sections of HHS Arabidopsis cells in days 1, 3, 5, and 7. Colored arrows were used to indicate strength of the signal; yellow to label adhesion between neighbouring cells and white to label newly formed young cell walls. Scale bar representing 10 µm.

5.5.3 Immuno-Labelling of Arabidopsis cell walls with monoclonal antibody LM6

The monoclonal anti-arabinan LM6 antibody could detect the presence of arabinose rich domains throughout the primary cell walls of the embedded suspension cells of Arabidopsis habituated to 300mM NaCl (Fig. 5.10. The distribution of (1→ 5)-α -L-arabinan was similar to that described for wild type Arabidopsis. The (1→5)-α-arabinan epitope was more abundant in older walls

and middle lamellae (yellow arrows) than in newly formed ones (white arrows).

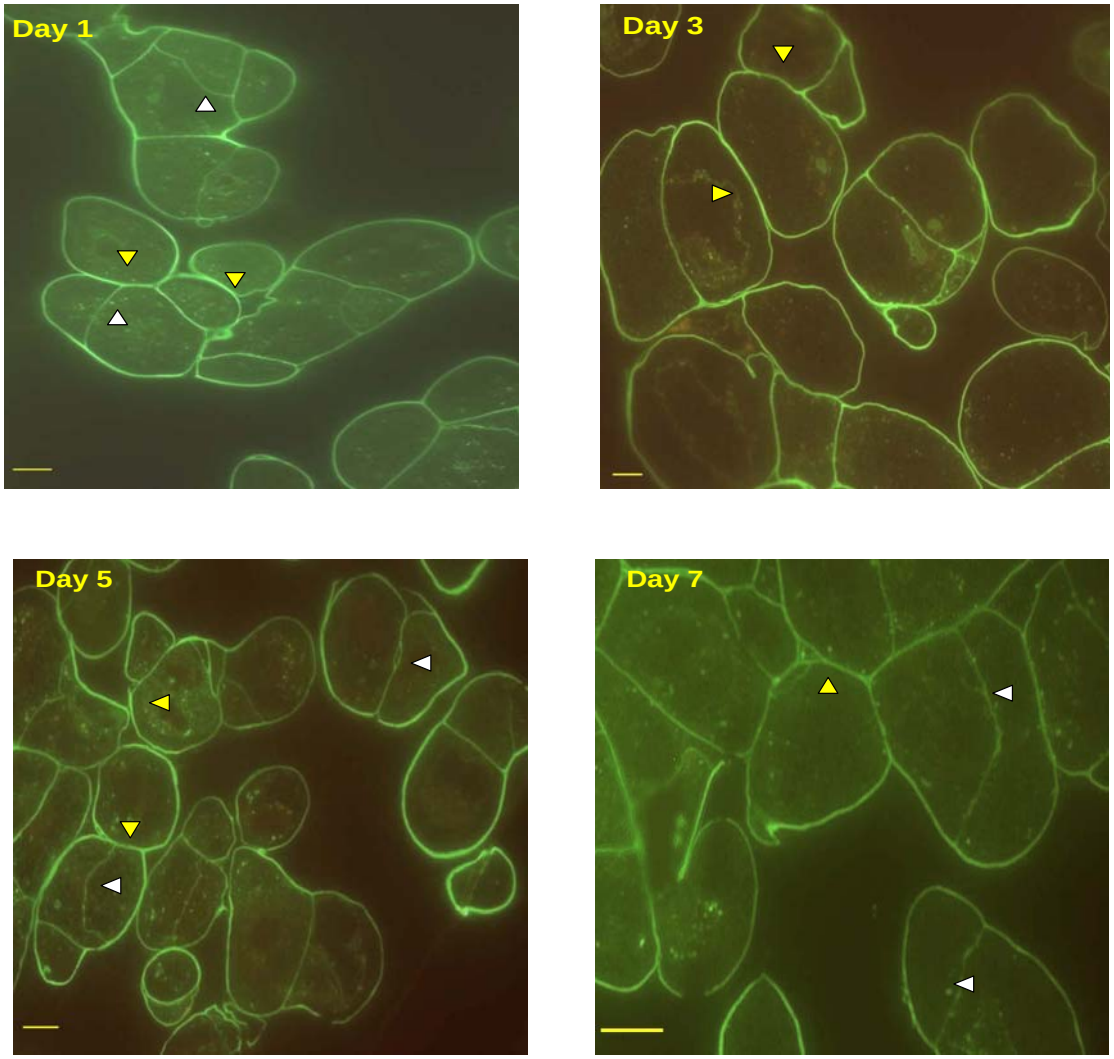

Fig. 5.10 Immunolocalization of LM6 recognized epitopes in resin embedded sections of HHS Arabidopsis cells in days 1, 3, 5, and 7. Colored arrows were used to indicate strength of the signal; yellow to label adhesion between neighbouring cells and white to label newly formed young cell walls. Scale bar representing 10 µm.

5.6 Discussion

Plant cell walls are dynamic entities that determine the morphology, growth, and development of plants. They form the borders between the plants and their environment and a site of communication through their structural and chemical adaptations. The HHS cell line *Arabidopsis thaliana* can tolerate over 300 mM NaCl while the non-habituated cultures do not survive in more than

80 mM. The growth curve showed that the packed cell volume (PCV) rapidly increased during the exponential phase between days 2 to 6 after subculturing, and then entered a phase of much slower growth on day 7. The HHS suspension cells were highly aggregated into large clusters.

The total EDTA-phosphate buffer is known to bring the water soluble loosely interacting polymers and calcium-interacting pectin into solution (Leboeuf *et al*., 2004). The increase in the amounts of the extracted pectins during the culture cycle could be related to the continuous biosynthesis of highly methylated pectic polysaccharides as a result of division and growth of cells (Willats *et al*., 2001), in addition to the ongoing demethylation of cell wall pectins by the action of pectin methylesterase (PME), which were subsequently complexed with Ca^{2+} to each other and thus becoming extractable by EDTA (Jarvis *et al*., 2003). The EDTA/phosphates extracts of the salt habituated cultures contained sugar levels close to those detected in the extracts of the non-habituated wild type cells, although the packed cell volume was much less. This could reflect the enrichment in the absolute amount of loosely bound pectic polysaccharides as the culture proceeded through its cycle. The total uronic acid fraction constituted 35% of the total pectic extracts during the early phase of active cell division, and increased up to 68% of the EDTA extract at day 6. Previous studies demonstrated the increase in total uronic acid exposed to different types of abiotic stress, for example, the total uronic acid content increased in cotton roots exposed to 150mM NaCl (Zhang and Lauchli, 1993). The proportion of total esters in walls of Arabidopsis salt habituated cells was 52% on day 2 at the early exponential phase and dropped to 35% at day 7. The decrease in the degree of methylesterification of the extracted pectin probably resulted from an *in muro* enzymatic de-esterification of GalA residues catalysed by PME. High salt concentration up to 300mM and 500 mM NaCl concentrations resulted in an increase in the *in vitro* activity of pectin esterase enzyme extracted from the cell walls of *Suaeda maritima* leaves and etiolated hypocotyls of *Vigna radiata (*Thiyagarajah *et al*., 1996).

The EDTA/phosphate extract of salt habituated Arabidopsis cells resolved into two discrete components when probed with JIM5, JIM7 and LM7, indicating that the HG epitopes were present in at least two distinct pectic components. Using immuno dot labelling of EDTA fractions, both of JIM5 and JIM7 recognizable epitopes were detected. The signal was more concentrated in the central dots, reflecting their abundance in the large immobile highly branched fraction of the extracted pectin, however, the highly methylesterified fraction constituted a higher proportion of the extract. The profile obtained suggests that the JIM5 epitope was increasing during the exponential phase (days 2 to 6) and decreased on days 6 and 7 when the degree of methyl-esterification reached 35%. The 2F4 antibody failed to detect the presence of calcium-associated homogalacturonan**.** The epitope recognized by LM7 was detected in small amounts that increase slightly towards the end of the culture cycle. The detection of LM7 epitope reflects the presence of non blockwise deesterified residues on the extracted HG fraction. A similar labelling pattern was observed in the immuno blots of pectic extracts of non-habituated wild type Arabidopsis probed with JIM7. A high level of esterification makes pectin unable to form rigid egg box structures through Ca^{2+} bridges, making the cell walls potentially flexible.

The immunoprofiles of pectin probed with JIM5 and JIM7 were complementary to the *in situ* histochemical immunolocalization of their epitopes. The embedded cells probed with JIM5 showed the presence of the epitope in discrete micro-domains as localized dot like structures through all the boundaries of the cells. The epitope was clearly visible between adjacent cells. The immunofluorescent labelling of embedded cells using JIM7 showed that the primary cell walls, middle lamellae and intercellular junctions are rich in the highly methylesterified HG.

The antibody reactions in immuno dot assays directed against RG-I side chains indicated the presence of high levels of arabinan and galactan mostly attached to the immobile large highly branched fraction of the extracted pectins. The LM5 binding was greater in the salt adapted cells compared to the wild type, possibly due to the increased accessibility of the antibody to the

epitope, as a result of some conformational changes because of the reduction in the degree of methylesterification. The restricted occurrence of the LM5 epitope at the primary cell walls located at the interface between the cells living in large clusters and the surrounding medium appears to be a marker for the increased tolerance of the cells to the high salt concentration. This epitope did not seem to be significantly involved in the intercellular adhesion between cells in a cluster. Higher levels of EDTA/Phosphate extract labelling was observed using LM5 in the bean cell suspensions habituated to 0.3 μM dichlobenil (Garcia-Angulo, *et al*., 2006). Intense signals were found in the primary cell walls of the resin embedded cells after the immuno reaction with the LM6 antibody, and the epitope was detected in the intercellular spaces and middle lamellae. Similar results were recorded in the non-habituated Arabidopsis embedded cells.

Studies involving *Nicotiana tabacum* suspension cultures adapted to 428mM NaCl revealed that the total amount of cell wall content was almost the half the cell wall content of unadapted cultures, the EDTA extracted pectins contained higher proportions of loosely bound uronic acids and rhamnogalcturonan, and rhamnose units of RG-I were more highly substituted with polymers containing arabinose and galactose (Iraki *et al*., 1989a, b, c). In *Aster tripolium* adapted to 260mM NaCl, the pectic network contained elevated levels of arabinose and less galacturonic acid content, which was highly methylesterified allowing an easy expansion and enlargement of cell walls (Binet, 1985).

The above biochemical and immunocytochemical analyses showed that the EDTA/buffer solubilised pectic sugars of Arabidopsis habituated to 300mM NaCl (HHS cell line) generally conformed to the pattern found in non-habituated Arabidopsis suspension cells. The HG domain is made up of a mixture of highly and low methylesterified micro-domains favouring the abundance of highly methylesterified GalA residues in which the hydrogen bond and hydrophobic interactions between the methoxy groups are involved in cell-cell adhesion. It seems that the roles of calcium associated pectin dimers are relatively insignificant in maintaining the adhesion between the

cells, although a set of JIM 5 reactive antigens were located between neighbouring cells suggesting the presence of unesterified micro domains that may be involved in the establishment of Ca^{2+} associated interactions. It seems that the primary cell wall is enriched with loosely bound RG-I carrying side chains of (1→4)-β-galactan and (1→5)-α-L-arabinan, with more galactan relative to arabinan in salt adapted cells compared to wild-type. The presence of galactan and arabinan could enable the cells to withstand the salinity that was imposed in the culture media. It has been shown that arabinan polymers act as plasticizers, increasing cell wall flexibility and diminishing strong interactions between homogalacturonan chains in pectin (Jones *et al*., 2003). Future studies involving the molecular biology and the expression patterns of pectin methylesterase could reveal the impact of salinity on the different PME isoforms annotated to occur during the cell cycle.

As a conclusion, the obtained data are showing that the cell walls and middle lamellae of Arabidopsis suspensions habituated to high salts, seemed to show an increase in the level of adhesion between cells in clumps, as a result of increase in the level of the random methyl esterification of HG detected by the monoclonal antibody LM7 in the phosphate/chelator extracts, along with the increased branching of the rhamnogalacturonan-I polymers.

Chapter 6

General Discussion

Dicotyledonous plants generally contain cell walls with about one-third each of cellulose, hemicellulose, and pectin components (Brett and Waldron, 1996). Pectins are established as adhesion molecules in somatic cells, reproductive tissues and transmitting tracts of pistils. After the deposition at the cell plate during cell division and later at the primary cell wall, high methylesterified homogalacturonan domains produced by the endomembrane system undergo several modulations in the pattern and degree of methylesterification. The pectin methylesterases are responsible for the removal of methylesters in diverse patterns. Although rice contains about two fold more genes than Arabidopsis, it has 55 genes encoding pectin methylesterase (PME) while Arabidopsis genome contains 66 genes (Yokoyama and Nishitani, 2004). The size of the gene family encoding PMEs in Arabidopsis reflects the importance, diversity and complexity of the biological processes in which the gene family is involved. So far, the presence of the PME multi-gene family has limited the progress in the study of the function of single PME genes (Brummell and Harpster, 2001). Pectin methylesterases are members of the plant esterases family which were reported to contribute to cell wall construction and modulation (Willats *et al*., 2001a, b and c). Esterases could serve as a marker of organism development and growth of tissues and cell suspension cultures. When a cell was dying or had been damaged, the activity of these enzymes was decreasing or non existent (Vítećek *et al*., 2004). Pectin esterase (U227174) was one of the cell wall carbohydrate hydrolases that were induced upon salt treatment of tomato seeds (Zhou *et al*., 2007).

As rapidly dividing cells are limited in whole plants and are often difficult to manipulate (Pathirana and Eason, 2006), synchronized Arabidopsis suspension cultures were assessed as a model to approach the HG role in the adhesion of cells living in suspensions without the complications of development and differentiation. The first part of the project aimed at characterising the changes in the HG fraction in the middle lamellae during the culture cycle. Then, once a reasonably reproducible synchrony was achieved, the changes in the levels of PME transcripts along with the biochemical and imunohistochemical data were used to refine the impact of PME genes on the onset of adhesion through the cell cycle, and the

development of cellular junctions and intercellular spaces as the culture proceeded through its cycle.

The growth of Arabidopsis suspension cultures of both the high salt habituated HHS and non-habituated cell lines followed a classical growth curve, which clearly showed the active reproduction phase to last between days 2 and 6, followed by reduced or zero growth 7 days after subculturing. Only viable cells could contribute to the linear increase in the packed cell volume during the exponential phase. Regardless of the osmolarity of the growth medium, Arabidopsis cells growing in a suspension tend to grow as individual cells and in aggregates of variable sizes due to the non-separation and adhesion between daughter cells during cytokinesis. However, salt habituated cultures were clumpier and cell clusters were of larger size.

The extracellular matrix of cells growing in a suspension culture is typically present as primary cell walls. The middle lamellae are pectin rich and microfibril-free, and are widely considered to play a key role in cell-cell adhesion (Jarvis *et al*., 2003). A compositional analysis of the EDTA extract from normal cells showed that it was rich in components characteristic of a mixture of HG, RG-I, and possibly xylogalacturonan (XGA) and xyloglucan. The UA fraction accounted for about 50 to 60% of the total extracted sugar during the culture cycle of non-habituated cells. However in the HHS cell line, the UA content increased from 35% on day 2 to 68% on day 6. The degree of methylesterification (DM %) of the pectic extracts in the normal non-habituated cultures ranged between 40 to 60%. Recently, it was reported that the %DM limits cell growth, and that a minimum level of about 60% was required for normal cell elongation in Arabidopsis hypocotyls (Derbyshire *et al*., 2007). Accordingly, our wild type normal culture seemed to go through a process of cell elongation on day 6. In contrast, in the salt habituated cells, the DM% went down from 52 to 35% during the active division phase suggesting a minimum number of cells undergoing elongation, possibly due to the increased levels of adhesion between the cells. The observed changes in the %DM of the EDTA extracts were in agreement with the results reported for

tobacco suspension cells; the DM% of NaCl adapted cells was less than the control un-adapted cells (McCann *et al*., 1994).

The immunodot assay and immunolabelling techniques were used to have an overview of the changes in the pectic epitopes in the middle lamellae during the culture cycle. The JIM7 monoclonal antibody binds to pectin with DM % ranging between 15 -80% (Willats *et al*. 2000a and b). The abundance of JIM7 recognized epitopes in the EDTA extracts could suggest roles for methylesterified pectins in maintaining the adhesion between the cells in clusters, possibly through hydrophobic interactions between methoxy groups and hydrogen bonds between un-dissociated carboxyl and secondary alcohol groups. Highly esterified HGs do not form stiff gels and are thought to help the expanding walls to remain pliant (Cosgrove, 2005). A similar observation was recorded in other suspension cultures of tobacco and wild type Arabidopsis, where highly methylesterified pectins were abundant throughout the cell walls (Iwai *et al*., 2001; Leboeuf *et al*., 2005).

In both salt habituated and non-habituated cells, lower levels of the JIM5 recognised epitopes were detected. The JIM 5 antibody binds weakly to completely de-esterified pectin, and binding is greatly increased by the presence of methylesterified GalA residues up to a level of about 40% (Willats *et al.*, 2000a and b). The low-methoxyl homogalacturonan epitopes were detected in the middle lamellae, suggesting a possible connection via Ca^{+2} bridges. However, 2F4 monoclonal antibody could not detect calcium-binding sites forming egg boxes, thus dismissing the idea of a strong role of Ca^{+2} bound matrices in maintaining adhesion between cells. In an earlier report, 2F4 labelling was observed at tri-cellular junctions of mature wild type Arabidopsis suspension cells (Leboeuf *et al*., 2005). The LM7 non-blockwise de-esterified HGs were shown to exist in the EDTA extracts from salt habituated cells, possibly playing a role in maintaining adhesion between cells in a cluster. They were almost undetectable in the pectic extracts of normal non-habituated cells**.** The occurrence of the LM7 epitope at the tricellular junctions and the corners of the intercellular spaces were associated with the capacity of cells to withstand stress and stability of cell adhesion level.

Compared to pectins of non-habituated cell, the EDTA/phosphate extracts of salt habituated cells seemed to be more branched and hairy as revealed by the immunoprofiles using LM5 and LM6. The Rhamnogalacturonan I (RG-I) of the salt habituated cells seemed to be more highly substituted with polymers containing galactose. The presence of galactan side chains have often been related with cell wall rigidity and with firmer textures of tissues and organs (McCartney *et al*., 2000; McCartney and Knox, 2002). Both cultures were rich in arabinose. The high levels of arabinose residues were suggested to play a role in the intercellular attachment in the carrot embryogenic cultures, tobacco callus cells, and Arabidopsis suspension cultures (Satoh, 1998; Iwai *et al*., 2001).

It is at the cell plate that the initial cell adhesion events take place that result in formation of the primary wall between cells and the cementing middle lamella (Verma, 2001). Both the semiquantitative PCR analysis of cell cycle genes including CDKB2, CycD3 and H4, and the determination of mitotic index indicated that a reasonable synchrony was achieved by the aphidicolin block. The EDTA soluble sugars increased during cytokinesis, which could be related to the formation of pectin rich cell plate, the enrichment of the entire cell wall with pectin and /or changes in the parental cell walls. Similar data were reported previously in synchronous cultures of *Catharanthus roseus* (Amino *et al*., 1984). Higher levels of PME activity were observed during the cell cycle of the cultures of *Catharanthus roseus* (Liners and Van Cutsem, 1992).

According to antibody localization, strong reactions of both of low esterified JIM5 detected and highly methylesterified JIM7 detected HGs reflected the abundance of both epitopes during the cell cycle. However, only small amounts of the Golgi synthesized pectins reactive to the JIM7 antibody were located in the cell plates of maize root meristems ((Baluska *et al*., 2005). The internalization of HG pectins characterised by a low level (up to 40%) of methylesterification was reported in the meristematic cells of maize (*Zea mays*) and wheat (*Triticum aestivum*) root apices (Yu *et al*., 2002).

During the cell cycle, RG-I was increasingly substituted with arabinose while galactose was not detected. In carrot root apices, the actively dividing cells of the meristems contain high levels of (1→5)-α-arabinan. When carrot suspension culture cells were induced to elongate, the (1→5)-α-arabinan epitope was depleted from the cell walls of proliferating cells (Willats *et al*., 1999b). By contrast, the cell proliferation zones in Arabidopsis root apices lack the (1→5)-α-arabinan epitope. It was more abundant in elongated cells and occasionally at the root caps (Willats *et al*., 2001a).

The cell cycle related PMEs shared similarity with other identified PMEs like AtPME3 (At3g14310: Micheli *et al*., 1998), AtPMEI-1 and AtPMEI-2 (At3g17220, At1g48020 respectively: Raiola *et al*., 2004) and AcPMEI (Scognamiglio *et al*., 2003). The amino acid residues at the active site are highly conserved; two aspartic acids, arginine, two glutamines ad most of the aromatic acids lining the active site cleft. A phylogenetic analysis of the cell cycle related PME isoforms clearly distinguishes type I from type II genes. The results suggested that At4g12390 might function as an invertase inhibitor as it groups with the functionally characterized tobacco invertase inhibitor. The observed similarity of the N-terminal extension of At4g02330, At1g02810, At2g26440 and At2g47550 PME isoforms with PMEI supports the suggested inhibitory role of the PME pro-peptide region.

Transcript profiling shows that the expressions of many PMEs were fluctuating in the cell cycle and between the different synchronizations. Although no decisive conclusion could be drawn on the role of a particular isoform, the At5g47500 encoded isoform showed more reproducibility compared to the other cell cycle related PMEs. The levels of its messenger RNA (mRNA) were examined by Northern blot analysis. The expression levels of this gene were reduced during the S and G2 phase and increased again during the M and subsequent G1 phase. The detected degree of methylesterification of the extracted pectin increased slightly during the cell cycle ranging between 28 to 38%. However, pectins are thought to be deposited in a highly methylesterified form almost 75 to 80%, so it seems that

there is a rapid demethylation as the Golgi derived vesicles are docking their cargo at the growing cell plate.

As a summary, the results of the present work suggest that due to the lack of the tensile forces favouring the formation of intercellular spaces in growing seedlings, the cells of the wild type Arabidopsis suspension culture did not show appreciable presence of intercellular spaces, and tended to deposit highly methylesterified HG rich pectic network in primary cell walls, middle lamellae and cellular junctions which may play a role in the adhesion between cells possibly by forming hydrogen bridges and hydrophobic interactions. Calcium mediated cross linking of HG appears to play a minor role in the establishment of the adhesions between cells in clumps. The abundance of arabinan reflected the proliferating state of cells. However in the HHS Arabidopsis salt tolerant cell line, intercellular spaces were scarce, and the pectic network of cell walls, middle lamellae and cellular junctions appeared to contain higher levels of low esterified pectins possibly indicating a stronger role for calcium bridges maintaining the adhesion between the cells. The rhamnogalacturonan is highly branched decorated with galactan, arabinan and arabinogalactan possibly as a response to osmotic stress. Out of the five putative cell cycle expressed PMEs, only At5g47500 encoding a type I PME showed a systemic consistency in its expression during M phase, possibly attributing to the deesterification of the pectins as they are laid down in the site of developing cell plate.

Although the expression profiles do provide useful starting points some further work is needed to elucidate the substrate specifity, product and physiological role of At5g47500. Future work could involve knocking out the At5g47500 gene, synchronize salt tolerant Arabidopsis and study the expression of the PME isoforms in cells adapted to living in high osmolarity.

Chapter 7

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