Pectins: Cross-linking and Cell Adhesion in Developing Plants

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Dedication

For my mum and dad. Thanks. The moral (and more than once financial!) support has made it possible for me to come this far without becoming a nervous wreck. Even if you still have absolutely no idea what it is I did!

Declaration

Except where specific reference is made to other sources, the work presented in this thesis is the original work of the author. It has not been submitted, in part or in whole, for any other degree.

Susan Briggs

Acknowledgments

My supervisor, Dr Mike Jarvis. I can only hope that one day I will be able to give the appearance of knowing as much as you actually do! Thank you for all your help and encouragement.

I would also like to thank Nicola Stacey from the John Innes Centre and especially Michael Beglan in A, E & A Chemistry, Glasgow for all the technical support and for always being ready to share a joke. Thanks for putting up with me!

To everyone else in the lab especially Geraldine, Laura, Vicki and Christine. Thanks for crosswords, coffee breaks and the odd Friday night in the pub!

I would also like to acknowledge the unending support of all my friends. Although none of you had clue what I was up to, you were always ready to help me with a few beers to commiserate or celebrate. That sacrifice has not gone unnoticed!

And last but by no means least, for my husband-to-be! I cannot imagine having done this without you. It has to be a good sign for our future that the whole process didn't drive you mad and that consequently you kept me sane! Thank you for your love, support and unending patience. Roll on the wedding!

Summary

A great deal of the remarkable properties of plants lie in the complex nature of adhesion of cells which is unparalleled in modern materials science. The exact nature of the polymer network that results in this is still to be completely understood. A basic view of the plant cell wall has a cellulose microfibrillar network embedded in a highly crosslinked pectin matrix. It is suspected that it is these crosslinks that give rise to the adhesion between cells and it is the aim of this thesis to examine the molecular features responsible for inter cellular adhesion in plants and how these might vary within the cell and between cells.

A variety of cell separation techniques were utilised including the development of a multi-step pectin extraction procedure and investigation into the use of enzymes to break specific linkages in order to effect cell separation. These experiments confirmed that crosslinks between pectic polymers were responsible for cell adhesion as breaking them resulted in separation. These experiments also showed that the mechanism responsible for cell adhesion differed between faces of the individual cell and also between different types of cell.

The existence and potential role in cell adhesion for non-methyl esters was also investigated. From this work it was discovered that non-methyl intermolecular esters existed.

Studies that combined enzyme treatment with tensile strength testing equipment at the Institute of Food Research, Norwich endeavoured to test the strength of cell-cell adhesion and these discovered that this varied according to the structure of the polysaccharides in the wall.

Table of Contents

List of Abbreviations

Abbreviation	Meaning
AAS	Atomic absorption spectroscopy
ANOVA	Analysis of variance
CDTA	Cyclohexandiamine tetra-acetic acid
DF	Degrees of freedom
EtOH	Ethanol
Gal	Galactose
Gal*p*A	Galactopyranosyluronic acid
GalUA	Galacturonic acid
HG	Homogalacturonan
HRGPs	Hydroxyproline-rich glycoproteins
KDO	2-keto-3-deoxy-d-mannooctulosonic acid
LM	Light microscopy
LSD	Least significant difference
MCB	Mixed cation buffer
MHDP	meta-Hydroxydiphenyl
NMR	Nuclear magnetic resonance
PG	Polygalacturonase
RG I	Rhamnogalacturonan I
RG II	Rhamnogalacturonan II
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UV	Ultra violet
XG	Xyloglucan
Xyl	Xylose

Chapter One

Introduction

1.1 Introduction

The cell walls of higher plants perform many important roles. The wall provides the cell with shape, as without its presence turgor pressure from within would force the cell to be spherical. The structural strength that allows the plant to take on a distinct, specific form is also a product of the cell wall and where a cell wall is absent, eg cork cells, a spherical shape is adopted. The wall also provides protection from pathogens, fungi, etc. (Bowles, 1990) and regulates the permeability of the cell by acting as a sieve excluding large proteins but allowing the entry of sugars, amino acids and hormones into the cell (Bacic et al., 1988).

The structure of the cell wall can comprise up to three layers: the primary cell wall, secondary wall and the middle lamella. Primary walls are those in which the long cellulose chains known as microfibrils were laid down when the wall was still capable of growing and are generally around 0.1 μm thick. Microfibrils that are deposited after the cell had stopped growing form the basis of the secondary cell wall which may be between 10-20 μm thick and provide additional mechanical support. A polysaccharide matrix surrounds the cellulose microfibrils in both walls though this varies in composition between primary

and secondary walls. The middle lamella is responsible for the adhesion between neighbouring primary walls and is the weakest point and consequently is that which is most intimately involved in cell separation (Ginzburg, 1958). It contains no microfibrils (Fry, 1999). Although described here as belonging to three separate layers the pectic matrix is continuous across the walls and middle lamella.

1.2 Cell Wall Components

1.2.1 Cellulose

The most abundant carbohydrate polymer in nature, cellulose consists of thousands of β(1,4) linked D-glucose units at alternating 180° angles (figure 1.1) (Marx-Figini, 1982; Timpa, 1991) forming a flat ribbon-like structure. These bond to form semi-crystalline filaments known as microfibrils, which are typically 4-10 nm thick and roughly 30 nm apart. The microfibrils lie parallel to each other and encircle the cell, and their inflexible nature dictates the direction in which future expansion of the cell can occur.

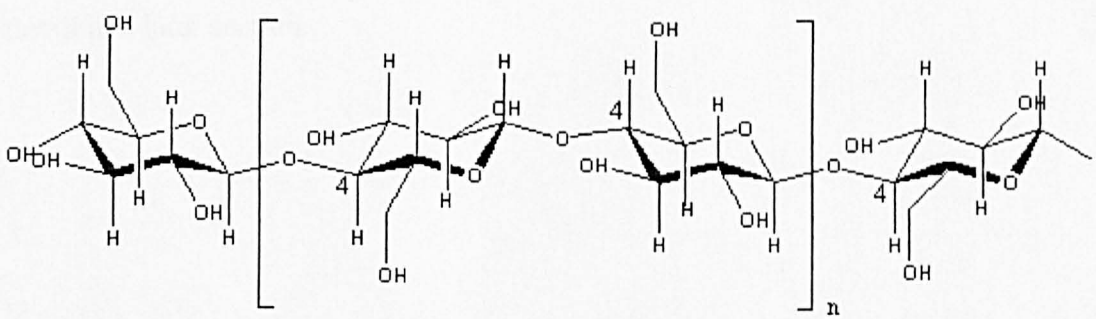


Figure 1.1 Structure of Cellulose

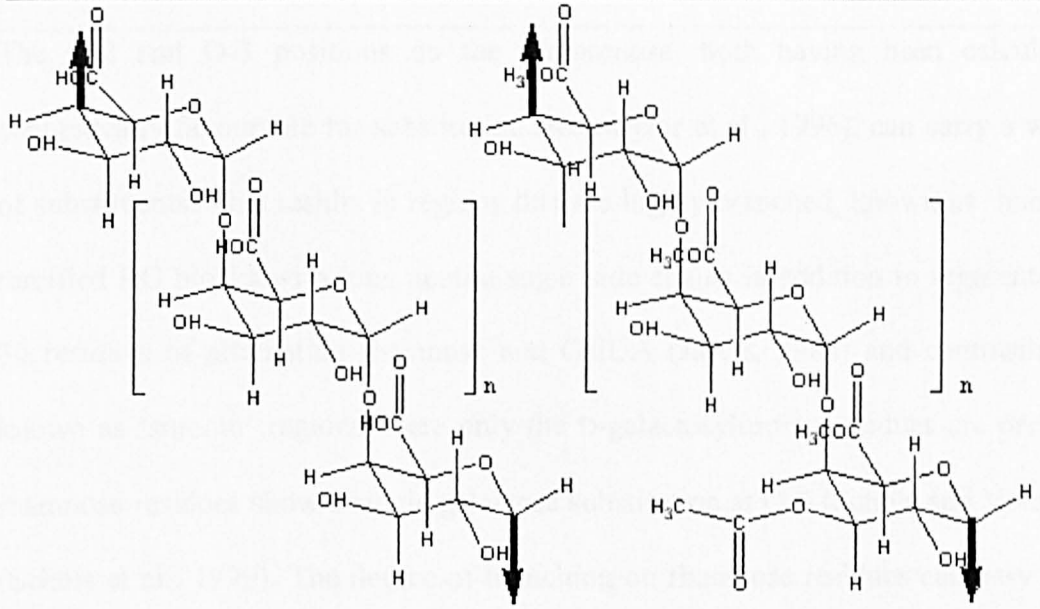
1.2.2 Pectic Substances

Commercially, pectins are widely used as a gelling agent in jams, jellies and confectionery and also in the stabilisation of acidified dairy-based drinks. In the human diet they form the important 'dietary fibre' group (Voragen, et al., 1995). In addition, many health benefits

have been suggested including anti-diarrhoea, anti-ulcer and anti-nephritic activity, the possible lowering of cholesterol, influencing glucose metabolism and also functioning as a detoxicant (Yamada, 1996; Yamada, 2000) and references in Voragen *et al* (1995). They also reportedly regulate and protect the gastrointestinal tract (Endress, 1991).

1.2.2.1 Homogalacturonan

This polymer consists solely of α-(1,4)-D-galacturonic acid residues which may be esterified. These are most commonly methyl-esters although the presence of acetyl esters at O-2 or O-3 depending on the plant material it originates from (O'Neill, et al., 1990; Voragen et al., 1995) has also been confirmed. Non-methyl galacturonoyl esters on homogalacturonan may cross-link the polymer either intramolecularly or with other wall polymers. The distribution of esterified galacturonic acid has been found to be non-random (Daas et al., 2000a; Daas et al., 2000b; De Vries, 1988; Kravtchenko et al., 1993; Mort et al., 1993) but the extent to which it is observed is highly variable. The unesterified homogalacturonan, found mainly in the middle lamella (Vreeland, et al., 1989), is capable of forming both inter- and intra-chain crosslinks with Ca^{2+}. This is discussed in greater detail in a later section.



(a) (b)

Figure 1.2 Structure of homogalacturonan. Figure a – basic repeating unit of 1,4-linked α-D-galactopyranosluronic acid (Gal*p*A), figure b shows methyl esterified site and an acetyl ester

Adapted from O' Neill, Malcolm A; Darvill, Alan G and Albersheim, Peter (April 1999) Pectic Substances. In: Encyclopedia of Life Sciences. London: Nature Publishing Group. http://www.els.net/

1.2.2.2 Rhamnogalacturonan I

Initially identified by Albersheim's group (McNeil et al., 1980; McNeil et al., 1984), rhamnogalacturonan has a highly complex structure. The name rhamnogalacturonan I covers a highly diverse group, regulated in its distribution within cell walls and between cells (Willats et al., 2001a). There is evidence emerging implicating RG I in a variety of different functions. The backbone consists of α(1→4) linked D-galacturonic acid and α(1→2) linked L-rhamnose (Lau et al., 1985) residues in repeating regions of around 100 - 300 units long (Albersheim et al., 1996) with the precise relationship to homogalacturonan depending on the species that the polymer originates from.

The O-2 and O-3 positions on the L-rhamnose, both having been calculated to be energetically favourable for substitution (Kouwijzer et al., 1996), can carry a wide variety of substituents. This results in regions that are highly branched, known as 'hairy' regions, ramified RG blocks with long neutral sugar side chains in addition to segments of around 30 residues of alternating rhamnose and GalUA (Jarvis, 1984) and contrasting sections known as 'smooth' regions. Here only the D-galactosyluronic residues are present. Some rhamnose residues show a single galactose substitution at O-4 (Schols and Voragen, 1994) (Schols et al., 1990). The degree of branching on rhamnose residues can vary from 20%-80% depending on the origin of the plant material and developmental state (De Vries, 1988; De Vries et al., 1982). No evidence exists to suggest that the Gal*p* residues in RG I are methyl esterified as those in homogalacturonan are (O'Neill, et al., 1990). The length of the side chains varies between 1 and 50 and these consist of arabinose, galactose or both. The side-chains of RGI in some plants may be esterified with phenolic acids, such as ferulic acid (Ishii, 1997). Despite the ambiguity in the precise location of substituents, the sugar residues of the backbone and side chains are not thought to be randomly distributed. However enzymic degradation of RG reveals unequal amounts of rhamnose and GalUA with a ratio that ranges from 0.05 to 1 (Schols and Voragen, 1996) but it has not been shown that more than one GalUA can be found between two rhamnose residues in the backbone. It must therefore be concluded that the extra GalUA are remnants of HG that cannot be degraded enzymically.

As previously stated, the precise structure of pectin in any individual plant species varies and summaries of the details specific for some plants can be found in the paper by Schols and Voragen (1996).

1.2.2.3 Arabinans

Arabinans (figure 1.3b) have α(1,5)- and α(1,3)-linkages. Further information and structural reviews are available from Beldman et al., (1997) and Whitaker (1984). They may also occur as sidechains of arabinogalactans.

1.2.2.4 Galactans

There are found in a broad range of higher plants (Clarke et al., 1979; Fincher, et al., 1983). An experiment with cultured carrot cells that had been induced to elongate showed galactan production to have been upregulated before any changes in the size of the cell which may indicate the requirement of a galactan epitope as necessary for cell elongation (Willats, et al., 1999b). The predominant type I galactans are β(1,4)-linked.

1.2.2.5 Arabinogalactans

These were classified as type I and II and are present as side chains or single polymers. Type II is found as a side chain of arabinogalactan protein.

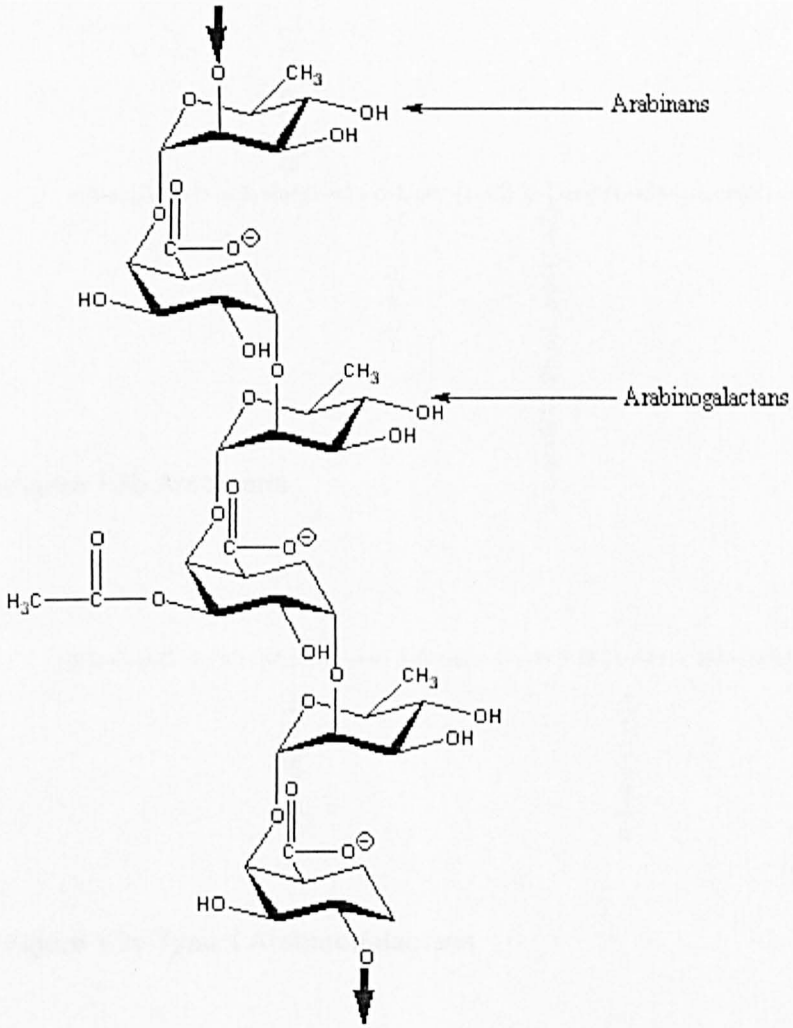


Figure 1.3 Structure of Rhamnogalacturonan I. One third of GalUA units are acetylated at secondary alcohols (Komalavilos and Mort, 1989).

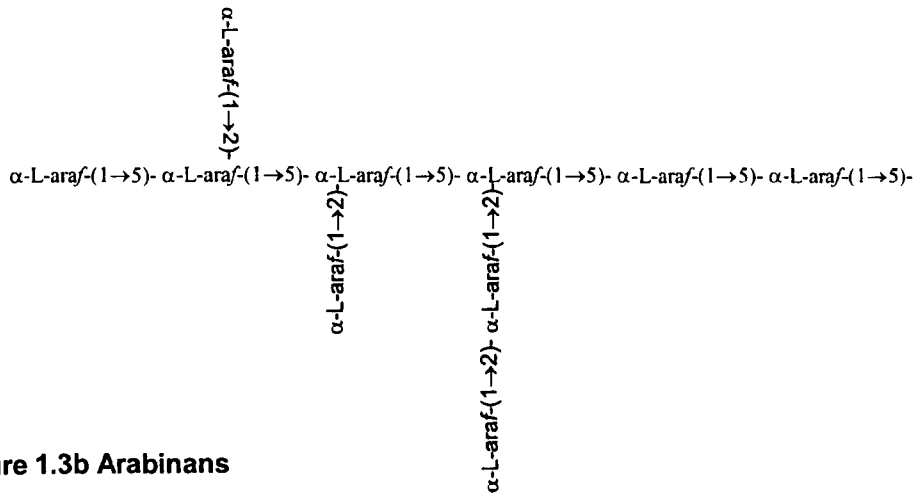


Figure 1.3b Arabinans

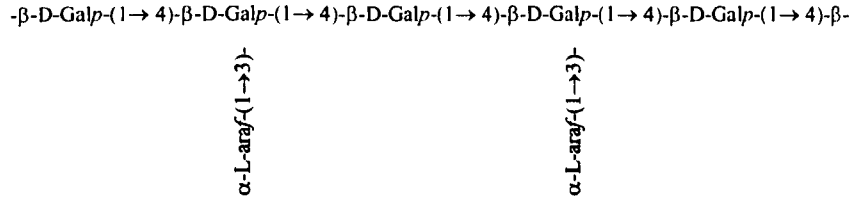


Figure 1.3c Type 1 Arabinogalactans

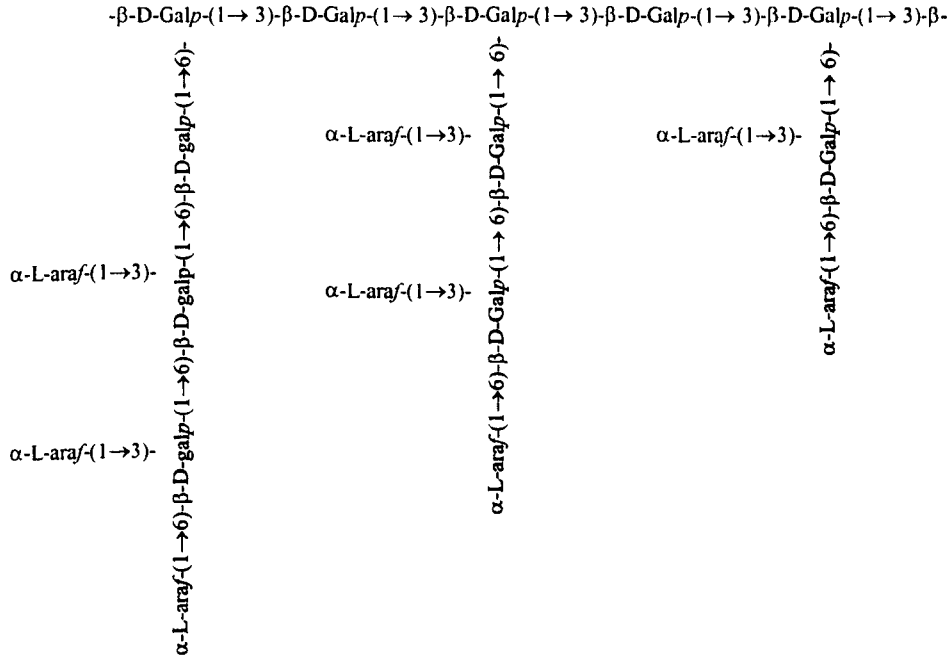


Figure 1.3d Type II Arabinogalactans

These three types of side group attach to about half of the rhamnosyl units of RG I

1.2.2.6 Rhamnogalacturonan II

First isolated in 1983 (Spellman, et al., 1983) from suspension cultured sycamore cells, RG II differs from the other two major pectic polysaccharides, which are heterogeneous in composition, in its highly conserved structure. The backbone is known to contain at least seven 1,4-linked α-D-Gal*p*A onto which four side chains are attached, although the precise positions of these in relation to each other has yet to be determined.

Although only a minor component of the cell wall, RG II is interesting as it exhibits two unusual features: the presence of the sugars apiose and 2-keto-3-deoxy-D-mannooctulosonic acid (KDO), and its ability to form crosslinks with boron through the apiose residues (Pellerin, et al., 1996; Vidal, et al., 2000) which are required for growth (O'Neill, et al., 2001) and will be discussed in a future section.

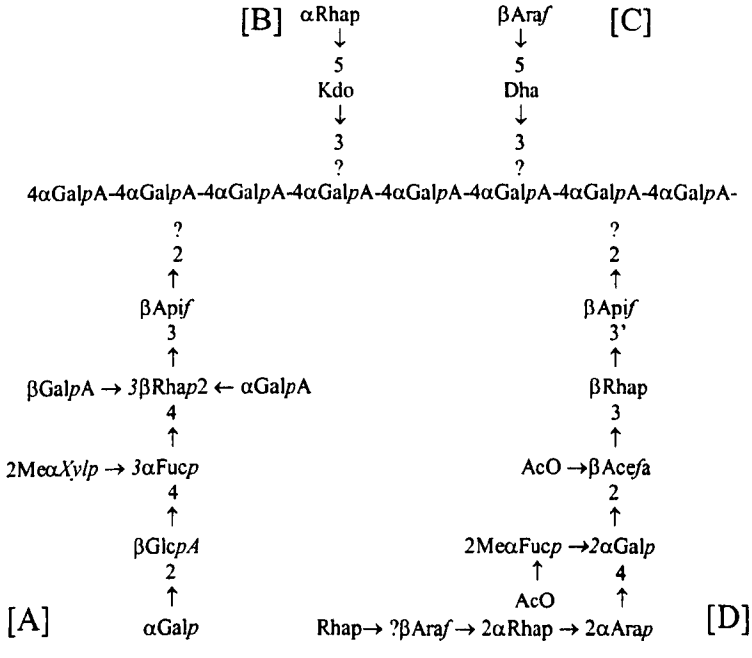


Figure 1.4 The structure of Rhamnogalacturonan II. Although the structure of the side-chains (A – D) are known, their positions relative to each other have not yet been determined. Oac, acetyl; Me, O-Methyl ether.

RG II has only been observed to occur in the primary cell wall and not the middle lamella (Williams, et al., 1996; Matoh, et al., 1998).

It has been proposed that RG II may be involved in the regulation of wall porosity (Fleischer, et al., 1999) and thickness (Ishii, et al., 2001).

1.2.2.7 Xylogalacturonans

Work with rhamnogalacturonan degrading enzymes on apple pectins has allowed the isolation of polymeric xylogalacturonan. This has an HG backbone and xylose side chains at the O-3 position. The level and precise pattern of substitution, again, varies with the origin of the plant tissue (Kikuchi, et al., 1996; Redgwell and Hansen, 2000; Renard, et al., 1997; Schols, et al., 1995; Weightman, et al., 1994).

1.2.3 Hemicellulose

Hemicelluloses, also known as cross-linking glycans, are defined as those polysaccharides with a 1,4-linked-D-pyranosyl residue with O-4 in the equatorial position, such as glucose, mannose and xylose. They are structurally similar to cellulose and this allows a strong non-covalent association with cellulose. Examples of hemicellulosic polysaccharides are xyloglucan, xylan, glucuronoxylan, arabinoxylan, mannan, glucomannan and galactoglucomannan.

1.2.3.1 Xyloglucan

The main hemicellulosic polysaccharide in the primary walls of dicots and non-graminaceous monocots, xyloglucan can be seen to be structurally similar to cellulose. This similarity may explain the decrease in diameter of the microfibrils when Acetobacter

xylinum - a cellulose synthesising bacterium - is grown in the presence of xyloglucan. This implies that xyloglucan is competing with the cellulose glucan chains in vitro during the synthesis and assembly of microfibrils (Atalla, et al., 1993). Thus microfibril assembly can be influenced by the presence of xyloglucan.

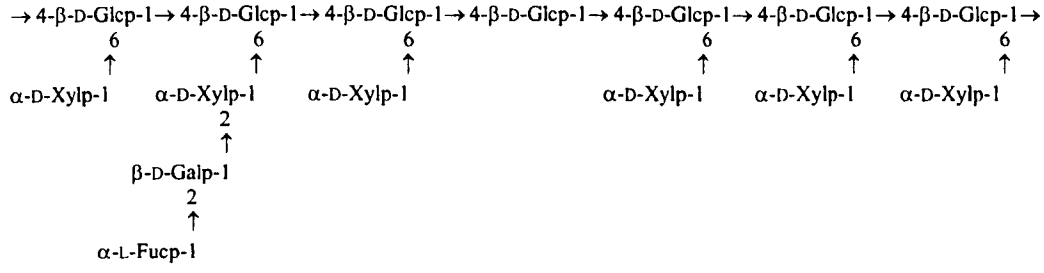


Figure 1.5 Example of the structure of xyloglucan adapted from Morrison, Ian M (July, 2000) Polysaccharides: Plant Noncellulosic. In: Encyclopedia of Life Sciences. London: Nature Publishing Group. http://www.els.net/

Both cellulose and xyloglucan have the same glucan backbone which most likely adopts a flattened 2_1 helix (Ogawa, et al., 1990; Taylor and Atkins, 1986) but unlike cellulose, up to 75% of these residues are substituted at the O-6 position with mono-, di-, or triglycosyl side chains in xyloglucan.

The conformation and pattern and degree of side-chain substitution affects the ability of the polymer to bind to cellulose. The terminal xylose residue and Fuc-Gal-Xyl side chains may fold over onto one face of the chain leaving the other face free for cellulose binding (Keegstra, et al., 1973; Levy, et al., 1997). However Gal-Xyl sidechains have been reported to sterically hinder the xyloglucan backbone and reduce binding to cellulose (Levy, et al., 1997).

The precise structure varies depending on the species it originates from, e.g. there is no fucose in the xyloglucans of Solanaceous plants like tobacco and tomato (York, et al.,

1996). They are thought to play an important role in the cohesion of the cell wall by tethering neighbouring microfibrils through hydrogen-bonding. This will be covered in more detail in a later section.

1.2.3.2 Xylans

Xylan-based polysaccharides are amongst the most abundant class of hemicellulose in the cell wall. They can be found as arabinoxylans, glucuronoxylans and arabinoglucuronoxylans with more than 50% of glucuronic acid residues being present as the 4-O-methyl ether. Glucuronic acid residues attached to the O-2 position on the xylose are present in the pyranose form whereas arabinose residues attach to O-3 and are in the furanose conformation.

Xylans are the principal non-cellulosic polysaccharide in the secondary cell wall of dicots while in grasses and cereals they are also abundant in primary cell walls.

1.2.3.3 Mannans, Glucomannans and Galactoglucomannans

Although mannans have been reported, their existence as a homogeneous polymer in the cell wall can be called into question due to the severity of the conditions required to isolate them. Most are present as glucomannans, present in the endosperm of seeds, and galactoglucomannans which are the major non-cellulosic polysaccharide to be found in the secondary cell wall of gymnosperms.

As can be seen in figure 1.6 the backbone of the structure consists of mannose and glucose residues connected by β-1→4 linkages with D-galactopyranose residues randomly attached onto the mannose residues that predominate. A proportion of O-acetylated groups are also evident.

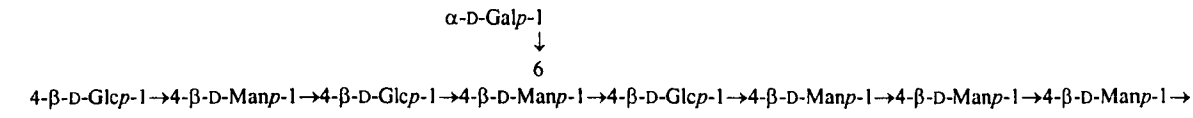


Figure 1.6 A typical galactoglucomannan

1.2.4 Structural Proteins

A further component of the cell wall that contributes to the overall structure are proteins. These have been comprehensively reviewed elsewhere (Showalter, 1993; Showalter, 2001) although they will be summarised here for completeness.

1.2.4.1 Extensin and Other Hydroxyproline rich Proteins

Hydroxyproline-rich glycoproteins (HRGPs) were the first family of proteins identified in the cell wall. Initially it was thought that they functioned in such as way as to facilitate growth, but more recent evidence suggests that either their role is in wall rigidification or slowing cell expansion (Kivirikko, et al., 1992; Cassab, 1998; Showalter 1993, 2001).

Examination of extensin under the electron microscope reveals a rod-like structure. This is composed of hydroxyproline residues, some glycosylated with one to four arabinosyl residues, and also the amino acids tyrosine, lysine, histidine and valine. They are considered to be structural proteins which may also function in wound healing, plant defence and development (Kivirikko et al 1992; Showalter 1993; Cassab 1998)

It has previously been postulated that cross-linking between extensin and some wall carbohydrates, especially pectin, may be possible. The 'warp-weft' model suggested the existence of covalent cross-links between structural proteins where the lattice of extensins hold microfibrils in place (Lamport, 1986). Biochemical evidence for this is now becoming apparent. The possibility of an ionic linkage between the protonated form of extensin –

specifically a positively charged lysine residue - and the negatively charged uronic acid of pectin may be regulated by changes in the cell wall pH and Ca^{2+} levels.

1.2.4.2 Solanaceous Lectins

Another group of proteins that resemble the extensin family is the solanaceous lectins. This is due to their serine, arabinose and hydroxyproline content, their extracellular location and the presence of identical carbohydrate-protein linkages. Various roles have been proposed including cell-cell interaction, sugar transport, stabilisation of seed storage proteins and control of cell division (Kivirikko et al 1992; Showalter 1993; Cassab 1998)

1.2.4.3 Glycine-Rich Proteins

This family is characterised by a repetitive primary structure consisting of up to 70% glycine. Glycine-rich proteins that exist in the cell wall are normally located in the vascular bundles, especially xylem tissue, tissue that will become lignified.

1.2.4.4 Arabinogalactan Proteins

As the name suggests arabinose and galactose are the predominant carbohydrates in these proteins. Analysis of the structure has indicated the presence of (1,3) β-D-galactopyranose backbone and (1,6) β-D-galactopyranose side chains. They are very soluble and have a high although variable degree of glycosylation which is reflected in the range of molecular weights found. Suggested functions include cell-cell recognition, wound healing and plant defence (Kivirikko et al 1992; Showalter 1993, 2001; Cassab 1998)

1.3 Crosslinking within the Cell Wall

The cross-linking of pectin within the middle lamella is essential for adhesion between neighbouring cells. The degree of cell adhesion is a controlling factor for common processes in plant development such as abscission and ripening and this is controlled partly through pectin modifying enzyme processes.

1.3.1 Covalent Linkages

An early model of the structure of the cell wall proposed that covalent linkages exist between pectin and xyloglucan and extensin. Keegstra, et al. (1973) and Monro, et al. (1976) questioned this and proposed non-covalent interactions. They attempted clarification of the possible covalent interaction but were unable to obtain large quantities of xyloglucan linked to pectin. However it was suggested that a possible warm-alkali-labile covalent link might exist between extensin and hemicellulose. A xyloglucan-pectin interaction been identified by Thompson and Fry (2000) (discussed in more detail 1.3.1.3), but an extremely specific method of cleaving such a link would be required for its full characterisation.

Recently proposed models of the primary cell wall (Mccann and Roberts, 1991; Carpita and Gibeaut, 1993) suggest that covalent cross-links between the pectic and cellulose-xyloglucan networks do not exist or do not make any mention of them as a possibility, as there is no agreement on whether they exist or what form such linkages might take.

The nature of many covalent interactions which might possibly exist remains unknown. However, some are less unclear.

1.3.1.1 Ester and Amide Cross-links

Ester links are known to exist between the carboxyl groups of some GalUA to other polysaccharides (Fry, 1986). The evidence for this originates from the extractability of some pectins by Na_2CO_3 (Selvendran, 1985) which are not solubilised by chelators. A Na_2CO_3 treatment may cleave ester links, although this has never been directly demonstrated: while such bonds are, in theory, quite labile, they are not easily isolated intact. Oligomers which contain methyl esters of GalUA can be isolated relatively easily.

Maness et al. 1990 optimised the conditions required for the reduction of GalUA esters to galactose and compared GalUA and galactose levels before and after reduction. This allowed them to estimate the degree of esterification of the GalUA carboxyl group. This method was modified and applied to maize pectin (Kim and Carpita, 1992) and it was found that up to one third of the esters present were non-methyl. Further studies into the existence of non-methyl esters estimated them to occur at around 5% in spinach (Brown and Fry, 1993) and up to 60% in tobacco-culture (McCann, et al., 1994; MacKinnon, et al., 2002).

1.3.1.2 Boron

Kobayashi, et al., (1996) described a room temperature, 30-minute 0.1 M HCl treatment which cleaved two RG II residues. These are conditions which should not disrupt a glycosidic bond. The link could be regenerated by incubation in pH 3.5 phthalate buffer with 15 mM Boric Acid for three days at room temperature; this would appear to implicate boron as an essential factor in the linking of two RG II monomers. This has been confirmed and the specific details of the cross-link are known. As mentioned previously RG II, rather unusually, contains the sugar apiose and it is through this that the esters with boron form, resulting in a 1:2 borate-diol ester (O'Neill, et al., 1996). The structure of RG

II is highly conserved in vascular plants which may be explained by the distinct structural requirements for dimer formation, a feature suggested by the specificity and cation dependence of this type of cross-link (Matoh and Kobayashi, 1998). The boron requirement and pectin content of the walls in many plants are correlated (Hu, et al., 1996; Brown, P H and Hu, 1997; Matoh, et al., 1996). This cross-link is influential in the pore size of pectin-rich cell walls (Fleischer, et al., 1999).

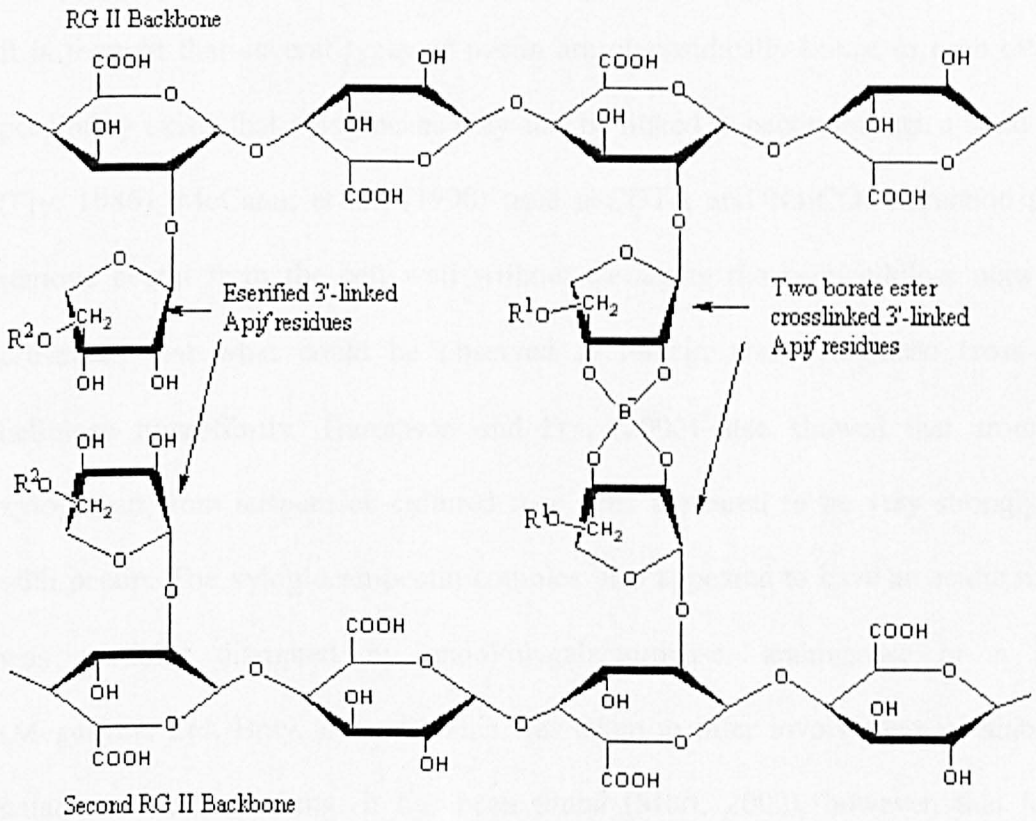


Figure 1.6 Crosslinking of two RG II molecules with a borate ester. The borate ester is believed to be crosslinked to th Api*f* residue in each of the two 2-O-Me-Xyl-containing side-chains (R_1 – see A in figure 1.4) but not the Api*f* residue in each of the two aceric acid-containing side chains (R_r – see D in figure 1.4).

1.3.1.3 Glycosidic Bonds

A bond of this sort can theoretically form through the reducing terminus of a polysaccharide to the non-reducing residue of another polymer. However, as each polysaccharide has only one reducing terminus, the resulting structure will be basically linear and not consistent with the three-dimensional network which might be expected if true crosslinking could be achieved.

It is thought that several types of pectin are glycosidically linked to each other, and the possibility exists that xyloglucans may also be linked to pectin through a bond of this type (Fry, 1986). McCann, et al., (1990) used a CDTA and Na_2CO_3 extraction sequence to remove pectin from the cell wall without damaging the hemicellulose network. It was presumed that what could be observed to remain was xyloglucan cross-linking the cellulose microfibrils. Thompson and Fry, (2000) also showed that around 30% of xyloglucan from suspension-cultured rose cells appeared to be very strongly associated with pectin. The xyloglucan-pectin complex also appeared to have an acidic nature which was partially disrupted by endoPolygalacturonase, arabinanase or a galactanase (Megazyme Ltd, Bray, Ireland). This was taken to infer involvement of arabinan and/or galactan in cross-linking. It has been found (Mort, 2002), however, that Megazyme's Arabinanase and Galactanase contain a small amount of endoxyloglucanase which will release xyloglucan fragments from a xyloglucan-pectin complex. Thus good evidence for such a cross-link in rose cell walls does exist but the exact details are as yet unknown.

A similar experiment (Mort, 2002) digesting cotton suspension-cultured cell walls with *endo*-polygalacturonase solubilised one quarter of their weight but only one eighth of the rhamnose. Therefore most of the RG I (the so called *hairy region*) of pectin is not released by cleavage of the majority of homogalacturonan. A cellulase treatment following on from the enzyme treatment solubilised the rhamnogalacturonan and the xyloglucan. Cellulase

will degrade xyloglucan so digestion of cellulose and xylogalacturonan will release rhamnogalacturonan from the cell walls.. The cellulase step can also be performed using 24 % KOH as an alternative (El Rassiz, et al., 1991). In that case chromatography indicated a link between some of the xylogalacturonan and rhamnogalacturonan.

1.3.1.4 Phenolic Coupling

Dimerization of various phenolics in the cell wall may also cross-link wall polymers. Tyrosine residues of proteins can become isodityrosine and feruloyl esters can form several different varieties of diferulate. The only known example so far of the successful isolation and characterisation of a cross-linked polymer fragment in the cell wall is a diferulic acid bridging two trisaccharides (Ishii, 1991).

1.3.2 Non-covalent Cross-links

Cell wall models generally stress the importance and occurrence of non-covalent interactions between polymers (Carpita and Gibeaut, 1993; Varner and Lin, 1989; Talbott and Ray, 1992) and present the pectin, structural protein and xyloglucan-cellulose domains as distinct but interacting networks.

1.3.2.1 Ionic Bonds

Divalent cations - most commonly Ca^{2+}, although Mg^{2+} can also act in a similar manner - can cross link the anti-parallel negatively charged homogalaturonan polymers. The cross-linked aggregates are known as junction zones and are shown in figure 1.7 (Rees, 1977). When many chains line up, the resulting structure became known as the 'egg-box' model (Powell, et al., 1982). The levels of methyl esterification may remain high so it is possible that a second type of gel will form with the highly esterified chains of homogalacturonan running parallel to each other (Morris, et al., 1980; Walkinshaw and Arnott, 1981).

Alternatively, a compromise can be made and a structure containing both the highly esterified acid-ester gel and the Ca^{2+} junction zones could form (Gidley, et al., 1980). In recent models pectins are shown either interacting in this way or unlinked (McCann and Roberts, 1991). In a few incidences, removal of these cations with chelating agents has been observed to result in cell separation.

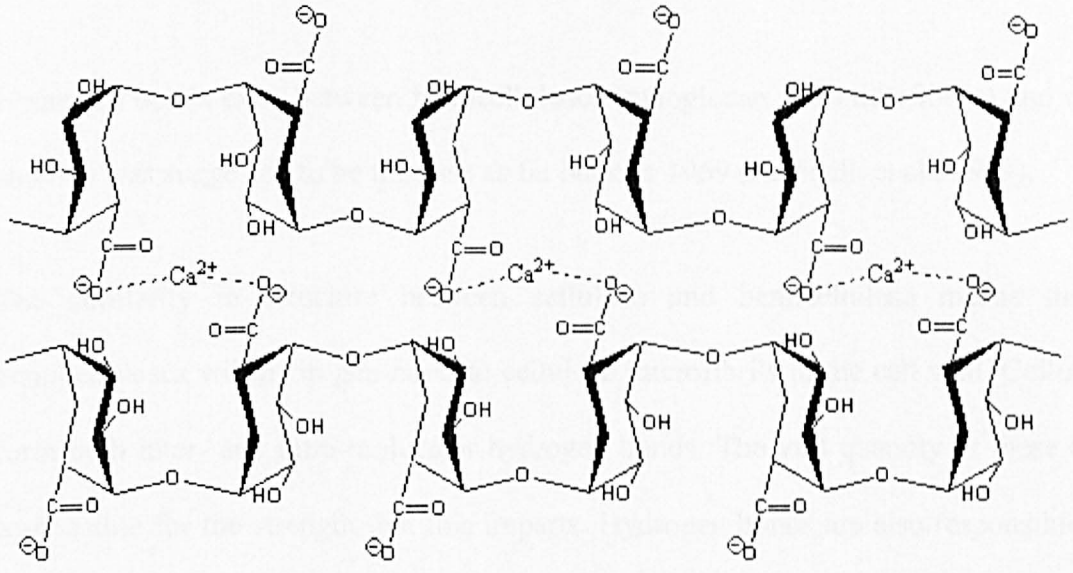


Figure 1.7 Ca^{2+} cross-links unesterified HG chains. The 'egg box' structure.

Amino groups and carboxyl groups can also form ionic bonds: for example, the lysine residue on extensin and the galacturonate in pectin.

$R—NH_3^+ \ ^-OOC—R$

There have been reports of pectin-protein links (Monro, et al., 1976). Previously Mort (1978) and O'Neill, later in 1980 (O'Neill and Selvendran, 1980) found that after the removal of 80% of the pectin, oxidation with acidic sodium chlorite solubilised extensin and pectin. The pectin and one third of the protein could not be separated by any method that was tested. Later experiments on runner bean pod parenchyma yielded similar results (Ryden and Selvendran, 1990) but a smaller proportion of extensin appeared to be linked to pectin. The poor solubility and low percentage of extensin-pectin fraction would seem to

imply a linkage via a polyphenolic that is not destroyed by chlorite. The link has been proposed to be protein-protein or protein-phenolic-protein (Qi, et al., 1995; Monro, et al., 1976). The stability to HF at -73 °C rules out the possibility of a linear or branched arabinan cross-link (Mort, 1978).

1.3.2.2 Hydrogen Bonds

Hydrogen bonds exist between hemicelluloses (xyloglucan and microfibrils) and cellulose and this was suggested to be the case as far back as 1969 (Aspinall, et al., 1969).

The similarity in structure between cellulose and hemicellulose means that most hemicelluloses will hydrogen bond to cellulose microfibrils in the cell wall. Cellulose can form both inter- and intra-molecular hydrogen bonds. The vast quantity of these bonds is responsible for the strength that this imparts. Hydrogen bonds are also responsible for the 'tethering' of cellulose microfibrils by xyloglucan, the fibres of which are generally ten times as long as the spacing between the microfibrils to which it binds allowing the xyloglucan to coat the microfibrils as well as cross-linking them (Hayashi, 1989). Electron microscopy has also confirmed this cross-linking behaviour (McCann, et al., 1990).

The cellulose/xyloglucan association occurs at or near the site of cellulose synthesis (Atalla, et al., 1993) and the binding is specific and unaffected by the presence of any other cell wall glucans, pectin or arabinogalactan (Hayashi, et al., 1987). Removing the pectin increases the pore size of the wall but does not affect the length or abundance of the cross-links (McCann, et al., 1990).

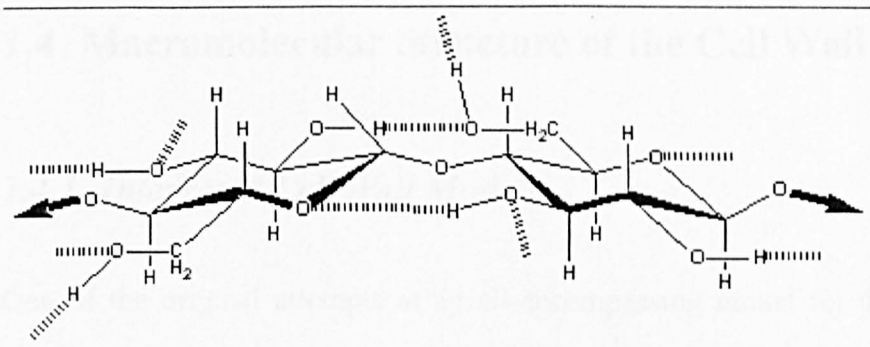


Figure 1.8 Cellobiose unit of cellulose showing possible hydrogen-bonding sites

1.4 Macromolecular Structure of the Cell Wall

1.4.1 Integrated Cell Wall Models

One of the original attempts at an all-encompassing model for the cell wall emerged in 1973 from Albersheim's group (Keegstra, et al., 1973). They proposed that the cell wall polymers were interconnected, forming an extensive network similar in construction to, if less complex than, the peptidoglycan of bacterial cell walls. The model tried to piece together the current knowledge and relate polysaccharides and proteins and what was known of their interactions. Unfortunately they relied on many assumptions that were unsupported by the data available and were recently proved incorrect. The effect of the model was, perhaps just as importantly, to stimulate wider interest in the area.

A few years later, work by Monro *et al* (1976) presented a less detailed but perhaps more accurate model. They summarised the inconsistencies in the first model with the results of their own work.

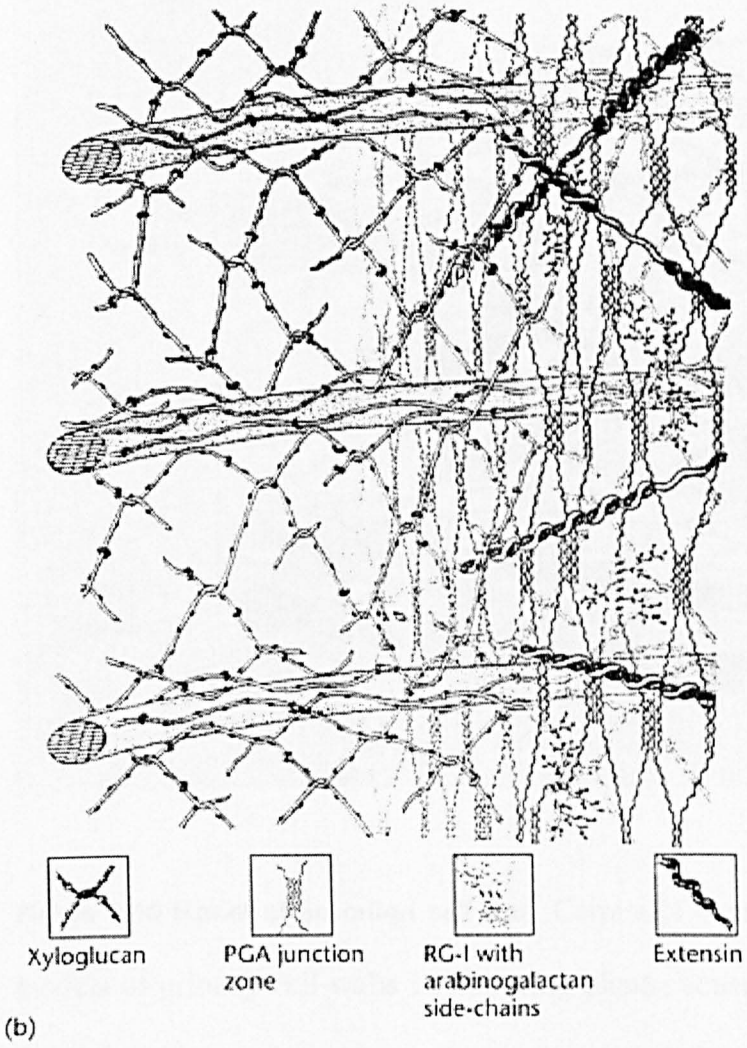


Figure 1.9 Model of a type I cell wall. Carpita N C and Gibeaut D M (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls growing growth. The Plant Journal 3(1), 1-30.

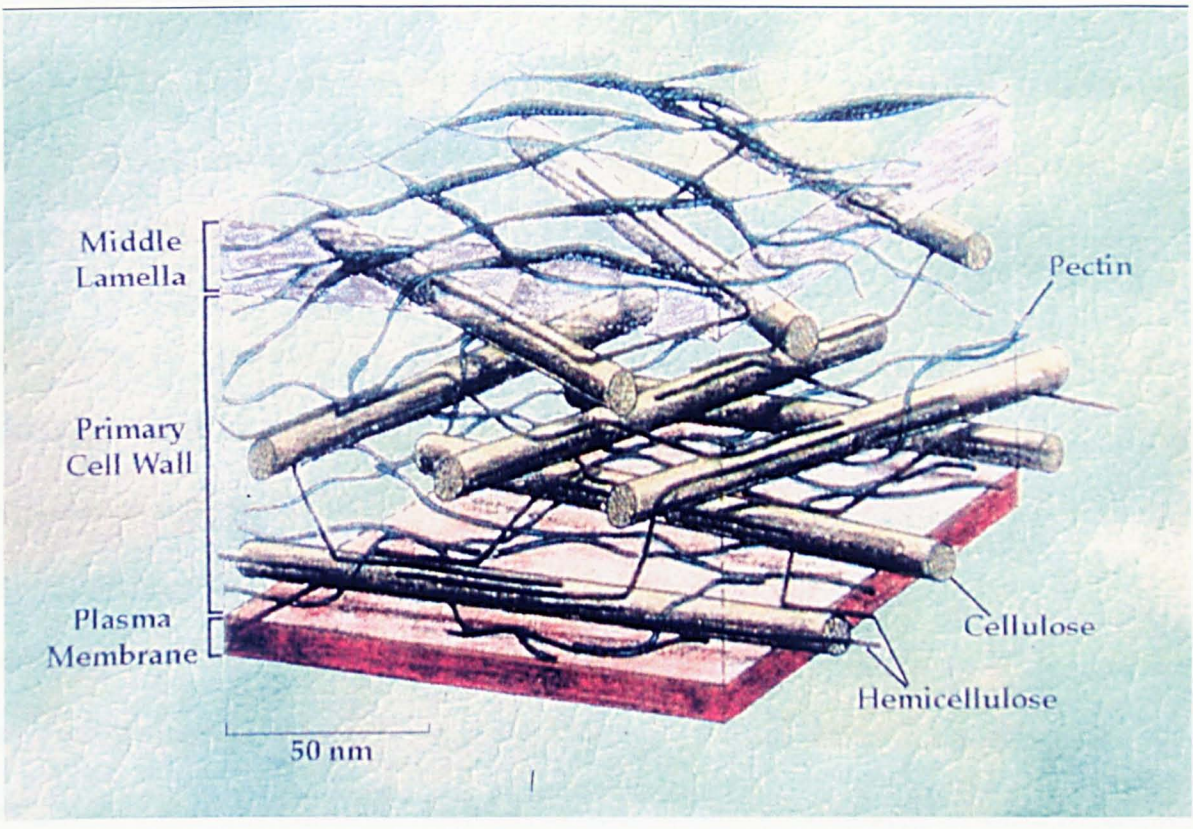


Figure 1.10 Model of an onion cell wall. Carpita N C and Gibeaut D M (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls growing growth. The Plant Journal 3(1), 1-30.

1.5 Origin of the Cell Wall

1.5.1 Transverse

Transverse and longitudinal walls have different developmental origins. The material in longitudinal walls is deposited as a result of cell elongation and transverse walls are initially formed as a division of the cell.

The life-cycle of the plant cell can be broken down into three main stages: Interphase, Mitosis and Cytokinesis. The 'interphase' period, responsible for cell growth and the

duplication of chromosomes in preparation for cell division, is further sub-divided into the G1, S and G2. During stage G1 the cell grows, stage S also sees growth and replication of the DNA contained in the chromosomes, and finally during G2 there is final growth and preparation for division.

Mitosis of the cell spans four stages: prophase, metaphase, anaphase and telophase. Over the course of these four stages the nucleus and chromosomes will divide equally to be allocated to their newly forming daughter cells. The first stage, prophase, consists of the initial development of the mitotic spindle. The spindle is composed of microfibrils and associated proteins that originate in the microtubules of the cytoskeleton. During metaphase/anaphase the cell elongates. This stage of growth is concentrated in the longitudinal walls and is dealt with more fully in the following section. The final stage of mitosis is termed telophase. During this the remnants of the spindle, now renamed the phragmoplast, will provide the basis for the newly forming wall. The spindle fibres, renamed phragmoplastic fibres, guide vesicles from the Golgi to the interzone of the phragmoplast. These vesicles contain polysaccharides for the new wall and eventually fuse to form the cell plate, which is surrounded by a double membrane and will eventually join with the existing plasma membrane. The pectin rich cell plate will eventually become the middle lamella between adjoining cells. Remnants of Endoplasmic Reticulum (ER) tubules trapped in the cell plate and new wall create holes called plasmodesmata that connect the cytoplasm of adjacent cells.

1.5.2 Longitudinal walls

Cell growth in the longitudinal direction can be facilitated by three known factors: turgor pressure, which typically causes pressure of between 0.3 – 1 MPa resulting in wall stress of 10 – 100 MPa (Cosgrove, 1997); expansins which act as a catalyst (Cosgrove, 1998); and

XET which will catalyse the endocleavage of the xyloglucan and ligation of the newly generated reducing end to the non-reducing end of another xyloglucan. Pectic polysaccharides play an important part in allowing the microfibrils of the walls to move apart (Cosgrove, 2000; Darley, et al., 2001).

The development of longitudinal walls can be divided into three processes: wall loosening, wall elongation and the deposition of new material. The latter two processes determine the rate of wall growth and must occur almost simultaneously for the thickness of the wall to remain constant. The direction of growth is controlled by the orientation of the cellulose microfibrils that loop around the cell. The exact mechanism of wall loosening is still much debated but it is likely that it can be attributed to one of three possible mechanisms. One such mechanism includes the protein family known as 'expansins'. These disrupt the hydrogen bonding between microfibrils and the hemicelluloses thought to tether them together causing the breakdown of the xyloglucan chains of hemicellulose and their subsequent movement and realignment of the fibrils by xyloglucan endotransferases (XETs). The targeting of xyloglucan crosslinks in particular appears to be an important part of the expansion of a cell and therefore plant growth (Cosgrove, 1999).

The synthesis of the constituent parts of the wall is necessary for growth. All noncellulosic polysaccharides are produced in the Golgi and delivered via a similar method to that observed in the transverse walls. The cellulose fibrils onto which the polysaccharides are attached are synthesised in the plasma membrane and are the only wall components to originate here.

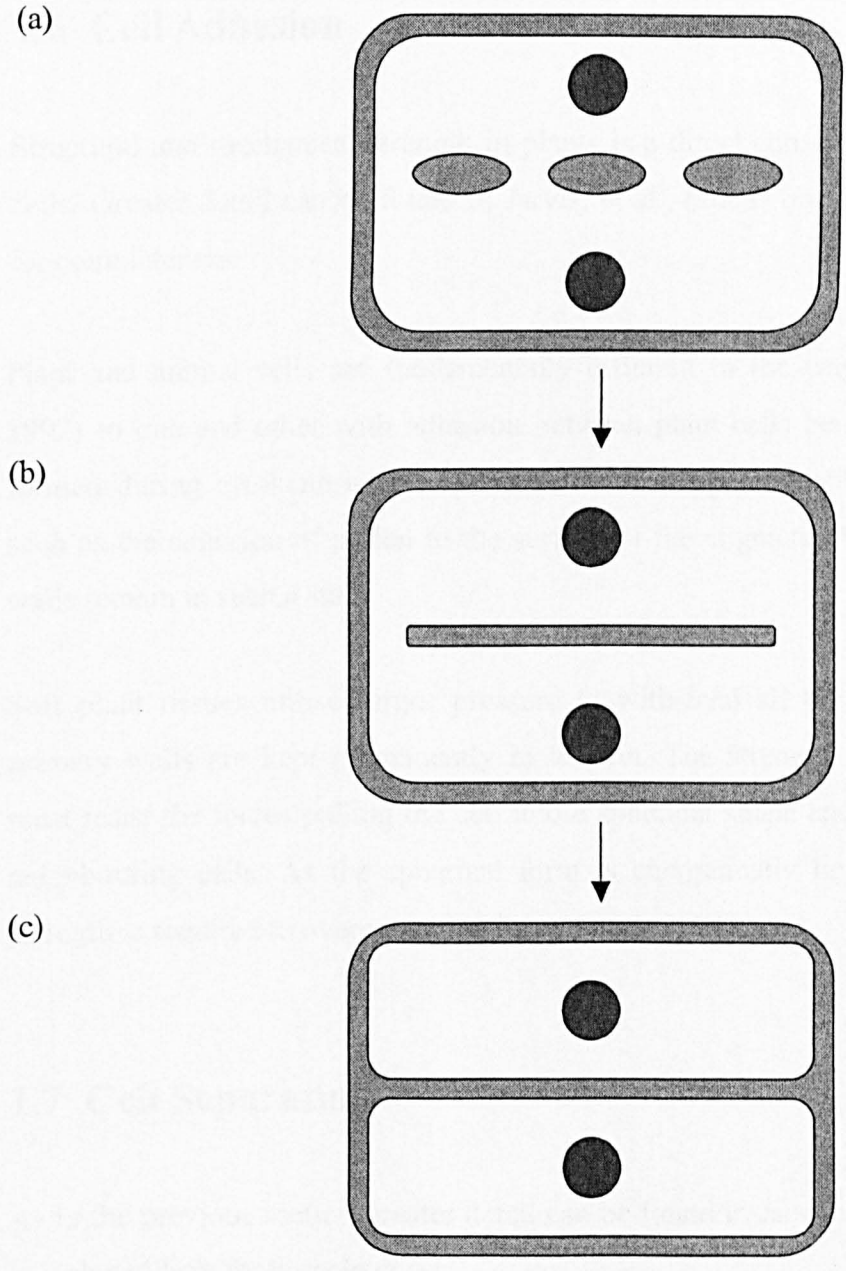


Figure 1.11 The formation of the new transverse wall. Initially the vesicles are shown aligning themselves at the location of the new wall (a). Eventually they coalesce, forming the cell plate which is enclosed by a double membrane (b) and finally the addition of new material allows the plate to join with the existing plasma membrane (c).

Finally cytokinesis, the final division of the cell resulting in two daughter nuclei, occurs completing the cycle before the process starts again.

1.6 Cell Adhesion

Structural and mechanical strength in plants is a direct consequence of adhesion between cells. Greater detail can be found in Jarvis, et al., (2003) but a summary is included here for completeness.

Plant and animal cells are fundamentally different in the way that they adhere (Knox, 1992) to one and other with adhesion between plant cells being established as cells are formed during cytokenesis and active adhesion happening only very rarely in instances such as the adhesion of pollen to the surface of the stigmata. As the cell grows, adherent walls remain in such a state.

Soft plant tissues utilise turgor pressure to withstand all the compressive stresses and primary walls are kept permanently in tension. The strength of adhesion between cells must resist the forces pulling the cell into a spherical shape and away from the corners of neighbouring cells. As the spherical form is energetically favoured enormous adhesive strength is required to overcome this force (Jarvis 1998).

1.7 Cell Separation

As in the previous section greater detail can be found in Jarvis et al (2003) but a summary is included here for completeness.

Limited turgor-driven cell separation at the cell corners (tricellular junctions) gives rise to intercellular spaces. Since cell separation under these circumstances is obviously a controlled process a mechanism must exist to precisely target the polymers for degradation. Enzymes target the cell periphery where the space will form, controlling the size (Kolloffel and Linssen, 1984; Jeffree, et al., 1986; Roland, 1978). Turgor pressure decreases as the space opens up (Jarvis, 1998) however this is not the limiting factor in the eventual size of the space. Throughout the development of the intercellular space a 'reinforcing zone' which is rich in galacturonan can always be found (Roy, et al., 1992). Antibody studies (Willats, et al., 2001b) indicate that a partially methyl esterified domain of HG is found in the region of the cell wall lining the intercellular spaces which leads to the conclusion that such a domain is associated with cell adhesion at cell junctions.

1.8 Aims and Objectives

In this thesis the aim was to examine the nature of cell adhesion, identifying the molecular features responsible and how they vary from cell to cell. Chapters 3 and 4 will deal with the question of whether crosslinks between pectins are responsible for cell adhesion and if this differs between cells. Chapter 5 will look at the strength of adhesion and how this might vary according to the structure of the polysaccharide network and Chapter 6 will investigate the possibility of the existence of non-methyl intermolecular esters.

Chapter Two

Materials and Methods

Glassware used for physical, chemical or microscopical examination was washed and cleaned in 2 % -5 % Decon solution, then rinsed three times in tap water and three times in deionised water and dried. All chemicals, unless otherwise stated, were Analar grade.

2.1 Growth and Preparation of Plant Material

2.1.1 Source of 'Commercial' Mung Bean Plant Material

Mung Bean (*Vigna radiata*) hypocotyls described as 'commercially obtained' were purchased from Safeway Stores Plc.

2.1.2 Growth of Mung Bean Plant Material

Seed trays were lined with several layers of paper towel and then covered with a layer of filter paper. This was then saturated with distilled water. Mung Bean seeds (Kings wholesale seed merchants, Kelvedon, Essex) were scattered on the filter paper, covered in aluminium foil to ensure dark conditions and placed in a growth cabinet with a constant temperature of 20 °C. Distilled water was added when necessary to maintain moist

conditions. After 7 days the hypocotyls, which had reached an average length of between 5cm and 6cm were removed and either stored at -20 °C until required or used immediately.

2.1.3 *Growth of Arabidopsis Plant Material*

This general method was used for the growth of both the wild types used and the mutants. Seeds of Arabidopsis were placed in a mixture of 3 % (v/v) hydrogen peroxide for two minutes to sterilise the surface and then rinsed well in sterilized water. The remainder of the procedure was carried out inside a flow hood to prevent any contamination with spores that may cause mould or fungus to grow and damage the seeds. Excess liquid was removed and the seeds were then transferred by pipette to agar plates, sealed and placed in the dark overnight at 4 °C. They were then placed in the light for 5 days.

2.1.4 *Preparation of Hypocotyl 'Ghost' Tissue*

40 thin strips of mung bean hypocotyls about 10 mm x 5 mm and about 1 mm thick were cut and weighed to obtain fresh weight. The prepared material was added to a sufficient quantity of boiling methanol and allowed to boil for not less than 10 minutes. After leaving to cool the methanol was drained off and the strips of mung bean placed in detergent buffer to kill the cells and extract the intracellular protein with gentle shaking for 15 minutes. The strips were then filtered through a 2 mm sieve, washing onto the sieve with buffer. Finally the strips were washed repeatedly with acetone and allowed to dry.

The ghosts were then weighed to obtain the dry weight.

2.1.5 *Preparation of Cell Walls*

The purpose of this method is to extract the primary cell walls of plants in as intact a form as possible. The method allows for the recovery of solubilised pectin and the prevention of enzymic degradation during extraction by carrying out the initial steps in ice cold

conditions if the plant material had not been enzymatically denatured, e.g. by heat treatment.

The procedure was carried out in a mixed cation buffer (MCB) that mimicked the ionic conditions of the apoplast. The MCB reduced any swelling in the cell wall and limited any solubilisation of the cell wall polymers caused by rapid changes in the ionic conditions. The concentrations of the salts in the buffer solution are given in table 2.1. The pH (Jardine, et al., 2002) of the buffer solution was then between 6.8 and 7.2.

Salt	Conc. (mM)
Sodium Acetate	10
Potassium Chloride	3
Magnesium Chloride	3
Calcium Chloride	1

Table 2.1 Salt concentrations of the Mixed Cation Buffer (MCB)

One hundred grams of fresh material (Mung Bean Hypocotyls, Safeway), 200 g of ice, 300 ml of chilled detergent buffer (2% MCB-triton X-100) and 4 ml of octanol to prevent the detergent foaming were blended (Waring Blendor) in 6 successive 8 second bursts. The addition of the ice was necessary to limit any enzymic activity. The sides of the blender were washed down with MCB between bursts to ensure all material received equal blending. The homogenised material was then transferred quantitatively to a two-stage sieving system. The topmost sieve was of mesh size 150 μm to separate the secondary cell walls from the primary ones which were then caught by the lower sieve which had aperture size of 54 μm. The cell wall material was then washed well with chilled buffer solution until no smell of octanol remained. One and a half litres was usually sufficient. The cell wall material was then boiled in methanol for 10 minutes in order to denature and therefore inactivate any enzymes present.

The isolated cell wall material was sequentially dried in 2 x 50 %, 70 % and finally 2 x 100 % acetone to remove any water present.

2.2 Extraction and Quantification of Pectin from Cell Walls

2.2.1 Pectin Extraction Sequence

The method for the extraction of pectins was based on that described by (Marry, et al., 2000). In this method cell wall material was extracted with 2 M pH7 imidazole at 20 °C for 6 hours followed by an overnight incubation in fresh 2 M imidazole. Both these steps were repeated prior to an overnight incubation in 0.05 M sodium carbonate at 4 °C and 20 mM $NaBH_4$ followed by a 3 hour incubation in fresh solution at 20 °C. The cell wall residue was further incubated in 1 M KOH and 10 mM $NaBH_4$.

To adapt this method for cell separation some modifications to assist cell separation were introduced and $NaBH_4$ was eliminated. A final 2 hour 4 M KOH incubation was also included. The initial 6 hour imidazole incubation was continuously stirred in order to provide energy to facilitate any separation induced by the removal of the Ca^{2+} bridging ions. The overnight incubation at 4 °C remained the same as the original method and both these steps were repeated. At the end of each step a vortexing treatment was introduced, again to facilitate separation by the introduction of mechanical energy. This consisted of 5 minutes vortexing, 45 seconds shaking and then a centrifugation step to allow the supernatant liquid to be removed. The overnight incubation in 0.05 M Na_2CO_3 at 4 °C and then 24 hours at room temperature also included the same vortexing treatment after each stage. The remaining cell wall residue was treated with 1 M and then 4M KOH and again subjected to vortexing, shaking and centrifugation.

2.2.2 Measurement of Galacturonic Acid Extracted from Cell Walls

0.5 ml of the extractant solution to be analysed ie imidazole supernatant, was added to a wide mouth boiling tube and 5 ml of concentrated sulphuric acid source (containing 0.0125 M sodium tetraborate) dispensed into the centre of the tube. Care was taken to ensure that the acid hit the centre of the solution and did not come into contact with the walls of the tube therefore maximising the heat of dilution that was necessary to drive the reaction. After the solution had been allowed to cool 0.08 ml of 0.15 % (w/v) *m*-hydroxydiphenyl (MHDP) in 0.5 % (w/v) NaOH was added and the solution vortexed. The colour was

allowed to develop and once a stable reading obtained, the absorbance read at 525 nm in 4.5 ml cuvettes. Appropriate blanks were used.

Standards of 0, 0.2, 0.4, 0.6, 0.8 and 1.6 mM monohydrate D-galacturonic acid were read in a similar manner.

2.2.3 Measurement of Total Sugars Extracted from Cell Walls

This method was modified from an existing protocol (Dubois, et al., 1956). 0.5 ml of extractant solution was added to a wide mouth boiling tube and 1ml of phenol added. 5 ml of sulphuric acid was then added to the tube. Care was taken to ensure that the acid hit the centre of the solution and did not come into contact with the walls of the tube therefore maximising the heat of dilution that was necessary to drive the reaction. After the solution had cooled and the colour developed the absorbance was read at 485 nm. Appropriate blanks were used.

Standards were measured in the range 0, 20, 40, 60, 80 and 100 μg/ml Glucose.

2.3 Enzyme Treatment of Cell Wall Ghosts

The basic method was applied as follows for the enzyme treatment of the plant tissue. Variations in the enzymes used, their source and concentrations were incorporated and these are discussed fully in Chapter Four. Other changes to the method are also included in Chapter Four.

Mung bean sprout ghosts prepared as described previously (Section 2.1.4) were immersed in enzyme solution (dissolved in 10 mM EDTA buffer at pH 5) at a variety of enzyme concentrations and incubated at room temperature for a given number of hours. The ghosts were then transferred to a 2 M imidazole solution and stirred for 2 hours to allow separation of the cells.

This method also formed the basis of the technique for the separation of the cells in the Arabidopsis hypocotyls. The changes necessary to successfully adapt this are described fully in Chapter Four.

2.4 Micrography of Cells

Micrographs were taken using a Nikon Coolpix 990 Digital Camera attached to a Leica ATC 2000 Microscope.

2.5 Tensile Strength Testing

2.5.1 Treatment of Arabidopsis Hypocotyls Prior to Stretching

Arabidopsis hypocotyls were first boiled in methanol for 10 minutes before being placed in the enzyme solution. The development of this procedure is discussed further in section 5.5.1.

2.5.2 Stretching

The method used for stretching the hypocotyls was used exactly as described in Ryden, et al., (2003).

2.6 Non-methyl Ester Analysis

2.6.1 Non-Methyl Esters by NMR

In addition to the usual cleaning procedure described previously, glassware used for this experiment was first cleaned with 1M HCL and then left to soak in 50 mM Na-EDTA (Sigma Aldrich, UK) overnight to remove any free cations which may interfere with the determination and affect the quality of any future H^+ NMR determination. Glassware was then rinsed with distilled water, dried in an oven at 120 °C for 4 hours and cooled in a dessicator to ensure as near to complete removal of any water as possible.

Approximately 200 mg of prepared cell wall material was placed in a Quick-fit test tube with 5 ml 50 mM NaOD (Sigma, UK) prepared in a 2:1 d_6 acetone/D_2O (Sigma, UK) mix, stoppered and left at room temperature for 1 hour. The contents of the tube were then transferred to a chromatography column fitted onto a Quick Fit Buchner Flask attached to a water pump to provide suction.

In order to limit the possibility of proton exchange that might interfere with the H^+ NMR spectrum, air being drawn into the column was passed first through a drying tube containing a cotton wool plug at each end and filled with granular $CaCl_2$. A drying tube was also attached between the Buchner flask and the water pump to prevent any protons entering the system at this point.

2.6.2 Non-esterified Carboxyl Acid Content

2.6.2.1 Copper Binding Method

The binding of Copper (II) ions to non-esterified galacturonic acid can be reversed with the addition of excess acid. By measuring the copper released, a value for the non-esterified carboxyl content can be established.

75 mg of cell walls were saturated by stirring twice in 30 ml of 2 % copper sulphate for 30 minutes. Any copper left unbound was removed by washing with deionised water and removal judged to be complete when the conductivity of the solution was less than 10 μS. The ionically bound copper was removed with 1 M HNO_3 in 60% ethanol and the volume of this filtrate made up to 50 ml and analysed by atomic absorption.

2.6.2.2 By Titration

Cells walls were initially acidified with 10mM HCl in 70 % ethanol at 4 °C overnight, to facilitate the protonation of all carboxylate sites. The acid was removed by repeated washing with 70 % ethanol. In order to ensure that the HCl was fully removed, and thus unable to adversely affect the subsequent titration, the pH of the filtrate was tested and

once it had reached pH 4, washing was judged to be complete. The cell walls were then acetone dried.

A quantity of the acidified cell walls were then transferred to a three-necked round-bottomed flask with 50 ml of degassed 0.1 M NaCl. The flask was also fitted with a pH meter and a nitrogen gas inlet and a microburette through which the alkali could be introduced. The cell walls were titrated to pH 7 with 10 mM degassed NaOH. Solutions used in the titration were degassed to prevent contamination with atmospheric CO_2. Titrations were carried out in triplicate.

2.6.3 Determination of Total Galacturonic Acid

Cell walls were saponified in 0.1 M NaOH in 70 % ethanol at 4 °C overnight and then treated similarly to the above method.

2.6.4 Methyl Ester Determination

Cell walls were saponified in a mixture of 6.25 ml of water and 1 ml 1 M KOH for one hour before neutralisation with 1 ml 0.49 M H_2PO_4. Blanks to correct for any free methanol contamination and controls with the order of addition of KOH and H_2PO_4 reversed were included. The samples were then centrifuged to release 1 ml of supernatant containing methanol.

1ml of alcohol oxidase (1 Unit/ml) from *Pichia pastoris* , (Sigma, where 1 unit oxidises 1 μmole of methanol to formaldehyde at pH 7.5 at 25 °C) in phoshate buffer as used for the standards and described below, was added to samples, blanks and controls to oxidise methanol to formaldehyde and incubated at 25 °C. Finally colour was developed by the addition of 0.02 M 2,4-pentadione (in 2 M ammonium acetate and 0.05 M acetic acid) and solutions incubated for 15 minutes at 60 °C (Klavons and Bennett, 1986).

Methanol standards in the range of 2, 4, 6, 8, 10, 12, 14 and 16 μM were made up in a phosphate buffer pH 7.5 (0.2 M monobasic and 0.2 M dibasic sodium phosphate)

Absorbance was then measured at 412 nm in plastic cuvettes.

2.7 Identification of Cell Types in Mung Bean Hypocotyls

To aid the identification of the various cell types, LR White Resin Embedded sections were examined using the light microscope and micrographs are presented in figures 2.1 to 2.4). The following types of cells were identified.

2.7.1 Cell Types

2.7.1.1 The Epidermis

Cells in the epidermis are long and thin in shape with no intercellular spaces visible. They appear present in the hypocotyl in sheets and can be 1, 2 or 3 layers deep (Figure 2.3).

2.7.1.2 The Cortex

Cortical cells are irregular in shape and are much longer and wider than those of the epidermis. Intercellular spaces can be observed and, from the micrographs of the transverse section, the cells are seen to be spherical (figure 2.1).

2.7.1.3 Vascular Tissue

The cells making the vascular tissue of the 7-day-old hypocotyls are heterogeneous in nature and are thus easily identified. A brief description of the constituent cell types will be given here.

Primary Phloem

These are small cells of which several different types exist and therefore there is a range of different shapes observable.

Primary Xylem

Xylem cells are also long and thin but not as much so as the cambium cells. They are larger than the phloem cells

2.7.2 Pith

Similar in shape to the parenchyma cells found in the cortex, these cells, also parenchyma, are much larger and by far the largest of all those found in the hypocotyl although they do vary in size.

(a) (b)

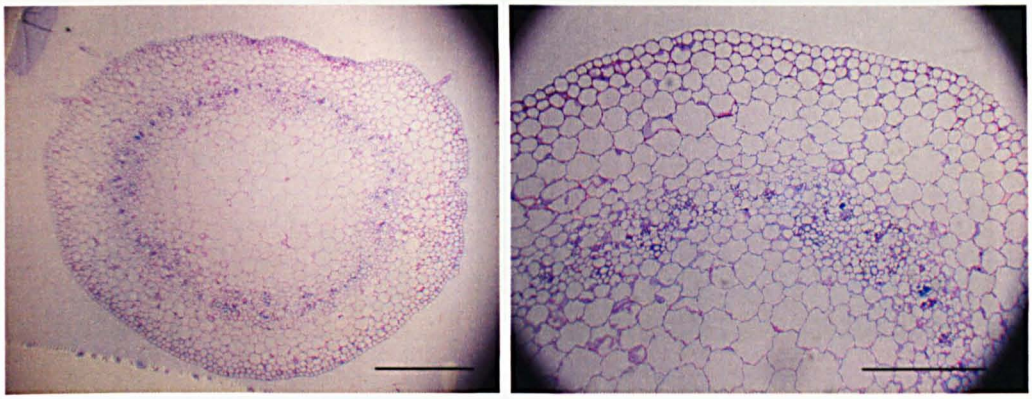


Figure 2.1 a and b. (a) Transverse section of a toluidene blue stained, resin embedded mung bean hypocotyl. (b) The same section under increased magnification. Scale bars are 500 μm (a) and 250 μm (b).

(a) (b)

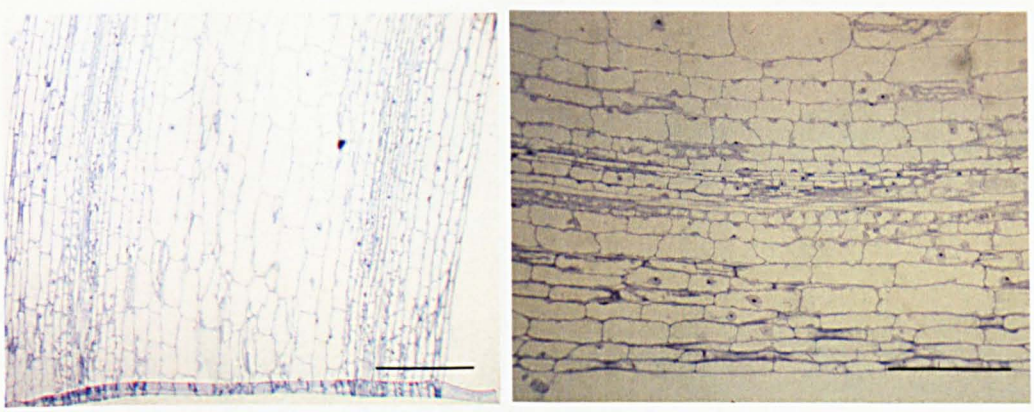


Figure 2.2 a and b (a) Longitudinal section of a toluidene blue stained, resin embedded mung bean hypocotyl. (b) A similar section under increased magnification and rotated through 90°. Scale bars are 500 μm (a) and 250 μm (b).

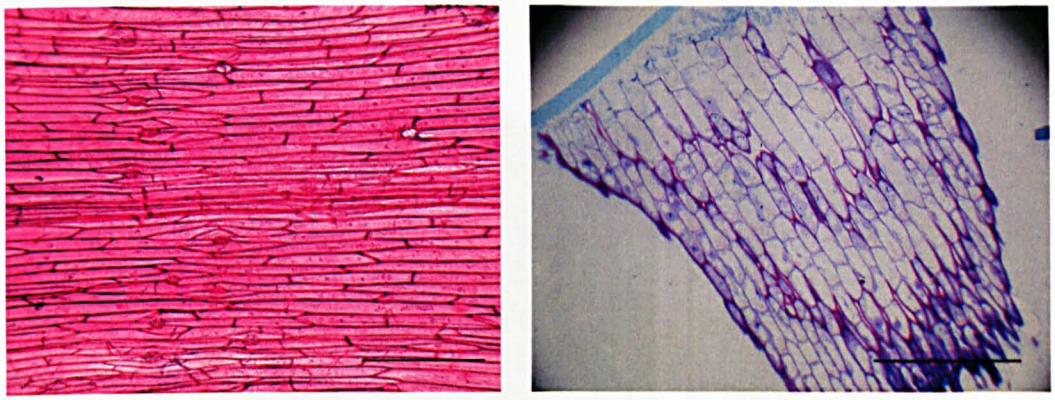
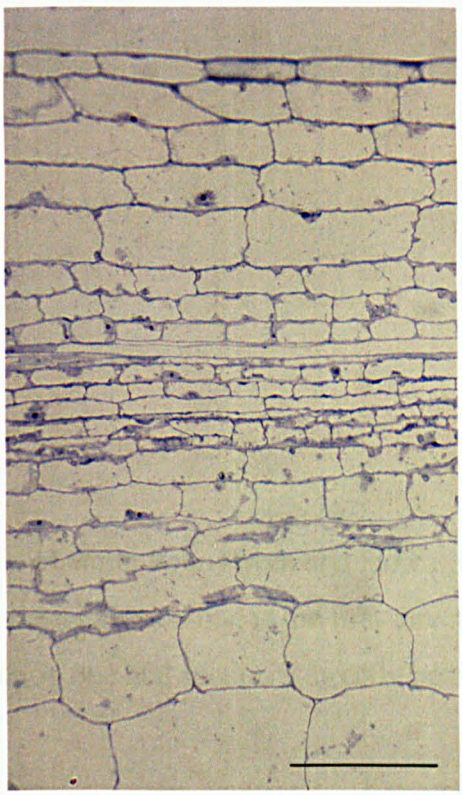


Figure 2.3 a (a) Single layer of epidermal tissue stained with tolduidene blue. (b) Section of single cell thickness taken at an angle through the cortex of the hypocotyl and in which several layers are visible. Scale bars are 250 μm (a) and 500 μm (b).

(a)



(b)

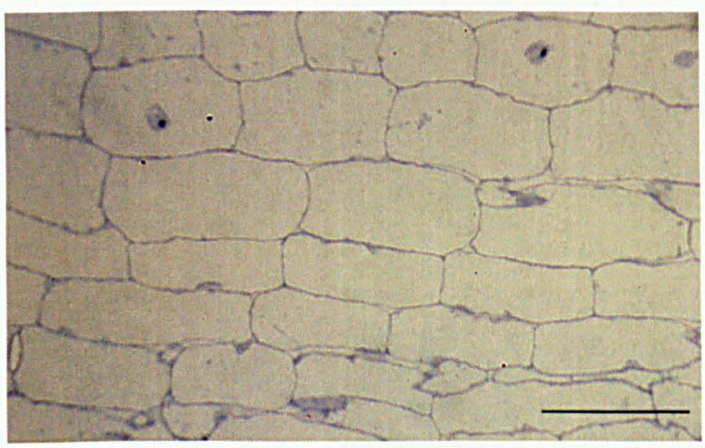


Figure 2.4 **(a)** Longitudinal Cross Section. Image obtained from a toluidene blue stained, resin embedded section through a single layer of cells. Scale bar is 50 μm. **(b)** Longitudinal Cross Section. Magnified Pith tissue. Scale bar is 50 μm.

2.8 Location of Sections 1, 2 and 3 in the Hypocotyl

Figure 2.5 indicates the positions of the sections that are investigated throughout the course of the thesis.

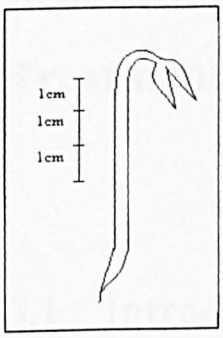


Figure 2.5 Diagram of Mung Bean Hypocotyl indicating the location of the sections referred to throughout the thesis. Section one is the first directly behind the apical hook, section two directly behind section one and section 3 directly behind section two.

Chapter Three

Separation of Mung Bean Hypocotyl Tissue by Chemical Treatments

3.1 Introduction

The use of calcium-chelating agents on dicot cell wall preparations to bring pectin into solution is well documented. However, normally much less than half of the total pectin present is solubilised and only limited cell separation is achieved under such circumstances (Cocking, 1960; McCartney and Knox, 2002). Files of cells that have elongated in parallel have been shown to be separable with chelators or alkaline extraction whereas transverse walls do not separate (Cocking, 1960). This would seem to indicate differences in the pectic network responsible for cell adhesion depending on the origin of the wall concerned and that this difference is identifiable using cell separation techniques.

This chapter aims to use and further develop such chemical pectin extraction methods to achieve selective cell separation and identify separation characteristics in relation to the particular cell wall, ie longitudinal or transverse, and tissue type, ie cortical, pith, etc.

3.2 Development of a Separation-Inducing Chemical Pectin Extraction Procedure

3.2.1 Initial pectin extraction sequence

A multi-step pectin extraction procedure based on that developed by Marry, et al. (2000) to extract pectic polysaccharides from sugar-beet cell walls rather than expressly for cell separation, was utilised to induce cell separation by removing the pectin from the middle

lamella. Imidazole, a chelating agent, will remove any Ca^{2+} ions linking chains of galacturonic acid, Na_2CO_3 will break any alkali-labile linkages, for example esters, and KOH will extract hemicellulosic polymers. By varying these extractants and the conditions under which they are applied the adhesion characteristics of the cell wall can be investigated

The initial pectin extraction method was carried out as per the method described in Chapter Two. However mung bean sprouts which had been grown commercially (Safeway Stores Plc) were used in the first instance. The degree of separation and correspondingly, the concentration of galacturonic acid and total sugars extracted also varied enormously with the chemical extraction procedure producing a range of results from no observable separation to a small degree of separation that could not be replicated. It was therefore decided to use mung bean sprouts that had been grown under known conditions in the laboratory for all future experiments. These behaved in a completely different manner and produced replicable results.

3.2.2 Results of the initial extraction and modifications to this

Even with using the laboratory grown hypocotyls, the method described previously proved to have limited success in achieving separation of the hypocotyl tissue into files of cells, instead large aggregates were still present. Since the final KOH extraction stages (1 M and 4 M) are known to be fairly non-selective in their extraction of pectin, it was decided to substitute these steps with two alternatives to ascertain to what extent, if any, the KOH extractions were necessary.

If adhesion between cells depends on molecular entanglement, then extended stirring might be enough to induce cell separation irrespective of the solvent. We therefore substituted the KOH extraction steps with a 6 hour stirring in imidazole and a 6 hour stirring in distilled water. If the reduced extractability was simply due to steric impedance, then a water extraction should have been all that was necessary to release solubilised pectins and separate cells adhering.

The 6 hour stirring with water left the aggregates that had been observed prior to the start of the stirring mostly broken apart although some smaller aggregates did remain and could

be clearly seen under the microscope. When imidazole was substituted for the water and the experiment repeated the ghost tissue could be seen to have been almost totally separated with only a few aggregates remaining. However it was notable that the aggregates were larger than those left after the water stirring step but the degree of separation into files was far greater.

The discovery that the inclusion of an extra imidazole stirring step facilitated greater cell separation led to the investigation of this phenomenon. It was found that the length of time the ghosts were stirred in imidazole for was crucial to the degree of separation achieved as the longer the stirring went on the shorter the files became. Cells also became more susceptible to breaking across the cell wall itself rather than the middle lamella.

Samples stirred for 2 hours exhibited the highest degree of separation into files and few aggregates were seen to remain. However those which were left for 4 hours began to break into much shorter chains and cells were observed to have begun to disintegrate. After 6 hours few files remained and these tended to be only 2 - 3 cells in length. The majority of the tissue observed was mainly single cells and cell breakage appeared to be much more widespread.

The sequential chemical separation procedure described previously was again used on the laboratory grown hypocotyls and differences in the separation characteristics were observable immediately. Ghost tissue could be seen to be disintegrating during the initial imidazole stirring step. Examination of separated cells under the microscope revealed some files but the tissue segments were mostly aggregates at this stage.

The readiness of the laboratory grown hypocotyl cells to separate could possibly have been caused by the ghosts from these hypocotyls being mechanically weaker. It was decided to test this by stirring a sample of them in Mixed Cation Buffer (MCB). MCB mimics the conditions in the cell and should not lead to separation as a result of a concentration gradient being set up and cell wall components being solubilised. Thus any separation observed should be a direct result of weakness in the cell to cell adhesion.

To eliminate the possibility that the more rapid onset of separation was due to an increased mechanical weakness in cell adhesion, a sample of hypocotyl ghosts were stirred in mixed cation buffer (MCB) concurrently with samples undergoing the initial imidazole treatment. Small aliquots of both the supernatant liquids were analysed under the microscope for

suspended cell ghosts. The imidazole treated samples contained, as expected, a high degree of suspended material in the form of aggregates and files as previously described whereas in the MCB stirred samples, no suspended material was visible. MCB was used in preference to water as this should prevent separation being induced by wall components being solubilised and any separation observed should be a direct result of mechanical weakness in the cell to cell adhesion.

It was thought possible that the lower degree of initial separation in the commercially available Mung Beans could be due to the growing region no longer being active as the beans would have been harvested and then stored at approximately 4 °C for a few days before used in experiments. To investigate this as a possibility, 3 x 1 cm long sections were taken from a selection of laboratory grown hypocotyls and the extraction sequence applied to them. Galacturonic acid and total neutral sugars extracted by each step in the sequence were measured by the MHDP method and Phenol-Sulphuric method respectively and, after the final stage, the cells examined under the microscope.

3.2.3 Final Extraction Sequence

The final extraction sequence is as follows:

The prepared ghosts were added to 15 ml of 2 M, pH7 imidazole and placed on a magnetic stirrer for 6 hours after which time they were subjected to vortexing for 5 minutes and shaking for a further 30 s. The supernatant liquid was separated from the solid cell wall ghost material by centrifuging for 15 minutes at 4500 rpm. At this point 15 ml of fresh imidazole solution was added and the solutions left overnight. This process was then repeated and all imidazole extracting solutions stored in the fridge to await analysis. The vortexing/centrifuge steps were included at each stage.

The cell wall material was then added to 15 ml 0.05 M Na_2CO_3 and left for 24 hours at 4 °C. This step was carried out twice before a final Na_2CO_3 extraction at the same concentration with the solutions then left overnight at room temperature. Finally the cell wall material was immersed in 15 ml 1 M KOH for 3 hours and then 15 ml 4 M KOH for a further 3 hours. Again the vortexing and removal of the supernatant liquid by centrifugation was incorporated into every step.

Finally, the cell wall material was stirred in 2 M Imidazole for 6 hours.

3.3 Galacturonic Acid and Total Sugar Concentrations - Cross Correction

The determination of uronic acids is always at risk from some degree of interference from neutral sugars although this can be minimised through adaptations of the MHDP method. If data is available from a phenol-sulphuric assay, it is possible to correct for this. The procedure for the cross-correction is relatively simple, providing that the calibration graphs for both the MHDP method and phenol-sulphuric are linear. Slopes of calibration graphs for the following must be obtained;

Uronic acid by MHDP	= W
Total Sugars by MHDP	= X
Uronic Acid by Phenol Sulphuric	= Y
Total Sugars by Phenol Sulphuric	= Z

Then calculate W/X and call this A, Equation 3.1
and Y/Z, calling this B. Equation 3.2

Absorbance of the sample analysed by phenol sulphuric	= Ap	Equation 3.3
Absorbance of sample analysed by MHDP	= Am	Equation 3.4

With these figures we can calculate the mMoles GalUA Equivalent;

$$= \frac{(Am/W)-A * (Ap/Z)}{1 - B * A} \qquad \text{Equation 3.5}$$

The figure for mM Glucose Equivalent is then calculated;

$$= (Ap/W) - B * (\text{mM GalUA Equivalent}) \qquad \text{Equation 3.6}$$

Both the values that will be obtained for glucose and galacturonic acid equivalents are in mM concentrations. They can then be converted through to μmol/g.

3.4 Results

3.4.1 Levels of Galacturonic Acid and Total Sugars Extracted

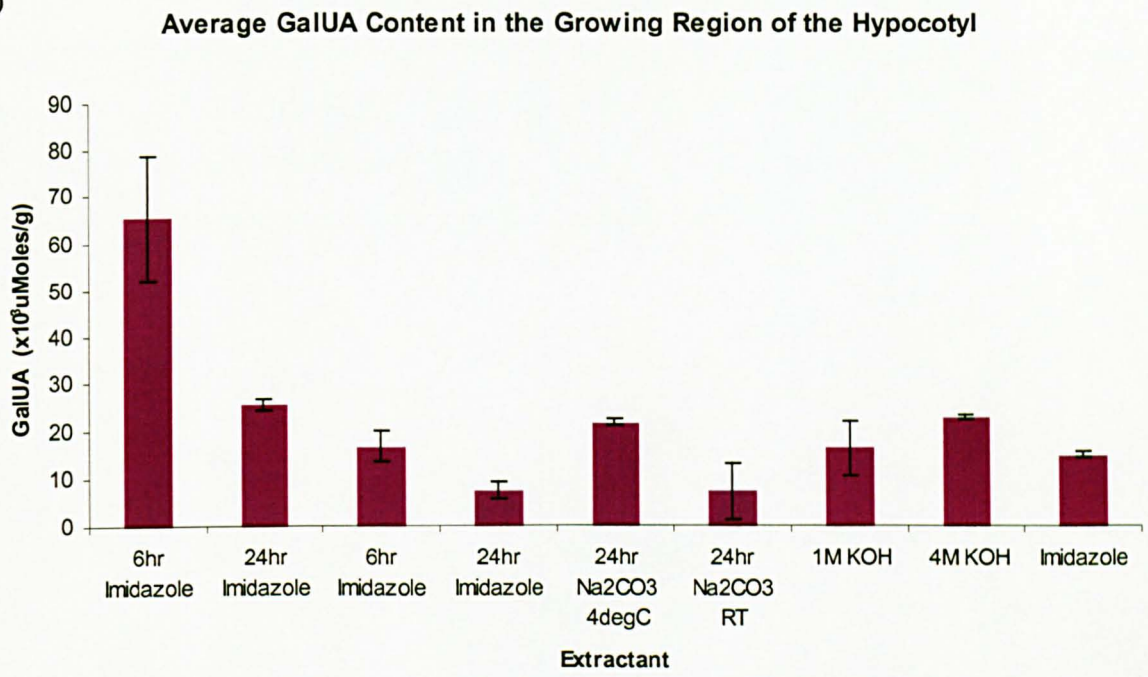
The galacturonic acid extracted from the growing region of the laboratory-grown hypocotyl by each stage in the separation procedure is show in figure 3.1 a and the total sugars in 3.1 b. The graphs show that imidazole-extractable GalUA levels are high and decrease gradually; increasing only as the extractant is changed and presumably a new set of cross-links are broken. This pattern is not repeated with the KOH extractions as increasing the concentration increases the quantity solubilised. The final imidazole treatment shows clearly that there is still galacturonic acid and sugars remaining in the cell walls as a significant amount can still be extracted at this stage. The pattern is repeated in the total sugar data.

The results of further similar experiments using 1cm sections further away from the growing section as indicated in the introduction are given in figures 3.2 a & b and also 3.3 a & b.

Figure 3.1 a Nine extraction conditions were applied to ghosts derived from the growing region of the mung bean hypocotyls as described in section 3.2.3. These resulting solutions were analysed for galacturonic acid that had been extracted from the cell wall. Values are mean concentrations in $x10^3$ μMoles/g. Bars on columns are the standard deviation where n = 6.

Figure 3.1 b Nine extraction conditions were applied to ghosts derived from the growing region of the mung bean hypocotyls as described in section 3.2.3. These resulting solutions were analysed for (total) neutral sugar that had been extracted from the cell wall. Values are mean concentrations in mMoles/g. Bars on columns are the standard deviation where n = 6.

(a)


Average GalUA Content in the Growing Region of the Hypocotyl
GalUA (x10⁶uMoles/g)
90
80
70
60
50
40
30
20
10
0
6hr Imidazole
24hr Imidazole
6hr Imidazole
24hr Imidazole
24hr Na2CO3 4degC
24hr Na2CO3 RT
1M KOH
4M KOH
Imidazole
Extractant

(b)

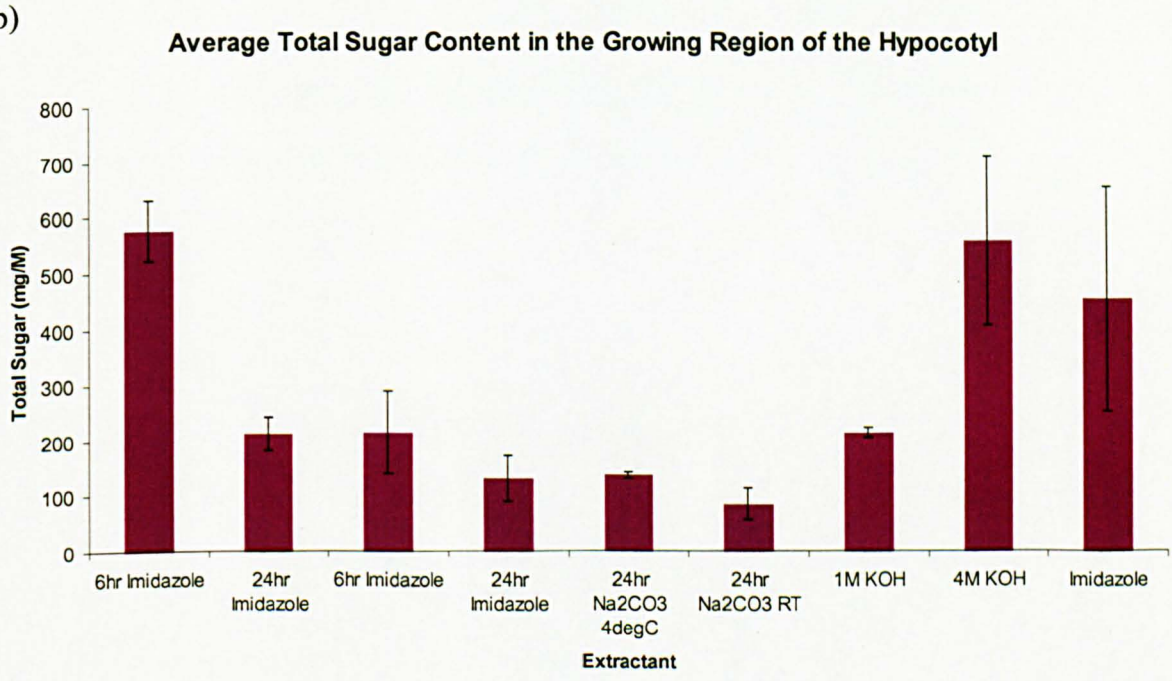
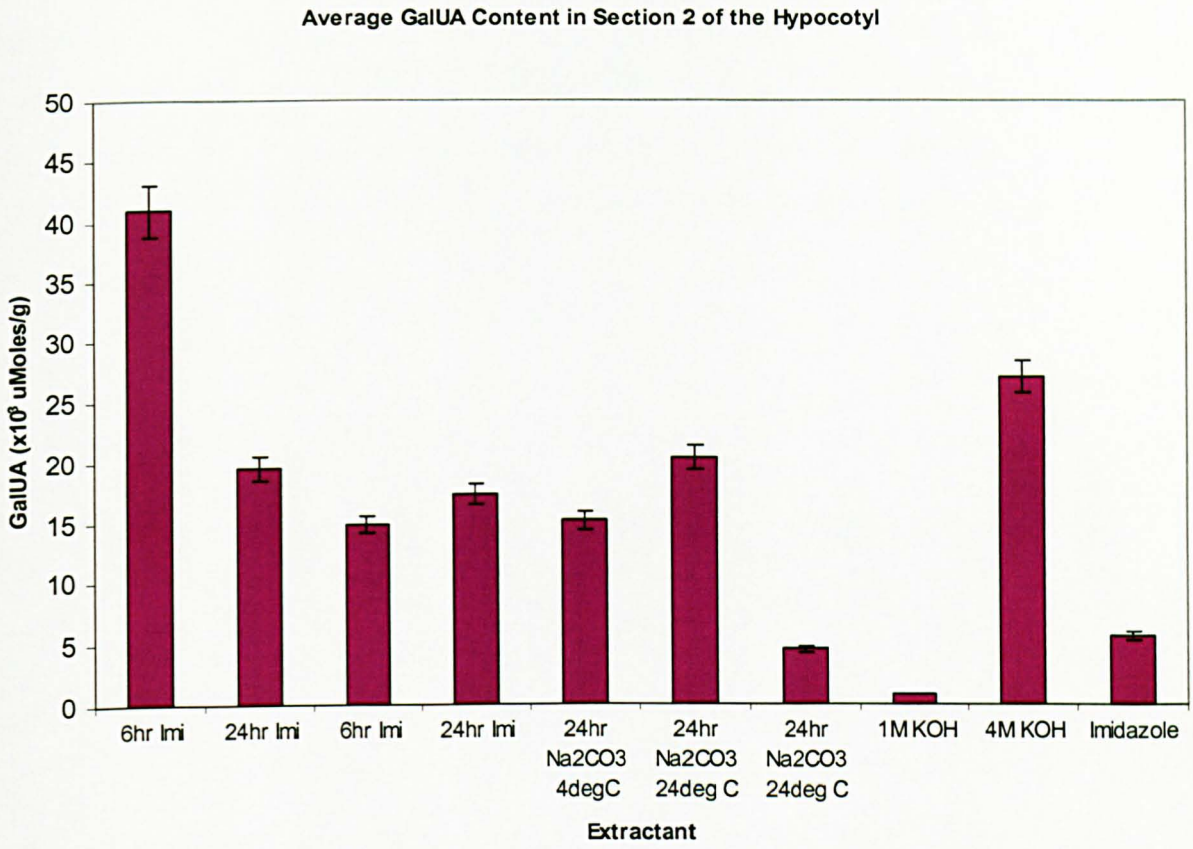

Average Total Sugar Content in the Growing Region of the Hypocotyl
Total Sugar (mg/M)
800
700
600
500
400
300
200
100
0
6hr Imidazole
24hr Imidazole
6hr Imidazole
24hr Imidazole
24hr Na2CO3 4degC
24hr Na2CO3 RT
1M KOH
4M KOH
Imidazole
Extractant

Figure 3.2 a Nine extraction conditions were applied to ghosts derived from a section taken from the 2 cm under the apical hook of the mung bean hypocotyl. Again the details of the extraction sequence are described in section 3.2.3. These resulting solutions were analysed for galacturonic acid that had been extracted from the cell wall. Values are mean concentrations in $x10^3$ μMoles/g. Bars on columns are the standard deviation where n = 6.

Figure 3.2 b Nine extraction conditions were applied to ghosts derived from a section taken from the 2 cm under the apical hook of the mung bean hypocotyls. Again the details of the extraction sequence are described in section 3.2.3. These resulting solutions were analysed for neutral sugar that had been extracted from the cell wall. Values are mean concentrations in mMoles/g. Bars on columns are the standard deviation where n = 6.

(a)


Average GalUA Content in Section 2 of the Hypocotyl
GalUA (x10⁶ uMoles/g)
50
45
40
35
30
25
20
15
10
5
0
6hr Imi
24hr Imi
6hr Imi
24hr Imi
24hr Na2CO3 4degC
24hr Na2CO3 24deg C
24hr Na2CO3 24deg C
1M KOH
4M KOH
Imidazole
Extractant

(b)

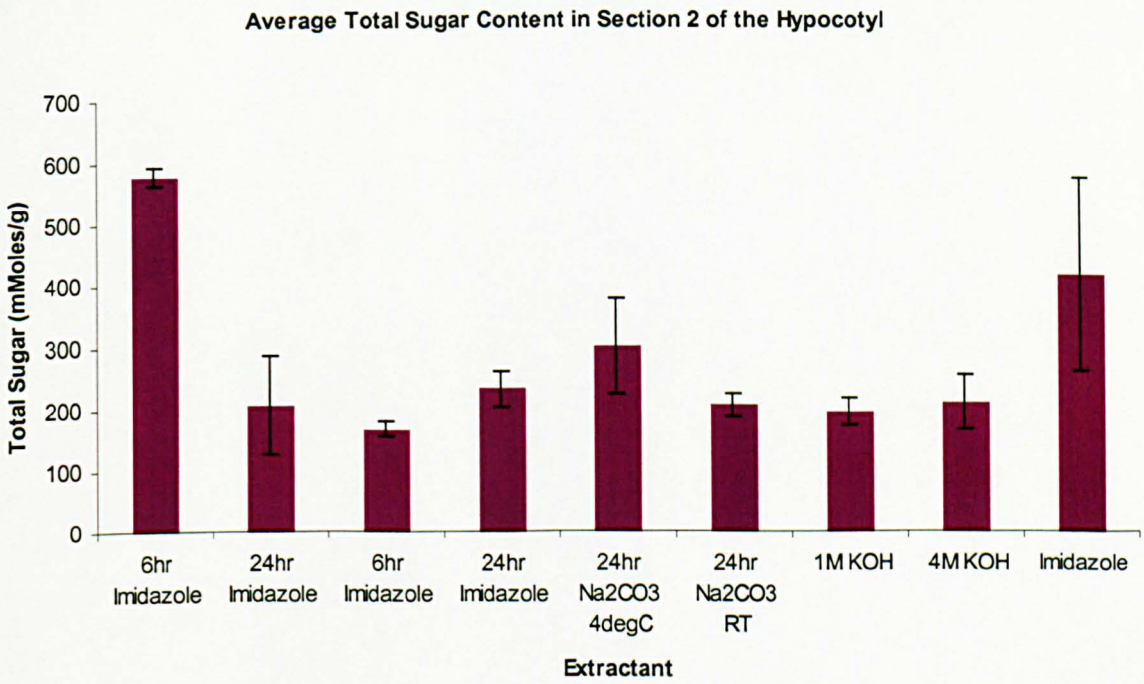
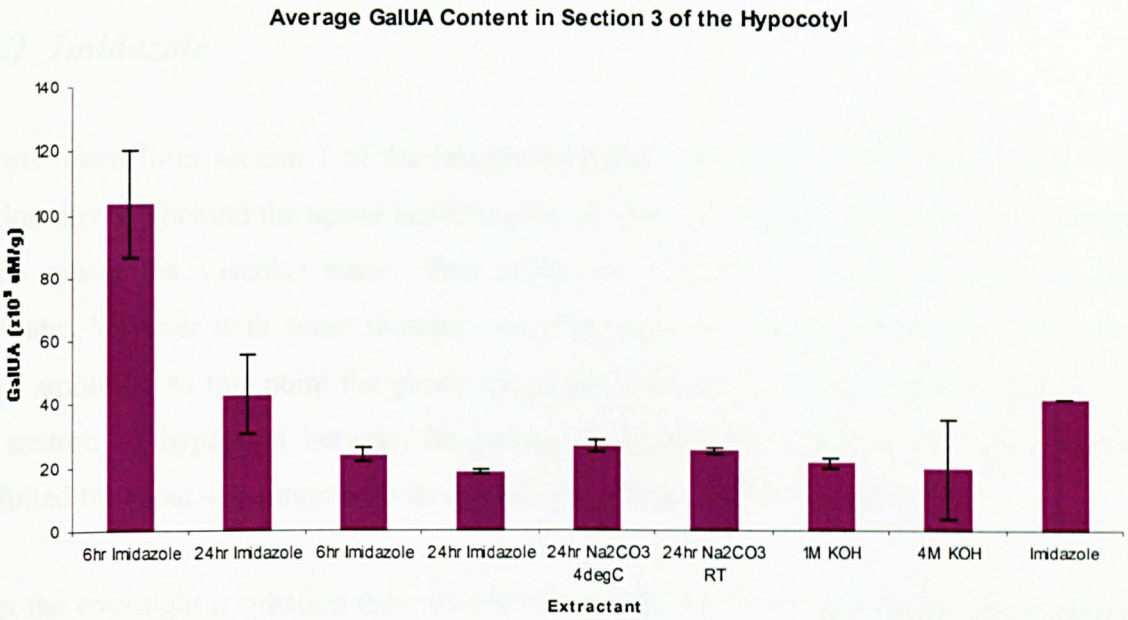
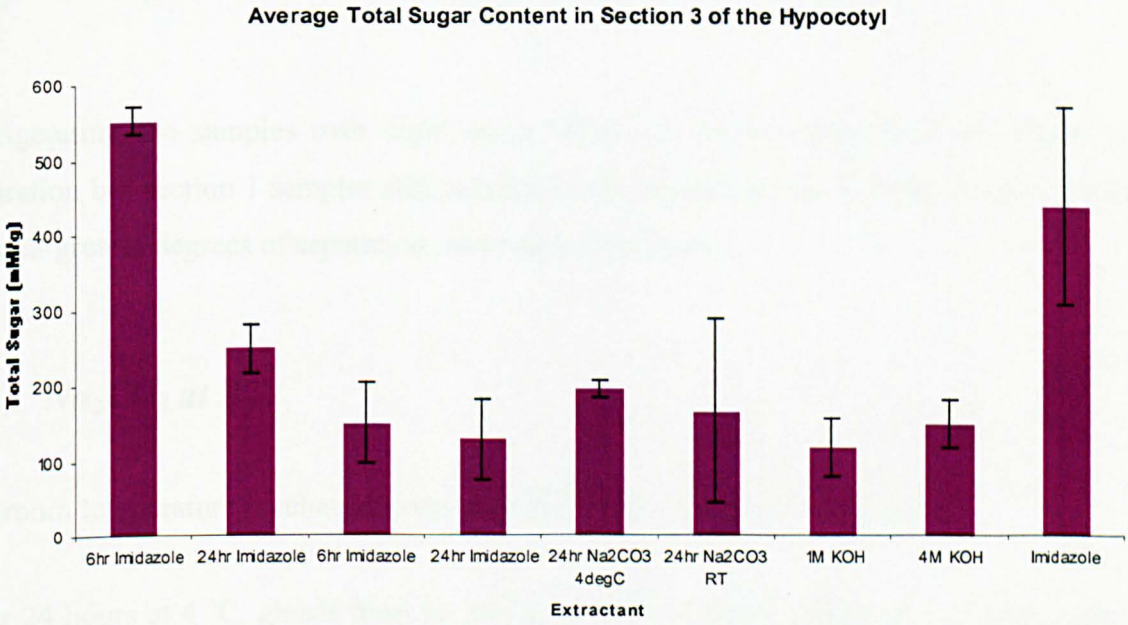

Average Total Sugar Content in Section 2 of the Hypocotyl
Total Sugar (mMoles/g)
700
600
500
400
300
200
100
0
6hr Imidazole
24hr Imidazole
6hr Imidazole
24hr Imidazole
24hr Na2CO3 4degC
24hr Na2CO3 RT
1M KOH
4M KOH
Imidazole
Extractant

Figure 3.3 a Nine extraction conditions as described in section 3.2.3, were applied to ghosts derived from the section of mung bean hypocotyl 3 cm below the apical hook. These resulting solutions were analysed for galacturonic acid that had been extracted from the cell wall. Values are mean concentrations in $x10^3$ μM/g. Bars on columns are the standard deviation where n = 6.

Figure 3.3 b Nine extraction conditions as described in section 3.2.3 were applied to ghosts derived from the section of mung bean hypocotyl 3 cm below the apical hook. These resulting solutions were analysed for neutral sugar that had been extracted from the cell wall. Values are mean concentrations in mM/g. Bars on columns are the standard deviation where n = 6.


Average GalUA Content in Section 3 of the Hypocotyl
GalUA (x10³ uM/g)
140
120
100
80
60
40
20
0
6hr Imidazole
24hr Imidazole
6hr Imidazole
24hr Imidazole
24hr Na2CO3 4degC
24hr Na2CO3 RT
1M KOH
4M KOH
Imidazole
Extractant


Average Total Sugar Content in Section 3 of the Hypocotyl
Total Sugar (mM/g)
600
500
400
300
200
100
0
6hr Imidazole
24hr Imidazole
6hr Imidazole
24hr Imidazole
24hr Na2CO3 4degC
24hr Na2CO3 RT
1M KOH
4M KOH
Imidazole
Extractant

3.5 Effect of Chemical Extraction Stages on Cell Structure Integrity

3.5.1 Imidazole

Ghosts taken from section 1 of the laboratory-grown hypocotyl (defined here as the 1cm section directly behind the apical hook) appear to have separated at the ends where cortical tissue meets the vascular tissue. This effect was observed to be uneven among the replicates however with some showing complete separation at this point and others only small amounts. At this point the ghosts taken from section 3 of the hypocotyl (defined as the section of hypocotyl between beginning 3 cm from the back of the apical hook) exhibited the most separation with the majority displaying partial separation.

After the overnight incubation there was an observable increase in the degree of separation visible in the section 3 ghosts. Only a minimal degree of separation was visible in the ghosts from section 1 with section 2 being intermediate but tending slightly more towards a degree of separation similar to that in the section 3 ghosts.

The second 6 hour stir in imidazole yielded an increase in the amount of separation in all samples although the differences between the 3 samples were maintained.

Refrigerating the samples over night again led to an overall increase in the degree of separation but section 1 samples still exhibited less separation than sections 2 and 3 which showed greater degrees of separation, increasing respectively.

3.5.2 Na_2CO_3 at RT

The room temperature incubation continued the steady increase in separation.

After 24 hours at 4 °C, ghosts from section 1, which had previously shown separation that was to a much lesser extent than sections 2 and 3, began to appear as though they were 'catching up'. Separation in the section 2 sample also began to look more in line with the degree of separation in section 3. The amount of separation that was observed in the sample with ghosts from section 3 continued to increase. However, the degree of

separation was already such that it was impossible to distinguish whether this was on the same scale as the separation of the ghosts from sections 1 and 2.

A further 24 hr Na_2CO_3 incubation, this time at room temperature, led to a slight but still noticeable difference in the amount of separation in each sample with section 3 appearing to have the most and section 1 the least but this was to a much lesser extent than was previously the case.

3.5.3 KOH

The effect previously described was seen to be exacerbated after a 3 hr stir in 1 M KOH. This trend described previously continued as a result of the 4 M KOH stirring stage..

3.5.4 Final 6hr Imidazole Stirring

Only after this final stage did all three samples appear to have separated to a similar degree by inspection with the naked eye.

3.6 Separation Characteristics of Different Tissue Types

3.6.1 Epidermis

In duplicate experiments with the chemical extraction process described on the previous page, on sections 1, 2 and 3 of the hypocotyl, there was no obvious difference between sections in the separation patterns of the epidermal cells. In each case the epidermis was seen to separate completely from all other plant tissue. After the cells became detached they could be seen in sheets of single-cell thickness. Since only a single layer of epidermis was observed after the separation-inducing process but, from observation it is known to be two layers deep at least some points then it is possible to deduce that separation may preferentially occur along the inner longitudinal walls of the cells. (Figure 3.4).

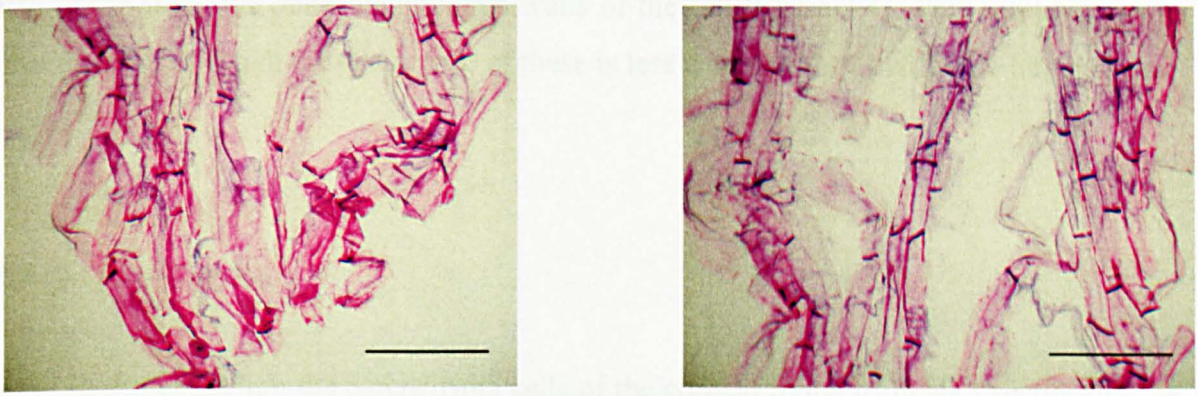


Figure 3.4 Epidermal cells show separating into files after being subjected to the multi-step pectin extraction sequences described I section 3.2.3. Scale bars are 100 μm.

Inspection under the light microscope of sheets of epidermis reveals that separation will also occur along the 'side' longitudinal walls of the cell (figure 3.4). This can give rise to files of cells, although the occurrence of these is less common than that of the larger sheets.

3.6.2 The Cortex

After pectin extraction the parenchyma cells of the cortical tissue from all 3 sections of the hypocotyl appeared to separate the most consistently of all cell types, into files (Figure 3.5 a & b) much more easily than those of the pith tissue.

3.6.3 Vascular Tissue

The vascular tissue as a whole appeared least apt to separate throughout the extraction process. Occasionally xylem vessels could be seen to have become detached from the rest of the tissue.

Identification of the individual tissue types of the vascular tissue, while possible in the intact hypocotyl sections could not be achieved with any degree of certainty in separated cells. After the extraction of pectin it was notable however, that the cells in the vascular region remained adhered (figure 3.6).

3.6.4 Pith Tissue

Cells from the pith of the mung bean hypocotyl were rarely found in files after the full extraction sequence. Although single cells could be seen (figure 3.7 a), and there were very rare files (figure 3.7 b) pith tissue was mainly found intact (figure 3.7 c & d).

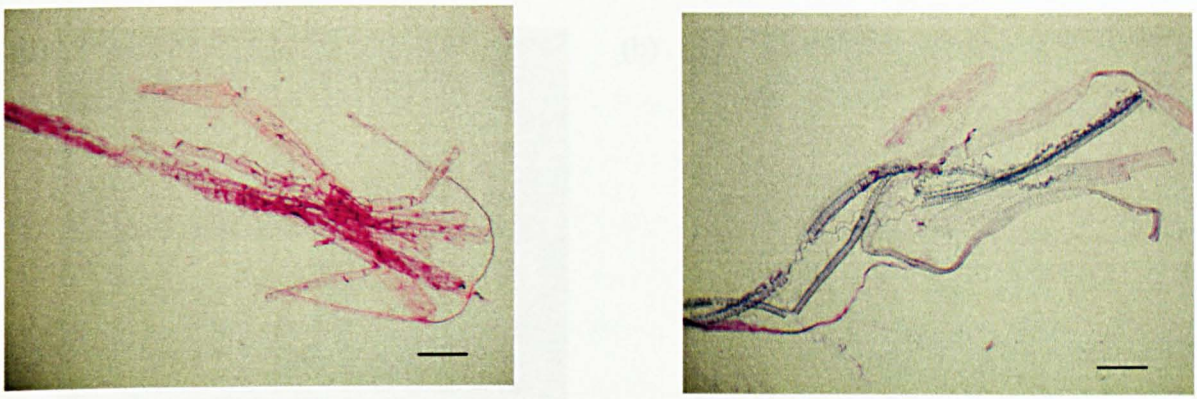


Figure 3.6 (a) & (b). Vascular cells having separated after being subjected to the multi-step pectin extraction procedure as described in section 3.2.3. These cells do not exhibit any conistent pattern to their separation. Scale bars are 100 μm.

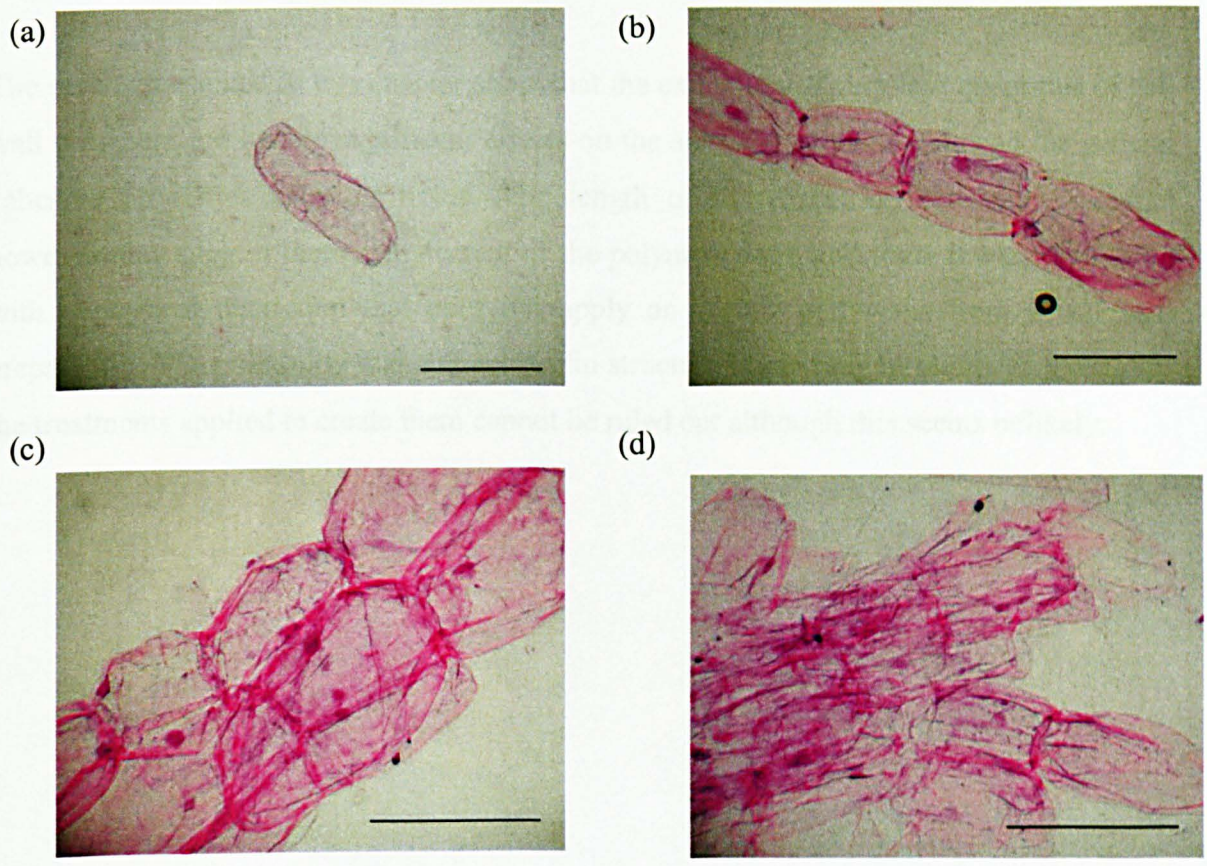


Figure 3.7 (a - d). Mung bean hypocotyl tissue was subjected to a multi-step pectin extraction sequence as described in section 3.2.3 and the resulting cells stained with toluidene blue. Figure **(a)** shows a single pith cell. **b)** File of Pith cells. Scale bar is 250 μm. **(c)** Pith cells which have remained in an adherent state along transverse and longitudinal walls. Scale bar is 100 μm. **(d)** Aggregated pith cells that have remained intact. Scale bars are 250 μm. (a) & (b) and 100 μm. (c) & (d).

3.7 Conclusions

The results presented in this chapter show that the extraction of very low quantities of cell wall polymers can lead to significant effects on the adhesion of plant cells and the general cohesive properties of plant tissue. The length of the extraction procedure required however may suggest that entanglement of the polymers does contribute towards adhesion with a physical restriction that does not apply on normal cell walls from a cell wall preparation. The possibility that a novel pectin structure found only in ghosts as a result of the treatments applied to create them cannot be ruled out although this seems unlikely.

Chapter Four

Enzymic Separation of Hypocotyl Ghost tissue

4.1 Introduction

The role of polygalacturonase (PG) and pectin lyase as cell wall degrading enzymes has been well characterised (Visser and Voragen, 1996). The PG enzyme splits the chain by hydrolysing the glycosidic linkage and pectin lyase splits this chain by the removal of a water molecule.

Initial studies with the enzymes polygalacturonase – *Aspergillus japonicus* and pectin lyase - *Aspergillus niger* indicated that cell separation would occur, with PG in particular separating cells along the longitudinal wall of the cell preferentially. From information supplied with the *Aspergillus japonicus* polygalacturonase enzyme it was noticed that there was a degree of contamination by other enzymes at a level that could possibly contribute towards cell separation. To eliminate the possibility that these impurities contributed to the observed separation it was decided that the experiments would be repeated with a more highly purified PG from *Aspergillus niger*.

Initial studies with the *Aspergillus niger* PG were carried out using the same concentration levels that had successfully induced separation when the *Aspergillus japonicus* enzyme was used. However this led to almost total separation of the tissue into single cells and a high degree of cell breakage. Enzyme activities when being used with 'solid' substrates such as those in hydrated cell walls are much lower than in solution and the magnitude of this discrepancy varies greatly from enzyme to enzyme. This makes prediction of the concentrations required very difficult. A series of experiments gradually reducing the enzyme concentration until cell breakage was minimised and separation was favoured was then planned and undertaken. It was also decided that a similar experiment but with galactanase or arabinanase should be carried out to confirm that the polymers cleaved by these enzymes are not involved in cell adhesion.

4.2 Results and Discussion

4.2.1 The Controls

The main function of the controls was to take account of separation induced by the chelating agents used in the preparation of the enzyme buffer and imidazole. If excess calcium is present then cross-linking of the pectic chains with calcium will increase the level of difficulty in separating the cells. Eliminating this allows the focus to be on what happens to the covalent bonding.

For each control ghost tissue was added to either distilled water or 10 mM EDTA (Sigma Aldrich) buffer at pH 5, identical to that which was used for the enzyme separation experiments. Following the 2 hour incubation at room temperature a 1 ½ hour stirring step in either distilled water or 2 M Imidazole (Sigma Aldrich).

The four controls included were as follows:

(i) 2 hour water incubation followed by a 1½ hour stir in water

(ii) 2 hour water incubation followed by a 1½ hour stir in imidazole

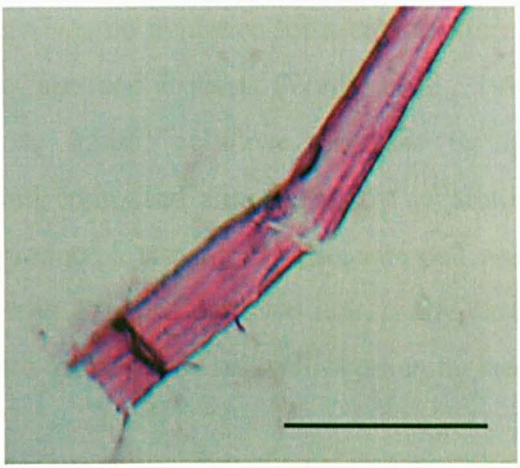
(iii) 2 hour EDTA incubation followed by a 1½ hour stir in water

(iv) 2 hour EDTA incubation followed by a 1½ hour stir in imidazole

As would be expected no separation was observed for control (i). The remaining three controls all exhibited varying degrees of separation and cell breakage.

Controls (ii) and (iii) were most notable for the amount of cell breakage. Evidence was present for the preferential separation along the longitudinal walls as would be expected after treatment with chelating agents. Separation characteristics that have been observed previously, such as the tendency of vascular tissue to remain adhered (figure 4.1 a) and the occurrence of single cells from the pith tissue, were evident in the controls also. However the walls of the cells that have had a water treatment at either stage would appear to be more susceptible to fracture (figure 4.1 b).

(a)



(b)

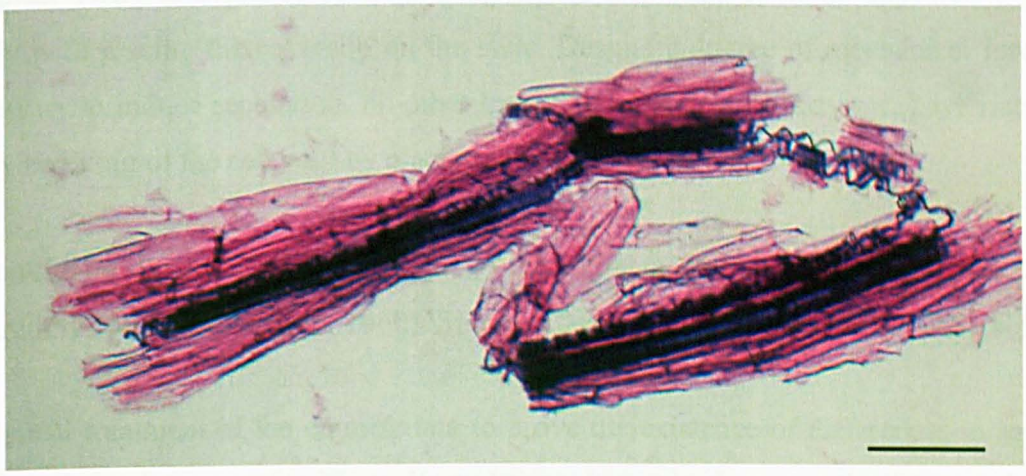


Figure 4.1 (a) & (b). The range ofcontrol treatments are described in section 4.2.1.All control treatments yielded fragments of tissue that had separated with approximately the same characteristics as their enzymically treated counterparts although to a lesser degree and with a great deal more breakage (figure (a) – 2 EDTA incubation followed by a 1 ½ hour stir in imidazole). The vascular tissue separated in the same way as had been observed with the enzymically treated samples earlier but again with a higher degree of cell breakage (figure (b) - 2 water incubation followed by a 1 ½ hour stir in imidazole). Scale bars are 100μm.

Work on the effect of pectins on the mechanical properties of cell walls (Whitney, et al., 1999) suggested that there was no evidence for a contribution by pectin. However this is disputed in work by McCann and Roberts (Wells, et al., 1994). These experiments, in which DCB was used to inhibit cellulose synthesis in the cell wall, allowed the development of a cell wall composed almost entirely of pectin, thus proving that *in this case* it was possible to produce a structurally adequate cell wall and therefore that pectin must have a mechanical role. It is unclear what link, if any, there would be between water treatment and the increased incidence of cell wall weakening leading to fracture.

Control (iv) (2 hour EDTA incubation followed by a 1 ½ hour stir in imidazole) appeared mostly free of breakage with the incidence being no greater than that observed in other samples. There is evidence of weakening of the cell walls as some appeared to fracture easily with placing the coverslip on the slide. Despite a degree of mechanical force being necessary to induce separation, no other treatment, chemical or enzymic, gave rise to such ready breaking of the cell wall by placing the cover slip on top.

As could be expected after treatment by chelating agent, there was evidence of some separation into files (Cocking, 1960). Single cells originating from the pith were also seen.

Statistical treatment of the control data to prove the existence of differences in separation characteristics between specific walls and tissue types in the enzymically treated samples was not necessary as the observable differences were so large. This is illustrated in the figures throughout the chapter.

4.2.2 Pectin Lyase is observed to produce more cell breakage than PG

Throughout the enyme experiments it was consistently observed that the level of cell breakage resulting from pectin lyase treament was higher than that observed after PG treatment. Pectin with a high degree of methyl esterification are found in the cell wall whereas low-ester pectins are typical of the middle lamella and cell junctions eg (Knox, et al., 1990; Roy, 1992; Liners, 1992; Willats, 1999; Parker, 2001). It is the high-ester pectins, located in the cell wall, that Pectin Lyase will attack thus weakening the cell wall. Evidence exists that demonstrates a role for pectin in attaching one layer of cells to another

(Jarvis, 1992) so weakening the wall structure would therefore cause a greater amount of cell breakage as applied forces pull the layers apart.

Polygalacturonase proved more effective at separating cells into files at lower concentrations than pectin lyase (see section 4.2.3 and onwards). As PG attacks pectins with a low degree of methyl-esterification such as those that are thought to be found in the middle lamella this was consistent with expectations.

However, the pectin lyase enzyme contains PG as an impurity. Although the contamination level is very low after dilution, PG is still present at concentrations that have previously been shown to induce separation. Thus the possibility that the separation observed in the pectin lyase treated samples may be due to PG contamination, cannot be eliminated. The calculation below demonstrates the levels of contaminant PG present:

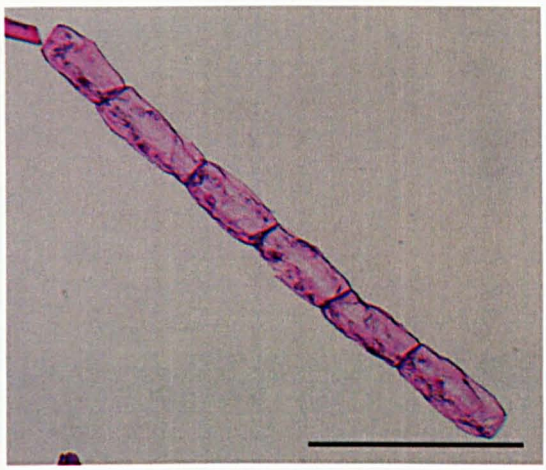
Range of Pectin Lyase Concentrations – 0.001 mg/ml to 0.0001 mg/ml
(0.092 Units/ml to 0.0092 Units/ml)

Concentration of PG in pectin lyase Enzyme = 1.4units/mg of protein (6.7mg/ml)
This can be represented as a concentration level of 9.38 Units/ml
When the pectin lyase is diluted this becomes a range of 0.0014 to 0.00014 U/ml

When PG *(Aspergillus japonicus)* is used at this concentration level separation is observed. This is also the case when the *Aspergillus niger* derived PG is used.

4.2.3 Separation of transverse walls and longitudinal walls is possible with enzymes

Both PG (*Aspergillus niger* and *Aspergillus japonicus)* and pectin lyase (*Aspergillus niger*) induced cell separation preferentially along longitudinal rather than transverse walls. This was confirmed by the presence of files of cells that remain adhered through the transverse walls (figure 4.2). However, single cells present were also observed to have separated rather than broken along the transverse walls so it is possible to conclude that both PG and pectin lyase enzymes are capable of inducing cell separation along both sets of walls given the correct conditions (figure 4.3).



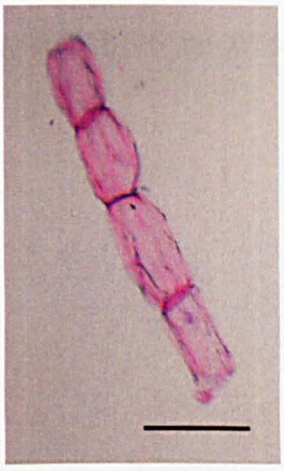
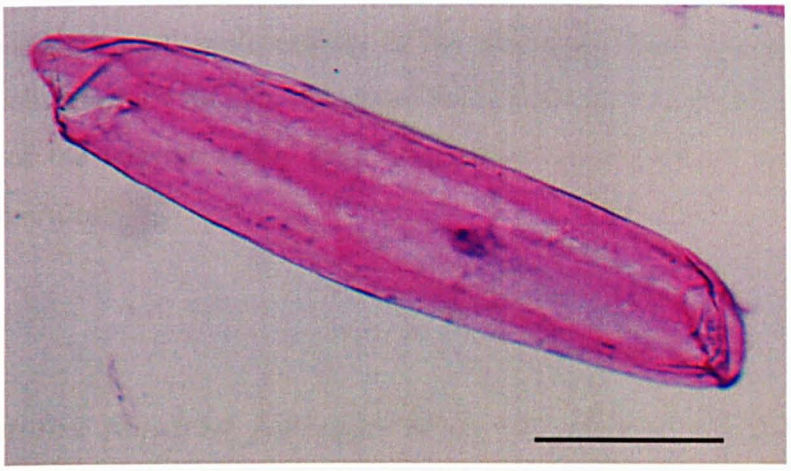


Figure 4.2 (a) and (b). Files of cortical cells obtained by treatment of mung bean hypocotyl ghosts with 10mM EDTA buffer at pH 5 incorporating 1.14 x 10^{-9} U/ml PG (figure a) or 1 x 10^{-5} U/ml PL (figure b) followed by a 2 hour stir immersed in 2M imidazole and then visualised by light microscope. Separation can be observed to have occurred preferentially along the longitudinal walls of the cells resulting in files. Scale bars are 250μm (figure a) and 100μm (figure b).

(a)



(b)

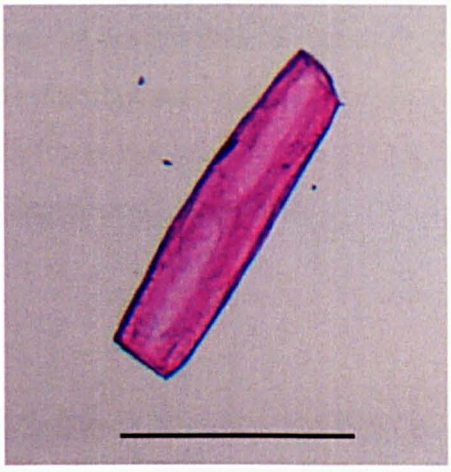


Figure 4.3 (a) & (b). Mung bean hypocotyl ghost tissue that had been treated with with 10mM EDTA buffer at pH 5 incorporating PG (figure a) or Pectin Lyase (figure b) followed by a 2 hour stir immersed in 2M imidazole and then visualised by light microscope. PG (figure a) or Pectin Lyase (figure b), also separated into single cells. This effect was observed both at high concentrations where separation into files did not occur and at concentrations where separation into files appeared to be favoured (cells show here were obtained by treatment with enzymes at concentrations of 1.14×10^{-9} U/ml PG (figure a) or 1×10^{-5} U/ml Pectin Lyase). Scale bars are 100μm.

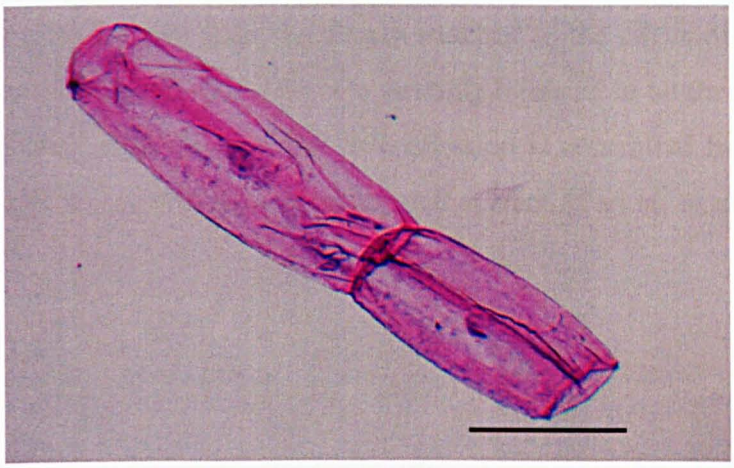
Results obtained from separation experiments using the more highly purified *Aspergillus niger* derived PG are used preferentially in the following sections although separation characteristics observed are broadly the same. This allows for the results to be attributable to the action of the enzyme rather than introducing an element of doubt through the presence of impurities.

4.2.4 Conditions required for separation are almost as severe as those inducing breakage

Separation (figure 4.4 a) and breakage (figure 4.4 b) can be seen to have occurred at every enzyme concentration used over an extremely wide range (4.15×10^{-9} U/ml to 0.415 U/ml - PG). However concentrations that favoured cell separation, most notably 4.15×10^{-8} U/ml to 4.15×10^{-6} U /ml had much lower incidences of breakage, although breakage of the cells was still observed to a small degree in all treatments.

4.2.5 Toluidene Blue staining is enhanced along the tricellular junction

One recurring observation was the 'tramline' like markings resulting from enhanced toluidene-blue staining along the cell edges. The toluidene blue dye attaches to anionic pectins and the darker colour that gives rise to the 'tramline' patterns observed indicates that these areas are of particularly high charge density. This observation is consistent with previously published data speculating that, in TEM these regions, represent sections of almost continuous arcs along the cell faces (Kolloffel and Linssen, 1984) and also published data showing 'tramlines' visualised by UV antofluorescence along the edges of the cell faces.



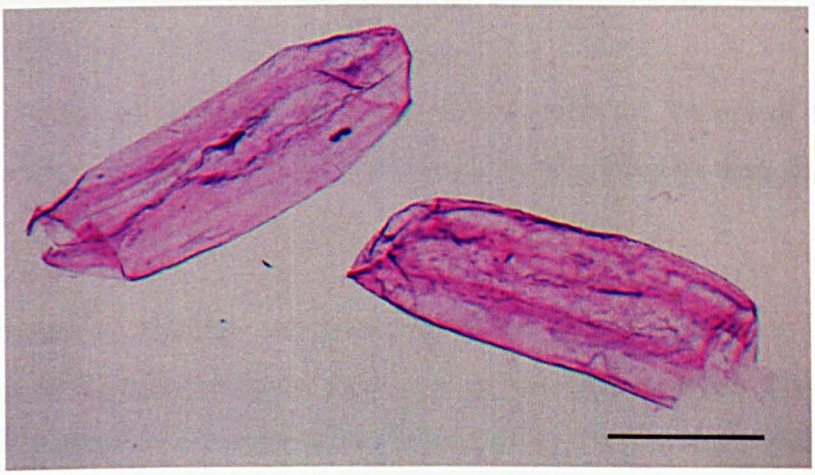


Figure 4.4 (a) & (b). Figure (a) clearly shows cells which have separated and figure (b) those which have broken apart as a result of the combined action of the enzyme (cells shown here were treated with 1.14 x 10^{-9} U/ml PG) and the 2 hour stirring in 2M imidazole (chelating agent). Although some incidences of breakage can always be detected, they are far less prevalent separation at concentrations where separation is favoured. Scale bars are 100μm.

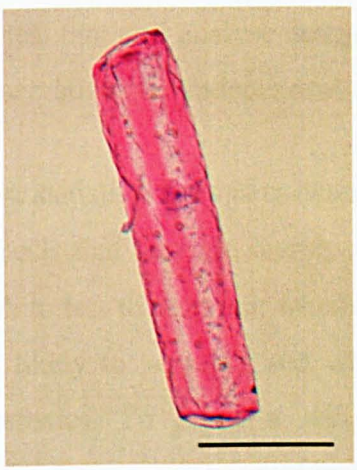
The enhanced-staining effect is particularly evident in single cells (figure 4.5 a) and also within files (figure 4.5 b). In figure 4.5 c – flattened out cell wall – a cell that has fractured allows the areas with increased staining to be easily observed. After anti-body labelling with JIM5, which reacts with pectins with a low degree of esterification, Parker *et al* observed patterns depicting the edges of the cell faces. They also observed a 'flap' of what appeared to be pectic material that was firmly attached to the edges of the cell but poorly attached to the rest of the cell face. This was strongly believed to be the middle lamella and is therefore consistent with the idea that cell adhesion is controlled from the cell corners rather than through the middle lamella at the cell surface (Parker, et al., 2001 and papers therein).

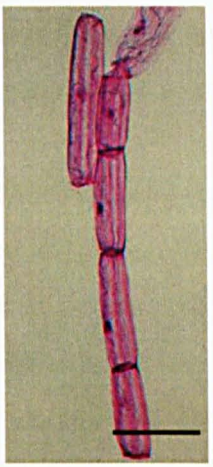
4.2.6 Anatomical differences in adhesion characteristics exist

Identification of the various cell types was carried out with the aid of the information presented in section 1.6 (Introduction). Cells could be identified by their shape and relative dimensions.

The small amount of vascular and epidermal tissue present relative to cortex and pith meant that statistical analysis was not possible on those tissue types. There were enough examples of pith and cortex tissue types to be able to obtain meaningful statistical results.

(a)





(b)

(c)

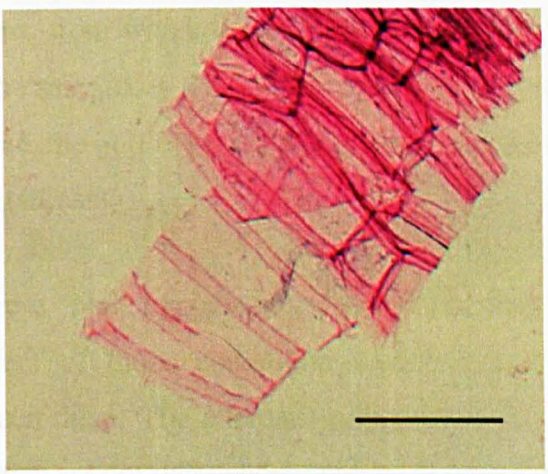


Figure 4.5 (a - c). Cells from a mung bean hypocotyl after treatment with 10mM EDTA buffer at pH 5 incorporating 1.14 x 10^{-9} U/ml PG followed by a 2 hour stir immersed in 2M imidazole and then visualised by light microscope. Figure (a) shows the enhanced toluidene blue staining along the cell corners in a single cell and the same effect in a file of cortical cells (figure b). Figure (c) shows a cell that has become flattened out and the staining on the corners can be observed clearly. Scale bars are 100μm.

4.2.6.1 Statistical Tests

This section aimed to test for statistically significant differences in separation characteristics of different tissue types and specific walls of the cell. The most appropriate was a Chi-squared test that will analyse categorical data. This is a non-parametric test that assumes that all observations are independent of other observations in the study.

The purpose of most statistical tests is to determine if the obtained results provide a reason to reject the hypothesis that they are merely a product of chance factors. For example, in this section we wish to test to ascertain whether walls of a certain type (i.e. longitudinal or cortical) are more likely to separate and whether different tissue types show differing separation characteristics. To prove a statistically significant difference it is always necessary to ask if the difference observed is among the differences that would be expected to occur by chance whenever two groups are randomly selected. Another way to say this is to assert that the investigator tests the null hypothesis that the difference between the means of the populations, from which the samples were drawn, is zero. If the difference between the means of the samples is among those that would occur rarely by chance when the null hypothesis is true, the null hypothesis is rejected and the investigator describes the results as statistically significant.

For this test we assume a 'Null hypothesis', denoted H_0: that the results described are independent of an effect of those conditions described and that no relationship or association exists between them. The alternative hypothesis H_1: a relationship does exist between them and that they are not independent will be assumed if H_0 is rejected.

An additional value known as phi is also calculated from the data obtained from the Chi-squared test. This is a 'power' value and gives an indication of how strong any association indicated by a chi-squared test is. It is calculated using the following equation:

$$\phi = \sqrt{(\chi^2 / N_{total})} \qquad \text{Equation 4.1}$$

The resulting value is between 0 and 1 with 0 indicating no association and 1 if there is perfect association. The scale generally used is 0.1, 0.3 and 0.5 to indicate small, moderate and large effects.

In both the pith and cortical tissue the condition tested was for the existence of an association between the type of connection between cells, i.e. through longitudinal or transverse walls of the cell and the likelihood of cells remaining attached to others through the connection of this type.

A separate treatment to investigate if the type of tissue affected whether cells from pith or cortical tissues were likely to remain connected through transverse or longitudinal walls was also considered.

For all treatments the notation used was the same. 'Longitudinal' relates to the walls of the cell that have formed by elongation and 'transverse' denotes the cross walls of the cell which are formed by the insertion of a cell plate and construction of the wall around this. In the case of the term 'none', this means no connections remained between cells through the walls investigated. 'Some' denotes a situation where connections existed between the walls being investigated

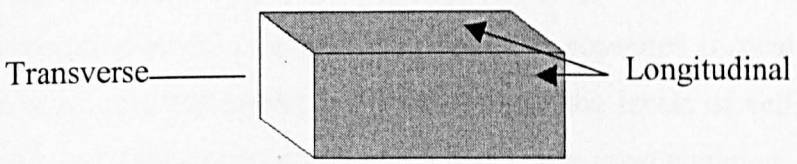


Figure 4.6. Diagram of a single cell with notation used for denoting walls as used in the text.

4.2.6.2 Hypothesis applicable to both Cortical and Pith Tissue

The aim is to assess whether the type of connection between cells influences if cells are likely to remain attached or not. For example, in cortical cells is it more likely that files will form through transverse walls or cells will have no transverse connections whatsoever?

The hypothesis to be tested is as follows;

H_0 : Differences are not observed in the number of cells remaining transverse/longitudinally connected other than those which can be reasonably expected due to chance rather than any increased likelihood of the cells to remain adhered through one type of wall or the other.

H_1 : Differences observed must be due to factors other than just chance variation

In both the cortical and pith tissue separation was more likely to occur along longitudinal walls

4.2.6.3 The Results and their Interpretation

Question 1: Does the pair of cell walls involved (i.e. longitudinal or transverse) influence whether or not cells remain attached after treatment designed to induce separation?

The Cortex

Cells originating in the cortex were similar in shape to the pith cells but smaller. As was also observed to be the case with the chemically separated cortical cells, once the enzyme concentration that minimised cell breakage and the levels of cells remaining aggregated was optimised, separation into files (figure 4.7) was most common.

Here we consider whether any difference is present, and hence an association existing between, the transverse and longitudinal conditions and the existence of adherent cells.

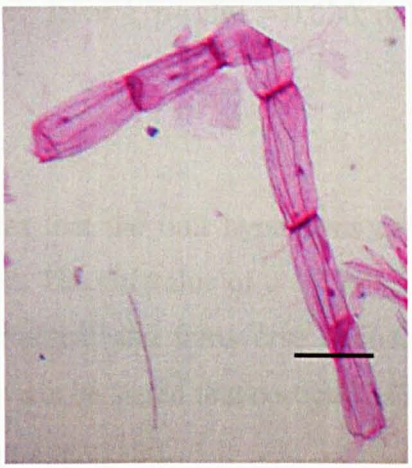


Figure 4.7. Cortical cells treated with 10mM EDTA buffer at pH 5 incorporating 1.14 x 10^{-9} U/ml PG followed by a 2 hour stir immersed in 2M imidazole appear to separate into files more easily than other tissue types and files also tend to contain more cells. Scale bar is 100μm.

Summarised results

Chi-squared statistic = 51.867, DF = 1, p-value = 0.000, phi = 0.66

A p-value of 0.000 indicates that the null hypothesis can be rejected indicating that an association does indeed exist. The phi value of 0.66 indicates a large degree of association between the cases of longitudinal and transverse connections and whether or not cells remain adhered. Therefore it can be stated that cortical cells are more likely to separate into files than single cells or other aggregates.

The Pith

Pith cells could be identified by their size, which was far greater than all other cell types and more commonly separated into single cells (figure 4.8 a). However occasionally files were seen (figure 4.8 b). This is in contrast to cells separated chemically where the pith remained mainly aggregated.

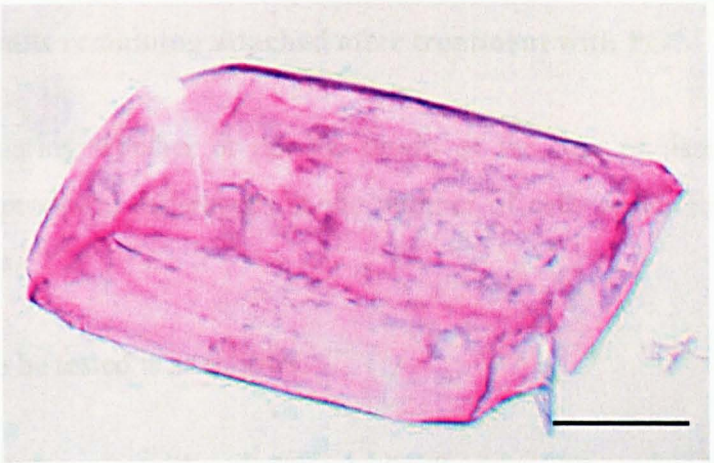
The relevant statistical question in the case of the pith tissue is identical to that described previously for the cortical tissue except with the data pertaining to the pith cells substituted for the cortical cells.

Summarised results:

Chi-squared statistic = 31.787, DF =1, p-value 0.000, phi = 0.44

These results can be interpreted similarly to those for the cortical data except with the association between each condition being at a moderate level (phi of 0.44).

(a)



(b)

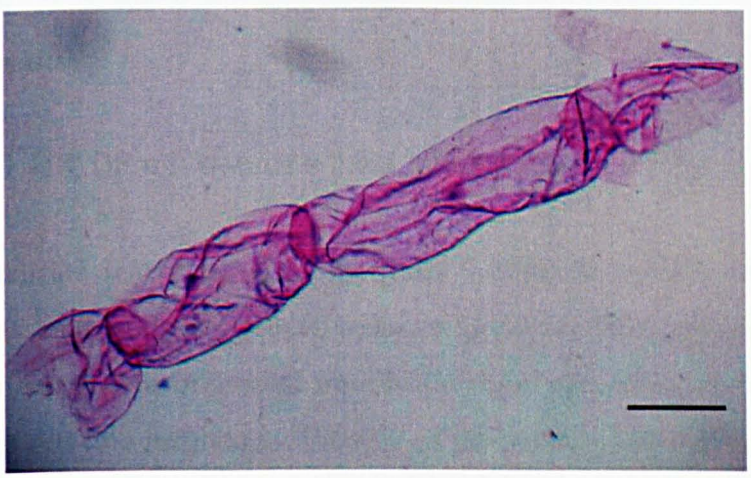


Figure 4.8. More commonly found as single cells after enzyme treatment (10mM EDTA buffer at pH 5 incorporating 1.14 x 10^{-9} U/ml PG) followed by a 2 hour stir immersed in 2M imidazole (figure a), pith cells are occasionally found in short files (figure b). Scale bars are 100μm

Question 2: Does the type of tissue involved make any difference to the likelihood of the transverse walls remaining attached after treatment with PG?

Hypothesis comparing whether or not the origin of the type of tissue being separated, cortical or pith, produced differences in the number of cells remaining adherent through longitudinal walls.

The hypothesis to be tested is as follows:

H_0 : there is no difference in the likelihood of cells from pith or cortical tissue to remain adhered through transverse sections

H_1 : such a difference exists

Summarised results:

Chi-squared = 11.718, DF = 1, p-value = 0.001, phi = 0.29

These results indicate that there is a significant difference in cells remaining adhered through the transverse walls in cortical verses pith tissue. This implies that the type of tissue that the adherent cells originated from had a significant effect on whether or not the cells remained joined to a neighbour. The size of phi implies that this difference is small, however. From observed data, transverse connections are more likely to separate in the pith tissue than cortical. Therefore H_0 can be rejected.

Combining this information with experimental observations then it would be possible to conclude that cortical cells are slightly more likely than pith tissue to remain adhered through transverse walls after treatment with PG.

Question 4: Does the type of tissue involved make any difference to the likelihood of the longitudinal walls to remain attached?

Hypotheses comparing whether or not the origin of the type of tissue being separated, cortical or pith, produced differences in the number of cells remaining adherent through longitudinal walls

H_0 : there is no difference in the likelihood of cells from pith or cortical tissue to remain adhered through longitudinal sections

H_1 : there is a difference

Summarised Results:

Chi-squared = 0.215, DF = 1, p-value = 0.643

From the data above it is possible to conclude that there is no significant difference in the behaviour of cells from the cortex or pith with regard to their separation characteristics along longitudinal walls. Whether or not the cells originate in the cortex or pith has no significant difference in their response to the separation-inducing treatment along the longitudinal walls of the cell; it is equally as likely in both cases.

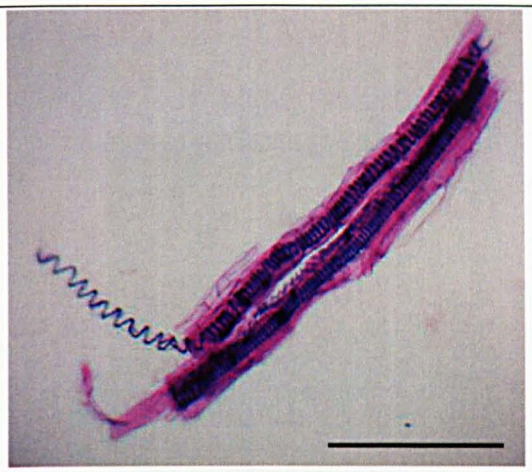
4.2.6.4 Vascular Tissue

Cells around the vascular region appeared more resistant to separation and were easily identifiable by the extreme heterogeneity or the aggregates observed. As previously mentioned even at the higher enzyme concentrations that had resulted in the complete separation to single cell level of pith and cortical tissue, aggregates could be observed (figure 4.9).

4.2.6.5 The Epidermis

Epidermal cells were observed to be relatively small and very densely packed with no visible intercellular spaces. In both chemical and enzymic experiments epidermal cells were be seen to behave quite differently and distinctly to all other cell types. Unlike epidermal tissues which have been separated by the chemical sequential extraction procedure where large, almost completely intact sheets of cells were generally observed, epidermal tissue subjected to enzymic separation conditions displayed a propensity to separate into much smaller fragments (figure 4.10 a) even under relatively severe enzymic conditions. Epidermal fragments were only one layer of cells thick and never observed to remain attached to the layer of cortical cells below, indicating that if they were ever in an

(a)



(b)

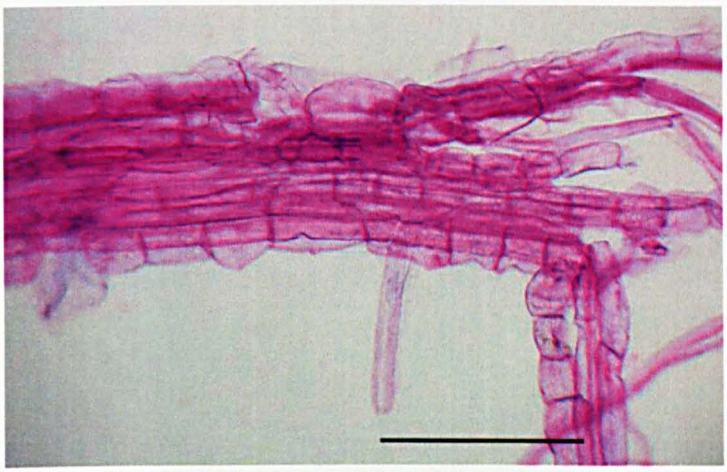
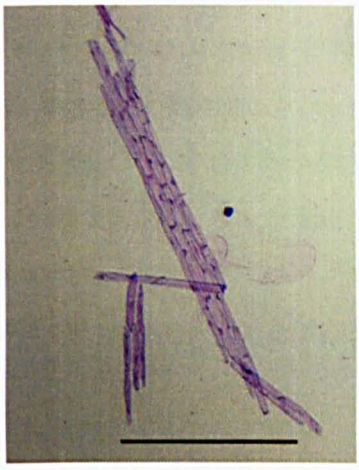


Figure 4.9 The result of mung bean hypocotyl tissue after treatment at any of the enzyme concentrations, 0.415 – 4.15x 10^{-9} U/ml, of PG made up in a 10mM EDTA buffer at pH 5 and then stirred for 2 hours in 2M imidazole, indicated that vascular tissue was more resistant to separation than tissue of other types. Only the epidermis was similar in its reluctance to separate. Scale bars are 250μm.

(a)



(b)

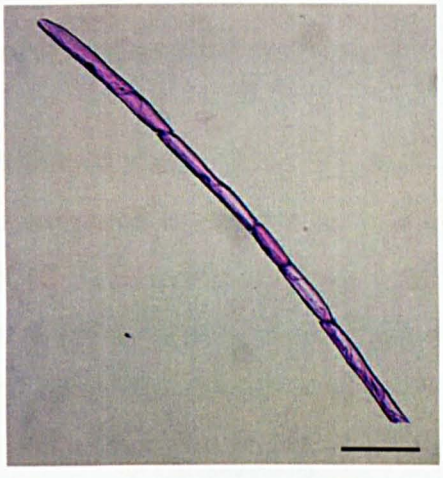


Figure 4.10 (a) & (b). Treatment of epidermal tissue from mung bean hypocotyl ghosts with 10mM EDTA buffer at pH 5 incorporating 1.14 x 10^{-9} U/ml PG followed by a 2 hour stir immersed in 2M imidazole resulting in epidermal tissue that appeared mainly to fragment (figure a) but occasionally separate into files (figure b). Scale bars are 500μm (figure a) and 100μm (figure b).

adherent state, then these links were first to break. This behaviour is similar into that observed in chemically separated ghosts.

When files of epidermal cells were seen to separate from the rest of the epidermal sheet, longer files were observed than those originating from other types of tissue at the same enzyme concentrations (figure 4.10 b). It was notable that whereas files of cells and intact fragments of epidermal tissue could be observed, under identical conditions the pith and cortical tissues were mainly reduced to single cells. Under these conditions vascular tissue was also significantly fragmented but remained adhered to a greater degree than that of the pith and cortex.

4.2.7 Do Galactanase or Arabinanase Influence Cell Separation?

In order to compare the contribution made, if any, by galactan or arabinan to cell adhesion, mung bean hypocotyl ghosts were treated with 4 x 10^{-7} U/ml galactanase *(Aspergillus niger* – Megazyme, Ireland*)* or 4 x 10^{-7} U/ml arabinanase *(Aspergillus niger,* Megazyme, Ireland*)* made up in the same buffer as previous enzyme treatments (10 mM EDTA at pH 5) and stirred in 2 M imidazole for 2 hours. The concentrations were chosen to be similar to those which had been used with PG and proven to achieve separation. To ascertain whether either of the enzymes had an effect over and above that of the mild chelating effects of the buffer solution or the stronger effect of the stirring in imidazole, the results were compared with those of the control condition which was identical except for the omission of the enzyme.

The comparison of the treatment including the galactanase or arabinanase enzyme with the control showed no discernable difference between either enzyme and the control. Both types of treatment gave rise to separation of cells rather than breakage in most cases. Cells that had separated had done so in a similar manner to those treated with polygalacturonase although this occurred to a lesser degree. In the case of galactanase vascular tissue remained largely adherent with no discernable patterns observable when separation did occur. However some separation did occur in the vascular tissue treated with arabinanase although this was not true for every instance.

4.3 Enzyme-induced Cell Separation using Arabidopsis Mutants and Wild Type

4.3.1 Mutant Selection

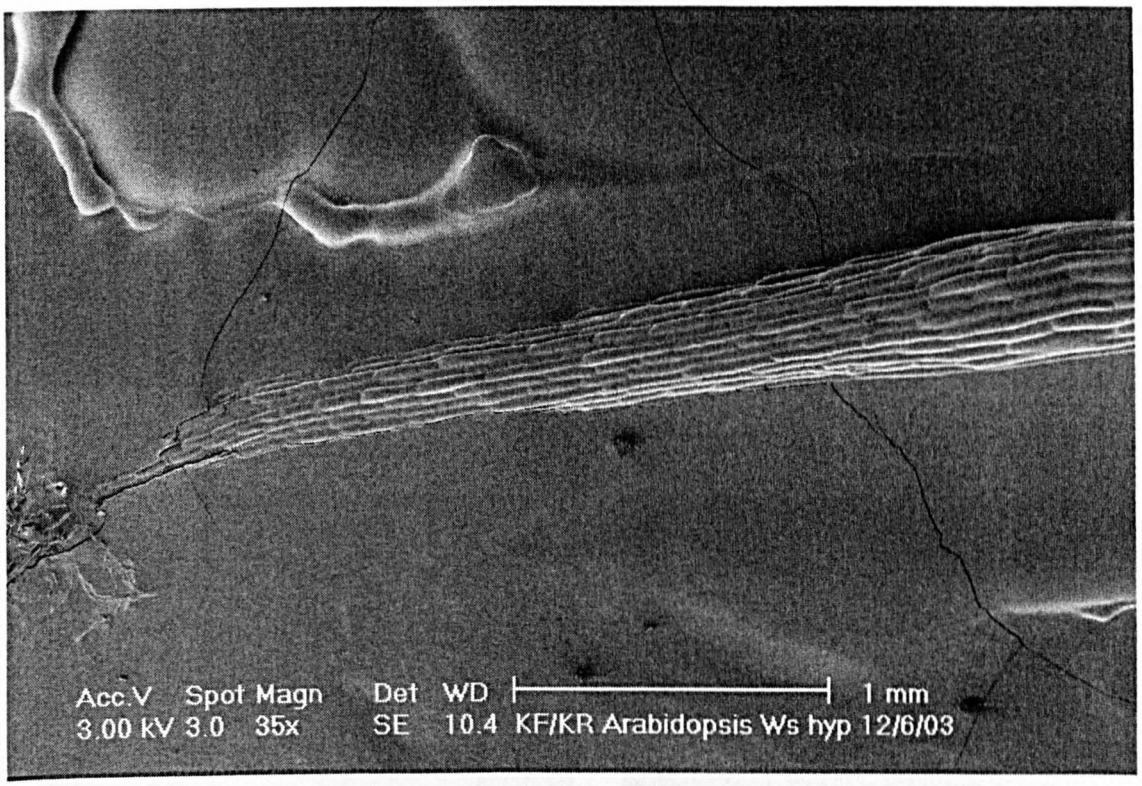
Arabidopsis mutants, *snakeskin* and '*things fall apart*' are known to exhibit a cell adhesion phenotype. They were chosen, along with their corresponding wild types, WS and Columbia respectively, to investigate the effects of polygalacturonase on cell-cell adhesion. (*Arabidopsis thaliana* wild type and mutant seeds were kindly provided by Professor Keith Roberts, John Innes Centre, Colney Lane, Norwich).

No published data is available on the specific details of the mutants and their abnormal adhesion characteristics. Electron microscope images (Figures 4.11 – 4.14) clearly show defects in adhesion in both mutant varieties.

Figure 4.13 a shows a WS hypocotyl. The cells can be seen to be firmly adhering to those adjoining it along both the transverse and longitudinal walls with no gaps or evidence of breakage apparent. This is confirmed in the higher magnification image (Figure 4.11 b). A comparison, however, of the *snakeskin* mutant with its wild type (WS) highlights significant differences (figure 4.12). The cells appear smaller and more rounded and of an irregular shape. Cell-cell adhesion is disrupted between both the transverse and longitudinal walls although this only is apparent in places. Cells that can be seen to have separated at the transverse walls showed no evidence of having broken apart: the cell is still intact and no holes or broken edges are apparent (figure 4.12 c & d).

The wild type, *Columbia* hypocotyl exhibits regularly shaped, firmly attached cells (figure 4.13). However, as is similar to the case described previously, the mutant *things fall apart* can be observed to have impaired cell-cell adhesion (figure 4.14). At higher magnification (figure 4.14 b) the transverse walls can be seen to have separated and large gaps are visible between the transverse wall of one cell and that of the next. Adhesion between longitudinal walls appears to be unaffected.

(a)



(b)

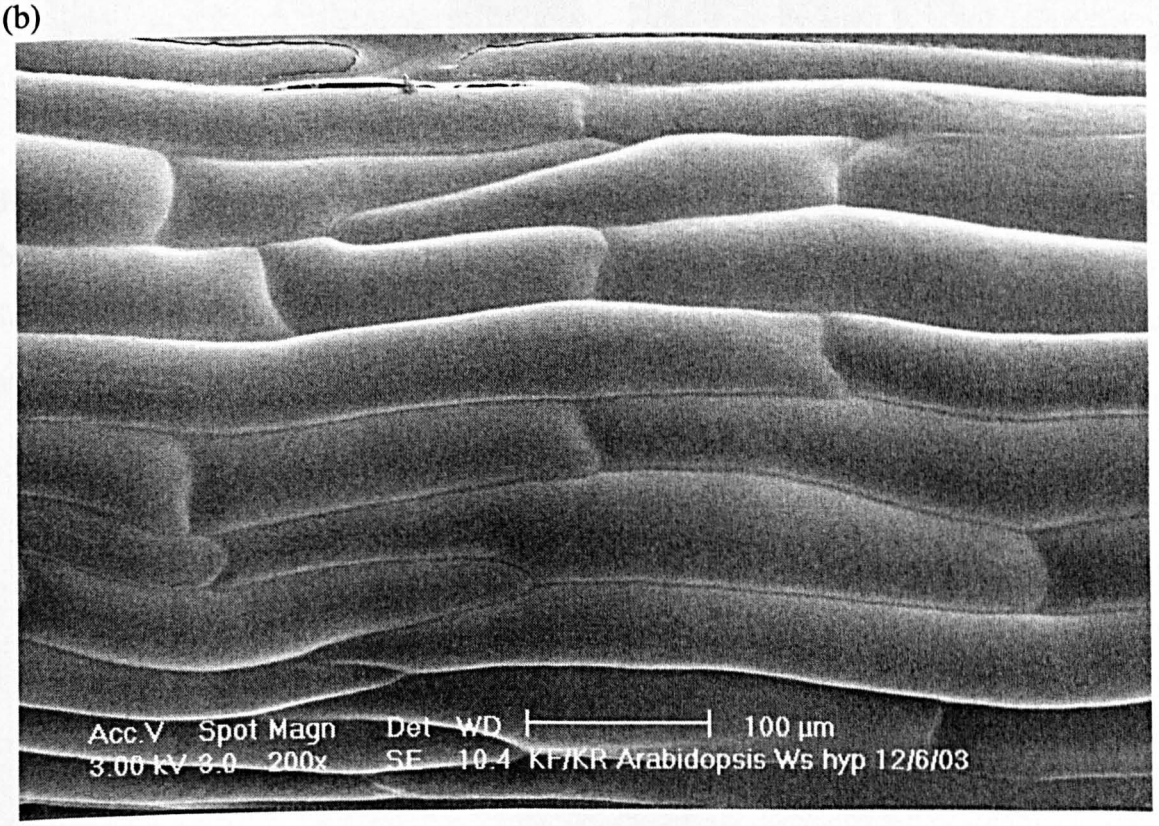


Figure 4.11 (a) and (b). Figure (a) shows a single Arabidopsis WS hypocotyl imaged by SEM at x 35 magnification. Figure (b) shows an Arabidopsis WS hypocotyl imaged by SEM at x 200 magnification. Detailed SEM image showing the adhering cells of the WS hypocotyl. Scale Bars are 1mm and 100µm.

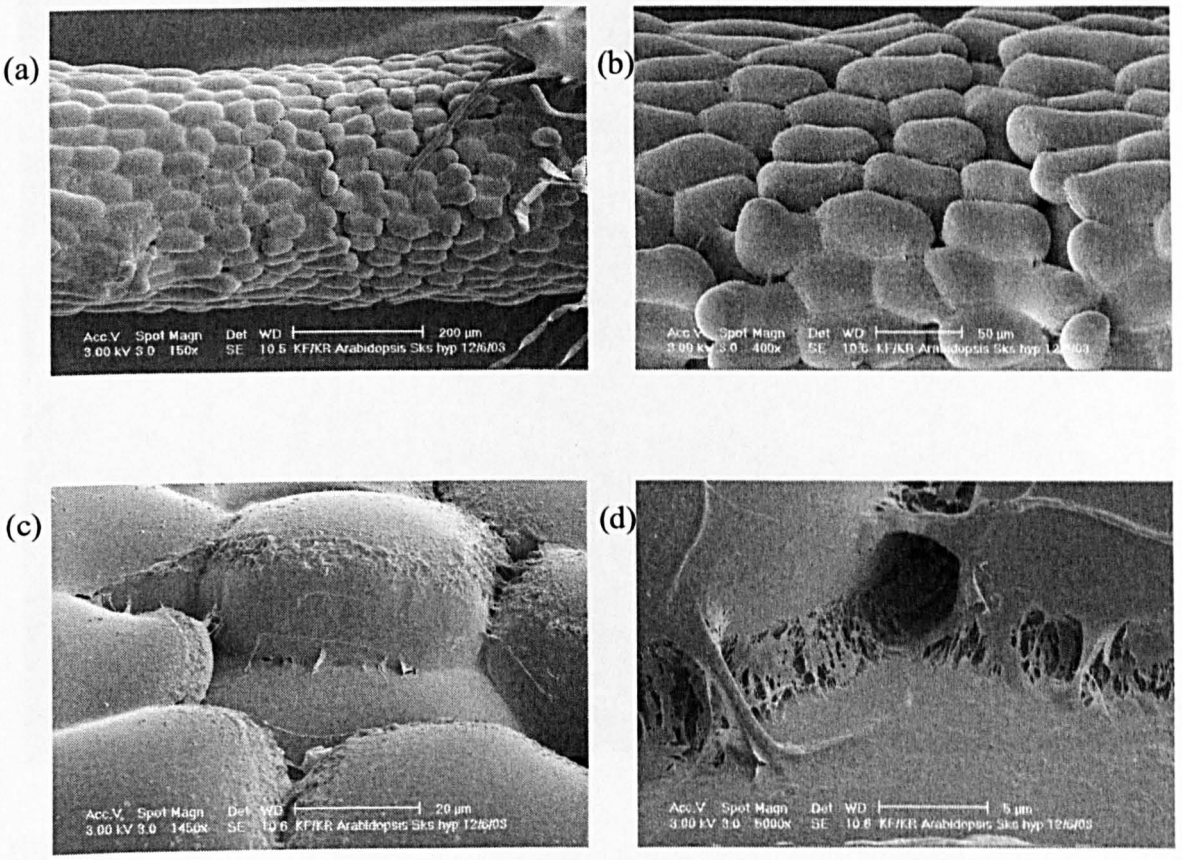
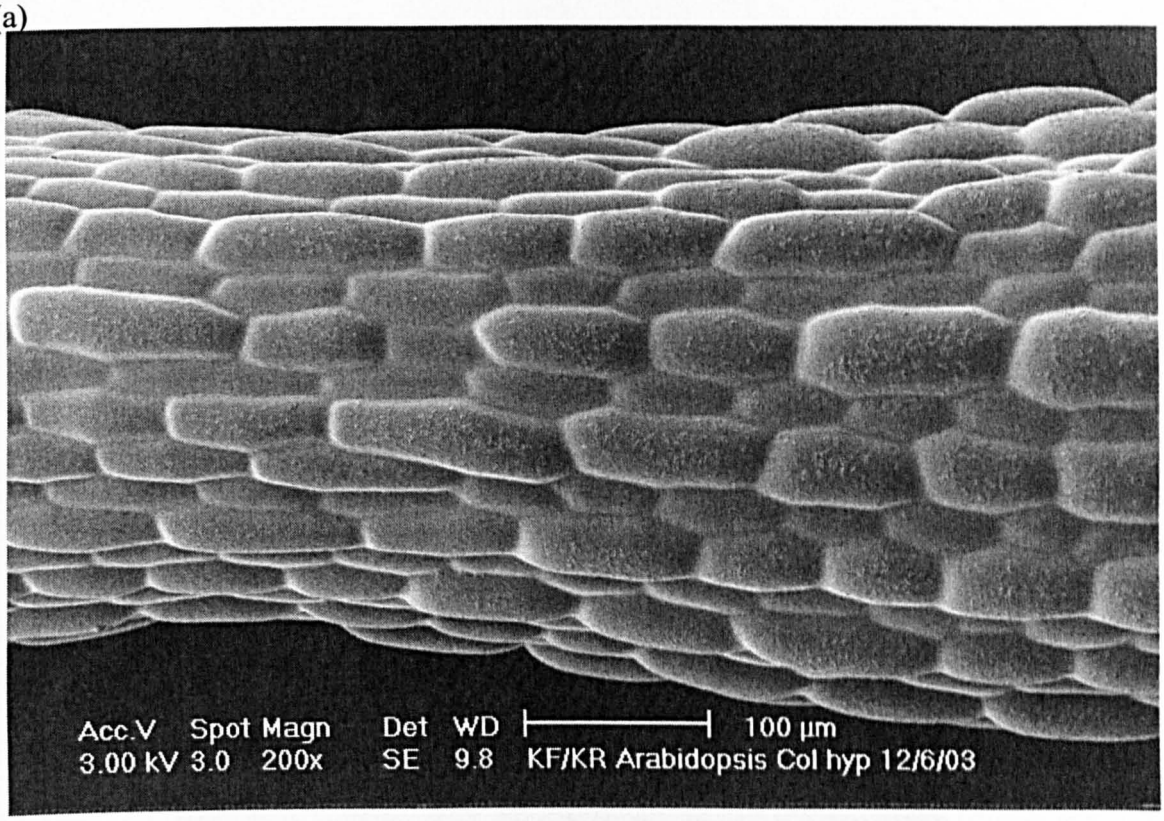


Figure 4.12 Figure showing (a) *snakeskin* hypocotyl at increasing magnification. Adhesion between to cells can be seen to be abnormal and irregular with neither transverse or longitudinal adhesion being more likely to fail. Scale bars are 200 μm (a) 50 μm (b) 20μm (c) and 5 μm (d).

(a)



(b)

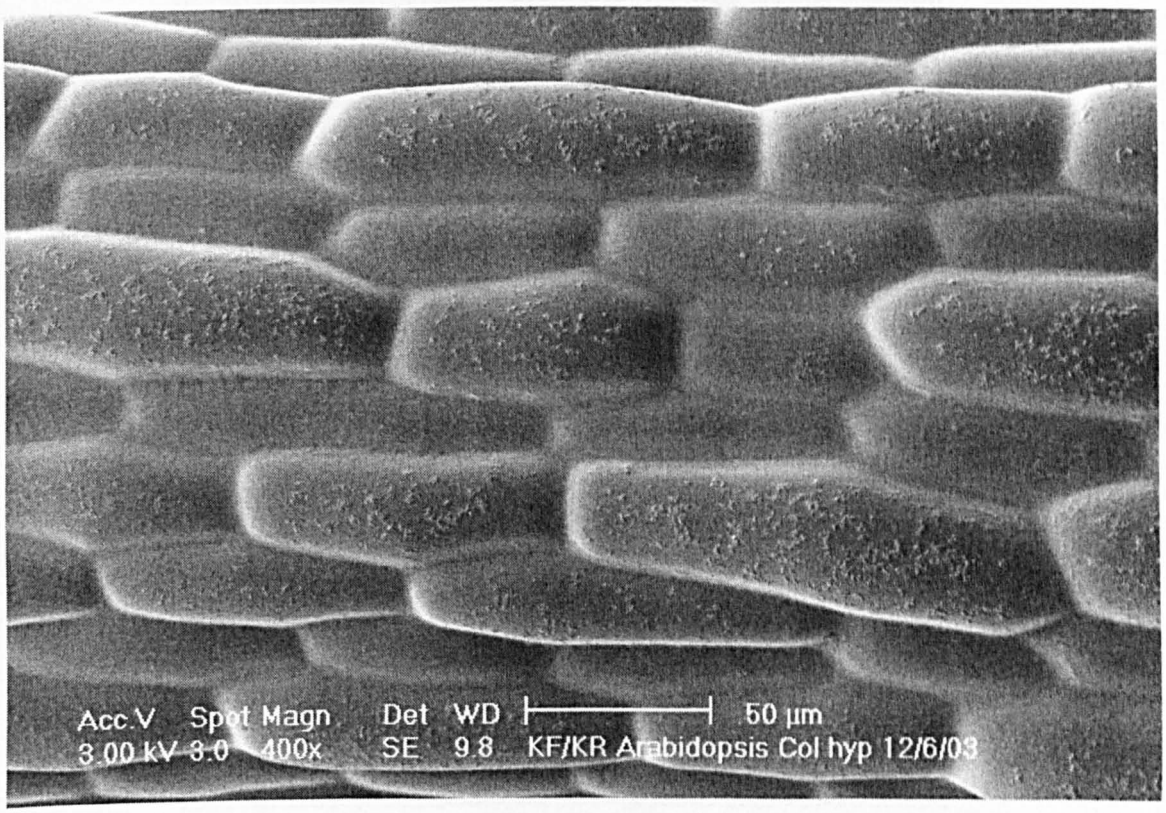
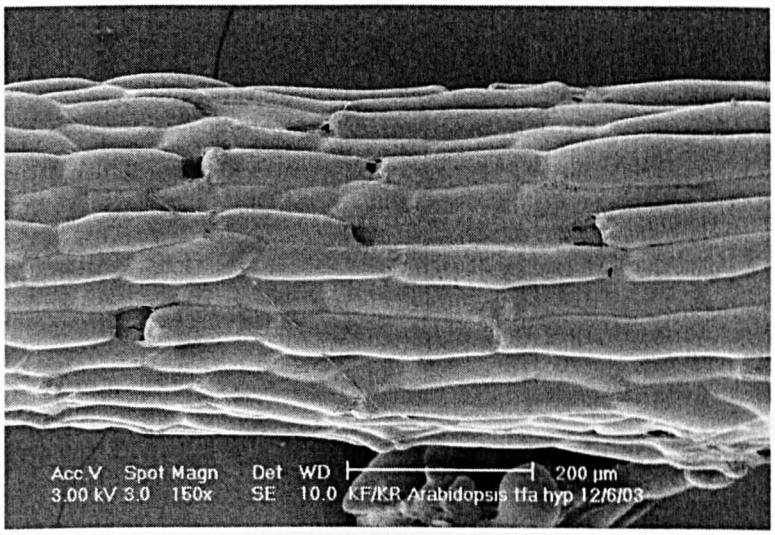


Figure 4.13 (a) & (b) Arabidopsis Columbia hypocotyl imaged by SEM at x 200 magnification. Scale bar is Arabidopsis Columbia hypocotyl imaged by SEM. Scale bars are 100µm and 50µm respectively.

(a)



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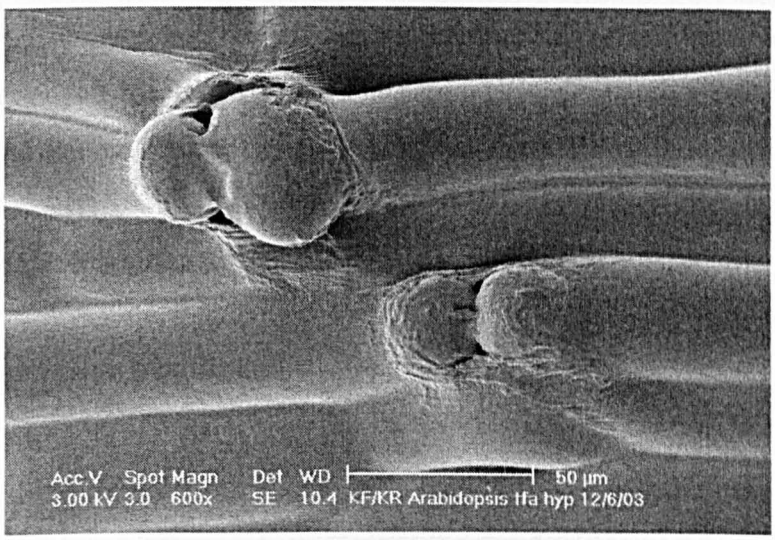


(c)

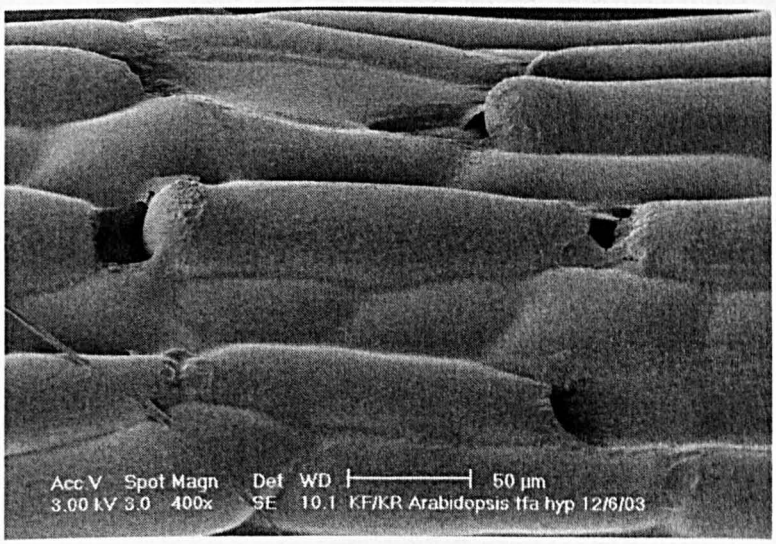


Figure 4.16 (a – c). (a) Arabidopsis *TFA* (Things Fall Apart) Hypocotyl imaged by SEM at x 150, (b) x 600 and (c) x 400 magnification. Scale bars are 200µm 50µm and 50µm respectively.

4.3.2 Adapting the Method

4.3.2.1 Preparation of Ghosts

The original method for the preparation of ghosts involved cutting the mung bean hypocotyls in half lengthways and then cutting a 1 mm thick section just above this cut. The aim of this was to remove the epidermis as a barrier to infiltration of the enzyme. However, the much smaller scale of the Arabidopsis hypocotyl made cutting sections of this sort by hand almost impossible. It was discovered that it was possible to cut the hypocotyl in half lengthways, but the amount of time this took compared with the amount of material generated made this method unsatisfactory. A comparison was made treating both whole and halved hypocotyls with enzyme levels that proved effective in initiating separation (see next section). No difference was observed in the degree of separation achieved therefore it was decided to use whole hypocotyls.

4.3.2.2 Separation Conditions

Experiments using mung bean hypocotyls had previously been carried out in conical flasks. However, the smaller scale necessary for the Arabidopsis experiment meant that a smaller container was required and Eppendorf microcentrifuge tubes proved suitable for this purpose.

The range of concentrations previously found to be successful in obtaining separation of mung bean hypocotyls was investigated (4.15×10^{-9} U/ml to 0.415 U/ml - PG) and, after careful examination and comparison under the light microscope, 4.15×10^{-8} U/ml was selected as the concentration providing the optimum level of separation and minimal cell breakage.

An essential part of the original mung bean method called for the input of mechanical force to assist in the separation of the cells. This was originally done by means of stirring using a stirrer bar in the imidazole solution. As it was not possible to put a stirrer bar into the Eppendorf tube an alternative was investigated and an Eppendorf Micropestle found to give the optimum amount of agitation to result in separation with breakages minimised.

4.3.3 Results

The following results are a qualitative indication of separation characteristics in wild types and mutants.

4.3.3.1 Columbia and TFA

The separation characteristics observed for the mutant and its wild type varied dramatically. In the wild type – Columbia – separation into files generally appeared to occur readily (Figure 4.15 a). As the whole hypocotyl had been treated with enzyme, many different cell types were present. The section of the hypocotyl above the roots and below the cotyledons was most likely to separate into files while cells in the root appeared to barely separate at all (Figure 4.15 b). Cells in the leaf could be seen to be separating but this appeared random and lacking in any pattern (Figure 4.15 c). Vascular tissue also appeared to separate readily although insufficient quantities could be observed to give an indication of any trends (Figure 4.15 d).

Contrasting with the behaviour of Columbia, Things Fall Apart showed very little tendency to separate into files and many single cells were visible along with a large number of transversely adhering pairs of cells (Figure 4.16 a). The root tissue remained mostly intact with some small amounts of separation (Figure 4.16 b). The leaves showed some degree of separation but, similarly to the wild type, no pattern to the separation could be observed (Figure 4.16 c).

4.3.3.2 WS and Snakeskin

The WS hypocotyl in the region above the roots and below the cotyledons appeared to separate preferentially along the longitudinal walls of the cells. The roots were mostly resistant to separation and this held the hypocotyl together giving a 'frayed' look. Files of cells could be seen here. There appeared to be little separation in the leaves.

The snakeskin mutant showed very different separation characteristics. No overall pattern for any tissue type could be seen.

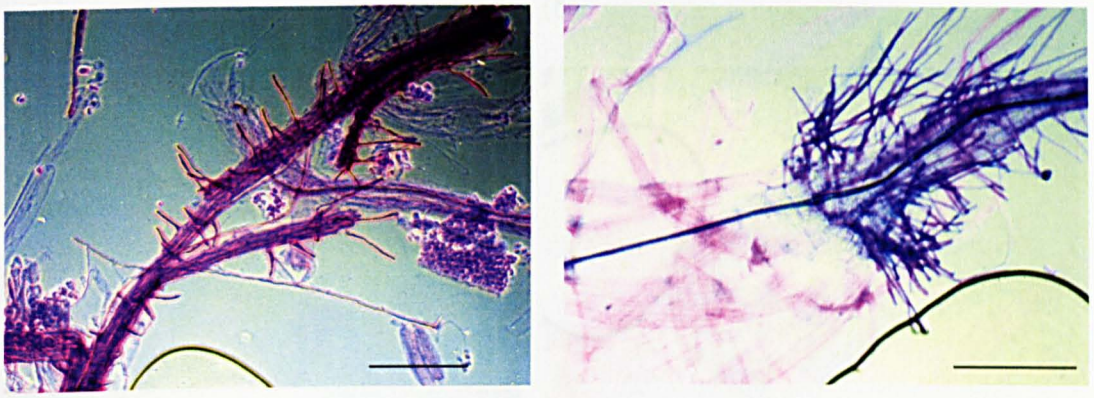


Figure 4.15 a and b. Figure (a) shows root tissue from the Arabidopsis wild type Columbia after treatment with 4.15 x 10^{-9} U/ml Polygalacturonase in 10mM EDTA buffer at pH 5 and 2 hour stirring in 2M imidazole. No separation is observed. Figure (b) shows the part of the hypocotyl above the root after the same treatment. Here separation into files appears to be common. Scale bars are 100 μm and 250 μm respectively.

(a) (b)

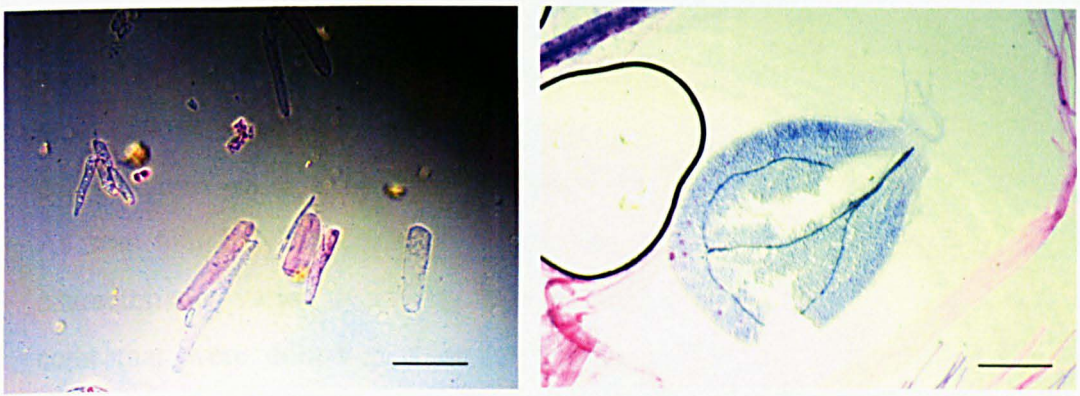


Figure 4.16 (a) and (b). Figure a shows cells from the Arabidopsis mutant *things fall apart* after treatment with 4.15 x 10^{-9} U/ml Polygalacturonase in 10mM EDTA buffer at pH 5 and 2 hour stirring in 2M imidazole. One feature of this experiment was the cell pairs that remained attached through the transverse walls. This appeared to be unique as it was not observed in any other experiments with such frequency. Figure b shows that some degree of separation occurred in the leaf tissue although there was no obvious pattern to this. Scale bars are μ100m.

4.4 Conclusion

In this chapter it was shown that enzymes were capable of producing separation in cells and could do so preferentially with respect to developmental origin. Pectin lyase which targets the highly methyl-esterified pectins such as those found in the wall gave rise to more cell breakage whereas PG, known to attack pectins found in the middle lamella and at cell junctions which exhibited a low degree of esterification, resulted in more files of cells. As was found in the chemical treatements described in the previous chapter, adhesion characteristics varied depending on both the nature of the wall involved and the type of cells that were adhering. Cortical tissue was found to most readily separate along longitudinal walls to result in long files of cells. Pith tissue was observed to behave similarly but to a lesser degree. These qualitative observations were confirmed by the statistical analysis.

Work duplicating the experiments with the inclusion of galactanase or arabinanase instead of PG or pectin lyase found that these enzymes had no effect on cell separation.

Finally, two *Arabidopsis* mutants with known cell adhesion phenotypes were selected to undergo the same enzyme treatment in an attempt to replicate the preferential separation effects observed previously in another system. SEM images of *Snakeskin* and its corresponding wild type WS showed the presence of cell-cell adhesion defects along both longitudinal and transverse walls in the mutant. SEM images of *things fall apart* and its wild type, *Columbia* indicated that defects in adhesion were present along transverse walls only. Files could be observed after enzyme treatment of the *things fall apart* hypocotyl but no pattern could be distinguished in the enzyme treated *snakeskin* mutant. This could be due to the severly disrupted adhesion in the hypocotyl before enzyme treatment.

Chapter Five

Tensile Strength of Polygalacturonase-treated Arabidopsis Hypocotyls

5.1 Introduction

The plant cell wall is composed of the two major structural networks responsible for its cohesion. The cellulose network dominates the mechanical response of the cell walls in small deformation rheology measurements (Whitney, et al., 1999). Work on onion (*Allium cepa*) cell walls, a non-graminaceous monocot with the cell wall composition of a dicot by Redgewell and Selvendran (1986) and Wilson, et al. (2000) generated evidence for the independence of the cellulose and pectin networks in epidermis and this work showed pectin to be mechanically important in its own right and that it had a role in adhesion between neighbouring cells

The work in this chapter aims to investigate the effect of enzymes, shown previously to affect cell adhesion by effectively cutting the pectic chains involved in the adhesion of cells resulting in cell separation, on the tensile strength of Arabidopsis hypocotyls. As any test of cell separation involves tensile forces this chapter attempts to quantify these forces.

5.2 Mechanical Properties of Plant Material

In order to properly place the experimental work presented in this section in context it is necessary to provide an overview of the theory behind tensile strength testing and the data such experiments generate. For this reason a brief description of the terms used and an explanation of the origin of data calculated will follow.

5.3 Stiffness

The effect of an applied tensile force is measured as the change in length of the whole specimen. The change in length is conveniently assumed to be uniform which is likely to be the case in the analysis of more conventional materials but likely to be only an approximation in biological materials such as plant tissue. The fractional change in length of the specimen which is subjected to a force is termed 'strain' and the force per unit area required for this strain to be achieved is termed 'stress'.

$$\text{Strain} = \frac{\text{Change in length}}{\text{Original length}} \qquad \text{Equation 5.1}$$

This figure can be expressed as a fraction or %

$$\text{Stress} = \frac{\text{Force (N)}}{\text{Area } (m^2)} \qquad \text{Equation 5.2}$$

This figure is expressed in Pascals (Nm^{-2})

The ratio of these two measurements defines the stiffness and is named Young's Modulus.

$$\text{Modulus (E)} = \frac{\text{Stress}}{\text{Strain}} \qquad \text{Equation 5.3}$$

The units of the modulus are Pascals.

In some relatively stiff materials where compression or extension is limited to only 1% of the bond length, length increases proportionally to increasing force leading to a constant value for the modulus. In other cases the stress-strain relationship is curvilinear.

5.3.1 Structure, Strength and the Plant Cell Wall

Intercellular strength is known to be connected with the tricellular junction thus implicating the pectic network. Cooking plant tissue leads to easier breakage of cells proving more evidence for pectic involvement in cell cohesion as nothing else is degraded in such circumstances. But to date no one has been able to provide a satisfactory

explanation for the strength of the primary cell wall in its own plane. High osmotic pressure facilitates cell separation but this tells us nothing about what's happening inside walls when they fracture. Fracture characteristics are also not the same in all directions therefore this must be in some way connected with polymer orientations (Khan and Vincent, 1993).

In this section it is aimed to investigate the forces required for cell separation.

5.4 Tensile Strength Testing of the Arabidopsis Hypocotyl

The following results were obtained on a visit to the Institute of Food Research, Norwich research Park, Colney Lane, Norwich, NR4 7UH. In particular I would like to acknowledge the help of Dr Peter Ryden.

There are two main problems to be overcome before credible data can be obtained by using tensile testing to measure the effect of enzymes on cell-cell adhesion. Firstly we must consider that the enzyme may have an effect on the wall stiffness and that this will be the main feature of the hypocotyl's mechanical properties before the point of fracture. Secondly, in many systems cell-cell adhesion is stronger than the cell wall material, so the application of force is more likely to result in the breakage of cells rather than separation at the middle lamella. In order to be certain that cells separate at the middle lamella and not through weakening of the cell wall to result in breakage across the cell, the ends are photographed before and after stretching to failure.

The experiment required 3 treatments. (1) Completely untreated, fully turgid hypocotyls, (2) Hypocotyls that had been boiled in methanol to inactivate any endogenous enzymes followed by a control treatment in which the hypocotyls were incubated in the buffer but without the presence of any enzyme and (3) hypocotyls boiled in methanol and then incubated in buffer which contained polygalacturonase.

5.5 Results

5.5.1 Development of the Enzyme Incubation Process

The method used to treat the hypocotyls with the enzyme was largely based on the method previously described for the enzyme cell separation experiments on mung bean and arabidopsis. Methanol boiling was necessary in order to remove turgor pressure and inactivate endogenous enzymes and both the control treatment and the enzyme treated hypocotyls were subjected to this. As with the cell separation experiments the polygalacturonase was made up in 10 mM EDTA buffer at pH 5 although the concentration of enzyme was increased from that used in previous experiments from 4 x 10^{-10} U/ml to 4 x 10^{-7} U/ml as it was expected that the presence of the epidermis would inhibit the passage of the enzyme into the tissue. During the control treatment the hypocotyl was incubated with buffer solution only, omitting the enzyme.

The tensile strength testing was then carried out using the method published by Ryden, et al. (2003). This produced a force/displacement curve (figure 5.1).

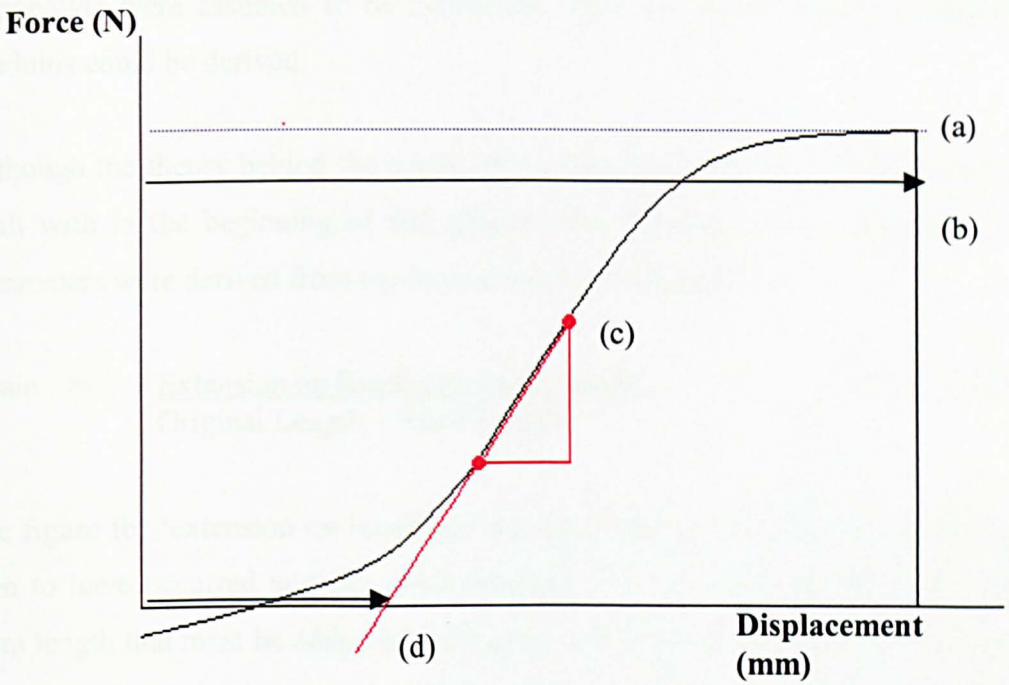


Figure 5.1 Schematic representation of a Force/Displacement Graph of the type generated by the tensile testing experiment. The main features of the graph are highlighted. The maximum force is labelled (a) and is obtained by reading the value for force (N) (y-axis – as indicated) at the highest point of the graph. This is indicated by the blue dashed line. Feature (b) shows the extension on breaking (mm) of the hypocotyls and this is indicated by the arrow. The maximum slope of the straight line segment of the graph is marked as (c) and this is the value for the modulus. The continuation of this line to the point at which it cuts the x-axis yields a value for the slack length (d) indicated by the arrow.

5.5.2 *Interpretation of the Force Displacement Curve*

From the force-displacement curve the maximum slope, the decrease in force on breaking, the extension on breaking and the slack length were recorded (Cleland, 1967). The hypocotyls were assumed to be cylindrical, thus the failure strain, tensile strength and modulus could be derived.

Although the theory behind the origin of the data obtained from this experiment has been dealt with in the beginning of this chapter, the following is an explanation of how the parameters were derived from the force displacement graph.

$$\text{Strain} = \frac{\text{Extension on Breaking} - \text{slack length}}{\text{Original Length} + \text{Slack Length}} \qquad \text{Equation 5.4}$$

The figure for 'extension on breaking' was the point at which the first breakage could be seen to have occurred and the graph dropped off. The value for the slack length, ie the extra length that must be added by pulling the hypocotyl apart before tension is introduced, was calculated from the equation of line of maximum gradient at the point at which $x = 0$. Measurements for the length of the hypocotyl were initially obtained using specialist software (Image pro, Media Cybernetics, Silver Springs, MD, at 40x magnification) that allowed accurate measurement of a photo taken of the hypocotyl.

$$\text{Strength} = \frac{\text{Maximum Force}}{\text{Cross sectional Area}} \qquad \text{Equation 5.5}$$

By inspection of the force/displacement graph at the highest value reached on the y-axis (Force), the value for maximum force could be ascertained. In order to calculate the cross sectional area, each hypocotyl was photographed and 10 measurements of the width taken using the same software process as for the determination of the length. These measurements were then averaged to give a value for the width of the hypocotyl. However, due to the turgor-removing effect of the methanol boiling the width of the hypocotyl decreased significantly after this stage. This loss of turgor which is essential to the rigidity of primary tissue – plasmolysed hypocotyls have no resistance to bending - may have a considerable effect on tensile properties. There is published evidence that the tensile modulus of turgid tissue is higher (Lin and Pitt, 1986). As no polymers involved in cell–cell adhesion or cell wall integrity were removed in this step the average value for the

diameter of the untreated hypocotyls was substituted in the case of the control and enzyme treated hypocotyls. This value was then used πr^2 to give the cross-sectional area (πr^2).

$$\text{Modulus} = \frac{\text{Stress}}{\text{Strain}} \qquad \text{Equation 5.6}$$

The values obtained from equations 5.5 and 5.4 were used in the above equation to give a value for the modulus.

5.5.3 Tensile Strength Testing of Wild Type Arabidopsis (WS) hypocotyls

Figure 5.2 Shows a graphical representation of the relationship between the three experimental conditions in terms of their tensile modulus and strength and allows comparison.

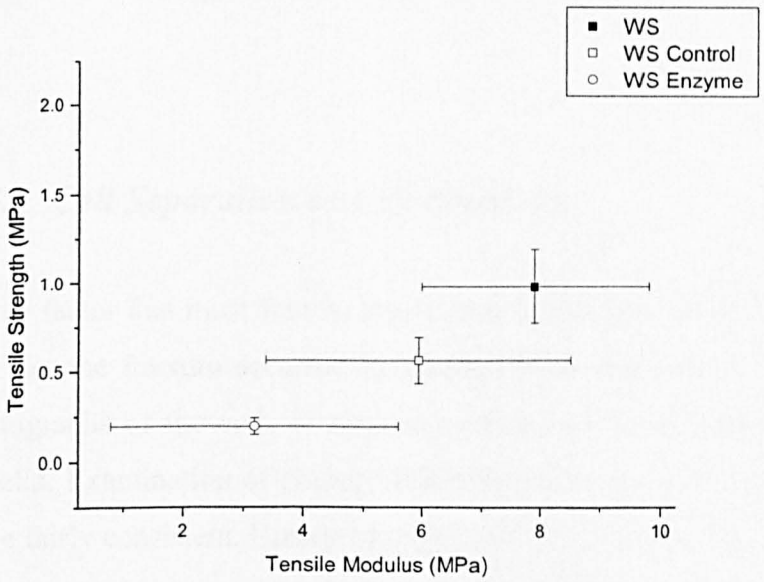


Figure 5.2. Graph of tensile strength versus modulus with error bars. The error in the modulus remains fairly constant while the error in the tensile strength appears to increase exponentially.

The untreated WS hypocotyls exhibited a higher modulus than those that had undergone any form of treatment. Increasing the severity of the treatment and removing increasing amounts of polymers from the wall lead to a progressive decrease in the modulus and an increase in the standard deviation as the variability in the results increased. The decrease in modulus may also be at least partly attributable to the effect of the loss of turgor described in the previous section.

In the control and other treatments the strength also was reduced compared to the untreated hypocotyls. This is to be expected as, though the chelating agent that makes up the buffer is of low concentration, it will still be able remove calcium from the walls and solubilise polymers.

Figure 5.2 also displays error bars representing one standard deviation either side of the mean for both tensile modulus and strength. As may be expected the untreated hypocotyls show the least degree of variability in the modulus although the difference between this and the control and enzyme treatments is not large. The error in the tensile strength increases in proportion to the tensile strength, thus for the purpose of statistical comparisons the $\log_e$ transformation was used.

5.5.4 Cell Separation or Cell Breakage

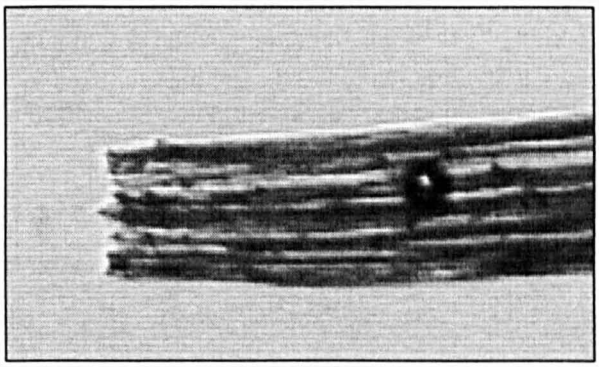
A key factor that must first be determined before the results can be declared meaningful is whether the fracture occurred by means of cell separation or breakage. Figure 5.3 shows photographs of the ends of hypocotyls that had either broken or separated at the middle lamella. Examination of photographs of broken hypocotyls showed the method of fracture to be fairly consistent. Untreated hypocotyls broke across the cell, whereas with the control treatment, where a weak chelating agent had been used, a mixture of breakage and separation at the middle lamella was observed. It was observed that the majority of hypocotyls that were treated with the polygalacturonase enzyme fractured by cell separation.

Figure 5.3 a An untreated hypocotyl that has been stretched until breaking. The cells fractured under stress rather than separate at the middle lamella.

Figure 5.3 b An hypocotyl that has been subjected to the control treatment and then stretched. The majority of the cells have broken to facilitate cell separation although there is some evidence of a small amount of cell separation.

Figure 5.3 c An hypocotyl that was treated with enzyme before stretching. The picture clearly shows that separation of the cells at the middle lamella occurred rather than breakage.

(a)



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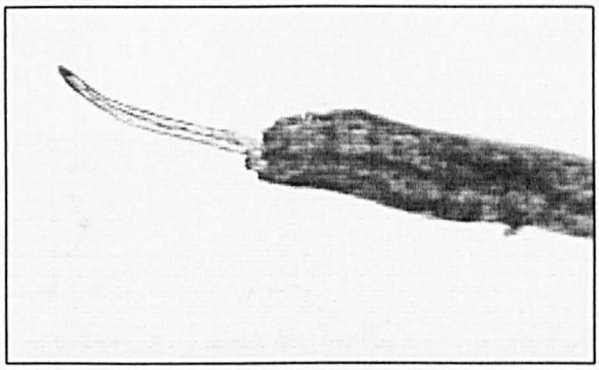
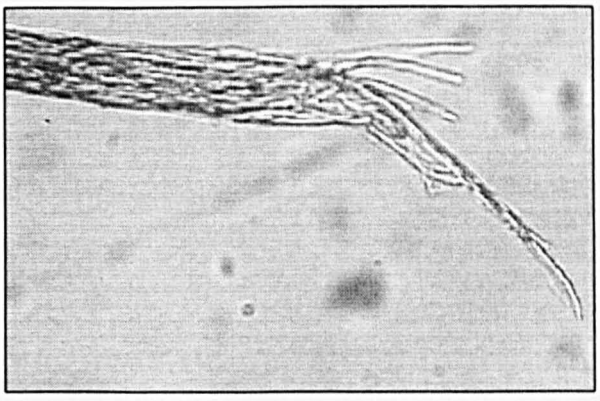


(c)



5.5.5 Statistical Comparisons

In order to test for the existence of a significant difference between the treatments a one-way ANOVA was carried out in conjunction with a Tukey LSD test to determine where these differences occurred.

A significant difference, as shown by the Tukey LSD, is denoted with a superscript letter. The same letter after each mean value should be interpreted as there being no significant difference between the results and it follows that different letters indicate the existence of a significant difference.

5.5.5.1 Modulus

Treatment	Mean	N
Wild Type	2.04^{a}	20
Wild Type Control	1.71^{a}	20
Wild Type Enzyme	0.86^{b}	20

Table 5.1 Comparison of untreated, control and enzyme treated WS hypocotyls for differences in tensile modulus with a superscript letter to indicate differences between treatments as determined by a Tukey LSD test. F test on ANOVA gave p-value of 0.000

The calculated p-value of 0.000 from the ANOVA indicated that a highly significant difference existed. From the experimental results it appeared that the enzyme treated condition could be significantly different from the others but it was unclear whether the control and untreated conditions were different. To confirm a difference in the enzyme treatment and identify any other differences a Tukey LSD test is also necessary.

5.5.5.2 Tensile Strength

Treatment	Mean	N
Wild Type	0.96^{a}	20
Wild Type Control	0.56^{b}	20
Wild Type Enzyme	0.2^{c}	20

Table 5.2 Comparison of untreated, control and enzyme treated WS hypocotyls for differences in tensile strength with a superscript letter to indicate differences between treatments as determined by a Tukey LSD test. F test on ANOVA gave p-value of 0.000

Again the p-value of 0.000 from the ANOVA indicates that a significant difference exists and the Tukey test confirms that all the conditions differ from each other.

5.5.6 Tensile Strength Testing of Wild Type Arabidopsis (Columbia) and Mutants (Snakeskin and Things Fall Apart)

Preliminary studies on mutants exhibiting a cell adhesion phenotype were done with only 3 replicates due to time constraints. It was suspected that meaningful results could not be obtained with such a small number of replicates but the information does provide an insight into potential trends for these hypocotyl types. The results are presented in figures 5.4 – 5.6.

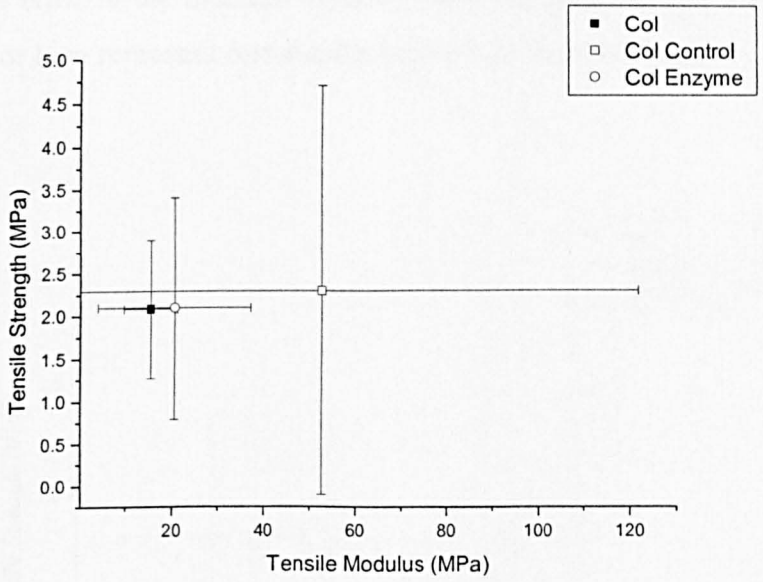


Figure 5.4 Graph of tensile strength versus modulus with error bars for Columbia. The errors in the modulus and tensile strength increase. Error bars represent one standard deviation from the mean.

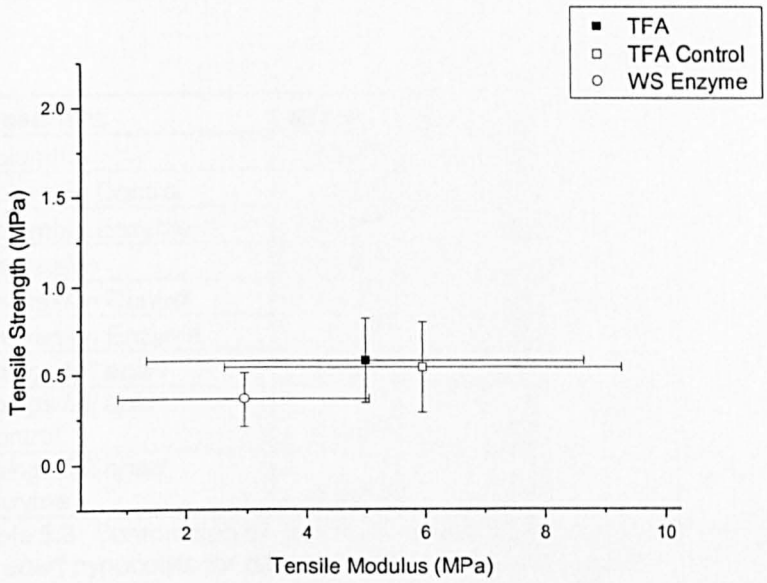


Figure 5.5 Graph of tensile strength versus modulus with error bars for *things fall apart*. The error in the modulus remains fairly constant as does the error in the tensile strength. Error bars represent one standard deviation from the mean.

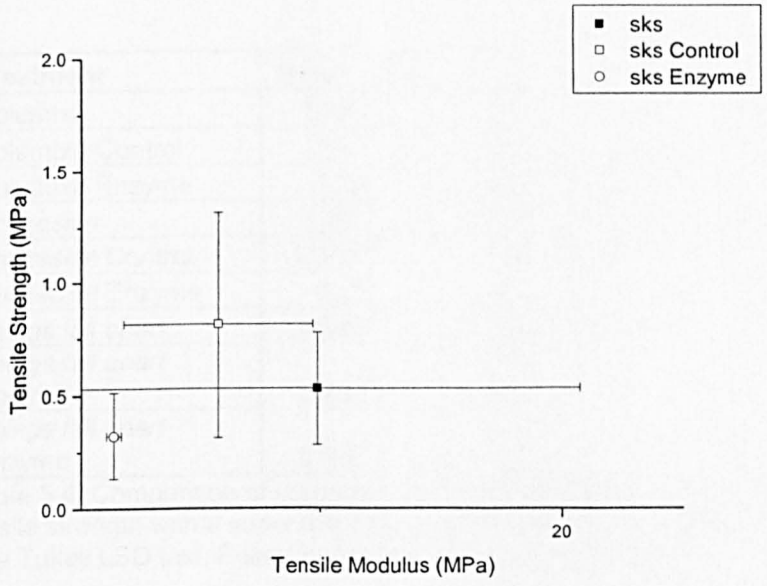


Figure 5.6 Graph of tensile strength versus modulus with error bars for *snakeskin*. The error in the modulus remains fairly constant while the error in the tensile strength appears to increase exponentially. Error bars represent one standard deviation from the mean.

5.5.6.1 Modulus

Treatment	Mean	N
Columbia	2.69[a,b]	3
Columbia Control	3.32[a]	3
Columbia Enzyme	2.46[a,b]	3
Snakeskin	1.86[a,b]	3
Snakeskin Control	1.63[a,b]	3
Snakeskin Enzyme	0.43[b]	3
Things fall apart	1.44[a,b]	3
Things fall apart Control	2.06[a,b]	3
Things fall apart Enzyme	0.87[a,b]	3

Table 5.3 Comparison of untreated, control and enzyme treated Columbia, *Snakeskin* and *Things fall apart* hypocotyls for differences in tensile modulus with a superscript letter to indicate differences between treatments as determined by a Tukey LSD test. F test on ANOVA gave p-value of 0.032

The p-value of 0.032 indicates that a significant difference does exist somewhere and the Tukey test shows it to be between the enzyme treated *Snakeskin* and the Columbia control.

5.5.6.2 Tensile Strength

Treatment	Mean	N
Columbia	1.98[a,b]	3
Columbia Control	1.6[a,b]	3
Columbia Enzyme	1.79[a]	3
Snakeskin	0.49[a,b]	3
Snakeskin Control	0.72[a,b]	3
Snakeskin Enzyme	0.27[b]	3
Things fall apart	0.54[a,b]	3
Things fall apart Control	0.49[a,b]	3
Things fall apart Enzyme	0.34[a,b]	3

Table 5.4 Comparison of untreated, control and enzyme treated WS hypocotyls for differences in tensile strength with a superscript letter to indicate differences between treatments as determined by a Tukey LSD test. F test on ANOVA gave p-value of 0.007

Here a p-value of 0.007 indicates that a significant difference exists somewhere and again we can tell from the Tukey test that this difference is between untreated Columbia and enzyme treated *Snakeskin* and also enzyme treated Columbia and enzyme treated *Snakeskin*.

5.5.7 Comparison of Wild Types and Mutants

To allow easy comparison of the results from both wild types and mutants figures 5.3 – 5.6 can be combined and the results displayed on one chart as shown below in figure 5.7. This highlights the large difference in errors exhibited by the *Columbia* wild type with the error increasing with the severity of the treatment. Differences in the others are more difficult to discern.

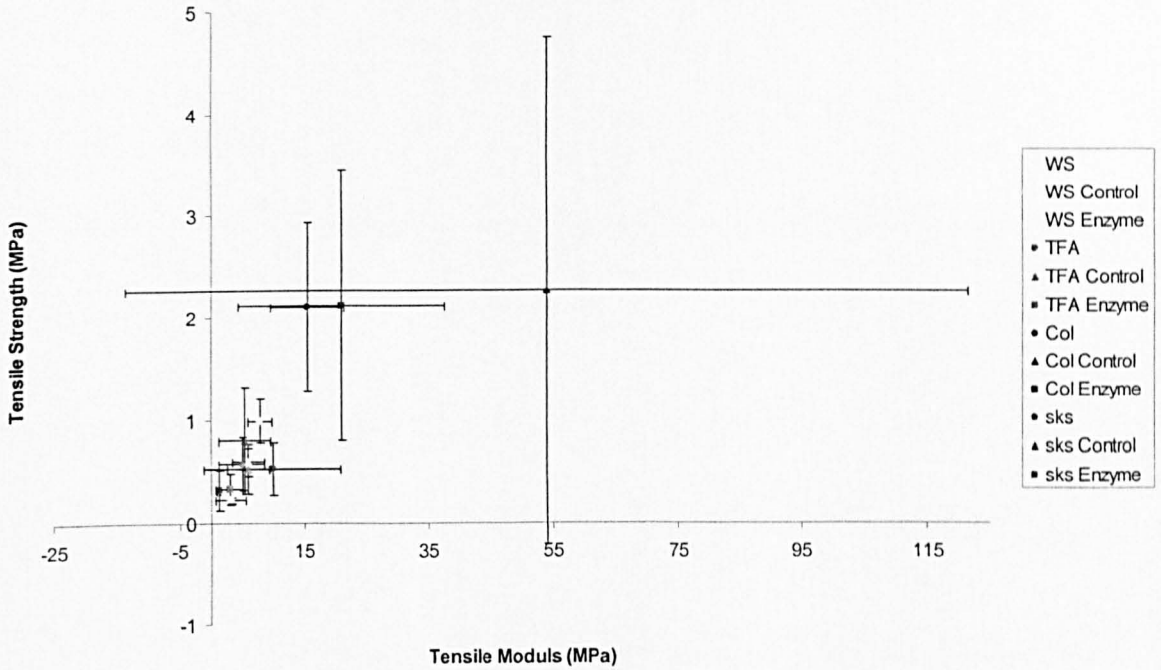


Figure 5.7. Combined graph for *Columbia*, *WS*, *snakeskin* and *things fall apart* of tensile strength verses modulus with error bars of one standard deviation. The error in both the modulus and tensile strength in the *Columbia* wild type is greater than those of the other wild type and mutants.

5.6 Conclusions

From the statistical analysis we can see that differences in cell adhesion between the wild type and the mutant *Snakeskin* do exist but it is only possible to see these when there has been enough degradation done to make sure that cells are not separating by fracture.

The results of this experiment when applied to mutants displaying a cell adhesion phenotype indicate that, as would be expected, their tensile strength is markedly lower, especially for snakeskin, and decreases further with the addition of enzyme. However the

small number of replicates and large error bars make further interpretation difficult. The error arising from the treatment of the *Columbia* hypocotyls is markedy larger than for the other wild type or either of the mutants although the reason for this is unclear.

Chapter Six

Pectic Methyl and Nonmethyl Esters in Mung Bean Hypocotyl Cell Walls

6.1 Introduction

During synthesis of cell wall pectin, galacturonic acid, the major constituent monomer, is methyl esterified after polymerisation. This is possibly to prevent premature cross-linking with Ca^{2+} as esterification will eliminate charge and therefore the binding site. Methyl esterase will then cleave the methyl group and cross-linking can occur.

It has also been suggested that alcohols other than methanol can form galacturonoyl esters in a manner similar to methanol. Acetic acid is known to form esters with the O-2 and O-3 hydroxyl groups of galacturonosyl residues but such esters are ethanoyl not galacturonoyl.

Here we use the methods published by MacKinnon et al 2002. to quantify the level of both methyl and non-methyl esterification in mung bean hypocotyl cell walls. Cell walls of three differing origins were analysed; the whole hypocotyls that had been grown in the laboratory under controlled conditions; the commercially purchased hypocotyls; and a section from the laboratory grown hypocotyls from the first centimetre behind the apical hook (Roland, et al., 1982).

6.2 The History of Nonmethyl Ester Research

Since the early days of pectin research it has widely been known that not all pectins are extractable by chelation. This implies that something in addition to Ca^{2+} cross-linking is important in the binding of pectins within the cell wall.

Studies that attempted to determine the structure of pectin in situ were hindered by the lack of availability of methods that allowed pectin to be extracted from the wall without altering the structure. A hot alkaline extraction, of the type that was commonly use at this time to extract pectin, would lead to depolymerisation by β-elimination. Work on this problem in the 50's by Albersheim (Keegstra, et al., 1973) concluded that there was no easy way to determine the structure as severe conditions are necessary for the extraction and these result in the destruction of the structure under examination.

In theory a glycosidic bond can form from the reducing terminus of the polysaccharide to the non-reducing sugar of another polymer. However no polysaccharide has more than one reducing end which means that although a branched structure may form, glycosidic cross-linking is impossible. Treatment with chelators under non-depolymerising conditions, to remove Ca^{2+} and hence break the crosslinks between homogalacturonan chains, does not result in the removal of all pectin from the cell wall. Thus the possibility of ester crosslinks was proposed. Also it was known that a cold mild alkali treatment could extract pectin without depolymerising it and it was hypothesised that ester bonds existed but at the time this could not be proven experimentally (Jarvis, 1984) and references therein.

The first people to demonstrate the existence of ester linkages in pectin were Kim and Carpita (1992) using maize as the plant material, an odd choice since maize is not a pectin rich material. However the method that they published was complicated and not without its problems. It relied on $NaBD_4$ to selectively reduce methyl esterified uronic acid groups while leaving the free acid groups unreduced and then a conjugation reaction to reduce the free acid. $NaBD_4$ is only stable at alkaline pH however the purity is critical as ordinary $NaBD_4$ degrades to borate which has a pH of around 11. To slow the decomposition Kim and Carpita used an imidazole buffer with the $NaBD_4$ as adding acid to adjust the pH only leads to the release of more borate (Maness, et al., 1990). This would pose another potential problem. At severely alkaline pH there is the danger that esters will be hydrolysed rather than reduced. If this happens there will be less deuterated galactose than expected and as the method quantifies the total amount of esterified galacturonan from galactose there will therefore be a lower figure than there ought to be. Consequently the problem of requiring a quantitative yield to give accurate results may affect the accuracy of the method.

Hou and Chang later published a colorimetric method for the determination of ester linkages in pea sprouts (Hou and Chang, 1996). However this lacked specificity and there

were many possibilities for interference from non-galacturonoyl esters which leads to doubts concerning the believability of the results. They did show that non-methyl esters were present and that changes in their quantities occurred in the presence of pectin esterase released during cooking. This provided the first indication of non-methyl ester occurrence during cooking.

6.3 Methods for Analysis

6.3.1 Nonesterified Carboxyl Acid Content

Two methods were used to measure the amount of the carboxylic acid groups in the cell wall that were not esterified in any way. Both methods used in the measurement of non-esterified Carboxyl Acid content (titration & copper) measure the total content of ionic sites, the cation exchange capacity, in the CW and not just galacturonyl free carboxyl content.

6.3.1.1 Copper Binding Method

Copper (II) ions bind to non-esterified pectic galacturonans but once these are saturated any other anionic sites will do including proteins and phenolics (levels in Mung bean cell walls). They can be removed by the addition of excess acid. The pH of the copper solution used is critical to the success of the binding step. If it is greater than pH 6 then binding of the $CuOH^+$ ion will occur if the pH is significantly below 6 then there will be significant protonation of the carboxyl groups (Jardine, et al., 2002; Keijbets and Pilnik, 1974). Binding of Copper (II) ions to pectic galacturonans can exceed the stoichiometric ratio at neutral pH. Between pH 5.5 and 6.0, this problem is minimal, but pectic carboxylate groups are essentially fully ionised. The pH must be very closely monitored during the binding step to check that there is no change and also checked in the final solution.

Sample Calculation

(i) the copper concentration as measured by Atomic Absorption) AA was in μg/ml.

(ii) this was converted to μMoles/ml (mM) by dividing by the atomic mass of copper

(iii) by multiplying the figure obtained above by 50 (the volume of the solution in the volumetric flask), the number of μMoles of copper obtained from the cell walls was ascertained.

1 μMole of copper → 2 μMoles carboxyl

(iv) the figure was divided by the mass of cell walls giving the answer in μMoles COOH/mg of cell wall

6.3.1.2 Measurement by Titration with NaOH

With this method (Mackinnon, et al., 2002) it is necessary for the pectin in the cells to be in the free acid form without any other free acid being present. The cell walls to be used must therefore be well purified as even acidic proteins can interfere. Glucuronic acid may still be present, in arabinoxylan, but only in relatively small quantities in the tissues used.

The initial acidification of the cell walls is done using a dilute acid in an aqueous ethanol solution. As the solution is only 70 % ethanol, water is present. If any hydrolysis of the polysaccharides occurs then they are not released into solution. High quality ethanol is essential to eliminate the potential for contamination.

The complete removal of the acid is essential and washing must be rigorous as there are not many protonated groups present. To monitor the progress of the acid removal, the washings were run into water and the pH checked. Once the pH had reached approximately 4, removal of the acid was judged to be complete. This method was considered to be more accurate than using silver nitrate which forms a fine precipitate that is not easily detected.

At the titration stage sufficient quantities of cell walls were used to allow replicate titrations to be carried out. Before use, the pH electrode was cleaned thoroughly by steeping overnight in HNO_3 and then rinsed in distilled water. The pH was closely monitored throughout and readings were stopped at pH 7. Approaching the end point, it was important that the additions were small and the reading allowed to stabilise, which took around 2 minutes after each addition, as allowing the pH to rise above 7 for any

length of time would cause deesterification. The total volume of addition was approximately 1 ml.

Calculation

(Titre value (ml) / 1000) * ((1/mass Cell Wall Material(g))*1000) Equation 6.3

6.3.1.3 Methyl Ester Determination

This method involved a saponification reaction to cleave the methyl-ester groups from the pectin and release methanol. Alcohol oxidase then converted the methanol to formaldehyde, the concentration of which could be measured colorimetrically by adding pentandione to give a coloured complex.

The method as set out by (Klavons and Bennett, 1986) was almost directly adhered to. In order to prolong the life of the enzyme aliquots were frozen in 2 ml Eppendorf tubes until ready to be used. Blanks were included to correct for free methanol contamination and controls, with the order of addition of KOH and H_2PO_4 reversed, were also run.

If the results of the colorimetry indicated that dilutions were necessary then the procedure was repeated with dilutions being made prior to the enzyme incubation.

Sample Calculation

(i) calibration graph drawn and the equation of the best fit line calculated

(ii) From the equation of the line, the concentration in μg/ml could be determined

(iii) this figure was then multiplied by 2 to account for the dilution (if there had been one)

(iv) the concentration was then divided by the molecular weight (32) to give a figure in μmoles/ml (v) In order to convert the previous figure to take the volume into consideration, a multiplication factor of 8.25 was applied. This gave a value in μmoles.

(vi) At this stage the average values for both the control and the blank were subtracted

(vii) Finally the figure was divided by the mass of cell walls to give a concentration in μmoles/g and an average taken.

6.4 Results

From table 6.1 we can see that good agreement between nonesterified galacturonate as determined by the copper binding and titrimetric methods is possible.

Table 6.1 Galacturonate content of cell walls with three different origins.

	Galacturonate Content μmol/g					
	Total Galacturonate	Nonesterified Galacturonate			Total Ester	Methyl Ester
	Titrimetric A	Titrimetric B	Copper C	Mean (B+C)/2	Mean D = A – (B + C)/2	Colourimetric E
Safeway	493	344	351	348	145	101
Whole Hyp	639	429	450	340	299	99
Growing	552	362	366	364	188	173

Table 6.2 Percentage esterification origins.

	Percent Esterification		
	Total Esters	Methyl Esters	Nonmethyl Esters
	100D/A	100E/A	100(D - E)/A
Safeway	28.4	20.5	8.9
Whole Hyp	46.8	15.5	31.3
Growing	34.1	31.3	2.7

6.4.1 Calculation of the Results

The whole hypocotyl of both laboratory and commercially grown mung bean sprouts, and a section 1 cm in length from the 'growing region' of the laboratory grown hypocotyls were analysed for the existence of putative nonmethyl esters. This required the measurement of the total uronic acid, nonesterified and methylesterified galacturonic acid content. A value for the total esterification of galacturonic acid was calculated by subtracting the nonesterified galacturonic acid content (eq 6.1) and the nonmethyl ester content could then be calculated (eq 6.2)

Total Esterified Galacturonic Acid — Equation 6.1

= Total GalUA - Nonesterified GalUA

Nonmethyl Esterified GalUA Equation 6.2

=Total Esterified GalUA - Methyl Esterified GalUA

A percentage value for the total esters, methyl and nonmethyl esters content of each sample is expressed in table 6.2

6.4.1.1 Esterification

GalUA is esterified when it is first formed to stop calcium cross-linking, it is then de-esterified once in the wall.

The mean total level of esterification for each of the three different samples varied. The results obtained for the 'Safeway' and laboratory grown hypocotyls should be directly comparable as the whole hypocotyl was used in each case. From table 6.2 we can see that the total level of esterification was 28 % and 47 % respectively. The hypocotyls from which the section constituting the growing region was taken were grown under identical conditions to the laboratory grown hypocotyls: therefore they should provide a comparison between an average of the whole hypocotyl and a specific section. The growing region showed total esterification to be 34 %.

The methyl and thus nonmethyl ester content of each sample again showed a great deal of variation. The Safeway and whole laboratory-grown hypocotyl data showed almost similar levels of methyl esterification – 21 % and 15 % respectively; however since their values for total ester content had been very different this meant that the level of nonmethyl esters was significantly higher in the whole laboratory-grown hypocotyl. Comparison of the whole hypocotyl with the growing region section reveals a bigger difference still. The whole hypocotyl contained half the number of methyl esters but ten times more nonmethyl esters.

6.5 Testing for the Presence of Nonmethyl Esters by H^+ NMR

Cell walls were subjected to a saponification reaction that would release anything held by an ester-type linkage. As methyl esters are known to exist in the cell wall, it was expected that methanol would be detectable by NMR. The presence of other alcohols would confirm the existence of nonmethyl esters and perhaps provide a clue as to their identities.

The reaction was carried out using deuterated solvents to eliminate as many proton peaks not originating from the cell wall material as possible.

The results showed that the following were present in the sample;

Table 6.3 Identities of NMR peaks

Peak Identity	Chemical Shift
Methanol	3.314
Acetic Acid	1.865
Acetone	2.077
Water (contaminant)	4.536

6.6 Conculsion

Thus the NMR results show that the only alcohol present was methanol, hence no other low molecular weight alcohol has been released in detectable amounts. Since we know from the chemical non-methyl ester experiments that non-methyl esters are present which may have been expected to be glyceryl, butyl, etc, these possibilities can be eliminated as their presence would have been detected in the NMR. Therefore, the non-methyl ester must be polymeric. This leads to the conclusion that there must be a crosslink between galacturonoyl groups and something else in the cell wall. However, this experiment cannot

distinguish between an inter-residue crosslink and a *crosslink from a pectic molecule to* another polymer molecule, pectic or otherwise.

Chapter Seven

General Discussion

It can be assumed that the pectins of the middle lamella/cell junctions and those of the cell wall are related, although not identical, in structure and are physically continuous with one another as illustrated in figure 1.10 (taken from Carpita and Gibeaut, (1993)). This is an important assumption as this thesis and a discussion of the results therein deals with cell adhesion (between middle lamella and cell junctions) and pectin solubilisation (whole cell wall). It is also equally reasonable to assume that all pectins are soluble unless cross-linked to one another or to other polymers. A soluble polymer cannot provide mechanical strength therefore it becomes likely that there is a link between solubility and cell adhesion with both being dependent on cross-linking. However it does not follow that the same number of cross-links would have equal effects on both. Two cross-links on one huge molecule are enough to make it insoluble (although PG will then solubilise it very fast). For mechanical strength, the more cross-links the better.

The results of the enzyme experiments detailed in chapter four where separation of cells was achieved with polygalacturonase and pectin lyase demonstrates that pectic galacturonan in the middle lamella is involved in cell-cell adhesion. The chemical separation experiments indicate that these pectins must be cross-linked as without cross-links pectins cannot provide mechanical strength to attach cells together (Jarvis, et al., 2003 and references therein). But why must they be cross-linked at all? The methods used to chemically separate the cells, which break cross-links between pectic molecules rather than within the molecules themselves, all resulted in pectin being brought into solution. This leads to the conclusion that whatever is responsible for cross-linking pectin is also responsible for making pectins insoluble. It is this insolubility that makes them capable of withstanding much more mechanical stress across the middle lamella. The evidence from

the chemical separation experiments provides an insight into what kind of cross-links might be present.

The chemical separation experiments conclude that at least three different types of cross-links must be present and involved in cell-cell adhesion; ionic Ca^{2+} cross-links; alkali-labile such as esters; and the hydrogen bonded hemi-cellulosic network. The varying degrees to which separation is observed in cells of different types and walls of different origin within the same cell implies that all types of cross-linking may be present in some and only one or two types in others. Only a very few incidences of adhesion rely on calcium cross-linking alone as described in the work by Cocking, (1960).

Enzyme degradation shows that pectin is involved in cell-cell adhesion. The chemical experiments show that pectins are cross-linked and that many different types of cross-link contribute. This is in agreement with the many references in the literature including (Fry, 1986; Selvendran, 1985; Thompson and Fry, 2000). It can be presumed that any type of cross-link will work to a certain degree in keeping cells adherent but observing the effect of imidazole alone, the removal of the calcium cross-links, did not generally result in cell separation. Thus these ionic cross-links are accompanied by others that are sufficient to hold cells together.

The removal of alkali-soluble pectins from tissue that has previously been treated with a chelating agent does not result in complete cell separation either. In at least some cells additional, alkali-stable cross-links must be present. That is, non-ester cross-links must be present also. The requirement of a stirring step adds a complication however. When cells are observed to separate after the destruction of most of the cross-links, the mechanical stress resulting from the stirring will break the few residual bonds. The lack of specificity here means that these bonds cannot be identified with certainty. They may be some of the alkali-stable ones that have been identified elsewhere or perhaps they could be something completely different.

The tensile strength testing experiments on Arabidopsis support the idea of mechanical force being able to break some degree of residual bonding; presumable predominantly located in the vascular tissue as this displayed large numbers of alkali-stable cross-links and was most difficult to separate. The longitudinal walls of the cells do not appear to contain cross-links of this type in sufficient quantity to greatly affect the ease of separation as most become part of a file. The remainder of the Arabidopsis experiments lead to the

conclusion that cross-linking is under genetic control although there is not enough evidence to be more specific.

It has therefore been shown that there are three main types of cross-link; ionic ie Ca^{2+}; alkali-stable; and alkali labile. This leads to a starting point for characterisation and the investigation of the alkali labile bonds. As intermolecular ester bonds are known to be present (Fry, 1986) these are assumed to coincide with ester cross-links. This assumption may lead to the conclusion that it is this that is making pectin insoluble in imidazole and soluble in alkali.

Also evident from the work presented in this thesis is the differing separation characteristics of transverse and longitudinal cell walls. The cell walls that do not separate as easily, the transverse walls, are formed in a different way from the longitudinal walls that do.

As described in section 1.5 different walls within the cell, ie transverse or longitudinal, are formed by different mechanisms. Transverse walls are formed by the insertion of a cell plate to divide an existing cell and the addition of new material to this to complete the process, and longitudinal walls are formed as a result of elongation of the cell and the deposition of new material.

Pith cells are known to expand laterally as well as lengthwise and therefore may contain amounts of each type of side-chain. This may provide an explanation for the reluctance of such cells to separate and in particular, to separate into files as observed as, for example the cells of the cortex are observed to do.

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