Identification and characterisation of the Arabidopsis thaliana cell wall proteome: unravelling novel cell wall proteins and new potential functions of the plant extracellular matrix

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Identification and Characterisation of the *Arabidopsis thaliana* Cell Wall Proteome: Unravelling Novel Cell Wall Proteins and New Potential Functions of the Plant Extracellular Matrix

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A thesis submitted to the University of Durham
for the degree of Doctor of Philosophy

Department of Biological Sciences

2001

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Abstract

The application of the proteomic approach has facilitated efforts directed toward the mapping of the entire *Arabidopsis* cell wall proteome. Proteins were sequentially extracted from purified cell walls using 0.2 M $CaCl_2$ followed by a urea buffer. The extracts were resolved via large format two dimensional polyacrylamide gel electrophoresis (2-D PAGE) and were visualised via Coomassie brilliant blue staining. The aim was to identify and characterise as many cell wall proteins as possible, with the hope of identifying novel cell wall proteins. Out of 325 spots visualised on the 2-D polyacrylamide gel, 144 gave a positive protein identification representing 104 different proteins. The identified proteins were divided into 3 categories. The first category included proteins that have been previously identified as plant cell wall proteins. The second category was designated to include novel cell wall proteins (hypothetical proteins) and the third category was made up of proteins, which had recognised functions, but had never hitherto been known to be secreted to the extracellular matrix. Among the identified novel cell wall proteins there were several that shared high homology with protein kinases. These proteins possessed all the characteristics of secreted polypeptides, such as the cleavable N-terminal signal peptide, and were found to lack both the transmembrane domain and the endoplasmic reticulum retention tetrapeptides (HDEL and KDEL). These observations suggested that, as in animal cells, plant cells had extracellular protein kinase activity (phosphorylation). This was supported by the recent discovery that plant cells secrete ATP to the extracellular matrix (Thomas et al., 2000). Verification of the occurrence of extracellular protein kinase activity was further strengthened by the identification of phosphorylated *bona fide* cell wall proteins and stress responses caused by the depletion extracellular ATP.

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List of Abbreviations

1-D PAGE	One-dimensional gel electrophoresis
2-D PAGE	Two dimensional gel electrophoresis
AA	Ascorbic acid
ADP	Adenosine 5'-diphosphate
AGP	Arabinogalactan-rich protein
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
BLAST	Basic local alignment search tool
BMS	Black Mexican Sweetcorn
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CID	Collision induced dissociation
CF	Culture filtrate
CW	Cell wall
CWBAP	Cell wall biosynthesis associated protein
DAB	Diaminobenzidine
DTT	Dithiothreitol
EB	Evans blue
EM	Electron microscopy
ENR	enoyl-ACP-reductase
ER	Endoplasmic reticulum
ESI-MS-MS	Electrospray ionisation tandem mass spectrometry
FDA	Fluorescein diacetate
FITC	Fluorescein isothiocyanate
GRP	Glycine-rich protein
GST	Glutathione *S*-transferase
HPLC	High Performance Liquid Chromatography
IEF	Isoelectric focusing
kDa	kilo Daltons
kVh	kilo Volt-hours
LC Q-TOF	Liquid chromatography quadrupole-time of flight mass (MS)
LRR	Leucine-rich repeats

MALDI-Tof	Matrix assisted laser desorption ionisation time of flight.
MAPK	Mitogen activated kinase
MIPS	Munich Information Center for Protein Sequences
MOWSE	Molecular weight search
MS-MS	Tandem mass spectrometry
m/z	mass per charge
NCBI	National center for biotechnology information
NEPHGE	Non-equilibrium pH gradient electrophoresis
NMR	Nuclear magnetic resonance
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAL	Phenylalanine ammonia-lyase
PBS	Phosphate buffered saline
PCV	Packed cell volume
PCWME	Possible cell wall modifying enzymes
PGIP	Polygalacturonidase inhibiting protein
pI	Isoelectric point
PLT	Potato tuber lectins
PR	Pathogenesis-related protein
PRP	Proline-rich protein
PY20	Anti-phosphotyrosine antibody
rpm	revolutions per minute
SDS	Sodium dodecyl sulfate
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEM	Transmission electrom microscopy
TMHMM	Transmembrane hidden Markov model
TMV	Tobacco mosaic virus
TSP	Total soluble protein
UB	Urea buffer
UV	Ultra violet
WAKs	Plant cell wall associated protein kinases
WLKs	Plant cell wall located protein kinases

Declaration

I declare that the work within this thesis, submitted for the degree of Doctor of Philosophy, is my own original work and has not been submitted for a degree at this or any other University

Acknowledgements

First and foremost I would like to thank my supervisor, Prof. Toni Slabas for his guidance and support throughout this work. I would also like to thank Dr Steve Chivasa for all the time spent brainstorming and trouble shooting cell wall protein 2D PAGE and many other issues. Also I would like to thank Xiao lan Yu for maintaining the cell suspension cultures. I am indebted to Bill Simon for the MALDI-ToF and MS-MS data. I and also thankful to Prof. J.P Knox for the *in situ* immunolocalisation work. Many thanks also go to Paul Sidney and Christine Richardson for the photographic and electron microscopy work, respectively. Special thanks goes to Mrs Colleen Turnbull for all the general help, which she was always happy to give. To all my lab mates, thank you for making life in the lab fun and enjoyable.

Last but not least I would like to thank Jasper Rees and my friends in the University of the Western Cape for pointing me in the right direction. To my family and loved ones; Ndiyanibulela nonke ngothando, ngomonde nangokundipha lenxaxheba. Kuwe Jambas'omkhulu (Sneyman ka-Tshifana) no mamVala, ndithi Camagu!

Finally, I would like to acknowledge the financial help provided by the Association of Commonwealth Universities and the British Council.

Chapter 1

General Introduction

1.1 Brief historical background of the study of plant cell walls.

The study of the cell wall probably dates back as far as the development of the microscope by Robert Hooke in 1667 (Preston, 1952). Although he was not a botanist, Hooke, was fascinated with plant cell anatomy and became intrigued by the structure described as the cell wall. Following his lead two botanists, Malpighi and Grew in 1682 produced the first detailed drawings of the plant fibrils that form the framework of the cell wall. For a further two centuries, scientific study of the cell wall remained limited to the realm of mere observations and drawings (Preston, 1952). With the dawn of the 19th century, however, a welcome resurgence in scientific enquiry led to several key developments in the study of this structure.

The first major contribution of the 19th century came from Kieser, a German plant scientist, in 1815. Kieser was the first to reveal that the cell wall is the structure responsible for giving plant cells their distinctive rhombic dodecahedral shape. In 1850, van Mohl, a German botanist, made the next major contribution by making use of a polarised microscope. This instrument enabled van Mohl to recognise that the plant cell (protoplasm) is surrounded by two cell wall structures, referred to as the primary and secondary cell wall. Following in the footsteps of van Mohl's work, a Swiss scientist, Nageli, also made use of the polarised microscope and in 1877 came to recognise that plant cell walls were made up of thin crystalline fibres. These fibres were noted to be quite tough, but were yet flexible enough to allow for cell growth and expansion. At this time it soon became established that the cell wall consists mainly of a mixture of cellulose and pectose (pectin and hemicellulose). In view of this discovery, scientific enquiry into the nature of cell walls has been

dominated, from the late 19th century up until the present, by studies investigating the biochemistry and biophysics of the cell wall carbohydrate (Preston, 1952; Brett and Waldron, 1996). Given that carbohydrates make up approximately 90% of the total macromolecular content of plant cell walls and can be purified much easier than other cell wall macromolecules (like proteins and phenolics; Brett and Waldon 1996), scientific research, throughout the years, has understandably been focused on its role in matters of cell wall function.

1.2 Plant cell walls

The plant cell wall is the outermost structure of the plant cell and it is mainly made up of carbohydrates and proteins (Showalter, 1993; Jose-Estanyol, 2000). The cell wall provides strength, shape and rigidity to plant cells, tissues, and organs. There are two types of cell walls: the primary cell wall and the secondary cell wall. It is mainly the latter that plays a major part in the cell wall functions mentioned above. The primary cell wall however is a complex and dynamic structure that is deposited at the cell surface forming a thin (50 nm – 100 nm) polymeric network of crystalline cellulose, hemicellulose, pectin and proteins (Cosgrove, 2000b). The primary cell wall is secreted during cell growth and is more flexible than the secondary cell wall. It must be clarified therefore that throughout this study the term 'cell wall' refers to the primary cell wall (unless stated otherwise).

In the past the cell wall was regarded as an inert structure whose role is to simply provide rigidity and physical protection to the protoplast (Preston, 1952). However it

is now well established that the plant cell wall is an active and highly dynamic compartment (Braam, 1999; Showalter 1993). While providing plant strength and rigidity, the cell wall also controls cell growth and division. It also participates in cell-to-cell communication, environmental sensing, signalling and pathogen defence (Showalter, 1993; Braam et al 1999). In addition to these various roles the cell wall is the most peripheral compartment of the cell, thus providing the first point of contact between the cell and the external environment. Plant cell walls are also precursors of various commercial products such as paper, textiles, thickeners, films and explosives. Despite these diverse and intriguing functions and applications, very little is known about the true diversity of cell wall proteins. It is mainly for this reasons that the work described in this thesis was embarked upon.

The intention of this chapter is to:

- Evaluate of the mechanisms by which primary cell walls are synthesised and assembled.
- Review all known cell wall structural proteins.
- Describe known cell wall associated enzymes.
- Explain the thesis objectives and the experimental strategies.

1.4 Cell Wall Biogenesis

The biosynthesis of plant cell walls is remarkably similar to that of most algae, fungi and bacteria (Cosgrove, 2000a). Matrix glycans, hemicelluloses and pectins are synthesised in the Golgi apparatus and are secreted to the extracellular matrix via secretory bodies (Figure 1.1). Cellulose, the largest cell wall macromolecule, on the other hand is synthesised by cellulose synthase complexes located in the plasma membrane. The synthesised cellulose is also secreted to the extracellular matrix where in the association with the secreted matrix glycans it forms long and thin cellulose microfibrils (Cosgrove, 2000b). Microfibrils cluster together to form a strong but flexible network of fibres surrounding the cell surface. The individual cellulose fibrils are held together by strong hydrogen bonds between complex branches of hemicellulose polysaccharide chains. Pectins on the other hand tend to form ionic bonds, which also participate in cellulose-matrix bonding (Cosgrove, 2000b) forming a primary extracellular matrix. It is believed however, that the strength of the cell wall network is provided by cross-linkings between xyloglucans (predominant hemicelluloses) and cellulose microfibrils (Reiter, 1998). Hemicellulose and other cell wall glucans are made up of at least 9 different sugars: glucose, galactose, mannose, xylose, arabinose, fucose, rhamnose, galacturonic acid and glucuronic acid. Apiose and aceric acid are occasionally found in some cell wall polysaccharides (Perrin et al, 2001). Presently little is known about the mechanism by which these sugars are organised in the Golgi apparatus, where glucans are made, and also no glucan synthases have been fully characterised to date (Perrin et al, 2001). This implies that little is known about glucan biosynthesis. It is known however that a xyloglucan synthase is involved in xyloglucan biosynthesis

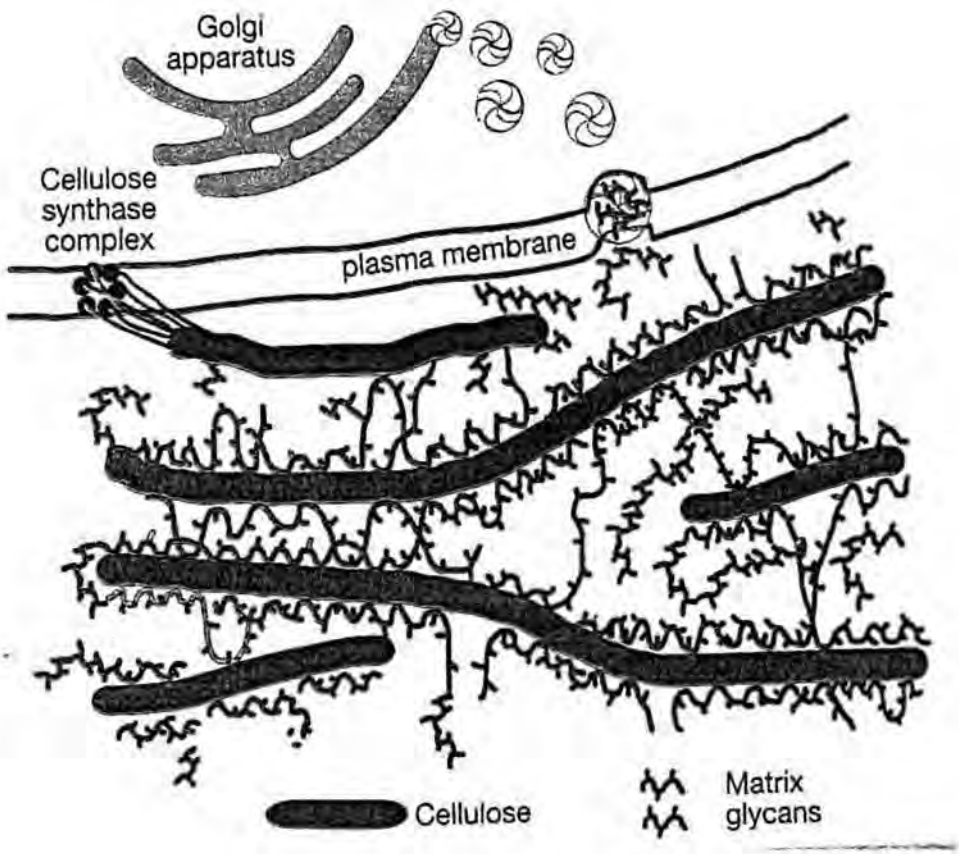


Figure 1.1 **Schematic diagram of cell wall structure and cell wall biogenesis.**

A schematic diagram showing a model of cell wall assembly. Cellulose microfibrils are synthesised by the plasma membrane bound cellulose synthase complex. The synthesised cellulose associates with hemicellulose and pectins, which are synthesised in the Golgi apparatus.
(Figure is adapted from Cosgrove, 2000b)

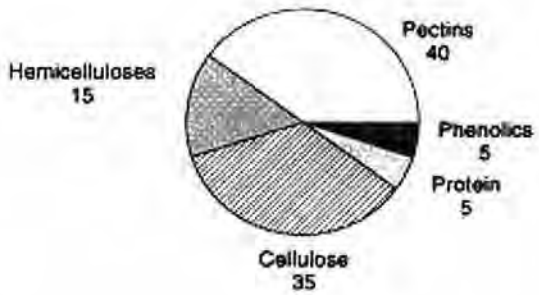
(formation of 1→4 β-D-glucan backbone). However this xyloglucan synthase can only function in combination with a xylosyl-transferase, an enzyme that catalyses the addition of xylose side chains to a polysaccharide (White et al., 1993). Whilst data is available to explain the mechanisms utilised by enzymes responsible for carbohydrate chain elongation, at this stage nothing is known about the biochemistry and enzymology of chain termination. It is however speculated that chain termination takes place once the polysaccharide chain is delivered to the extracellular matrix due to physical separation of the enzyme from the newly synthesised polysaccharide chain (Perrin et al., 2001).

Cell walls of monocots (particularly grasses) are different in structure compared to dicotyledonous species. Grasses possess little xyloglucan and pectin. Therefore, xylans and mixed-linked glucans form a major part of the cell walls in grasses (Carpita and Gibeaut, 1993). Mixed-linked glucans are composed of short blocks of β(1→4)-linked glucose molecules that are connected by β(1→3) glycosidic bonds.

1.5 The Linkage between Major Cell Wall Carbohydrates

The three dimensional structure of cell walls is formed by a complex network of carbohydrates, proteins, glycoproteins and phenolics. Carbohydrates alone constitute about 90% of the cell wall macromolecules, and its abundance varies in different plant species (Figure 1.2). Hemicelluloses (mainly xyloglucans and arabinoglucans) form hydrogen bonds with cellulose to create a complex network that is thought to provide the main structural support of the primary cell walls. Pectin, a mixture of

A



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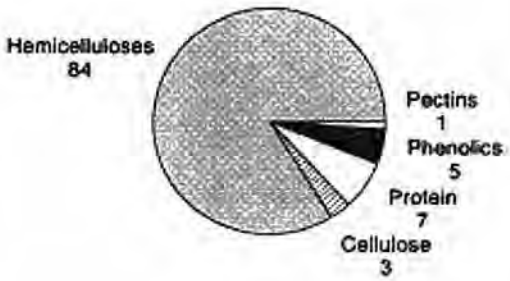

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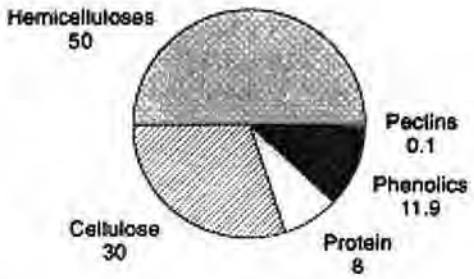


Figure 1.2. **Relative proportions (by weight) of the main cell wall macromolecules in various plant species.**

A. Fruit and vegetable
B. Cereal endosperm
C. Cereal bran

(Figure is adapted from Brett and Waldron, 1996)

sugar acids such as D-galacturonic acids that are connected by α1-4 glycosidic links, also forms a polymer network that is mostly independent of the cellulose-hemicellulose network. A schematic diagram showing the arrangement of various cell wall macromolecules is shown in Figure 1.3. Pectic polysaccharides can (in some instances) replace the cellulose-hemicellulose complex by cross-linking its polymers to form a tight cell wall-like structure (Brett and Waldron. 1996). Lignin, a phenolic polymer is laid down last after cell elongation and is believed to form ester bonds with other polysaccharides although the exact biochemistry remains obscure (Brett and Waldron, 1996). Cell wall polymers are also known to form hydrogen bonds, ionic bonds and other non-covalent bonds with cell wall structural proteins. The protein component of the cell wall is thought to be responsible for most of the catalytic roles assigned to the cell wall. It will therefore be reviewed in more detailed below.

1.6 Biosynthesis and transportation of cell wall proteins

Evidence of the presence of proteins in the cell walls of higher plants began to surface in the 1960's. Sadava and co-workers used autoradiographic detection following labelling with radioactive proline to show that hydroxyproline-rich proteins were *bona fide* resident proteins of plant cell walls (Sadava et al., 1969). Cell wall proteins like other cellular proteins are synthesised in the cytoplasm. However, the mechanism of their secretion to the extracellular matrix was unknown. In the early 1970's it was discovered however that eukaryotic proteins destined for

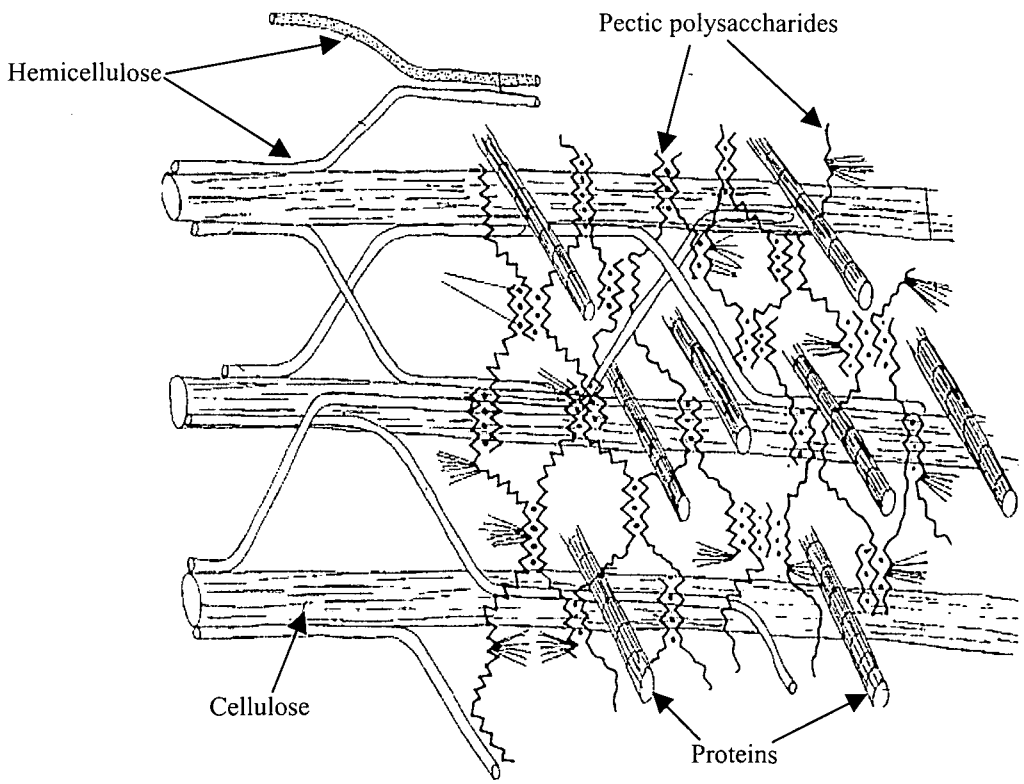


Figure 1.3 **A schematic diagram showing possible interactions between various cell wall macromolecules.**
(Figure is adapted from Brett and Waldron, 1996)

secretion segregated in the lumen of the endoplasmic reticulum (ER) before travelling to the cell surface (Palade, 1975). It is now known that cell wall proteins in the ER are transported across the plasma membrane via the Golgi apparatus (reviewed by Hadlinghton and Denecke, 2000).

Since all proteins are synthesised in the cytoplasm, those that are destined for secretion remain unfolded until they are translocated to the ER (Vitale and Denecke, 1999). In 1971, Blobel speculated that polypeptides that were destined for secretion possessed a "signal" for ER translocation. Blobel also postulated that the signal containing proteins crosses the membrane of the ER through special protein channels that are located along the ER membrane (Figure 1.4). In 1980, almost ten years later, Blobel and co-workers came to report that all the proteins that were destined for ER contained a specific amino acid sequence in their N-termini that functioned as a "tag" for secretion. It was soon discovered that proteins destined for other cellular compartments also carried a specific "address tag" in their polypeptides (Figure 1.5). This discovery had a major impact in cell biology research and for his efforts Gunter Blobel was awarded a Nobel Prize in 1999.

It is now known that the signal peptide is recognised during translation by ER luminal binding proteins that guide the newly synthesised polypeptide to the ER lumen. Following translation and ER translocation the signal peptide is cleaved by a signal peptidase to allow proper folding (Vitale et al., 1999). It is also known that the structure of the N-terminal signal peptide is defined by a positively charged n-region, followed by a hydrophobic h-region and a polar c-region (Nielsen et al.,

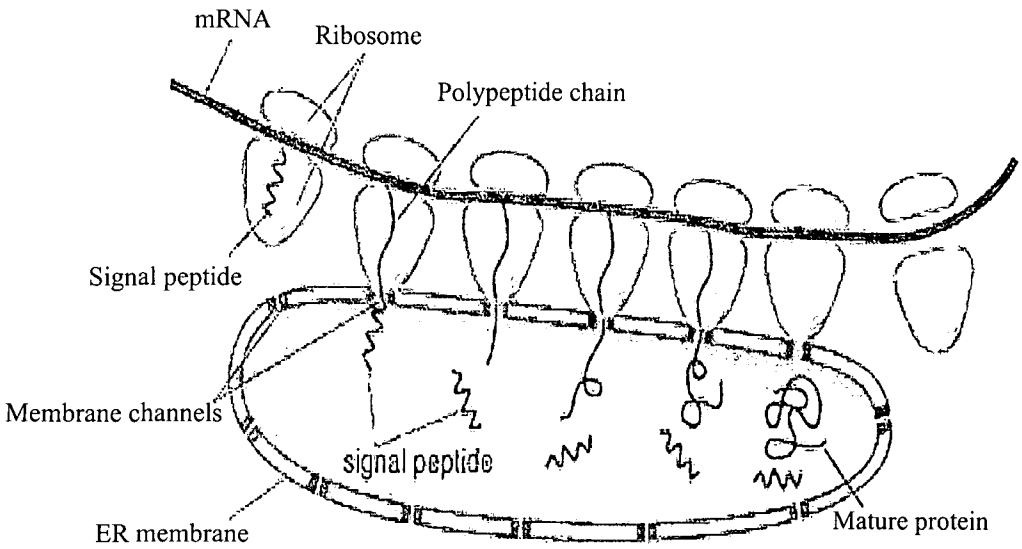


Figure 1.4. **The endoplasmic reticulum (ER) protein translocation model.**

The proteins destined for secretion have a N-terminus leader sequence (signal peptide) which is recognised by special ER located signal peptide binding proteins. After the polypeptide has translocated to the ER, the signal peptide is cleaved and the premature protein is folded and modified into a mature protein. (Figure adapted from The Nobel Foundation for Physiology and Medicine's Press Release, 2001)

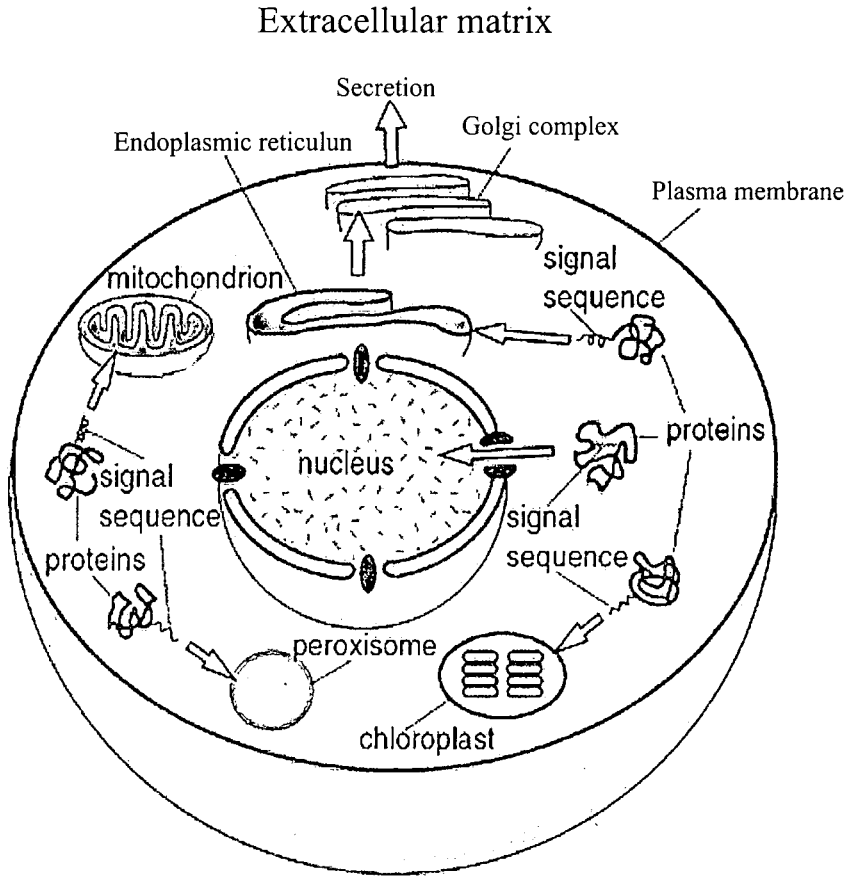


Figure 1.5. **Cellular protein trafficking.**

This figure shows a schematic cell with some of its organelle. Proteins destined for each organelle possess a special "tag" that signals its transportation to their destination. Note that proteins destined for extracellular secretion go via the endoplasmic reticulum and the Golgi complex. (Figure adapted from The Nobel Foundation for Physiology and Medicine's press release, 2001)

1997). Computer based algorithms that are designed to recognise these regions are also available (www.cbs.dtu.dk/services/SignalP). Once in the lumen the proteins are folded and modified into matured proteins. The most common post-translational modifications that take place in the ER lumen include proteolysis and glycosylation.

1.7 Protein glycosylation

The glycosylation of cell wall proteins takes place in the lumen of the ER and Golgi apparatus. Many proteins that are destined for secretion are glycosylated at N (asparagine) residues. There are structural motifs that are mostly preferred for glycosylation. These include a tetrapeptide sequence N-X-S/Y. In this sequence 'X' can be any amino acid but proline (Vitale et al., 1993). The enzyme that is known to catalyse this reaction is a multi subunit oligosaccharyl transferase. The protein glycosylation in the ER is thought to be involved in monitoring proper protein folding (Vitale and Denecke, 1999). Proteins that are partially folded are recognised and modified by glycosytransferases in combination with other ER chaperones (calnexin, calreticulins and lectins; Trombetta and Helenius 1997). Protein glycosylation and restricted folding mechanisms ensures the stability and the functionality of the mature protein (reviewed by Vitale and Denecke, 1999). Following correct folding and modification the cell wall destined proteins are delivered to the Golgi apparatus. The Golgi complex then transports the proteins across the plasma membrane to the extracellular matrix (see Figure 1.5)

It must be noted however that not all ER translocated proteins end up in the extracellular matrix. Some proteins contain ER retention tetrapeptides, such as, HDEL and KDEL, which signals their retention by the ER. In some instances the

Golgi apparatus vesicles are able to return proteins back to the ER via the retrograde transport system (Vitale and Denecke, 1999).

1.8 Structure and function of cell wall proteins

Cell wall located proteins can be divided into two families, i.e. the structural proteins and enzymatic proteins. Structural proteins of the cell wall are the most studied cell wall protein family and the first part of this section is aimed at reviewing them. Cell wall structural proteins are divided into 5 classes: extensins, glycine-rich proteins (GRP), proline-rich proteins (PRP), solanaceous lectins and arabinogalactan proteins (Reviewed by Showalter et al., 1993).

1.9 Extensins

Extensins, also known as hydroxyproline-rich proteins, are found in almost all plant tissues (Jose-Estanyol and Puigdomenech, 2000). All extensins contain a signal peptide that allows them to be exported to the cell wall. These proteins have a distinctive SPPPPPP amino acid repeat motif. The hydroxyproline residues of extensins are mostly glycosylated with up to 4 arabinosyl residues, and the hydroxylation of their prolines is performed by prolyl hydroxylases. Some extensin serine residues are also glycosylated with galactose (Reviewed by Showalter, 1993). Extensins are basic proteins. This basic nature is conferred by their high lysine content. These proteins also have polyproline helical structure and appear to have a

cylindrical shape when viewed via electron microscope (Reviewed by Showalter, 1993). The expression of the extensin genes is regulated by development, wounding and fungal elicitor attack (reviewed by Jose-Estanyol and Puigdomenech, 2000, Garcia-Muniz et al., 1998). Following their secretion, extensins are cross-linked to the cell wall. This process of cross-linking, followed by insolubilisation, is thought to occur via isodityrosine intermolecular bridges, mediated by extensin specific peroxidases (Schnabelnauch et al., 1996).

Extensins have also been reported in monocots (Jose-Estanyol and Puigdomenech, 2000). Maize extensins are mainly expressed in meristematic and vascular tissues and as in dicots, they are also induced by wounding (Stiefel et al., 1990). Most of the work reported on extensin studies has been carried out at the DNA and mRNA level. Progress in protein purification studies has been hampered by the lack of protein solubility. The lack of protein makes it extremely difficult to study protein expressions and post-translational modifications like hydroxylation, glycosylation and intermolecular isodityrosine linkages, which can only be determined on pure protein samples (Jose-Estanyol and Puigdomenech, 2000).

1.10 Glycine-rich Proteins

Glycine-rich proteins (GRP) are signal peptide containing proteins that are mostly dominated by Gly-X repeat units, where X is mostly Gly/Ala or Ser. Some GRP have up to 67% short Gly-X repeat units (Showalter, 1993). GRP gene has been isolated in many plant species including *Arabidopsis* (de Oliviera et al., 1990;

Quigley et al., 1991), corn (Goddemeier 1998; Matsuyama et al 1999), tobacco (Yasuda et al., 1997; Brady et al., 1993), petunia (Cheng et al., 1996). Not all GRPs possess a signal peptide for secretion. Two GRPs identified from maize and rice had no signal peptide (Mundy and Chua, 1988) and immunolocalisation studies localised these GRP to the cytoplasm. The precise function of cell wall GRP is yet to be reported. It is however believed that GRP are mainly structural proteins that are presumably involved in vascular system activities and wound healing. Their secondary structure assumes β-pleated sheets made up of a number of antiparallel strands. These structures are predicted to provide elasticity and tensile strength during vascular development (Showalter, 1993). Similar to extensins, GRP are often cross-linked and insolubilised in the cell wall matrix. Most recently GRPs have been found to be linked to cell wall associated kinases (WAKs; Anderson et al., 2001). It is also interesting to note that some GRPs are tightly bound to phosphorylated WAKs and pectins. These pectin-protein-carbohydrate cross-links are thought to play a significant part in the control of cell expansion (Kohorn, 2001). It has also been shown recently that GRP form specific protein complexes with WAKs and kinase-associated protein phosphatases, which are induced by pathogen elicitors (Park et al., 2001). These observations suggest that GRP's might be involved in signal transduction.

1.11 Proline-rich proteins

Proline-rich proteins (PRP) are also cell wall structural proteins. Their N-terminae have a signal peptide for secretion (Showalter et al.,1993). The signal peptide in

PRP is followed by PPVYK, which give them their characteristic repetitive motifs (Jose-Estanyol and Puigdomenech, 2000). Like GRPs and extensins, PRP are rapidly insolubilised in the extracellular matrix following their secretion. The PRP and extensin repeat motifs are very similar e.g. P-P-V-Y-K of PRP is similar to Ser-hyP$_4$-V-Y-K of extensins (Showalter et al., 1993). PRP are mainly expressed in proxylem and xylem structures in contrast to extensins, which are normally found in actively growing cells (primary cell walls). PRP genes have been found in many plant species including *Arabidopsis* (Fowler et al., 1999), soybean (Hong et al., 1989), alfalfa (Deutch and Winicov, 1995), wheat (Raines et al., 1991) and maize (Vignols et al., 1999).

PRP are developmentally expressed (Showalter, 1993) but their genes are also induced by salt (Deutch and Winicov, 1995) and fungal elicitors (Showalter, 1993). The rapid insolubilisation of PRP may function as a physical barrier that protects the cell against pathogen invasion (Bradley et al., 1992). PRP are also thought to play a role in nodule morphogenesis in legumes, specifically in the composition of cell wall oxygen barrier for oxygen sensitive nodules (Scheres et al., 1990). PRPs have been recently purified from the cell walls of chickpeas and were shown to crosslink *in vitro* (Otte and Barz, 2000).

1.12 Arabinogalactan proteins

Arabinogalactan proteins (AGP) are secreted hydroxyproline-rich-proteins. These proteins however differ from extensins in that they are also rich in serine, alanine,

threonine and glycine. AGPs are proteoglycans with an estimated carbohydrate content of 90-95%. Their main carbohydrates are mainly arabinose and galactose. Other minor carbohydrates are also present including, rhamnose, glucuronic acid and other monosaccharides (Jose-Estanyol and Puigdomenech, 2001). The sugar groups are O-linked to hydroxyproline serine and threonine amino acids of the core protein (Fincher et al., 1983). Some AGP are shown to be associated with the plasma membrane while others are associated with the intravascular bodies (Jose-Estanyol and Puigdomenech, 2001). AGP are found in various plant tissues including vascular bundles, schlerenchyma cells, pollen and pollen tubes (Showalter, 1993). AGP genes, like other cell wall structural proteins described above, have been isolated in many plant species, including *Arabidopsis* (Willats and Knox, 1996), maize (Samaj et al., 1999) carrot (Baldwin et al., 2001), wheat (Shroder et al.,1999) and tomato (Gao and Showalter, 2000).

Arabinogalactan proteins are thought to function in plant growth and development (Gao and Showalter, 2000). Although AGPs are highly soluble and acidic they have been shown to undergo oxidative cross-linking in the cell wall matrix (Kjellbom et al., 1997). Kjellbom et al., (1997) has also predicted that AGPs play a role in cell to cell signalling during development. Like GRPs, AGPs have been shown to form associations with wall-associated kinases (WAKs) thus supporting the theory that these cell wall proteins are involved in signalling (Gens et al., 2000). AGP and WAKs form a polyhedral array at the external face of the plasma membrane and these arrays appear to anchor the wall tightly to the plasma membrane (Gens et al., 2000).

1.13 Solanaceous lectins

Solanaceous lectins are a very diverse group of glycoproteins and are exclusively expressed in solanaceous plants. They are predominantly located in the cell wall and have a distinctive carbohydrates structure mostly made up of hydroxyproline and arabinose (Showalter, 1992). Potato lectins, the most well studied solanaceous lectin are made up of a lectin domain fused to a hydroxyproline-rich glycoprotein domain (Kieliszewski et al., 1994). This glycoprotein consists of approximately 50% protein and 50% carbohydrate by weight and the linkage of tri and tetra-arabinosides and galactose is with serine residues of the core protein (reviewed by Showalter, 1993). The core protein is mostly rich in hydroxyproline, serine, glycine and cysteine.

The core protein of solanaceous lectins is made up of two distinct polypeptides: a serine/hydroxyproline-rich glycopeptide and a glycine/cystein-rich polypeptide (Showalter, 1993). The serine-hydroxyproline-rich glycopeptide domain of solanaceous lectins shares a significant homology to extensins. Both solanaceous lectins and extensins have a high abundance of serine, arabinose and hydroxyproline. These proteins also have identical carbohydrate-protein linkages (Showalter, 1993). Their hydroxyprolines are attached to tri and tetra arabinoses and serines. This suggests that the serine-hydroxyproline-rich domain of solanaceous lectins share the same ancestry with extensins.

Potato tuber lectins (PTLs) have been shown to accumulate in response to wounding and pathogen invasion (Millar et al., 1992). The actual function of PTLs is still

obscure however its response to wounding and pathogen invasion suggests that lectins might be involved in wound healing and defence against pathogens.

1.14 Cell Wall located Enzymes

In addition to structural proteins, described above, plant cell walls harbour proteins with recognised enzymatic functions (Table 1.1). The cell wall enzymes can be divided into two categories: those, which are involved in cell wall modification and those whose precise role in the cell wall is still obscure.

1.15 Cell wall modification associated enzymes

Cell wall located proteins that are involved in cell wall modification include α-mannosidases, β-mannosidases, β-1.3 glucanases, β-1-4glucanases, polygalacturonases, pectin methylesterases, arabinosidases, α-galactosidases, β-galactosidases, β-glucuronosidases, β-xylosidases, arabinose transferase, xyloglucan endotransglycosylase, exolyase, allergens, expansins (Showalter, 1993; Brett and Waldon, 1996; Jose-Esanyol and Puigdomenech, 2000) and cell wall associated receptor-like kinases (He et al., 1996). These enzymes however occur in lower abundances in the cell wall compared to structural proteins. There is very limited information documented about the *in vivo* roles played by these cell wall enzymes, however the most studied are the expansins. In addition to expansins this section

Table 1.1 A list of known cell wall proteins

Protein	**Role *In vivo***	**Reference**
Extensin	Structural protein	Showalter, 1993
Glycine -rich protein	Structural protein	Showalter, 1993
Proline-rich protein	Structural protein	Showalter, 1993
Solanaceous lectin	Structural protein	Showalter, 1993
Secondary cell wall specific glycoprotein	Structural protein	Wojtaszek and Bolwell, 1995
α-mannosidase	PCWME	Varner and Lin, 1989
β-mannosidase	PCWME	Varner and Lin, 1989
Arabinosidase	PCWME	Showalter, 1993
Pectin methylesterase	cell wall metabolism	Wen et al., 1999
β-1.3 glucanase	PR protein	Showalter, 1993
β-1-4glucanase	PR protein	Showalter, 1993
Polygalacturonase	PCWME	Varner and Lin, 1989
α-galactosidase	PCWME	Varner and Lin, 1989
β-galactosidase	PCWME	Varner and Lin, 1989
β-glucuronosidase	PCWME	Varner and Lin, 1989
β-xylosidase	PCWME	Varner and Lin, 1989
Arabinose transferase	CWBAP	Brett and Waldron, 1996
Xyloglucan endotransglycosylase	CWBAP	Thompson and Fry, 2001
Exolyase	PCWME	Brett and Waldron, 1996
Allergens	PCWME	Jose and Puigdomenech, 2000
Cell wall associated receptor-like kinases	Structural protein/ Putative signalling	He et al., 1996
Chitinase	PR protein	Brett and Waldron, 1996
Ascorbic acid dehydrogenase		Showalter, 1993
Peroxidase		Showalter, 1993
ATPase		Kiba et al., 1996
Invertase		Showalter, 1993
Protease		Varner and Lin, 1989
Malate dehydrogenase		Brett and Waldron, 1996
Arabinogalactan protein		Showalter, 1993
Cellobiohydrolase		Brett and Waldron, 1996
Cellulase	PCWME	Brett and Waldron, 1996
Endoglycanase	PCWME	Brett and Waldron, 1996
Polygalacturonases inhibiting protein	Pathogen defense	Yao et al., 1999
Endolyase	PCWME	Brett and Waldron, 1996
Endoxylanase	PCWME	Brett and Waldron, 1996
Exoglucosidase	PCWME	Brett and Waldron, 1996
Galactosidase	PCWME	Brett and Waldron, 1996
Galacturonyltransferase	PCWME	Brett and Waldron, 1996
Glucosyltransferase	PCWME	Brett and Waldron, 1996
Glucuronyltransferase	PCWME	Brett and Waldron, 1996
Lectins	Structural protein	Showalter, 1993
Pectinase	PCWME	Brett and Waldron, 1996
Phosphatase		Varner and Lin, 1989
Pectin methyltransferase	Virus movement protein receptor	

Key: PCWME – Possible cell wall modifying enzyme
CWBAP – Cell wall biosynthesis associated protein

will also review cell wall associated receptor-like protein kinases and their possible involvement in signal transduction.

1.16 Expansins

Expansins are cell wall enzymes that have been shown to catalyse plant cell wall expansion (Cosgrove, 2000b). Expansins have been particularly associated with acid induced cell growth and are thought to facilitate this by loosening non-covalent associations formed between polysaccharides (Reviewed by Jose-Estanyol and Puigdomenech 2000). The first expansin cDNA was sequenced in 1995 (Shcherban et al., 1995) and it was found to encode a protein which had a signal peptide N-terminal domain for secretion, a conserved cysteine-rich central domain, a basic region and a conserved tryptophan-rich C-terminus (Jose-Estanyol and Puigdomenech 2000). The conserved cystein-rich region is thought to be involved in the formation of intra-molecular bridges (Cosgrove, 1997). Expansins have been purified from rapidly growing plant tissues and were found to be less glycosylated than most cell wall proteins and were not soluble in low ionic strength solutions (Cosgrove, 2000).

Expansins have been found in several plant tissue types, including meristematic tissues and pericarp of ripening fruit (Jose-Estanyol and Puigdomenech, 2000). These enzymes play a significant role in fruit ripening (Cosgrove, 2000). When over expressed, expansins hydrolyse the cell wall polysaccharides during the final stages of fruit development (Rose et al., 1999). In tomato, expansin expression was shown to be stimulated by ethylene, a plant development associated hormone (Rose et al., 1997). Experimental work involving constitutive expression of expansins in tomato

resulted in slower fruit growth and also caused a rubbery texture to the flesh of the fruit (Brummell et al., 1999).

Expansins share up to 25% similarity with Group 1 allergens. Group 1 allergens are hay fever causing cell wall proteins that are found in grass pollen grains (Cosgrove, 1997). Following the completion of the *Arabidopsis* genome-sequencing project, more genes coding for putative expansins have been discovered. A model showing an expansins in action is shown in Figure 1.6. It has been shown using nuclear magnetic resonance (NMR), however, this model does not account for the stretching that allows expansion of the cell wall (Whitney et al., 2000). This therefore means that the exact mechanism underlying expansin function still requires further elucidation. However, despite these loop holes in the present understanding of expansins, it is clear that they offer many potential applications especially in bioengineering and biomedicine.

1.17 Cell wall associated receptor-like protein kinases.

Receptor-like protein kinases that are tightly associated with the plant cell wall have been reported (He et al., 1996). The discovery of these cell wall associated kinases (WAKs), suggested that plant cell walls are directly involved in signal perception (He et al., 1998). These proteins, although they are tightly associated with the cell wall they also possess transmembrane domains (Figure 1.7). These characteristics suggested that WAKs could be the part of the physical continuum between the cytoplasm and the extracellular matrix. Also, WAKs are thought to be involved in sending the cytosolic derived cell wall expansion cues to the cell wall modifying enzymes that are located in the extracellular matrix (Wanger and Kohorn, 2000).

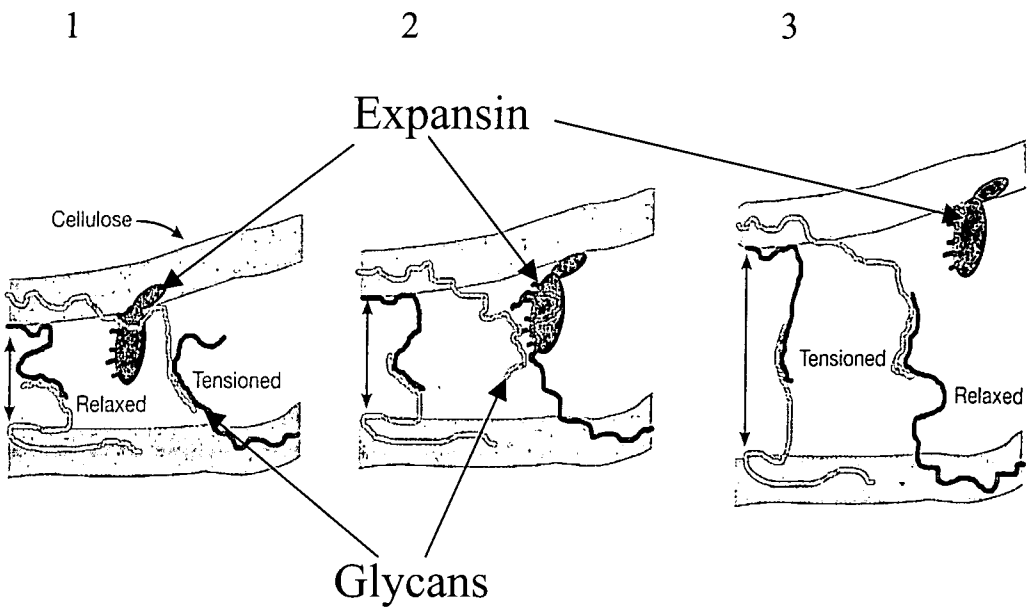


Figure 1.6 **A model for expansin mediated cell wall loosening**.

It is believed that expansins relieve tension in the cell wall by disrupting the cellulose-glycan and glycan-glycan bonds.
(This figure is adapted from Cosgrove, 2000).

WAKs are not the only proteins known to physically link the cell wall and cytoplasm wall. Others include, an arabinogalactan protein (Oxley and Bacic, 1999), cellulose synthase (Pear et al., 1996) and a putative endo-1,4-β-D-glucanase (Nicol et al., 1998). Additionally, the *Arabidopsis* genome has over 500 putative receptor-like protein kinases, which possess at least one transmembrane domain (Figure 1.7). The function of receptor proteins is to perceive stimuli and convey the signal to the downsteam elements (Trotochaud et al., 1999). Cell wall receptor-like kinases are therefore thought to be involved in extracelullar stimuli perception (He et al., 1998). The mRNA of WAKs is induced following pathogen invasion (He et al., 1998). This induction suggests that WAKs are involved in pathogen defence (He et al., 1998). Most recently, a novel chitinase-related receptor-like kinase was discovered (Kim et al., 2000). This novel chitinase-related receptor-like kinase has a transmembrane domain and due to its chitinase-like structure it is likely to have some association with the cell wall matrix. Kim, et al, (2000) also demonstrated that their chitinase-related receptor kinase was an autophosphorylating kinase. Although this protein resembles chitinases it has no chitinase activity, but its mRNA in induced by fungal elicitors (Kim et al., 2000). It can therefore be speculated that this receptor-like protein evolved from plant cell wall chitinases to become a chitin binding receptor protein kinase.

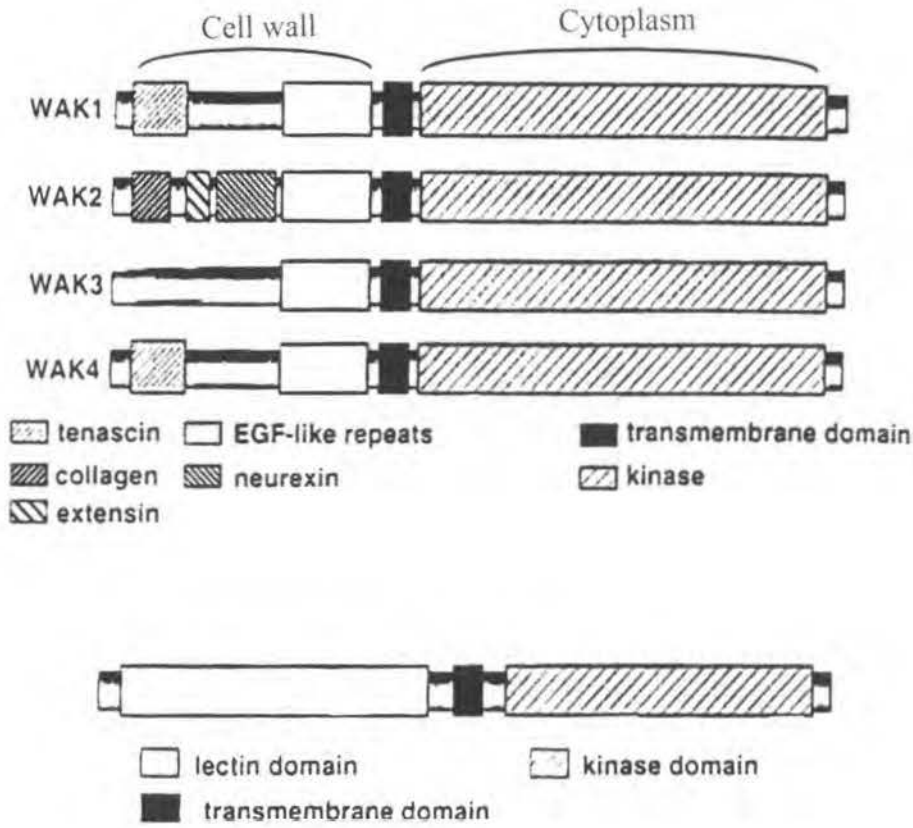


Figure 1.7 **Schematic diagram of various cell wall associated receptor-like kinases**.
All the documented putative receptor-like protein kinases with an extracellular receptor-like domain possess at least one transmembrane domain. The patterns indicate the BLAST sequence similarities to various proteins. The predicted transmembrane domain is also indicated. (Figure adapted from Hardie, 1999)

1.18 Other cell wall enzymes

In addition to structural proteins and cell wall modification associated proteins, plant cell walls harbour enzymes whose physiological function in the cell wall remains controversial. This class of cell wall enzymes include, ascorbic acid dehydrogenase (Showalter, 1993), peroxidase (Bolwell et al., 1999), ATPase (Kiba et al., 1996), invertase (Showalter, 1993), malate dehydrogenase (Brett and Waldron, 1996) and several more (see Table 1.1). Plants cell walls are not the only cell walls that harbour enzymes with, hitherto, no justified extracellular functions. This phenomenon has also been observed in fungal cell walls (Chaffin et al., 1998).

Plant cell walls are similar to fungal cell walls. In both organisms the major cell wall constituents are polysaccharides (90%) and proteins (10%; Chaffin et al., 1998; Robertson et al., 1997; Cosgrove, 2000). One of the major differences between the two is that fungal cell wall carbohydrates are mainly composed of β-glucans. Due to their medical importance, the study of fungal cell wall proteins is far more advanced than that of plants. Many plant cell wall proteins identified to date are also found in fungal cell walls, these include β-(1-3)-glucanases, mannosidases, chitinases, xylosidases and polygalacturonidases (Wegener et al., 1999; Showalter, 1993). However in addition to these common enzymes, fungal cell walls harbour many other enzymes (Table 1.2). The diversity of cell wall proteins that are localised to the fungal extracellular matrix is reviewed by Chaffin et al., (1998). In addition, Sepulveda et al., (1996) also presented tantalizing evidence of the presence of unexpected proteins in the cell walls of *Candida albicans*. The question that has not yet been answered is whether the complement of plant cell wall proteins differs in

Table 1.2 Proteins identified from fungal cell walls (adapted from Chaffin et al., 1998)

Protein	Role *In vivo*
Exo-β-(1,3)-glucanase	Cell wall morphogenesis
β-1,3-glucan transferase	Cell wall metabolism
Chitinase	Hydrolytic enzyme, cell wall morphogenesis
β-*N*-acetylglucosaminidase	Hydrolytic enzyme, virulence factor?
Transglutaminase	Covalent cross-links?
Secreted aspartyl proteinase	Putative virulence factor, gene expression condition dependent
Phospholipase A	Hydrolytic enzyme
Phospholipase B	Hydrolytic enzyme, putative virulence factor
Phospholipase C	Hydrolytic enzyme
Lysophospholipase	Hydrolytic enzyme
Lysophospholipase-transcylase	Hydrolytic enzyme, putative virulence factor
Esterase	Hydrolytic enzyme
Glucoamylase	Hydrolytic enzyme
Hemolytic factor	Hydrolytic enzyme
Acid phosphatase	Hydrolytic enzyme
Lipase	Hydrolytic enzyme
Hyaluronidase	Hydrolytic enzyme, virulence factor?
Chondroitan sulfatase	Hydrolytic enzyme, virulence factor?
Metallopeptidase	Hydrolytic enzyme
Trehalase	Hydrolytic enzyme
4C12 antigen (180 kDa, 260 kDa)	Structural protein ?
3D9 antigen (110-170 kDa)	Structural protein ?
DC3 :H10antigen	Present in complex with other proteins
Hwp1p (34 kDa)	N-terminal signal sequence, tandem repeats, C terminus ST rich
Hyr1p (34 kDa)	N-terminal signal sequence, tandem repeats, C terminus ST rich
30 kDa cell wall protein	Probable post translation regulation of location
Hsp70 (70 kDa)	Chaperone, Translocation, Immunogenic
Hsp90 (48 kDa fragment)	Hsp90 product, immunogenic, Ab protective
Enolase	Binds to glucan, lacks N-terminal signal peptide
Phosphoglycerate kinase	lacks N-terminal signal peptide, immunogenic
Fibrinogen binding protein	Fibrinogen binding, ubiquinated, collagen-like
Laminin binding proteins	Yeast cell wall, ubiquinated, collagen-like
Glyceraldehyde-3-phosphate dehydrogenase	Enzyme activity, immunogenic
Protein-protein CR2	
iC3b binding protein	Epithelial, endothelial cell adhesin
Glyceraldehyde-3-phosphate dehydrogenase	Enzyme activity, immunogenic
Collargen binding protein	60 kDa species reacts with MAb
Entactin binding protein	
Vitronectin binding protein	Cell wall protein
Ala 1p	Laminin and fibronectin binding protein
Fucose binding protein	Buccal, vaginal epithelial cell adhesin
GlcNAc binding protein	Buccal, vaginal epithelial cell adhesin
Protein-glycosphingolipid frimbrial protein	Epithelial cell adhesin

Table 1.2 Proteins identified from fungal cell walls (adapted from Chaffin et al., 1998)

Secreted aspartyl proteinase	Endothelial binding protein
Plastic binding protein	Binding to many plastic materials
b-1,2 mannotetraose	Mannose receptor
Acid-stable mannan factor 6	Adhesion

complexity to fungal cell wall proteins. Many of the challenges presented to the fungal cell are similar to those faced by plant cells. For example, both cell types use the cell wall to: provide structural support, perceive environmental stimuli, facilitate cell-to-cell adhesions, protect the protoplast, and facilitate cell division, growth and development. It is therefore quite conceivable that the protein population of plant cell walls could be much more diverse than it is thought to be at the present time.

1.19 Thesis objectives and experimental strategies

- The main aim of this thesis is to extract, identify and characterise as many cell wall proteins as possible.
- Mapping of the entire cell wall proteome is likely to reveal novel cell wall proteins.
- Novel cell wall proteins will lead to the formulation and testing of hypotheses on novel cell wall functions.

Mapping of the entire organelle proteome became possible since the introduction of the proteomic approach towards mass protein characterisation (Garin et al., 2001). In the past attempts of mapping the entire cell wall proteome yielded less than optimum results. This was due to various complications: (1) Cell wall proteins are difficult to solubilise, (2) Since one dimension gel electrophoresis was the method used for protein separation, the protein-to-protein resolution was very low (Robertson et al.,

1997), (3) The available protein identification method then, the Edman N-terminal sequencing, was unable to sequence proteins with blocked N-termini (Robertson et al., 1997) and the throughput was very low, that is, very few proteins could be sequence per day. (4) Prior to the end of the year 2000 there was no full plant genome data, therefore protein identification was only possible via direct amino acid sequencing.

All these problems have virtually disappeared with the completion of the *Arabidopsis* genome sequence (December, 2000) and the development of a more reproducible large format two-dimension polyacrylamide gel electrophoresis protein resolving technique (reviewed by Gorg et al., 2000). The identification a large number of proteins was facilitated by the optimisation of a novel high throughput protein identification technique (mass spectrometry; Reviewed by Pennington and Dunn, 2001).

The plant material chosen for the cell wall proteomic studies was *Arabidopsis thaliana* cell suspension cultures. *Arabidopsis* was chosen because it is presently the only plant with a fully sequenced genome. Cell suspension cultures were chosen because they are composed of one cell type, thus avoiding cell type to cell type variations often seen in whole plant tissues. Also cell cultures are advantageous over whole plant tissues, because they can be grown in a tightly controlled physiochemical environment. The growth of cell cultures in a precisely controlled environment provides a highly reproducible experimental system. Additionally, cell cultures provide an unlimited amount of experimental material, because they can be

grown *en masse* in days rather than weeks or months. Finally, the cell walls of undifferentiated plant cell cultures resembles the primary cell walls of all cell types found in whole plant tissues.

1.20 Study of plant cell wall proteins using the proteomic approach

It is clear that detailed studies of the whole protein population of plant cell walls is still lacking. Research on the diversity of cell wall proteins has never been done in one plant species, i.e. all the cell wall protein data that is available has been extrapolated from several plant species (Showalter, 1993; Jose-Estanyol and Puigdomenech, 2000). However, Showalter, (1993) warned that caution should be taken when such extrapolations are made. It is known that there are many variations between plant species, tissue types and even cell types, therefore the extrapolation of data from one plant species to represent a situation in another plant species might lead to inaccurate conclusions.

Most of the data on cell wall proteins has been obtained from cDNA and genomic clone studies (Showalter 1993; Jose-Estanyol and Puigdomenech, 2000). The cell wall protein data that has been obtained at the protein level is very limited (Robertson et al., 1997; Showalter 1993). The limitations in direct protein studies have been mainly due to the low solubility of cell wall proteins and the unavailability of an efficient system that allows separation and identification of the cell wall proteome on a large scale.

1.21 Proteomics

The term "proteomics" refers to the characterisation of proteins expressed by the genome (Reviewed by Pennington and Dunn, 2001). Presently, the proteomic approach has been adopted as the main tool for studying complex protein mixtures (Hoving et al., 2000; Rechinger et al., 2000; Issaq, 2001). In most proteomic studies complex mixtures of proteins are resolved via two-dimensional gel electrophoresis (2-D PAGE) and the resolved protein spots are identified and characterised by mass spectrometry based methods (Pennington and Dunn, 2001).

1.22 Two dimension polyacrylamide gel electrophoresis (2D PAGE)

The resolution of complex mixtures of proteins via two dimensions (isoelectric point and molecular weight) was pioneered by O'Farrell (1975). The first dimension of 2D PAGE involves isoelectric focusing of proteins, which is carried out on a thin polyacrylamide gel.

1.23 Isoelectric focusing (IEF). Polypeptides are amphoteric molecules, i.e. they are both positively charged or negatively charged molecules. The total charge of any polypeptide is the sum of all negative and positive charges of their amino acid side chains. The charge of the polypeptides is affected by the pH of its environment, i.e. a protein is positively charged at pH below its pI and negatively charged at pH above its pI. For example, a protein with pI 8 will be negatively charged at pH 9 and will be positively charged at pH 6. When a mixture of proteins with various pI's are loaded into a pH gradient gel they acquire a positive, neutral or negative charge. When

subject to an electric field, positively charged proteins migrate toward the basic end direction and the negatively charged proteins migrate toward the acidic direction (Figure 1.8).

Historically, the IEF step was carried out on a gel rod with carrier ampholyte generated pH gradients. However, in order to gain full protein separation, this method required very long focusing time period. Unfortunately, prolonged focusing times at the required high levels of voltage, adversely affected the stability of the pH gradient. This disruption in the stability of the pH gradient can be described by the phenomenon known as 'cathodal drift', which refers to voltage induced carrier ampholyte changes. For example, the gradient range that started as pH 3 to 10, becomes a final gradient of pH 4 to 7, thus losing all proteins in the range of pH 3-4 and pH 7 to 10. The cathodal drift was however remedied by using a non-equilibrium pH gradient electrophoresis (NEPHGE), where a sample is loaded on the acidic end of the gradient and the voltage applicator allows the basic protein samples to migrate towards the cathode. However in order to gain a better reproducibility a precise focusing time had to be applied (O'Farrell et al., 1977). Although this modification led to improved reproducibility, the protein resolution was compromised (Westermeier, 2000).

The biggest leap in 2D PAGE came with the development of immobilised pH gradients (Gorg et al., 1988). The gel tubes were replaced by a thin (0.5 mm) dehydrated gel strips supported by a strong plastic film, which allowed easier handling. The immobilised pH gradients confer several distinct advantages over the old system: (1) they are highly reproducible; (2) they allow separation of proteins

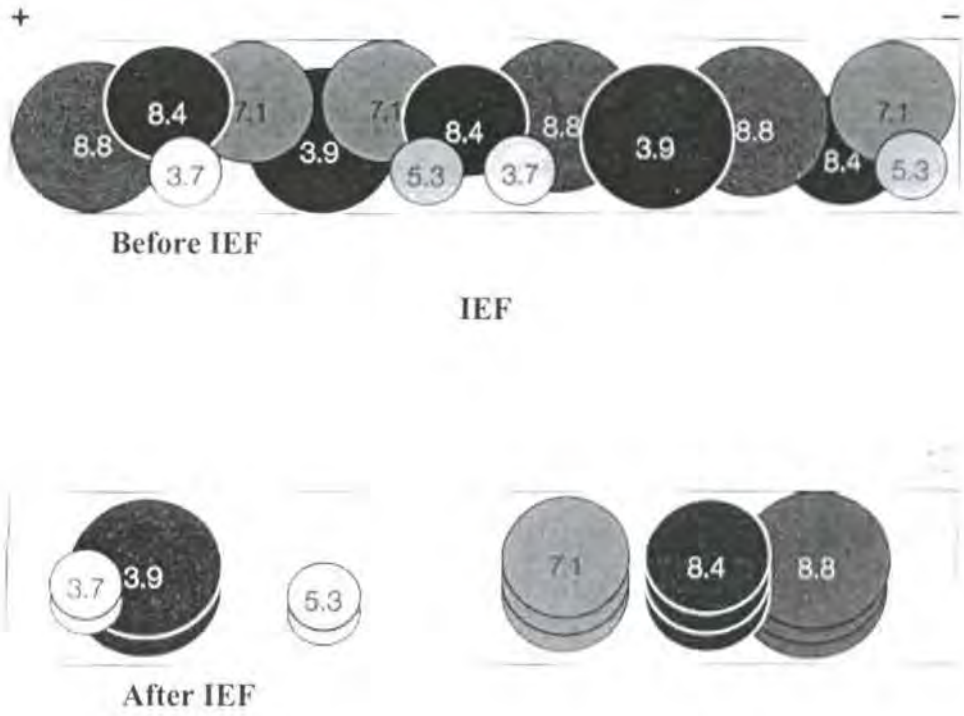


Figure 1.8 **A sample of protein mixture before and after isoelectric focusing.**

When a mixture of proteins with various pI's are loaded onto a pH gradient gel, they acquire a positive or a negative charge. When subject to an electric field, positively charged proteins migrate toward the basic end direction and the negatively charged proteins migrate toward the acidic direction until they reach their isoelectric point.

over both wide and narrow pH gradients and (3) they virtually eliminated gradient drifting (Westermeier, 2000). In addition to all these advantages, dried IEF gels of immobilised pH gradients can be rehydrated with the sample solution. The in-gel rehydration allows large sample loadings and also allows a uniform sample loading throughout the pH spectrum.

1.24 The Second Dimension of 2D PAGE. Following IEF the proteins are mixed with SDS containing equilibration buffer (see chapter 2) and transferred to a second polyacrylamide gel for a second dimension PAGE. In principle the second dimension of 2D PAGE involves the separation of proteins according to their molecular weights. The purpose of the equilibration step following IEF is to supply the proteins with negatively charged sodium dodecyl sulfate which coats all the proteins in proportion to their size (Laemmli, 1970). The SDS coated proteins migrate with a uniform negative charge-to-mass ratio under the electric field. The migration distance of these proteins has a logarithmic relationship to the protein mass (Westermeier, 2000).

1.25 Methods Used for Protein Detection (reviewed by Westermeier, 2000)

After the second dimension of 2D PAGE the resolved protein spots can be detected using a wide range of techniques. These techniques include Coomassie Brilliant Blue staining, Silver staining, negative staining, fluorescence staining and fluorescence labelling.

1.25.1 Coomassie Brilliant Blue Method. Coomassie Brilliant Blue method is the easiest and most widely used method for staining proteins. It stains almost all polypeptides with an efficient quantitative linearity and is compatible with mass spectrometry. The limitation of Coomassie lies with its low sensitivity. This stain is known to detect proteins from 0.1μg upwards.

1.25.2 Silver staining. Silver is a very sensitive method of protein detection. It can visualise protein spots from 0.2ng. There are two silver staining procedures: the silver nitrate method and the silver diamine method. Silver diamine is the most sensitive of the two methods and is particularly effective in the case of basic proteins. In the silver nitrate method, the silver binds proteins more weakly, but it can be made compatible with mass spectrometry after several modifications. These modifications however can lead to a reduction in sensitivity. Both methods require tedious multiple steps which are likely to increase the risk of contamination. Unlike Coomassie Brilliant Blue, the silver staining methods are not quantitative.

1.25.3 Negative (Zinc) Staining. This technique stains polyacrylamide gels and not protein spots. It is also a highly sensitive method that can visualise protein spots as low as 15ng. Since the proteins themselves are not stained, there is a high protein yield for further mass spectrometry. Similar to silver staining, zinc negative staining is not quantitative.

1.25.4 SYPRO Ruby Red Fluorescence Staining. This method is also highly sensitive, although less sensitive than silver staining. SYPRO Ruby Red has a linear quantitative range of 10^4 (2ng to 20μg) and is compatible with mass spectrometry. However visualisation of the spots require a special fluorescence scanner/camera, making this technique an extremely expensive option.

1.25.5 Fluorescence Protein Labelling. CyDyes (or Bimane) can be conjugated to protein samples prior to IEF. Different derivatives of CyDyes can be excited and emitted at different wavelengths thus allowing analysis of two different samples in one gel i.e. sample A and sample B can be labelled with CyDye-a and CyDye-b respectively. Since CyDyes label proteins prior to IEF, sample A and sample B can be pooled and loaded in the same gel. The different excitation and emission wavelengths allow visualisation of sample A independent of sample B, although they are both mixed in one gel, making direct sample comparisons much easier. CyDyes are highly sensitive and are compatible with subsequent mass spectrometry analysis.

Other widely used protein visualising techniques can also be applied in 2D PAGE resolved proteins including radioactive labelling, stable isotope labelling and western blotting.

1.26 Protein Identification: Mass spectrometry

A mass spectrometer is an instrument that can detect the mass (m) and the charge (z) of any ionisable molecule. This instrument has three main components: (1) a source of ionisation; (2) a mass analyser and (3) a detector. The most widely used mass spectrometers in proteomics are the mass assisted laser desorption ionisation time of flight (MALDI-ToF; Figure 1.9) and the tandem mass spectrometer (Reviewed by Pennington and Dunn, 2001)

1.26.1 Matrix assisted laser desorption ionisation time of flight (MALDI-ToF).

In proteomic studies the MALDI-ToF mass spectrometer is employed as a peptide mass measuring instrument. The application of MALDI-ToF was first described in 1988 by Karas and Hillenkam. Peptides, which are normally generated from protelytically digested 2D PAGE spot are mixed with a low molecular weight matrix, which has an absorption maximum at the wavelength of the ultra-violet laser (Westermeier, 2000). The matrix normally used for peptide analysis is either a α-cyano-4-hydroxy cinnamic acid or 2,5-dihydroxybenzoic acid (Beavis and Chait, 1989). The mixture of the matrix and peptides is applied and dried on a special mass spectrometer metal slide. As the matrix solvent dries, the peptide molecules are crystallised with the matrix. A pulsed UV laser beam of 337nm wavelength is fired into the matrix-peptide mixture. The energy of the UV pulse is absorbed by the matrix and this effect induced the ionisation of both the matrix and the peptide molecules. The excited peptide ions of different masses are accelerated in an electric field at high voltage towards a high vacuum tube of the mass spectrometer. The peptide ion times of flight (ToF) are detected by the mass spectrometer detector normally in the reflection mode (Figure 1.9). The lighter (smaller) ions fly faster than the heavier (larger) peptide ions and therefore the recorded times of flight can be used to calculate the masses of the peptide ions. The peptide mass data is normally recorded as the mass/charge ratio (m/z). The peptide mass ion spectra generated from the proteolytic digests is used to interrogate the genomic databases, which contains theoretical tryptic digests (Westermeier, 2000). This method however depends heavily on the availability of the genome sequence data, therefore its application is limited to those organisms whose genomes have been fully sequenced (e.g. *Arabidopsis* and Human).

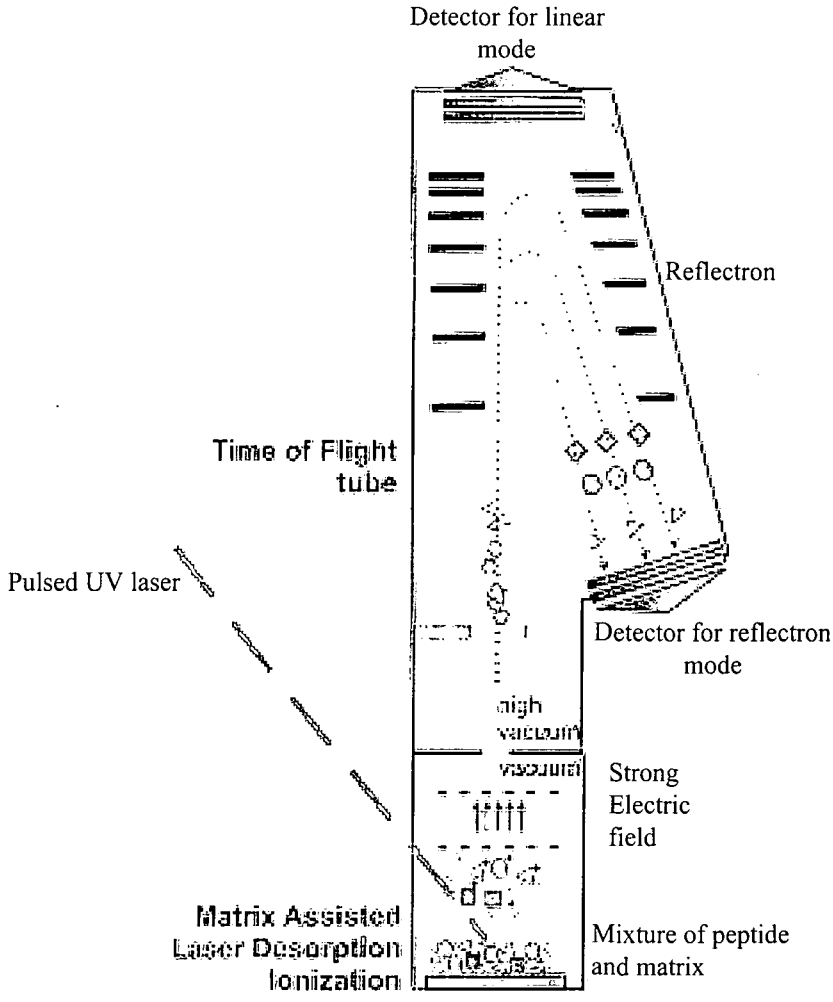


Figure 1.9 **A schematic diagram showing the basic principle of MALDI-Tof.**

Peptides to be identified are mixed with an ultra-violet (UV) absorbing matrix. When a UV laser is fired into the matrix-peptide complex, the matrix is excited and the peptides are ionised. The ionised peptides are subjected to a strong electric field, which accelerates the peptide ions through a vacuum towards the detector. Lighter ions fly faster than the heavier ions. The time-of-flight of the peptide ions is converted into mass per charge (m/z).
(This figure is adapted from Westermeier, 2000)

1.26.2 Tandem Mass Spectrometry. Improvement in mass spectrometry came with the development of tandem mass spectrometry (reviewed by Pennington and Dunn, 2001). In tandem mass spectrometry (MS-MS) the protein peptides are ionised twice in the same experiment. For example, MALDI-ToF generated peptide ions are further fragmented to produce amino acid ions. The primary ionisation however, does not have to be generated via MALDI-ToF. An electrospray method can also be used to ionise peptide digest. In the electrospray method the tryptic digests are compressed through a metal capillary needle at high potential. Small volumes (0.2 - 2μl) tryptic digests are sprayed for an extended period (60 seconds) at atmospheric pressure. In the presence of an electric field the spray droplets become highly charged and fly towards the inlet of the mass spectrometer which is kept at lower potential (Figure 1.10). The charged spray droplets are evaporated by the application of heated dry nitrogen at the inlet of the mass spectrometer. The evaporated ions are channelled through an orifice into the vacuum of the mass analyser. At the first quadrupole filter stage, the chosen peptide ion(s) either from MALDI-ToF or electrospray are selected for further fragmentation. The selected ion is accelerated into the second quadrupole containing a heavy gas such as Argon or Nitrogen. This acceleration leads to collisions between the peptide ions and the gas molecules. These collisions induce the fragmentation and dissociation of the peptide covalent bonds. The ionic products of the fragmentation reaction are called fragment or product ions (Rinter and Nicholas, 2000). The fragment ion forming process described above is called collision-induced dissociation (CID). The CID generated fragment ion spectrum is measured via a mass detector and the data given is as a mass per charge (m/z) ratio. The fragment ion data can be given either as b or y

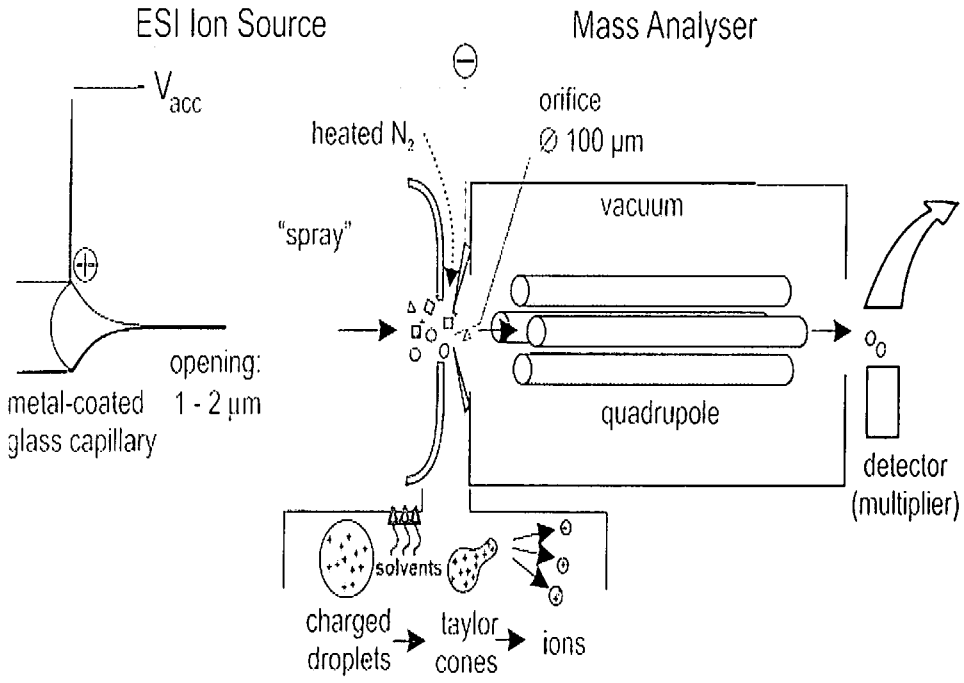


Figure 1.10. **A schematic diagram of peptide ionisation via electrospray.**

As an alternative to MALDI-ToF (see figure 1.9), peptides can be ionised via an electrospray. The charge is created when microdroplets are ejected from a microneedle at high potential to atmospheric pressure. These charged droplets are attracted to the mass spectrometer inlet, which is kept at low potential. The charged droplets become dehydrated and protonated by heated nitrogen as they enter the mass spectrometer orifice. The protonated peptide ions are analysed and/or further fragmented into amino acid ions.
(The figure is adapted from Westermeier, 2000).

series depending on the terminus retained in the ionic species that are detected. All the fragment ions that retain a C-terminus are given as y-ions and all those that retain the N-terminus are given as b-ions (Kinter and Nicholas, 2000).

1.26.3 MS-MS Data Interpretation. The fragment ions generated via collision induced dissociation of a MALDI-ToF or electropsray generated peptide ion are assigned b or y series nomenclature as described above. Each fragment ion m/z corresponds to one of 20 standard amino acids (Table 1.3). Therefore from the m/z spectrum, the amino acid residues can be obtained. However in the instance of a leucine and isoleucine, it is impossible to tell which is which because their masses are identical. Often the MS-MS data interpretation is automatically calculated using a computerised algorithm programme developed by Yates and co-workers (Yates et al., 1995), but sometimes a manual interpretation of the CID might be necessary (Pardo et al., 2000). In addition to amino acid sequence data, MS-MS also gives post translational amino acid modifications like methione oxidation, glycosylation and phosphorylation (Pennington and Dunn, 2001; Yates et al., 1995).

1.27 Aims of this study.

In the light of the limited data on the diversity of cell wall proteins, the aim of this thesis was to extract and characterize as many *Arabidopsis thaliana* cell wall proteins as possible. The cell wall protein mapping exercise was likely to reveal novel cell wall proteins. The identified novel cell wall proteins led to the formulation of hypotheses on novel cell wall roles. The formulated hypotheses were tested.

Table 1.3. Amino acid residue masses

Amino acid	One-letter code	Residue mass (Da)	Immonium ion (m/z)
Glycine	G	57.02	30
Alanine	A	71.04	44
Serine	S	87.03	60
Proline	P	97.50	70
Valine	V	99.07	70
Threonine	T	101.05	74
Leucine	L	113.08	86
Isoleucine	I	113.08	86
Asparagine	N	114.04	87
Aspartate	D	115.03	88
Glutamine	Q	128.06	101
Lycine	K	128.09	101
Glutamine	E	129.04	102
Methionine	M	131.04	104
Histidine	H	137.06	110
Oxidised Methionine	Mo	147.04	120
Phenylalanine	F	147.07	120
Arginine	R	156.10	129
Carbamidomethylcysteine	C*	160.03	133
Tyrosine	Y	163.06	136
Acrylocysteine	C^a	174.04	147
Tryptophan	W	186.08	159

Table 1.3 is adapted from Kinter and Sherman, 2000

Chapter 2

Materials and Methods

2.1 Generation of cell suspension cultures (all chemicals used were purchased from Sigma Chemical Co. St Louis, MO, unless stated otherwise). All the tissue culture manipulation experiments were performed aseptically in a flow cabinet wiped with 80 % ethanol.

2.1.1 Sterilization and germination of Arabidopsis seeds. *Arabidopsis thaliana var Erecta* seeds were surface-sterilized by soaking in 70% (v/v) ethanol for 5 minutes and rinsed 3 times with sterile distilled water. This was followed by a 30 minute incubation and shaking in 5 % hypochlorite solution with Tween 20 (1 drop/100 mL). The seeds were washed once again with sterile deionised water as above. The seeds were left overnight to dry in a laminar flow cabinet. The dried sterilised seeds were then plated onto Murashige and Skoog (MS) medium (pH 5.8) containing 3% sucrose and 0.8% agar. The plates were incubated at 20 – 24 °C in a 16-hour photoperiod provided by white fluorescent lights.

2.1.2 Callus induction. Seedlings large enough to handle were transferred to sterile Petri dishes and were cut into 1-2 mm pieces using sterile forceps and scalpel blade. The cut tissues were plated onto callus induction medium (Gamborg B5 1X, 2% (w/v) Sucrose, 0.5 g/L Mes, 0.5 mg/L 2,4-D, 0.05 mg/L Kinetin, 0.8% (w/v) agar, 0.8% (w/v) agar adjacted to pH 5.7) and incubated incubated at 20 – 24 °C in a 16-hour photoperiod provided by white fluorescent lights. The callus tissue was subcultured once every month.

2.1.3 Initiation of cell suspension culture. Suspension cell cultures are established by inoculating approximately 300 mg of rapidly dividing white callus into 100 mL of

liquid medium (M&S 4.43g per litre, 0.5 ml lmg/ml naphthalene acetic acid, 0.05 ml lmg/ml kinetin, 30 % w/v sucrose) and incubating at 24 °C in a 16-hour photoperiod provided by white fluorescent lights on a rotary shaker at 100-120 rpm or in the dark. Cell suspensions were subcultured weekly.

2.2 Growth and maintainance of *Arabidopsis thaliana* cell cultures

The *Arabidopsis thaliana var Erecta* seeds cell line was kindly provided by Mike May (May and Leaver, 1993), on Murashige and Skoog (M&S) Basal salts with minimal organics (MSMO; Sigma Chemicals Company, Dorset, UK). The M&S medium (M&S 4.43g per litre, 0.5 ml lmg/ml naphthalene acetic acid, 0.05 ml lmg/ml kinetin) pH 5.7, was supplemented with 30 g sucrose and autoclaved. Working aseptically in a laminar flow, *Arabidopsis* suspension cultures were initiated by supplying a 90 ml sterile fresh media in a 250 ml flask with a scoop of callus (growing in solid media). The inoculated flasks were given a brief shake to break the clumped cells and incubated at 25°C in continuous white light on a shaking platform at 150 rpm. The cells were subcultured on a weekly basis using an inoculum density of 10% (v/v) in a total volume of 100 ml in 250 ml glass conical flasks. All the chemicals were purchased from Sigma.

2.3 Growth and maintainance of Black mexical sweetcorn cell cultures

Black Mexican Sweetcorn (BMS) cells were kindly provided by Prof. Stephen C. Fry (University of Edinburgh). These cells were grown in the dark at 25 °C on a shaking platform (150 rpm.) in sterile M&S medium (20 g/L sucrose, 2 mg/L 2,4-D, 4.7g/L

M&S). The cells were subcultured on a weekly basis using an inoculum density of 10% (v/v) in a total volume of 100 ml in 250 ml glass conical flasks. All the chemicals were purchased from Sigma.

2.4 Preparation of solution used in extracellular ATP experiments. (all the tissue culture manipulation experiments were performed in a flow cabinet wiped with 80 % ethanol)

2.4.1 Apyrase. 1000 units of freeze-dried apyrase purchased from Sigma were solubilised in 2 mL deionised water. The mixture was vortexed hard for 10 to 20 minutes. The apyrase solution was spun down at 30000 g for 30 minutes. The 500 units per millilitre apyrase solution was filter sterilised using a 0.2 μm filter from Millipore in flow cabinet and divided into 250 mL aliquots which were subsequently stored at - 20 °C until needed.

2.4.2 Hexokinase (37 units/mg). 0.05 g hexokinase was dissolved in 1 mL deionised water to make a 1850 units/mL (1.850 units/μL) enzyme. The enzyme was filter sterilised through a 0.2 μm filter from Millipore in a flow cabinet and stored at –20 °C until needed.

2.4.3 Glucose (925 mM). 0.5 g of glucose was dissolved in 3 mL deionised water to give 925 mM. Glucose was filter sterilised using a 0.2 μm filter in a flow cabinet and stored at –20 °C until needed.

2.4.4 Adenosine triphosphate (ATP, 181 mM). 0.2 g ATP purchased from Sigma was dissolved in 2 mL of deionised water. This solution was filter sterilised under a flow cabinet. The filter sterilised ATP samples were stored at –20 °C until needed.

2.4.5 β,γ ATP 45.5 mM. Twenty five milligrams of non-hydrolysable β,γ ATP purchased from Sigma was dissolved in 1 mL deionised water to make a final concentration if 45.5 mM stock. This solution was sterilised by filtration through a 0.2 μm Millipore filter in a flow cabinet. The filtered solution was stored at – 20 °C until needed.

2.5 Preparation of a chitosan elicitor

Chitosan (from crab shells; Sigma Chemical co) was dissolved in 0.1 M acetic acid to a final concentration of 10 mg/mL by stirring for 24 hours at 50 °C. The acidic chitosan solution was dialysed 3 times over night against 4 L of distilled water at 4 °C. After dialysis the pH of the chitosan solution was 6.5 and the final concentration was 5.3 mg/mL. The chitosan solution was autoclaved and stored at 4 °C in several aliquots.

2.6 Preparation of a *Fusarium moliniforme* elicitor.

Fusarium moliniforme 010 (strain Mo33) were grown in culture medium 60 S YPD (1 % w/v DIFCO yeast extract, 2 % w/v bactopeptone, 0.05 % dextrose (or D-glucose), 1.5 % Bacto-Agae) for 7 days in the dark. The fungal suspension culture was then sterilised by autoclaving at 122 °C for 30 minutes. The fungal hyphae were

harvested by filtration through a Whatman filter paper. The hyphae were washed 3 times on the filter paper with sterilised deionised water. The washed fungal hyphae were transferred to a Falcon tube and lyophilised for 48 hours. The lyophilised fungal material was ground into a powder with pestle and mortar. The fungal powder was mixed with water to a final concentration of 50 mg.mL and autoclaved once more. The fungal elicitor was aliquoted into 1 mL microcentrifuge tubes and that stored at – 20 °C.

2.7 Growing tobacco (SR1) and black-eyed bean plants and infiltration experiments.

The seeds of tobacco and black-eyed bean were planted in a Multi-Purpose compost (Premier) in several pots and were grown at 20 °C in a 16-hour photoperiod provided by white fluorescent lights. Then 6 to 10 weeks after germination the fully expanded leaf apoplasts were infiltrated with either, 0.5 units/μL of apyrase, or 1.85 units/μL hexokinase or 10 mM βγ ATP or10 mM ATP or 10 mM ADP, or 10 mM AMP or100 μg BSA, or 10 mM glucose. This was done by injecting the appropriate solution under the abaxial cuticle of the leaf using a sterile hypodermic needle attached to a 1 mL plastic syringe.

2.8 Acetone Protein Precipitation

Dilute protein samples were adjusted to pH 8.8 by spiking with 1 M Tris-HCl pH 8.8. These protein samples were then mixed with acetone to a final concentration of 80 % acetone v/v. The protein samples with 80 % acetone were incubated at – 20 °C

for up to 24 hours. The protein precipitates were collected by centrifugation at 10 000 **g** for 15 minutes at 4 °C and dried using filtered air.

2.9 Determination of Cell Viability

2.9.1 Evans blue method. The method used for staining dead cells is based on that of Baker et al. (1994). This technique is based on the fact that living cells are capable of regulating the type of molecules that get through their plasma membrane while dead cells have lost this selective permeability. Thus, Evans Blue stain is excluded from intact, live cells but it can penetrate dead cells. Cells can be viewed under the microscope to check their staining status. Cells with a fresh weight of 20 mg were resuspended in 180 µl of distilled water. A 20 µl aliquot of 0.5% (w/v) Evans Blue solution (Sigma) was added and the cells incubated at room temperature for 15 minutes with shaking. The cells were spun for 1 minute to remove the supernatant and then resuspended in 1 mL water. After 5 minutes incubation (with shaking) at room temperature the cells were pelleted again and this washing was repeated three times. In order to quantify the qualitative data obtained by viewing these cells under microscope, the amount of stain taken up by cells (which is proportional to the number of dead cells) was determined using a spectrophotometer at 600 nm. The stained cells were homogenised in 1.2 mL of 1% SDS in 50% methanol and incubated at 50^{o}C for 6 hours to release the stain taken up by cells. The cell debris was pelleted and the optical density (OD) of the supernatant determined at wavelength 600 nm (Pharmacia LKB ultraspec III). For the determination of cell viability, at each time point during growth of cell cultures, 4-replicate samples of

cells were stained separately. The cells that had been deliberately killed by boiling for 5 minutes in a water bath were stained in parallel and served as the 0% viability baseline. Cell viability in the other samples is given by the modulus of the difference between the means of OD values of the sample and the heat killed cells expressed as a percentage of the latter value.

2.9.2 Fluorescein Diacetate (FDA). Confirmation that Evans Blue-stained cells were dead and that non-stained cells were alive was obtained by counter-staining with another stain, fluorescein diacetate (FDA). This approach relied on the esterase-dependent conversion of FDA from the non-fluorescent form to a fluorescent product inside living cells. Dead cells lose this enzyme activity and, therefore, fail to fluoresce when illuminated with UV light. Twenty milligrams (fresh weight) of cells was resuspended in 200 µl of 0.2 M calcium chloride ($CaCl_2$). Ten microlitres of 0.5% FDA was mixed with the cells and incubated at room temperature for up to 15 minutes. Cells were washed with 1 mL of deionised water to remove the excess stain. The cells were examined using bright-field and dark-field (ultra-violet light) microscopy and photographs were taken.

2.10 Conductivity and pH

pH and conductivity of the external medium were measured using a Combination pH electrode (Phillip Harris) connected to a Jenway 3010 pH meter and HI 3292 ATC conductivity probe (Hanna instruments) respectively.

2.11 Packed Cell Volume (PCV).

Cell growth in cell cultures was monitored by measuring packed cell volume (PCV) in 5 mL aliquots withdrawn from the cultures at different time points. With the help of Dr S. Chivasa, a low tech, but highly accurate cell culture PCV measuring was developed (Figure 2.1). This device is made up of two parts: a 15 mL Falcone tube with a cut bottom and a graduated 2 mL microcentrifuge tube. The two tubes were joined as shown in Figure 2.1 and sealing the junction with a parafilm prevented the possible leakage. Five millilitres of cell suspension cultures were loaded into the PCV measuring device and the cells were collected in the graduated microcentrifuge tube by centrifugation in a swinging bucket rotor (Sigma 204, Nr 11030) at 1000 revolutions per minute for 5 minutes. The volume of the packed cells was measured and was given as a percentage of the 5 mL aliquot.

2.12 Isolation of cell walls from cell suspension cultures

Cells cultures grown for 3, 4 or 5 days post subculture were separated from the culture medium through 2 layers of miracloth 3using vacuum-assisted filtration. The filtered cells were washed three times with 500 mL of cold deionised water. After washing the cells were resuspended in deionised water and passed twice through a mechanical cell disruptor (Constant Systems, Warwick, U.K) at 25 pressure units, cooled down to 4 °C. In a cold room maintained at 4 °C, the homogenised cells were layered on to a 29 cm high 10% glycerol column (i.e. 500 mL 10% glycerol in 4.5 cm-diameter dialysis tube) overnight. The cell walls sediment to the bottom of the glycerol column within 24 hours while leaving the rest of the cellular debris at the top. After sedimentation, the supernatant was carefully discarded and the isolated

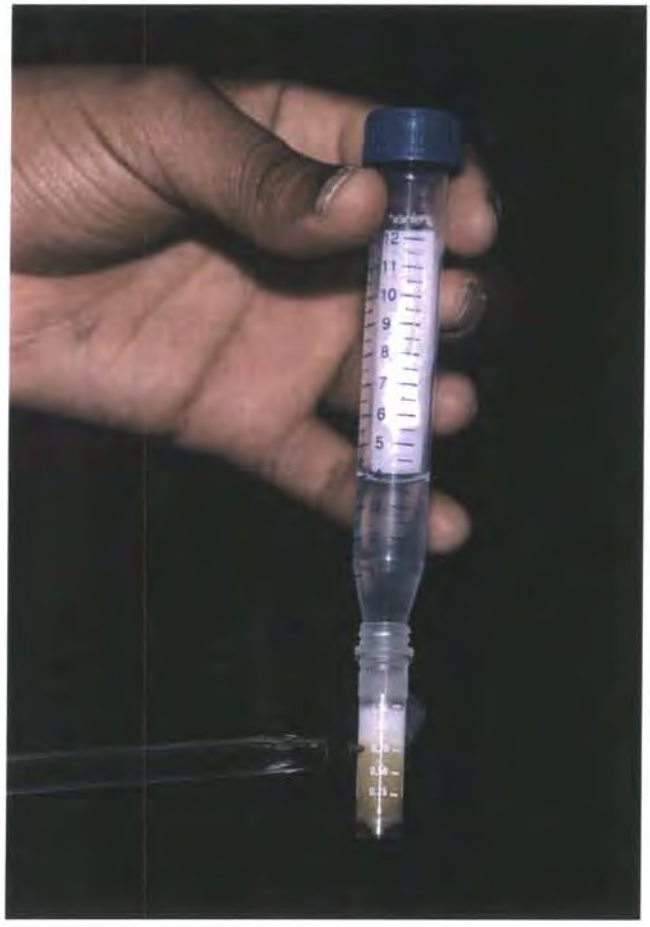


Figure 2.1 Makeshift packed cell volume (PCV) measuring device.

cell walls were then transferred to 50 mL Falcone tubes and collected by centrifugation for 10 minutes at 12 000 **g**. The excess glycerol supernatant was discarded and the walls were resuspended in 30 mL cold (4 °C) deionised water and pelleted again. The water washing stage was repeated three times. After washing, the cell wall proteins are either extracted immediately or stored at-80 °C waiting for protein extraction.

2.13 Electron Microscopy

Three days post subculture cells were harvested and washed three times with deionised water. Also purified cell walls were washed with deionised water. The samples to be analysed (intact cells of purified cell walls) were mixed with one volume of 1% low melting point agar immediately before it set. Agarose gel was then sectioned into 1 mm cubes with a razor blade and fixed in 20 volumes of 2.5% glutaraldehyde in 50 mM phosphate buffer, pH 7.0 for 2 hours at room temperature. The glutaraldehyde was removed by washing twice for 5 minutes in the same buffer before being fixed for 2 hours in 1% osmium tetroxide. Intact cells and purified cell walls were then dehydrated via several washes with absolute ethanol. The dehydrated material sections were impregnated with 25% LR white (London Resin, Reading, UK) in ethanol for 1 hour followed by 50% LR white in ethanol, and finally 100% LR white. The samples were allowed to equilibrate in full strength resin for 4 hours with 3 changes before being polymerised overnight at 60°C. Ultra-thin sections were examined using a transmission electron microscope (Philips 400T, Holland).

2.14 Immunolocalisation (done in collaboration with Prof. J.P. Knox)

Cell walls that were purified from *Arabidopsis* cell cultures were fixed in 50 mM phosphate buffer (pH 6.9) containing 4% (w/v) glutaraldehyde. The fixed cell wall material was pipetted onto a glass slide and allowed to dry. The fixed and dried cell wall specimen were incubated for 1 hour with phosphate buffered saline (PBS) containing a 5-fold dilution of red monoclonal antibody hybridoma (antibodies to homogalacturonan, β-1.4-galactan or glycolipids) supernatant in the presence of 1% milk powder w/v to prevent non-specific binding. The specimen was washed 3 times in PBS and then incubated for 1 hour with 100-fold dilution of rabbit anti-rat IgG (whole molecule) linked to a fluorescein isothiocyanate (FITC) in PBS containing 1% (w/v) milk powder. Specimens were washed with PBS and then mounted in a PBS based fluorescence antifade solution (Citifluor AF3). The mounted specimen was examined microscopically using an olympus microscope equipped with an epifluorescence filter.

2.15 Cell wall protein extraction.

The purified cell walls were resuspended in 30 mL of 0.2 M $CaCl_2$ in a 50 mL Falcone tube and proteins were extracted by shaking on a rocking platform at 4 °C for 30 min. The $CaCl_2$ protein extracts were separated from the cell walls by centrifuging at 10000 g for 20 minutes at 4 °C. The cell walls were washed 3 times with 30 mL cold deionised water. The washed $CaCl_2$ extracted cell walls were extracted with 30 mL of urea buffer (7M urea, 2 M thiourea, 4 % CHAPS, 1 % DTT, 0.8 % pharmalytes (pH 3-10) at room temperature for 1 hour on the rocking plantform. The urea buffer extracts were separated from the cell wall also by

centrifugation as above. The $CaCl_2$ and urea buffer extracts were precipitated with 80 % acetone method (see acetone precipitation). The protein precipitates were washed 3 times with 80 % acetone and dried using filtered air. The protein pellets were resolubilised in 2-D sample buffer (9 M urea, 2 M thiourea, 4 % CHAPS, 1 % DTT and 2 % pharmalytes.

2.16 One-dimension SDS Polyacrylamide gel electrophoresis (SDS–PAGE)

The protein was resuspended in 35 µL of SDS sample loading buffer [2% (w/v) SDS, 100 mM DTT, 0.01% Bromophenol Blue, 0.63M Tris-HCl.Cl, 10% glycerol] and boiled for 10 minutes before loading on to SDS gels. Proteins were resolved by SDS-PAGE essentially according to the method of Laemmli et al. (1970) using stacking gels of 5% (w/v) acrylamide and resolving gels of 10% acrylamide. Mini gels, cast using the Mini-Protean II Cell (Bio-Rad, Hercules, CA) gel rigs, were electrophoresed in a Tris-glycine buffer (0.025 M Tris-HCl, 0.19 M glycine, 0.1% (w/v) SDS) at 50 V until the bromophenol blue dye front crosses the stacking gel-resolving gel border at which point the voltage was increased to 100 V.

2.17 Staining mini gels with Coomassie Brilliant blue

Staining of gels was effected by heat-treating them in a microwave oven for 3 minutes at full power while immersed in Coomassie Brilliant Blue (CBB) stain I (0.025% CBB w/v, 25% (v/v) isopropanol, 10% (v/v) acetic acid). From the oven the gels were incubated for 10 minutes on a shaking platform, after which the staining cycle was repeated using CBB stain II (0.003% CBB, 10% (v/v)

isopropanol, 10% (v/v) acetic acid) and CBB stain III (0.025% CBB and 10% (v/v) acetic acid). The gels were destained by incubating in a destain solution (10% (v/v) acetic acid, 1% glycerol). The gels were incubated on a shaking platform until the bands were visible against a clear background.

2.18 Immunoblotting with anti enoyl-ACP-reductase (ENR)

Twenty micrograms of protein total soluble protein extracted in Tris-HCl EDTA buffer (10 mM Tris-HCl pH 8.8, 1 mM EDTA, 2 mM DTT) or cell wall protein extracts were loaded and electrophoreses in 10 % 1-dimension polyacrylamide gels, in Tris-glycine buffer (0.025 M Tris-HCl, 0.19 M glycine, 0.1% (w/v) SDS), at 50 V for 30 minutes followed by 100 V until the bromophenol blue front reached the bottom of the gel. The stacking gel was carefully removed from the electrophoressed gels. The electrophoressed gels were layered to a nitrocellulose membrane and sandwiched in a Western blot kit (Biorad, Hercules, CA). The protein samples were blotted from the polyacrylamide gels to the nitrocellulose membrane overnight at 4 ^{o}C, at 30 V in Tris-glycine buffer (with out SDS). The nitrocellulose membranes were stained for 5 minutes with Ponceau to assess the transfer efficiency. Following staining the membranes were rinsed with deionised water and immersed and incubated in 10 mL blocking solution (5 % w/v dried milk, 0.1 % Tween 20 in 1 X TBS pH 7.6) on a rocking platform for 1 hour at room temperature or overnight at 4 ^{o}C. The blocking solution was replaced with 10 mL primary antibody solution (1/10000 dilutions anti rape enoyl-ACP-reductase antibody in blocking solution and this was incubated at room temperature for 1 hour. The membrane was washed 3 times for 5 minutes with 10 mL of washing buffer (0.1 % Tween 20 in 1 X TBS pH

7.6) at room temperature. The washed membranes were incubated in 10 mL of secondary solution (1/20000 dilution of goat anti-rabbit IgG (H+L)-HRP conjugate (Biorad) in washing buffer) for 1 hour at room temperature. The membrane was then washed 2 X 5 minutes and 1 X 10 minutes in 1X TBS pH 7.6. The washed membrane was immersed in a 10 mL chemilumnisence developing solution (50 % luminol enhancer in 50 % stable peroxide solution (Pierce). Finally the membrane was mounted in a chemilumnisence cassette and exposed to an X-ray film in the dark for various lengths of time starting from 30 seconds. The X-ray film was developed via an Compact X 4 automatic X-ray film Processor (X-graph imaging system).

2.19 Western blot staining using a Ponceau stain

Ponceau staining method is normally used for reversibly staining proteins that are Western blotted onto a Nitrocellulose membrane (Amersham Pharmacia Biotech). The stain is made up of 0.1 % Ponceau S (w/v) in 5 % acetic acid.

After Western blotting the membrane was submerged in a Ponceau stain for about 5 minutes at room temperature. The membrane was then rinsed with deionised water until the red protein bands or spots were visible and the background was white.

2.20 Immunoblotting with anti-phosphotyrosine.

Twin gels were electrophoresed for each set of protein samples. For 1-D PAGE 20 µg of protein total soluble protein extracted in Tris-HCl EDTA buffer (10 mM Tris-HCl pH 8.8, 1 mM EDTA, 2 mM DTT) or cell wall protein extracts were loaded and electrophoresed on 10 % 1-D SDS-PAGE, in Tris-glycine buffer (0.025 M Tris-HCl,

0.19 M glycine, 0.1% (w/v) SDS), at 50 V for 30 minutes followed by 100 V until the bromophenol blue front reached the bottom of the gel. The second gel was electroblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech) over night, at 30 V in Tris-glycine buffer (with out SDS). The nitrocellulose membranes were stained for 5 minutes with Ponceau to assess the transfer efficiency.

The membrane was blocked with 5 mL blocking buffer (1 % w/v BSA in Tris-HCl buffered saline; 10 mM Tris-HCl pH 7.5, 100 mM sodium chloride with 0.1 % Tween 20) at room temperature for 1 hour on a shaking platform. The blocked membrane was rinsed briefly with two changed of Tris-HCl buffered saline. The anti-phosphotyrosine antibody (PY20; Amersham Pharmacia Biotech) was diluted 1:1000 dilutions in Tris-HCl buffered saline containing 0.1 % tween 20 and incubated with the membrane for 1 hour at room temperature on a rocking platform. The membrane was washed twice for 5 minutes with 10 mL Tris-HCl buffered saline containing 0.1 % Tween 20 and once with 10 mL of fresh Tris-HCl buffered saline for 15 minutes at room temperature. All the washing steps were done on the rocking platform.

2.21 Large format 2-dimension Gel Electrophoresis

One milligram of protein in 350 μL 2-D sample buffer was loaded into 18 cm, pH 3-10 or pH 6-11 isoelectrofocusing (IEF) gel strips (Amersham-Pharmacia Biotech) by the rehydrating the dry strips (with the protein sample) overnight at room temperature, using a Amersham-Pharmacia Biotech rehydration tray. The hydrated IEF strips were rinsed briefly with a flow of deionised water. The proteins in the IEF strips were focused for 70 kVh at 20 ^{o}C using the Multiphor II system (Amersham

Pharmacia Biotech). The focused protein samples were equilibrated by shaking for 15 minutes in a equilibration buffer [6 M urea, 30% (v/v) glycerol, 10% (w/v) SDS, 50 mM Tris-HCl pH 8.8] with 1% (w/v) DTT and for another 15 minutes in the same buffer containing 4.8% (w/v) iodoacetamide at room temperature. The equilibrated IEF gel strips were rinsed by a stream of deionised water and aligned on top of large format 12 % polyacrylamide gels (200 x 200 x 1 mm) for second dimension SDS-PAGE. The aligned strips were immobilised with a 1 % low melting point agarose (in Tris-glycine buffer) mixed with bromophenol blue. The protein sampled were electrophoresed at 20 mA per gel at 10 °C using the Hoefer Dalt 2-D gel system (Amersham Pharmacia Biotech) until the bromophenol blue front reached the bottom of the gel. The gels were transferred to large clean staining dishes and stained with Coomassie brilliant blue I, II and III each at 40 °C. The gels were destained with 10% (v/v) acetic acid, 1% glycerol.

2.22 Mini format 2-dimension Gel Electrophoresis

Protein (200 μg) in 125 μL 2-D sample buffer was loaded into 7 cm, pH 3-10 or pH 6-11 isoelectrofocusing (IEF) gel strips (Amersham-Pharmacia Biotech) by rehydrating the dry strips (with the protein sample) overnight at room temperature, using an Amersham-Pharmacia Biotech rehydration tray. The hydrated IEF strips were rinsed briefly with a gentle flow of deionised water. The proteins in the IEF strips were focused for 6.5 kVh using the Multiphor II system (Amersham Pharmacia Biotech) at 20 °C. The focused protein samples were equilibrated for shaking for 15 minutes in a equilibration buffer [6 M urea, 30% (v/v) glycerol, 10% (w/v) SDS, 50 mM Tris-HCl pH 8.8] with 1% (w/v) DTT and for another 15 minutes in the same

buffer containing 4.8% (w/v) iodoacetamide at room temperature. The equilibrated strips IEF gel strips were rinsed in deionised water and aligned on top of 12 % mini Biorad polyacrylamide gels without a stacking gel. The gel strip was held in to place by covering with a 1 % low melting point agarose (in Tris-glycine buffer) mixed with bromophenol blue. The gels were were electrophoresed in a Tris-glycine buffer (0.025 M Tris-HCl, 0.19 M glycine, 0.1% (w/v) SDS) at 50 V for 30 minutes followed by 100 V until the bromophenol blue dye front reached the bottom of the gel. The proteins in the gels were either stained with Coomassie brilliant blue or blotted onto a nitrocellulose membrane for immunoprobing.

2.23 Matrix assisted laser desorption ionisation time of flight (MALDI-ToF mass spectrometry.

All the MALDI-ToF mass spectrometry reported here were analysed on a PE Voyager DE STR MALDI-ToF instrument (Figure 2.2; Perkin Elmer Biosystems in Boston, MA) operated by J.W. Simon. After electrophoresis and staining with Coomassie brilliant blue, the spots were picked, either manually or via a ProPick robot (Figure 2.2; Genomic solutions). The picked protein spots were first digested using an automated long trypsin digestion method on a ProGest digestion robot (Genomic solutions; Figure 2.2). The protein-acrylamide plugs were first washed in 25 mM bicarbonate buffer. The washed plugs were then destained and dehydrated in a concentrated acetonitrile solution. The plugs were then rehydrated once again in 50 mM bicarbonate buffer. The rehydrated gel plugs were alkylated with DTT and iodoacetamide. This was then followed with a bicarbonate wash. After washing

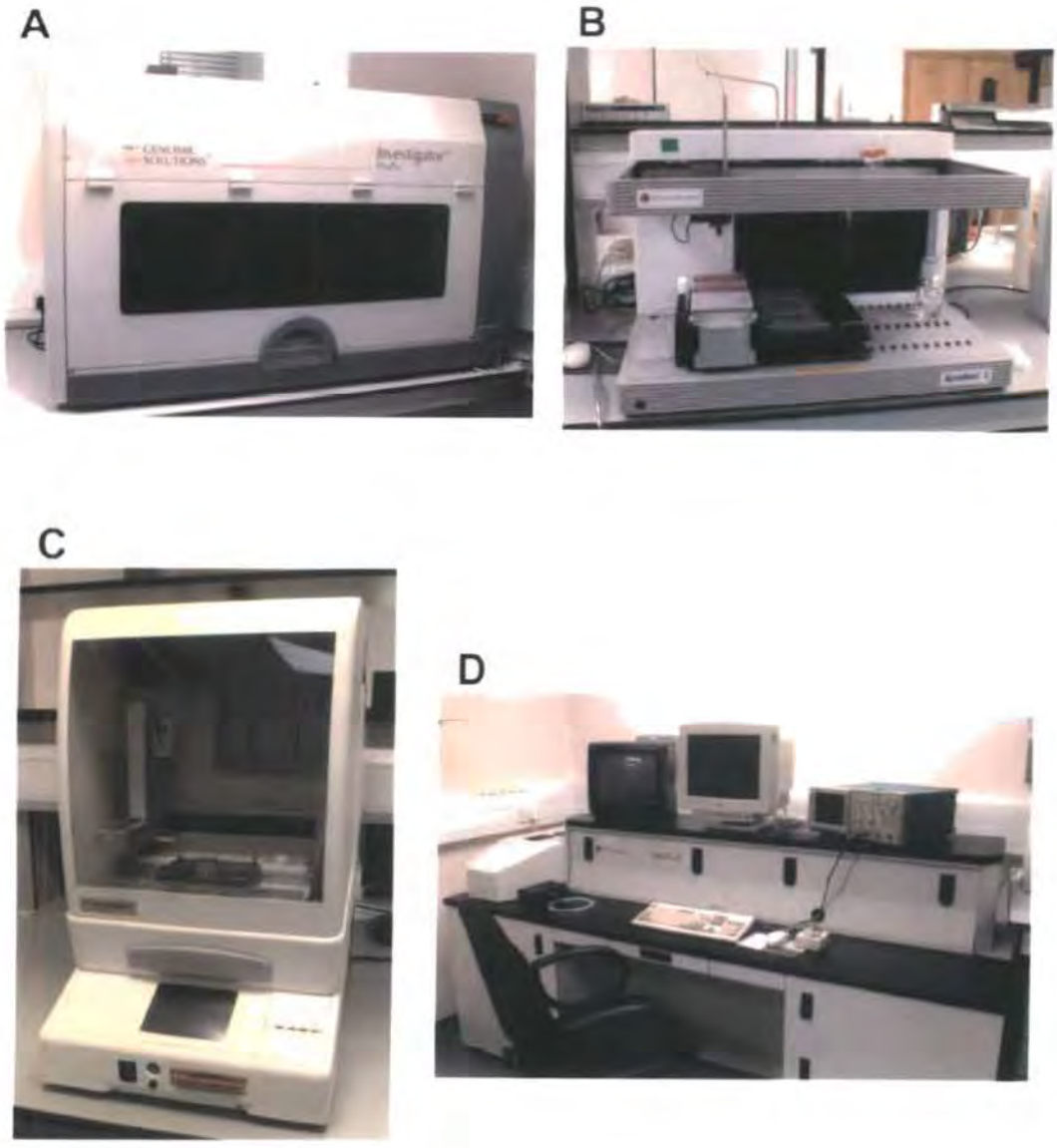


Figure 2.2 **Some of the in-house facilities in the proteomic unit.**

A. ProPick robot (Genomic solutions), for automatic spot picking.

B. Symbiot robot (Perkin Elmer Biosystems), for applying the peptide matrix complex onto a MALDI-ToF target plate.

C. ProGest polypeptide digestion robot (Genomic solutions).

D. PE Voyager DE STR MALDI-ToF instrument (Perkin Elmer Biosystems).

each protein plug was digested with 20 ng trypsin (Promega, Madison. Wi) for 8 hours at 37 °C. The tryptic digests were extracted from the gel plugs with 100 µL of 50 % acetonitrile and 0.1 % formic acid and transferred to micro titre plates. Half of a microliter of each sample was mixed with 0.5 µL of alpha-cyano-4-hydroxy-cinnamic acid matrix. The peptide matrix complex was applied onto a MALDI-ToF target plate using a Symbiot robot (Perkin Elmer Biosystems; Figure 2.2). A laser was fired to the peptide-matrix complex and the peptides were ionised. The MALDI-ToF 's reflectron mode detector measured the masses of the generated peptide ions. The measured mass spectra were acquired at an automated mode for mass peak detection, noise reduction and deisotoping. The peptide masses were used to interrogate the *Arabidopsis* database with theoretical tryptic digest of all open reading frames in the *Arabidopsis* genome using an automated MS-Fit (Protein Prospector). The data reported in this study was obtained from the *Arabidopsis thaliana* genome database that was down loaded in the public domain by the Munich Information center for Protein Sequences (MIPS; http://mips.gsf.de/proj/thal/). The primary searches were done using a peptide mass tolerance of 150 parts per million (ppm). The primary search was followed by a recalibration of the acquired spectra using a Proteomic Solutions I (PSI) Intelical software from PE Biosystems, based on the top matches detected in the primary round of searches.

The Intelical software takes the spread of the mass errors for the best matches obtained at 150 ppm and uses them to re-calibrate the peptide masses before they are re-submitted at 50 ppm accuracy. Protein Prospector database searches were done with an allowance for peptide mass change arising from amino acid modifications, like oxidation of methionines, N-terminal acetylations, acrylamide induced cystine

residue modifications and conversion of N-terminal glycine to pyroglycine. The database searches gave an output of 5 matched matched polypeptides ranked according to the MOWSE score, the peptide mass coverage and the mass error margin (Pappin et al., 1993). The top ranking protein was considered as the probable positive hit.

2.24 Phosphate Determination.

All the chemical compounds used for this assay were purchased from Sigma. This assay is based on the formation of a yellow mixed heteropoly acid, vanadomolybdophosphoric acid. Stock solutions of 0.2 M ammonium metavanadate, 0.04 M ammonium molybdate, and 35 % (v/v) nitric acid were prepared. Samples (300 µl) were mixed with 100 µl HNO_3, 100 µl ammonium metavanadate, and 100 µl ammonium molybdate, in that order (mixing after every step). The samples were allowed to stand for at least 10 minutes and the absorbance determined at 470 nm against a reagent blank (sample substituted with 300 µl of water). 1M KH_2PO_4 was used to construct a calibration curve.

2.25 Estimation protein concentration in urea containing protein samples.

For protein samples that were solubilised in urea containing buffers the concentrations were estimated as follows:

Five microlitre of the protein sample was mixed with 80 µL deionised water and 10 µL 0.1 M hydrogen chloride. Eight hundred and ninety five millilitres of 20 % Bradford reagent (Biorad, Hercules, CA) was added to the mixture by pipetting up

and down few times. The proteins were allowed to stand for 10 minutes at room temperature. The absorbance at 595 nm is then measured. The protein (Bovine serum albumin; BSA) standards were prepared simultaneously as follows:

Final Concentration	BSA (5mg/mL)	Lysis buffer	HCl	H_2O
0 μg/μL	0 μL	10 μL	10 μL	80 μL
5 μg/μL	1 μL	9 μL	10 μL	80 μL
10 μg/μL	2 μL	8 μL	10 μL	80 μL
20 μg/μL	4 μL	6 μL	10 μL	80 μL
40 μg/μL	8 μL	2 μL	10 μL	80 μL
50 μg/μL	10 μL	0 μL	10 μL	80 μL

These BSA dilutions are mixed with 900 μL Bradford reagent and allowed to stand at room temperature for also for 10 minutes. The absorbance at 595 nm is then measured and these values are plotted against the BSA concentration. The generated standard is used to estimate the concentration of the protein extract.

2.26 ATPase activity assay

The following stock solution and cell wall material were used: 0.28 mg/mL purified cell wall; 1 M Tris-HCl pH 7.5; 0.2 M $CaCl_2$; 100 mM ATP; 5.3 mg/mL chitosan; 10 % w/v TCA.

One hundred microliters of purified cell wall material was mixed with 10 mL 1 M Tris-HCl pH 7.5; 5 μL 0.2 M $CaCl_2$ and 60 μL deionised water. The control cell

walls were treated with 5 µL water and the chitosan treatments were mixed with 5 µL 5.3 mg/mL chitosan. The ATPase activity was ignited by spiking the experiment mixtures with 20 µL 100 mM ATP and the reaction was carries out in the 37 °C incubator for 20 minutes. At the end of the incubation period the reaction was stopped by the addition of 200 µL 10 % TCA and incubation on ice for 5 minutes. The blanks in both experiments were made by adding TCA prior to the addition of ATP. The phosphate liberated from ATP was measured using the phosphate determination method (2.24), and the specific activity of ATPase was given as the amount (mM) of phosphate generated per mg cell wall ATPase per minute at 37 °C.

2.27 Phenylalanine ammonia lyase activity.

Stock solutions (all chemicals were purchased from Sigma chemical Co.): 0.1 M Borate pH 8.8. 0.25 M Borate pH 8.8 with and without 5 mM β-mercaptoethanol; 0.1 µCi/µL ^{14}C-phenylalanine; 20 mM L-phenylalanine; 10 N sulphuric acid; cyclohexane; diethylether; Solution 1: 2.5 µCi ^{14}C-phenylalanine, 2 mM L phenylalanine, 895 mM Borate pH 8.8 with no β-mercaptoethanol.

Arabidopsis cells were subcultured and grown in suspension culture for 3 days after which they were treated with 200 µg/mL chitosan for 24 hours. The soluble protein from the treated and non treated cells (controls) were extracted by grinding the cells in liquid nitrogen and the cell powder resuspended in 0.1 M borate containing β-mercaptoethanol. The solubilised protein was separated from the cell debris by centrifugation at 10000 g for 15 minutes at 4 °C. Two hundred and fifty microliters (approximately 38 µg protein) of the supernatant was mixed with 100 µL of solution 1 and 1.5 mL 0.1 M borate (with no β-mecaptoethanol) and incubated at 37 °C for 2

hours. The reaction was terminated by addition of 100 µL of 10 N sulphuric acid. ^{14}C-cinnamic acid was extracted from the reaction with 6 mL ether: cyclohexane (1:1). Three millilitres of water and 3 mL of scintillation fluid were then added to the terminated reaction mixture. The background noise was normalised by the addition of sulphuric acid to the reaction mixture prior to the addition of total soluble protein. The relative activity of PAL was estimated from the ^{14}C-cinnamic acid scintillation counts.

2.28. Electrospray ionisation tandem mass spectrometry (ESI-MS-MS)

The protein spots to be analysed were carefully excised from the Commassie brilliant blue stained gel using a sterile wide bore yellow pipette tip attached to a 200 µL pipette. Pipetting approximately 50 µL of sterile deionised water up and down several times facilitated the release of the gel plug from the pipette tip. The gel plugs were digested using an automated long trypsin digestion method on a ProGest digestion robot (Genomic solutions). The protein-acrylamide plugs were washed in 25 mM bicarbonate buffer. The washed plugs were destained and dehydrated in a concentrated acetonitrile solution. The concentrated gel plugs were then rehydrated once again in 50 mM bicarbonate buffer. The rehydrated gel plugs were alkylated with DTT and iodoacetamide. This was then followed with a bicarbonate wash. After washing each protein plug was digested with 20 ng trypsin (Promega, Madison. Wi) for 8 hours at 37 °C. The tryptic digests were extracted from the gel plugs with 100 µL of 50 % acetonitrile and 0.1 % formic acid. These samples were loaded onto a reverse phase capillary HPLC running at 120nl minutes. The HPLC was interfaced to a Micromass Q-ToF triple quad mass spectrophotometer. The four most intense

peptide ions at each time were monitored and automatically extracted into the second chamber of the quadrapole where they were accelerated and collided against a heavy inert gas (Argon or Nitrogen) under low kinetic energy conditions (10 eV to 50 eV). This gas collision induced fragmentation of the peptide ion covalent bonds. The resulting amino acid ions were detected and analysed in the third quadrapole for their mass and charge. Both b and y amino acid mass ion spectra were automatically interpreted into amino acid sequences using a program developed by Yates et al, (1995). These amino acid tags were subsequently used to interrogate the *Arabidopsis* genome database.

Chapter 3

Establishment of a Stable Cell Suspension Culture System.

3.1 Introduction

Plants respond to pathogen attack by mounting a coordinated, genetically determined defense that is designed to limit pathogen growth, multiplication and spread. The response includes the early pathogen-induced changes in ion fluxes across the plasma membrane, the most important of which is an influx of Ca^{2+} ions which serve to activate cytoplasmic components of the signalling cascade. Recognised events that occur downstream of the pathogen-induced Ca^{2+} influx include an increase in reactive oxygen species (Levine et al., 1994), induction of the phenylpropanoid pathway as indicated by an increase in activities of enzymes such as phenylalanine ammonia lyase and chalcone synthase (Lawton et al., 1983), and the subsequent synthesis of pathogenesis-related proteins (van Loon and van Kammen, 1970).

Plant cell suspension cultures can mimic whole plant tissues in their response to pathogen attack (Romeis et al., 1999; de Marco et al., 1999). As a result, cultured cells have been used to characterise cell wall responses to attempted pathogen invasion (Bradley et al., 1992. Brisson et al., 1994). Thus, for the study described herein the cell suspension cultures of *Arabidopsis thaliana* and Black Mexican Sweetcorn (BMS) were used to examine the role of the cell wall in pathogen defense. The choice of species in both cases was determined in relation to the availability of genomic sequence data. The genome sequence data of *Arabidopsis thaliana* is freely available via public databases on the Internet (http://mips.gsf.de/proj/thal/ and www.ncbi.nlm.nih.gov/). For BMS, the genomic sequence data is held privately by Pioneer Hi-Bred International and our laboratory had a privileged access to it.

The use of cell suspension cultures has several distinct advantages over whole plant tissues. Firstly, cells grown in suspension cultures are often derived from a single tissue type and are, therefore, uniform. Consequently, these cells are more likely to give reproducible experimental results in comparison to cells of various tissue types, as often seen in whole plant experiments. Secondly, the physico-chemical environment of the cell suspension system is tightly controlled, thus serving to diminish the variability of responses between experiments. Thirdly, cells grown in suspension culture provide an unlimited source of research material (e.g. proteins and secondary metabolites which are secreted to the extracellular medium) given that they can be grown in large quantities in a matter of days rather than weeks or months. Lastly, in the case of extracellular matrix studies, it is almost impossible to use whole plant tissues without damaging cells and thus increasing the risk of intracellular contamination. Cell suspension cultures, therefore, offer an excellent system for the study of events that take place in the apoplasm, especially the role of the extracellular matrix in general plant cell growth and defense against pathogens. However, although cell cultures are an ideal experimental system for some biological studies, certain experiments, especially those that involve phenotypic observations can only be done in intact tissues or whole plants.

One of the objectives of this thesis was to perform a proteomic study of the cell wall. Cell cultures were considered more suitable for this purpose since the homogenous nature with respect to cell type would probably result in less complex protein mixtures than is found in whole plants. Additionally, cell walls of cultured cells resemble primary cell walls common to all cell types of the whole plant. Moreover,

plant cell suspension cultures have previously been used to study the cell wall and extracellular matrix (Smith and Fry, 1991; Robertson et al., 1997; Blee at al., 2001).

In order to perform a proteomic study of the plant cell wall and determine the role of cell wall proteins in disease resistance, it was necessary to establish a highly reproducible cell suspension culture system. To achieve this, a series of experiments were conducted to establish the biological and chemical uniformity of *Arabidopsis* and BMS cell cultures by measuring:-

- Cell growth and multiplication monitored as packed cell volume as a function of time,
- Cell viability throughout the growth period,
- Uptake of nutrients from the medium with particular reference to phosphate levels
- pH and conductivity of the external medium
- Reproducibility of intracellular and extracellular protein profiles

3.2 Results

3.2.1 Growth of *Arabidopsis* and BMS Cell Cultures

Cell growth in *Arabidopsis* and BMS cell cultures was monitored by measuring packed cell volume (PCV) in 5 mL aliquots withdrawn from the cultures at different time points. The growth of both *Arabidopsis* and BMS cell cultures followed a typical sigmoid growth pattern (Figure 3.1). Upon subculturing, *Arabidopsis* cells entered a short lag phase that lasted for about 31 hours. After this period, the cell cultures grew exponentially until about 120 hours post subculturing at which point growth slowed down and the cells entered the stationary phase (Figure 3.1A). BMS cultures, however, had a longer lag phase (48 hours) followed by an exponential growth that declined around 180 hours and was replaced by stationary growth (Figure 3.1B). In both cultures, the variation of PCV between replicates was minimal as reflected by the narrow standard deviations. Moreover, three independent experiments produced very similar results. Therefore, the *Arabidopsis* and BMS cell cultures had highly reproducible growth rates and patterns as observed from the plots of PCV as a function of time.

3.2.2 Viability of *Arabidopsis* and BMS Cell Cultures During Growth

Having established the growth pattern of the cell cultures (3.2.1) it became necessary to monitor the levels of cell viability (and cell death) throughout the duration of the 7-day growth period. This was especially important given that growth

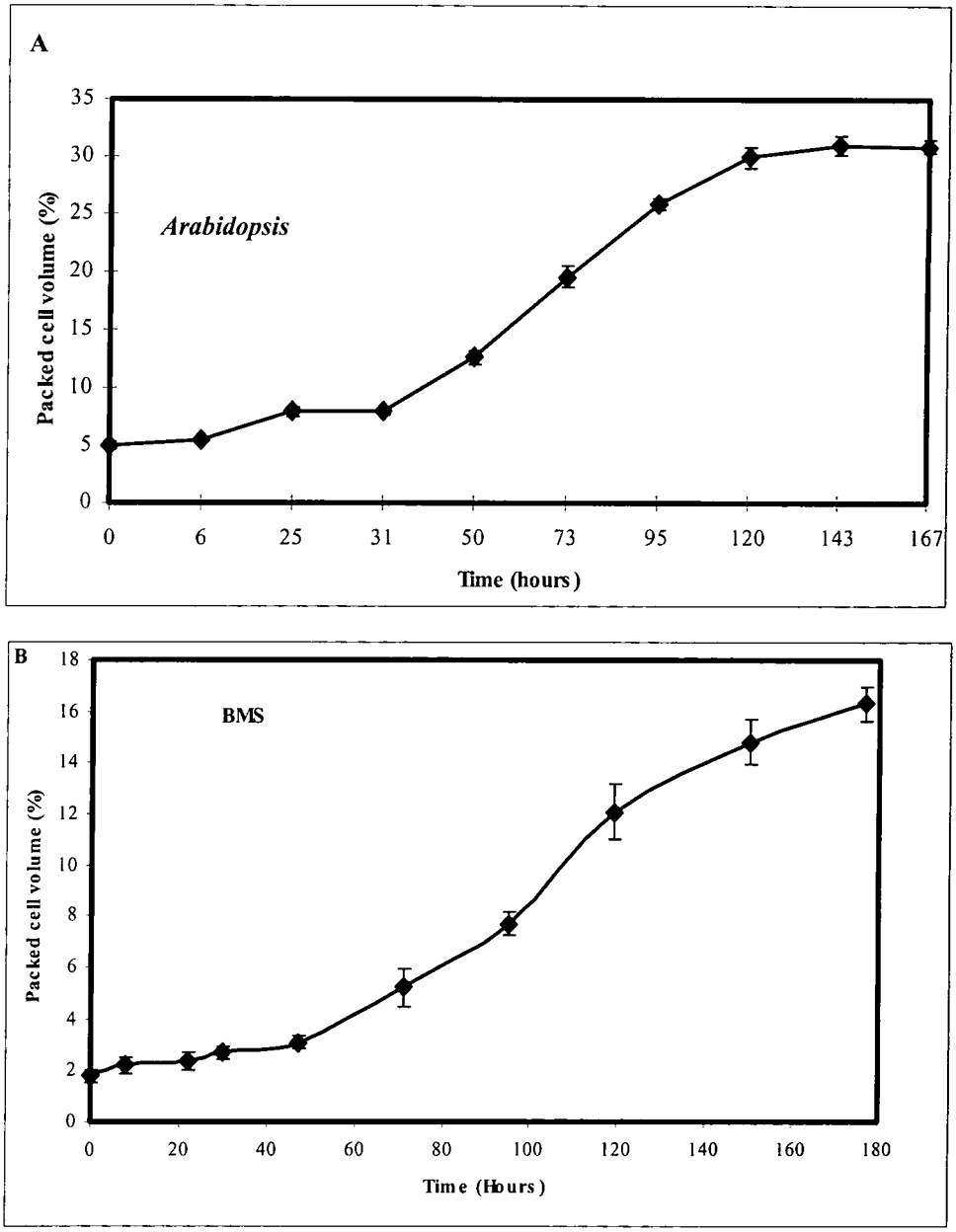


Figure 3.1 **Growth of *Arabidopsis* (A) and BMS (B) cells.**

Seven-day old cell suspension culture was diluted 10-fold (v/v) in 4-replicate flasks each containing 100 mL of culture. Five milliliter liquots were withdrawn from each replicate at the indicated times after subculturing and the PCV of the cells was measured. The volume of the packed cells is expressed as a percentage of the total withdrawn cell suspension culture aliquot. The data in each panel represent 1 of 3 independent experiments. The graphs represent the average PCV obtained from the 4 replicates and error bars indicate standard deviation from the mean.

was measured as cumulative PCV, which does not indicate the viability status of the cell cultures at any given point in time.

For cell death and viability analysis, a double stain strategy that used Evans blue and fluorescein diacetate (FDA) was employed. The rationale behind this approach is based on the fact that FDA penetrates cells and is converted to a fluorescent compound by esterase enzymes active only in viable cells. On the other hand, Evans blue is taken up by dead cells due to their plasma membrane's loss of selective permeability, which characterises viable cells (Figure 3.2). Thus, only dead cells are stained when visualised by bright field microscopy, while only live cells fluoresce under ultraviolet (UV) light (Figure 3.3).

Using this viability test, both *Arabidopsis* and BMS were found to consistently have very few dead cells throughout the duration of the 7-day growth period. These visual observations were then confirmed quantitatively. This was achieved by eluting the Evans blue stain taken up by stained cell samples and determining its absorbance at a wavelength of 600 nm. The quotient of this figure and the absorbance of stain similarly eluted from 100 % dead cells (i.e. boiled cells) expressed as a percent was the level of cell death. Percent viability was the difference between 100 and the recorded percentage of cell death. Using this method cell viability in both *Arabidopsis* and BMS cultures was over 90 % throughout the growth period (Figure 3.4). Therefore, not only did the cell suspension cultures grow very rapidly (section 3.2.1), but the cells also had high levels of viability from the lag phase through to the stationary phase when they were diluted into fresh medium to generate the subsequent daughter cell cultures.

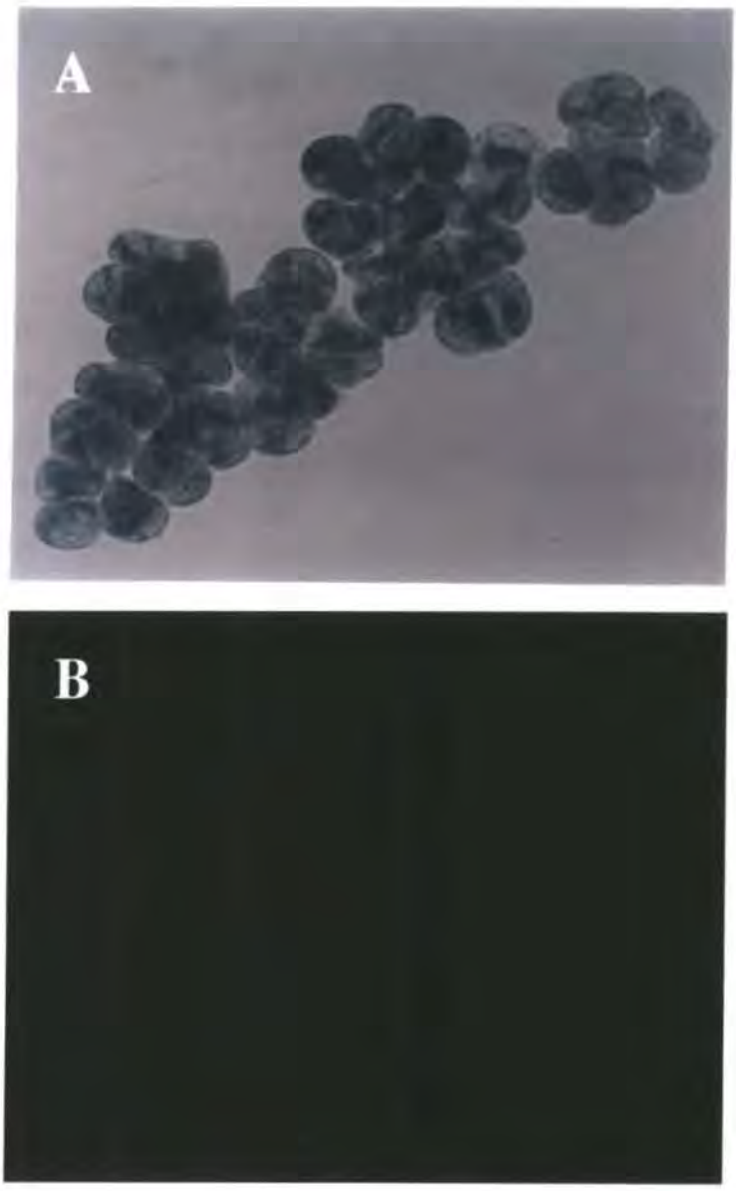


Figure 3.2 ***Arabidopsis*** **cells stained with Evans blue and fluorescein diacetate for cell viability analysis.**

Arabidopsis cells were deliberately killed by incubating in a boiling waterbath for 5 minutes. The killed cells were subsequently stained with Evans blue (EB) and counter-stained with fluorescent diacetate (FDA). Panel A (white light microscopy) shows that EB diffused to all cells. Panel B (UV light microscopy) shows no green FDA fluorescence indicating that FDA has not been converted to the fluorescent compound.

3.3 Uniformity of the Chemical Environment of Cell Suspension Cultures

In intact plants, the apoplast is an important compartment with a regulated pH and inorganic ions. In suspension cultures, the culture medium serves as a continuous apoplasm for all the cells. However, cell growth and viability do not reflect variations in the chemical nature of the culture medium. Therefore, the variation in the chemical nature of the culture medium during growth was monitored. The pH of the growth medium started at 5.7 immediately after inoculating the medium with *Arabidopsis* cells (Figure 3.5A). There was a sharp decrease to pH 5.2 within the first 6 hours. The pH then gradually increased until it had reached pH 6.7 by the time the experiment was terminated at day 7. For BMS, the pH declined sharply from pH 5.4 to pH 4.3 in the first 5 hours, but remained at an average of pH 4.5 for most of the time until 150 hours (Figure 3.5B). However, the pH slightly rose to an average of pH 5.5 at the end of 7 day growth period. The standard deviation shows that the variations between 4 replicate cultures were minimal in both *Arabidopsis* and BMS cell cultures.

Conductivity of the growth medium with *Arabidopsis* cells declined gradually from 5.3 mS/cm to approximately 1.8 mS/cm over the 7 day growth period. For BMS cell cultures, the conductivity of the medium decreased from 6.0 mS/cm to about 4.8 mS/cm over the growth period of 7 days. The variations in the conductivity between 4 replicate cultures were very minor as shown by narrow error bars (Figure 3.6).

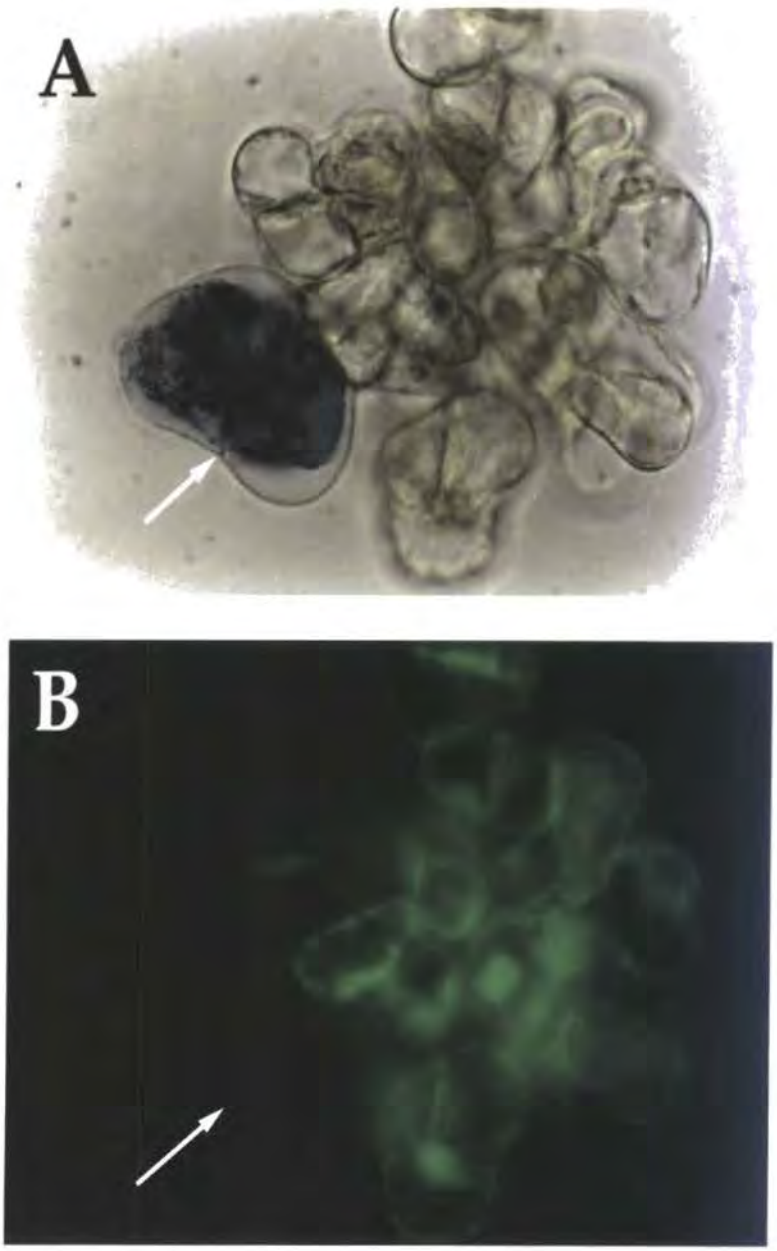


Figure 3.3 **Evans blue and fluorescein diacetate double stained cells for viability.**

Three-day old suspension cultured cells stained with EB and counter-stained with FDA. Panel A, is the bright light field view and Panel B, is the ultraviolet view of the same cells. The dead EB stained cell (pointed with an arrow) does not fluoresce under ultraviolet light.

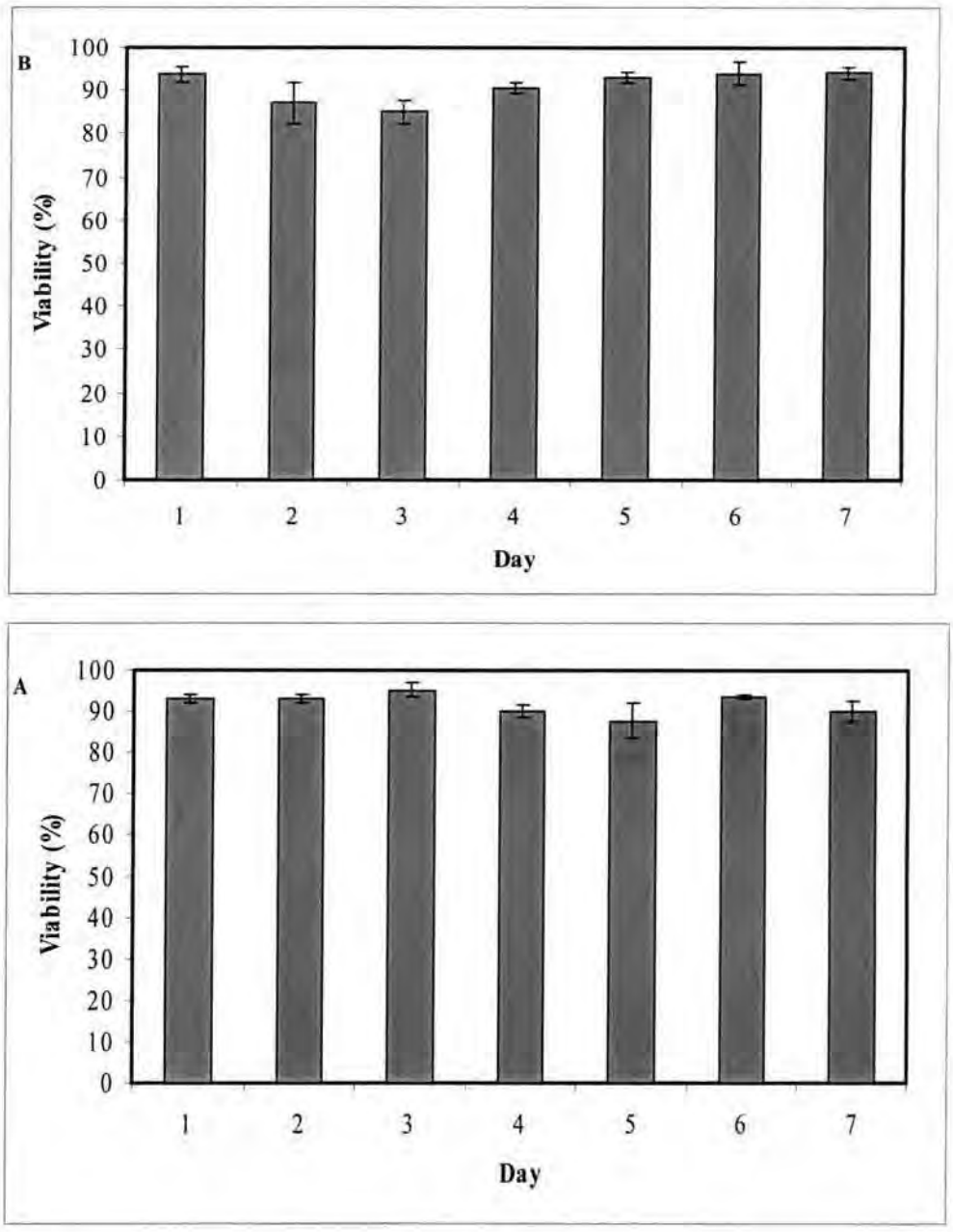


Figure 3.4 **Viability of *Arabidopsis* and BMS cell suspension cultures.**

Cells of *Arabidopsis* (A) and BMS (B) were diluted 10-fold in fresh media and grown for 7 days. At the specific time-points an aliquot of cells was stained with Evan's blue to determine their viability spectrophotometically. The optical density (OD) at 600 nm of the stain eluted from the dead cells was determined and the viability was expressed as the difference between this value and the OD of heat-killed cells as a percentage. Each histogram represents a mean of 4 independent replicates and the error bars are standard deviation from the mean.

As shown in Figure 3.7, there was a rapid uptake of phosphate by *Arabidopsis* cells that led to a sharp decline in medium phosphate concentration from about 1.029 mM to 0.808 mM within 6 hours post subculturing. Between 6 hours and 24 hours post subculture, the concentration of phosphate in the medium remained at about 0.734 mM. There was a second sharp decrease of phosphate in the external medium from 0.734 mM to 0.073 mM in the period between 24 hours and 48 hours post subculture. Between 72 and 144 hours post subculturing, the phosphate concentration levels were below the detection limit of the assay. The phosphate levels started to rise gradually after 143 hours (6 days) (Figure 3.7A). For BMS, the time-course of phosphate content of the external medium followed a similar pattern to *Arabidopsis* cell cultures. There was an initial slight drop in medium phosphate from 1.32 mM to about 1.029 mM in the first 24 hours of growth. From 24 hours to 36 hours post subculture the overall phosphate uptake by the cells was very low. There was a second sharp decline in the phosphate levels from approximately 1.029 mM to 0.220 mM between 36 and 96 hours post subculture. The phosphate levels rose gradually after 96 hours post subculture from about 0.220 mM to about 0.588 mM at the end of the 7 day growth period (Figure 3.7B).

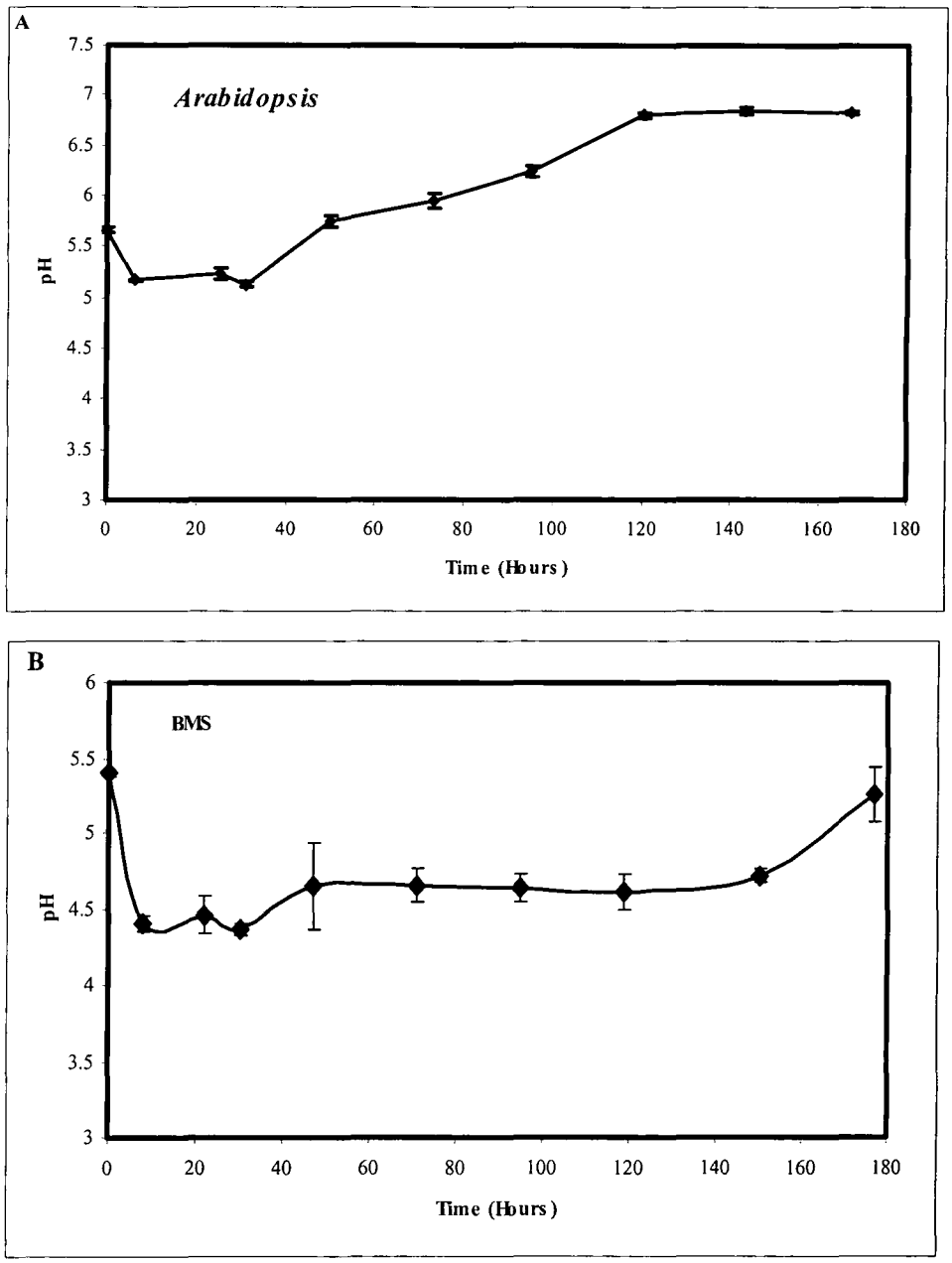


Figure 3.5 **pH in the external medium of *Arabidopsis* (A) and BMS (B) cells.**

Seven-day old cell suspension culture was diluted 10-fold (v/v) in 4-replicate flasks containing fresh growth medium to a final volume of 100 mL. Aliquots were withdrawn at the indicated times after subculturing and pH of the external medium was measured. Data in each panel represent 1 of 3 independent experiments. The error bars are the standard deviation from the mean of the four replicates.

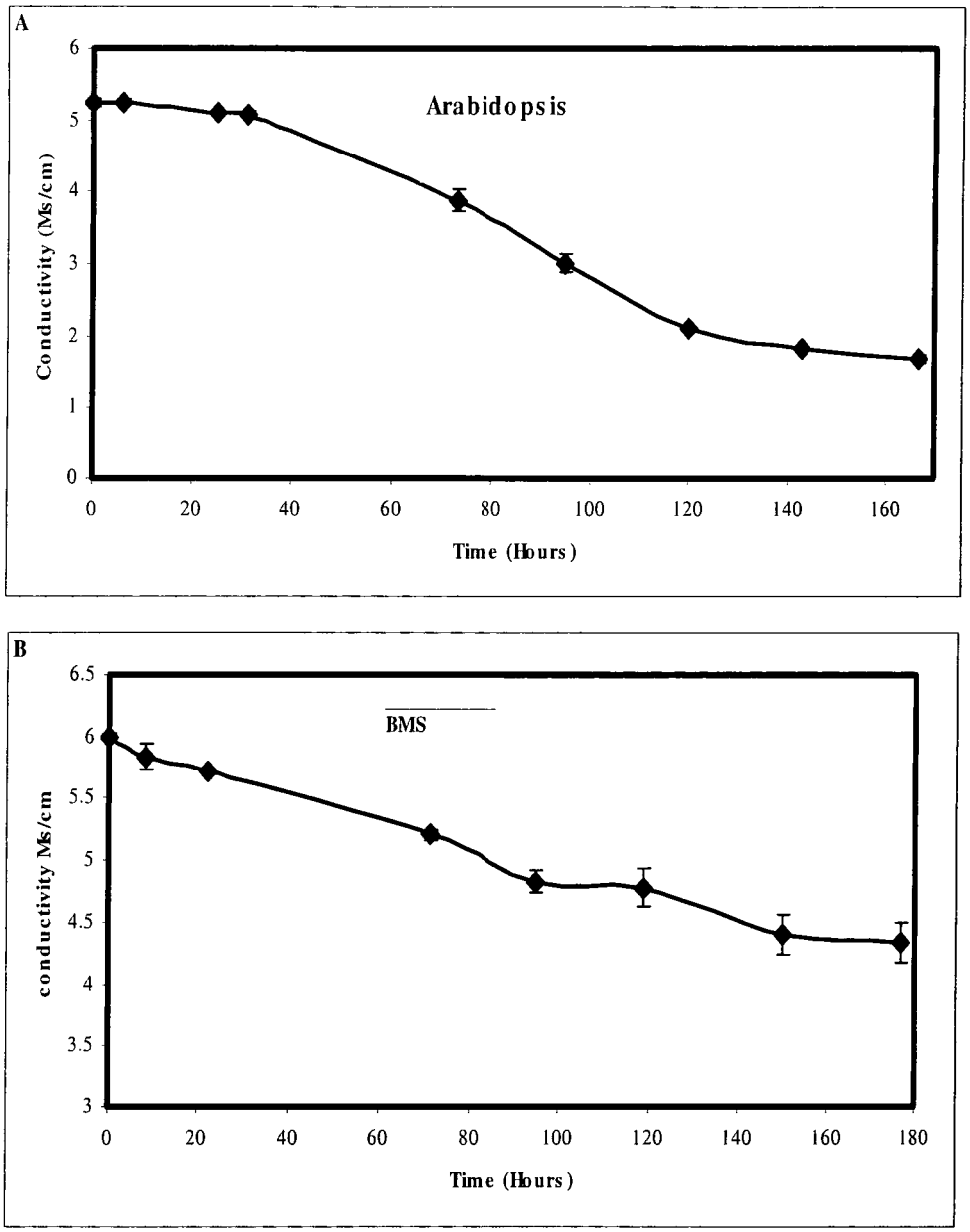


Figure 3.6 **Conductivity of the culture filtrate of *Arabidopsis* (A) and BMS (B) cultures.**

Seven-day old cell suspension culture was diluted 10-fold (v/v) in 4-replicate flasks each to make a final volume of 100 mL. Aliquots were withdrawn at the indicated times after subculturing and the conductivity of the external medium was measured. Data in each panel represent 1 of 3 independent experiments. The error bars are the standard deviation from the mean of the four replicates.

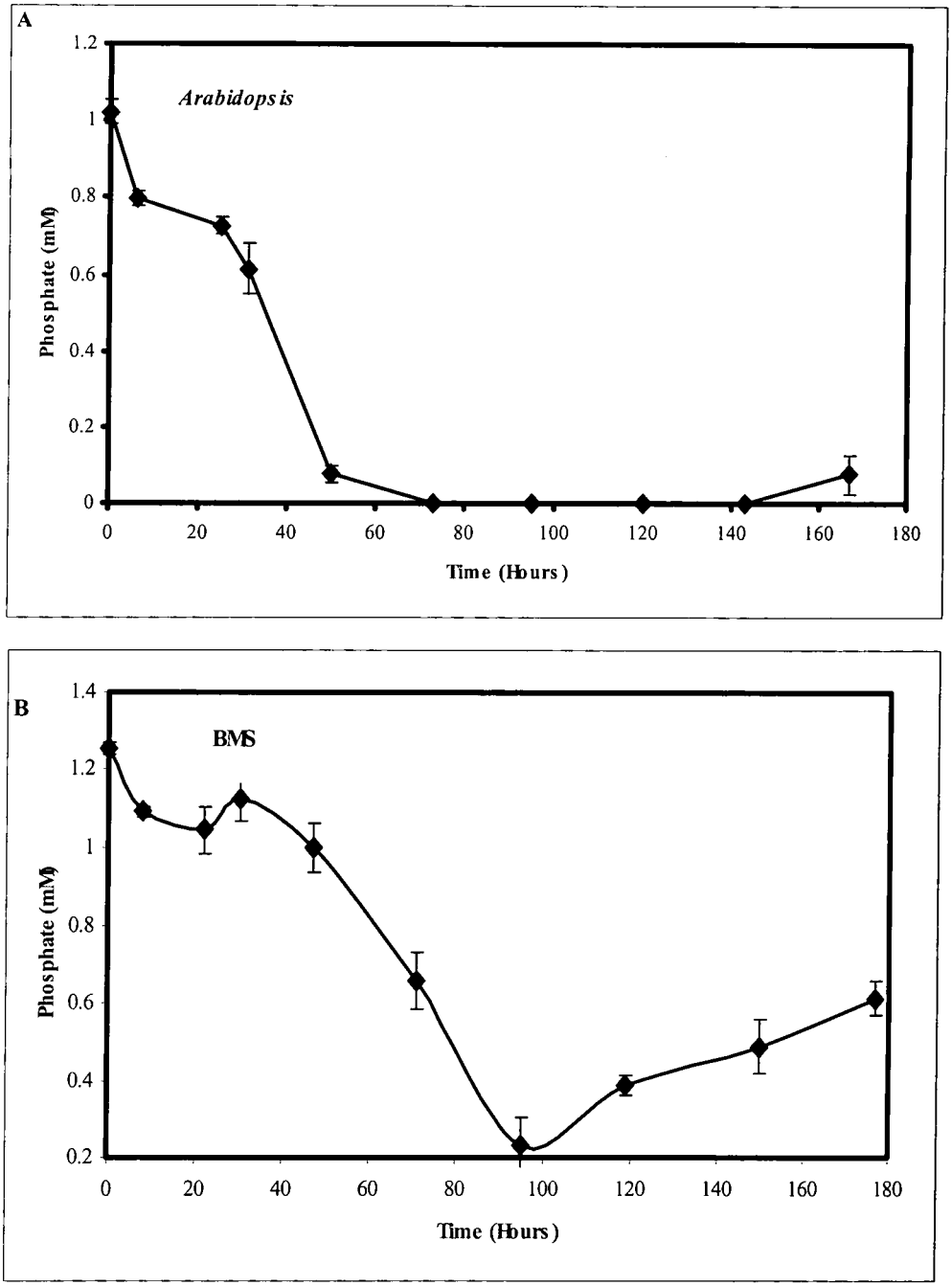


Figure 3.7 **Phosphate content of the culture filtrate of *Arabidopsis* (A) and BMS (B) cultures.**

An aliquot 10 mL seven-day old suspension culture was mixed with 90 mL of fresh growth medium in four replicate experiments. Aliquots were withdrawn from each replicate at the indicated times after subculturing and phosphate content of the external medium was determined. The data in each panel represent 1 of 3 independent experiments. The error bars are the standard deviation from the mean of the four replicates.

The same pH, conductivity and phosphate content profiles were observed in 3 independent experiments, showing that the chemical environment or the "apoplasm" of the *Arabidopsis* and BMS cell cultures was uniform from one generation of cells to the next.

3.4 The Biological Uniformity of the *Arabidopsis* and BMS Cell Cultures

The growth pattern and changes in the chemical environment of *Arabidopsis* and BMS cell cultures proved to be highly reproducible from one generation of cells to the next (see sections 3.2.1, 3.2.2 and 3.3). However, it was important to establish whether cell cultures in different generations were uniform at molecular level. To determine this, proteins secreted to the extracellular matrix, the total soluble protein and the cell wall proteins from three different cell culture generations were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For both plant species, proteins extracted from different subcellular compartments (proteins secreted to the apoplast, total soluble proteins or cell wall proteins) had distinct profiles. The total soluble proteins from both *Arabidopsis* and BMS had the most complex but highly reproducible protein profile in SDS-PAGE (Figure 3.8). The protein bands in culture filtrate and cell wall protein extracts were less complex but also highly reproducible (Figure 3.9 and 3.10 respectively).

In all three compartments the protein profiles were unique to each plant species and were highly reproducible across three different generations of cell cultures. These

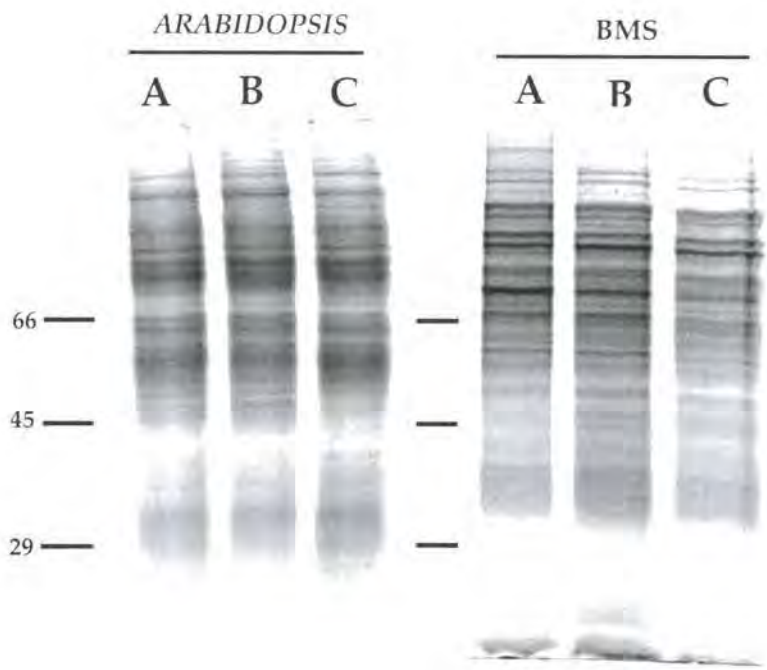


Figure 3.8 **Total soluble protein profiles showing high reproducibility between experiments**

Total soluble proteins were extracted from three independent experiments (marked A, B and C) of 3 day old *Arabidopsis* and BMS cell suspension cultures. The protein extracts were denatured and separated using 10 % SDS-PAGE and the gels were stained with Coomassie Brilliant Blue to visualise the protein bands.

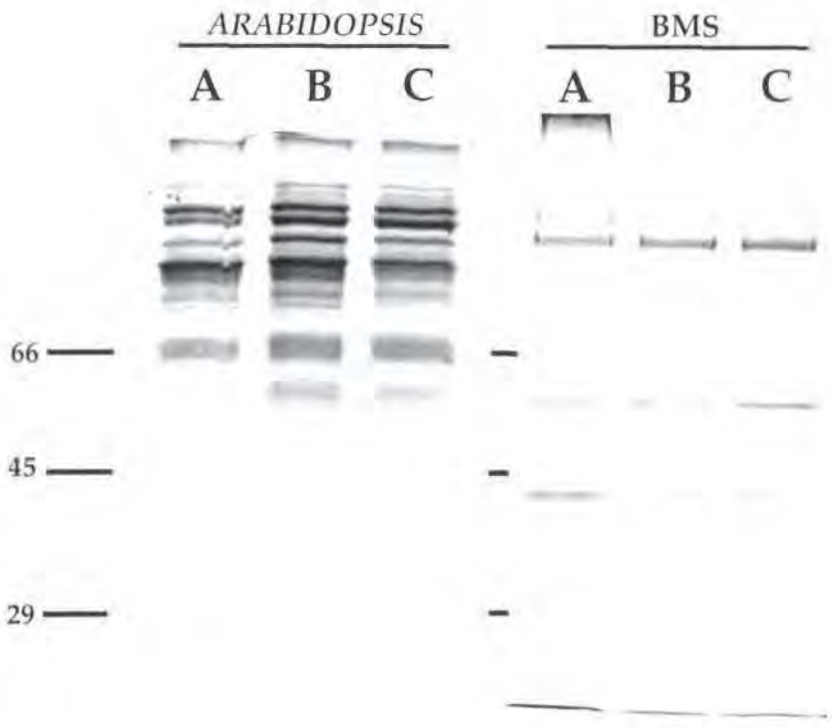


Figure 3.9 **Profiles of proteins that are secreted into the culture filtrate of cell suspension cultures.**

Proteins were extracted from the culture filtrates of *Arabidopsis* (A) and BMS (B) cell suspension cultures using 80 % acetone. The protein extracts were denatured and separated using 10 % SDS-PAGE and the protein bands were visualised by Coomassie Brilliant Blue staining. Lane A, B and C in both panels represent protein profiles from 3 separate experiments.

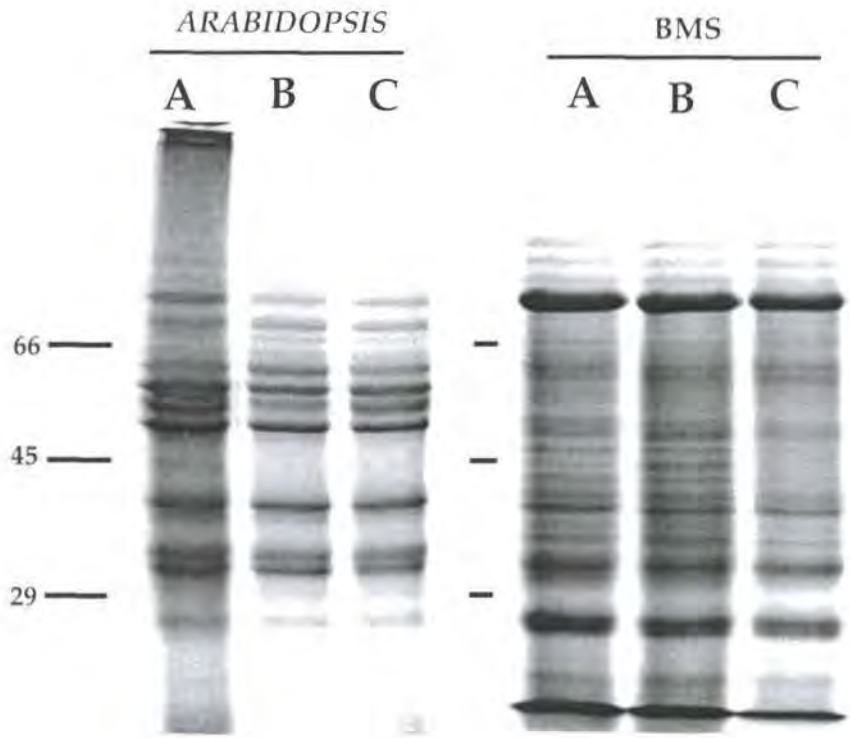


Figure 3.10 **Profiles of *Arabidopsis* and BMS cell wall proteins extracted with $CaCl_2$ from purified cell walls.**

Proteins were extracted from purified cell walls of 3-day old *Arabidopsis* and BMS cell suspension cultures using $CaCl_2$. The protein extracts were denatured and separated using 10 % SDS-PAGE and were stained with Coomassie Brilliant Blue. Lanes A, B and C in each panel represents the protein profiles from three independent experiments.

results revealed that the *Arabidopsis* and BMS cell cultures used in this study were reasonably stable as defined by their physiological and biochemical behavior during growth.

3.5 Discussion and Conclusion

3.5.1 Cell Growth and Viability

Aspects of plant growth, development, and responses to environmental cues can be recapitulated in cells grown in suspension culture within short periods of time (Romeis et al., 1999; de Marco et al., 1999; Bradley et al., 1992; Felix et al., 1991). This makes a suspension culture system suitable to use for understanding the mechanisms underlying responses of plants to various stimuli. However, before suspension cell cultures can be used in this way, a stable and reproducible cell culture system needs to be established. In order to exploit the advantages offered by cell suspension cultures as an experimental system, the stability and reproducibility of *Arabidopsis* and BMS cell cultures down to molecular level were investigated. The growth of both *Arabidopsis* and BMS cells followed the sigmoidal growth pattern commonly seen in all cell cultures. The sigmoid curve is roughly defined by three phases of growth: the lag phase, the exponential phase and the stationary phase. These phases are mainly controlled by the balance between the availability of nutrients and the accumulation of waste products, that is, cells grow faster when more nutrients are available and the growth slows down when nutrients are depleted

from the growth medium (Murashige and Skoog. 1962). The reproducibility of the cell growth pattern across three independent experiments was a good indicator of the reproducibility of the rate at which the nutrients are taken up from the growth medium by the cells, thus showing that the nature of the cells was the same from one generation of cells to the next. Within the 7 day growth period the *Arabidopsis* cultures increased by 6-fold (from 5% to 30 %) while BMS increased in PCV 8-fold (from 2 % to 16 %). This explosive growth obviously makes these cultures an ideal source of massive amounts of experimental material that can be generated in a short space of time. In both cell culture systems cells were growing at the fastest rate on day-3 (i.e. between 72 and 108 hours) post subculture, making this an ideal period for testing cell responses to various treatments. According to Collin and Edwards (1998), cell senescence starts soon after the cell cultures enter the stationary growth phase. Prolonged incubation of cells that have entered the stationary phase is followed by a rapid decline in cell viability and cell lysis eventually results in negative growth of the cell culture. Repeated use of inoculum from the decline phase for propagation of any cell suspension culture leads to a gradual loss in cell density in the successive daughter cell cultures (Collin and Edwards, 1998). In order to prevent this from happening, cell viability was monitored during the cell culture growth and, for both *Arabidopsis* and BMS cultures, cell viability was above 90 % from subculturing to day 7 when the cells have just entered the stationary phase. Based on these findings, both cultures were subcultured into fresh medium once every week in order to maintain viable and vigorous growing cell cultures.

3.5.2 Uniformity of the Cell Culture Medium pH, Phosphate and Conductivity

The chemical nature of the growth medium was monitored at two levels. First, two individual chemical species, protons and inorganic phosphate, were measured during cell growth. Second, variation in the totality of chemical species in the medium was monitored by measuring conductivity of the growth medium. All the three parameters, i.e. changes in pH, inorganic phosphate, and conductivity were highly reproducible across replicates in individual cell culture generations, and also, across three different generations of cell cultures. During growth, cells take nutrients from the medium and release organic and inorganic molecules, which modify the chemical nature of the growth medium. These may, in turn, influence the molecular nature of the cell, especially the cell wall, which is the most peripheral cellular compartment consistently in contact with the growth medium. For example, the pH of the medium can affect the activity of cell wall peroxidases, which produce hydrogen peroxide (Bolwell et al., 1998) that may change the association of soluble cell wall structural proteins from a non-covalent to a covalent association (Bradley et al., 1992). In addition, the culture medium is equivalent to the apoplastic fluid that bathes both the cell walls and protoplasts in intact tissues.

The pH of the external medium is dependant mostly upon the activity of the membrane bound H^+-ATPases. The membrane bound H^+-ATPases are responsible for the proton extrusion by the cells and this activity polarises the electrical charge across the cell membrane (Young et al., 1998). The changes in pH of the external medium in both *Arabidopsis* and BMS was highly reproducible throughout the growth period. These results therefore indicated that the activities of H^+-ATPase

were also highly consistent and reproducible as highlighted by the reproducibility of the changes in the extracellular pH. The gradual decrease in conductivity reflected an overall uptake of electrolytes (nutrients) into the cells. When the experiments were terminated, the downward trend of medium conductivity was still continuing. This was also mirrored by the medium phosphate concentration. The decrease in medium phosphate and conductivity was consistent with the observed increase in the growth of the cell cultures. Phosphate is rapidly taken up and incorporated into nucleic acids, cell membranes and high energy molecules such as ATP and NADPH, therefore phosphate uptake has a direct impact on cell growth (Stafford and Warren, 1991).

3.5.3 The Molecular Homogeneity of Cell Cultures

Cellular proteins are affected by various abiotic and biotic environmental factors such as pH and microbial pathogen invasion (Bradley et al., 1992, Wojtaszek et al., 1997). Hence the last criteria used for assessing the stability and the uniformity of *Arabidopsis* and BMS was the reproducibility of the protein profiles. Proteins from three major plant cell culture compartments were analysed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These included the intracellular matrix proteins (represented by the total soluble protein) and the extracellular matrix proteins (represented by the cell wall and the culture filtrate proteins). The proteins extracted from three independent three day old cell cultures were compared. The SDS-PAGE profiles of these proteins gave a remarkable reproducibility. These results were taken as supplementary evidence for showing

that the cell suspension cultures used here were uniform and reproducible.

The reproducibility of the cell growth, the chemical environment and the molecular homogeneity at protein level show that the *Arabidopsis* and the BMS cell cultures tested in this study are stable. These criteria therefore qualified the cell cultures as suitable plant material to be employed in the proteomic studies described in the subsequent chapters.

Chapter 4

Characterisation of the *Arabidopsis thaliana* Cell Wall Proteome

4.1 Introduction

The cell wall is the outermost compartment of the plant cell and until recently its role has been mainly implicated only in the maintenance of cell shape and rigidity. It is now known that the cell wall is a dynamic structure and it is involved in many cellular roles including the perception of external stimuli (Lally et al., 2001; He et al., 1996), pathogen defence (Mellersh and Heath. 2001), cell growth and cell development, (Burk et al., 2001; Lally et al., 2001).

The plant cell wall is mainly composed of carbohydrates (90 % by weight) and less than 10 % (by weight) proteins. Although proteins make up only about 10 % of the cell wall, most of the recognised cell wall activities are associated with the cell wall enzymes and structural proteins. For example the cell wall proteins have been shown to protect the protoplasm by cross-linking with each other and with other cell wall structures in response to pathogen invasion (Bradley et al., 1992; Brisson et al., 1994; Wojtaszek et al., 1995; El-Gendy et al., 2001). The xyloglucan endo-transglycosylases and expansins have been shown to be involved in cell growth and elongation (Fry et al., 1992 and McQueen-Mason et al., 1992). Also the arabinogalactan proteins have been shown to be involved in defence-associated apoptosis (Gao and Showalter. 1999). However, despite these interesting roles, only a fraction of the total number of cell wall protein population has been identified and characterised to date.

A large proportion of the present data on plant cell wall proteins is derived from genomic studies (Sheng et al., 1991; Scheres et al., 1990; Keller et al., 1988; Condit and Meagher. 1987), however, in certain cases it has been found that there is low or

no correlation between the messenger RNA (mRNA) abundance and protein levels (Penninghton and Dunn, 2001). Moreover, posttranslational modifications like glycosylation, phosphorylation and proteolysis of polypeptides can often alter the protein function, therefore, simply looking at the cellular mRNA profile will not always give a true reflection of the protein diversity and their modifications (Pennington and Dunn. 2001).

Studying cell wall proteins using the traditional methods such as 1-dimension polyacrylamide gel elecrophoresis (PAGE) and Edman N-terminal sequencing had been proven to be difficult and to have a low throughput (Robertson et al., 1997). Compared to other cellular proteins cell wall proteins are highly insoluble and therefore they tend to become less abundant in total cellular homogenates. In addition, a number of cell wall proteins are N-terminally blocked and therefore the traditional Edman N-terminal protein sequencing approach results in a lower success rate of identification (Marques et al., 1998; Robertson et al., 1997). Therefore, an application of a more efficient protein identification method was necessary.

A novel proteomic approach has been applied in an attempt to overcome some of the problems faced when dealing with cell wall protein studies. The term proteomics was invented approximately 5 years ago by Wilkins et al., (1996). It is generally defined as the study of the protein complement expressed by the genome of an organism. The primary tool of this technique is the 2-dimension polyacrylamide gel electrophoresis (2-D PAGE). In 2-D PAGE the proteins are resolved both according to their charge (pI) and molecular weight (MW). In most proteomic studies the 2-D SDS PAGE resolved proteins are identified using matrix assisted laser

desorption/ionisation time of flight (MALDI-ToF) mass spectrometry, although western blotting and N-terminal sequencing technique can still be applied. Proteomics is now regarded as the fastest, most sensitive and robust method of studying complex mixtures of proteins. Proteins that are to be analysed via MALDI-ToF mass spectrometry are proteolysed into peptides (e.g. using trypsin). The peptides are normally mixed with a matrix (small aromatic molecules) and dissolved in an organic solvent. The mixture of peptides and matrix are placed and dried/crystallised on a metallic probe. The peptide/matrix co-crystals are then subjected to a high voltage and a short laser pulse inside a vacuum chamber of the mass spectrometer. This treatment turns the dry peptides into gas ions which are in turn repelled and accelerated at high voltage into a field-free drift tube of the mass spectrometer towards a detector which records the time of flight (ToF) as mass/charge (m/z) ratio. The smaller ions fly faster that the bigger ions and therefore m/z ratios can be calculated directly from the ToF. The experimental peptide masses are used to search the databases containing peptide masses generated theoretically from known polypeptides and the positive "hits" are assigned with a molecular weight search (MOWSE) score. The MOWSE score algorithm was developed by Pappin et al., (1993) and it gives results as a list of positive protein assignments ranked according to their MOWSE scores, and the highest scoring protein is normally the positive hit. Peptide-mass fingerprint, the protein molecular weight range and the cleavage enzyme are the only parameters used in the MOWSE scoring algorithm. The more peptide masses match with theoretical peptide masses generated from a given polypeptide, the higher the MOWSE score. In this study only proteins with high MOWSE scores ($>10^2$) were considered as positive hits.

The unique feature of the proteins destined for secretion is that they posses a cleavable N-terminal signal peptide that targets them to the endoplasmic reticulum (ER) where some are modified, folded and assembled before travelling via the Golgi apparatus on their way to the extracellular matrix (Vitale et al., 1993; Palade. 1975). The N-terminal signal peptide (15-30 amino acids long) is generally defined by a positively charged n-region, a hydrophobic h-region, and a polar c-region. A signal peptidase cleavage site defines the end of the N-terminal signal peptide (Emanuelsson et al., 2000). However, not all signal peptide possessing and ER translocated proteins end up in the extracellular matrix, some plant proteins have a C-terminus KDEL, HDEL, RDEL, KQEL, SDEL, or KEEL retention tetrapeptide which stops them from secretion (reviewed by Vitale et al., 1993). However, among these, KDEL and HDEL are the most common retention tetrapeptides found in both plant and mammalian ER proteins (Vitale and Denecke. 1999).

The simplest method of extracting cell wall proteins from plant cells grown in suspension cultures is by washing the intact cells in a protein extracting reagent (Blee et al., 2001; Robertson et al., 1997), However, in an attempt to minimise cell wall protein contamination by cytosolic proteins, the cell walls were isolated and purified from the rest of the cellular components using a method optimised in our laboratory. This cell wall purification technique involves 100 % cell disruption using a French press and purification of the cell walls are purified from the cell lysate by sedimentation by gravity through a glycerol column. The cell walls of the suspension-cultured cells represent the primary cell walls of all plant cell types and thus minimises the possibility of cell type to cell type protein variations. The cell suspension cultures of *Arabidopsis thaliana,* shown to be highly reproducible in the chapter 3, were used in this study to map, identify and analyse the protein composition of the plant cell walls.

The main aim of this chapter was to apply a proteomic approach in an attempt to identify and characterise as many cell wall proteins as possible. Using this approach both classical cell wall proteins and a number of novel cell wall proteins have been identified. In addition, a group of unexpected proteins have also been identified.

4.2 Results

4.2.1 Isolation Of Pure Cell Walls from *Arabidopsis* Cell Suspension Cultures

Cell walls of high purity were obtained by completely disrupting *Arabidopsis* cells using a French press cell disrupter at 4 °C. Cells grown for 5 days were removed from the growth medium using a vacuum assisted filtration. The cells were then washed 3 times with 100 mL of deionised water at 4 °C. The washed cells were then passed through a French at least two times until all the cells were completely disrupted. The cell homogenates were layered onto 500 mL of ice-cold 10 % glycerol in a plastic tube (4 cm in diameter). The cell walls being the most dense structures in the cell homogenate, sedimented through the glycerol column under gravity overnight at 4 °C, leaving the cellular debris at the top. Complete disruption of cells was the most essential step in this procedure since the purification of the cell walls was entirely dependent on the density of individual components the homogenate, therefore after French press disruption a sample of the disrupted cells was examined with a light microscope prior to glycerol gradient purification. The sediment was white and completely free of the green chlorophyll coloration, which remained at the top of the column (Figure 4.1). The lack of any green colour in the sediment confirmed the lack of intact cells and chlorophyll containing organelles. The lack of green chlorophyll coloration in the cell wall sediment also served as a first marker for the lack of cytoplasmic contamination.

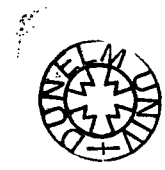


Figure 4.1 **Purification of *Arabidopsis* cell walls.**

(**A**) *Arabidopsis* cells disrupted using a French press were layered onto a 10% glycerol column. The cell walls being the most dense structures in the cell lysate sediment by gravity as a snow white cotton wool-like material to the bottom of the column while the rest of the cellular structures remain at the top. Chlorophyll, a natural plant cell pigment is used as a marker for both intact cells that might have survived cell disruption and soluble cytoplasmic contamination. (**B**) Intact (undisrupted) cell were also layered on a glycerol column to demonstrate that the chlorophyll inside the cells in a reliable marker for intact cells.

4.2.2 The Purity Of The Cell Wall Preparations

4.2.2.1 Cell Wall Purity Examined Via Transmission Electron Microscopy and In situ Immunolabeling

Following glycerol gradient purification the isolated cell wall material was transferred to 50 mL Falcone tubes and the excess glycerol was removed by centrifugation at X 10 000 **g** for 10 minutes followed by 3 times washes with 30 mL of ice-cold deionised water. The purified cell walls were embedded in agarose, fixed, dehydrated and then impregnated with resin before sectioning and examination by transmission electron microscopy (TEM). The examination of purified cell walls via TEM showed no sign of cytoplasmic organelles such as mitochondria, chloroplast, nucleus, endoplasmic reticulum, etc. This observation was the first evidence to show that the cell wall material was free of cytoplasmic organelle contamination. When the cell walls were viewed at high TEM magnification no plasma membrane contamination could be detected (Figure 4.2). This electron micrograph also shows the characteristic fibrous nature of the cell walls. As a point of reference for *Arabidopsis* cellular structures, an electron micrograph of intact cell was also prepared. Cellular organelles including mitochondria, chloroplast and Golgi apparatus were clearly visible (Figure 4.2). From EM perspective the cell wall preparations were free of these components and had no visible plasma membrane contamination. In order to confirm the absence of membrane structures in the purified cell wall material, immunofluorescent probes were used. A monoclonal

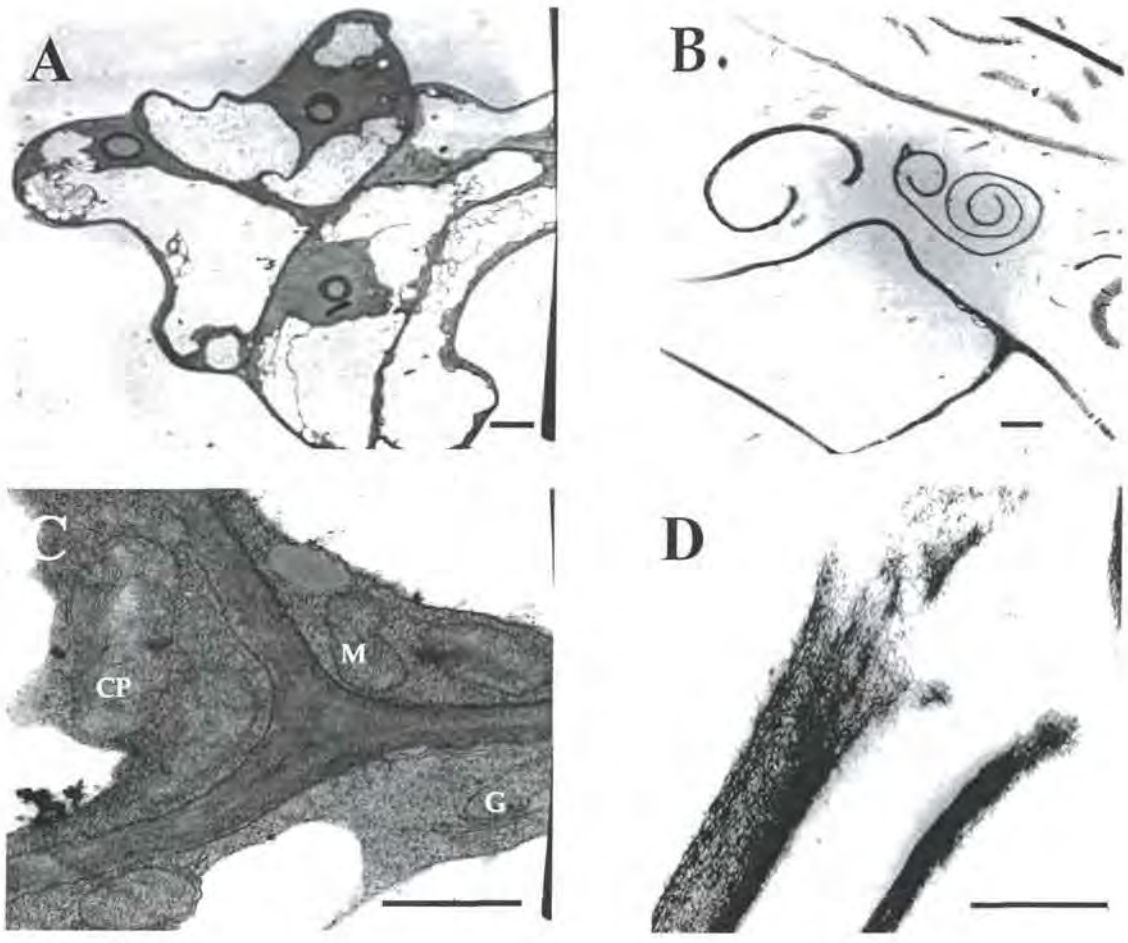


Figure 4.2 **Purified cell wall viewed via Electron Microscopy (EM).**

Intact *Arabidopsis* cells (A and C) and purified cell walls (B and D) were embedded in agarose, fixed, dehydrated and then impregnated with resin before sectioning and examination by electron microscopy at low magnification (A and B) and high magnification (C and D). Intact *Arabidopsis* cells showing as (a point of reference) the cellular structures and organelles that are likely to contaminate the cell wall preparations. The bars at the bottom right hand corner are 1 μm long in A and B, and 0.5 μm long in C and D. CP = Chloroplast, M = mitochondria and G = Golgi apparatus.

antibody JIM 18, which recognises plasma membrane abundant glycolipid epitopes (Knox et al., 1995) was applied as a probe to the purified cell walls. This antibody failed to detect any plasma membrane epitopes in the purified cell wall material as shown by the lack immunofluorescence (Figure 4.3). As positive controls the purified cell wall material was immunolabelled with JIM 5 and LM5 antibodies which are specific for an unesterified homogalacturonan protein (Knox et al., 1989) and a β-1,4-galactan (Jones et al., 1997) respectively, the results showed positive fluorescence (Figure 4.3).

Additionally, an assay for callose synthase, a membrane bound enzyme was performed on the purified cell walls by Dr S Chivasa. This assay showed that the cell wall preparations had no callose synthase activity. Therefore the immunofluorescent probing with plasma membrane component specific antibodies and the assay for the activities of membrane bound enzyme (callose synthase) confirmed that the cell wall isolation method used produced cell walls that were free from any detectable plasma membrane contamination.

4.2.2.2 Proteins extracted from the purified cell walls lack any detectable soluble cytoplasmic protein contamination

Having tested the absence of plasma membrane contamination, it was important to prove that the proteins extracted from the purified cell walls were also free from soluble cytosolic protein contamination. This analysis was required as the cytosolic proteins released during the cell homogenisation step of cell wall purification could co-sediment through the glycerol with the cell wall material. In order to address

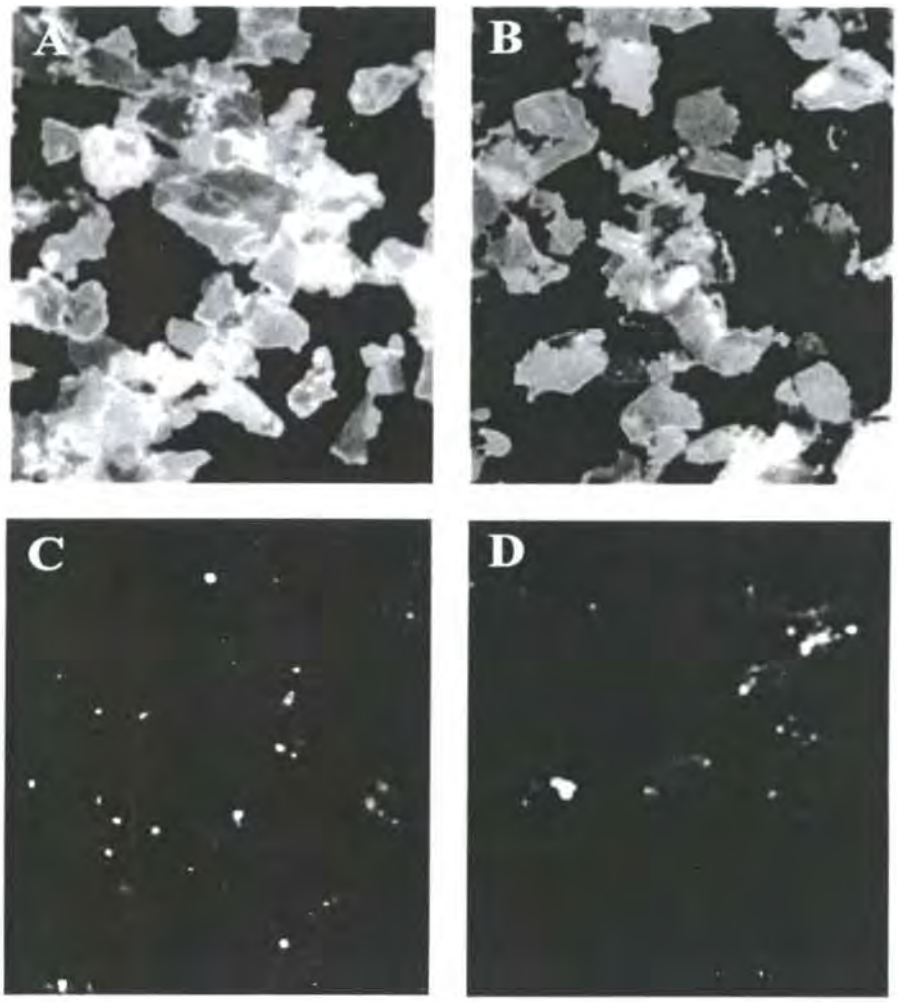


Figure 4.3 **The analysis of purified cell walls using immunofluorescent antibodies.**

Purified cell walls were fixed and incubated with 1% milk powder with various monoclonal antibody (A-C). The presence of the antigen was detected by using a fluorescein isothiocyanate-tagged secondary antibody and microscopic examination of epifluorescence.

A. JIM 5 antibody recognising unesterified homogalacturonan.

B. LM 5 antibody recognising ß-1,4-galactan.

C. JIM 18 antibody recognising membrane glycolipids.

D. No antibody to serve as negative control.

this, an antibody of enoyl-ACP-reductase (ENR), was used to probe for the presence ENR in the cell wall protein preparations. ENR was chosen because it is one of the highly a soluble proteins in the cell homogenate and therefore would be one of the proteins that might form non-specific associations with the cell wall preparations.

The protein extracts from total soluble proteins (TSP) and purified cell walls were resolved via 1-dimension polyacrylamide gel electrophoresis (PAGE) and were analysed by western blotting using a polyclonal antibody raised against *Brassica napus* ENR. The antibody recognised the ENR in the TSP lane but did not detect the antigen in the cell wall protein extracts (Figure 4.4). Since the cell wall protein extracts were free of any enoyl-ACP-reductase, it was concluded that cell wall proteins were free of soluble cytoplasmic protein contamination. These results showed that the stringent washing steps during cell wall purification process were sufficient to remove the soluble proteins that might have had non-specific associations with the cell walls. This analysis was used as the last quality control check before embarking on the cell wall proteomic exercise.

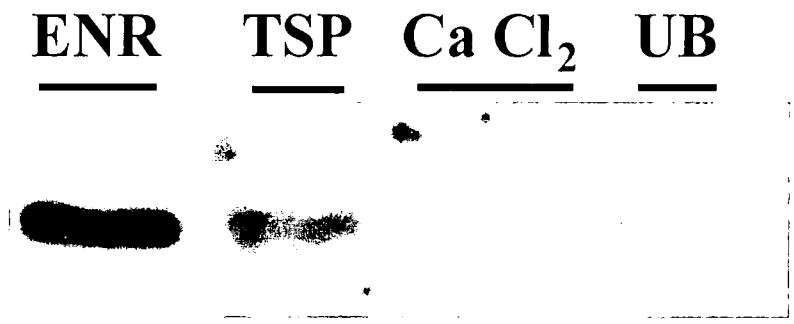


Figure 4.4 **The analysis of *Arabidopsis* proteins using western blotting.**

Twenty micrograms of pure enoyl-ACP reductase (ENR), the *Arabidopsis* total soluble protein (TSP), and *Arabidopsis* cell wall proteins extracted with $CaCl_2$ and urea buffer (UB) were separated via 10 % SDS-PAGE. These proteins were Western blotted onto a Nitrocellulose membrane and probed with an antibody raised against oil seed rape enoyl-ACP reductase.

4.3 Protein Identification Via 2-D SDS Polyacrylamide Gel Electrophoresis And Matrix Assisted Laser Desorption/Ionisation Time-Of-Flight (MALDI-ToF).

4.3.1 2-dimension Polyacrylamide Gel Electrophoresis

Having tested the purity of the cell walls, the next requirement was to extract and separate the cell wall proteins via 2-dimension polyacrylamide gel electrophoresis (2-D PAGE). The test for all potential contaminants such as cytosolic and membrane proteins was crucial because the cell wall localisation of the proteins to be identified in this study relied on a cell wall preparation of high purity. The cell wall harbours hundreds of proteins, therefore, proteins were extracted sequentially to minimise protein complexity on the 2-D PAGE. Proteins were extracted from the purified cell walls sequentially with 0.2 M $CaCl_2$, followed by a Urea buffer (7 M Urea, 2 M Thiourea, 4 % Chaps, 1 % DTT and 0.8 % Ampholytes). The protein extracts were precipitated by 80 % acetone at –20 °C over night. The precipitated proteins were then dried with filtered air and resolubilised in a 2-D sample buffer (9 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% Pharmalyte 3-10). The protein concentration was estimated using a modified Bradford assay (see general materials and methods). One milligram of each extract was loaded by reswelling the IEF gel strips (18 cm pH 3-10 and pH 6-11 IEF strips) over night at room temperature. The reswelled strips were subjected to isoelectric focussing overnight at 70 kVh. Following IEF the proteins were then transferred onto large format 12 % polyacrylamide gels and were subjected to electrophoresis at 20 mA per gel at 10 °C until the bromophenol blue reached the bottom of the gel. The electrophoresed gels were stained with Coomassie brilliant blue.

4.3.2 MALDI-ToF Identification of Cell Wall Proteins.

The Coomassie stained gels (Figure 4.5, 4.6 and 4.7) were imaged and all the detected protein spots were picked from the gel into 96-well microtitre plates using a Genomic Solutions ProPick robot. The picked protein spots were proteolysed with trypsin and the tryptic digests were analysed via MALDI-ToF. The mass spectra given by the MALDI-ToF were used to interrogate the nonredundant MIPS *Arabidopsis* database (http://mips.gsf.de/proj/thal/) using MS-Fit Protein Prospector database search program interacting with the Intelical programme (PE Biosystems, Boston, MA) in automated mode. This resulted in 144 spots giving a positive identification and proteins with MOWSE score of 10^2 and above. These spots represented 104 proteins that were selected for further characterisation. Some spots were identified as hypothetical proteins and some were proteins of known biological functions. The term "hypothetical protein" refers to peptide sequences which are generated by computer assisted theoretical translation of nucleotide sequences of *Arabidopsis* genes, sometimes these polypeptides are referred to as putative proteins, unknown proteins, or just a gene identification number.

In order to differentiate between proteins extracted from the cell walls with $CaCl_2$ and urea buffer, the spot numbers for urea buffer extracts are preceded by a letter u (e.g. spot u-9; u-79; u-145 and so on; Figure 4.7). As seen on figure 4.5 some of the $CaCl_2$ cell wall protein extracts are extremely basic and therefore could not be resolved properly with IEF range of pH 3-10. This prompted the use of a narrow basic IEF range, pH 6-11 and the protein spot numbers are preceded with a letter b (e.g. b-2; b-10; b-41 etc.) to differentiate these from the $CaCl_2$ pH 3-10 and urea buffer protein extracts.

Some matured proteins are made up of more than one domain (e.g. the glyceraldehyde 3-phosphate dehydrogenase) and some polypeptides undergo post-translational modifications, such as; glycosylation, phosphorylation and proteolysis. The multiple domain nature and posttranslational modifications can alter the isoelectric point (pI) and/or the molecular weight of the protein. Multiple protein spots in some instance were assigned to one protein for example, spot b-21, b-22 and b-23 have different pI's but they were all identified as glyceraldehyde 3-phosphate dehydrogenase C subunit Also spot 58 and 62 have different molecular weights and pI's, and yet they were both identified as stearoyl ACP desaturase (AT2g43710).

4.3.3 Improving the Resolution of *Arabidopsis* Cell Wall Proteins by the use of Overlapping pH Isoelectric Focusing (pH 3-10 and pH 6-11).

The extreme basic protein spots in the *Arabidopsis* $CaCl_2$ extracts resolved by pH 3-10 in the first dimension (isoelectric focusing) were vertically streaked (Figure 4.5). The vertical streaking is mainly due to protein precipitation at their isoelectric point (pI). The application of the additional pH 6-11 IEF range eliminated the vertical streaking of the extreme basic proteins seen in pH 3-10 IEF range SDS-PAGE (figure 4.6).

One of the sources of artefacts in protein identification via 2-D SDS PAGE and MALDI-ToF is the possibility of two or more proteins migrating to the same position in the gel because they have very close molecular weights and isoelectric points. This possibility is also minimised by applying narrow pH range IEF. The application of additional pH 6-11 IEF greatly improved the resolution in the basic end of the SDS PAGE (Figure 4.6). For example, spot 61 identified as a polygalacturonase

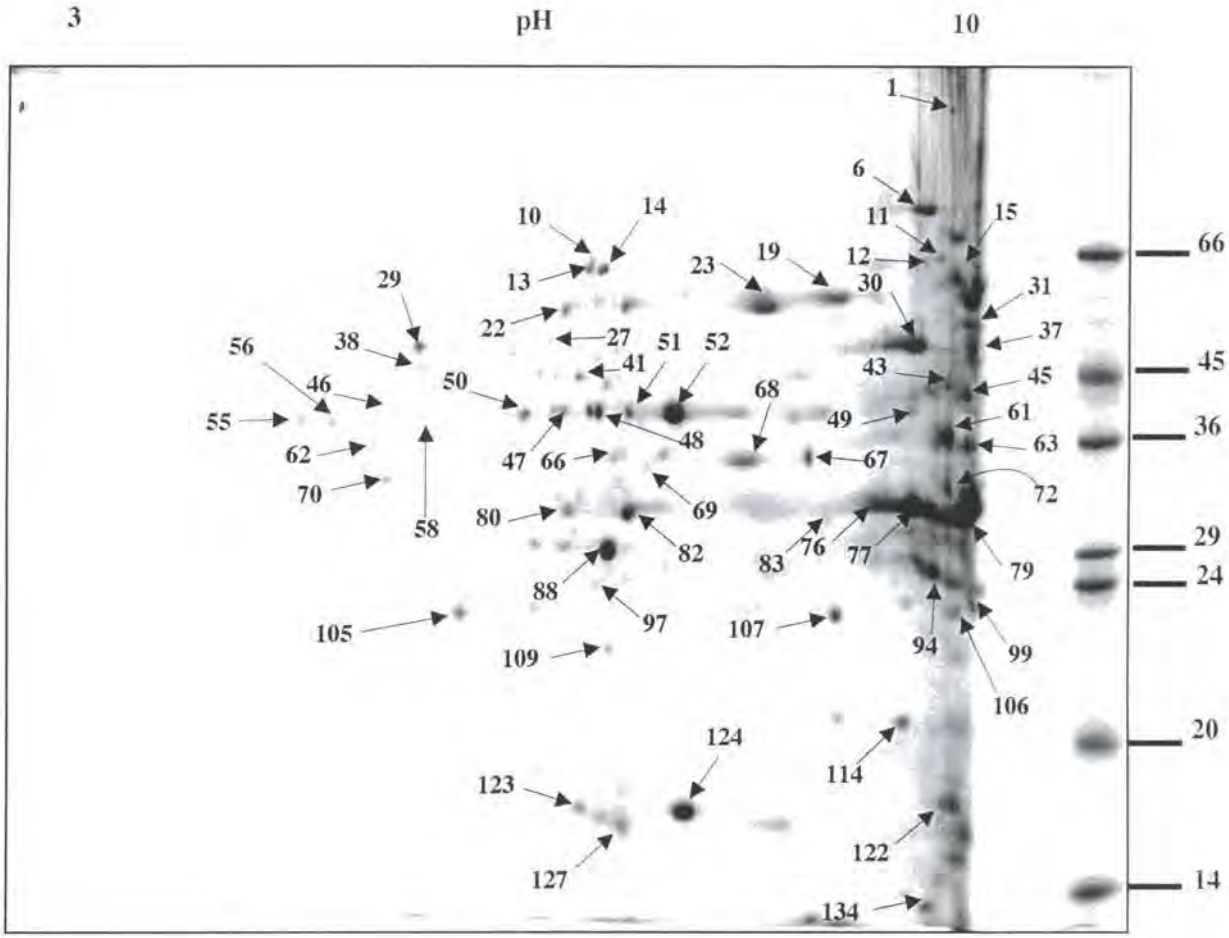


Figure 4.5. **Two dimension SDS Polyacrylamide gel electrophoresis profile of *Arabidopsis* cell wall proteins extracted using a $CaCl_2$ solution.**

Cell wall proteins were extracted from purified cell walls with a $CaCl_2$ solution. One milligram of the protein extract was loaded into the dry gel strip using the in-gel reswelling method. The proteins were resolved in the first dimension (horizontal) by isoelectric focusing (IEF) on a pH 3-10, 18 cm gel strip and the second dimension (vertical) was performed in a large format 12 % SDS-PAGE gel. The resolved protein spots were stained with Coomassie brilliant blue. The markers indicate the protein molecular weights in kilo Daltons (kDa).

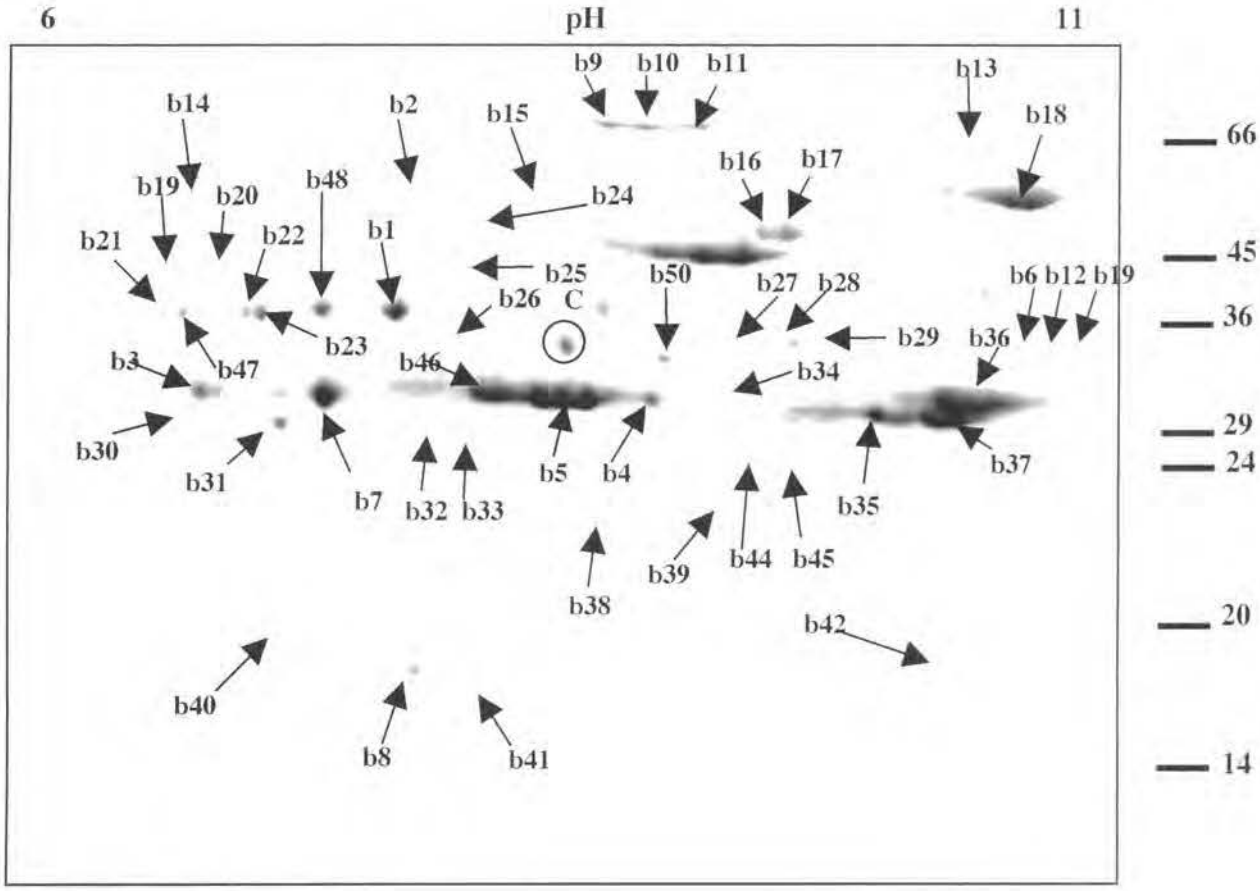


Figure 4.6. **Two dimension SDS-page separation of $CaCl_2$ extracted cell wall protein using pH 6-11 isoelectric focusing in the first dimension.**

Proteins were extracted with $CaCl_2$ from the purified cell walls of *Arabidopsis*. One milligram of the cell wall protein extracts was subjected to pH 6-11 first dimension (horizontal) isoelectric focusing (IEF), and large format 12 % SDS-PAGE for the second dimension (vertical) resolution. The resolved protein spots were visualised via Coomassie brilliant blue staining.

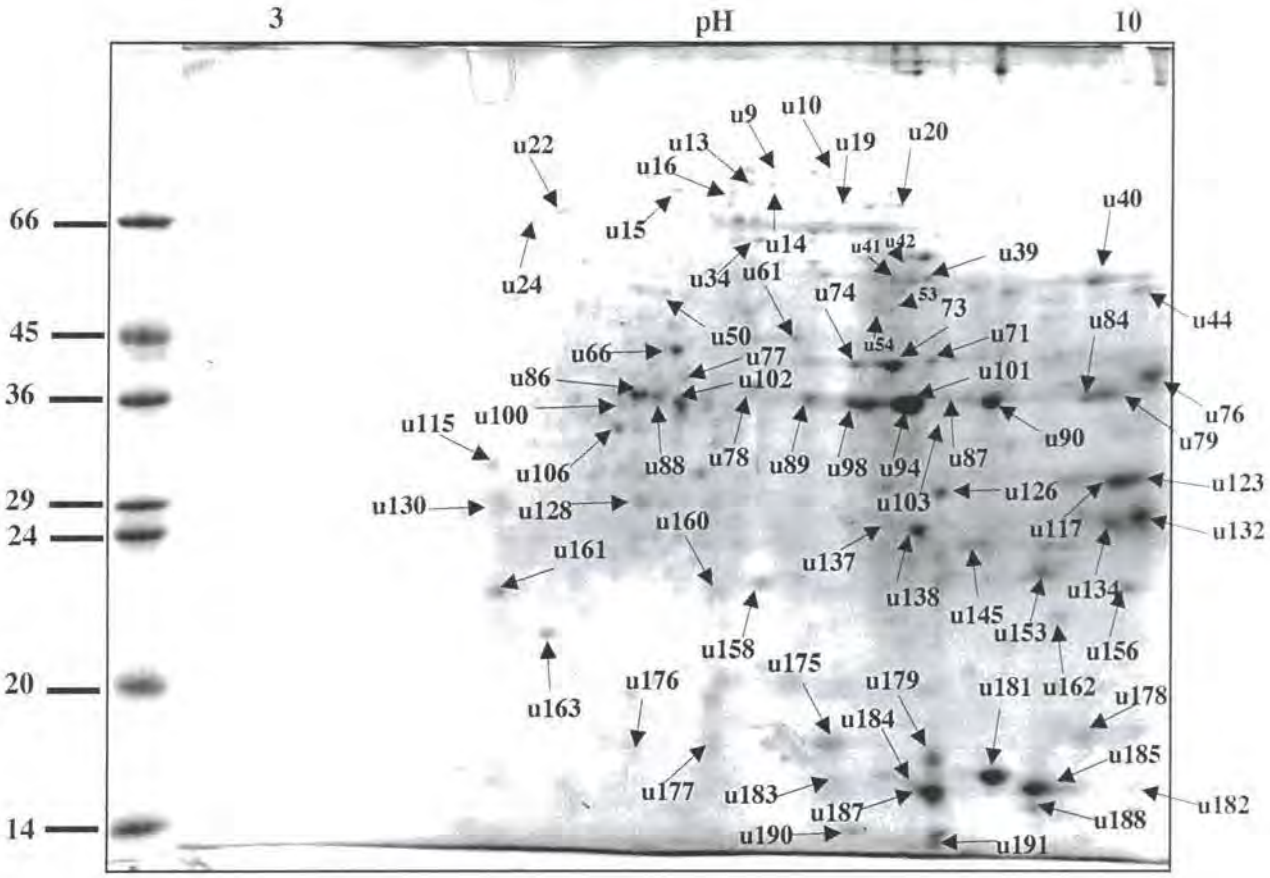


Figure 4.7. **Two dimension SDS Polyacrylamide Gel Electrophoresis profile of urea buffer extracted *Arabidopsis* cell wall proteins.**

Proteins were extracted with a urea buffer from *Arabidopsis* cell walls previously extracted with $CaCl_2$. One milligram of urea buffer protein extract was loaded into the dry gel strip using the in-gel reswelling method. The reswelled 18 cm pH 3-10 gel strips were subjected to isoelectric focusing (IEF) for the first dimension (horizontal) separation and the second dimension (vertical) was performed in a large format 12 % SDS-PAGE gel. The protein spots were visualised with Coomassie brilliant blue staining and the molecular weight markers indicate the estimated protein size in kilo Daltons (kDa).

inhibiting protein (AT5g06870) seemed to be one protein spot in pH 3-10 IEF. However, when resolved via pH 6-11 IEF this turned out to be made up of two spots b-28 and b-29 which were identified as a putative serine carboxypeptidase II (AT2g33530) and polygalacturonidase inhibiting protein (AT5g06870) respectively.

Using the narrower pH range (pH 6-11) IEF also revealed more proteins not seen when pH 3-10 IEF was applied. The recovered proteins include β-D-glucan exohydrolase-like protein (spot b-13; AT5g20950), a putative serine carboxypeptidase II (spot b-28; AT2g33530), a putative phi-l-like phosphate induced protein (Spot b-37; AT4g08950) and a disease resistance-like protein (spot b-18; AT5g23400) and more novel cell wall proteins (Table 4.1).

4.4 The cell wall proteomic analysis reveals both classical and unexpected cell wall proteins

4.4.1 Classical Cell Wall Proteins

As expected the cell wall protein extracts contain a variety of proteins whose cell wall localisation has been established (Table 4.1). These include a large collection of cell wall modifying enzymes like, polygalacturonases (spot 31), putative beta-glucanases (spot 12), xyloglucan endo-1,4-beta-D-glucanase precursor (spot 83), putative pectin esterase (spot 43) β-glucosidases (spot 19, b-15 and u-40), putative endoxyglucan glycosyltransferase (spot b-35), putative pollen-specific protein (spot 6), a polygalacturonase inhibiting protein (spot 61) and expansin (spot 99).

Table 4.1. List of identified *Arabidopsis thaliana* cell wall proteins.

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
6	Putative pollen-specific protein	AT4g12420	66/9.16	9.03E+04	+	0
10	Beta-1,3-glucanase-like protein	AT5g58480	51/7.19	1.03E+03	+	0
12	Putative beta-1,3-glucanase precursor	AT3g07320	49/8.84	3.42E+03	+	0
14	Phosphoenolpyruvate carboxykinase (ATP)-like protein	AT4g37870	73/6.61	1.56E+08	-	0
15	Putative pollen-specific protein	AT4g12420	66/9.16	3.99E+03	+	0
19	Putative beta-glucosidase	AT2g44450	57/7.56	7.58E+04	+	0
22	Hypothetical protein (Receptor-like ser/thre protein kinase)	AT1g78850	49/7.82	2.85E+05	+	0
23	Hypothetical protein (Receptor-like ser/thre protein kinase)	AT1g78850	49/7.82	2.85E+05	+	0
30	Hypothetical protein	AT1g21670	77/9.33	1.52E+03	+	0
27	Putative calreticulin	AT1g08450	48/5.61	8.12E+03	+	1
31	Putative polygalacturonidase	AT4g01890	49/7.87		+	0
37	Putative protein (Aspartyl protease; periplasmic space)	AT3g61820	51/8.75	8.36E+04	+	1

Table 4.1 continued

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
41	Putative GTP-binding protein	AT1g30580	44/6.35	4.13E+05	-	0
43	Putative pectinesterase	AT2g47050	24/9.16	1.88E+03	+	0
45	Unknown protein	AT2g41800	40/9.22	1.30E+08	+	0
46	Putative stearoyl ACP desaturase	AT3g02630	45/5.87	1.96E+06	-	0
47	Hypothetical protein (Glyceraldehyde-3-phosphate dehydrogenase)	AT1g13440	40/6.67	7.93E+04	-	0
48	Glyceraldehyde 3-P dehydrogenase C subunit	AT3g04120	37/6.67	7.93E+04	-	0
49	AAF68827.1 disease resistance Cf-2 like protein	AT5g06860	37/8.24	2.86E+04	+	0
50	Lactate dehydrogenase	AT4g17260	37/8.24	6.56E+05	-	0
51	Unknown protein	AT3g08030	39/7.17	1.43E+07	+	0
52	Unknown protein	AT3g08030	39/7.17	1.43E+07	+	0
53	Unknown protein	AT3g08030	49/7.82	1.43E+07	+	0

Table 4.1 continued

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
55	Hypothetical protein (Receptor-like ser/thre protein kinase)	AT1g78830	49/7.82	5.85E+04	+	0
56	Hypothetical protein (Receptor-like ser/thre protein kinase)	AT1g78830	49/7.82	5.85E+04	+	0
58	Stearoyl ACP desaturase	AT2g43710	46/6.05	1.96E+06	-	0
61	Polygalacturonidase inhibiting protein	AT5g06870	37/9.02	3.27E+04	+	0
62	Stearoyl ACP desaturase	AT2g43710	46/6.05	1.96E+06	-	0
63	Possible apospory-associated-like protein	AT4g25900	38/9.83	3.27E+04	+	0
67	Putative protein kinase	AT1g53070	30/8.49	1.14E+08	+	0
76	Putative protein (Putative expansin)	AT3g45970	29/8.28	1.25E+03	+	0
77	Putative protein (Putative expansin)	AT3g45970	29/8.28	1.25E+03	+	1
82	Putative protein (Ubiqutin-specific protease)	AT4g22410	40/9.19	3.28E+03	+	1
83	Xyloglucan endo-1,4-beta-D-glunanase precursor	AT4g30270	31/8.36	6.13E+03	+	0
88	Cytochrome-b5 reductase-like protein	AT5g20080	34/6.96		-	0

Table 4.1 continued

Spot no.	Protein ID		Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
94	Putative protein	(SPT7)	AT5g46550	57/9.38	2.27E+03	+	0
106	Putative protein	(Putative expansin)	AT3g45960	29/9.20	7.50E+04	+	0
114	AAC26009.1	(Calcineurin B-like protein)	AT5g55990	26/4.89	1.21E+04	+	0
122	Putative peroxiredoxin		AT3g26060	24/9.53	1.93E+03	-	0
b-1	Unknown protein		AT3g08030	39/7.17	4.41E+03	+	0
b-2	Hypothetical protein	(Receptor-like ser/thre protein kinase)	AT1g78850	49/7.82	2.57E+04	+	0
b-3	Hypothetical protein	(Receptor-like ser/thre protein kinase)	AT3g12500	36/7.81	2.55E+03	+	0
b-4	Putative phospholipid cytidyltransferase		AT2g38670	47/7.27	1.15E+03	+	1
b-5	Putative phospholipid cytidyltransferase		AT2g38670	47/7.27	1.15E+03	+	1
b-6	Possible apospory associated protein-like protein		AT4g25900	36/7.81	4.97E+06	+	0
b-9	Zwille/pinhead-like protein		AT5g21030	96/8.96	1.07E+03	-	0
b-10	Putative protein		AT3g56750	46/9.43	7.16E+02	+	1

Table 4.1 continued

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
b-11	Cytochrome P450-like protein	AT3g48310	56/7.68	1.84E+02	+	3
b-12	Possible apospory associated protein-like protein	AT4g25900	46/7.17	4.97E+06	+	0
b-13	β-D-glucan exohydrolase-like protein	AT5g20950	70/9.14	9.07E+04	+	0
b-14	Hypothetical protein (Receptor-like ser/thre protein kinase)	AT1g78850	49/7.82	6.47E+03	+	0
b-15	Putative beta-glucosidase	AT2g44450	57/7.56	5.77E+03	+	0
b-16	Unknown protein	AT1g03220	46/8.97	1.47E+04	+	0
b-17	Unknown protein	AT1g03220	46/8.97	1.47E+04	+	0
b-18	Disease resistance-like protein	AT5g23400	64/9.65	3.50E+03	+	0
b-19	Possible apospory associated protein-like protein	AT4g25900	36/7.17	4.97E+06	+	0
b-20	Putative GTP-binding protein	AT1g30580	44/6.35	1.29E+06	-	0
b-21	Glyceraldehyde 3-P dehydrogenase C subunit	AT3g04120	37/6.62	2.74E+04	-	0
b-22	Glyceraldehyde 3-P dehydrogenase C subunit	AT3g04120	37/6.62	2.74E+04	-	0

Table 4.1 continued

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
b-23	Glyceraldehyde 3-P dehydrogenase C subunit	AT3g04120	37/6.62	2.74E+04	-	0
b-24	Hypothetical protein	AT1g23210	55/7.64	2.68E+03	+	0
b-25	Hypothetical protein	AT3g04890	17/4.94	5.26E+03	-	0
b-26	Hypothetical protein	AT1g53070	30/8.49	1.28E+03	+	0
b-27	Putative exportin, tRNA	AT1g72560	111/5.48	4.05E+02	-	0
b-28	Putative serine carboxypeptidase II	AT2g33530	51/8.66	2.15E+03	+	0
b-29	Polygalacturonase inhibiting protein	AT5g06870	46/6.05	6.49E+07	+	0
b-30	Putative protein	AT5g10090	65/9.08	9.00E+02	-	0
b-31	Putative protein	AT5g10090	65/9.08	9.00E+02	-	0
b-32	Putative exportin, tRNA	AT1g72560	111/5.48	4.05E+02	-	0
b-33	Hypothetical protein	AT1g28530	44/6.35	1.55E+03	-	1
b-35	Putative endoxyloglucan glycosyltransferase	AT2g06850	NG	1.67E+09	+	0

Table 4.1 continued

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
b-36	Putative protein	AT4g45960	29/9.20	2.24E+03	+	0
b-37	Putative phi-1-like phosphate induced protein	AT4g08950	34/9.39	1.17E+04	+	0
b-38	Putative protein kinase	AT2g05940	52/9.47	7.97E+02	+	0
b-41	Hypothetical protein	AT1g66960	88/6.37	8.99E+02	-	?
b-42	Hypothetical protein	AT1g32900	67/8.76	9.96E+01	+	0
b-44	Hypothetical protein	AT2g41800	40/9.22	3.67E+05	+	0
b-45	Hypothetical protein	AT2g41800	40/9.22	3.67E+05	+	0
b-46	Putative protein	AT3g45970	29/8.28	9.44E+03	+	1
b-47	Hypothetical protein	AT1g13440	37/6.67	2.74E+04	-	0
b-48	Unknown protein	AT3g08030	39/7.97	4.41E+03	+	0
b-50	Hypothetical protein (serine carboxypeptidase)	AT1g28110	51/6.86	6.10E+03	+	0
u-9	Elongation factor EF-2	AT1g56070	95/5.83	1.06E+05	-	0

Table 4.1 continued

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
u-10	Elongation factor EF-2	AT1g56070	95/5.83	1.06E+05	-	0
u-13	Elongation factor EF-2	AT1g56070	95/5.83	1.06E+05	-	0
u-14	Putative9-cis-epoxycarotenoid dioxygenase	AT3g14440	66/5.90		-	0
u-15	Phospholipase D	AT3g15730	92/5.53	2.68E+03	-	0
u-16	Unknown protein (Receptor-like ser/thre protein kinase)	AT1g61500	90/6.06	1.07E+03	+	1
u-19	Putative ubiquitin-like protein	AT2g17190	57/4.96		-	0
u-20	Glutamyl-tRNA synthetase	AT5g26710	81/6.61	1.26E+04	+	0
u-22	Luminal binding protein	AT5g28540	74/5.08	2.29E+07	+	0
u-34	Aspartate-tRNA ligase-like protein	AT4g31180	63/5.91	5.01E+07	-	0
u-39	Phosphoenolpyruvate carboxykinase (ATP)-like protein	AT4g37870	73/6.61	1.04E+07	-	0
u-40	Putative beta-glucosidase	AT2g44450	57/7.56	2.21E+05	+	0
u-41	Putative purple acid phosphatase precursor	AT2g16430	54/7.30	3.84E+06	+	1

Table 4.1 continued

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
u-42	Putative purple acid phosphatase precursor	AT2g16430	54/7.30	3.84E+06	+	1
u-48	Unknown protein	AT1g67880	46/7.95	7.40E+04	+[#]	1
u-50	Enolase (2-phospho-D-glycerate hydrolase)	AT2g36530	48/5.54	4.04E+04	-	0
u-53	Purple acid phosphatase-like protein	AT2g16430	54/7.30	3.84E+06	+	1
u-54	Purple acid phosphatase-like protein	AT2g16430	54/7.30	3.84E+06	+	1
u-61	Citrate synthase	AT2g44350	53/6.41	8.55E+03	-	0
u-66	Putative tryptophenyl-tRNA synthetase	AT3g04600	46/5.67	9.96E+03	-	0
u-71	Putative GTP-binding protein	AT1g30580	44/6.35	1.84E+07	-	0
u-73	Putative GTP-binding protein	AT1g30580	44/6.35	1.84E+07	-	0
u-74	Putative GTP-binding protein	AT1g30580	44/6.35	1.84E+07	-	0
u-76	Peroxidase C2 precursor-like protein	AT4g08770	38/7.55	9.92E+03	+	0
u-77	Putative GTP-binding protein	AT1g30580	44/6.35	1.84E+07	-	0

Table 4.1 continued

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/Pi	MOWSE score	Signal-P[y]	TM[z]
u-78	Putative GTP-binding protein	AT1g30580	44/6.35	1.84E+07	-	0
u-79	Peroxidase ATP4a	AT1g71695	NG	8.75E+03	+	0
u-84	Peroxidase ATP4a	AT1g71695	NG	8.75E+03	+	0
u-86	Putative ACP desaturase	AT1g43800	44/6.10		+	0
u-88	Putative ACP desaturase	AT2g43710, AT1g43800	44/6.10		+	0
u-87	Unknown protein	AT3g08030	39/7.17	6.47E+04	+	0
u-89	Lactate dehydrogenase (LDH1)	AT4g17260	38/6.07	6.98E+08	-	0
u-90	Unknown protein	AT3g08030	39/7.17	6.47E+04	+	0
u-94	Glyceraldehyde-3-phosphate dehydrogenase C subunit (GAPC	AT3g04120	37/6.62	2.74E+04	-	0
u-95	Glyceraldehyde-3-phosphate dehydrogenase C subunit (GAPC	AT3g04120	37/6.62	2.74E+04	-	0

Table 4.1 continued

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
u-98	Glyceraldehyde-3-phosphate dehydrogenase C subunit (GAPC	AT3g04120	37/6.62	2.74E+04	-	0
u-100	Chloride channel-like protein	AT5g33280	84/8.84	2.03E+03	-	9
u-101	Glyceraldehyde-3-phosphate dehydrogenase C subunit (GAPC	AT3g04120	37/6.62	2.74E+04	-	0
u-102	steroyl ACP desarturase	AT2g43710	46/6.05		-	0
u-103	Latex abundant-like protein	AT5g04200	36/5.81	1.99E+03	-	0
u-106	steroyl ACP desarturase	AT2g43710	46/6.05		-	0
u-117	Anthranilate phosphoribotransferase-like protein	AT3g57880	89/9.11	3.47E+03	-	2
u-123	Putative protein	AT3g45970	29/8.28	2.10E+03	+	2
u-128	steroyl ACP desarturase	AT2g43710	46/6.05		-	0
u-130	Putative protein	AT5g40290	52/5.68	1.30E+03	-	0

Table 4.1 continued

Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
u-134	Putative 3-oxoacyl [acyl-carrier protein] reductase	AT1g24360	32/8.99	3.26E+04	-	0
u-137	ARR1 protein, putative, 5' partial	AT3g16857	62/6.36	1.04E+03	-	0
u-138	Cytochrome-b5 reductase-like protein	AT5g20080	34/6.96	1.87E+07	-	0
u-145	Glyceraldehyde-3-phosphate dehydrogenase C subunit (GAPC	AT3g04120	37/6.67	2.74E+04	-	0
u-153	Putative protein	AT3g57400	53/9.70	5.28E+03	-	1
u-160	Putative protein	AT5g06450	23/4.92	1.36E+06	-	0
u-162	Unknown protein	AT3g11770	23/5.26	1.57E+03	-	0
u-175	ABC transporter-like protein	AT3g52310	82/9.12	1.46E+03	-	4
u-178	Hypothetical protein	AT1g07030	36/8.15	3.34E+03	-	0
u-182	Putative protein	AT3g57400	53/9.70		-	0
u-183	Unknown protein	AT1g69290	94/6.92	1.55E+03	+	0

Table 4.1 continued

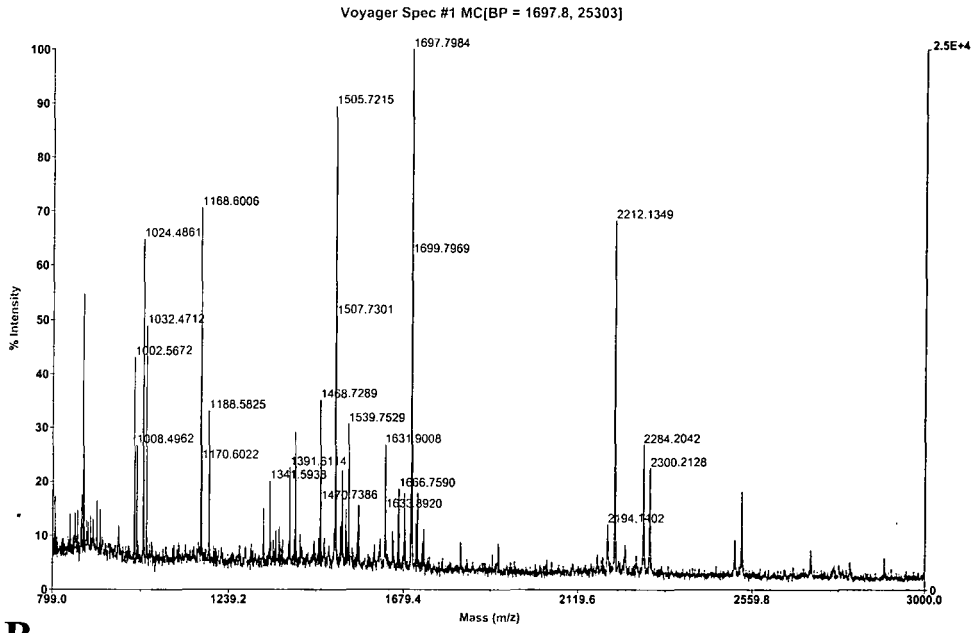
Spot no.	Protein ID	Accesssion No.[w]	MW[x]/pI	MOWSE score	Signal-P[y]	TM[z]
u-184	Hypothetical protein	AT3g32350	48/8.44	1.54E+03	-	0
u-185	Hypothetical protein	AT1g52960	106/8.64	5.57E+04	-	0
u-188	Hypothetical protein	AT1g52960	106/8.64	5.57E+04	-	0
u-190	Putative protein	AT3g57400	53/9.70	5.28E+03	-	0
u-192	Hypothetical protein	AT1g52960	106/8.64	5.57E+04	-	0

[w]Accesion number in the MIPS database
[x]Molecular weight in kilodaltons (kDA)
[y]Result of Signal-P analysis. The symbol (+ and -) indicates presence or the absence of a signal peptide respectively
[z]TM- depicts the number of transmebrane domains predicted from the protein sequence
[#] Signal anchor.
NG = Not given in the MIPS database.

Also, other cell wall localised enzymes like a putative peroxidase (spots u-79, and u-84) and purple acid phosphatase-like protein (spots u-53 and u-54) have been identified in this study. An example of a MALDI-ToF spectrum and MALDI-ToF peptide coverage of one of the identified classical cell wall enzymes (putative endoxyglucan glycosyltransferase) is shown in Figure 4.8.

Major cell wall proteins mainly involved in cell wall extension (cell wall loosening) and cell protection against pathogens and other mechanical stresses were also identified. For example, polygalacturonases are involved in cell wall polygalacturonic acid hydrolysis, which lead to the cell wall loosening (Sitrit et al., 1999). Glucanases, glucosidases and glucosyltransferases are believed to be involved in the hydrolysis of major cell wall structures like glucans (see general introduction for details). Expansins are one of the newest class of cell wall modifying enzymes. These enzymes were first isolated in 1992 and were shown to act as mediators of "acid growth" which refers to the increase in growth rate when plant cells are grown in an acidic medium (Reviewed in Cosgrove. 1998). Nothing much is known about the biochemical activities of expansins, however some evidence suggest that they may be involved in the weakening of glucan-glucan bonds, thus contributing to cell wall loosening (Cosgrove. 2000). Polygalacturonase inhibiting proteins, pectin methylesterases and peroxidases alter the wall components so as to make it resistant to cell wall degrading enzymes. For example, polygalacturonase-inhibiting proteins binds and inhibit polygalacturonidases, enzymes that are involved in plant cell wall degradation (Bergmann et al., 1994). Finally, cell wall peroxidases are believed to be involved in the production of huge amounts of reactive oxygen species (ROS), and the ROS have

A.



B.

MTVSSSPWALMALFLMVSSTMVMAIPPRKAIDVPFGRNYVPTWA
FDHQKQFNGGSELQLILDKYTGTGFQSKGSYLFGHFSMHIKLPA
GDTAGVVTAFYLSSTNNEHDEIDFEFLGNRTGQPAILQTNVFTGG
KGNREQRIYLWFDPSKAYHTYSILWNMYQIVFFVDNIPIRTFKNAK
DLGVRFPFNQPMKLYSSLWNADDWATRGGLEKTNWANAPFV
ASYKGFHIDGCQASVEAKYCATQGRMWWDQKEFRDLDAEQWR
RLKWVRMKWTIYNYCTDR TRFPVMPAECKRDRDA

Figure 4.8 **A classical cell wall protein identified via MALDI-ToF.**

(**A**) A MALDI-ToF peptide ion spectrum of (spot b-35, Figure 4.6) identified a putative endoxyglucan glycosyltransferase (AT2g06850) giving a MOWSE score (1.67×10^9) in the *Arabidopsis* database. (**B**) The MALDI-ToF peptide coverage on the identified polypeptide is highlighted (bold). The predicted signal peptide for secretion is also highlighted (bold and italic).

been shown to catalyse cell wall protein cross-linking as an early defence strategy (Reviewed by Bolwell et al., 1999; Wojtaszek et al., 1997).

4.4.2 Identification of Novel Cell Wall Proteins

Following the completion of the *Arabidopsis* genome sequencing, a large number of hypothetical proteins have been generated using computer assisted theoretical translation of DNA open reading frames. The hypothetical proteins are just theoretical polypeptides with no information of whether they are expressed or not. Among the 104 proteins identified in this study 52 proteins were identified as hypothetical, putative or unknown proteins (Table 4.1). This was the first evidence that these hypothetical proteins were expressed. Additionally, these hypothetical proteins are shown to be located to the cell wall, a first step towards understanding their possible functions. Almost all of these new proteins have an N-terminal signal peptide, a tag for protein secretion. Additionally, following a series of basic local alignment search tool (BLAST) searches for homologous sequences in the Munich Information Center for Protein Sequences (MIPS) and National Center for Biotechnology Information (NCBI) protein databases it was discovered that some of the hypothetical proteins identified here share significant homologies to proteins known to be associated with the cell wall. These include spot 76, 106 and 77, which have 86 % identity to pollen allergens, a subgroup of the beta-expansin family of cell wall proteins. Spot b-50 is 84 % homologous to a putative serine carboxypeptidase, a plant cell surface receptor for brassinosteroids. Spot b-24 is 85 % homologous to an endo-1,4-β-glucanase a classical cell wall protein.

4.4.3 Proteins Not Expected To Be Localised In the Cell Wall

Some proteins identified here however, have never before been known to be associated with plant cell walls. These include some fatty acid metabolism associated proteins like steroyl ACP desaturase (spot 58, 62, u-86, u-88, u-102 u-106 and u-128), putative phospholipid cytidyltransferase (b-4 and b-5) and phospholipase D (spot u-15), some glycolysis related proteins like putative glyceraldehydes-3-phosphate dehydrogenase (spot b-21, 48, b-21, b-22, b-23, b-47, b- u89, u-101, and u-145) and citrate synthase-like protein (spot u-61). Proteins that are traditionally associated with nucleic acids, including a glutamyl-tRNA synthetase (spot u-20), a putative aspartate-tRNA ligase (spot u-34) were also identified in the cell wall protein preparations. There are also other proteins that share some homology to enzymes that are involved in various metabolic pathways known to be located only in the cytosol and other organelles (Table 4.1). Some of these peculiar proteins were found to possess an N-terminal signal peptide for secretion (Table 4.1), thus confirming the results of this study. In some instance another signal peptide possessing isoform of the identified protein was found in the genome databases (Table 4.2). Additionally, some of these proteins have been shown (via immunolocalisation) to reside in the extracellular matrix of other organism (see the discussion for details). The possible alternative functions of some of these proteins are also explained in the discussion section.

An example of a protein, which has both the secreted and a non-secreted isoform, is demonstrated in Figure 4.9 and 4.10. A tryptic digest MALDI-ToF spectrum generated from spot u-22 identified a putative luminal binding protein (BiP; AT5g28540). As expected the polypeptide of this protein possessed a signal peptide

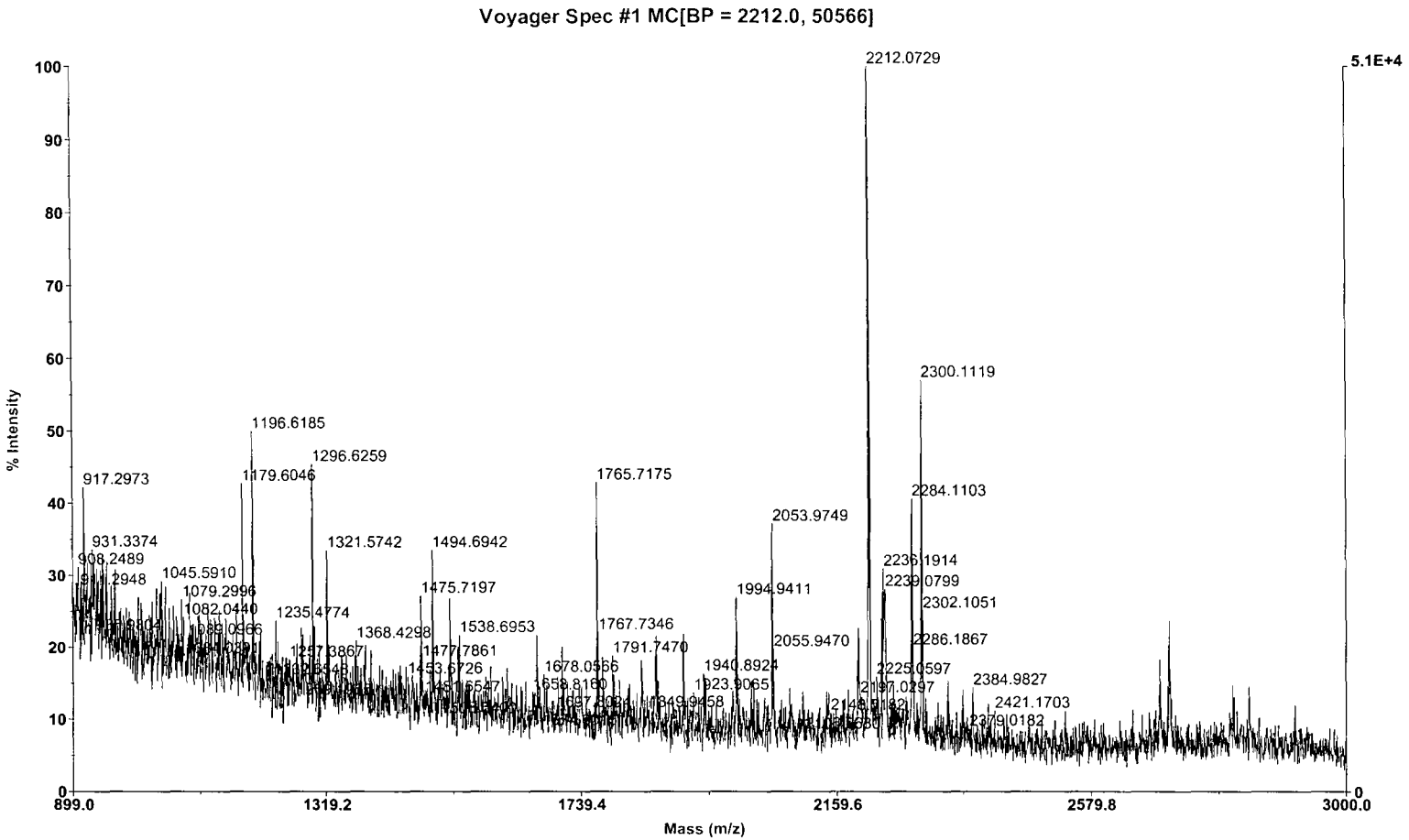


Figure 4.9 **MALDI-ToF peptide ion spectrum of a tryptic digest of spot u-22.**

The MALDI-ToF spectrum generated from the tryptic digests of spot u-22 was used to interrogate the *Arabidopsis* database. Fifteen of these peptide ions matched with 15 out of 205 theoretical tryptic digests generated from a putative luminal binding protein AT5g28540, giving a MOWSE score of 2.29E+07.

A.

AT5g28540

MARSFGANSTVVLAIIFFGCLFALSSAIEEATKLGSVIGIDLGTTYSCVGVYKN
GHVEIIANDQGNRITPSWVGFTDSERLIGEAAKNQAAVNPERTVFDVKRLIG
RKFEDKEVQKDRKLVPYQIVNKDGKPYIQVKIKDGETK**VFSPEEISAMILTK**
MKETAEAYLGKK**IKDAVVTVPAYFNDAQRQATKDAGVIAGLNVARI**INEPT
AAAIAYGLDKKGGEKNILVFDLGGGTFDVSVLTIDNGVFEVLSTNGDTHLGGE
DFDHR**VMEYFIKLIK**KKHQKDISKDNKALGKLRRECERAKRALSSQHQVR**V**
EIESLFDGVDFSEPLTRARFEELNNDLFRKTMGPVKKAMDDAGLQKSQIDEI
VLVGGSTRIPKVQQLLKDFFEGKEPNKGVNPDEAVAYGAAVQGGILSGEGGD
ETKDILLLDVAPLTLGIETVGGVMTKLIPRNTVIPTKKSQVFTTYQDQQTTVSI
QVFEGERSLTKDCR**LLGKFDLNGIPPAPR**GTPQIEVTFEVDANGILNVKAEDK
ASGKSEKITITNEKGRLSQEEIDRMVKEAEEFAEEDKKVKEKIDAR**NALETYV**
YNMKNQVNDKDKLADKLEGDEKEKIEAATKEALEWLDENQNSEKEEYDEK
LKEVEAVCNPIITAVYQRSGGAP GGAGGESSTEEEDES***HDEL***

B.

AT1g09080

MIFIKENTAKMTRNKAIACLVFLTVLDFLMNIGAALMSSLAIEGEEQKLGTVIG
IDLGTTYSCVGVYHNKHVEII**ANDQGNRITPSWVAFTDTE**RLIGEAAKNQAA
KNPERTIFDPKRLIGRKFDDPDVQRDIKFLPYKVVNKDGKPYIQVKVKGEE**KL**
FSPEEISAMILTKMKETAEAFLGKK**IKDAVITVPAYFNDAQRQATKDAGAI**
AGLNVVRIINEPTGAAIAYGLDKKGGESNILVYDLGGGTFDVSILTIDNGVFEV
LSTSGDTHLGGEDFDHR**VMDYFIKLVK**KKYNKDISKDHKALGKLRRECELA
KRSLSNQHQVR**VEIESLFDGVDFSEPLTRARFEELNMDLFKK**TMEPVKKAL
KDAGLKKSDIDEIVLVGGSTRIPKVQQMLKDFFDGKEPSKGTNPDEAVAYGA
AVQGGVLSGEGGEETQNILLLDVAPLSLGIETVGGVMTNIIPRNTVIPTKKSQV
FTTYQDQQTTVTINVYEGERSMTDNR**ELGKFDLTGILPAPR**GVPQIEVTFEV
DANGILQVKAEDKVAKTSQSITITNDKGRLTEEEIEEMIREAEEFAEEDKIMKE
KIDAR**NKLETYVYNM**KSTVADKEKLAKKISDEDKEKMEGVLKEALEWLEEN
VNAEKEDYDEKLKEVELVCDPVIKSVYEKTEGENEDTMEM ITMSYRIV

Figure 4.10 **The peptide coverage on the MALDI-ToF identified luminal binding protein.**

(A) The amino acid sequence of the MALDI-ToF identified luminal binding protein (AT5g28540), and (B) the sequence of another luminal binding protein isoform (AT1g09080) found in the *Arabidopsis* genome sequence database. The signal peptide for secretion (Bold italics), the MALDI-ToF peptide coverage (Bold) and the retention tetrapeptide (Bold, italic and underlined) are highlighted.

Table 4.2. List of protein isoforms predicted to have a cleavable N-terminal signal sequence.

Protein name	Accession no.[w]	MW[x]/pI	Signal-P[y]	TM[z]
Stearoyl ACP desaturase	AT1g43800	44/6.10	+	0
Citrate synthase-like protein	AT3g58750	57/8.73	+	0
NADH cytochrome-b5 reductase	AT5g17770	31/8.75	+	1
Phospholipase D	AT2g42010	93/8.47	+	0
Putative aspartate-tRNA ligase	AT4g33760	71/6.73	+	0
Putative GTP-binding protein	AT1g56050	45/5.61	+	0
Putative glyceradehyde-3-phosphate dehydrogenase	AT1g16300	45/8.73	+	0
Putative ubiquitin	AT5g42220	95/5.14	+	0
beta-ketoacyl-ACP reductase - like protein	At3g55310	32/7.52	+	0

[w]Accesion number in the MIPS database

[x]Molecular weight in kilodaltons (kDA)

[y]Result of Signal-P analysis. The symbol (+) indicate presence of a signal sequence

[z]TM -depicts the number of transmebrane domains predicted from the protein sequence

sequence in its N- terminus, which targets it to the secretory pathway (see Figure 4.10). The MALDI-ToF spectrum of the BiP tryptic digests is shown in Figure 4.9. However the identified polypeptide also had a C-terminus endoplasmic reticulum lumen retention tetrapeptide (HDEL; see figure 4.10). In this case, the cell wall localisation was not expected (see introduction). Further analysis of the *Arabidopsis* database revealed another copy of the luminal binding protein gene (AT1g09080) in the *Arabidopsis* genome with 78 % homology to the one identified in this study (AT5g28540). This homologue, however, contained no HDEL or KDEL endoplasmic reticulum lumen retention tetrapeptides (see Figure 4.10). The MALDI-ToF generated peptides sequences that are identical between the two polypeptides are underlined in Figure 4.10B showing that both polypeptides share a similar tryptic peptide coverage. This example therefore highlights one of the shortcomings of using MALDI-ToF mass spectrometry for protein identification, in that, often it is unable to distinguish the difference between closely related isoforms of the same protein. In such cases tandem mass spectrometry (MS-MS), which is able to generate the amino acid sequences of individual peptides, is often applied (see general introduction). This evidence, therefore, strongly argues that the luminal binding protein is also secreted to the cell wall.

4.4.4 The Signal Transduction Associated Proteins.

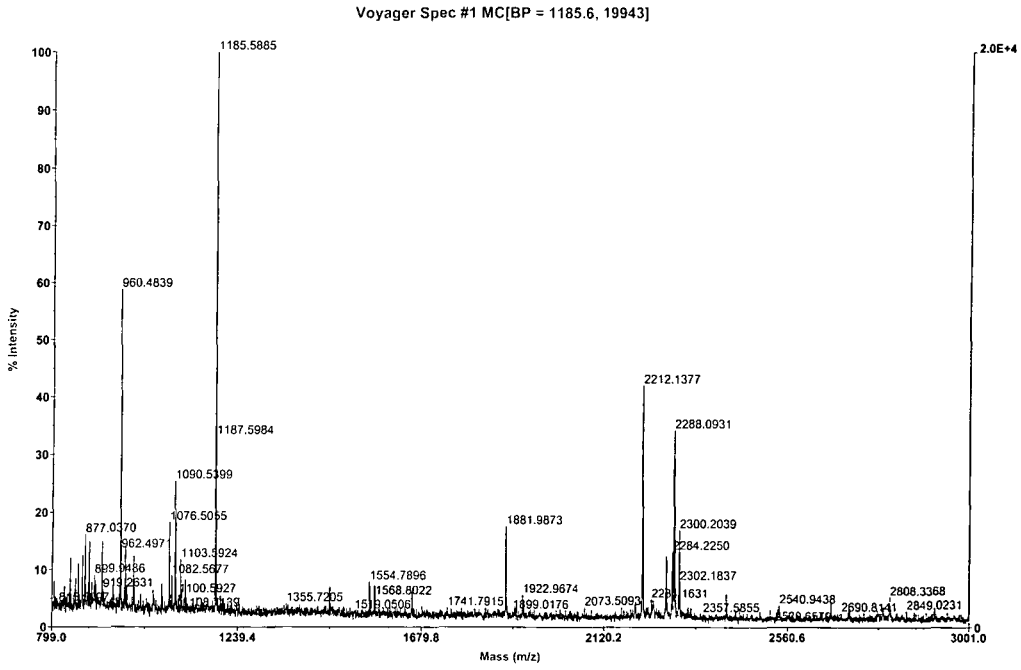
Among the proteins identified in this study, there were various proteins sharing significant homology to signal transduction related proteins. These included 8 novel receptor-like serine threonine kinases (e.g. spot 22, 23, 55, 67, b-2, b-3, b-14 and u-16). A putative calcineurin B-like protein (spot 114) and a calreticulin (spot 27) were

also spotted. Calcineurin-B like proteins and careticulins are both known to be involved in Ca^{2+} signalling (Kudla et al., 1999). All these proteins possessed a signal peptide for secretion, some with no transmembrane domains (Table 4.1). The extracellular localisation of calreticulin is not unprecedented. Although it is traditionally associated with endoplasmic reticulum, it has been localised on the surface of many mammalian cell types, including human peripheral blood T lymphocytes (Arosa et al., 1999), human fibroblasts (Gray et al., 1995), and human T-cells (Basu et al., 1999). The data presented in this section strongly suggested that these signalling associated proteins reside in the extracytoplasmic matrix and are strongly associated with the *Arabidopsis* cell wall. At this stage however, the concept of extracellular phosphorylation and signal transduction in plants was unprecedented, and therefore further investigation was necessary. Chapter 5 is an attempt to investigate this issue in more detail. As an example, a MALDI-ToF spectrum and the peptide coverage of one of the identified putative extracellular protain kinases with no transmebrane domain is shown in Figure 4.11.

4.4.5 Cell Wall Bound Transmembrane Proteins

Some of the proteins identified in this study possessed a predicted transmembrane domain(s) (protein spots: 27, 37, 82, b-5, b-11, b-35, u-41, u-16, u-41, u-123; Table 4.1). Although most of these are new (hypothetical) proteins, some have recognised putative functions, including a putative purple acid phosphatase precursor, a putative phospholipid cytidyltransferase and a NADH cytochrome-b5 reductase (Table 4.1).

A.



B.

MKFSITLALCFTLSIFLIGSQAKVPVDDQFR**VVNEGGYTDYSPIEYNPD**
VRGFVPFSDNFRLCFYNTTPNAYTLALRIGNRVQESTLR**WVWEANR**
GSPVKENATLTFGEDGNLVLAEADGRLVWQTNTANKGAVGIKILENGN
MVIYDSSGK**FVWQSFDSPTDTLLVGQSLK**LNGRTKLVSRLSPSVNTNG
PYSLVMEAKKLVLYYTTN**KTPKPIAYFEYEFFTK**ITQFQSMTFQAVED
SDTTWGLVMEGVDSGSKFNVSTFLSRPKHNATLSFIRLESDGNIRVWSY
STLATSTAWDVTYTAFTNADTDGNDECRIPEHCLGFGLCKKGQCNACP
SDKGLLGWDETCKSPSLASCDPKTFHYFKIEGADSFMTKYNGGSSTTES
ACGDKCTRDCKCLGFFYNRKSSRCWLGYELKTLTRTGDSSLVAYVKAP
NANKKST L

Figure 4.11 **MALDI-ToF peptide ion spectrum of a putative ectokinase, spot 22.**

(A) The MALDI-ToF peptide ion spectrum generated from tryptic digests of spot 22 was used to interrogate the *Arabidopsis* database. The experimental tryptic digests masses matched the theoretical tryptic digests generated from a putative protein kinase, AT1g78850. (B) The identified polypeptide is shown. The signal peptide for extracellular secretion (Bold italics) and the peptide mass coverage (Bold) are also highlighted. The TMHMM transmembrane predicting program showed no possible transmembrane domain in this polypeptide.

Also an unknown protein that shares significant homology to receptor-like serine/threonine kinases had a transmembrane domain. The discovery of cell wall bound proteins possessing a transmembrane and a cytosolic domain is not unprecedented. The first case was reported about 5 years ago (He et al., 1996). He et al., (1996) reported a kinase that has an N-terminal sequence that is tightly bound to the cell wall and also possesses a domain that spans the plasma membrane to have a cytosolic catalytic C-terminal sequence. Following the completion of the *Arabidopsis* genome sequencing, it is estimated that there are 600 genes coding for receptor-like kinases in the *Arabidopsis* genome. This figure represents approximately 2.5 % of the entire *Arabidopsis* gene pool and thus shows that there could be many more cell wall associated transmembrane proteins in plants. Proteins that are bound to the cell wall and the plasma membrane are thought to play a significant role in both structural linkage between the two compartments and provide a signalling connection between the extracellular matrix and the cytoplasm (see the discussion for details).

4.5 Discussion and Conclusions

4.5.1 The Purity of the Cell Wall Preparations.

Preparation of cell walls of highest purity was the primary prerequisite for the analysis of the proteomics of this compartment. The cell walls purified using the method described above were therefore subjected to rigorous tests to assess the presence of contamination. The contaminating elements were likely to come from three main sources, the plasma membrane, cytosolic organelles and proteins of cytoplasmic origin. In order to eliminate the possibility of intact cells co-sedimenting with the cell walls in the glycerol purification column, a powerful cell disrupter (French press) that forces the cell material through an orifice with a diameter smaller than the cells was employed. The cell material was passed through the cell disrupter at least twice to ensure that no cell escapes the disruption. Chlorophyll, the most abundant pigment in plant cells remained at the top of the glycerol column while the cell wall material sedimented to the bottom. Electron microscopy was used to investigate whether any organelles were present in the purified cell wall material. There was no evidence of the presence of any organelle or plasma membranes in the purified cell wall material. Electron microscopy might be viewed as less sensitive for the detection of plasma membranes, therefore, immunological screening for plasma membrane-bound glycolipids was applied. This test failed to detect any glycolipids (one of the most abundant plasma membrane lipids) epitopes in the cell wall preparations therefore ruling out the presence of cell membrane contamination. The final test was to evaluate the possibility that some proteins of cytosolic origin might be non-specifically bound to the purified cell wall material and that the washing steps

could have failed to remove. For this western blotting was applied using antibodies raised against enoyl-ACP reductase (ENR) a protein that is abundant in the disrupted cells. The choice of using ENR was deliberate since the *Arabidopsis* database showed that this protein has a single copy, and it lacked the signal peptide leader sequence for secretion. This test showed that the ENR was absent in the proteins extracted from the purified cell walls. These results are also complemented by the results of Corke and Roberts (1997) who showed that the mung bean cell walls obtained using a similar method were free of a cytosolic abundant actin. In addition, another independent group working with rice coleoptile cell walls prepared using a similar method, showed by activity assay, that their cell wall preparations were not contaminated by alcohol dehydrogenase (Atwell and apRees, 1986).

4.5.2 Identification of Novel Cell Wall Proteins

Among the classical cell wall proteins identified in this study, a number of novel proteins (hypothetical proteins) have also been discovered. The existence of these proteins had been predicted from the *Arabidopsis* genome sequence project and up to now there had been no proof whether these genes are expressed. All "classical" cell wall proteins or proteins destined for the extracellular matrix possess a unique N-terminus leader sequence that targets them toward the endoplasmic reticulum for post-translational modification, folding and secretion (Vitale and Denecke, 1999). No less than 36 out of 52 novel proteins identified in this study possessed a cleavable signal peptide in agreement with their cell wall localisation. Some novel proteins however, were found to lack the predicted signal peptide. Several of these shared high homology to proteins that have been localised to the cell wall (e.g. spot 47 is

homologous to glyceraldehyde-3-phosphate dehydrogenase). Although glyceraldehyde-3-phosphate dehydrogenase has been localised to the cell wall (Gozalbo et al., 1998; also see section 4.6.7 below) its sequence does not have the signal peptide and its established function has never been reported to take place in the extracellular matrix. The possible explanation for this unexpected localisation is discussed below (4.5.4).

4.5.3 Cell Wall-Plasma membrane Continuum

A number of cell wall proteins possessing membrane-spanning domain(s) have been identified in this study (Table 4.1). Such proteins are thought to play a major role in both physical and signal transduction connections between that cell wall and the plasma membrane (Anderson et al., 2001; He et al., 1996). For example, wall associated kinases (Waks) have a tight connection with both the cell wall and the plasma membrane, thus providing a structural connection between the two organelles (He et al., 1996). Waks also have a receptor-like extracellular N-terminal sequence, therefore they have a potential to play some role in signal transduction (Anderson et al., 2001, He et al., 1999; Kohorn et al., 2000). Waks are not the only class of known cell wall bound transmembrane proteins, others exist, such as arabinogalactans that are reversibly attached to the plasma membrane via glycosyl phosphadyl inositol anchors (Oxley and Basic, 1999), putative endo-1,4–β-D-glucanases (Nicol et al., 1998) and cellulose synthatases (Pear et al., 1996). These "wall-to-membrane linker" proteins are thought to play a significant role in plant cell expansion and plant development. Among the wall-to-membrane linker proteins listed above, Waks have received the most attention, as they have been implicated in defence against

pathogens (Reviews by He et al., 1998 and Anderson et al., 2001). Waks have also been viewed microscopically and shown to act as strong physical cell wall-plasmamembrane anchors (Gens et al., 2001).

4.5.4 Identification of Non-classical Cell Wall Proteins

Some proteins, although not classified as classical cell wall proteins have been shown to exist in the cell wall. Glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme has also been shown by immunolocalisation to exist in the cell walls of *Candida albicans* (Gozalbo et al., 1998). Calreticulin, an endoplasmic reticulum protein has been shown to localise on the external surface of human blood T lymphocytes (Arosa et al., 1999), human fibroblast (Gray et al., 1995) and B16 cells (White et al., 1995). Ubiquitin, an enzyme traditionally known to play a vital role in a variety of cellular processes, including an ATP-dependent selective degradation of cellular proteins, protein modification, gene transcription, organisation of chromatin structures, ribosome biogenesis, and stress response (Finley et al., 1991; Finley et al., 1987) was also identified here. In *Arabidopsis* ubiquitin has been shown via immunoblotting to localise in the nucleus, in the microsomes and in the vacuoles (Beers et al., 1992). Beers et al., (1992) also identified some ubiquinated proteins in the *Arabidopsis* apoplast however, they recorded their data as a possible cytosolic contamination. It is now known that ubiquitin is a *bona fide Candida albicans* cell wall protein (Sepulveda et al., 1996a). Ubiquitin has also been shown to interact with cell surface receptors such as, T-cell antigen receptor (Cenciarelli et al., 1992), growth hormone receptors (Leung et al., 1987); platelet-derived growth factor b-receptor (Mori et al., 1992); immunoglobulin

E receptors (Paolini and Kinet. 1993, C3d- binding protein of *Candida albicans* (Saxena et al., 1990). Moreover, recently ubiquitin has been shown to conjugate with plasma membrane-bound glycine receptors and to regulate their endocytosis and subsequent proteolysis in *Xenopus* oocytes (Büttner et al., 2001). The possible roles of ubiquitin in the apoplast are discussed in Sepulveda et al., (1996b), and are reported to include the regulation of ligand binding, cell adhesion, and cell signalling. In addition, Büttner et al., 2001 recently discussed that cell surface ubiquitination might be one of the mechanisms by which some cell surface proteins are targeted for endocytosis and lysosomal degradation thus regulating the numbers and types of cell surface proteins during cell development. Additionally, most of the proteins discussed above have at least one signal peptide possessing homologue.

However, 29 out of 104 proteins that were identified here possessed no signal peptide and no signal peptide possessing homologue was found in the *Arabidopsis* database. These proteins with no signal peptide include, an elongation factor-1α, a putative peroxiredoxin, and an enolase. The elongation factor-1α, a DNA replication associated protein has also been found to be a *bona fide* protein resident of *Mycobacterium leprae* cell walls (Marques et al., 1998), also recently the elongation factor has been found to reside in the cell wall of *Trichoderma reesei* mycelia (Lim et al., 2001). In the same study an enolase was also identified among the cell wall protein extracts. Enolase has also been previously immunolocalised to the *Saccharomyces cerevisiae* cell walls (Edwards et al., 1999). Peroxiredoxin has been immunolocalised to the extracellular surface of the potato cyst nematode cells (Robertson et al., 2000). These examples support the findings that elongation factor-1α, putative peroxiredoxin, and an enolase are *bona fide* cell wall proteins although they lack the signal peptide for secretion suggesting that there may be other

alternative protein secretory pathway(s), which are still to be discovered. This possibility is discussed by Rapport, (1992).

New evidence of multifunctional enzymes are beginning to surface, for example, glyceraldehyde-3-phosphate dehydrogenase a classical glycolytic enzyme has been implicated in the mediation of cellular adhesion to fibronectin and laminin, therefore contributing to the fungus-host interaction (Gozalbo et al., 1998). Cell surface calreticulins that also function as mannose lectins have been shown to be involved in the spreading of mouse melanoma cells (White et al., 1995).

4.5.5 Concluding remarks

A parallel study of Black Mexican Sweetcorn cell walls was performed by Dr Chivasa et al, (unpublished). The results obtained in this study are remarkably similar to the data presented in this chapter. Almost all the classes of proteins identified in *Arabidopsis* were also identified in corn including the non-classical cell wall proteins. This observation strongly suggests that many cell wall functions are evolutionally conserved between monocots and dicots. The ultimate proof for the existence of these unexpected proteins in the cell wall however, would require more sensitive methods like *in situ* immunolocalisation. The next challenge would be the investigation of the role of these novel and non-classical cell wall proteins. In conclusion, this chapter has given new insight into the diversity of plant cell wall proteins. The elucidation of the exact functions of these proteins will require further experimentation using gene knockouts, gene silencing and other appropriate molecular biology methods.

Chapter 5

Extracellular Phosphorylation In Plants

5.1 Introduction

Signal transduction is the mechanism by which the stimuli from the environment are perceived and conveyed to the cytoplasm. In animals, extracellular signalling molecules like growth factors, hormones, soluble molecules, pathogens and other environmental cues are recognised by transmembrane receptor proteins such as receptor tyrosine kinases. The recognition of the external stimulus generally stimulates membrane associated GTP binding proteins to activate the phosphorylation of cytosolic mitogen activated protein kinase kinase kinases (MAPKKKs), which in turn phosphorylate mitogen activated protein kinase kinases (MAPKKs). The dual specific MAPKKs are known to phosphorylate mitogen activated protein kinases (MAPKs). This phosphorylation cascade leads to the activation of transcription factors, which in turn catalyse the transcription of relevant messenger RNA(s) and result in the production of the stimulus related polypeptide (Figure 5.1).

In mammalian and yeast systems various signal transduction pathways have been well studied (Reviewed by Yang 1996). In plants however, the progress has been much slower. The first plant receptor kinase ZmPK1 was cloned from maize (Walker and Zhang, 1990) but hitherto, only 7 receptor kinases have been characterised in plants; five from *Arabidopsis* (CLAVATA1, Clark et al., 1996; BRI1, Clouse et al., 1996; ERECTA, Torii; et al., 1996; FLS2, Gomez-Gomez and Boller., 2000; HAESA, Jinn et al., 2000), one from maize (CRINKLY4, Becraft et al., 1996) and one from rice (Xa21, Ronald et al., 1992). The protein targets of these receptor kinases however, are still to be uncovered (Peck et al., 2001).

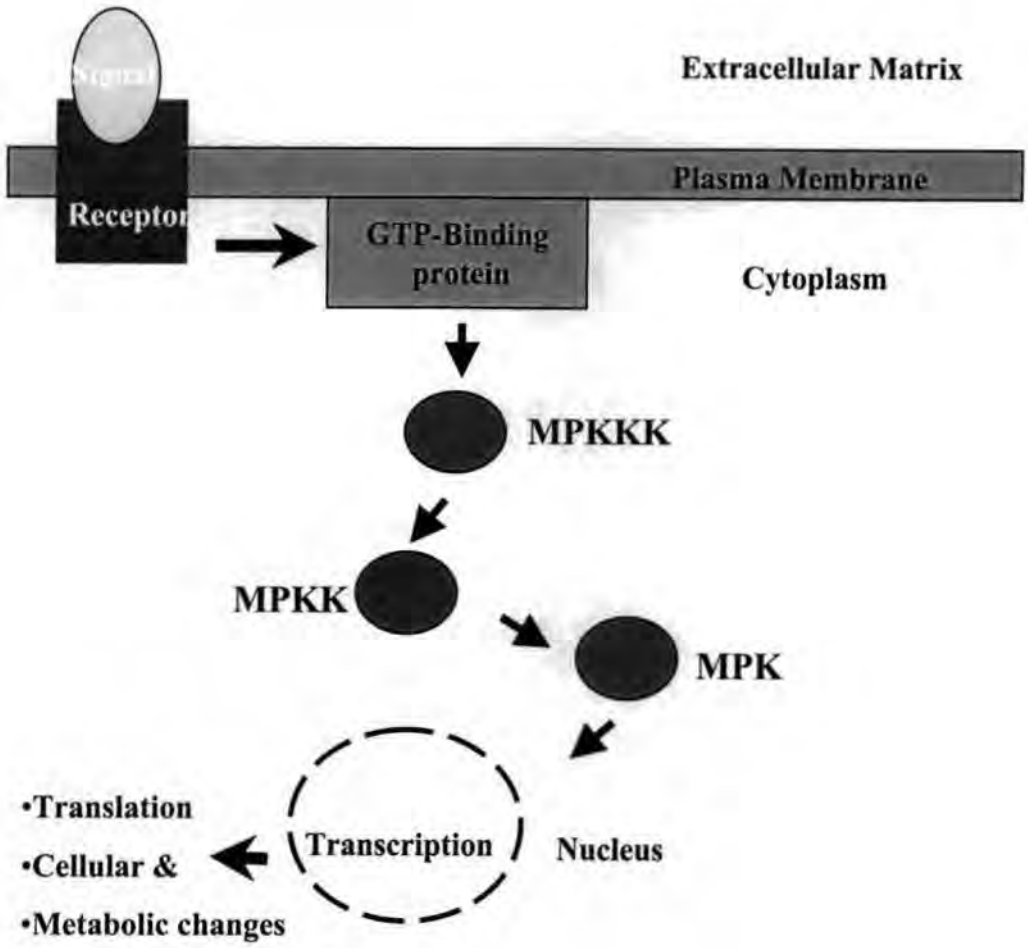


Figure 5.1 **Signal transduction cascade model.**

A schematic diagram highlighting the accepted signal transduction pathway model found in animal and fungal systems. A ligand (signal) from the extracellular matrix binds the transmembrane receptor kinase. The receptor kinase then activates a membrane associated GTP binding protein. The activated GTP binding protein in turn activates a cascade of mitogen activated protein kinase kinase kinases (MAPKKKs). MAPKKKs phosphorylate MAPKKs, which in turn phosphorylate MAPKs. The phosphorylation of MAPKs lead to the activation of transcription factors located in the nucleus. (adapted from Yang, 1996)

Protein phosphorylation is the central feature of signal transduction (Reviewed by Hunter, 1995), however, it has never been shown to exist in the extracellular matrix of plant cells. The signal transduction model that is applied in plant studies is derived from animal systems (Review by Yang, 1996). This approach, however, has a danger of overlooking the potential role of the cell wall in signal transduction. Unlike animal cells, the cell wall is the first contact between the plant cell and the outside environment, making it a strategic location for the deployment of receptors. The identification of cell wall located putative receptor-like protein kinases and protein phosphatases (in chapter 4) led to the hypothesis that protein phosphorylation/dephosphorylation events might be taking place in the plant apoplasm. Previously this possibility was inconceivable because energy-rich compounds like adenosine 5'-triphosphate (ATP) were generally presumed non-existent in the plant apoplasm (Eisenhaber and Bork, 1998).

The main aim of the work described in this chapter was to investigate whether protein phosphorylation takes place in the plant extracellular matrix. ATP has been recently reported to be secreted to the apoplasm of plant tissues (Thomas et al., 1999). This data combined with the identification of putative protein kinases, which are located entirely in the cell wall with no transmembrane domains, supported the hypothesis of plant extracellular phosphorylation. In an attempt to verify this theory, various extracellular protein kinase inhibition experiments were employed. Protein kinases catalyse the reaction between ATP and a target protein to produce a phosphoprotein plus an adenosine 5'-diphosphate (ADP): -

ATP + target protein ----------$^{\text{protein kinase}}$----------> phosphoprotein + ADP

To investigate whether the extracellular putative protein kinases (described in chapter 4) are enzymatically active, methods capitalising on the phosphorylation principle described above were applied. Various extracellular kinase-blocking techniques were considered. The main challenge was to ensure that the method did not interfere directly with the cytosolic protein kinase activities. The techniques considered included the treatment of the cell suspension cultures with non-cell permeable protein kinase inhibitors, the application of non-hydrolysable ATP analogues to compete out the hydrolysable ATP in the apoplast and the destruction of the apoplastic ATP with ATP hydrolysing enzymes. All these methods were potentially able to block or retard the activities of the extracellular protein kinases, however, the application of "non-cell permeable" protein kinase inhibitors was not ideal. The only well documented and commercially available cell impermeable protein kinase inhibitor (to my knowledge) is K252b. However, although it is described as "cell impermeable" it has been reported to permeate some cells including Sf9 insect cells and PC12 rat pheochromocytoma cells (Ross et al., 1995), although to a lesser extent than its cell permeable analogue, K252a. The two remaining choices involved (1) the removal of the apoplastic ATP and (2) the application of the non-hydrolysable ATP analogue in the apoplasm to compete out the hydrolysable ATP.

This chapter aims to investigate the effects of blocking extracellular protein kinase activities using two approaches: (1) By removing the extracellular ATP, and (2) by replacing the apoplastic ATP with its non-hydrolysable homologue. Also in order to verify that extracellular kinase activities (phosphorylation) take place in the apoplasm, extracellular phosphoproteins will be investigated, identified and

characterised using 2-dimension polyacrylamide gel electrophoresis (2D PAGE) followed by immunoblotting with anti-phosphotyrosine antibodies. The anti-phosphopeptide antibodies were preferred over the conventional radioactive protein-labelling technique because the low abundance of cell wall proteins required a large amount (Litres) of experimental cell material.

5.2 Results.

5.2.1 The Removal of Extracellular ATP Induces Oxidative Burst in Cell Suspension Cultures

Novel cell wall located putative protein kinases were identified in Chapter 4, however, unlike other cell wall associated kinases (Waks) reviewed by Kohorn, (2001), some of the reported kinases lacked the transmembrane domain. The lack of a transmembrane domain implied that the catalytic domain of these novel protein kinases was located in the extracellular matrix. This observation led to the hypothesis that, protein phosphorylation events take place in the plant extracellular matrix. The first step towards proving this possibility was to block the activity of all the extracellular kinases by removing extracellular ATP. If extracellular phosphorylation is a phenomenon that exists in plant cells, it would be expected that "blockage" of this event would induce some metabolic changes (including stress response). One of the common stress responses in plant cells is the sudden accumulation of reactive oxygen species (Bolwell et al., 1998).

Two methods of removing extracellular ATP from the cell cultures were applied. First, 5 millilitres of 3-day old Black Mexican sweetcorn (BMS) cultures were treated with 125 units apyrase (an ATP hydrolysing enzyme). The accumulation of hydrogen peroxide (H_2O_2; one of the reactive oxygen species) was observed in the apyrase treated cells within 30 minutes (Figure 5.2). The accumulation of hydrogen peroxide was visualised using the diaminobenzidine (DAB) method (see Materials and Methods).

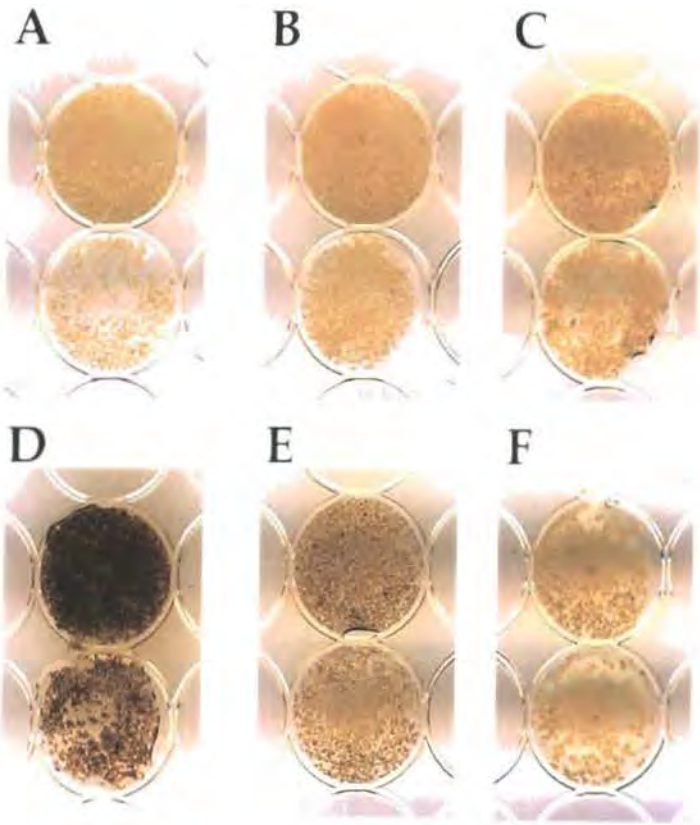


Figure 5.2. Apyrase induced oxidative burst in BMS cell cultures.

(A – F) Black Mexican Sweetcorn (BMS) cells were grown for three days in suspension culture. Five millilitre aliquots of the cell cultures were transferred to sterile flask and were treated with diaminibenzidine (DAB; a reagent that turns brown in the presence of hydrogen peroxide) and one of the following compounds: - **(A)** Water, **(B)** ATP, **(C)** ascorbic acid (AA), **(D)** Apyrase, **(E)** a combination of 1mM ATP and Apyrase, **(F)** a combination of AA and Apyrase.

In the presence of H_2O_2, DAB polymerises and turns brown (Hamby-Mason et al., 1998). The apyrase-induced cell browning was delayed when cells were treated with a combination of apyrase and 1mM ATP (Figure 5.2). Also, when cells were pre-treated with ascorbic acid (a H_2O_2 scavenger) the cell browning was completely eliminated, thus confirming that the cell browning was due to the accumulation of H_2O_2. Second, the cells were treated with a combination of hexokinase (925 units) and 1 mM glucose. Hexokinase converts glucose into glucose-6-phosphate and in the process the available ATP is hydrolysed to yield adenosine 5'-diphosphate (ADP). The application of hexokinase and glucose to the cell cultures also induced oxidative burst (Figure 5.3). The main products of the reaction between hexokinase, glucose and ATP, include ADP and glucose 6-phosphate. BMS cells were treated with glucose 6-phosphate and ADP and both treatments failed to induce oxidative burst therefore confirming that the oxidative burst was due to the removal of ATP from the external medium. The apyrase and hexokinase experiments showed that the removal of extracellular ATP induces oxidative burst. However the biological significance of this oxidative burst was unclear, therefore the next experiment was to investigate the events that take place down stream of (ATP removal induced) oxidative burst.

5.2.2 The Extracellular ATP is Essential for Cell Survival.

Having shown that the elimination of ATP from the external medium of cell cultures induces the accumulation of reactive oxygen species, the next step was to investigate the events that take place down stream of oxidative burst.

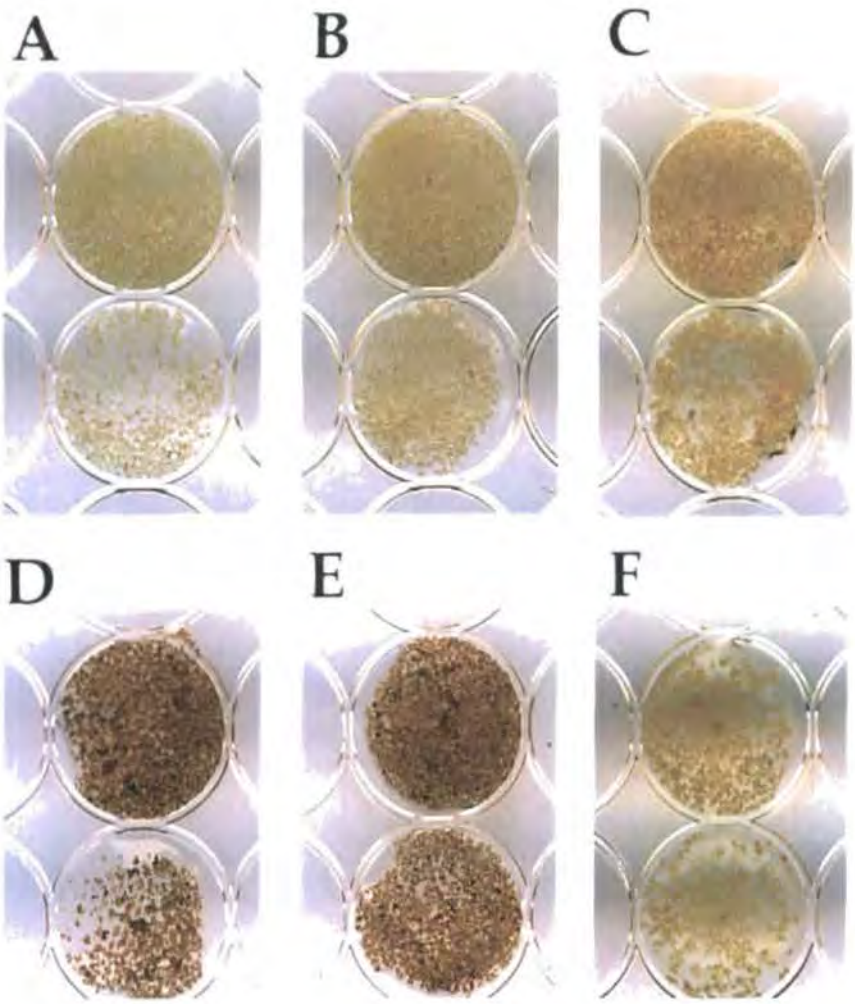


Figure 5.3. Hexokinase/Glucose induced oxidative burst in BMS cell cultures.

(A – F) Black Mexican Sweetcorn (BMS) cells that were grown for three days in suspension culture were transferred (5 mL aliquots) to sterile flasks. The cell aliquots were treated with diaminibenzidine (DAB; a reagent that turns brown in the presence of hydrogen peroxide) and one of the following reagents: - **(A)** Glucose **(B)** ADP, **(C)** Glucose-6-phosphate, **(D)** Hexokinase and glucose, **(E)** Hexokinase only **(F)** a combination of AA and hexokinase.

For this, 3-day old BMS cell cultures were treated with apyrase for 24 hours in sterile specimen flasks. At the end of the 24-hour incubation period 200 μL aliquots of cell cultures were transferred to sterile microcentrifuge tubes and were stained with Evan's blue solution to measure cell viability. Evan's blue stain permeates dead cells and is excluded by viable cells. Following staining the cells were washed 3 times with 1 mL of deionised water at room temperature. The stain was eluted from the cells with 40 % methanol in 1 % sodium dodecyl sulfate solution (see materials and methods). The absorbance of the eluted stain was measured with a spectrophotometer at 600 nm. The apyrase treated cells absorbed significantly more stain than the control cells (Figure 5.4). This implied that the application of apyrase induced cell death in the BMS cell cultures. When the application of apyrase in the cells was coupled with the exogenous ATP application the cells were rescued from cell death showing that the removal of ATP from the external medium induced cell death. These results therefore showed that the extracellular ATP is vital for sustaining the viability of BMS cells.

5.2.3 The Removal of Apoplastic ATP Induces Necrotic Lesion Development in Whole Plant Leaves.

The removal of apoplastic ATP induces oxidative burst followed by cell death in cell suspension cultures, however at this stage it was not evident whether these effects can be reproduced in whole plant tissues. In order to examine this, the effect of the removal of apoplastic ATP was tested on the leaves of the black-eyed beans and tobacco.

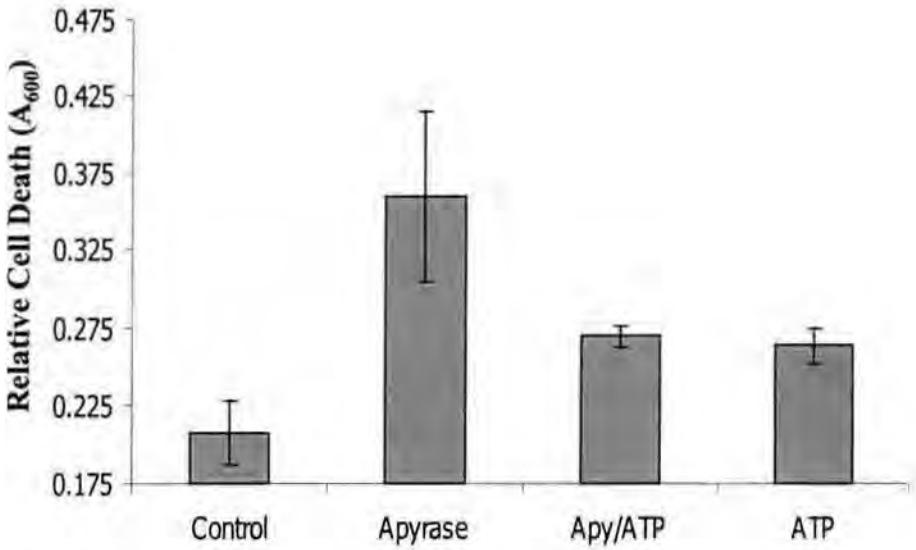


Figure 5.4 Evans blue test for apyrase induces cell death in cell cultures.

Cell cultures of Black Mexican sweetcorn (BMS) were subcultured and grown for 3 days. The cells (5 mL aliquots) were transferred to sterile flasks and were treated with water (control), Apyrase, a combination of Apyrase and ATP (Apy/ATP) and ATP for 24 hours. Two hundred millilitre aliquots were removed from the culture and were stained with Evan's blue to test for viability. The absorbance of the stain taken up by the cells was measured at 600nm. Error bars indicate the standard deviation from the mean of three replicates.

These two plant species were chosen because the thick texture of their leaves was suitable for the apoplastic needle infiltration experiments. As in cell cultures two methods of removing ATP in the leaf apoplasts were applied. First, apyrase (a strong ATP hydrolysing enzyme; as described before), was infiltrated into the apoplast of the black-eyed bean and tobacco leaves. The second method involved the injection of a mixture of hexokinase and glucose into the apoplast of the plant leaves.

Consistent with the cell culture results (5.1.2) both the apyrase and hexokinase experiments induced the formation of cell death lesions at the site of infiltration (Figure 5.5). As a control, an equivalent amount of bovine serum albumin (BSA), glucose, glucose 6-phosphate and ADP were also infiltrated. The cell death lesions on the leaves did not spread beyond the site where the ATP hydrolysing enzymes were infiltrated, showing that the enzymes were confined to the apoplasm and did not enter the symplasm. These results showed for the first time that the apoplastic ATP is vital for the sustained plant cell viability. At this stage however, it was still not clear whether ATP was needed as a receptor-binding factor, or whether its hydrolysis was coupled with protein phosphorylation as hypothesized in this study. In order to verify this a non-hydrolysable ATP analogue was used to compete out the hydrolysable ATP.

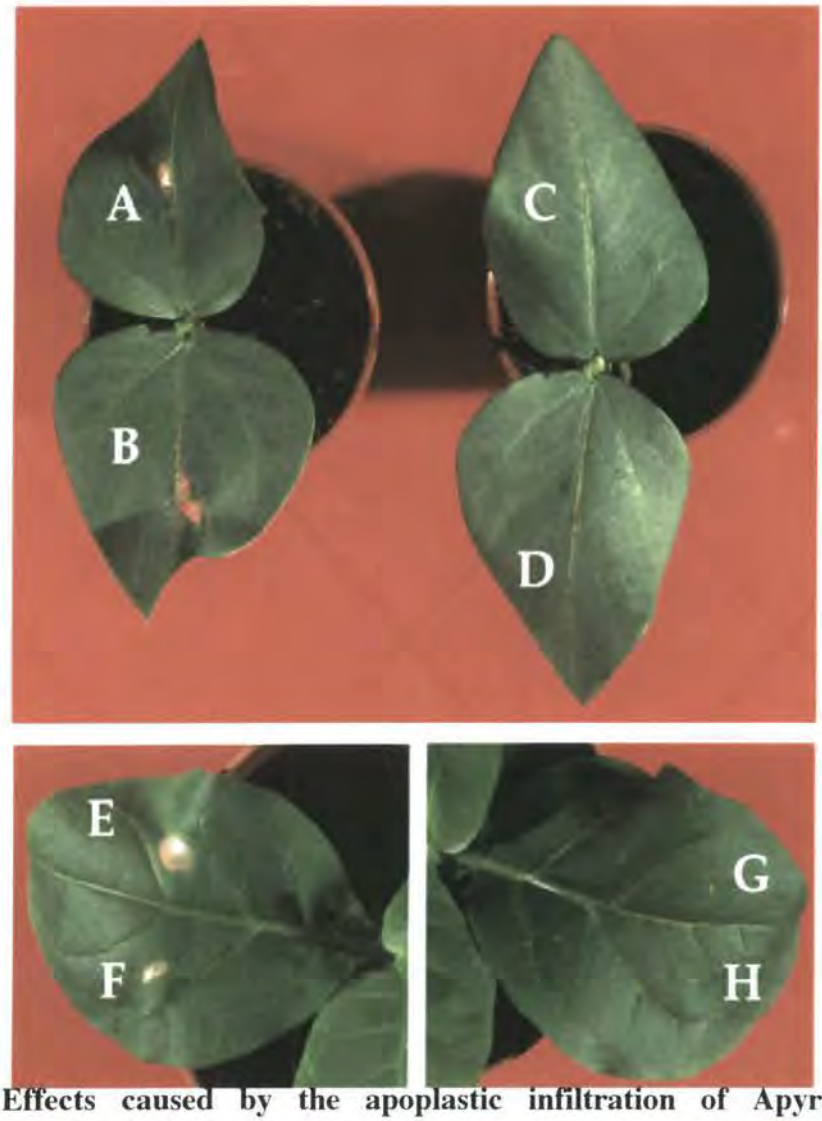


Figure 5.5. Effects caused by the apoplastic infiltration of Apyrase and Hexokinase/Glucose in whole plant leaves.

Apyrase was infiltrated into the apoplast of the leaf blades of Black-eyed bean (**A**) and tobacco (**E**). Also a combination of hexokinase and glucose was administered into the leaf apoplast of Black-eyed bean (**B**) and tobacco (**F**). The lesions of cell death can be seen in all the enzyme treated leaf blades (**A, B, E and F**). As controls water and a mixture of glucose/glucose-6-phosphate/ADP/AMP (**C**), and Bovine serum albumin (BSA) (**D**) were infiltrated to the apoplasm of the black eyed beans. Water and a cocktail of ADP/AMP/ glucose-6-phosphate/glucose (**G**), and BSA (**H**) were also infiltrated as controls to the apoplasm of the tobacco leaf blades.

5.2.4 The Non-hydrolysable ATP Induces Cell Death in the Plant Apoplast.

The precise role of extracellular ATP in plant cell growth and development is still unclear. In plants the extracellular ATP has mainly been thought to act as an alternative source of inorganic phosphate (Thomas et al., 1999). Extracellular ATP has also been shown to be involved in the binding and activation of receptor-like transmembrane enzymes and channels (Haines et al., 2001). However, the identification of novel extracellular located putative protein kinases in this study led to the hypothesis that, the extracellular ATP is an active substrate for protein phophorylation. In order to verify the validity of this hypothesis a non-hydrolysable ATP analogue (βγ-ATP) was infiltrated into the apoplasm of the tobacco leaves to perturb the activity of extracellular kinases without affecting the ATP binding roles. The results showed a rapid development of massive cell death on the area of the leaf that was infiltrated with βγ-ATP and as a control the same amount of hydrolysable ATP was infiltrated in the separate area of the leaf and no cell death was observed (Figure 5.6).

These results therefore showed that the role of ATP in the plant apoplast is more than just binding to the transmembrane and/or membrane bound receptors. Its hydrolysis is also essential for sustaining cell viability. The evidence shown so far indicates that protein phosphorylation might be taking place in the plant extracellular matrix, however, hitherto no phosphoproteins (products of protein phosphorylation) have ever been reported to reside entirely in the extracellular matrix of the plant cells. This led to the investigation of whether the plant cell extracellular matrix harbours any phosphorylated proteins.

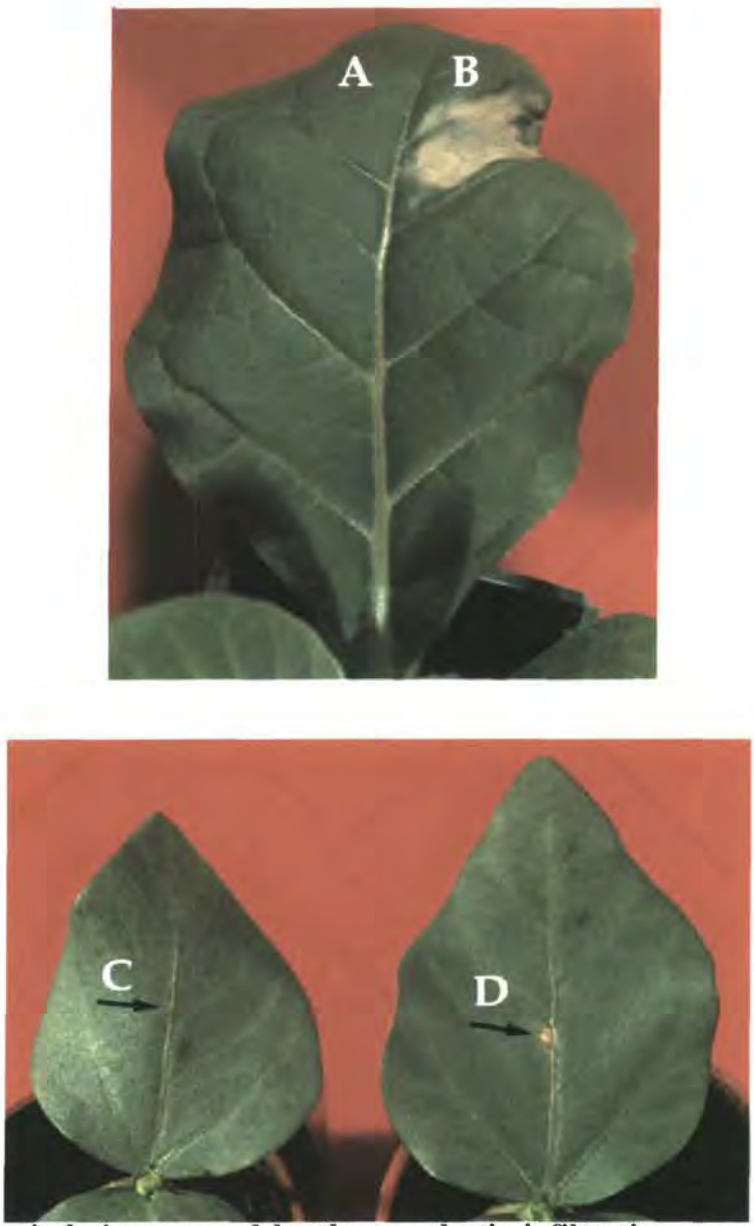


Figure 5.6. Necrotic lesions caused by the apoplastic infiltration a non-hydrolysable ATP analogue in plant leaves.

(**A**) Tobacco leaf blade apoplastically infiltrated with 10 mM ATP

(**B**) Tobacco leaf blade apoplastically infiltrated with 10 mM non-hydrolysable ATP (βγATP)

(**C**) Black-eyed bean leaf apoplastically infiltrated with 10 mM ATP

(**D**) Black-eyed bean leaf apoplastically infiltrated with 10 mM βγ ATP

5.2.5 Identification and Characterisation of Novel Cell Wall Phosphoproteins

The discovery of novel extracellular putative protein kinases (chapter 4), and the realisation that non-hydrolysable extracellular ATP induces cell death (5.1.4), led to the possibility that the plant extracellular matrix (as in animal systems) contains phosphorylated proteins. In order to verify this possibility, 500 mL *Arabidopsis* cells in suspension culture were grown for 3 days in 5 separate flasks (each containing 100 mL). The culture medium was separated from the cells using vacuum assisted filtration through two layers of miracloth layered on a Buchner funnel ensuring that no intact cells go through with the filtrate. The proteins secreted to the culture medium were concentrated by precipitation with 80 % acetone at –20 °C and the protein precipitates were resolubilised in PAGE loading buffer. Twenty micrograms of culture filtrate proteins, $CaCl_2$ cell wall protein extracts (prepared as in chapter 4) and total soluble proteins were separated via polyacrylamide gel electrophoresis, western blotted onto a nitrocellulose membrane and were probed with an anti-phosphotyrosine antibody (Amersham Pharmacia Biotech) that recognise peptides that posses phosphorylated tyrosine residues. The anti-phosphotyrosine antibodies recognised several protein bands in both culture filtrate and cell wall protein extracts (Figure 5.7). The profile of the culture filtrate proteins and cell wall protein extracts were different from those of cellular soluble proteins. This showed that the proteins of cytosolic origin did not contaminate the culture filtrate and the cell wall proteins. The competition assays with phosphotyrosine and onblot phosphotyrosine dephosphorylation experiments with a tyrosine phosphatase confirmed that the electrophoressed proteins were specifically labelled with the anti-phosphotyrosine

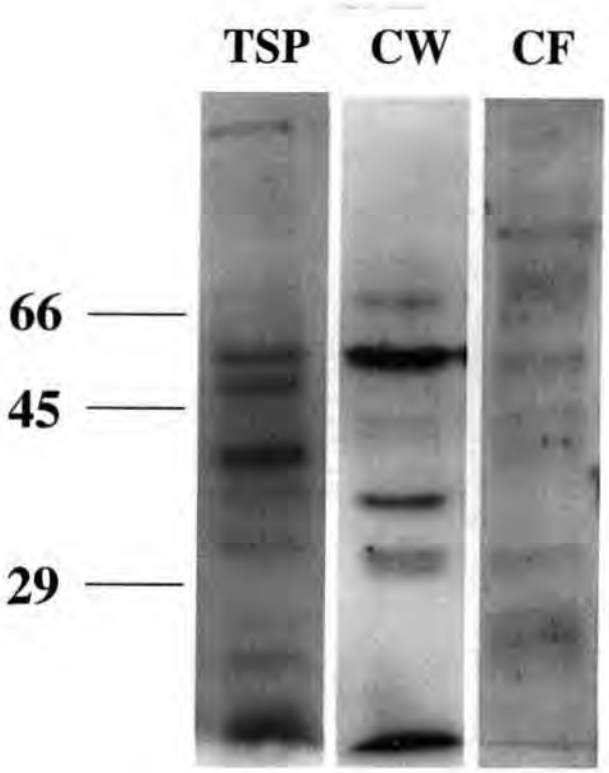


Figure 5.7. **Western blot of anti-phosphotyrosine against *Arabidopsis* protein extracts**

Twenty micrograms of *Arabidopsis* total soluble protein (TSP), pure cell wall extracts (CW), and culture filtrate (CF) were separated via 10 % 1-D PAGE, Western blotted, probed with anti-phosphotyrosine antibodies and detected via chemiluminescence. The lanes indicate the tyrosine phosphoprotein band profile from each protein extract. Molecular weight markers (kDa) are indicated.

antibodies (Results not shown because the autorads were blank). These results therefore strongly suggested that the extracellular matrix of plant cells harbours several phosphoproteins. However, 1-dimension PAGE has a low protein resolving ability, and therefore it was almost impossible to estimate the number of phosphorylated protein in these protein extracts.

Proteins that were extracted from purified cell wall with $CaCl_2$ were resolved via two dimension PAGE. Two hundred micrograms of the protein sample was loaded into 7 cm pH 6-11 2-D gel strips using an in-gel reswelling method (see Materials and Methods). The reswelled gel strips were subjected to isoelectric focusing (using a Pharmacia Amersham Multiphor) followed by 12 % PAGE using a BioRad minigel electrophresis apparatus (see Materials and Methods). The electrophoressed proteins were western blotted onto a nitrocellulose membrane and probed with anti-phosphotyrosine antibodies and exposed to an X-ray film and detected via chemiluminescence. The autorad with phosphotyrosine labelled protein spots was superimposed on a duplicate polyacrylamide gel with the same protein sample stained with Coomassie brilliant blue. The application of this method visualised about 20 protein spots that were labelled with the anti-phosphotyrosine antibody (Figure 5.8). The labelled protein spots were excised from the minigels manually and were digested with trypsin. The tryptic digests were analysed via MALDI-ToF as in chapter 4. So far 5 spots representing 3 proteins; 2 hypothetical proteins with MIPS accession numbers AT3g12500 and AT1g78850 and an unknown protein, AT3g08030 were identified. The MALDI-ToF peptide ion spectra and the corresponding peptide ions of the identified polypeptides proteins are shown in

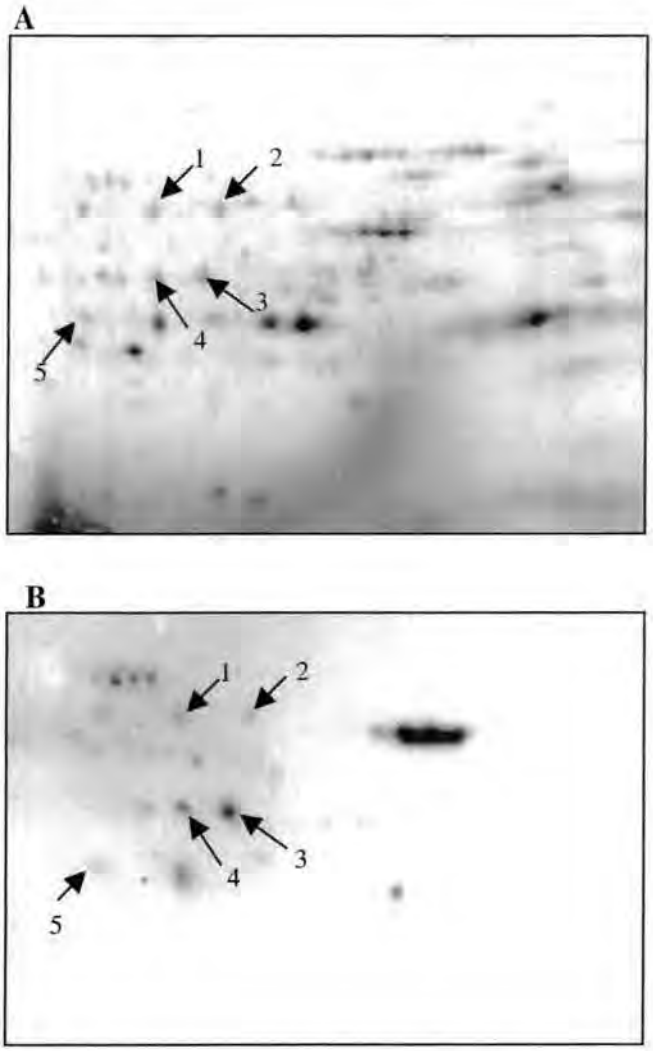
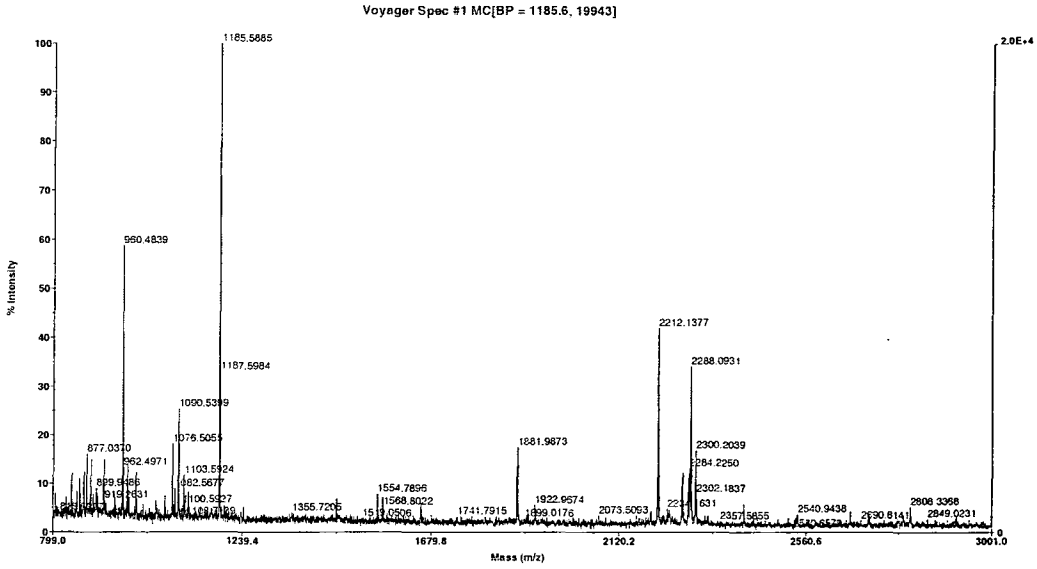


Figure 5.8 **Two dimension PAGE profile of proteins extracted from *Arabidopsis* pure cell walls**

Two hundred micrograms of protein sample extracted from purified *Arabidopsis* cell wall using $CaCl_2$ were separated (in duplicate) via 7 cm pH 6-11 IEF strips in the first dimension followed by 12 % PAGE in the second dimension. One fraction (200 µg) was stained with Coomassie brilliant blue **(A)** and the second fraction (200 µg) was Western blotted, probed with anti-phosphotyrosine antibodies and detected via chemiluminescence on an X-ray film **(B).** The numbered arrows are pointing at the proteins identified via MALDI-ToF mass spectrometry.

Figures 5.9, 5.10 and 5.11. All the polypeptides identified so far possess a cleavable N-terminal signal peptide (Figure 5.12), which targets the polypeptide towards the secretory pathway (signalP: www.cbs.dtu.dk/services/SignalP/). According to the TMHMM transmembrane prediction program (www.cbs.dtu.dk/services/TMHMM/) these proteins possess no transmembrane domains, thus indicating that they are located entirely in the extracellular matrix. All the phosphoproteins described in this chapter so far are among the proteins that were already identified in chapter 4. One of the hypothetical proteins identified (spot 1 and 2; AT1g78850) shares 50 % homology with a carrot S-like receptor protein kinases. The second hypothetical protein is 91 % homologous to basic endochitinases (spot 5). Basic endochitinases are one of the well-studied groups of plant pathogen related proteins and their 3-D structure has been solved (Reviewed in Swapan and Muthukrishnan, 1999). Chitinases catalyse the hydrolysis of β-1.4-linkage between *N*-acetylglucosamine residues of chitin, a structural polysaccharide component of many fungi, insects and nematodes. These enzymes (chitinases) however, have never hitherto been reported to be phosphoproteins, therefore the identified polypeptide was subjected to further analysis for possible phosphorylated amino acid residue(s) using antibodies that recognise tyrosine phosphorylated motifs.

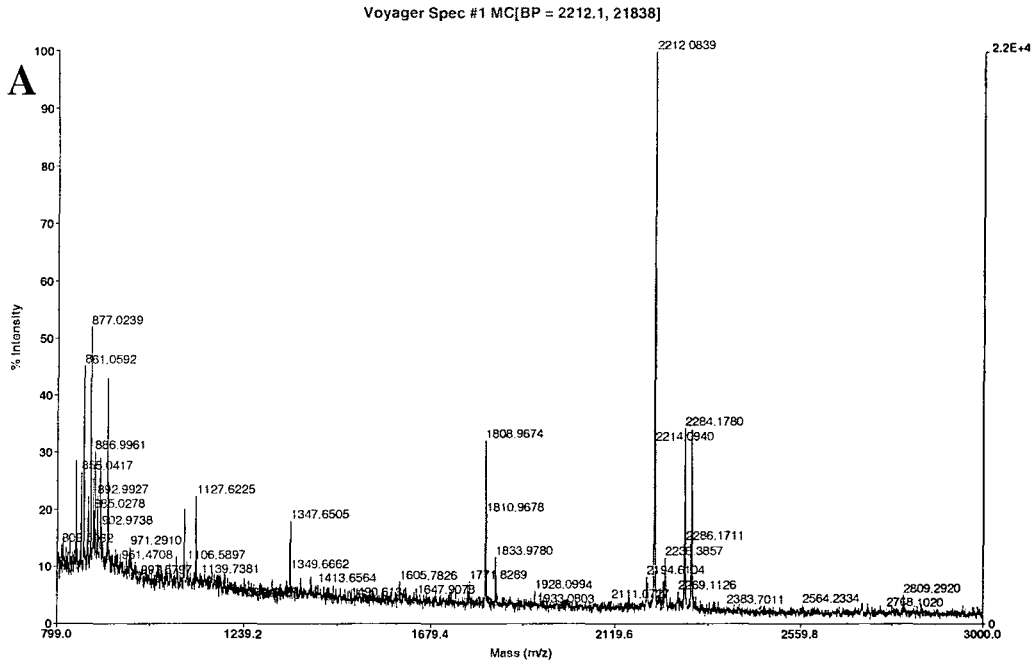


Hypothetical protein (At1g78850)

MKFSITLALCFTLSIFLIGSQAKVPVDDQFR**VVNEGG(Y)TDYSPI EYNPDVRGFVPFSDNFRLCFYNTTPNAYTLALRINR**VQESTLR **WVWEANR**GSPVKENATLTFGEDGNLVLAEADGRLVWQTNTAN KGAVGIKILENGNMVI(**Y**)DSSGK**FWQSFDSPTDTLLVGQSLKL** NGRTKLVSRLSPSVNTNGPYSLVMEAKKLVLYYTTNK**TPKPIAY FEYEFFTK**ITQFQSMTFQAVEDSDTTWGLVMEGVDSGSKFNVST FLSRPKHNATLSFIRLESDGNIRVWSYSTLATSTAWDVTYTAFTN ADTDGNDECRIPEHCLGFGLCKKGQCNACPSDKGLLGWDETCK SPSLASCDPKTFHYFKIEGADSFMTKYNGGSSTTESACGDKCTRD CKCLGFFYNRKSSRCWLGYELKTLTRTGDSSLVA(**Y**)VKAPNAN KKSTL

Figure 5.9 **MALDI-Tof mass fingerprint generated from spot 1 and 2** (see Figure 5.8).

(A) The tryptic peptide mass ion spectrum of spot 1 and 2 identified a putative protein kinase with no predictable transmembrane domain. (B) The signal peptide (Bold italics), MALDI-Tof peptide coverage (Bold) and the putative tyrosine phosphorylation sites (**Y**) are all highlighted in the identified polypeptide.



B

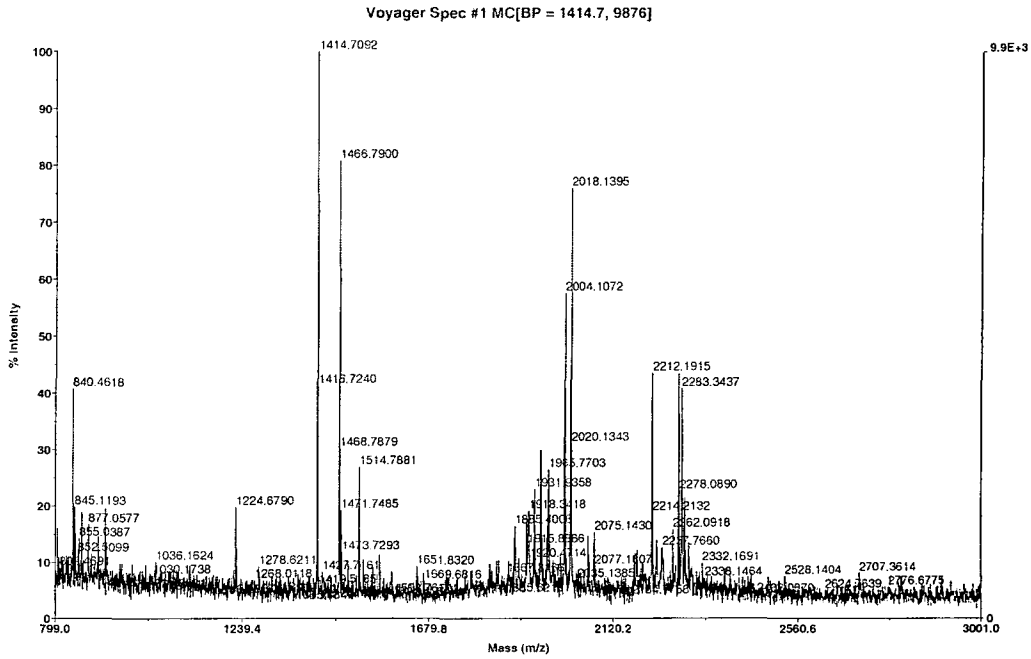
Unknown protein (AT3g08030)

MAVPKAIILPILLLICGAALGAPASEG(**Y**)LRNGNFEESPKKTDMKK
TVLLGKNALPEWETTGFVEYIAGGPQPGGMYFPVAHGVHAVRLG
NEATISQK**LEVKPGSLYALTFGASR**TCAQDEVLRVSVPSQSGDLP
LQTLYNSFGGDVYAWAFVAKTSQVTVTFHNPGVQEDPACGPLLD
AVAIK**ELVHPI(Y)TR**GNLVK**NGGFEEGPHR**LVNSTQGVLLPPKQ
EDLTSPLPGWIIESLKAVKFIDSK(**Y**)**FNVPFGHAAIELVAGK**ESAI
AQVI**RTSPGQTYTLSFVVGDAK**NDCHGSMMVEAFAARDTLKVP
HTSVGGGHVKTASFKFKAVEARTR**ITFFSGFYHTK**KTDTVSLCGP
VIDEIVVSHVA

Figure 5.10 **MALDI-ToF mass fingerprint generated from spot 3 and 4** (see Figure 5.8).

(A) The tryptic peptide mass ion spectrum of spot 3 and 4 identified a polypeptide, which shares no homology to any known protein (unknown protein). (B) The signal peptide (Bold italics), MALDI-ToF peptide coverage (Bold) and the putative tyrosine phosphorylation sites (**Y**) are all highlighted in the identified polypeptide.

A.



B.

Putative endo chitinase (AT3g12500)

MPPQKENHRTLNKMKTNLFLFLIFSLLLSLSSAEQCGRQAGGALC
PNGLCCSEFGWCGNTEP(**Y**)CKQPGCQSQCTPGGTPPGPTGDLSGII
SSSQFDDMLKHRNDAACPAR**GFYTYNAFITAAKSFPGFGTTGDT**
ATRKKEVAAFFGQTSHETTGGWATAPDGP(**Y**)SWGYCFKQEQNP
ASD(**Y**)CEPSATWPCASGKRYYGRGPMQLSWNYNYGLCGR**AIGV**
DLLNNPDLVANDAVIAFKAAIWFWMTAQPPKPSCHAVIAGQWQ
PSDADRAAGR**LPGYGVITNIINGGLECGR**GQDGRVADRIGFYQR
YCNIFGVNPGGNLDCYNQRSFVNGLLEAAI

Figure 5.11 **MALDI-ToF mass fingerprint generated from spot 5** (see Figure 5.8).

(A) The tryptic peptide mass ion spectrum of spot identified a putative endochitinase. (B) The signal peptide (Bold italics), MALDI-ToF peptide coverage (Bold) and the putative tyrosine phosphorylation sites (**Y**) are all highlighted in the identified polypeptide.

5.2.6 Bioinformatic Analysis Reveals Potential Phosphorylation Sites of the Novel Cell Wall Phosphoproteins

The potential phosphorylation sites can be predicted from the polypeptides using the NetPhos 2.0 Prediction (Centre of Biological Sequence Analysis: www.cbs.dtu.dk/services/NetPhos/). This program is able to predict the potential phosphotyrosine, phosphoserine and phosphothreonine residues within a given polypeptide to a sensitivity of up to 96 % (Blom et al., 1999). This program predicted that the putative endochitinase-like protein identified here (AT3g12500) has 3 potential phosphotyrosine sites: - Y63, Y164 and Y180. The potential tyrosine phophorylation sites of the unknown protein (AT3g03080) were predicted to be: - Y28, Y190 and Y246 and those of the identified receptor-like protein kinase-like protein (AT1g78850) are: - Y38, Y146 and Y427 (see Figure 5.9, 5.10 and 5.11).

5.2.7 Tandem Mass Spectrometry Confirms the Identity of The Chitinase.

The basic endochitinase identified here via MALDI-ToF represents the first classical cell wall protein which is phosphorylated. However often MALDI-ToF protein identification is unable to distinguish between closely related protein isoforms, therefore the identity of the endochitinase was confirmed using tandem mass spectrometry. In tandem mass spectrometry one or more peptide ions (produced via LC Q-TOF electrospray or MALDI-ToF MS) is further fragmentated by gas collision to produce specific ions (b and y ions; depending on terminus [C or N] retaining the charge), which give the amino acid sequence data (Kinter and Sherman, 2000). The

process of generating amino acid ion data from a given peptide ion is generally referred to as "collision induced dissociation" (CID). The peptide dissociation is induced by accelerating the selected peptide mass ion (under high pressure) in an inert gas (nitrogen or argon) chamber of the mass spectometer. As the peptide ion collides with the gas molecules its kinetic energy is converted into internal energy. This energy conversion makes the ion unstable and this lead to fragmentation of the peptide ion into amino acid ions. The mass (m) and the charge (z) of the amino acid fragment ions generated from a given peptide ion are measured by the detector of the mass spectrometer and the detected ion data is given as a spectrum of m/z ratios of all the detected amino acids (Kinter and Sherman, 2000).

The tryptic digests prepared from spot 5 (Figure 5.8) were sprayed and ionised in the Q-TOF instrument (ABI) via an elecrospray needle. The micro droplets of the sample were ionised by the direct flow of inert gas in the vacuum. Two doubly protonated ions (1413.62 and 1465.73) were selected (in automated mode) in the first quadrupole (Q1) of the electrospray mass spectrometer and transferred to the second quadrupole (Q2) where they were subjected to CID (one by one). The ions generated from the fragmentation of the peptide ion were selected and channelled to the third quadrupole (Q3). The masses of the ion fragments were analysed in Q3 and their m/z ratio was registered. The recorded m/z spectrum of two selected peptide ions are shown with their corresponding amino acid tags (Figure 5.12). Both the b and y ion series corresponded to the following amino acid sequence tags: SFPGFGTTGDTATR and GFYTYNAFITAAK for peptide ions 1413.62 and 1465.73 respectively. The ion spectra obtained from the tandem mass spectrometer were used to interrogate Mascot and NCBI databases. Both the Mascot (a search

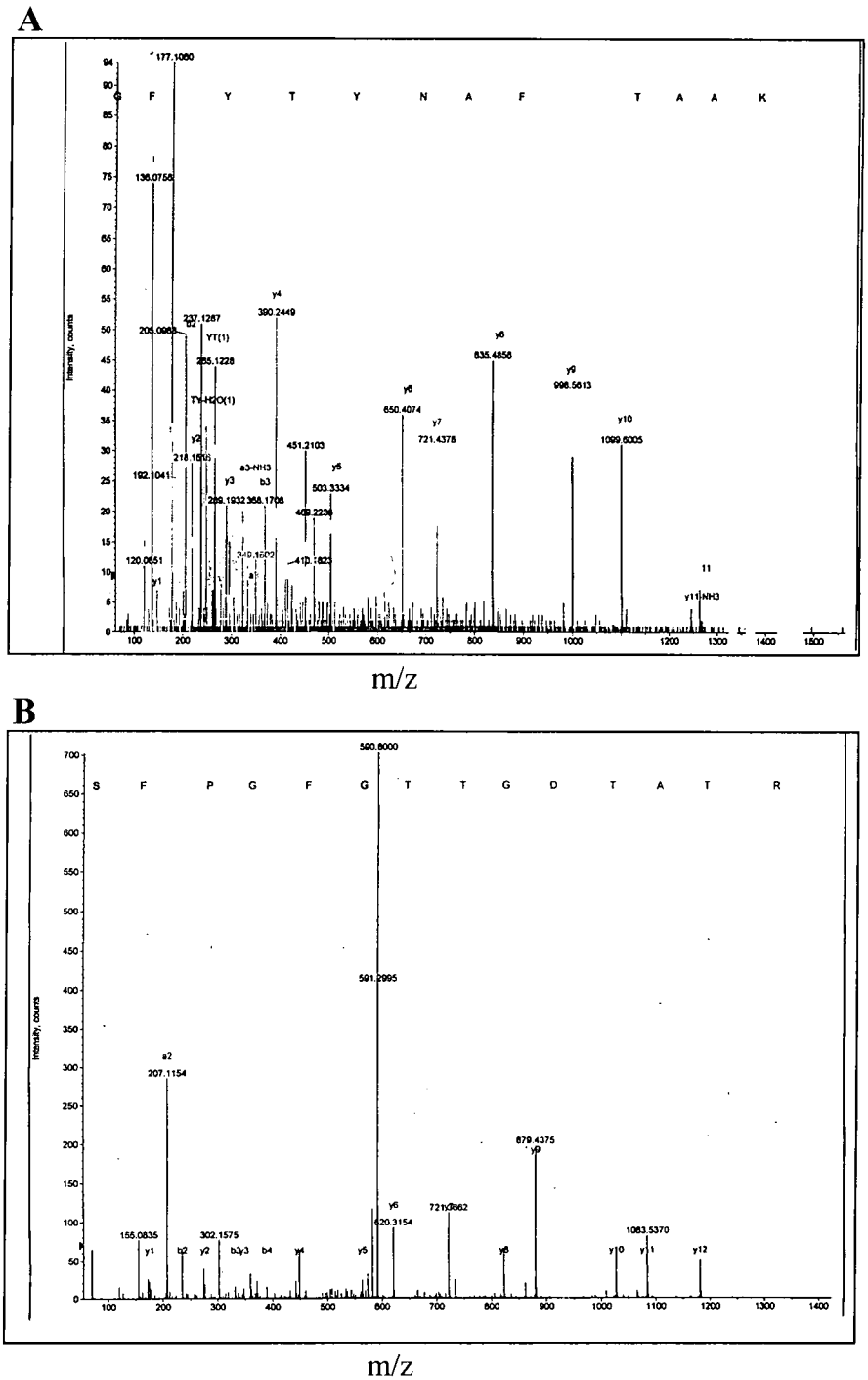


Figure 5.12 **The b and y spectra of two endochitinase doubly charged ions.**

Two doubly charged ions were selected among the tryptic digests generated from spot 5 (Figure 5.8) using an LC Q-TOF instrument (ABI). The selected ions (1413.62 m/z **A** and 1465.73 m/z **B**) were further fragmented in the Q-TOF MS's second quadrupole (Q2) and the spectra of the b and y series are shown together with the corresponding amino acid tags. The peptide ions and the amino acid sequence tags belong to an *Arabidopsis* endochitinase.

engine which uses massspectrometry data to identify proteins from primary sequence databases: www.matrixscience.com) and the NCBI (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov/BLAST/) databases confirmed that these amino acid sequences are part of a basic endochitinase. These amino acid tags were also identical to two of the peptide ions generated via MALDI-ToF (Figure 5.9) thus confirming the MALDI-ToF results.

5.2.8 The Biological Significance of Extracellular ATP and Extracellular Protein Phosphorylation.

The levels of extracellular ATP seem to be tightly regulated in plant tissues. Recently Thomas et al., (1999) showed via trypsin proteolysis followed by activity assays that the ATPase activity of the *Arabidopsis* membrane bound apyrase is on the extracellular side of the plasma membrane. Kiba et al, (1996) also reported cell wall bound ATPases in pea (*Pisum sativum*) and cowpea (*Vigna sinensis*). These reports led to an investigation of whether the purified cell walls of *Arabidopsis* possessed any apyrase-like activities. For this, ATPase enzyme activity was assayed with *Arabidopsis* cell walls that were free from any detectable cytoplasmic and membranous contamination (see chapter 4) following a method described by de Aguiar Matos et al., (2001). A sample of purified cell walls (0.143 mg) was mixed with ATPase assay buffer (200 mM $CaCl_2$, in 50 mM Tris pH 7.5), the assay was initiated by adding 1mM ATP at 37 ^{o}C and was stopped after 20 minutes with 5 % trichloroacetic acid on ice. The amount of inorganic phosphate released from ATP was measured using the sodium metavanadate/sodium molybdate method (see

Materials and Methods). The results showed that purified *Arabidopsis* cell walls have some ATPase activity (Figure 5.13). The ATPase activity of the purified cell walls was induced by up to 10 % when the cell walls preparations were spiked (*in vitro*) with 25 μg chitosan (a fungal elicitor) showing that the extracellular ATPase functions independent of plasma membrane and cytoplasmic influence. These results are consistant with the cell wall ATPase activity described by Kiba et al, (1996) in peas and cowpeas. The function of extracellular ATPases is likely to (directly or indirectly) affect the extracellular phosphorylation events. Having observed that chitosan increases the activity of the extracellular ATPases, it was expected that this treatment could affect the phosphorylation status of the extracellular proteins. This was tested by treating *Arabidopsis* cell growing in suspension culture with chitosan for 10 minutes. The cell wall proteins were extracted from purified cell walls of the treated cells using $CaCl_2$. The protein extracts were separated via 2-D PAGE, Western blotted onto a nitrocellulose membrane and probed with anti-phosphotyrosine antibodies. This brief treatment of *Arabidopsis* cells with chitosan induced a significant change in the profile of the cell wall phosphoproteins (Figure 5.14). Some (but not all) cell wall proteins were dephosphorylated following cell treatment with chitosan. These results taken together with the observed apyrase induced oxidative burst and cell death argue strongly that in plants, there exists a myriad of apoplastic ATP/apyrase regulated events (including protein phosphorylation) some of which are vital for survival, growth and development.

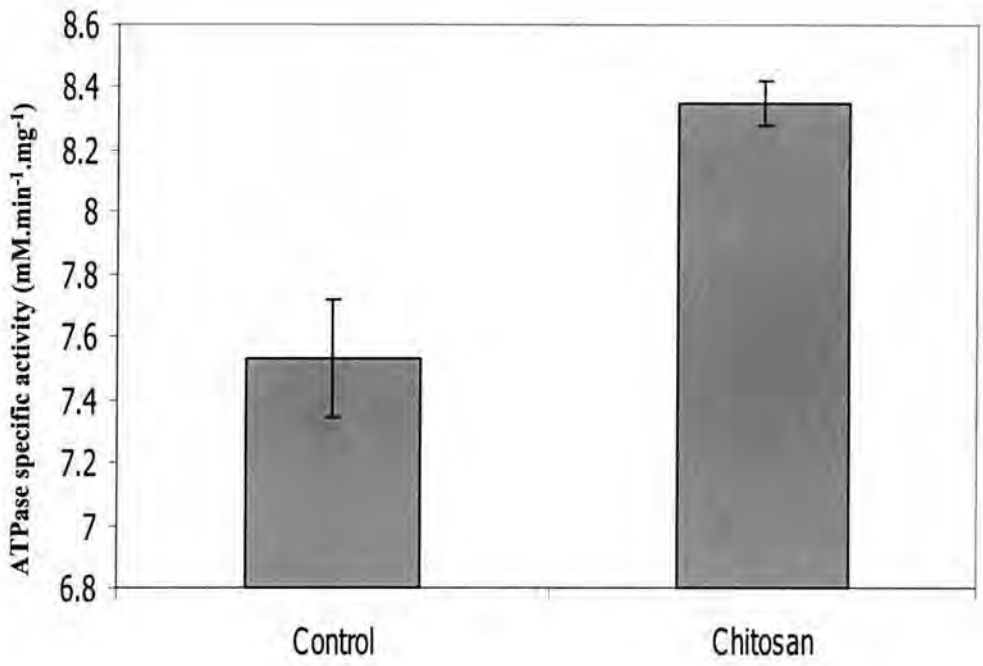


Figure 5.13 **The ATPase activity assay on pure cell walls**

The ATPase activity was measured in purified *Arabidopsis* cell walls before and after treatment with chitosan (in vitro). The specific activity of cell wall ATPase increased by up to 10 % when cell walls were spiked for 10 minutes with 25 μg chitosan. The error bars indicate the standard deviation of the mean.

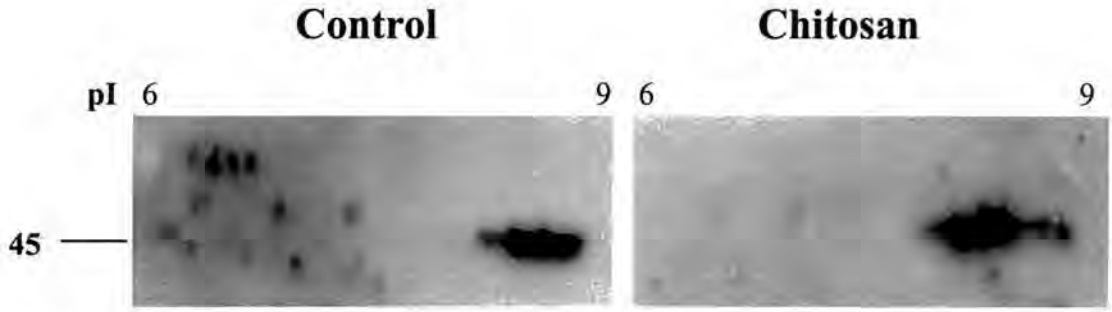


Figure 5.14 **Chitosan induces dephosphorylation to some cell wall proteins**

Proteins were extracted with $CaCl_2$ from the cell walls that were purified from *Arabidopsis* cells that were treated with 50 μg/mL chitosan for 10 min. The cell wall protein extracts were Western blotted, probed with anti-phosphotyrosine antibodies and detected via chemiluminescence on an X-ray film. The comparison of the 2-D phosphoprotein profile of cell walls from the treated control cells shows that chitosan induces dephosphorylation to some proteins.

5.3 Discussion

5.3.1 Plant Signal Transduction Cascade Begins Beyond The Plasma Membrane

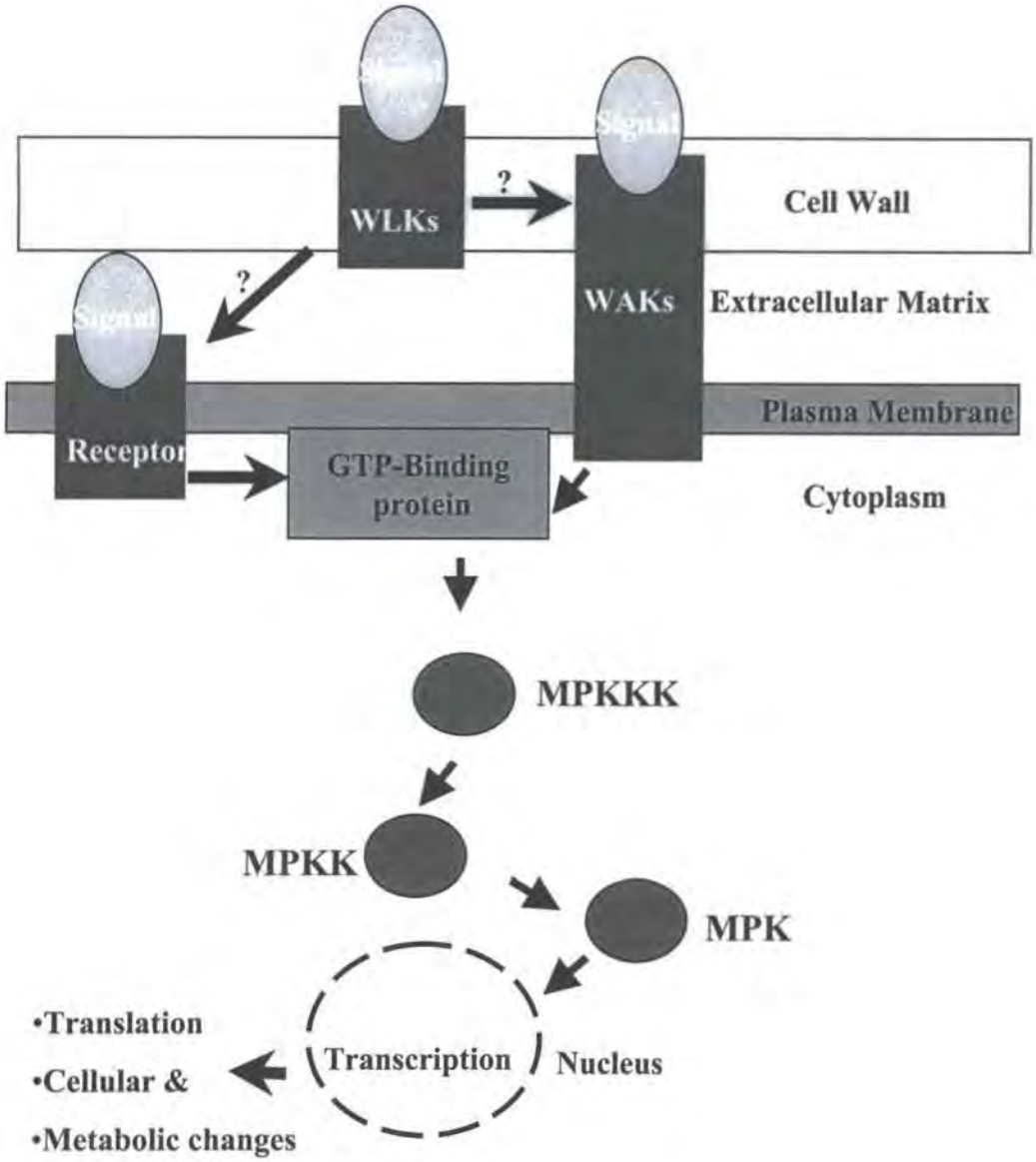
The generally accepted model for the signal transduction cascade shows that stimuli from the environment are perceived by transmembrane receptor protein kinases and these in turn trigger a cascade of kinase phosphorylation events (via GTP-binding proteins). This in turn activates transcription factors that are located in the nucleus. However, the discovery of cell wall located putative receptor-like protein kinases led to the speculation that in plant cells the extracellular stimulus is perceived by the cell wall and conveyed to membrane bound receptors. In the past this possibility was viewed as inconceivable because energy-rich compounds like ATP were generally presumed non-existent in the plant apoplasm (Eisenhaber and Bork, 1998). Additionally, the generally accepted signal transduction pathways are based on studies done in animal cells, and therefore this paradigm led to a bypass of the cell wall (because in animal cells the plasma membrane is the most peripheral compartment). The results shown in this chapter propose that in plants the origin of some signalling pathways might be at the cell wall level.

This proposed paradigm is consistent with the identification of cell wall associated receptor-like kinases (WAKs) reported by He et al., (1996) and reviewed by Kohorn, (2000) and Anderson et al., (2001). The evidence provided by the studies on WAKs indicate that they (WAKs) provide a direct signal from the cell wall to the cytoplasm in many plant cell types during cell development. WAKs have also been shown to play a major part in the regulation of cell expansion and defence against the

detrimental effects that are sustained during the pathogenesis response (He et al., 1998). The cell wall located kinases (WLKs described in chapter 4) and WAKs have similar features. Both kinases have a receptor-like domain that is associated with the cell wall. Also these kinases are predicted to be serine/threonine kinases and both are phosphoproteins. Their co-localisation in the cell wall suggests possible protein-protein communication between these two receptor-like cell wall associated kinase classes because, although it has been shown that WAKs are phosphorylated, their kinase(s) is (are) still to be uncovered. Although it is speculated that WAKs might be autophosphorylating (Anderson et al., 2001) it is equally possible that a separate class of kinases (e.g. WLKs) might be responsible for the phosphorylation of WAKs. A schematic diagram summarising this new concept is shown in Figure 5.15. Historically, however protein phosphorylation has never been known to occur in the extracellular matrix of plant cells. Hence, the work described in this chapter provides grounds for questioning this dogma.

Figure 5.15 **Proposed signal transduction cascade model.**

A schematic diagram showing a proposed plant signal transduction pathway model. A ligand (signal) from the extracellular matrix either binds the cell wall located or transmembrane receptor kinases. The cell wall located receptor-like protein kinases transduce the signal to the GTP binding proteins either via WAKs (He at al., 1996) and/or via other plasma membrane associated receptor kinases which then activates membrane associated GTP binding protein. The activated GTP binding protein in turn activates a cascade of mitogen activated protein kinase kinase kinases (MAPKKKs). MAPKKKs phosphorylate MAPKKs, which in turn phosphorylate MAPKs. The phosphorylation of MAPKs lead to the activation of transcription factors located in the nucleus (adapted from Yang, 1996).


Signal
WLKs
?
Signal
Cell Wall
?
WAKs
Extracellular Matrix
Signal
Receptor
Plasma Membrane
GTP-Binding protein
Cytoplasm
MPKKK
MPKK
MPK
•Translation
•Cellular &
•Metabolic changes
Transcription
Nucleus

5.3.2 The Plant Apoplasm Harbours ATP And This ATP is Vital For Cell Survival

Thomas et al, (1999) demonstrated using transgenic plants expressing extracellular ATPases that ATP is naturally secreted to the plant extracellular matrix. This demonstration, and the fact that the cell wall is the outermost compartment of the plant cell that harbours putative protein kinases, inspired the hypothesis of extracellular protein signal transduction in plants. The approach applied in this study towards proving this hypothesis was the destruction of the extracellular ATP pool in an attempt to disable or perturb the extracellular kinase activities. This was achieved by injecting two ATP hydrolysing systems, (1) the apyrase and (2) the hexokinase/glusose systems in the plant apoplasm. Few minutes after the application of these enzymes (apyrase or hexokinase/glucose) in the culture medium (which is equivalent to the apoplasm of the whole plant tissues) an increase in hydrogen peroxide (oxidative burst) was observed. In plant cells the oxidative burst phenomenon is normally associated with stress response (Bolwell et al., 1998). This observation confirmed the results of Thomas et al, (1999), that ATP is indeed secreted to the apoplasm of plant cells. Prolonged incubation of plant cells with an ATP removing agent (apyrase or hexokinase) led to cell death (in both suspension cultures and whole plant leaves). This observation indicated that extracellular ATP is required for the survival of plant cells. This observation might also explain the reason why exponentially growing cells enter a lag growth phase when transferred to a fresh growth medium. The fresh medium has no ATP and therefore the lag phase might allow the cell to make and secrete enough ATP required for cell growth. This is also in agreement with the sharp uptake of inorganic phosphate from the medium

during the first few hours following cell subculture to fresh growth medium (chapter 3).

5.3.3 The ATP Hydrolysis is Vital for Sustaining Plant Cell Viability

Extracellular ATP has been shown to be involved in the binding and activation of receptor-like transmembrane enzymes and channels (Haines et al., 2001). In order to verify that it is ATP hydrolysis, and not necessarily ATP binding that is required for cell survival, a non-hydrolysable ATP analogue was applied in the apoplast of the plant leaves to compete out the hydrolysable ATP. If ATP binding was the main requirement for the sustained cell viability, therefore the replacement of ATP with a non-hydrolysable analogue should not affect the cell viability. The application a non-hydrolysable ATP analogue in the apoplast of the plant leaves however, led to the development of necrotic cell death lesions. This showed that ATP hydrolysis is vital for cell survival. The cell death lesions were only confined to the area of the leaf where the non-hydrolysable ATP was infiltrated. This confinement of cell death was crucial because it indicated that the apoplastic-infiltrated agents (apyrase, hexokinase and non-hydrolysable ATP) did not access the cell symplasm (where all cells of a given tissue are interconnected via structures called plasmodesmata and are isolated from the apoplast by a plasmamembrane). Since the symplasm is a continuous interconnection of cells, then if the infiltrated agent accessed it, the induced necrotic lesions should spread beyond the infiltrated area of the leaf. The non-hydrolysable ATP induced cell death meant that ATP hydrolysis and perhaps protein kinase induced extracellular protein phosphorylation takes place in the plant

apoplasm. Hence, the identification of an extracellular located phosphoprotein would provide compelling evidence for demonstrating that protein kinase induced protein phosphorylation takes place in the plant extracellular matrix.

Thomas et al, (2000) showed in plant and yeast cells that the secretion of ATP and its subsequent hydrolysis was necessary for xenobiotic resistance. They demonstrated this by showing that mutant cells with lower ATP efflux and no subsequent ATP hydrolysis ability were more sensitive to xenobiotics. Those results support the results presented here. The mutants that have an impaired ATP secretion ability will have less than optimal extracellular phosphorylation, which might be required for resistance against the xenobiotics. When excess ATP was administered to the cell cultures a slight increase in cell death was observed, suggesting that cells are also sensitive to high extracellular ATP levels. It can therefore be speculated that plant cells might also be using extracellular ATP as a pathogen invasion (or wounding) surveillance mechanism. When a pathogen invades a plant tissue, the invaded group of cells are wounded and their cytosolic contents spill to the environment. This spillage will contain among other things, ATP. The neighbouring cells will sense the sudden rise in the ATP levels and this cue will instruct them to commit suicide (hypersensitive response). However, this speculation might be difficult to prove since other wounding products, like cell wall polysaccharide fragment and small peptides are also known to induce pathogen responses in plant cells (Reymond et al., 1995). In plants, cell death is also an integral part of growth and development. The best example is given by the function of the xylem vessels. Xylems are the specialised cells of the plant vascular system, which start off as living cells and when they reach maturation they die to form tube like structures that are responsible for

conducting water (and other water soluble inorganic ions) from the roots to the rest of the plant body, and also provide mechanical strength required for plant rigidity. Since the xylem is part of the apoplasm it is possible that some of the control for its development is located within the apoplast.

5.3.4 The Identification of the First Classical Cell Wall Tyrosine Phosphoprotein

A proteomic approach was applied to search for cell wall phosphoproteins. Due to the low abundance of cell wall proteins relative to cytosolic proteins in plant cells, a large amount of cell material was necessary for the cell phosphoproteome studies and in order to prevent artefacts due to inorganic phosphate spiked samples, radioactive phosphate protein labelling was not a practical option. As an alternative a phosphoprotein specific antibody technique was applied. Phosphoprotein antibodies have been applied in many animal signal transduction studies (Susa and Marre, 1999; Lavin et al., 1994; Bangalore et al., 1992). A phosphotyrosine basic endochitinase was identified (via MALDI-ToF) among 20 cell wall phosphoprotein protein spots visualised via an autorad of a mini 2-D PAGE. This represents the first report of the existence of a phosphorylated bona fide cell wall protein. The identity of this endochitinase was verified by tanderm mass spectrometry, which is by far the most informative protein identification method, in that it is not only able to provide the amino acid data, but also their post-translational modifications, like phosphorylation, glycosylation, oxidation etc (Kinter and Sherman, 2000). The predicted phosphorylation sites will be confirmed with tanderm mass spectrometry and may provide new targets for site directed mutagenesis. Chitinases are among one of the

well-characterised pathogen related proteins and this additional post-translational modification may provide a contribution towards the understanding of plant-pathogen interactions. The second cell wall located phosphoprotein identified in this study share 50 % homology to receptor-like protein kinases and has no transmebrane domain. Similar to the mitogen activated protein kinase 6 (MAPK 6), the first phosphoprotein to be identified in *Arabidopsis* (Nühse et al., 2000) and the autophosphorylating cell wall-associated kinases (WAKs; Anderson et al., 2001), this putative protein is likely to play a major part in primary stimulus perception and signal transduction, possibly by phosphorylating transmembrane protein kinases such as WAKs.

5.4 Concluding Remarks

The evidence provided in this chapter has proved that in plants (as in animals) extracellular phosphorylation does take place. The evidence given, also shows that the extracellular protein phosphorylation is imperative for cell survival, and probably cell growth and development.

Many cellular activities are regulated by protein phosphorylation/dephosphorylation and therefore the characterisation of the *Arabidopsis* extracellular protein kinases, phosphatases and their target proteins will without a doubt bring about more understanding of extracellular and intracellular plant metabolic activities. Despite the enormous potential roles that can be played by these proteins only two *Arabidopsis* phosphoproteins have been identified (Peck et al., 2001; Nühse et al., 2000). Hence the identification of 3 tyrosine phosphoproteins in this study is a

significant contribution to plant biology studies. Tyrosine specific protein kinases are still to be identified in plants, therefore the identification of the novel cell wall located tyrosine phosphoprotein supports the theory of the existence of these kinases. However, some kinases such as mitogen activated kinase kinases (MAPKKs) have been shown to have dual specificity, that is, they are capable of phosphorylating both the serine/threonine and tyrosine amino acid residues (Yang, 1996). The exact role of extracellular ATP in maintaining cell viability and the possible involvement of apoplastic signalling will require further experimentation, but it clearly represents additional targets of controlling cellular events, which have not been reported previously.

Chapter 6

Identification and characterisation of rapid elicitor responsive *Arabidopsis* cell wall proteins

6.1 Introduction

Pathogenesis-related proteins are one of the most studied plant protein families. These proteins were first observed in interactions between tobacco and tobacco mosaic virus (TMV) (van Loon et al., 1970). They were initially thought to be the products of proteolytic breakdown of larger proteins, which accompanied pathogen invasion. Subsequently these proteins were found to be matured proteins that are induced in a highly co-ordinated and consistent manner. In order to facilitate their study a unifying nomenclature "pathogenesis-related" (PR) was introduced (reviewed in Swapan and Muthukrishnan, 1999). The term pathogen-related proteins refers to a collective set of defence associated proteins that are coded by the host plant, but are only induced during pathogen invasion particularly in incompatible interactions (Swapan and Muthukrishnan, 1999).

PR proteins are divided into 11 families, namely: - PR-1 to PR-11 (Table 6.1). All the recognised PR proteins are secreted to the extracellular matrix upon elicitation (except PR-10). The localisation of the PR proteins to the extracellular matrix is a logical strategy that ensures their exposure to the invading pathogen before it is able to penetrate the cell. Most PR proteins are pathogen cell wall degrading enzymes (chitinases and glucanases) and it was therefore expected that their over expression in the host plant would render the plant more resistant to the pathogen invasion. Over the past decade a number of transgenic plants carrying a PR gene(s) have been constructed. The over-expression of a chitinase gene under the constitutive control of 35S promoter from cauliflower mosaic virus in tobacco and *Brassica napus* has

Table 6.1. Families of pathogenesis-related proteins (adapted from Swapan and Muthukrishnan, 1999)

Family	Properties
PR-1	Unknown
PR-2	b-1,3- glucanase
PR-3	Chitinase type I, II, IV, V, VI andVII
PR-4	Chitinase type I and II
PR-5	Thaumatin-like protein
PR-6	Proteinase-inhibitor
PR-7	Endoproteinase
PR-8	Chitinase type III
PR-9	Peroxidase
PR-10	Ribonuclease-like protein
PR-11	Chitinase type I

been shown to reduce the formation of pathogen symptoms caused by *Rhizoctonia solani* infection (Broglie et al., 1991). Constitutive expression of chitinase in *Nicotiana sylvestris* increased resistance to *Rhizoctonia solani* (Swapan and Muthukrishnan, 1999). More recently, tomato plants that were transformed with a combination of chitinase and β-1.3-glucanase were shown to be more resistant to *Fusarium oxysporium* (Reviewed in Swapan and Muthukrishnan, 1999).

It has however, been shown that over expression of PR protein in a host plant does not always suppress pathogen colonisation (Reviewed in Swapan and Muthukrishnan, 1999). PR proteins are not universally effective in conferring resistance to pathogen attack, for example, while some chitinases are effective as a defence mechanism in some hosts, they are not effective against all chitin-containing fungi (Swapan and Muthukrishnan, 1999). Questions of whether the constitutive expression of chitinase alone is responsible for the increased resistance against fungal pathogens are beginning to surface. These questions have been formulated because in nature, the induction of PR proteins is the final product of a complex signal transduction cascade that is normally initiated by the interactions between pathogen elicitors in the extracellular or intracellular matrix and receptor proteins. For example, it is now known that a number of molecules, like chitin fragments, glucans, avirulence gene products, oligosaccharides and small peptides derived from either the invading pathogen or the host plant during plant-pathogen interactions can serve as elicitors for PR gene induction (Peck et al., 2001). Unlike the number of known elicitors, very few plant extracellular signal receptors have been characterised in detail. Thus far, only about 7 plant receptor-like proteins have been studied to some detail (including Xa21, Ronald et al., 1992; CLAVATA1, Clark et al., 1997;

BRI1, Clouse et al., 1996; ERECTA, Torii et al., 1996; CRINKLY4, Becraft et al., 1996; FLS2, Gomez-Gomez and Boller. 2000; HAESA, Jinn et al., 2000). Despite the attempts that have been made over the past few years, the downstream targets of most these transmembrane receptors are still to be established (Peck et al., 2001).

Much attention has been devoted to the study of pathogen induced responses of cell wall structural proteins (Bradley et al., 1992; Showalter, 1993; Brisson et al., 1994; El-Gendy et al., 2001), however, the emerging evidence strongly suggests that the cell wall does not solely act as a physical barrier in response to pathogen attack. He et al, (1996) reported the first autophosphorylating cell wall associated receptor-like protein kinase (WAK), suggesting that the cell wall might be an integral part of the signal perception and signal transduction. The potential roles of WAKs in signal transduction have since been gaining more scientific attention (He et al., 1998, 1999; Kohorn, 2000; Anderson et al., 2001; Lally et al., 2001).

The work reported in this chapter attempts to investigate early protein responses to pathogen invasion. In order to simplify the complexity of this interaction, *Arabidopsis* cell cultures and chitosan (a general fungal pathogen elicitor) were employed. In the light of the results in chapter 4 and 5 of this thesis, which show novel cell wall located putative receptor-like kinases and phosphoproteins, special attention has been directed to early (within 10 minutes) responses of cell wall located proteins to treatment with chitosan.

6.2 Results

6.2.1 Fungal pathogen elicitors induce oxidative burst and alkalinisation of the external medium in *Arabidopsis* cell cultures

The oxidative burst, a sudden accumulation of reactive oxygen species, is one of the earliest events that occur in many plant species following invasion by avirulent pathogen attack (Wojtaszek, 1997; Lamb and Dixon 1997; Bolwell et al., 1999; Baier et al., 1999; Heath, 2000). In order to evaluate whether a pathogen response was initiated in *Arabidopsis* cell cultures following a simulated pathogen invasion using chitosan and a *Fusarium moliniforme* derived elicitor, accumulation of H_2O_2 was monitored. The H_2O_2 levels in the cells were monitored with diaminobenzidine (DAB), a reagent that turns brown in the presence of H_2O_2. Within 30 minutes following elicitation the cells started to turn brown (Figure 6.1). In order to demonstrate that the brown coloration in *Arabidopsis* cells was due to the accumulation of H_2O_2, the cells were treated with ascorbic acid (AA; a H_2O_2 scavenger) and the fungal elicitor simultaneously. When the cells were challenged with elicitor and AA concurrently the appearance of brown coloration was blocked (Figure 6.1D). This observation confirmed that chitosan and a *Fusarium* elicitor induced an oxidative burst in cell cultures of *Arabidopsis*.

Fungal elicitors are also known to induce an increase in the pH of the external medium (alkalinisation) in some plant cell cultures (Felix et al., 1991, 1993). Following treatment with chitosan and *Fusarium* the pH of the external medium was measured. The observations showed a significant increase in the pH of the cell

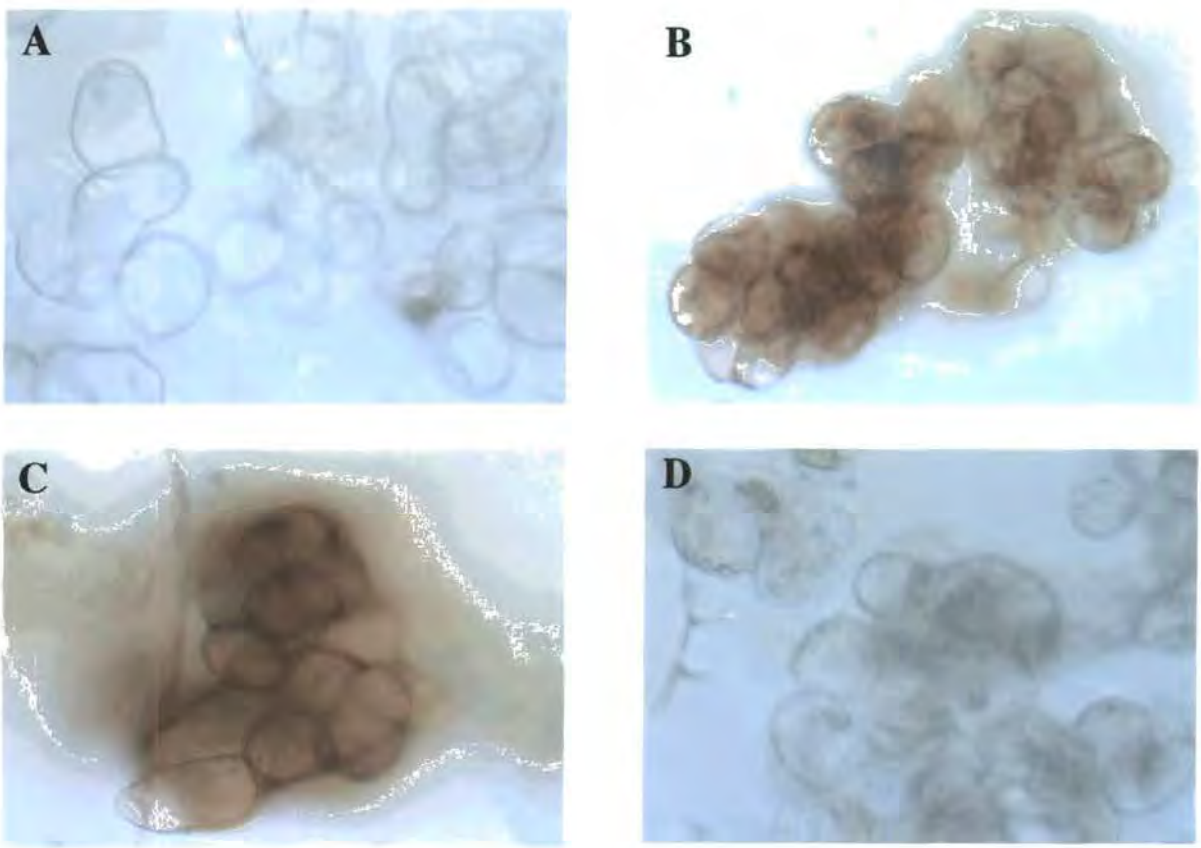


Figure 6.1. **Elicitor induced generation of H_2O_2 in *Arabidopsis* cell cultures**

Three day old *Arabidopsis* cells were treated with a combination of diaminobenzidine (DAB) and (**A**) water, (**B**) chitosan elicitor (**C**) *Fusarium moliniforme* elicitor and (**D**) a combination of *Fusarium moliniforme* elicitor and ascorbic acid (AA) for 1 hour. DAB turns brown in the presence of hydrogen peroxide (H_2O_2).

culture medium (Figure 6.2). Alkalinisation was also observed when *Arabidopsis* cells were spiked with 4 mM of exogenous H_2O_2 (Figure 6.2). This observation suggested that the oxidative burst might be upstream of alkalinisation. Downstream of oxidative burst and alkalinisation of the external medium, genes that are associated with pathogenesis are normally induced during pathogen invasion. Therefore the activity of phenylalanine ammonia-lyase and the induction of glutathione-*S*-transferase was measured in chitosan treated *Arabidopsis* cells.

6.2.2 Chitosan induces phenylalanine ammonia-lyase activity and the accumulation of phi glutathione *S*-transferase in *Arabidopsis* cell cultures.

The accumulation of phytoalexins has been established as the most common plant cell response to microbial pathogen attack. Phytoalexins are defined as low molecular weight, antimicrobial compounds that accumulate in plant cells following exposure to pathogen elicitors. The first enzyme of the phytoalexin biosynthesis pathway is L-phenylalanine ammonia-lyase (PAL). PAL is induced by a number of environmental stimuli (Bolwell et al., 1985), and is also a reliable maker of pathogen responses in plant cells (Bolwell and Rodgers, 1991). The activity of PAL, was used here as a marker for gene activation following *Arabidopsis* treatment with chitosan. Cells were treated with chitosan and allowed to grow for 24 hours. Cells were harvested 24 hours post treatment and the activity of PAL in the total soluble protein extracted measured. Activity was monitored by measuring the rate of [^{14}C]-phenylalanine conversion to [^{14}C]-cinammic acid. The activity of PAL increased approximately 2.5 fold when the cells were treated with chitosan for 24 hours compared to control cells (Figure 6.3). The increase in PAL activity confirmed

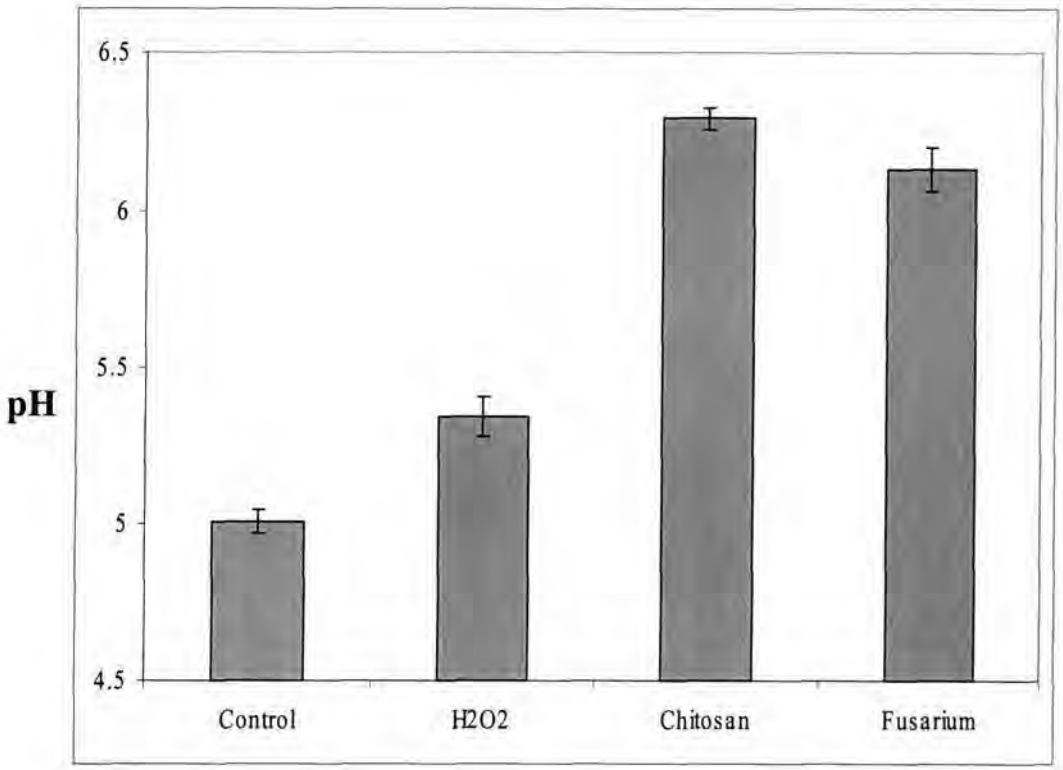


Figure 6.2 **Elicitor induced alkalinisation in *Arabidopsis* cell suspensions.**

Three days post-subculture *Arabidopsis* cell suspension cultures were treated with water (Control), 4mM hydrogen peroxide (H_2O_2), 200 μg/mL chitosan and 200 μg/mL *Fusarium Moniliforme*. The pH of the external medium was measured 1 hour post-elicitation and is represented by the bars in the panel above. Each bar represents the average of three independent replicates and the error bars are standard deviation from the mean.

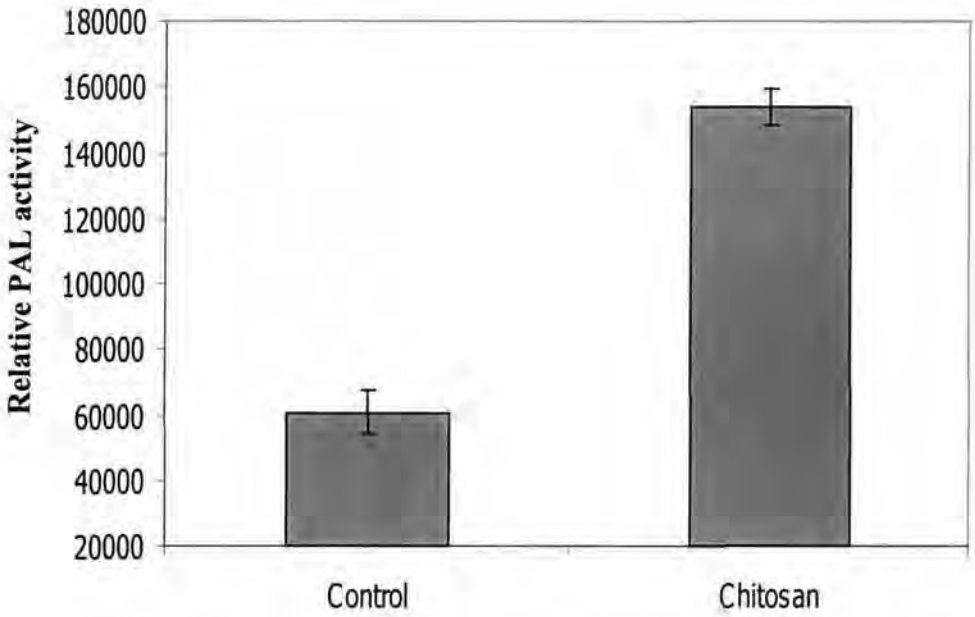


Figure 6.3 **PAL activity in chitosan treated *Arabidopsis* cell cultures**

Arabidopsis cells were treated with water (control) and 200 μg/mL chitosan for 24 hours. The activity of phenylalanine lyase (PAL) in 20 μg of total soluble protein was measured by monitoring the rate of [^{14}C]-phenylalanine conversion to [^{14}C]-cinammic acid. The relative increase in PAL activity is 2.5 fold compared to the control.

the use of chitosan as an elicitor of pathogen response in *Arabidopsis* cell cultures in this study.

Glutathione *S*-transferases (GST), are enzyme responsible for detoxifying xenobiotics by catalysing their conjugation with the tripeptide glutathione (Edwards et al., 2000). These enzymes are coded by a diverse gene family and are divided into 5 classes (phi, tau, theta, zeta and lambda) according to gene sequence identity (reviewed in Dixon et al., 2002). Phi and tau GST's are known to be highly induced and act as antioxidants during an incompatible interaction between plants their pathogens (reviewed in Dixon et al., 2002; Mullineaux et al., 2000). The accumulation of GST has been used in these studies as a maker for pathogen invasion. *Arabidopsis* cells were treated with chitosan and *Fusarium moliniforme* elicitor for 24 hours. The soluble protein was extracted from the treated and control cells and resolved via 2-D PAGE, the gels were stained with Coomassie brilliant blue (Figure 6.4). These results show that both chitosan and the *Fusarium moliniforme* induced the accumulation of a putative phi GST. The identification of two spots for the phi GST is a typical dimerisation characteristic of most GST's (Dixon et al., 2002). The results presented here, such as the oxidative burst, alkalinisation, PAL and GST inductions demonstrate that the interaction between *Arabidopsis* cells and chitosan give the predicted plant-pathogen interaction responses.

6.2.3 Chitosan induces a dose-dependent hypersensitive response in *Arabidopsis* cell cultures

The hypersensitive response, i.e. pathogen induced cell death, is one of the natural responses of plant cells to microbial pathogens (Lamb et al., 1989). The induction of

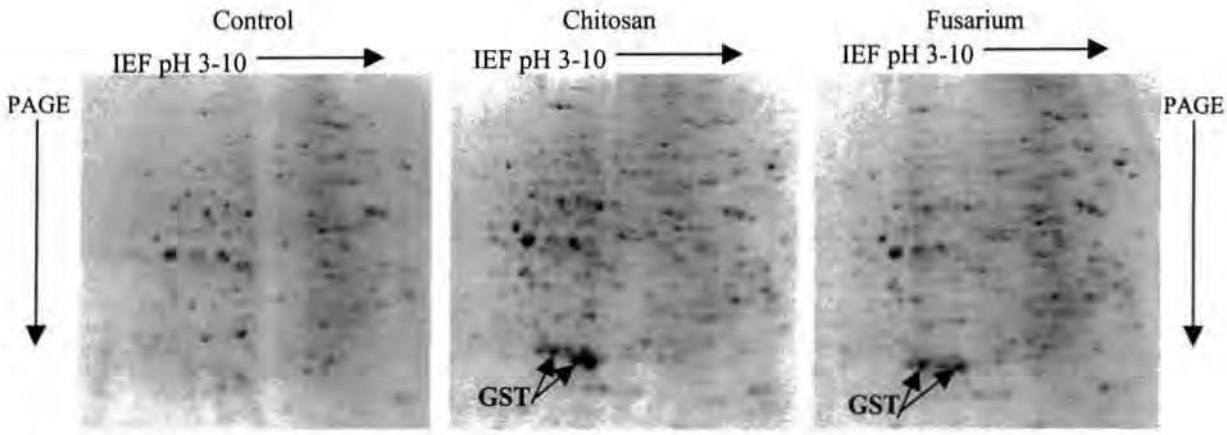


Figure 6.4 **Coomassie stained 2-D gel of *Arabidopsis* soluble proteins**

Arabidopsis cell cultures were treated with water (control), 50 μg/mL chitosan and 200 μg/mL *Fusarium moliniforme* elicitor for 24 hours. From each treatment 1 mg of soluble proteins was resolved via Large format 2-D PAGE, pH 3-10 in the first dimension and 12 % PAGE in the second dimension. All the gels were stained equally with Coomassie brilliant blue and phi glutathione *S* transferase spots identified via MALDI-ToF are indicated with arrows.

phi GST have all been shown in chitosan treated *Arabidopsis* cells (6.2.1; 6.2.2). The oxidative burst however, has been shown to initiate the development of hypersensitive response (Levine et al., 1994). Cell death was therefore measured in *Arabidopsis* cells that were treated with 200 µg/mL chitosan for 24 hours. The cell death was quantified by measuring the amount of Evans blue that was taken up by the cells (Levine et al., 1994). A drastic increase in cell death was observed in chitosan treated cells (Figure 6.5). The evidence shown here indicated therefore that *Arabidopsis* cells are responding to chitosan in a typical plant pathogen interaction response fashion.

The aim of this chapter was to use the *Arabidopsis*-chitosan experimental system as a model to study the response of cell wall proteins to pathogen invasion. In order to reduce the non-specific protein changes following elicitation, a chitosan dose assay was conducted. The aim of the assay was to find the amount of chitosan that was high enough to induce pathogen responses without causing cell death. For this the cells were treated with 10 µg/mL, 20 µg/mL and 50 µg/mL of chitosan for 24 hours. The cell viability cells and the pH of their external medium were determined. The viability of the cells and the pH of the external medium were not affected when cells were treated with 10 µg/mL and 20 µg/mL chitosan. However, 50 µg/mL chitosan induced external medium alkalinisation although cell viability was not affected (Figure 6.6). These results showed that 50 µg/mL chitosan was sufficient to elicit pathogen response in *Arabidopsis* cells without affecting the viability of the cells.

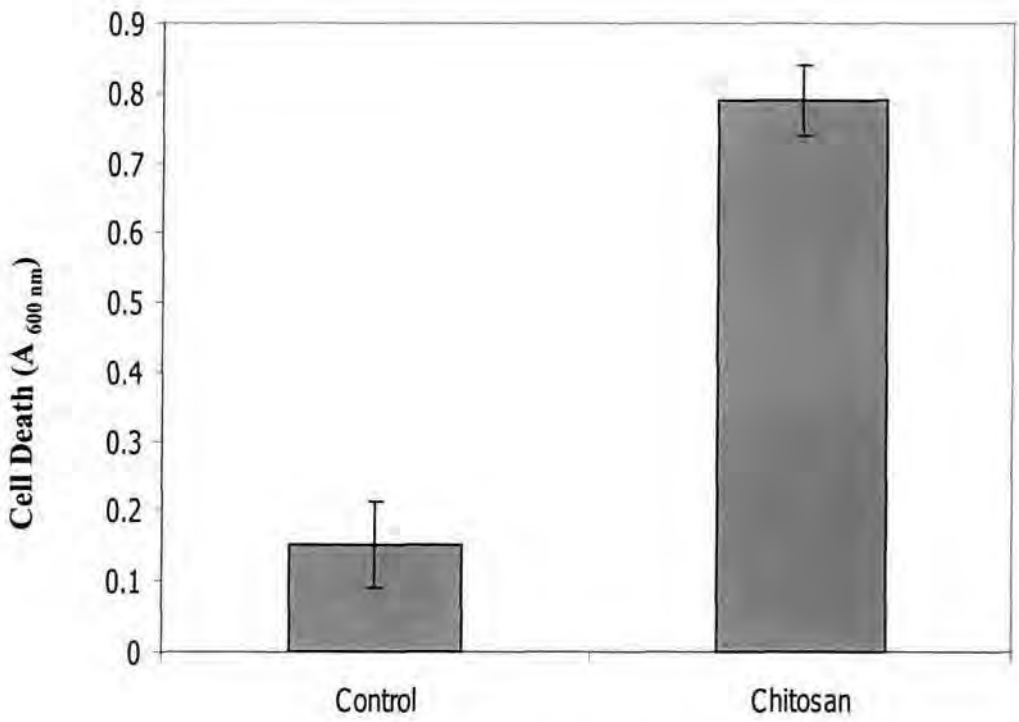


Figure 6.5 **Effect of chitosan on *Arabidopsis* cell viability**

Three days post subculture *Arabidopsis* cells were treated with 200 μg/mL chitosan for 24 hours. The treated and the control cells were stained with Evan's blue. The Stain was eluted and its absorbance at 600 nm, which is proportional to cell death, was measured. The error bars are standard deviation from the mean of three replicate experiments.

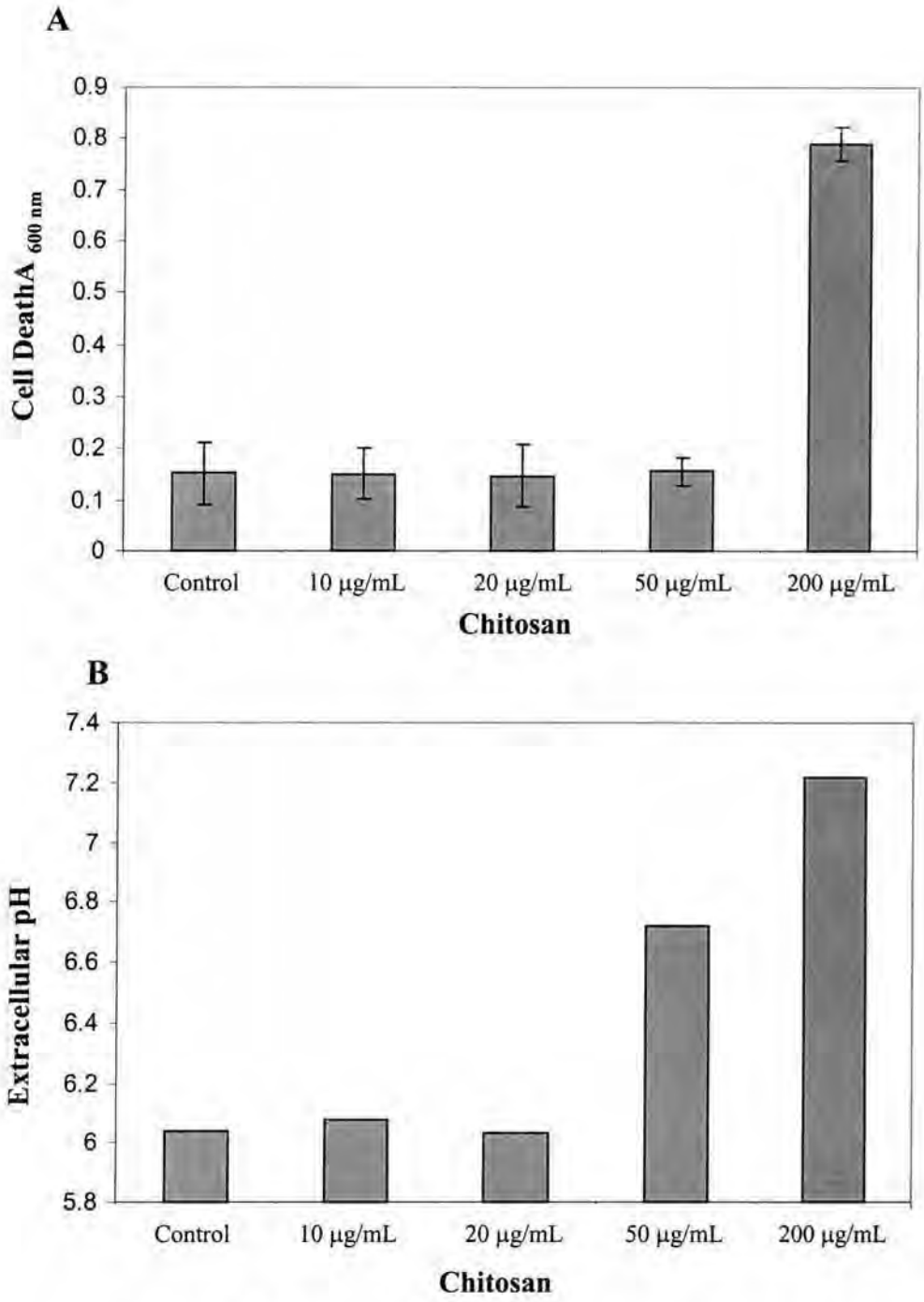


Figure 6.6 **Effect of chitosan on *Arabidopsis* cell viability and medium alkalinisation.**

Arabidopsis cells that were subcultured and grown for 3 days were treated with water (control) and various concentrations of chitosan (indicated in the panels above). (A) Cell death at 24 hours post elicitation and (B) extracellular medium pH measured 1-hour post elicitation using Evans blue staining method and a pH probe respectively. The error bars indicate a standard deviation from the mean of three replicates.

6.2.4 Effects of chitosan in the proteome and phosphoproteome of *Arabidopsis* cell walls

Having demonstrated that the chitosan elicitor induces the expected pathogen responses in *Arabidopsis* suspension cultures, the response of the cell wall proteome to chitosan treatment was examined. *Arabidopsis* cells were subcultured and grown for 3 days. The 3 day old cells were treated with 50 μg/mL chitosan for 10 minutes. The cell walls were purified from the treated cells and their proteins were extracted with $CaCl_2$. The protein extracts were precipitated with 80 % acetone at –20 °C overnight. The protein precipitates were collected by centrifugation and the protein pellets were dried with filtered air. The dried protein pellets were resolubilised in 2-D lysis buffer (see materials and methods). $CaCl_2$ protein extracts were loaded onto isoelectric focusing (IEF) strips using the in-gel reswelling method. The reswelled strips were subjected to isoelectric focusing. Following IEF the proteins in the strips were transferred to 12 % PAGE for the second dimension. The polyacrylamide gels were stained with Coomassie brilliant blue. The 2-D profile of the cell wall proteins extracted from the chitosan treated cells was compared to the profile the cell walls extracted from the control cells (spiked with equivalent volume of water for 10 min). A duplicate set of treated and non-treated cell wall protein extracts were also separated via 2-D SDS-PAGE. The duplicate gels were Western blotted onto nitrocellulose membranes and probed with phosphotyrosine specific antibodies. Chitosan induced rapid (within 10 minutes) changes in the abundance of some protein spots (Figure 6.7A). Also the status of phosphorylation in tyrosine phosphoproteins was affected (Figure 6.7). The proteins that responded reproducibly

(in at least 3 independent experiments) to chitosan elicitation are marked with numbered arrows. These were excised from the gels manually, proteolysed with trypsin and analysed via MALDI-ToF. So far 7 chitosan responsive cell wall proteins and 4 chitosan responsive cell wall tyrosine phosphoproteins have been positively identified with MALDI-ToF (Table 6.2 and 6.3). The abundances of some protein spots were induced. This includes the receptor-like protein kinase-like protein (spot 1 and 2; AT1g78850), the endochitinase (spot 5; AT3g12500), the polygalacturonase inhibiting protein (spot 6; AT5g06870) and the probable apospory-associated protein (spot 7; AT4g25900). The abundance of an unknown protein (spot 3 and 4; AT3g08030) however, was partially suppressed.

The phosphorylation status of some proteins responded to chitosan treatment proportionally to their Coommassie staining abundances. For example, the endochitinase and the polygalacturonase inhibiting protein (PGIP) were induced at Coomassie level and their phosphorylation was also increased. Also as the abundance of the unknown protein was suppressed its phosphorylation status was also reduced. The receptor-like protein kinase-like protein (spot 1 and 2) however responded differently. While the protein was dephosphorylated by chitosan treatment its abundance at Coomassie level was induced. Also a number of (so far unidentified) proteins were affected by the chitosan treatment, some both at Coommassie and at phosphorylation level (Figure 6.7).

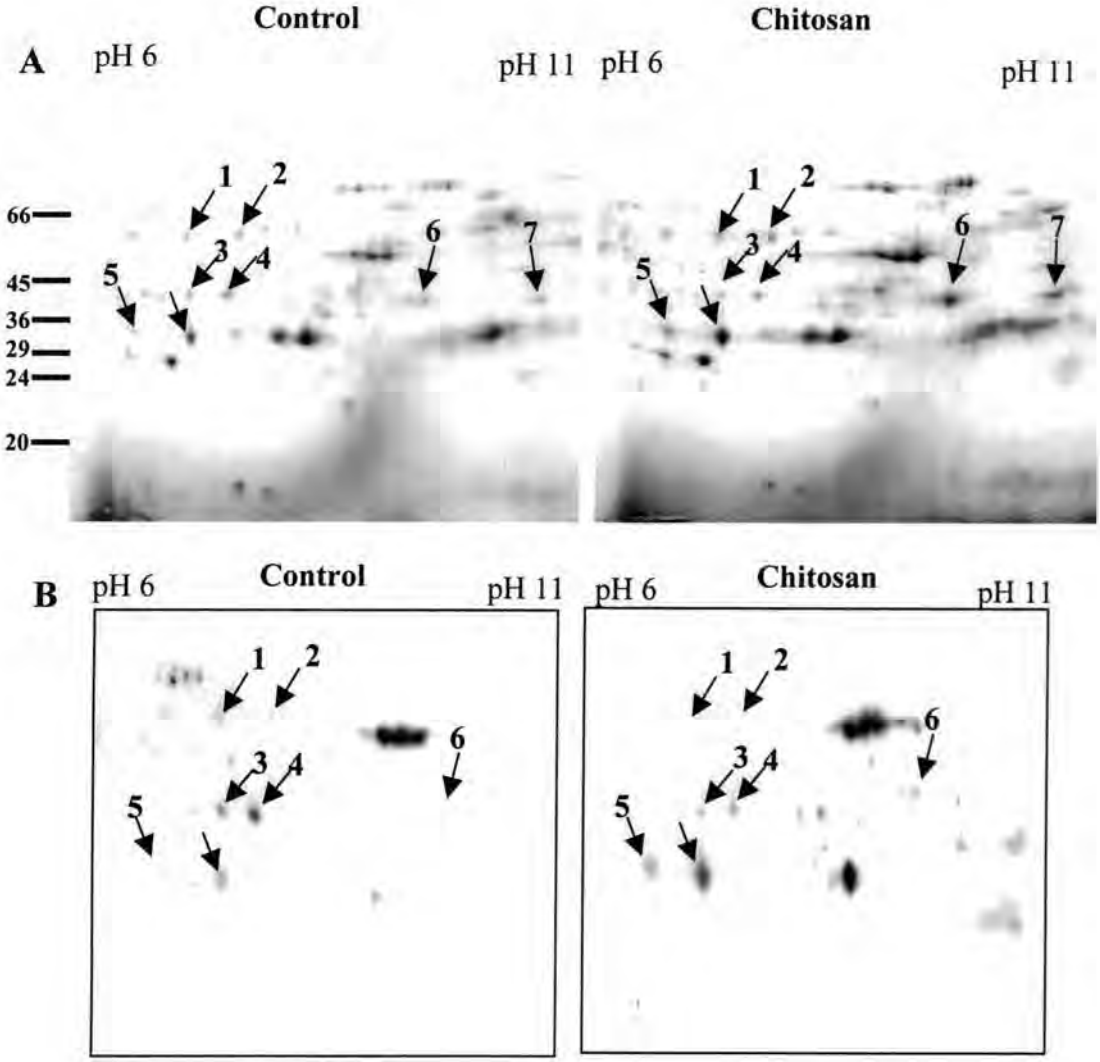


Figure 6.7. **Cell wall proteome and phosphoproteome of chitosan treated *Arabidopsis* cell cultures**

(A) *Arabidopsis* cells cultures were grown for 3 days followed by treatment with chitosan for 10 min. The cell walls were purified from the treated and control cells and their proteins were extracted using $CaCl_2$. The $CaCl_2$ extracts were separated via 2-D PAGE, using pH 6-11 IEF in the first dimension and 12 % PAGE in the second dimension. The gels were then stained with Coommassie brilliant blue.

(B) A duplicate of cell wall proteins separated via 2-D as in (A), Western blotted and probed with anti-phosphotyrosine antibodies. The labelled protein spots detected via chemilumniscence on an X-ray film. The molecular weight markers are in kilodaltons (kDa), and the numbered arrows are marking the identified proteins.

Table 6.2. Cell wall proteins whose abundance and/or phosphorylation status rapidly increase or decrease following elicitation with a fungal pathogen elicitor

Spot No.	Protein Identity, and MIPS Accession No.	MOWSE Score	Induced/ Suppressed	Phosphorylation status
1 & 2	Receptor-like protein kinase-like protein AT1g78850	7.03E+02	Induced	Complete dephosphorylation
3 & 4	Unknown Protein, AT3g08030	2.36E+04	Suppressed	Partial dephosphorylation
5	Basic endochitinase, AT3g12500	2.53E+03	Induced	Phosphorylation induced
6	Polygalacturonase Inhibiting Protein, AT5g06870	1.14E+03	Induced	Phosphorylation induced
7	Probable apospory-associated protein, AT4g25900	1.43E+06	Induced	N/A

Table 6.3. Chitosan induced and suppressed cell wall proteins

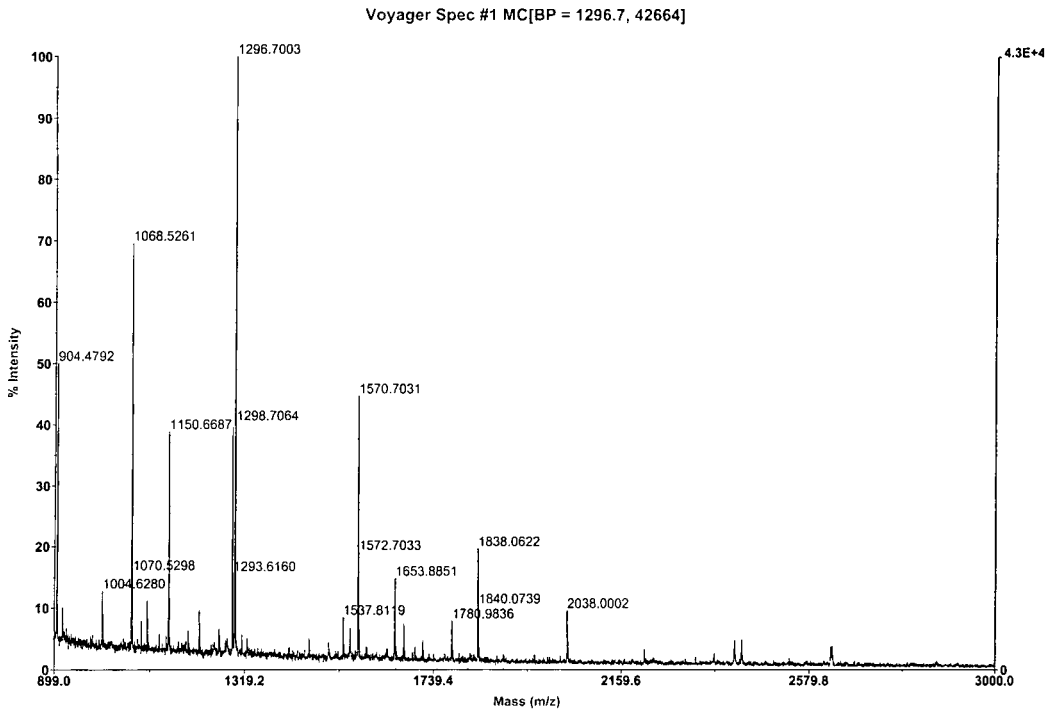
Spot No.	Protein Identity, and MIPS Accession No.	MOWSE Score	Induced/Suppressed
1 & 2	Receptor-like protein kinase-like protein AT1g78850	2.57E+04	Induced
3 & 4	Unknown Protein, AT3g08030	4.28E+05	Suppressed
5	Basic endochitinase, AT3g12500	2.53E+03	Induced
6	Polygalacturonase Inhibiting Protein, AT5g06870	1.14E+03	Induced
7	Probable apospory-associated protein, AT4g25900	4.97E+02	Induced
8	Hypothetical protein (serine carboxy peptidase-like protein), AT1g28110	6.10E+03	Suppressed
9	Unidentified		

6.2.5 Polygalacturonase inhibiting protein is rapidly induced following chitosan treatment.

Polygalacturonase inhibiting protein (PGIP) is a classical cell wall protein (Powell et al., 2000) and it is induced rapidly (within 10 minutes) in the cell walls of *Arabidopsis* following chitosan treatment (spot 6; Figure 6.7). PGIP's are among the most studied cell wall proteins and have been found in all dicotyledonous plants that have been examined (Yao et al 1999; Mattei et al., 2001). Genes coding for PGIP have been cloned from several species including apples, soybean and tomato (Yao et al 1999; Favaron et al 1994; Stoz et al 1994; Toubart et al 1992) however, the rapid changes in phosphorylation and abundance in the cell wall (which seem to be independent of transcription) have never been reported to date. The MALDI-ToF peptide mass ion spectrum of the identified PGIP is shown in Figure 6.8 and the corresponding peptides are shown in Table 6.2. The peptide coverage in the identified PGIP is shown in Figure 6.8B.

The identified PGIP was confirmed via tandem mass spectrometry. The PGIP spot (spot 6 Figure 6.7 A) was excised from the gel manually and digested with trypsin. The tryptic digests prepared from spot 6 were sprayed and ionised in the Q-TOF instrument (Genomic Solutions) via an elecrospray needle. Seven doubly charged peptide mass ions (1067.5036; 1089.5818; 1144.5223, 1218.6608, 1290.5669, 1536.7824 and 1837.0097) were selected for further fragmentation and the mass spectra of the generated b and y ions including the corresponding amino acid sequence tags are shown in Figure 6.9.

A.



B.

MDKTMTLFLLLSTLLLTTSLAKDLCHKDDKTTLLKIKKSLNNPYHLASW
DPKTDCCSWYCLECGDATVNHRVTSLIIQDGEISGQIPPEVGDLPYLTSLIF
RKLTNLTGHIQPTIAK**LKNLTFL**RLSWTNLTGPVPEFLSQLKNLE**(Y)**IDLS
FNDLSGSIPSSLSSLRKLE**(Y)**LELSRN**KLTGPIPESFGTFSGKVPSLFLSH**
NQLSGTIPKSLGNPDFYRIDLSRNKLQGDASILFGAKKTTWIVDISRN
MFQFDLSKVKLAKTLNNLDMNHNGITGSIPAEWSKAYFQLLNVSYNRL
CGR**IPKGE(Y)IQRFDS(Y)SFFHNK**C LCGAPLPSCK

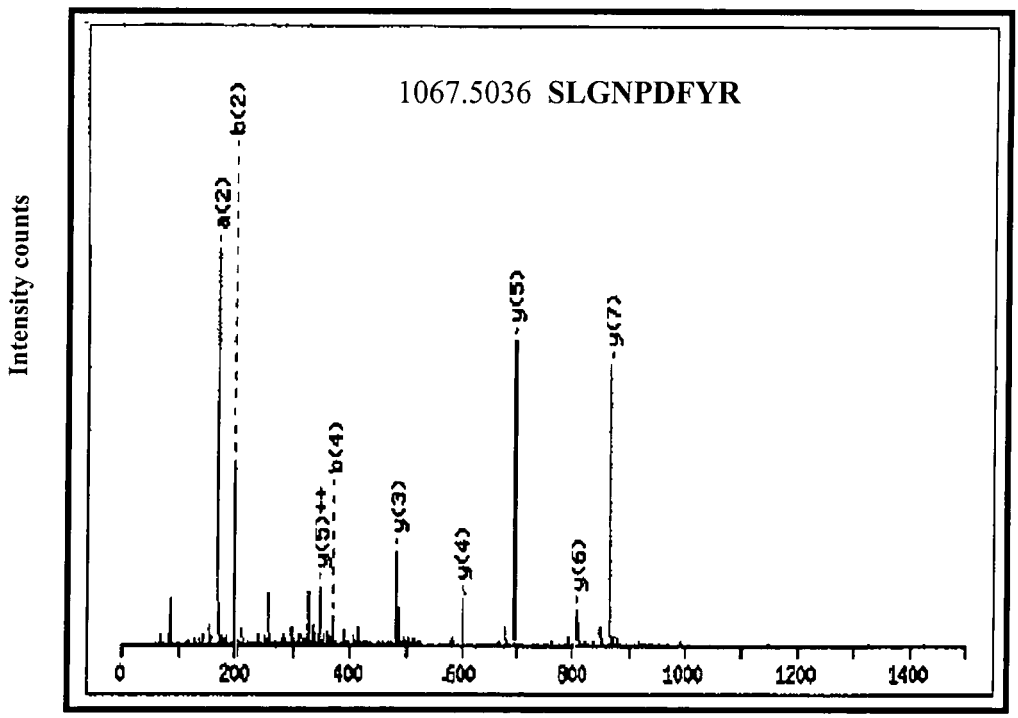
Figure 6.8 **MALDI-ToF spectrum of polygalacturonidase inhibiting protein**

Spot 6 (Figure 6.7) was picked from the gel and digested with trypsin. The tryptic digests were analysed via MALDI-ToF and the detected peptide mass spectrum is shown in (A). The corresponding peptide sequences are shown in Table 6.4. (B) The identified polypeptide and the peptide coverage is shown (bold). The predicted signal peptide (bold italics) and tyrosine phosphorylation sites **(Y)** are also highlighted.

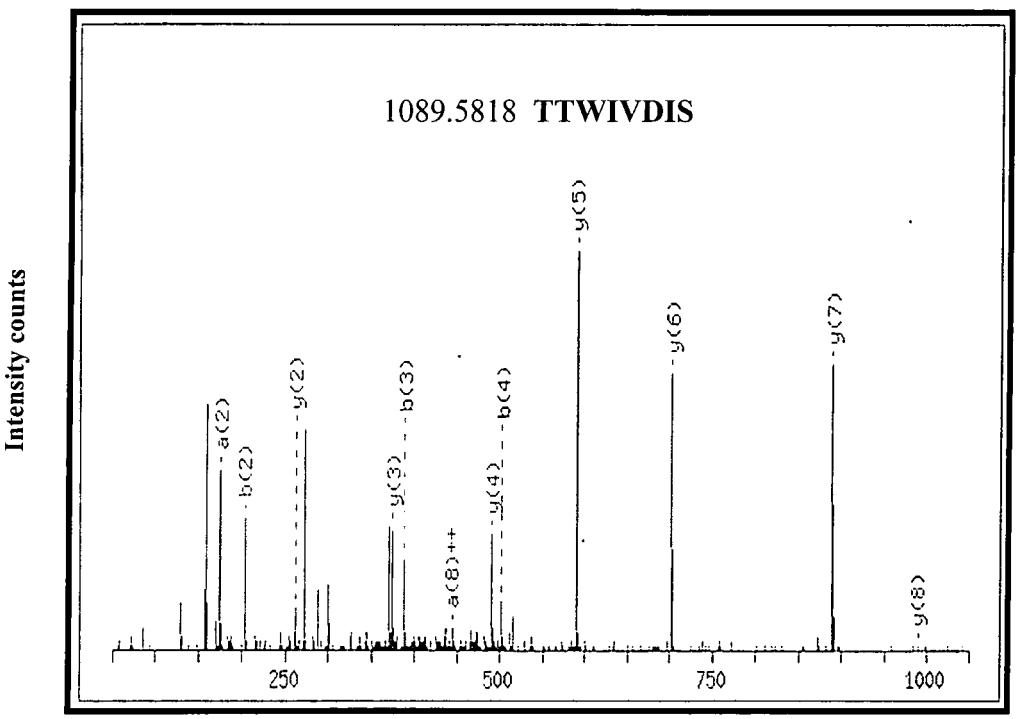
Figure 6.9. The b and y tandem mass spectra of doubly charged ions from spot 6.

Seven doubly charged ions were automatically selected among the tryptic digests prepared from spot 6 (Figure 6.7) using an LC Q-TOF instrument. **(A-G)** The selected ions were further fragmented in the Q-TOF MS's second quadupole (Q2) and the spectra of their b and y series are shown together with the corresponding amino acid tags. The peptide ions and the amino acid sequence tags belong to an *Arabidopsis* polygalacturonidase inhibiting protein identified via MALDI-ToF (Figure 6.8).

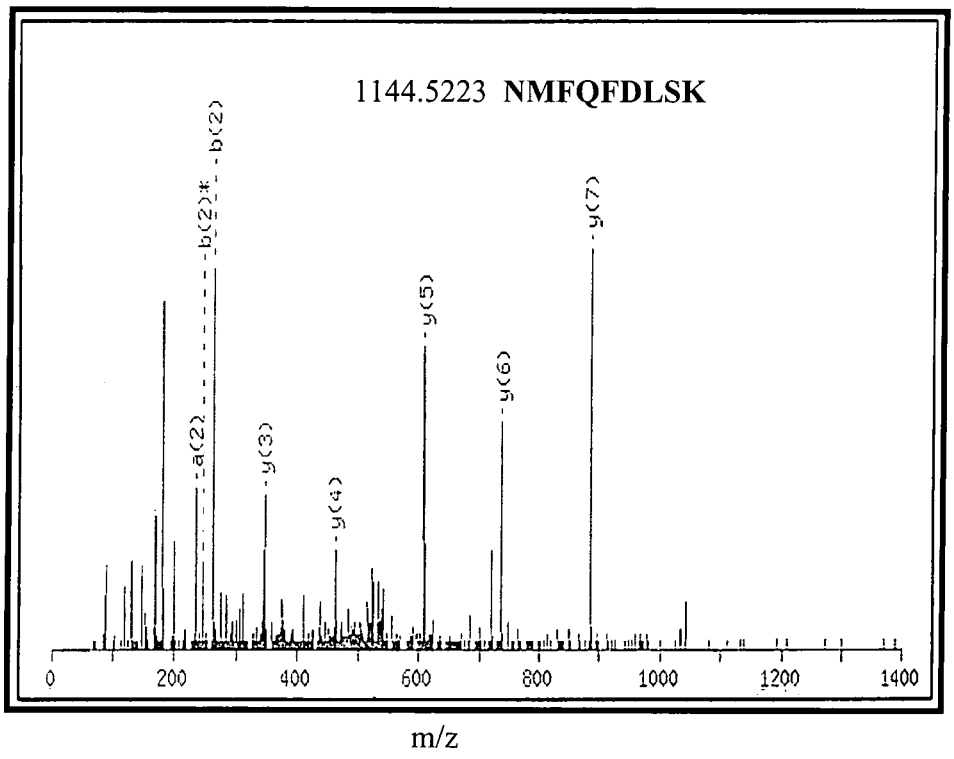
A


1067.5036 SLGNPDFYR
Intensity counts
a(2)
b(2)
y(5)++
b(4)
y(3)
y(4)
y(6)
y(5)
y(7)
0
200
400
600
800
1000
1200
1400
m/z

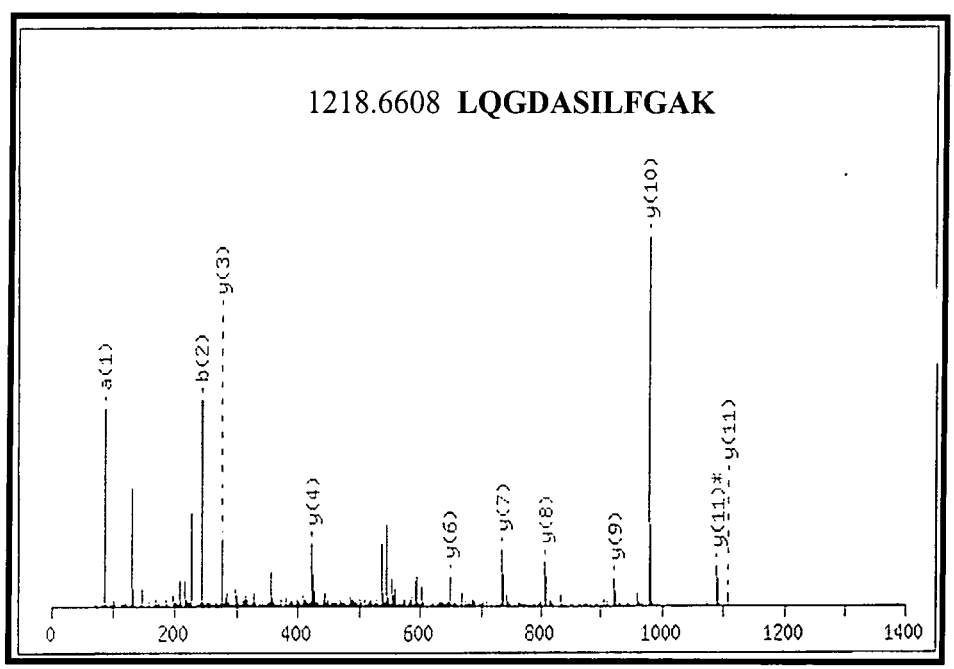
B


1089.5818 TTWIVDIS
Intensity counts
a(2)
b(2)
y(2)
y(3)
b(3)
a(8)++
y(4)
b(4)
y(5)
y(6)
y(7)
y(8)
250
500
750
1000
m/z

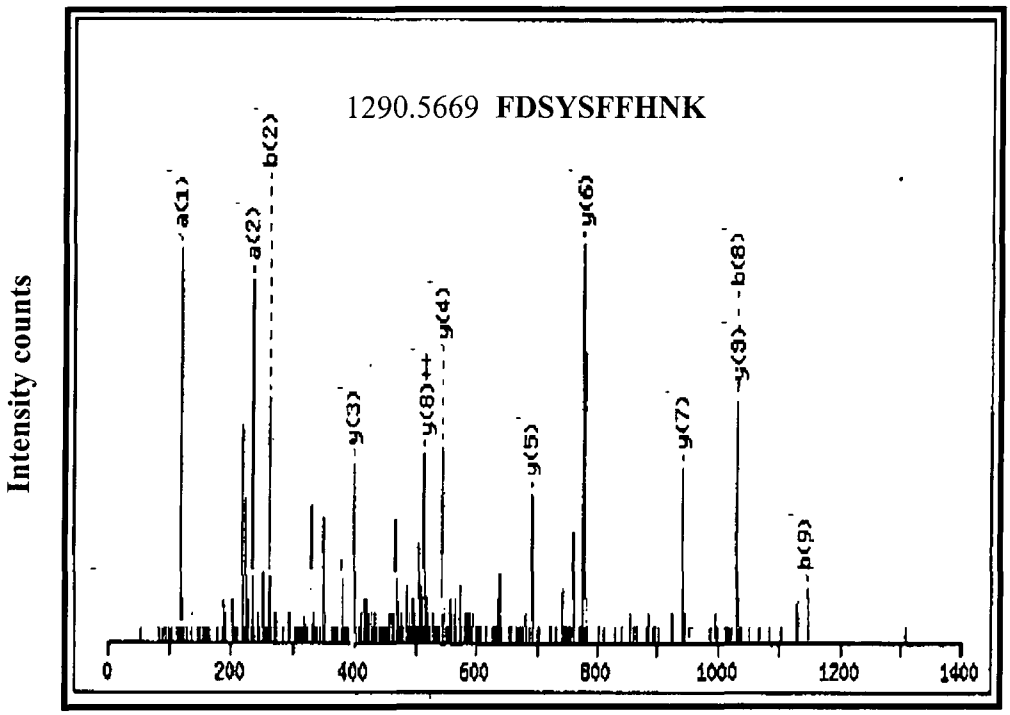
C


1144.5223 NMFQFDLSK
Intensity counts
a(2)
b(2)*
b(2)
y(3)
y(4)
y(5)
y(6)
y(7)
0
200
400
600
800
1000
1200
1400
m/z

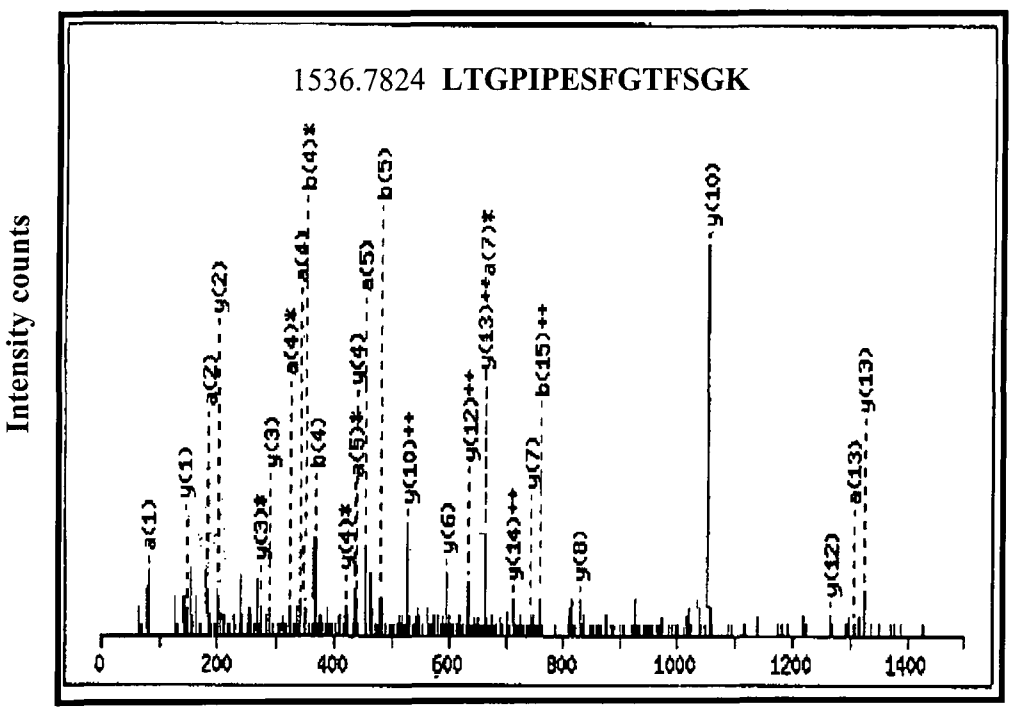
D


1218.6608 LQGDASILFGAK
Intensity counts
a(1)
b(2)
y(3)
y(4)
y(6)
y(7)
y(8)
y(9)
y(10)
y(11)*
y(11)
0
200
400
600
800
1000
1200
1400
m/z

E


1290.5669 FDSYSFFHNK
Intensity counts
a(1)
a(2)
b(2)
y(3)
y(8)++
y(4)
y(5)
y(6)
y(7)
y(9)
b(8)
b(9)
0
200
400
600
800
1000
1200
1400
m/z

F


1536.7824 LTGPIPESFGTFSGK
Intensity counts
a(1)
y(1)
a(2)
y(2)
y(3)*
y(3)
a(4)*
a(4)
b(4)*
b(4)
y(4)*
y(5)*
y(4)
a(5)
b(5)
y(10)++
y(6)
y(12)++
y(13)++a(7)*
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b(15)++
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0
200
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m/z

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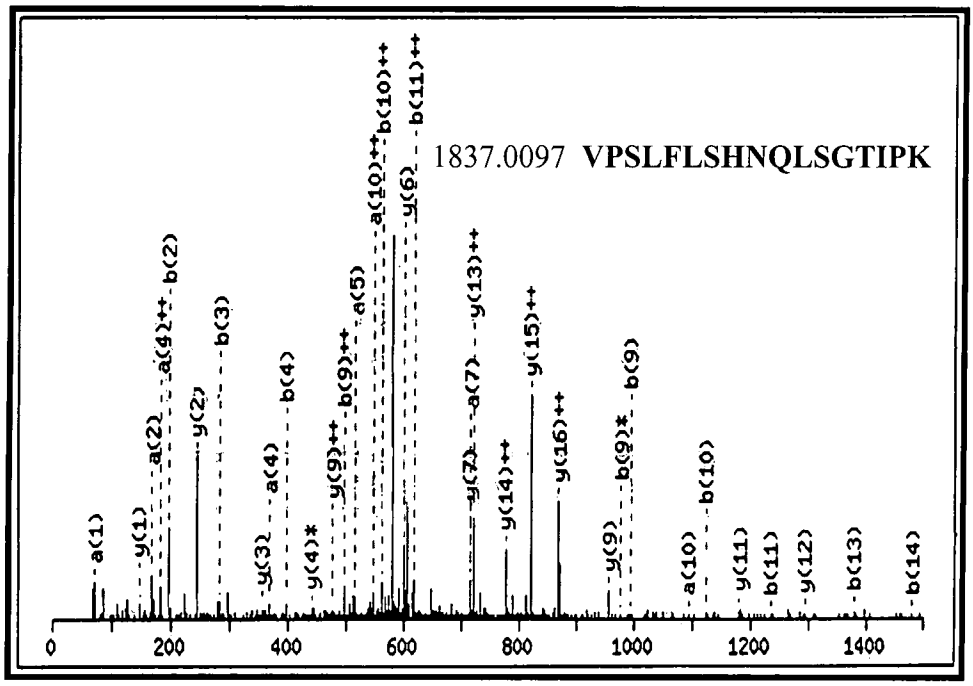
1837.0097 VPSLFLSHNQLSGTIPK
Intensity counts
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y(9)++
b(9)++
a(5)
a(10)++
b(10)++
y(6)
b(11)++
y(7)
a(7)
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y(15)++
y(16)++
y(9)
b(9)*
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y(11)
b(11)
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b(13)
b(14)

Table 6.4 Maldi-ToF peptide ions of a polygalacturonidase inhibiting protein (PGIP)

Submitted Mass (m/z)	Matched Mass	Delta ppm	Start-End	Peptide Sequence
1004.6104	1004.6257	15.1573	128 - 135	(K)**LKNLTFLR**(L)
1068.5079	1068.5114	-3.3026	228 - 237	(K)**SLGNPDFYR**(I)
1090.5888	1090.5897	-0.872	260 - 268	(K)**TTWIVDISR**(N)
1150.6482	1150.6472	0.8361	182 - 191	(R)**KLEYLELSR**(N)
1218.6993	1218.6846	11.9983	259 - 268	(K)**KTTWIVDISR**(N)
1291.5745	1291.5748	-0.201	335 - 345	(R)**FDSYSFFHNK**(C)
1461.7953	1461.8066	-7.7280	244 - 258	(R)**NKLQGDASILFGAK**(K)
1537.7789	1537.7902	-7.3852	194 - 209	(K)**LTGPIPESFGTFSGK**(V)
1652.8471	1652.8396	4.5232	228 - 243	(K)**SLGNPDFYRIDLSR**(N)
1779.9252	1779.9281	-1.6545	192 - 209	(R)**NKLTTG PIPESFGTFSGK**(V)
1838.0247	1838.0176	3.8718	210 - 227	(K)**VPSLFLSHNQLSGTIP**(K)
2037.9558	2037.9459	4.8711	329 - 345	(K)**GEYIQRFDSYSFFHNK**(C)
2412.1592	2412.1618	-1.0825	285 - 308	(K)**TLNNLDMNHNGITGSIPAEWSKA**(A)
2428.1516	2428.1567	-2.1066	285 - 308	(K)**TLNNLDMNHNGITGSIPAEWSKA**(A)*

Six out of seven amino acid ion tags (1067.5036; 1089.5818; 1218.6608; 1290.5669; 1536.7824 and 1837.0097) obtained from the tandem mass spectrometer were identical to 6 of the mass peptide ions generated via MALDI-ToF (Table 6.4). Therefore when used to interrogate Mascot (www.matrixscience.com) and NCBI (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov/BLAST/) databases they identified the same PGIP polypeptide. It was also subsequently confirmed that the seventh tandem mass spectrometry generated tag (which was not among the MALDI-ToF data) also belonged to the identified PGIP polypeptide (see Figure 6.8B).

6.2.6 PGIP phosphotyrosine residues predicted via the NetPhos 2.0 prediction program

The phosphorylation of PGIP is induced only when the cells are treated with chitosan (Figure 6.7B) and this explains why it was not seen among the phosphoproteins identified in chapter 5. The potentially phosphorylated tyrosine residues of the PGIP were predicted using the NetPhos 2.0 program provided by the Centre of Biological Sequence Analysis (CBS; www. cbs.dtu.dk/services/NetPhos). This program is able to predict the phosphorylated amino acid residues in any given polypeptide. The PGIP is predicted to posses 4 tyrosine phosphorylated residues (Y147, Y171, Y306 and Y313; Figure 6.8B).

6.2.7 Effects of chitosan on the phosphorylation status of the cell wall tyrosine phosphoproteins identified in chapter 5

The treatment of *Arabidopsis* cells with chitosan led to the rapid increase in both the abundance and the phosphorylation of the endochitinase identified in chapter 6.5 (spot 5, Figure 6.7). The increase in the abundance of the chitinase is not unexpected, however the speed of the induction and the phosphorylation induction has never hitherto been shown. The phosphorylation status of the unknown protein (spot 3 and 4) was reduced following the chitosan treatment. However, the receptor-like kinase-like protein (spot 1 and 2) was almost completely dephosphorylated by the chitosan treatment whereas the abundance of the protein (at Coomassie stain level) increased. Following the early responses (proteins that are responding to chitosan within 10 minutes), the longer effects of chitosan in the profile of the *Arabidopsis* cell wall proteome was investigated.

6.2.8 The longer term effects of chitosan elicitation on the proteome of the *Arabidopsis* cell walls.

Following the establishment of the rapid effects of chitosan in the profile of the cell wall proteins, the longer term effects of chitosan in the profile of the cell wall proteins was investigated. For this *Arabidopsis* cells were subcultured, and grown as above (6.2.4). The cells were then treated for 24 hours with 50 μg/mL chitosan. A control experiment was done by spiking the cells with equivalent volume of water for

24 hours. The cell walls were purified from the treated cells and their proteins were also extracted with $CaCl_2$. The protein extracts were precipitated with 80 % acetone at –20 °C, the precipitates collected by centrifugation and were dried with filtered air. The dried protein pellets were resolubilised in 2-D lysis buffer and analysed by 2D polyacrylamide gel electrophoresis. The Coomassie stained gels containing cell wall proteins from the treated cells were once again compared to the profile of the control cell wall proteins (Figure 6.10). The proteins that respond to chitosan treatment reproducibly (in at least 3 independent experiments) are marked with numbered arrows and their identities are listed in Table 6.3. Almost all the proteins that are induced when *Arabidopsis* cells are treated with chitosan for 24 hours are the same as the protein seen in the 10-minute treatments. It is interesting to see that the induction of a receptor-like protein kinase-like protein by chitosan increases with time, i.e. their abundance relative to control is higher than at the 10-minute time-point (Figure 6.8). The induction of PGIP (spot 6) however, seems to be transient, that is, although it was induced at the 10-minute time-point, the protein abundance at 24 hours appears to have gone back to basal levels. Consistent with the 10-minute responses, at 24 hours the abundance of the unknown protein (AT3g08030) spots (2 and 3) was markedly reduced following chitosan treatment. In addition, a protein identified as a serine carboxypeptidase-like protein (AT1g28110) was suppressed by chitosan treatment (spot 8 Figure 6.10). Another (so far unidentified) protein spot (spot 9; Figure 6.10) was also suppressed when the cells were treated with chitosan for 24 hours).

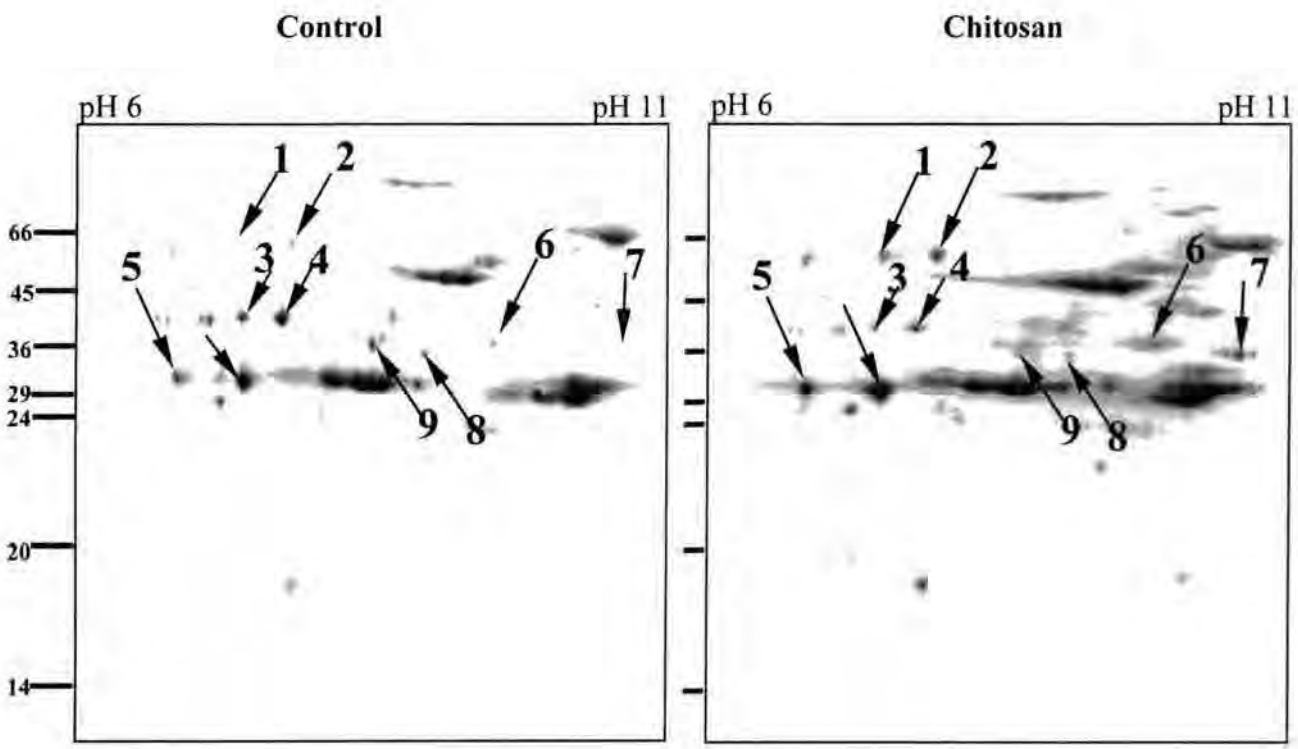

Figure 6.10. **Cell wall proteome of chitosan treated *Arabidopsis* cell cultures**

Arabidopsis cells cultures were grown for 3 days followed by treatment with chitosan for 24 hours. The cell walls were purified from the treated and control cells and their proteins were extracted using $CaCl_2$. The $CaCl_2$ extracts were separated via 2-D PAGE, using pH 6-11 IEF in the first dimension and 12 % PAGE in the second dimension. The gels were then stained with Coomassie brilliant blue. The molecular weight markers are in kilodaltons (kDa).

6.3 Discussion and conclusions

6.3.1 Use of *Arabidopsis* cell cultures in plant pathogenesis studies

The treatment of cell cultures with pathogen elicitors has proved to be an ideal experimental system for pathogenesis studies (Atkinson and Baker, 1989; Felix et al., 1991; Brisson et al., 1994). The completion of the *Arabidopsis* genome sequencing project has made *Arabidopsis* cell cultures a more attractive model for pathogen related proteome studies (Peck et al., 2001; Nuhse et al., 2000; Kunkel, 1996) hence its application in this study. The cell line that was employed in the study of the pathogen related proteome described here was subjected to rigorous tests for its ability to deploy the full pathogen response complement when challenged with chitosan, a well-established pathogen response elicitor (Vasiukova et al., 2001). The treatment of *Arabidopsis* cell cultures with chitosan induced a large variety of defense responses, including a rapid induction of reactive oxygen species (oxidative burst), alkalinisation of the external medium, and accumulation of glutathione S-transferase (GST). Chitosan also induced the activity of phenylalanine ammonia-lyase (PAL) the first enzyme in the phenylpropanoid pathway that leads to the biosynthesis of phytoalexins. A chitosan dose dependent hypesensitive response (programmed cell death) was observed in the *Arabisopsis* cell line used in this study. These responses are all typical plant reactions to pathogen invasion and are all aimed at stopping or limiting the growth of the pathogen in the host plant during an incompatible interaction (Baier et al., 1999; Romeis et al., 1999; Brisson et al., 1994; Vasiukova et al., 2001).

6.3.2 Effects of chitosan treatment on the *Arabidopsis* cell wall proteome

The changes in the 2-D profile of *Arabidopsis* cell walls have been highly reproducible. At least 5 Commasie brilliant blue stained proteins showed a rapid and consistent response to chitosan treatment in at least three independent experiments. The proteins whose abundances are rapidly induced include a putative receptor-like protein kinase, an endochitinase, a probable apospory associated protein and a polygalacturonidase inhibiting protein. The induction of a chitinase with a pathogen elicitor was not unexpected (Inui et al., 1996; Mason and Davis, 1997; reviewed in Swapan and Muthukrishnan, 1999). However, the chitinase reported in this study responds to chitosan treatment within 10 minutes. Whether or not this speed reflects the dynamics in whole plant tissue will require *in planta* studies. The other interesting feature of the identified chitinase is that it is also a tyrosine phosphoprotein and its phosphorylation status is rapidly induced by chitosan treatment. The phosphorylation status of this protein seems to be emulating the protein abundance trend after treatment with chitosan. However, at this stage it will be premature to conclude whether the protein phosphorylation status and the protein abundance are connected or are two separate events. Parallel to the increase in phosphorylation status of the chitinase, a putative receptor-like protein kinase was also induced. It is therefore tempting to speculate that the two events might not be just a coincidence but rather a well co-ordinated response. The rapid induction of putative receptor-like protein by chitosan treatment is a very interesting phenomenon and has never been reported hitherto.

A tobacco chitinase, which has a homologue in the *Arabidopsis* genome, was found to be a receptor-like protein kinase (CHRK1), and its autophosphotylation capability has been demonstrated (Kim et al., 2000). This evidence is consistent with the results presented here. Looking closer to the sequence of CHRK1 it was discovered (using the TMHMM v. 2.0 prediction program of transmembrane helices in proteins; www. cbs.dtu.dk/services/TMHMM) that its polypeptide (739 amino acids) has two transmembrane domains located towards the centre of the polypeptide (286-308 and 362-384) and the only portion of the polypeptide that is exposed to the cytoplasm is 52 amino acids long (309 to 361). This orientation predicts that both the N-terminus and the C-terminus of the CHRK1 are in the extracellular matrix. A simplified diagram of the protein arrangement (using the given information) with respect to its association with the membrane is shown in Figure 6.11. Kim et al, (2000) however, applied only one transmembrane domain (362-384), which locates the demonstrated C-terminus kinase domain inside the cell (Figure 6.11). Kim et al., (2000) does not explain the reason behind their application of only one transmembrane of the predicted domain in their report. However, it is possible that they omitted the second domain for the sake of convenience, because applying both domains to the polypeptide would imply that the active kinase domain is in the extracellular matrix, a possibility that is not yet accepted in plant studies. It is also worth highlighting that the receptor kinase-like chitinase reported in Kim et al, (2000) has lost its ability to hydrolyse chitin, therefore suggesting that CHRK1 may have evolved to form a chitin binding receptor kinase. Consistent with the results presented in this chapter, *CHRK1* mRNA accumulation is significantly stimulated by fungal pathogen elicitors (Kim et al., 2000).

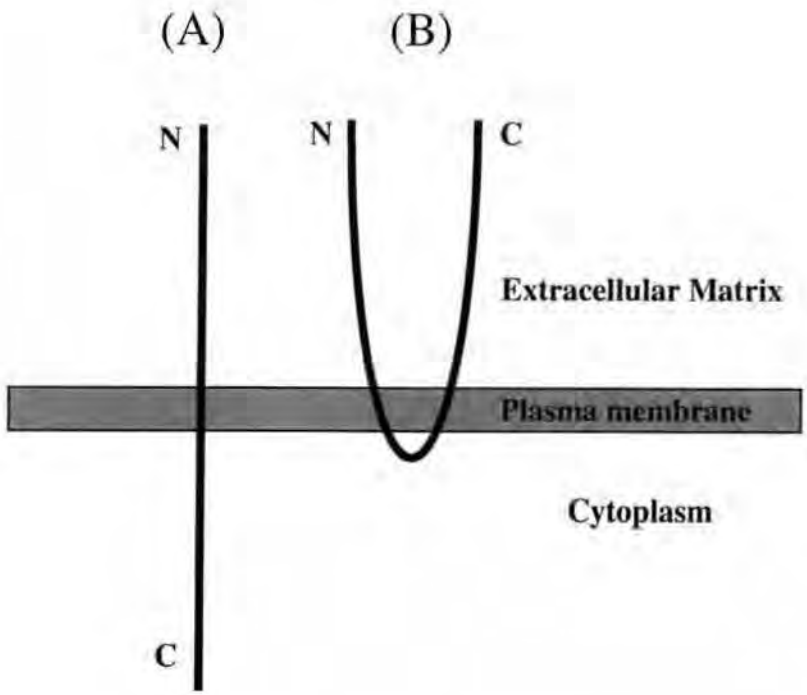

Figure 6.11 **A predicted orientation of the membrane associated CHRK1 polypeptide.**

A. Kim's CHRK 1 model (Kim et al., 2000)

B. The CHRK 1's association with the membrane, when both predicted transmembrane domains are applied.

Another intriguing result reported in this chapter is the identification of a polygalacturonidase inhibiting protein (PGIP), which is both rapidly induced and phosphorylated following chitosan treatment. PGIP is a strong inhibitor of the fungal polygalacturonase (enzymes that hydrolyses the link between galacturonic acids of plant cell wall homogalacturonans). PGIP's are now known to be leucine rich repeat (LRR) proteins that are located entirely in the extracellular matrix. In plants LRR are the peptides, which are known to be involved in pathogen signal perception, and they are associated with the largest group of *Arabidopsis* receptor kinases (reviewed by Hardie, 1999). The induction of PGIP by pathogen elicitors has been reported before (James and Dubery, 2001). The possible involvement of PGIPs in cell signalling has also been reported (Komjanc et al., 1999; Yao et al., 1999). The elicitor-induced phosphorylation of PGIP however has never been reported. These results therefore are also are in agreement with the extracellular protein phosphorylation hypothesis proposed in chapter 5.

The treated cells were allowed to grow for 24 hours with the elicitor in order to investigate whether the elicitor induced effects were transient or enduring. The hope of this strategy was also to identify the down stream effects of the signal (chitosan) perception. The profile of control versus elicitor did not change very much from the 10 minute pattern, except that the protein abundance of the chitosan treated cells was higher particularly in the case of the putative receptor kinase (AT1g78850; spot 1 and 2). A protein identified as a probable apospory-associated protein (AT4g25900) was also rapidly induced when *Arabidopsis* cells were treated with chitosan, and their induction was even higher when the cells were elicited for 24 hours. The

function of this protein is so far unknown, but it is shown to have a possible protein phosphatase domain (http://mips.gsf.de/proj/thal/). A hypothetical protein (AT1g28110; spot 8), which shares some homology to a serine carboxypeptidase, was partially suppressed by chitosan treatment, however the biological significance of this effect is as yet not clear. The unknown protein (AT3g08030; spot 3 and 4) remained suppressed even at 24 hours post elicitation. The lowered abundance of this protein could be the result of chitosan induced protein insolubility. Fungal elicitors are known to induce hydrogen peroxide mediated cross-linking and insolubilisation of some cell wall proteins, and this is a strategy used to reinforce the wall to create a physical barrier against the pathogen invasion (Brisson et al., 1994; Bradley et al., 1992).

6.4 Concluding remarks

The evidence presented in this chapter reveals that plant cell walls harbour a variety of dynamic receptor-like proteins whose abundances and phosphorylation status is pathogen elicitor responsive. This rapid response to chitosan suggests that these proteins might be acting upstream (at elicitor perception level) of pathogenesis and this also hints that the induced abundances might be transcription and translation independent. This speculation will require verification with transcription and translation inhibitors. Also, [^{35}S] labelling experiments will establish whether the accumulating proteins are synthesised post elicitation. The response speed of these protein changes will however, need to be verified in intact plants, and their role in pathogenesis will require molecular genetic approaches, such as antisense RNA techniques and gene knockouts. In addition, identifying interacting signalling components may also provide insights into cellular functions of these proteins.

Chapter 7

Summary and References

7.1 Summary and final comments

The main aim of this thesis was to extract and characterise as many cell wall proteins as possible, with the hope of identifying novel cell wall proteins. Out of 325 spots visualised on the large format 2-dimension polyacrylamide gel, followed by Coomassie brilliant blue staining, 144 spots gave a positive protein identification representing 104 different proteins. The identified proteins were divided into 3 categories. The first category included proteins that have been previously identified in plant cell walls. Proteins assigned to the second category were novel *Arabidopsis* cell wall proteins (hypothetical, putative and unknown proteins). Finally, the third category included those proteins whose cell wall localisation was unexpected (e.g. enolase and glyceraldehydes-3-phosphate dehydrogenase).

A large number of classical cell wall proteins were identified here. These include cell wall biosynthesis associated enzymes like glucan transferases, glucosidases, polygalacturonases, pectin esterases and expansins. Classical cell wall located pathogenesis relates (PR) proteins were also identified, including glucanases, chitinases and peroxidases (Datta and Muthukrisnan, 1999). Other plant enzymes whose cell wall localisation has been reported elsewhere were also identified here including, phosphatases, proteases (Showalter, 1993) and kinases (He et al., 1996). However, some of the previously documented cell wall located proteins were not detected in this study. These include extensins, proline-rich proteins and glycine-rich proteins. The absence of these candidates could be due to several reasons. First, the reagents used for protein extraction could have failed to extract these proteins from the purified cell wall. Second, the missing proteins could be expressed at certain

developmental stages (Robertson et al., 1997). Third, these missing proteins could be water-soluble proteins that are washed off during the stringent washing regime of cell wall purification. Fourth, these proteins that are missing could be present as minor components of the cell wall protein population that are not visible at Coomassie brilliant blue (CBB) level of staining, and lastly the missing characters could be heavily glycosylated proteins which are known to resist CBB staining (Robertson et al., 1997).

In the category of novel (hypothetical) cell wall proteins, there were those that shared some homology with classical cell wall proteins. However, the biggest revelation of this work was the identification of novel cell wall located putative protein kinases. Unlike previously reported cell wall associated protein kinases (He et al., 1996), this new group of cell wall putative kinases did not possess any predicted transmembrane motifs. This observation implied that the reported putative kinases are located in the extracellular matrix. Given that the phenomenon of protein phosphorylation was never hitherto reported in the extracellular matrix of plant cells, this observation suggested that cytosolic proteins could have contaminated the cell wall protein extracts. The structure of the identified putative kinases was examined further. Consistent with their cell wall localisation, these putative kinases possessed an N-terminal signal peptide for secretion. Although this was not taken as full proof of their cell wall localisation, this characteristic strongly suggested that these kinases were indeed located in the extracellular matrix. In animals, the concept of extracellular protein phosphorylation is well documented (Ehrlich and Kornecki, 1987; Reviewed by Ehrlich et al., 1990; Fujii et al., 2000). Extracellular phosphorylation in animal cells is thought to be involved in the regulation of

intercellular communication (Ehrlich et al., 1990). Ectokinases are also believed to play a role in the phosphorylation of ion channels (Ehrlich et al., 1990). More recently ectokinases have been directly implicated in neuron-to-neuron communications (Fujii et al., 2000).

The first step of the evidence gathering exercise was to investigate whether phosphoproteins exist in the extracellular matrix of plant cell. It was discovered that plant cells secrete ATP (Thomas et al 1999, 2000) and harbour phosphoproteins (this study). The inactivation of ectokinases, by removing the extracellular ATP or by replacing it with a non-hydrolysable ATP analogue, induced the hypersensitive response in both suspension cultures and whole plant tissues. This evidence was thought to provide overwhelming support for the proposed existence of active ectokinases in the plant extracellular matrix.

Other enzymes that have never hitherto been reported in the plant cell wall were also identified here. The evidence provided indicates that almost all of these enzymes are *bona fide* cell wall residents in other cell wall containing organisms such as fungi where the cell wall studies are much more advance than plant cell walls. These include enolase, glyceraldehydes-3-phosphate dehydrogenase and phospholipase D (reviewed by Chaffin et al., 1998). Although many reports have confirmed the cell wall localisation of these proteins, their biological roles in this compartment are largely unknown (Chaffin et al., 1998).

The work presented in this thesis is in line with the final comments made by Brett and Waldron, (1996) and Chaffin et al, (1998), that cell walls are full mysterious

challenges, and even 400 years since their discovery they still harbour mysteries that remain unresolved. One of the fundamental questions that still need to be answered is: How proteins are retained and distributed in the plant cell wall? From the model presented by Chaffin et al, (1998), it is shown that following secretion across the plasma membrane, some proteins localise in the periplasmic space, some form associations with cell wall glucans and others are released to the apoplasm. It is speculated that those that colonise the periplasmic space are likely to be involved in enzymatic roles. Those that are associated with the cell wall glucans are most likely to cater to both enzymatic and structural functions. And finally those that are released to the apoplasm are likely to be in equilibrium with other bound cell wall proteins, therefore, contributing to the cell wall surface layout either as enzymatic or structural entities (Chaffin et al., 1998).

The work described in this thesis can in no way be thought to provide a full answer to all of the questions involved in the scientific study of the plant cell wall, but, it is likely to provide a foundation for a new wave of plant research. The application of more sensitive research methods like immunocytochemistry, and green fluorescent protein labelling would establish whether or not these proteins are localised in the cell wall *in planta*. As with other *bona fide* cell wall proteins, a conclusive verification of protein localisation is only the beginning. The real challenge is to establish the *in planta* roles (Brett and Waldron, 1996). Future work will involve [^{35}S] labelling experiments will establish whether the accumulating proteins are synthesised post elicitation. As mentioned in the concluding remarks of Chapter 6, the response speed of the cell wall protein changes be verified in intact plants, and their role in pathogenesis will require molecular genetic approaches, such as

antisense RNA techniques and gene knockouts. These approaches will establish the phenotypic roles of the chosen novel protein(s) identified in this work. In addition, identifying interacting signalling components is also likely to provide insights into cellular functions of these proteins.

The involvement of cell wall proteins in signal transduction has been hypothesised before (Hoson, 1998), however the identification of phosphorylated pathogenesis related proteins, which are located in the extracellular matrix is novel. These novel pathogenesis related candidates are likely to provide a better understanding of the signalling events that take place during plant-pathogen interactions.

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