Characterization of cell wall proteome in *Medicago truncatula* suspension culture

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If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts, he shall end in certainties-**Francis Bacon**

.....to my Parents

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Abbreviations

AGPs	arabinogalactan proteins
APS	Ammonium persulphate
ATP	adenosine triphosphate
Avr	avirulence
Å	Angstrøm
bp	basepairs
$CaCl_2$	Calcium chloride
CDPK	calcium-dependent protein kinase
CWPs	Cell wall proteins
Da	Dalton
DIG	digoxigenin
DIGE	difference gel electrophoresis
DNA	deoxyribonucleic acid
DTT	dithiothreitol
e.g.	exempli gratia (for example)
EDTA	Ethylene diamine tetra acetic acid
EPS	exopolysaccharide
ESI	electro spray ionisation
EST	Expressed sequence tag
et al.	Et alii (and others)
Fig.	figure
g	gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GHs	Glycosyl hydrolase
h	hours
HPLC	high performance liquid chromatography

HR	hypersensitive response
HRGPs	hydroxyproline-rich glycoprotein
Hrp	hypersensitive response and pathogenicity
i.e.	Id est (that means)
IEF	isoelectric focusing
l	liter
LC	liquid chromatography
LiCl	lithium chloride
LPS	lipopolysaccharide
LRR	leucine-rich receptor
MALDI	matrix-assisted laser desorption/ionisation
MAMPs	Microbe-associated molecular patterns
MAPK	mitogen-activated protein kinase
MASCOT	Multiple-Access Space-Time Coding Testbed
Mb	Mega base pairs
M. truncatula	*Medicago truncatula*
µg	microgram
min	minute
MOWSE	MOlecular Weight SEarch
MS	mass spectrometry
M_r	molecular weight
NaCl	Sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National center for biotechnology information
nm	nano meter
ORF	Open reading frame
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern

PCR	polymerase chain reaction
pI	isoelectric point
PMF	peptide mass fingerprint
PRPs	Pathogenesis-related proteins
PRPs	Proline rich proteins
PRRs	Patten-recognition receptors
PSD	post source decay
PTMs	Post translational modifications
PVDF	Polyvinylidene Difluoride
R	resistance
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	Rounds per minute
SAMS	Sequence Analysis and Management system
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
TEMED	N,N,N',N' tetramethylethylene-diamine
TIGR	The institute for genomic research
TOF	time of flight
Tris	N-tris-(hydroxymethyl)-amino methane
V	Volt
v/v	Volume per volume
W	watt
w/v	Weight per volume
1-D	One-dimensional
2-DE	Two-dimensional gel electrophoresis

Summary

Aim of this work was a comprehensive analysis of the cell wall proteome of *Medicago truncatula* suspension cell cultures. Three different tasks included the establishment of an optimised protocol for the extraction of the cell wall proteins (CWPs), development of an optimised method to establish the cell wall proteome map of *M. truncatula* and comparative proteome profiling of CWPs during elicitation with yeast invertase and suppression with *Sinorhizobium meliloti* lipopolysaccharides (LPS), representing the pathogenic and symbiotic interactions respectively.

The CWPs extracted using living cells and/or cell wall fragments were analysed using different techniques. Using a combination of either one-dimensional, preparative isoelectric focussing, two-dimensional gel electrophoresis (2-DE) or LC-MS/MS and MALDI-TOF-MS analysis, established the proteome reference map of *M. truncatula* CWPs. In addition to establishing methods for proteome analysis of CWPs of the model plant *M. truncatula*, the presented work highlights the different composition of several protein classes in cell culture. 2D-PAGE analysis for the CWPs extracted from living cells enabled to characterise approximately 48 CWPs. An alternative approach using a preparative free-flow electrophoresis technique for first dimension separation to obtain 20 fractions with proteins differing in their isoelectric points which were subsequently separated by SDS-PAGE was established. This resulted in identification of 59 out of 100 additional proteins, resulting in 107 identified proteins in total. The proteins analyzed were related to defence, oxidative stress, cell wall modification and signal mediation. In order to overcome the limitations caused because of the nature of the CWPs in proteome analysis, a new extraction protocol (using cell wall fragments) and alternate method (LC-MS/MS) to identify more proteins was established. Furthermore CWPs extracted from purified cell wall fragments and using 2D-PAGE or LC-MS/MS approach, 46 and 65 proteins were identified respectively with a total of 111 proteins. Among the identified were the proteins involved in various process like cell wall modifications, signaling, defence mechanism, membrane transport, protein synthesis & processing, as well as wall construction processes.

Also the comparative proteome analysis using yeast elicitor (Invertase) in response to pathogen defence and suppressor using *S. meliloti* were also established. 2D-PAGE analysis for the CWP samples extracted with elicitor and LPS treated cells resembled the

same as the proteome map of the elicitation with invertase alone, with the few upregulated proteins involved in defence and from LPS treated cell wall proteome map there was no significant difference. Using this approach, proteins involved in defence like L-ascorbate peroxidase, specifically targeted proteins to the cell wall during defence response which include glyceraldehydes-3-phosphate dehydrogenase and proteins that play an important role during growth and development were identified. Also some of this defence related proteins are absent in the same gel after elicitation conferring that oxidant protection is regulated by these proteins. For most CWPs the biological functions are yet to be identified. Bioinformatic analysis helps to have a clue to design an experiment, to understand the biochemical and biological functions of these proteins.

1.0. Introduction

Plants have the capacity to recognize a wide range of pathogens like bacteria, viruses, oomycetes, nematodes, fungi and insects. During their course of development both the partners interact together and develop mechanisms either to benefit from the interaction as in symbiosis or defend themselves as exemplified in pathogenic interactions.

1.1. Plant microbe interactions

The specificity of many plant microbe interactions implies some mechanism of recognition, one of which may be the exchange of molecular signals between host and microbe (Halverson and Stacey 1986). Depending on such signals, plant microbe interactions can be beneficial, as in symbiotic Rhizobium and mycorrhizal associations to legumes, but in other cases microbes represent potential pathogens with the ability to parasitize plants causing disease (Hirsch 1992, Djordjevic et al. 1987). The exchange of signal molecules appears to be necessary for many plant microbe interactions. Signal exchange in plant-microbe interaction involves the recognition and exchange of specific molecules by the host or microbe or both that trigger biochemical, physiological, or morphological responses that affect the development of the plant-microbe interaction. The signal molecules may be deoxyribonucleic acid (DNA), ribonucleic acid (RNA), protein, lipid, or polysaccharides. Lacking the adaptive immune system, plants depend on innate immunity to defend against most potential pathogens (Halverson et al. 1986).

The first line of plant innate immune response is triggered upon the detection of many common pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) that are not found in host cells (Boller 1995, Nümberger et al. 2004, Ausubel 2005, Zipfel et al. 2005). The term MAMPs is used here since both non-pathogenic and pathogenic microbes produce effective MAMPs to activate immune responses (Ausubel 2005, He et al. 2006, Jones et al. 2006). Moreover, some pathogens actively suppress MAMP triggered immunity (Kim et al. 2005, Li et al. 2005, He et al. 2006). The perception of different MAMPs by specific pattern-recognition receptors (PRRs) induces defence responses that contribute plant immunity to both pathogenic and non-pathogenic microbes. Plant-microbe interaction offers an oppurtunity to elucidate the basic mechanisms governing signal

perception and transduction in plants, as well as the mechanisms by which microbes respond to plant signals (Niehaus et al. 1998, Tellström et al. 2007).

1.2. Plant pathogen interactions

When a plant is infected by a pathogen, several possible outcomes can occur. Once a pathogen is recognized, the plant responds by induction of apoplastic defence for inhibition of microbial enzymes, for cell wall strengthening, or for poisoning of the pathogen. As a second attempt of defence, the hypersensitive reaction (HR) including cell death restricts pathogens in infected area. Also, the plant may be able to resist pathogen invasion. In some cases, resistance is passive: the plant fails to provide the appropriate environment to support pathogen colonization. In other cases, resistance is an active process in which the plant produces a cascade of defence responses. Studies of plant microbe interactions have shown that active resistance usually is initiated by a plant resistance gene, whose product directly or indirectly recognizes a race-specific avirulence determinant produced by the pathogen (Gonzalez et al. 2006, Keen 1990, Scofield et al. 1996, Tang et al. 1996). One of the most visible signs that a plant is resisting pathogen attack is the development of a hypersensitive response (HR). The HR is characterized by small necrotic lesions that form around the infection site (Matthews 1991). These lesions help to restrict the growth and spread of the pathogen (Slusarenko et al. 1991). In addition, antimicrobial compounds (phytoalexins) often are produced (Dixon 1986) and the cell wall is strengthened as a result of lignification and cross-linking of cell wall proteins (Bowles 1990).

In the early 1970's, it has been noted that small moleculer proteins and oligosaccharides produced by the plant pathogens act as elicitors. Initial elicitation studies in the plant cell system and the name ‘elicitor’ were introduced by Keen in 1975 and later Albershein et al (1977) investigated the interaction of microbial oligosaccharides interaction on plants. Elicitors are compounds secreted by microorganism or liberated from the cell walls by hydrolases and are responsible for the activation of defence responses. It is a microbial signal which is recognised by a plant receptor. Elicitors are classified as physical or chemical, biotic or abiotic and complex or defined elicitors depending on the origin and the molecular structure (Radman et al. 2003). Biotic elicitors are molecules of either pathogen or host origin that can induce defence responses such as phytoalexin

accumulation or hypersensitive response in plant tissue. Often complex biological preparations have been used as elicitors, where the molecular structures of the active ingredients are unknown. Examples of such elicitors are yeast extract and microbial cell-wall preparations. Recently, the exact molecular structure of an increasing number of elicitors has been elucidated, including various polysaccharides, oligosaccharides, proteins, glycoproteins, and fatty acids (Anderson 1989, Hahn 1996). The use of abiotic elicitors in plant cell cultures has received less attention compared with the biotic elicitors (Radman et al. 2003). Some heavy metal salts are often found to trigger phytoalexin production. Examples include $AgNO_3$ and $CdCl_2$.

The plant's innate immunity is based on recognition of microbial signal molecules, termed general elicitors or pathogen-associated molecular patterns (PAMPs). Many bacteria, fungi or oomycete-derived cell surface components have been shown to act as PAMPs that trigger immune responses in various plant species (Nürnberger et al. 2004, Zipfel and Felix 2005). PAMPs that trigger plant innate immune responses include the lipopolysaccharide (LPS) fraction of Gram-negative bacteria (Meyer et al. 2001), peptidoglycans from Gram-positive bacteria, eubacterial flagellin (Felix et al. 1999), bacterial DNA fragments, glucans, chitins (Ren and West 1992), yeast (*Saccharomyces cerevisiae*) invertase (Basse et al. 1992) and proteins that are derived from the fungal cell wall (Aderem and Ulevitch 2000, Imler and hoffmann 2001, Underhill and Ozinsky 2002).

The PAMP triggered immunity (Chisholm et al. 2006, Nürnberger et al. 2004, Zipen and Felix 2005) is initiated upon recognition of conserved microbial structures (PAMPs) by plant surface pattern recognition receptors (Gomez-Gomez and Boller 2000, Zipfel et al. 2006). Importantly, such PAMP-induced immune responses are crucial for nonhost resistance and also contribute to the basal resistance of host plants (He et al. 2006, Kim et al. 2005, Zipfel 2004 and 2006). Also, PAMP-triggered immunity by microbial effectors is one of the strategies that pathogens use to colonize host plants (Alfano and Collmer 2004, Chisholm et al. 2006).

Biochemical and genetic approaches have established that the interaction of plants and microorganisms is complex. Plants produce a defensive response to some pathogens after recognising the presence of a pathogen through the physical interaction of pathogen-derived elicitor molecules with the plant receptors (Leister et al. 1996, Dangl and Jones

2001, Nurnberger and Brunner 2002). But in some cases of cultivar-specific resistance, which is expressed only by particular plant cultivars against some races of a pathogen species, conforms to the gene-for-gene-hypothesis and is genetically determined by complementary pairs of pathogen-encoded avirulence (AVR) genes and plant resistance (R) genes.

In this gene-for-gene interaction (Flor 1971), absence of either the avirulence gene or the resistance gene leads to the failure by the plant to recognize the pathogen and the pathogen successfully colonizes the host and produce disease. Nevertheless, induced resistance by plants to some pathogens is not based on the gene-for-gene interaction, but still involves surveillance systems of receptor molecules that can recognize diverse elicitor molecules of pathogen origin and is functional against a wide spectrum of pathogens (Nurnberger et al. 2004). Regardless of whether the elicitor is, race-specific or a general elicitor, the events that the elicitor-receptor binding triggers are the same.

1.2.1 Mechanism of elicitation in plant cells

During biotic elicitation in plants or plant cell culture, a rapid array of biochemical responses occurs. According to Radman et al (2003), the general mechanism of elicitation in plants includes (Figure 1):

- Binding of the elicitor to a plasma membrane receptor.
- Changes in ion fluxes across the membrane: Ca^{2+} influx to the cytoplasm from the extracellular environment and intracellular Ca^{2+} reservoirs and stimulation of K^{+} and Cl^{-} efflux.
- Rapid changes in protein phosphorylation patterns and protein kinase activation mitogen-activated protein kinase (MAPK) stimulation, G-protein activation.
- Synthesis of secondary messengers such as Ins(1,4,5)P_3 and diacylglycerol (DAG) mediating intracellular Ca^{2+} release, nitric oxide and octadecanoid signalling pathway.
- Cytoplasm acidification caused by H^{+}-ATPase inactivation and decrease in membrane polarization; extracellular increase of pH has been reported in elicitor treated plant tissues.
- Activation of NADPH oxidase responsible for the production of reactive oxygen

species (ROS) and cytosol acidification.

- Cytoskeleton reorganization.
- Production of ROS, such as the superoxide anion and H_2O_2 might have a direct antimicrobial effect as well as contributing to the generation of bioactive fatty acid derivatives and being involved in the cross-linking of cell-wall-bound proline-rich proteins. H_2O_2 can act as a secondary messenger and it is involved in the transcriptional activation of defence genes.
- Accumulation of defence-related proteins (pathogenesis-related proteins such as chitinases and glucanases, endopolygalacturonases that contribute to the release of signalling pectic oligomers (endogenous elicitors), hydroxyproline-rich glycoproteins, protease inhibitors.

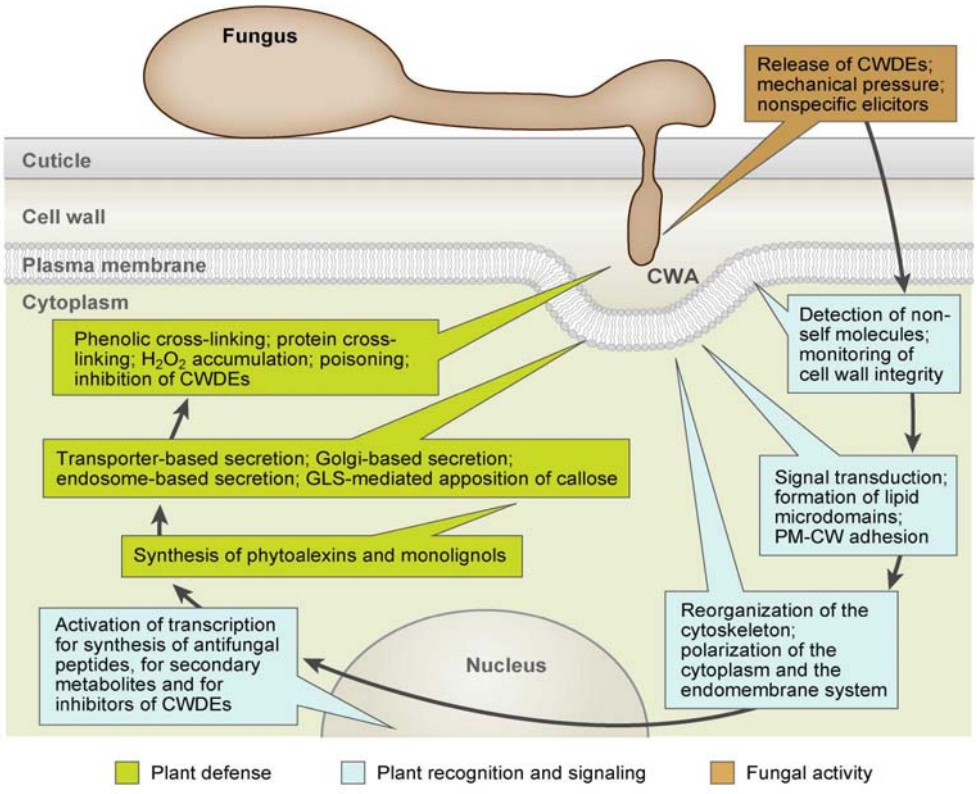


Figure 1: Biochemical and molecular mechanisms for cell wall-associated defence. CW, cell wall; CWA, cell wall apposition; CWDE, cell wall–degrading enzyme; GLS, glucan synthase; PM, plasma membrane (Huckelhoven 2007).

- Cell death at the infection site (hypersensitive response).
- Structural changes in the cell wall (lignification of the cell wall).

- Transcriptional activation of the corresponding defence-response genes.
- Plant defence molecules such as tannins and phytoalexins are detected 2-4h after stimulation with the elicitor.
- Synthesis of jasmonic and salicylic acids as secondary messengers (Katz et al. 2002, Memelink et al. 2001).
- Systemic acquired resistance (SAR).

However, not all elicitors cause this sequence of events. Some peptide elicitors act through plasma-membrane receptors (Nennstiel et al. 1998). Defence gene activation, which in some cases occurs within 2 to 3min of elicitor treatment, is a part of massive change in the pattern of mRNA and protein synthesis underlying the induction of defence responses (Cramer et al. 1985). Elicitors have also been used as tools to understand the complex pathways and interaction of plant secondary metabolites. Previous results reported by Hahn et al (1978) showed that an autolysate of brewer's yeast (*Saccharomyces cerevisiae*) possesses an elicitor very similar to the Phytophthora elicitor.

Plant-pathogen interactions are extremely complex and dynamic, and the interactions between the pathogen and the plant are difficult to monitor with traditional genetic and biochemical methods. With the advent of the large-scale genomic sequencing and EST (expressed sequence tag) projects, and with the development of proteome techniques, it is now possible to monitor the expression of hundreds or thousands of genes and their products simultaneously. This can be done under different defence-related treatments and over different time periods. The technologies open up tremendous opportunities to identify new pathogenesis-related genes, co-regulated genes and associated regulatory systems, as well as to reveal interactions between different signaling pathways (Harmer et al. 2001, Kazan et al. 2001).

1.3. Plant symbiont interactions

Symbiotic soil bacteria classified as *Azorhizobium, Bradyrhizobium, Rhizobium* and *Sinorhizobium* are collectively called rhizobia. These rhizobia can form a symbiosis with the legumes. The infection of the legume by the soil bacterium leads to the formation of a new structure called root nodule, where nitrogen fixation occurs (Hirsch 1992). Aromatic compounds from legumes called flavonoids signal the rhizobial bacteria to produce

lipochitooligosaccharide compounds called Nod factors (Perret et al. 2000). Nodulation in legumes is activated in response to rhizobial nodulation factors. Though Nod factors are produced by the bacteria, they behave in many ways like plant hormones and they activate multiple responses in the host plant that prepare the plant to receive the invading bacteria. They are diffusible signals that activate diverse developmental processes in the plant. Nod factors possess species-specific modification that has important functions in defining the specificity of the interaction between the rhizobia and their plant hosts (Denarie et al. 1996, Downie 1998). During nodulation, three major infective phases are readily observable (Figure 2): (i) bacterial colonization of the root hair, (ii) bacterial invasion of nodule tissue via extracellular infection threads and (iii) uptake of bacteria into nodule cells at infection thread termini. There they penetrate into the plant cortical tissue and deliver the bacteria to their target cells. Plant cells in the inner cortex internalize the invading bacteria in host-membrane-bound compartments that mature into structures known as symbiosomes. Differentiated intracellular bacteria, termed bacteroids, proceed to reduce atmospheric nitrogen and deliver fixed nitrogen to the plant. The nodulation programme plays a central role in nitrogen cycling in many native and agricultural ecosystems, with legumes and their microbial symbionts providing nearly half of all biologically fixed nitrogen entering the soil (Zahran 1999).

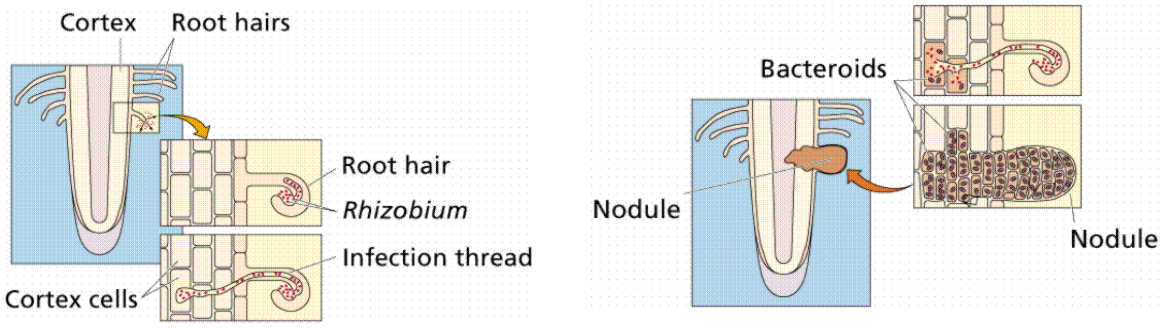


Figure 2: Root nodule formation by the symbiotic bacteria. Rhizobia become attached to emerging root hairs and as a consequence of Nod factor activity the root tip bend entrapping the Rhizobial colony within pockets formed by the walls of the curled root hairs. A new wall is synthesized at the infection site, forming a tunnel shaped structure, the infection thread. Once the Rhizobia enter the cortex region, cells in the root cortex divide under the influence of Nod-factor signal molecules or possibly a secondary plant factor.

The initiation of infection thread growth is a major stage for the discrimination of the appropriate bacterial species and involves a number of checkpoints: a high degree of Nod factor specificity, probably increased levels of Nod factors, and the presence of appropriate

polysaccharides on the surface of the bacteria. Rhizobial surface polysaccharide plays a critical role during infection and the best-understood interaction is the one between *Sinorhizobium meliloti* and *M. truncatula* (Jones et al. 2007). Most strains of *S. meliloti* produce two structurally distinct exopolysaccharides called succinoglycan and EPSII; mutants lacking both are unable to produce normal infections but mutants lacking only one are able to initiate infection (Jones et al. 2007). The active components of these polysaccharides are low molecular-weight forms, because *S. meliloti* mutants unable to produce these forms are defective for infection (Gonzalez et al. 1996, Leigh et al. 1985, Urzainqui and walker 1992). This reports points toward the possibility of polysaccharide fragments acting as signals, which fits with reports that addition of polysaccharide fragments can complement exopolysaccharide mutants (Djordjevic et al. 1987, Leigh et al. 1985). Initiation of infection in *S. rostrata* by its symbiont *Azorhizobium caulinodans* also requires surface polysaccharides, but in this case purified lipopolysaccharides can restore infection in an *A. caulinodans* mutant with pleiotrophic alterations to diverse surface polysaccharides (Mathis et al. 2005). One possible role for these surface polysaccharide signals could be to affect plant cell defence responses (D'Haeze and Holsters 2004, Niehaus et al. 1993), although a positive signaling role is also possible (Scheidle et al. 2005).

Genes from both the plant and rhizobia play an important role in the establishment and maintenance of this interaction (Bladergroen and Spaink 1998, Schultze and Kondorosi 1998) in which the plant supplies reduced carbon to the bacteroid (the nodule-residing form of the bacteria) in exchange for fixed nitrogen. For example, *S. meliloti* nodulates *Medicago truncatula* and other legumes including *M. sativa* and *Melilotus alba*. Also *S. meliloti* strain 1021 has been proposed as a model microsymbiont and *M. truncatula* as a model legume by Cook in 1999.

Bacterially derived secreted and cell-surface components have received a great deal of attention, since these molecules come into direct contact with the host during infection. In *Sinorhizobium meliloti*, important symbiotic roles have been attributed to cyclic glucans, outer membrane lipopolysaccharides (LPS), extracellular polysaccharides (EPS) and the lipid-linked oligosaccharide known as Nod factor (Niehaus and Becker 1998). All these molecules are synthesized by the bacterial symbiont and exported to the periplasm,

the outer membrane or the extracellular milieu. Various models have been proposed to account for how these secreted compounds might direct the symbiotic programme as developmental signal, suppressors of host defence responses or as contributors to cell envelope integrity (Fraysse et al. 2003, Gage 2004, Jones et al. 2007, Oldroyd and Downie 2006, Tellström et al. 2007).

For various Rhizobium species, it was found that EPS play an important role in the infection of the host plant (Albus et al. 2001). The rhizobial cell surface components involved in the plant-bacterial interaction are the LPS bound to the outer membrane of gram-negative bacteria (Zevenhuizen et al. 1980, Carlson 1984). The response to these bacterial LPS is different in most plant species (Dow et al. 2000). Inoculation of a leaf with purified LPS from a plant pathogenic strain can often induce localized resistance in the treated tissue to subsequent infection by that strain (Dow et al. 2000). In many cases, LPS pretreatment can prevent a plant hypersensitive response that results in severe tissue damage (Dow et al. 2000). This may be due to the antimicrobials produced in the LPS-treated leaf tissue prevent the bacteria from proliferating enough to activate a hypersensitive response (Dow et al. 2000). But in contrast, plant cell cultures often respond to LPS fractions from bacteria with an oxidative burst and transcription of defence genes (Zeidler et al. 2004). For example, *S. meliloti* LPS core oligosaccharide can induce an oxidative burst in cultured cells of the nonhost plant tobacco (*Nicotiana tabacum*) (Scheidle et al. 2005). However, *S. meliloti* LPS can suppress both an oxidative burst and the expression of defence genes in host plants *M. truncatula* and *M.sativa* (Scheidle et al. 2005, Tellström et al. 2007). Also, *S. meliloti* LPS act as a specific signal molecule in promoting the symbiotic interaction and suppressing a pathogenic response in the host plant, alfalfa (Albus et al. 2001).

In order to understand the plant microbe interaction, it is necessary to know which genes are expressed; their functions, how the gene products interact and how these processes depend on biotic and abiotic conditions. For example extensive alteration in the CWP pattern caused by stress-inducing compounds such as elicitors during infection processes can be modelled. As plant cells cultured in suspension have cell walls comparable to primary cell walls found in the meristematic cells of the plant, they can be used to study some of the signal dependent changes in their composition. Figure 3

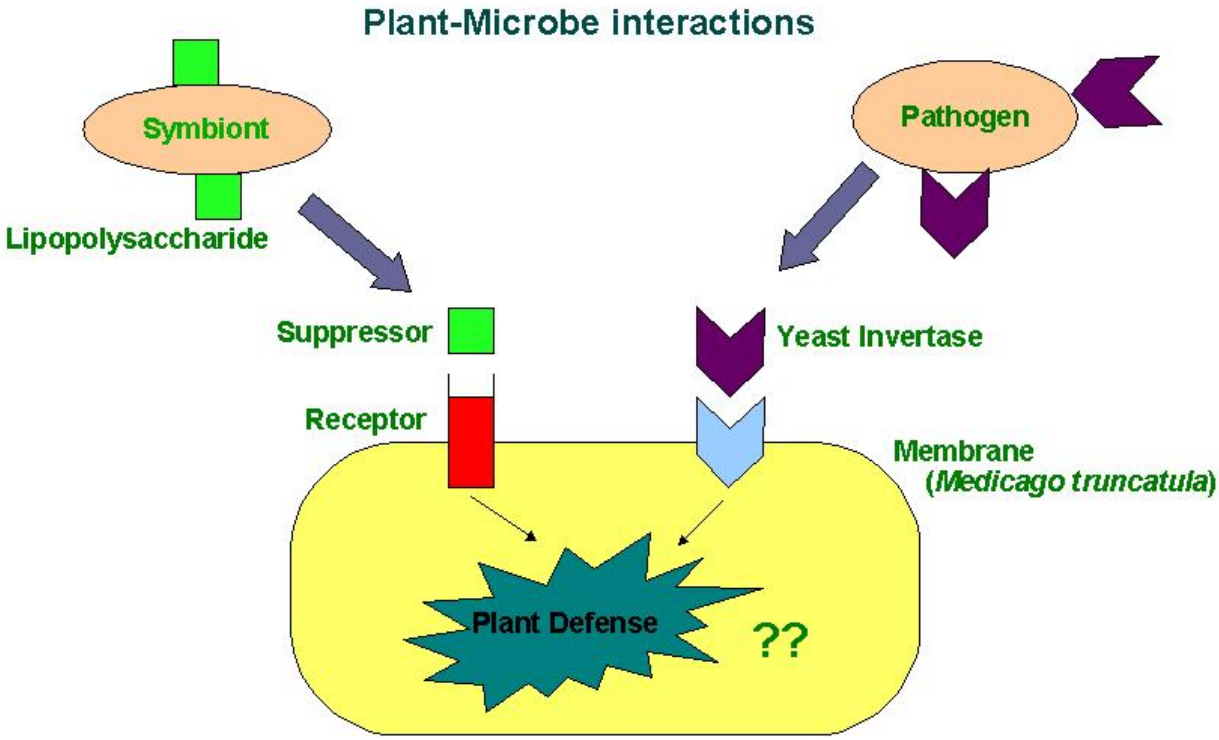


Figure 3: Hypothetical model for plant-microbe interaction, depicting the main aim of this study comparing the symbiotic and pathogenic interactions. The symbiotic interactions are represented by the *Sinorhizobium meliloti* lipopolysaccharide (Left) and the pathogenic interaction represented by elicitation with yeast invertase (Right) in *Medicago truncatula*.

indicates the proposed model of this work in concern with the plant microbe interactions.

1.3.1. *Medicago truncatula*

M. truncatula, a close relative to alfalfa, has been used as a model legume because of its small diploid genome, it is self fertile and easily transformed (Cook 1999). The importance of legumes to global agriculture and the need to understand these important biological functions not found in existing plant models make *M. truncatula* exceptionally interesting for sequencing. Also these large scale sequencing of *M. truncatula* ESTs gives us a chance to study variation of the plant cell wall proteomics in response to both symbiotic and pathogenic interaction.

Among cultivated plants, legumes are unique in their ability to fix atmospheric nitrogen through a symbiotic relationship with nitrogen-fixing bacteria. Among legumes, *M. truncatula* (www.medicago.org) is widely considered to be one of the pre-eminent

model for genomic research as a reference representing most legume-specific properties beside *Lotus japonicus* (Handberg et al. 1992). *M. truncatula* has a small compact diploid genome and is well suited for the study of the cell wall proteome during pathogenic or symbiotic condition (Barker et al. 1990, Cook et al. 1997, Cook 1999). Since the establishment of *M. truncatula* as a model legume, an international sequencing project has so far generated DNA sequences of approximately 227,000 expressed sequence tags (ESTs), which is the third largest EST data base next to *Arabidopsis* and soybean (Brink et al. 2002). Recent achievements in genome and EST analysis have provided a lot of information for model plants but sequence information alone is not sufficient for gene function prediction and developmental biology. In this context, proteomics is a powerful tool to characterize which proteins encoded by the genome actually perform enzymatic, regulatory and structural functions (Watson et al. 2003).

2.0. Structure and functions of plant cell walls

2. 1. General structure of the cell wall

The cell wall is the rigid, outermost covering of the plant cell. It is a complex molecular entity made of polysaccharides, lignin, suberin, waxes, proteins, enzymes, calcium, boron and water that has the ability to self-assemble. During growth, development, environmental stresses and infection, the cell wall is continuously modified by enzyme action (Cassab and Varner 1987). Cell walls consist of 3 types of layers: **Middle lamella**, this is the first layer formed during cell division. It makes up the outer wall of the cell and is shared by adjacent cells. It is composed of pectic compounds and proteins. **Primary wall** is thin, flexible and formed after the middle lamella it consists of a rigid skeleton of cellulose microfibrils embedded in a gel-like matrix composed of pectic compounds, hemicellulose, and glycoproteins. **Secondary wall** is stronger, more rigid and formed after cell enlargement is completed. The secondary wall is extremely rigid and provides compression strength. It is made of cellulose, hemicellulose and lignin. The secondary wall is often layered. The cell wall surrounds the cell membrane (Figure 4).

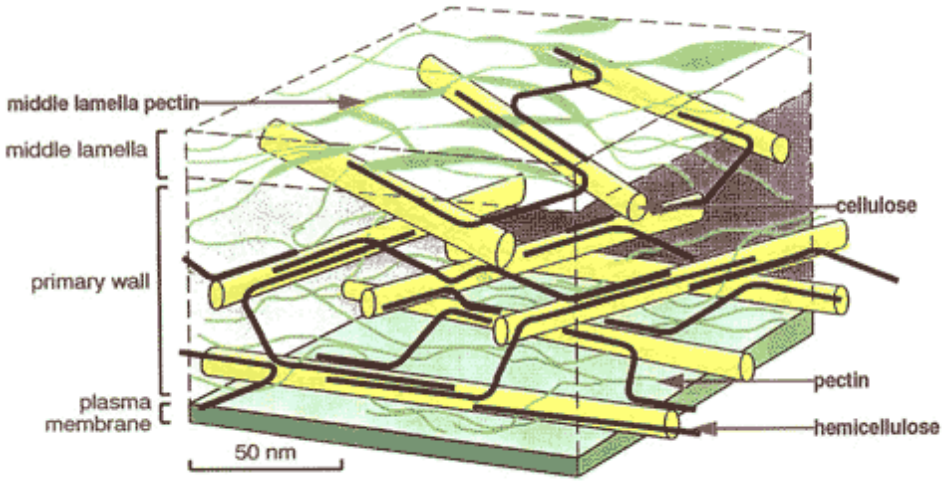


Figure 4: Model of a plant primary cell wall structure. It is formed after the middle lamella and consists of a rigid skeleton of cellulose microfibrils embedded in a gel-like matrix composed of pectic compounds, hemicellulose, and glycoproteins.

In plants, the cell shape is determined by the presence of a rigid cell wall separating the cell from the extracellular medium. In the past years, the cell surface of higher plants – the cell wall and the plasma membrane–was recognised as highly dynamic and complex, both in structure and function. It plays an essential role not only for the cell structure, but also in cell growth, expansion, development, positioning during morphogenesis, in response to environmental factors and in pathogen or symbiont interaction (Bolwell 1993, Cassab 1998, Roberts 1994, Sakurai 1998). The content of this compartment is rapidly changed in response to internal and external factors.

2.2 Cell Wall Proteins (CWPs)

Cell wall proteins are essential constituents of plant cell walls, involved in modifications of cell wall components, wall structure, signaling and interactions with plasma membrane proteins at the cell surface. The principle composition of the plant primary cell wall is well known (90% polysaccharides and less than 10% proteins, phenols, lipids and other compounds), but aspects such as assembly and remodeling remain to be investigated (Carpita et al. 2001). Proteins localized in cell walls are ubiquitous and relatively abundant in land plants and green algae. Accordingly, cell wall proteins of higher

plants can generally be categorised into three major groups of ubiquitous associated proteins: cell wall modifying proteins (e.g. glycosidases), defence related proteins (e.g. peroxidases) and structural proteins (e.g. extensins, proline-rich proteins) (Cassab 1998).

Several proteins are found to be associated with the cell walls (Cassab 1988, Fry 1995). Some can modify the major polysaccharides of the plant wall, e.g. endoglucanases, xylosidases, pectinases, pectin methyl esterases, and xyloglucan endotransglycosylases. Also many glycosyl hydrolases and transferases are known to be present in plant cell walls. Some of these are exoglycosidases such as ß-glucosidase, ß-xylosidase, ß-galactosidase, alpha-galactosidase, and ß-fructofuranosidase (McNeil et al. 1984). Other proteins can act on the wall of bacterial and fungal pathogens, classified as defence related proteins, e.g. chitinases and (1-3) ß-glucanases, or modify other substrates in the wall, e.g. invertase, peroxidases, phosphatases, various dehydrogenases (Cosgrove 1997).

Structural proteins localized in cell walls are unusually rich in one or two amino acids, contain highly repetitive sequence domains and may be highly or poorly glycosylated (Cassab 1998). However, the majority belong to the hydroxyproline-rich glycoproteins (HRGPs) or extensins, the proline-rich proteins (PRPs), the glycine-rich proteins (GRPs) and the proteoglycan arabinogalactan proteins (AGPs). With the exception of the glycine-rich group, all these proteins have a predominance of the unusual amino acid hydroxyproline (Showalter 1993). Recent characterization of new cell wall proteins show that the classification of proline-rich, hydroxyproline-rich, and glycine-rich proteins may be more relevant to sequence domains within proteins than to the proteins themselves because there are proteins that have a mixture of these domains (Carpita et al. 1996). Although these proteins have structural roles, they also appear to function in other equally important and novel functions which have yet to be investigated (Domingo et al. 1999, Hunter et al. 1999, Hay et al. 1998, Reiter 1998, Showalter 2001). The alkaline extensins (pI~10) which belong to the group of the HRGP contain the repeating pentapeptide motif Ser-Hyp_4. They are arabinose or galactose glycosylated and can be extensively cross-linked in the cell wall.

It has been proposed that extensin is slowly insolubilized in the cell wall by a covalent link (Cooper and Varner 1983, Lamport 1986). Extensins are generally rich in Lys,

making them basic proteins, possibly interacting with acidic pectic blocks in the cell wall. The abundant Tyr residues might be involved in isodityrosine cross-links (Keller 1993). One proposed covalent link is isodityrosine formed between two Tyr residues from different extensin molecules (Fry 1986). This proposition, however, seems to be vague, since the evidence is circumstantial. Isodityrosine is present in many but not all cell walls, and to date no intermolecular cross-link has been characterized. A novel amino acid, di-isodityrosine, has been isolated from hydrolysates of cell walls of tomato cell culture (Brady et al. 1996). This compound could form an interpolypeptide linkage between cell wall proteins such as extensin. It has been found that, at least in cotton suspension culture; there is a covalent linkage between pectin and most or all of the extensin that has been incorporated into the cell wall matrix (Qi et al. 1995).

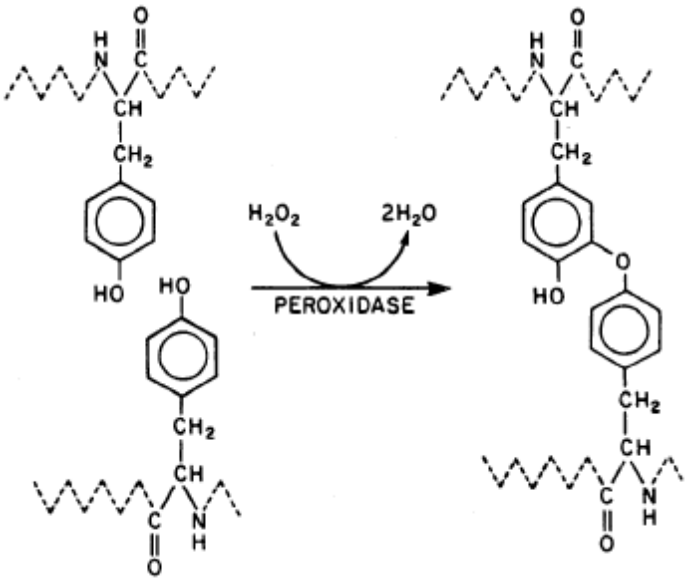


Figure 5: A possible mechanism for the formation of isodityrosyl residues that are believed to cross-link extensin molecules.

Another possible cross-link could be derived from the peroxidization of extensin (Varner 1994). Proteins can be peroxidized by exposure to ROS, such as hydroxyl free radicals produced from hydrogen peroxide and Fe^{2+} ions (Gebicki and Gebicki 1993). The most likely mechanism for the synthesis of isodityrosyl residues are the peroxidase-catalyzed oxidative coupling of tyrosyl residues (Figure 5) (Fry 1982, 1983). The kinetics of this reaction appears to differ widely. A small amount of the extensin precursors was insolubilized very soon after secretion, and the rest more slowly (Cooper and Varner 1983). The slow insolubilization was inhibited by peroxidase inhibitors, e.g. ascorbic acid

(Cooper and Varner 1983), suggesting that at least this slow reaction was due to the peroxidase catalyzed formation of isodityrosyl bridges. The expression of extensins is regulated during wounding, infection and development (Showalter 1993). The PRPs show two broad subclasses characterised by repeating Pro-Pro units. They are only lightly glycosylated and may be involved in various aspects of development. GRPs are another class of structural wall proteins that probably provide elasticity and may serve as nucleation sites for lignin synthesis. The protein is 68% Gly and 12% Ser and is slightly glycosylated. They usually contain an amino terminal signal peptide and, thus, are believed to be cell wall proteins. AGPs are not covalently linked to the cell wall and therefore do not have a structural function. Upon wounding, AGPs are secreted in large amounts and they might act as a physical barrier by producing a gel plug. However, AGPs are present in many different tissues and are not exclusively produced upon wounding, and thus they may display other functions. A role of AGPs in plant differentiation has been proposed (Knox et al. 1991, Kreuger and Holst 1993, Schindler et al. 1995). The acidic AGPs (pI~2 to 5), initially membrane-bound by glycosylphosphatidyl inositol anchors, appear to be a generally water soluble and highly glycosylated (2 to 10% protein by weight) family of proteins with significant homology to extensins (Frueauf et al. 2000). No clear function for AGPs has been established so far but they are supposed to play a role during embryonic development.

2.3. Dynamics of the plant cell walls

The plant cell wall is a dynamic structure whose content of the compartment is changed in response to internal and external factors. Three major polymeric components of the cell wall, the polysaccharides, proteins and phenolics have been shown to change quantitatively and qualitatively in response to a variety of signals both host and environmentally derived (Figure 6) (Sakurai et al. 1998, Bolwell et al. 1993, Carpita et al. 1993).

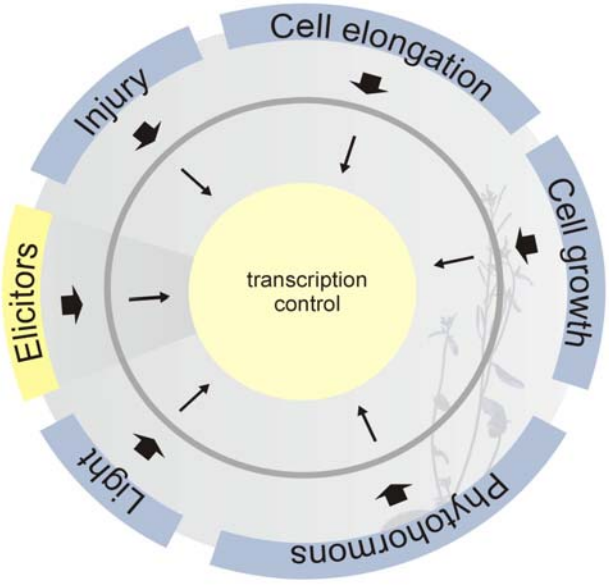


Figure 6: A model-depicting transcriptional regulation of the dynamic cell wall in response to various factors like light, elicitors, phytohormones, injury, cell growth and elongation.

The cell wall changes in composition during expansion growth, differentiation and during responses to environmental stress and pathogen attack (Fry et al. 1998, Bolwell et al. 1993, Sakurai et al. 1998). Plants exhibit natural resistance to disease, and attempted infection by an avirulant pathogen or non-pathogen induces a number of defences, which involves phytoalexin synthesis, wall toughening, accumulation of the cell wall HRGPs and increase in the activity of lytic enzymes such as chitinases and other antimicrobial proteins (Lamb et al. 1989, Bowles 1990, Sequeria 1983). Such responses can be induced not only by infection but also by microbial elicitors and endogenous plant elicitors (Darvill et al. 1984, Ayers et al. 1976, Templeton et al. 1988). Cell wall dynamics are achieved by two distinct steps: 1) the biosynthesis of cell wall components by the action of membrane-bound enzymes at the plasma membrane and endoplasmic reticulum-Golgi apparatus; 2) by the assembly and rearrangement of cell wall structures in muro through the actions of extracellular proteins.

3.0. Proteomics

Proteomics is a high-throughput experimental analysis of hundreds of proteins particularly their expression, structures and functions.

3.1. Two-dimensional gel electrophoresis (2D PAGE)

Two-dimensional gel electrophoresis (2-D electrophoresis) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples (O'Farrell et al. 1975). This technique separates proteins in two steps, according to two independent properties: the first-dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI), the second-dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MW). By this method, complex mixtures consisted of thousands of different proteins can be resolved and the relative amount of each protein can be determined.

The procedure involves placing the sample in gel with a pH gradient and applying a potential difference across it. In the electrical field, the protein migrates along the pH gradient until it carries no overall charge. This location of the protein in the gel constitutes the apparent pI of the protein.

There are two alternative methods to create the pH gradient - carrier ampholites and immobilized pH gradient gels (IPG). The IEF is the most critical step of the 2-D electrophoresis process. The proteins must be solubilized without charged detergents, usually in high concentrated urea solution, reducing agents and chaotrophs. To obtain high quality data, it is essential to achieve low ionic strength conditions before the IEF itself. Since different types of samples differ in their ion content, it is necessary to adjust the IEF buffer and the electrical profile to each type of sample. The separation in the second dimension by molecular size is performed in slab SDS-PAGE. SDS is an anionic detergent that denatures proteins, masks the charges and moves in a negative SDS-protein-complex into the direction of the electrophoresis anode. Thus the proteins are separated in the polyacrylamide matrix proportional to their size. To ensure transfer of the proteins from the first dimension matrix to second dimension gels, equilibration of the IPG gel strips with a buffer containing SDS and a reducing agent is necessary to cleave the disulfide bonds between cysteine residues by, e.g. dithiothreitol (DTT) (Görg et al. 2000). The protein spots on the 2-D gel can be visualized by staining methods like Coomassie Blue staining and silver staining. Coomassie Blue is an organic dye (anionic triphenylmethane) that binds non-covalently to the lysyl residues of proteins, which are stained in proportion to the amount of their basic and aromatic amino acids and the amount of protein in the spot. In

contrast to the silver staining method, at least 0.1µg of protein per spot is required. The colloidal Coomassie staining reveals increased sensitivity (ca. 30ng per band) and shows no background staining (Neuhoff et al. 1985). Silver staining is a very sensitive method limited to protein concentrations between 1 and 10ng. The silver (Ag^+) ions form complexes with glutamine, asparagine and cysteine residues (Coligan et al. 1995). The application of new silver staining protocols in which the silver reducing agent formaldehyde is used instead of glutaraldehyde allows a MALDI compatible analysis (Shevchenko et al. 1996).

3.2. Preparative isoelectric focussing (Rotofor system- Bio-Rad)

The Rotofor system fractionates complex protein samples in free solution using preparative isoelectric focusing. The Rotofor system is used for the initial clean up of crude samples and in purification schemes for the elimination of specific contaminants from

Figure 7: Scheme representing the Biorad's Rotofor System and their components used to fractionate complex protein samples in free solution using preparative isoelectric focusing.

proteins of interest that might be difficult to remove by other means. The Rotofor cell provides up to 500-fold purification for a particular molecule in less than 4 hours. Since the isoelectric focusing is carried out in free solution, fractions from an initial run can be collected, pooled, and refractionated. Purification using isoelectric focusing is especially

advantageous when protein activity must be maintained. Bioactivity is maintained because the proteins remain in solution in their native conformation.

The main component of the Rotofor cell is the cylindrical focusing chamber with an internal ceramic cooling finger. The membrane core, with nineteen parallel, monofilament polyester membranes divides the focusing chambers into 20 compartments, each holding one fraction (Figure 7). Rotation of the chamber at 1rpm stabilizes against convective and gravitational disturbances. After focusing, the solution in each compartment is rapidly collected without mixing using the harvesting apparatus supplied with the unit. The use of interchangeable focusing chambers allows the Rotofor System to accommodate a range of sample volumes. The Mini Rotofor chamber is used for sample volumes of 18ml containing µg to mg of total protein. The Standard Rotofor chamber is used for samples of 35 to 60ml containing mg to g of total protein.

3.3. Mass Spectrometry

Mass spectrometry (MS) is an analytical tool used for measuring the molecular mass of a sample. MS for protein identification depends on the digestion of protein samples into peptides by a sequence-specific protease such as trypsin. After the proteins are digested, the peptides are delivered to a mass spectrometer for analysis via chromatographic separation coupled online to electrospray ionization (LC-MS for liquid chromatography mass spectrometry) or by Matrix-assisted laser desorption/ionization (MALDI) as an alternative ionization method (Aebershold et al. 2003, Hoog et al. 2004, Mann et al. 2001).

Mass spectrometers can be divided into three fundamental parts, namely the ionisation source, the analyser, and the detector. The sample is introduced into the ionisation source of the instrument. Once inside the ionisation source, the sample molecules are ionised, these ions are extracted into the analyser region of the mass spectrometer where they are separated according to their mass (m)-to-charge (z) ratios (m/z). The separated ions are detected and this signal is sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of a m/z spectrum. The analyser and detector of the mass spectrometer, and often the ionisation source too, are maintained under high vacuum to give the ions a reasonable chance of travelling from one end of the instrument to the other without any hindrance from

air molecules.

3.4. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS)

MALDI-MS is an efficient method introduced in 1987-1988 by Karas et al and Taneka et al (1988), reporting UV-laser desorption of bioorganic compounds above 10 kDa for the first time. This is a 'soft ionization' method which ionizes and sublimates the samples out of a dry and crystalline matrix via laser pulses usually from nitrogen lasers with a wavelength of 337nm (Figure 8).

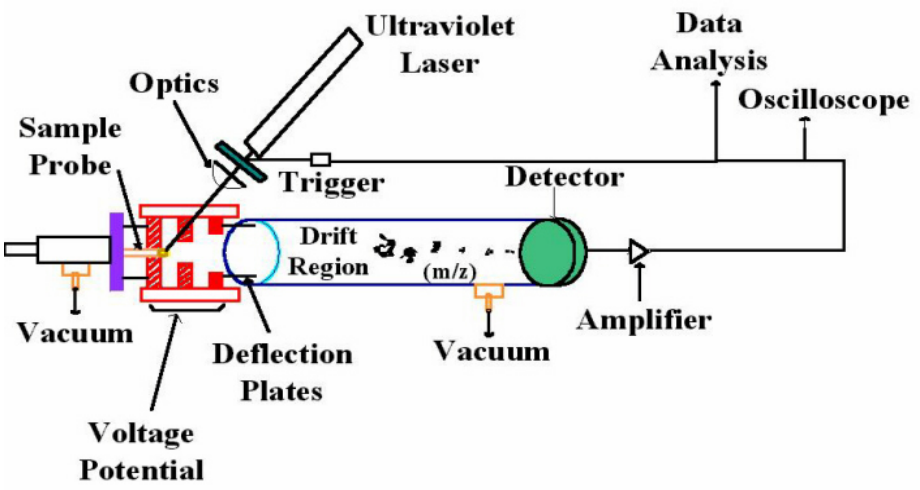


Figure 8: Scheme of a MALDI-TOF Mass Spectrometer. Cocrystallized sample with the matrix is irradiated by a laser beam, leading to sublimation and ionization of peptides. The ions generated were essential at a point source in space and time then enter a vacuum where they were accelerated by a strong electric field in a 'flight tube' where they are then separated in time and finally hit the detector. An analyser measures the time-of-flight (TOF) taken for particular ions to hit the detector. The mass-to-charge ratio is related to the time it takes an ion to reach the detector; the lighter ions arrive first.

Samples mixed with an organic compound like α-cyano-4-hydroxycinnamic acid, have a strong absorption at this laser wavelength. The irradiation by the laser induces a rapid heating of the crystallized sample matrix mixture which generates gas phase, protonated molecules. Matrices minimize the high sample fragmentation by absorbing the incident energy and increase the efficiency of energy transfer from the laser to the biomolecules. To accelerate the ions into the mass analyzer a high voltage (15-25 kV) electric field is applied between the sample slide (Kussmann et al. 2000) and the samples

are separated in a field-free flight tube and detected as an electrical signal at the end of the flight tube. Several advantages have been demonstrated with MALDI, including spectral simplicity due to singly charged ions, a high mass range up to >900 kDa (Nelson et al. 1994), low noise levels, high sensitivity, little sample consumption, short measurement times, average salt tolerance and minimal fragmentation.

3.5. Electrospray Ionization

Electrospray Ionization Mass Spectrometry (ESI-MS) has been developed by Fenn et al (1989). ESI is a method by which ions, present in solution, can be transferred to the gas phase by applying a voltage at atmospheric pressure to the sample droplet, which rapidly evaporates and which impart their charge onto the analyte molecules (Figure 9).

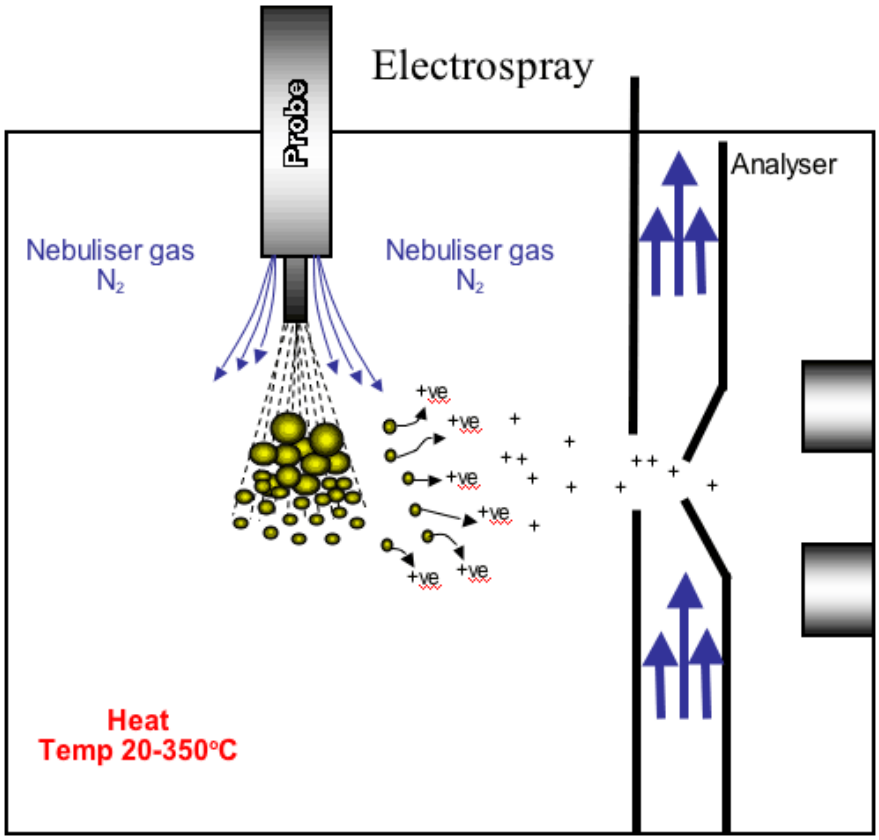


Figure 9: The electrospray ionisation process. A strong electrical charge is applied to the capillary resulting in charged droplets. To assist the evaporation of the eluent a hot bath gas (normally nitrogen) flows around the tip of the capillary. Once the droplet leaves the capillary and enters the nitrogen it continues to lose solvent until the charge density exceeds the surface tension, i.e. the Raleigh constant is exceeded, the droplet explodes resulting in smaller charged droplets. This process continues until the droplets are small enough for ion desorption. The creation of these ions facilitates the transfer of the sample molecules from the source into the MS as the ions are attracted and accelerated into the mass analyser.

Since the ionization process takes place in atmosphere, it is very gentle (without fragmentation of analyte ions in the gas phase). To stabilize the spray, a nebulizer gas or some other device is often employed. This gas, usually nitrogen, helps to direct the spray emerging from the capillary tip towards the mass spectrometer. The molecules are transferred into the mass spectrometer with high efficiency for analysis. It is one of the most effective interfaces for liquid chromatography (LC) and capillary zone electrophoresis.

3.6. Liquid chromatography and Tandem mass spectrometry

When liquid chromatography and Tandem mass spectrometry are coupled, MS analysis of the components of the sample takes place online as the samples elute from the chromatography column. By this so called online method, the samples are cleaned up, separated, and concentrated in a single step. The resulting spectra (MS/MS) generated by this method are used for the database search. Even the crude complex mixtures containing hundreds of proteins can be analysed with high sensitivity by this method (Yates et al. 1997).

3.7. Protein identification and databases

Peptide mass fingerprint (PMF) uses the molecular weights of the peptides resulting from digestion of a protein by a specific enzyme (Trypsin) (James et al. 1993, Pappin et al. 1993, Yates et al. 1993). The principal advantage of PMF is, it is faster and the analysis and database search can be fully automated. The experimental workflow for database matching of MS/MS data is similar to that for PMF, but with an added stage of selectivity and fragmentation.

A large number of programs are now available for the identification of proteins by using uninterpreted MS/MS data. Examples of these include MASCOT (Perkins et al. 1999) and SEQUEST (Eng et al. 1994). The masses of peptides obtained from the proteolytic digestion of an unknown protein are compared to the predicted masses of peptides from the theoretical digestion of proteins in a database. A protein identification can be made if sufficient peptides from the real mass spectrum and the theoretical one overlap. The TIGR MtGI EST, Medicago_SAMS or NCBI nr database were used to identify the proteins. Identification was considered positive comparing the MOWSE (MOlecular

Weight SEarch) score and sequence coverage.

3.8. Aim of this work

The aim of this work is to characterize the cell wall proteome of *Medicago truncatula* in suspension culture. Consequently *M. truncatula* suspension cell culture was adopted as a model for the establishment of the reference CWP map for following investigations of changes in response to pathogen or symbiotic influences (Figure 10).

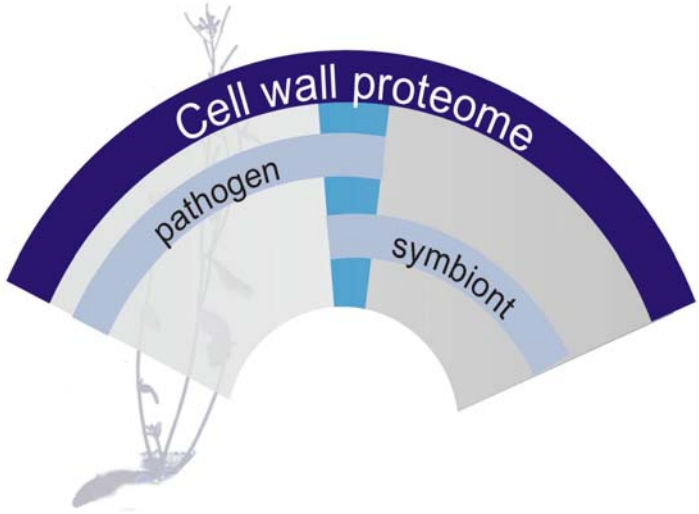


Figure 10: Proposed model of this work. Comparative proteome analysis during elicitation with yeast invertase as a plant pathogen interaction and suppression by *Sinorhizobium meliloti* LPS represented as a symbiotic interaction.

This work contributes to three different tasks: (i) to describe an efficient protocol for extracting CWPs using living cells and/or cell wall fragments. (ii) to evaluate the efficiency of several methods to analyse the CWPs. (iii) to compare the proteome analysis upon elicitation and suppression with yeast elicitor and *Sinorhizobium meliloti* LPS respectively.

Further the CWP extracts using living cells and/or from cell wall fragments will be analysed using techniques like preparative isoelectric focusing, 2D-PAGE, LC-MS/MS and the PMF by MALDI-TOF-MS will be used for the identification of proteins.

2.0. Materials

2.1. *Medicago truncatula*

M. truncatula ‘Jemalong’ cell suspension cultures were used for this study (Scheidle et al. 2005).

2.2. Growth media for cell culture

Murashige & Skoog (MS) medium (pH 5.7)

4.4g	MS medium
30g	Sucrose
0.1mg	Kinetin (1mg/ml, 100µl/l)
1 mg	2,4-Dichlorophenoaceticacid (1mg/ml in EtOH, 1ml/l)
	Autoclaved

Trypton yeast medium (TY medium)

5g	Trypton
3g	Yeast extract
0.4g	$CaCl_2x2H_2O$

2.3. Buffers and solutions

2.3.1. Buffers and solutions for extraction of cell wall proteins.

Living cell extraction:

200mM	$CaCl_2$
1M	NaCl
1M	LiCl
10mM	DTT
50mM	EDTA
80%	Ethanol
80%	Acetone
0.5M	Tris/HCl (pH 6.8)
300µl	Water saturated phenol
1M	DTT
70%	Ethanol
8M	Ammonium acetate

Cell wall Fragments:

50mM	Sodium acetate, pH 5.5
50mM	NaCl
30mM	Ascorbic acid
100mg	PVPP
0.1M	NaCl
10mM	Sodium acetate
200mM	$CaCl_2$
3.0M	LiCl
8M	Urea
4%	CHAPS

2.3.2. Buffers and solutions for oxidative burst measurements

Pre incubation medium		
	3% (w/v)	Sucrose
	40ml	MS medium
Kpi buffer (pH 7.9)		
	50mM	KH_2PO_4
	50mM	K_2HPO_4
Luminol		
	1.2mM	5-Amino, 2, 3-dihydrophthalazin-1-4-dion
	14mM	Potassium-hexacyanoferrate

2.3.3. Buffers and solutions for 2D-PAGE gels (Bio-Rad system)

Tricine gel buffer (pH 8.5)	3M	Tris-HCl
	0.3%	SDS
Anode buffer (pH 8.9)	0.2M	Tris
Cathode buffer (pH 8.2)	0.1M	Tris
	0.1M	Tricine
	0.1%	SDS
Overlay solution (pH 8.5)	1M	Tris
	0.1%	SDS
Agarose sealing solution	100ml	Tricine gel buffer
	0.5%	Agarose
	0.01%	Bromophenol Blue
DTT	28%	Dithiothreitol
SDS solutions	10%	Sodium dodecyl sulfate
Equilibration buffer (EB)	50 mM	Tris-HCl (pH 8.8)
	6M	Urea
	30% (v/v)	Glycerol (87%)
	2% (w/v)	SDS
	0.01%	Bromophenol Blue
EB 1	5ml	Equilibration buffer
	2%	DTT
EB 2	5ml	Equilibration buffer
	2.5%	Iodoacetamide

2.3.4. Buffers and solutions for 1D-PAGE gels

4x stacking buffer (pH 6.8)	500mM	Tris
	0.1%	SDS
4x separating buffer (pH 8.8)	1.5M	Tris
	0.1%	SDS
Protein sample buffer	100mM	Tris
	4%	SDS
	0.2%	Bromophenol Blue
	20% (v/v)	Glycerol (87%)
Cathode buffer (10x)	250mM	Tris
	1.9M	Glycine
	1%	SDS
Anode buffer (10x) (pH 8.4)	250mM	Tris

2.3.5. Buffers and solutions for Western Blot analysis DIG Glycan Detection Kit (Roche)

TB buffer	500mM	Tris
	500mM	Boric acid
TBS buffer (pH 7.4)	10mM	Tris
	150mM	NaCl
DIG Kit contains	5mg	Sodium metaperiodate
	3.75g	Sodium disulfite
	0.5ml	DIG-3-O-Succinyl-aminocaproic acid hydrazide
	300µl	Anti-digoxigenin- AP
	1.1ml	NBT/X-Phosphate solution
	1mg	Transferrin
	1mg	Creatinase
	100ml(10X)	Blocking reagent
Dissolving buffer (pH 5.5)	0.1M	Sodium acetate
Staining solution	0.2%	Ponceau S solution (in 3% acetic acid)
Washing buffer	50mM	Potassium phosphate
	150mM	NaCl

2.3.6. Buffers and solutions for Prepartive isoelectric focussing

Equilibration buffer A (Anion)	0.1M	NaOH
Equilibration buffer B (Cation)	0.1M	H_3PO_4
Ampholytes	40%	Bio-Lyte

2.3.7 Staining buffers and solutions

2.3.7.1. Coomassie Blue staining

Fixing solution	10% (v/v)	Acetic acid
	30% (v/v)	Ethanol
	60%	H_2Odist
Staining solution	2g/L	Coomassie brillant blue CBB-G250 (Roth)
	0.5/L	Coomassie brillant blue CBB-R250 (Roth)
	5%	Methanol
	42.5%	Ethanol
	10%	Acetic acid
	42.5%	H_2Odist

2.3.7.2. Colloidal Coomassie staining (Roti-Blue)

Fixing solution	20ml	Methanol
	1ml	Ortho-phosphoric acid (85%)
	79ml	H_2Odist
Staining solution	60ml	H_2Odist
	20ml	Methanol
	20ml	Roti-Blue (5X)
Washing solution	75ml	H_2Odist
	25ml	Methanol (99.8%)

2.3.7.3. Silver staining for protein gels (MS compatible)

Silver staining solution (prepared fresh)	0.4g	$AgNO_3$
	200ml	H_2Odist
	150µl	Formaldehyde (37%)
Developer (prepared fresh)	15g	Na_2CO_3
	1mg	$Na_2S_2O_3$ x 5 H_2O
	250ml	H_2Odist
	125µl	Formaldehyde (37%)
Sensitizing solution (prepared fresh)	0.1g	$Na_2S_2O_3$ x 5 H_2O
	500ml	H_2Odist

Fixation solution	50%	Ethanol
	10%	Acetic acid
	40%	H_2Odist
	0.5ml/L	Formaldehyde (37%)
Washing solution	50%	Ethanol
	50%	H_2Odist
Stopping solution	44%	Ethanol
	44%	H_2Odist
	12%	Acetic acid

2.3.8. Buffers and Solutions for tryptic digest

Washing solution	0.1%	Trifluoroacetic acid (TFA)
	60%	Acetonitrile (CH_3CN)
Solution A	50%	CH_3CN
	50%	H_2O
Solution B	50%	CH_3CN
	50mM	NH_4HCO_3
Solution C	50%	CH_3CN
	10mM	NH_4HCO_3
Solution D	10mM	NH_4HCO_3
Trypsin solution (SIGMA) proteomic grade	100µl	HCl (1mM)
1 vial (20µg/ml)	900µl	NH_4HCO_3 (10mM)

3.0. Methods

3.1. Cultivation

3.1.1. Cultivation of *Medicago truncatula* cell culture

Medicago truncatula 'Jemalong' cell suspension cultures were obtained by placing sterile root explants of one week old seedlings on sterile MS medium (Murashige and Skoog, 1962) supplemented with the phytohormones, 2,4-dichlorophenoxyacetic acid and kinetin . Eight week-old explants frequently produced callus tissue and non necrotic calli were subcultivated to establish callus cell cultures of *M. truncatula* root tissue. Cell suspension cultures were then obtained by transferring small amounts of callus tissue into liquid MS medium and agitating on a shaker. Cell cultures consisting of single cells and small aggregates of cells were used for sub cultivation. *Medicago truncatula* 'Jemalong' cell suspension cultures were maintained in MS medium and sub cultured every 7 days (Baier et al. 1999).

3.1.2. Bacterial strain and culture conditions

For the isolation of lipopolysaccharides, *Sinorhizobium meliloti* wild-type strain 2011 (Casse and Boucher 1979) was grown in liquid TY medium plates supplemented with 0.4% glucose (w:v) at 30°C for 3 days.

3.1.2.1. Hot phenol extraction and purification of LPS

Sinorhizobium meliloti, wild-type strain 2011 cells were grown on TY agar plates for 3days and washed from the plates with 0.9% NaCl (w:v). After centrifugation at 5000g for 20 min, cell pellets of approx. 60g wet weight were resuspended in H_2O and LPS was extracted using the hot phenol–water method according to Westphal et al (1965). The water phase was dialysed extensively against water, proteins and nucleic acids were removed by treatment of the dialysate with 100µg/ml DNAse I (Boehringer Mannheim, Germany), 15µg/ml RNAse (Boehringer Mannheim) and 150µg/ml proteinase K, redialysed and lyophilized. The LPS was resuspended and purified by ultracentrifugation at 100 000g. Subsequently, the LPS was further purified by gel-permeation chromatography using a Sephadex G-50 matrix in a pyridine acetate solvent (0.4% pyridine, 1% acetic acid).

3.2. Determination of the oxidative burst reaction in plant cell suspension cultures

Yeast invertase (elicitor) was used as an inducer of defence response of the plant. And for the suppression of elicitor induced oxidative burst activity, the same concentration of elicitor is added to the cells along with the *S. meliloti* LPS. The *Medicago truncatula* cell cultures were treated with water, 25*µ*g/ml yeast invertase (Sigma), 20*µ*g/ml *S. meliloti* LPS, or a combination of 25*µ*g/ml invertase and 20*µ*g/ml *S. meliloti* LPS, incubated for 90 min and 12h with constant shaking. The detection of the oxidative burst was performed using the H_2O_2-dependent chemiluminescence reaction described by Warm et al (1982). Three to five days after sub cultivation, 2g of cell material from the cell suspension cultures were diluted in 8ml of preincubation medium (3% w:v sucrose in 0.04× MS; Murashige et al. 1962) and incubated for 3–4h. For the measurement of the oxidative burst 200 µl aliquots of these suspensions were mixed with 700µl phosphate buffer (50mM K-phosphate; pH 7.9) and 100µl 1.2mM luminol in the same phosphate buffer. The reaction was started by the addition of 100µl of 14mM potassium-hexacyanate. The luminescence was measured with a Sirius Luminometer from Berthold Detection Systems (Pforzheim, Germany).

3.3. Extraction of cell wall proteins.

3.3.1. Extraction of cell wall proteins from living cells

Subsets of cell wall proteins were extracted directly from living cells. For this purpose, *M. truncatula* cells of three to five days old suspension cultures (200mL) were washed with deionised water in a sieve, resuspended in an equal volume of different extraction solutions without plasmolysis of cells and agitated gently without disruption. For the extraction solutions, 200mM $CaCl_2$, 1M NaCl, 1M LiCl, 10mM DTT or 50mM EDTA containing protease inhibitors (Roche, Complete) were used. Control extractions were performed with distilled water. The cell wall proteins were extracted in parallel for 30min by gentle shaking at 4°C. The suspensions were centrifuged at 10 000g for 20min (4°C) and the pellet was discarded. The extracts were either dialyzed against distilled water in cellulose dialysis tubing (Zellutrans, Roth, MWCO 8 to 10kD) cut bags or subjected to phenol extraction. After dialysis, the proteins were concentrated by 80% acetone precipitation. The precipitate was collected by centrifugation at 10 000g for 15min and washed three times with 80% methanol or ethanol at 4°C.

3.3.2. Phenol Extraction Method

Protein extracts (1ml) were mixed with 100µl of 0.5M Tris/HCl (pH 6.8), 300µL of water saturated phenol, 10µl of 1M DTT and centrifuged for 15min at 8000g. The phenol phase (250µl) was incubated for 1h at -20°C after addition of 10µL 1M DTT, 15µl 8M ammonium acetate and 1ml ethanol. The solution was centrifuged (10 000g, 20min, 4°C) and the supernatant was discarded. The collected pellets were washed twice with 70% ethanol. The sample was lyophilized and resolubilized in rehydration buffer (9M Urea, 2M Thiourea, 4% CHAPS, 1% DTT, and 0.01% Bromophenol-blue).

3.3.3. Isolation of cell wall proteins from cell wall fragments

Seven to eight grams of frozen and thawed cells was ground with a mortar and pestle in ice-cold grinding buffer (50mM Na acetate, pH 5.5, 50mM NaCl, and 30mM ascorbic acid) with 100mg PVPP according to the method published at: http://cellwall.genomics.pudue.edu/techniques/index.html

The cell walls were isolated by filtering through nylon mesh membranes and washing sequentially with grinding buffer (100ml), 0.1M NaCl (50ml), dH_2O (100ml), acetone (250 ml), dH_2O (100ml), and 10mM sodium acetate (50ml) in 50ml increments. Cell wall proteins were extracted sequentially, twice with 6–8ml of 200mM $CaCl_2$, 50mM sodium acetate (incubated on ice for 30min–1h). The $CaCl_2$ extracts were combined and the supernatants were centrifuged at 950g to remove any particulate matter. Proteins were concentrated, desalted and processed using the 2D Cleanup Kit (Biorad). Samples were resuspended in 8M urea and 4% CHAPS. Protein concentrations were determined using the Bradford method with BSA as the standard.

3.4. Protein Estimation (Bradford Method)

The total protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when binding to protein occurs. For the assay, to 10µl of protein solution 790µl of water was added. 200µl of concentrated Biorad reagent is added and incubated at room temperature for 5 min. Assayed for the absorbance at 595nm (Bradford 1976).

3.5. Analysis of *M. truncatula* CWPs

3.5.1. SDS- PAGE analysis of Protein Extracts

Protein samples prepared as described above were denatured at 95°C for 5min in sodium dodecyl sulphate mercaptoethanol buffer (1.5M Tris/HCl, pH 6.8, 20mM EDTA, 10% SDS, 10% glycerol, 10% ß-mercaptoethanol, 0.1% bromophenolblue) and subjected to analysis by electrophoresis on an 8.5% / 4% SDS-homogeneous polyacrylamide gel (8cm x 10cm or 16cm x 18cm; 30min at 50V, 200V until stop) as described by Laemmli et al (1970). Proteins were detected by Coomassie staining or silver staining after separation of the mixture (Table 1).

	Resolving gel				**Stacking gel**
	8.5%	9%	10%	11%	4%
H_2O	4.47 ml	4.35 ml	4.1 ml	3.85 ml	2.89 ml
Tris/HCl-Buffer, pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml	-
Tris/HCl-Buffer, pH 6.8	-	-	-	-	1.26 ml
10 % SDS	100 µl	100 µl	100 µl	100 µl	50 µl
Acrylamide 40% 29:1	2.13 ml	2.25 ml	2.5 ml	2.75 ml	0.5 ml
APS (1.5%)	0.8 ml	0.8 ml	0.8 ml	0.8 ml	0.3 ml
TEMED	5 µl	5 µl	5 µl	5 µl	5 µl
Total Volume	10 ml	10 ml	10 ml	10 ml	5 ml

Table 1: Components of SDS-PAGE for protein separation

3.5.2. 2-Dimensional Electrophoresis of the Cell Wall Proteins

For isoelectric focussing (IEF) 750µg to 1.0mg of protein in 400µl rehydration buffer with 5µl of 28% w/v DTT and 1% of IPG buffer pH 3-10 (Amersham Biosciences) was loaded directly on pH 3-10 IEF 24cm gel strips (Immobiline DryStrip, Amersham Biosciences). IEF was performed for 20-26h to 75 000Vh using the IPGphor™ (Amersham Biosciences).

IPGphor running protocol for 24 cm strips:

20°C, max. 50µA per strip 1h 0V (Rehydration)

12hrs 30V

2hrs 60V

1h 500V

1h 1000V

9hrs 8000V

Total: 26hrs, 57980Vh

After focusing, the proteins were reduced for 15min in 5ml of EB I containing 50mg of DTT and alkylated for another 15min in 5ml of the same buffer with 225mg of iodoacetamide. The gel strips were loaded on top of 12.5% polyacrylamide gels (200x200x1mm) for the subsequent SDS-PAGE using an Ettan Dalt 2D gel system (Amersham Biosciences) and run at 15W per gel. After electrophoresis gels were fixed in fixing solution for 30min, for protein spot visualization stained for 90min in a Coomassie brillant blue (CBB) staining solution and destained in fixing solution twice for 60min. The gels were further destained in 7% v/v acetic acid until the background was clear. For visualization of low abundant proteins CBB stained gels were scanned, subsequently destained completely in fixing solution and used for silver staining modified according to Shevchenko et al (1996).

3.5.3. Preparative Isoelectric Focusing of the Cell Wall Proteins

Separation of cell wall proteins were achieved within a pH 3-10 ampholyte gradient using a Rotofor Cell (Bio-Rad Laboratories). After precipitation with 80% acetone, 10mg of protein sample was loaded in a 1.25% ampholyte solution into the electrofocusing Rotofor Cell and focused for 4h at 4°C at constant 12W. The final parameters of 1400V and 8mA were held for at least 2h. After focusing, 20 fractions were harvested and the pH of each fraction was determined. Each sample was then concentrated by 80% acetone precipitation and used for SDS-PAGE.

3.5.4. LC-MS/MS analysis of cell wall proteins

The cell wall proteins extracted from cell wall fragments of *Medicago truncatula* were digested with trypsin and analyzed using LC-MS/MS, an automated nano-electrospray (Eksigent Technologies, USA) coupled with a Thermo Finnigan LCQ Deca ion trap mass spectrometer (Thermo Electron Corporation, USA). After adding 200µl 60%

acetonitrile and 0.1% TFA the samples were kept at room temperature for 60min. This step was repeated after samples had been transferred into prewashed tubes and the mixtures had been dried in a Speedvac. The dried samples were dissolved in 10µl of 5% acetonitrile, 0.05% TFA. 2µl of this solution was separated chromatographically on an RP-18 capillary column (50µm i.d., Dionex, USA) at a flow rate of 200nl/min. The gradient profile consisted of a linear gradient from 98% A (acetonitrile/H_2O/formic acid, 5/95/0.1) to 50% B (acetonitrile/H_2O/formic acid, 80/20/0.1) over 40 min followed by a linear gradient to 98% B over 5min. The eluted peptides were analyzed by nano-spray MS/MS using the LCQ Deca ESI-ion trap MS. The MS instrument was operated with the following settings: spray voltage 1.3kV, heated capillary voltage and temperature 14V and 165°C, respectively. Collision energy was set to 35%. Upon a full scan a zoom scan was recorded to determine the charge state of the peptide, followed by the isolation of the particular mass and an MS/MS scan. The instrument executed one full scan, followed by a zoom in scan and MS/MS scan of each one of the three most intense peaks from the MS scan. The generated peptide sequence tags were analyzed by the MASCOT database query. Proteins were identified by two or more tryptic peptide matches and MOWSE scores of more than 40 were reported.

3.6. Protein staining methods

3.6.1. Coomassie Blue

Gels were incubated in fixing solution for at least 45min and Coomassie stained overnight. Destaining was performed for 2h with fixing solution and then overnight with 7% acetic acid.

3.6.2. Colloidal Coomassie Blue (Roti-Blue; Carl Roth GMBH & Co)

Gels were incubated for 1h with fixing solution and then stained with colloidal Coomassie for 3h to overnight. Destaining was carried out only with redistilled water (Mahon and Dupree 2001).

3.6.3. Silver staining method

The low abundant cell wall proteins in 2D-PAGE gels were visualized with the MALDI compatible silver staining method (Shevchenko et al. 1996). The MALDI-TOF-MS analysis was carried out immediately after the staining process, because of the reducing agent

formaldehyde, which leads to cross-linking of proteins. The silver staining process was carried out as followed:

- After electrophoresis, the gels were fixed for 20 minutes using the fixing solution.
- Gels were washed for 2x25 minutes with the washing solution.
- Gels were washed again for 10 minutes with distilled water.
- Gels were sensitized by incubating for 1 minute in the sensitizing solution.
- Rinsed with two changes of distilled water for 1 minute each.
- Stained with chilled 0.1% silver nitrate solution for 20 minutes.
- Rinsed with two changes of distilled water for 1minute each.
- The gels were developed with developing solution with intensive shaking.
- It is critical that the developing solution be replaced with fresh solution once it turns yellow. Therefore, the solution was typically replaced every few minutes until the desired staining intensity is reached.
- Staining was terminated by discarding the developing solution and replacing it with 1% acetic acid.
- Gel was stored in 1% acetic acid at 4°C until analyzed.

3.7. Detection of glycoproteins using DIG Glycan detection kit

3.7.1. SDS-PAGE and blotting

The SDS-PAGE gel for the CWPs of *M. truncatula* cell culture were transferred electrophoretically onto a hydrophobic, microporous polyvinylidine (PVDF) membrane, which was chosen for its high binding capacity of proteins and its mechanical stability compared to other membranes. Because of its hydrophobic character, the membrane had to be activated prior to usage with methanol to make it hydrophilic. The CWPs are transferred using a vertical tank-blot system (Hoefer), where the gel and the blotting membranes was clamped in grids between filter papers (Whatman 3MM) and sponge pads suspended in the tank filled with TB buffer (Figure 11). Before loading the tank blot, the blotting membranes and the gel were prepared as followed:

- The membrane and the filter papers were cut according to the size of the SDS gel
- Unstained gel was incubated with the TB buffer for 15min
- PVDF membrane was activated by soaking 15sec in methanol, 2min in distilled water and 5min in TB buffer.

3.7.2. Oxidation and digoxigenin labeling of glycoproteins

Blotted membrane was incubated gently by agitation at 15 to 25°C, except color development which was done without shaking. Membrane was washed approximately with 50ml PBS, pH 6.5; TBS was avoided since Tris interferes with the subsequent digoxigenin labelling. Oxidation was carried out using 10mM sodium metaperiodate, in sodium acetate buffer, pH 5.5. Further the filter was incubated with 10 ml of this solution for 20 min at room temperature. And washed 3 times for 10min each with approximately 50ml PBS. Later the membrane was incubated with 1µl DIG-0-3-succinyl-aminocaproic acid hydrazide dissolved in 5ml sodium acetate buffer pH 5.5, for 1h at 15 to 25°C. Finally washed again 3 times for 10min each with approximately 50ml TBS. At this step, proteins were stained with Ponceau S.

3.7.3. Protein staining

The proteins were stained specifically with the Ponceau S solution. The membrane was incubated in Ponceau S solution for 5min and then rinsed with distilled water until the bands were visible. The filter was then photographed or the standard proteins marked with a pencil for documentation. The Ponceau S staining disappeared during the incubation in the blocking solution.

3.7.4. Glycoprotein detection

The membrane was incubated for at least 30min in the blocking solution. If necessary, the glycoprotein detection was interrupted at this stage and the filter stored at at 2-8°C. Washed 3 times for 10min each with approximately 50ml TBS, incubated with anti-digoxigenin-AP and added 10µl conjugate to 10ml TBS and incubated the filter in this solution for 1h and washed again 3 times for 10min each with approximately 50ml TBS.

Staining reaction: Staining solution (prepared just before use): 10ml Tris buffer, pH 9.5, 200µl NBT/X-phosphate solution.

The filter was immersed without shaking in the staining solution and observed for the development of the grey to almost black color. This normally completes within a few minutes, but can take for up to one hour or overnight if very little glycoprotein is present. Further the filter was rinsed several times with redistilled water to stop the reaction and dried the filter on paper towels. The filter was photographed or photocopied and stored for documentation.

The cell wall proteins are highly glycosylated. In order to detect the extent of glycosylation the extracted CWPs were blotted and stained using DIGE Glycan detection kit. The main principle of this method is the adjacent hydroxyl groups in sugars of glycoconjugates are oxidised to aldehyde groups by mild periodate treatment. The spacer linked steriod hapten digoxigenin (DIG) was then covalently attached to these aldehydes via a hydrazine group. Digoxigenin labeled glycoconjugates were subsequently detected in an enzyme immunoassay using a digoxigenin specific antibody conjugated with alkaline phosphatase (Figure 11).

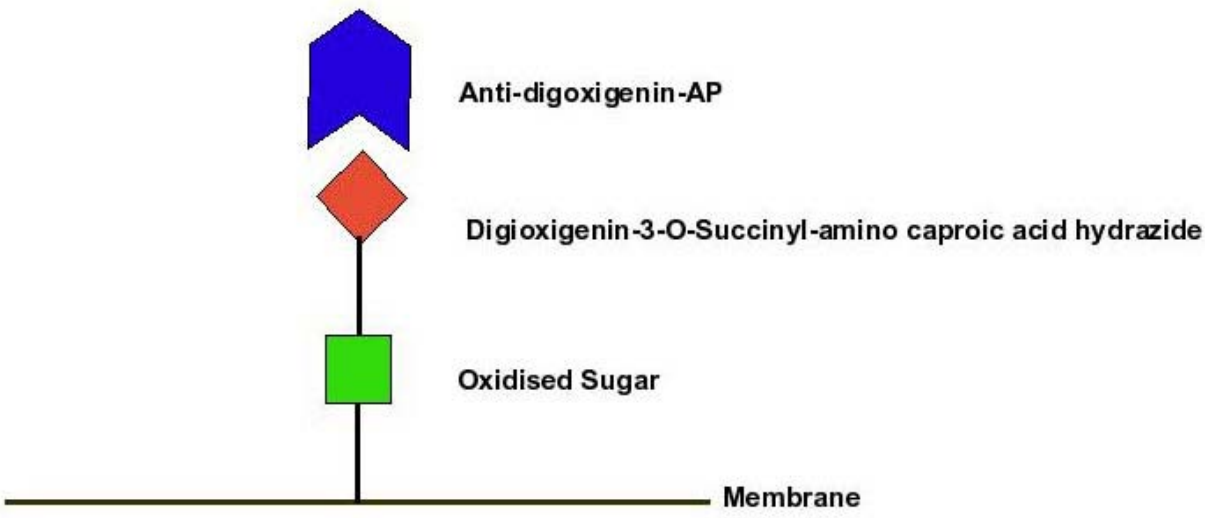


Figure 11: Scheme showing the principle of detecting the glycoproteins. Glycoproteins were detected on PVDF filters by using method B of the Roche Molecular Biochemicals digoxigenin (DIG) glycan detection kit according to the manufacturer's recommendations. Proteins were separated by SDS-PAGE and blotted to PVDF membrane as described above. Subsequently, membranes were washed in phosphate-buffered saline (50 mM potassium phosphate, 150 mM NaCl [pH 6.5]), and carbohydrates were oxidized with sodium metaperiodate. Oxidized carbohydrates were labeled with DIG-conjugated hydrazide, and PVDF membrane was stained with Ponceau S to confirm that equal amounts of proteins had been loaded in each well and that electroblotting had occurred evenly. DIG-labeled proteins were visualized with alkaline phosphatase-conjugated anti-DIG antibodies followed by a color reaction with nitroblue tetrazolium and X-phosphate.

3.8. Mass Spectrometry and Identification of proteins

3.8.1. In-gel Tryptic Digest of Proteins and Mass Spectrometry

The stained protein spots were excised with a 2mm inner diameter Pasteur pipette and placed into a microtiter plate well, which was previously washed twice with TFA:ACN:water 0.1:60:40 (v/v). Each gel piece was washed successively with 150µl of 50% acetonitrile for 5min, with 50mM ammonium hydrogencarbonate, 50% acetonitrile for 30min and with 10mM ammonium hydrogencarbonate, 50% acetonitrile for 30min. After drying for 1h at 37°C, the gel pieces were rehydrated with 10µl of 10µg/ml modified trypsin

(Promega, sequencing grade) in 10mM ammonium hydrogencarbonate and digested over night at 37°C. Gel pieces were sonicated for 5min in order to extract digested peptides. Solution containing the peptides that are released into the buffer were analysed by MALDI-TOF.

3.8.2. Automated sample spotting for MALDI-TOF-MS analysis

Following tryptic digestion analyte samples were spotted, washed and recrystallized on AnchorChip 600/384 targets for Bruker Ultraflex mass spectrometers. A Genesis RSP200 workstation (Tecan, Maennedorf, Switzerland) was employed for pipetting. Matrix solution was prepared from 0.05g of alpha-cyano-4-hydroxycinnamic acid (Bruker) by suspension in 10 ml acetone and subsequently mixed with 20ml ethanol. Of each analyte sample 0.5µl was mixed with 1µl of matrix solution. After spotting on the target the analyte/matrix mixture was dried by evaporation. The dried samples were washed on target. For this purpose, for each sample 1.5µl of 0.1% trifluoroacetic acid washing solution was added and subsequently removed after 5 seconds of incubation. Small remains of the washing solution were evaporated. Then the samples were recrystallized by adding 0.5µl of a mixture of 6 volumes ethanol, 3 volumes acetone and 1 volume of 0.1% trifluoroacetic acid. The samples were evaporated at room temperature. Analyses were performed on Ultraflex MALDI-TOF mass spectrometer (Bruker) which was operated in positive reflector mode at the following parameters: accelerating voltage, 25kV, extraction delay time, 120ns. Acquisition mass was between 500 and 3600Da. External mass calibration was performed using a peptide mass standard (Sigma). Internal mass calibration was performed using trypsin autolysis peaks (monoisotopic MH^+ 842.51 and 2211.10).

3.8.3. Protein Identification by MALDI-TOF and N-terminal signal-peptide prediction

Peptide mass fingerprinting data was analyzed by Flex Control, Flex Analysis and Biotools (Bruker). A search algorithm such as MASCOT (Perkins et al. 1999) is used to compare the experimentally digested and analysed proteins with the theoretically analysed proteins of the organism of interest in the database. The TIGR MtGI EST, Medicago SAMS or NCBI nr database were used to identify the proteins. Tolerance was set to 100 PPM, one missed cleavage was allowed and monoisotopic MH^+ ions were defined. Identification was considered positive when the difference in MOWSE score was more then 60.

For N-terminal signal-peptide prediction the SignalP software was used to search for signal peptides of identified proteins/TC sequences (Nielsen et al. 1997). The following parameters gave the most reliable results: (i) the organism type (t-option) were set to “Eukaryotes”, (ii) only the first 70 N-terminal amino acids were submitted, (iii) both the neural network and Hidden Markov model had to predict the same cleavage site, (iv) the prediction score had to be higher than 0.98.

4.0. Establishment of techniques to analyse the *M. truncatula* cell wall proteome from suspension cell culture

4.1. Extraction of cell wall proteins from the living cells or cell wall fragments using different salt solutions:

4.1.1. Extraction of cell wall proteins (CWPs)

One of the systematic approaches for extraction of CWPs was established by Robertson et al (1997). Also proteomics of *M. sativa* cell walls using stem tissue has been reported (Watson et al. 2004). Figure 12 indicates the microscopic view of individual *M. truncatula* cells. In this work, subsets of cell wall proteins from *M. truncatula* were obtained by washing whole cells with different substances without disturbing the cell integrity.

The analysis of *M. truncatula* cell wall proteins was performed in parallel using solutions containing $CaCl_2$, NaCl, EDTA, LiCl, or DTT. For this purpose, *M. truncatula* suspension culture cells were harvested 5 days after subculture and incubated separately with each of these different solutions. Thus, proteins were extracted from the cell line before extensive lignifications and consequently cross linking occurred.

Figure 12: Microscopic view of *M. truncatula* cells (40 x magnifications).

In this context, $CaCl_2$ was reported as a very efficient salt for the extraction of CWPs from purified cell walls while treatment with high salt concentrations (NaCl or LiCl) was expected to elute proteins associated through ionic interactions and HRGPs. EDTA as a calcium chelatant extracts proteins which are associated with pectins, and DTT may help to extract proteins that have a high content of disulphide bonds (Blee et al. 2001, Robertson et al. 1997, Smith et al. 1984, Voigt et al. 1985).

The concentration of the proteins extracted varied particularly depending upon the applied reagent (Figure 13). Extracted protein samples showed generally very low protein content and high concentrations of interfering substances (salts and polysaccharides). The highest yield could be obtained by extraction with $CaCl_2$ or NaCl (10mg and 12.5mg, extracted from 200ml cell culture). LiCl seemed to be as efficient as NaCl in cell wall protein extraction (12.5mg, extracted from 200ml cell culture). The lowest yield was observed after the incubation of the whole cells with DTT (6.5mg, extracted from 200ml cell culture).

After successful extraction, four different approaches were performed in order to remove disturbing compounds and to concentrate the extracted CWPs: acetone precipitation, TCA precipitation, extraction with phenol and subsequent precipitation with ammonium acetate and a no-precipitation extraction procedure with dialysis as first step. Further these concentrated proteins were lyophilised and the proteins were quantitated using the Bradford Method (Bradford 1976)**.** All the methods were very efficient in removing contaminations like polysaccharides, salts and concentrate proteins but each one resulted in loss of proteins. Best protein yields could be obtained by phenol extraction or dialysis. After optimising the protocol for the extraction of CWPs, different approaches were made in order to separate the extracted CWPs. The methods like one-dimensional and two-dimensional reference maps, preparative isoelectric focussing and LC-MS/MS were established for *M. truncatula* suspension cell cultures. Proteins visualized by standard one or two dimensional electrophoresis were analyzed by PMFs using MALDI-TOF mass spectrometry. A comparison of the obtained PMFs was performed with predicted PMFs of translated EST sequences of the MtGI at The Institute for Genome Research (TIGR, www.tigr.org).

4.1.2. Proteome Analysis of *M. truncatula* cell wall proteins by SDS-PAGE

There are several constraints in cell wall proteome like, the CWPs sticks to the polymeric cell wall compounds like cellulose, pectin or rhamogalactans, these CWPs embedded in a polysaccharide matrix and interact in different ways with other cell wall components, thereby making the extraction very challenging. For this reason the first and critical step was to develop extraction protocols for these proteins. Ionically

bound or hydrophilic proteins can be extracted from the cell wall of living cells by high ionic strength buffers, chelating compounds or reducing agents.

The differentially extracted CWPs from *M. truncatula* suspension cell culture using 200mM $CaCl_2$, 1M NaCl, 1M LiCl, 10mM DTT or 50mM EDTA were concentrated, separated by SDS-PAGE and stained by Coommasie. The tryptic digested protein samples were analyzed by MALDI-TOF-MS. The PMF obtained by MALDI-TOF-MS allowed the identification of proteins by MASCOT, (www.matrixscience.com). SDS-PAGE analysis of extracted cell wall subsets exhibited a striking difference in protein concentration for each of the employed reagents (Figure 13). Identified proteins were listed under the (Table 2). Each entry was indicated by a number which corresponds to the protein spot numbers as displayed in figure 13, accession number, score, number of matched peptides, percentage sequence coverage of the PMF, signal peptide, the relative molecular weight M_r (cal) calculated by the MASCOT database and the pI/M_r (gel) values estimated from the spot positions in the SDS gel. Approximately 40 proteins were selected from the Coomassie Brilliant Blue (CBB) stained SDS-PAGE and excised for PMF analysis after tryptic digestion. Out of these 40 proteins, 13 proteins could be

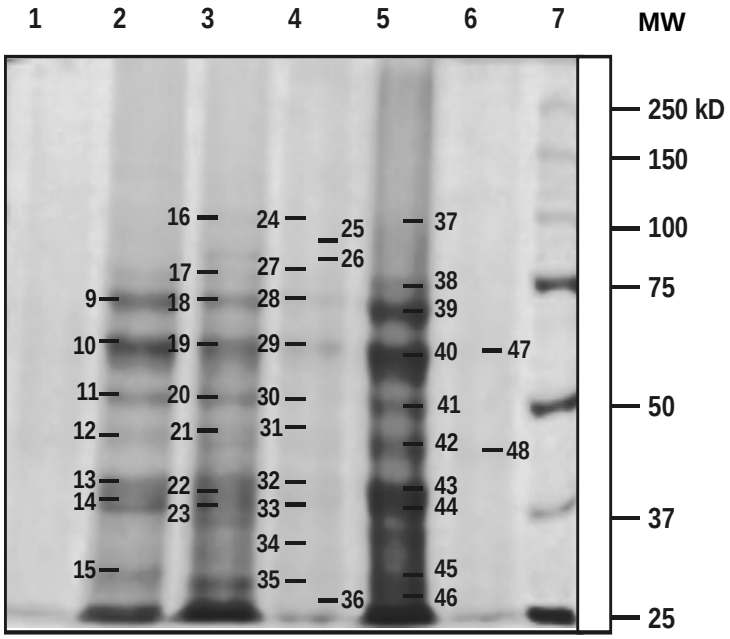


Figure 13: SDS-PAGE of *M. truncatula* cell wall proteins extracted from suspension cell culture using different solutions (1) H_2O (2) 200mM $CaCl_2$; (3) 1M NaCl; (4) 50mM EDTA; (5) 1M LiCl; (6) 10mM DTT; (7) molecular weight standard. Protein bands have been visualized by Coomassie staining. The numbers indicated on the left side corresponds to the bands used for tryptic digestion and MALDI-TOF-MS.

identified with significant scores. Identified proteins include 15% cell wall modifying enzymes, e.g. ß-1.3-exoglucanase. Furthermore, 39% of the proteins were defence related proteins, which include pathogen related protein, (Figure 13, Spot No 33), 23% of the proteins identified were grouped under miscellaneous and 23% were proteins of unknown function (Table 2 and Figure 14). 27 proteins did not show significant hits to any of the protein in the databases**.**

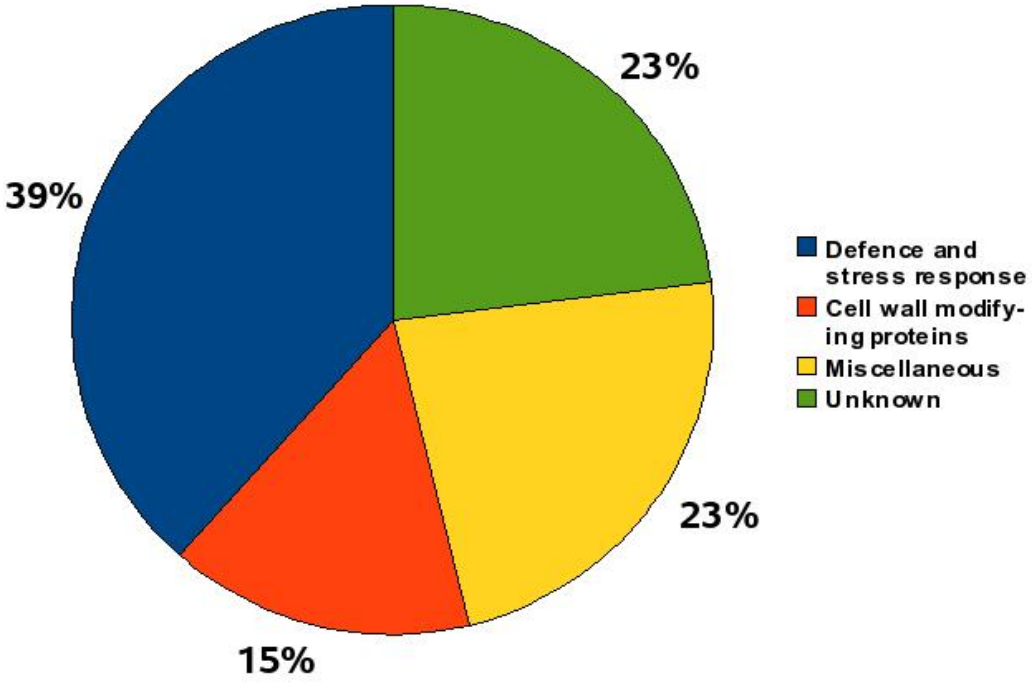


Figure 14: Graphical view of the classification of cell wall proteins separated by 1D-SDS-PAGE

However, this first approach failed to identify cell wall proteins in adequate amounts with sufficient diversity. This was probably in part due to the insufficient separation of proteins of similar molecular mass-more than one protein could be observed in a single protein band and a simple prolongation of the separation distance can not resolve this problem.

4.1.3. Detection of glycosylated *Medicago truncatula* cell wall proteins

Cell wall proteins are ubiquitous and are unusually rich in one or two specific amino acids, contain highly repetitive sequence domains, and are highly glycosylated

(Cassab 1998). Determining whether a protein is in fact glycosylated is also an important concern because glycosylated proteins often migrate as diffuse spots that differ in pI and/or apparent molecular mass. These are usually isoforms of the same protein and result from a variety of posttranslational modifications. Taking this as an important consideration and in order to identify the extent of glycosylation, a commercially available kit (DIG Glycan Detection kit from Roche) was used. The main principle of the method is: The adjacent hydroxyl groups in sugars of glycoconjugates were oxidized to aldehyde groups by mild periodate treatment. The spacer linked steroid hapten digoxigenin (DIG) was then covalently attached to these aldehydes via a hydrazide group. DIG labeled glycoconjugates were subsequently detected in an enzyme immunoassay using a digoxigenin specific antibody conjugated with alkaline phosphatase.

This method was used to detect glycoproteins upon separation by gel electrophoresis and blotting on nitrocellulose or on PVDF membrane and in principle was also suitable for the detection of other glycoconjugates that have been fixed appropriately and contain oxidizable OH-groups. The following methods were developed for the detection of glycoproteins on membrane which had been analyzed on an SDS-polyacrylamide gel and transferred. Glycoproteins which were transferred on a PVDF membrane by blotting are oxidized and labeled with digoxigenin.

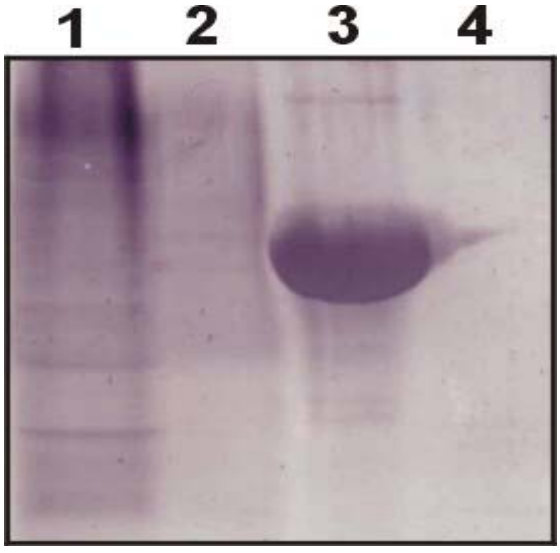


Figure 15: Western blot for detecting the glycosylated proteins (1) $CaCl_2$ extract (after phenol extraction) (2) $CaCl_2$ extract (after dialysis) (3) positive control (transferrin) (4) negative control (creatinase).

For glycoprotein analysis using DIG glycan detection kit, *M. truncatula* wall protein samples extracted with 200mM $CaCl_2$ and further concentrated by phenol extraction method (lane 1) and dialysis method (lane 2) were used for this method (Figure 15). Also transferrin and creatinase were used as a positive and negative control respectively (lane 3 and 4). It is obvious from the results that, there was no significant detection of glycoprotein in lane 1 and 2 comparing the positive control (lane 3). Nevertheless, very low bands for glycosylated proteins can be detected in phenol extracted sample as shown in lane 1 of the immunoblot assay.

4.2. Analysis of Cell Wall Proteins by Preparative Isoelectric Focusing in Combination with SDS-PAGE

In order to enhance the seperation of CWPs, preparative isoelectric focusing was used to fractionate complex CWP samples in free solution as an alternative to IEF.

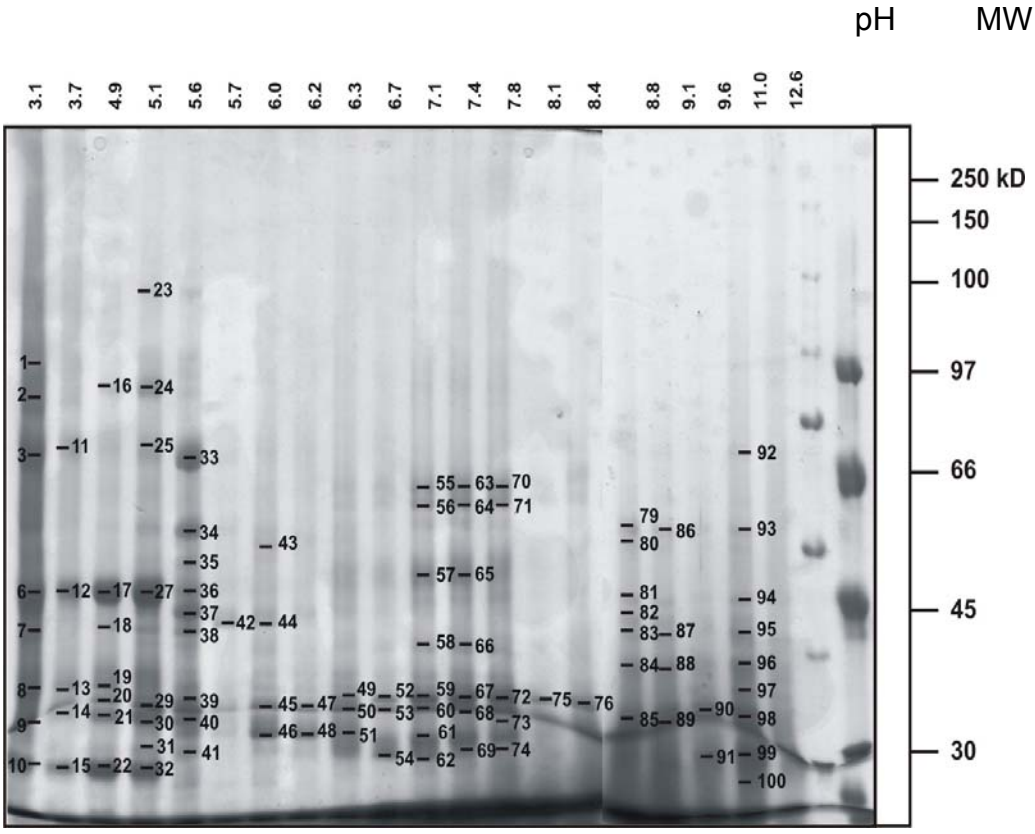


Figure 16: Image of cell wall proteins after separation by preparative isoelectric focusing in combination with 12.5% SDS-PAGE extracted with 0.2M $CaCl_2$ from *M. truncatula* cell culture. The numbers correspond to the bands selected for tryptic digestion and MALDI-TOF-MS peptide mass fingerprint analysis.

The CWP samples extracted from living cells with 0.2M $CaCl_2$ from *M. truncatula* cell suspension culture were lyophilised and concentration was measured. Approximately 10mg of protein samples were transferred to a preparative isoelectric focusing system (Rotofor System, Biorad). This system was designed for an initial cleanup of the crude samples and for use in purification schemes for the elimination of specific contaminants from proteins of interest that might be difficult to remove by other means.

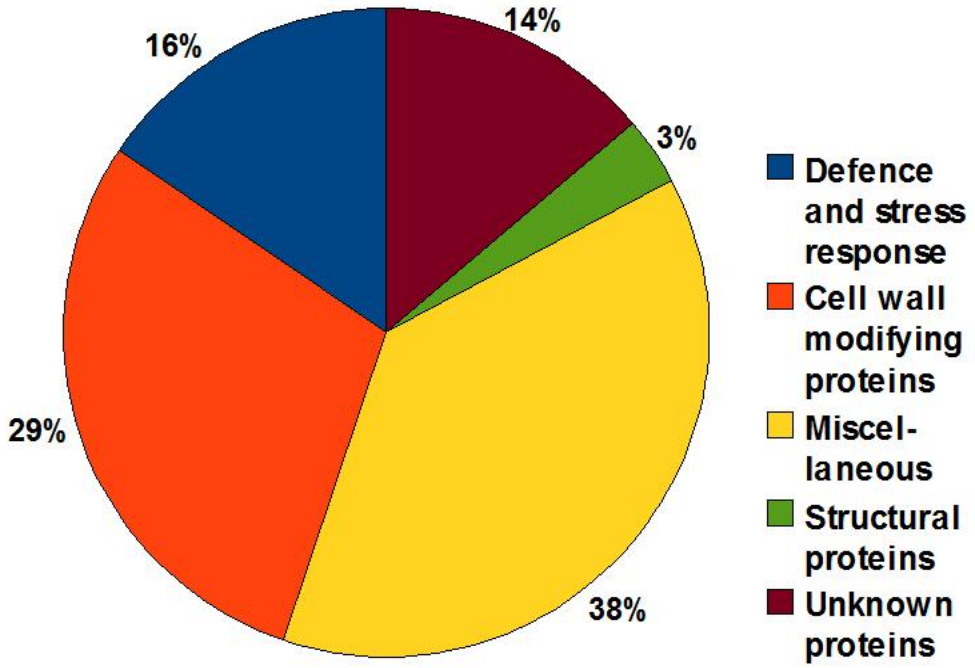


Figure 17: Graphical view of the classification of cell wall proteins separated by IEF in solution/SDS-PAGE.

In addition this system provides up to 500-fold purification because IEF was carried out in free solution. The combination of SDS-PAGE with this method showed that the proteins were successfully separated according to their pI from 3.1 to 12.6 and their molecular weight ranging from 10-250kD (Figure 16). Each lane showed a distinct protein spot identified by MALDI-MS analysis, indicated by a number which correspond to the protein spot numbers in figure 16 and listed in table 4. In total approximately 100 protein spots were digested and analysed by MALDI-TOF-MS. This resulted in the identification of 59 proteins; listed in table 4 and the classification of the corresponding proteins were shown in figure 17. They include 29% of cell wall modifying enzymes (e.g., endo-beta-1 4-glucanase, Figure 16 and Table 4, spot19), peroxidase (Figure 16 and Table 4, spot 62, 72), cellulose (Figure 16 and Table 4,

spot 65, 85, 98) and acid beta fructofuranosidase (Figure 16 and Table 4, spot No 36), which belongs to the glycosyl hydrolase 32 family and catalyses the hydrolysis of glycosidic bonds between carbohydrates. 16% were defence related proteins like glutathione S transferase (Figure 16 and Table 4, spot No 50, 54 61) and NADP dependent oxidoreductase (Figure 16 and Table 4, spot No 12). Hydroxyproline-rich glycoproteins (HRGPs), (Figure 16 and Table 4, spot No 49) as a candidate of structural proteins (3%) and 38% of the proteins identified were grouped under miscellaneous functions. These include phosphatase (Table 4 and Figure 16 and spot No 41) and elongation factor 1-alpha (Table 4 and Figure 16 and spot No 13). More than 14% are proteins of still unknown functions.

4.3. Comparision of CWPs from living cells and cell wall fragments by 2D-PAGE

The common extraction method for the loosely bound proteins from the cell wall using $CaCl_2$ has been reported previously by Borderies et al (2003) and Roberson et al (1997). Later, taking purity as a significant concern, Watson et al (2004) reported the extraction protocol from purified cell wall fragments using *M sativa* mature stem tissue. In order to optimize the extraction procedure, and to compare the cell wall proteome map of *M. truncatula*, cell suspension culture from both living cells and cell wall fragments were established.

4.3.1. 2D-PAGE analysis of *M. truncatula* CWPs extracted from living cells

The experimental protocol platform usually includes 2D gel electrophoresis followed by spot excision, trypsin digestion of the samples and subsequent identification by mass spectrometry. Therefore, CWPs extracted using $CaCl_2$ were separated by 2D-gel electrophoresis and analyzed by MALDI-TOF-MS. For verification, three sets of protein extraction and 2D gel electrophoresis were performed as technical replicates.

Seven hundred and fifty micrograms of *M. truncatula* cell wall proteins extracted with 0.2M $CaCl_2$ were separated on IEF 24 cm Immobiline DryStrip (pH ranging from 3-10) and by SDS-PAGE (12.5%) as second-dimension. The gels were subsequently stained with CBB (Figure 18). Numbers indicated on the protein spots were selected

for tryptic digestion and MALDI-TOF-MS analysis; the corresponding identified proteins are listed in table 3. From CBB stained 2D gels of the $CaCl_2$ extracts (Figure 18) about 20 prominent and 30 less abundant protein spots could be resolved in a pI range of 3 to 10 showing a molecular weight of 10 to 100kD. MALDI-TOF-MS and subsequent database searches using Mascot resulted in the identification of 34 protein spots from 50 in total, corresponding to 29 different proteins without redundancy. This discrepancy in numbers results from the co-migration of proteins in more than one visible protein spot revealing shifting in both isoelectric point and molecular weight.

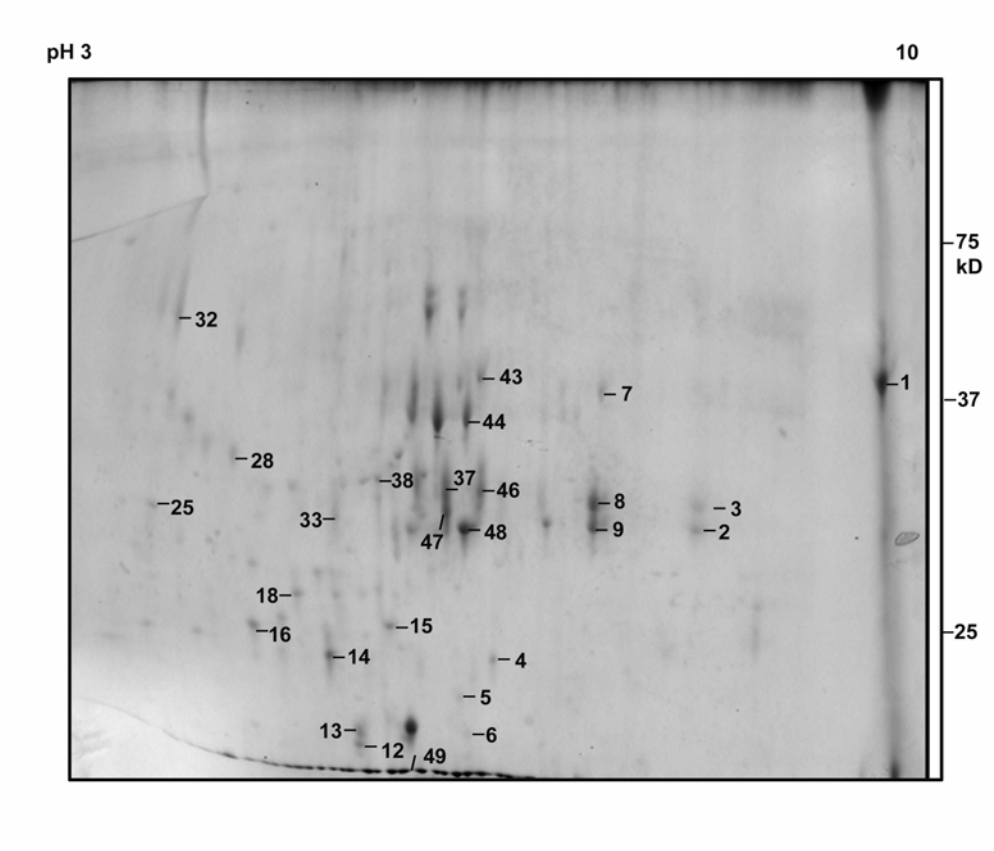


Figure 18: Two-dimensional map of the *M. truncatula* cell wall proteins extracted with 0.2M $CaCl_2$, separated on IEF using 24cm Immobiline DryStrip (pH 3-10) and by SDS-PAGE (12.5%) as second-dimension. The gels were subsequently stained with CBB. Numbers indicate the protein spots selected for tryptic digestion and finger print MALDI-TOF-MS analysis

Among the identified CWPs, 26% were cell wall modifying enzymes, 21% defence related enzymes, 6% structural proteins and 12% of unknown functions (Figure 19). Examples of identified cell wall modifying enzymes were the peroxidase (Table 3 and Figure 18, spot No 9, 1, 2) normally secreted pectin esterase (Table 3

and Figure 18, band No 43) and expansin (Table 3 and Figure 18, spot No 4). Members of the defence related proteins were, e.g., class II chitinase (Table 3 and Figure 18, spot No 25). Structural proteins include glycine-rich cell wall protein (Table 3 and Figure 18, spot No 14), and a proline rich protein (Table 3 and Figure 18, spot No 14). Also 35% of the identified proteins were grouped under miscellaneous function. These include phosphorylated proteins like acid phosphatase (Table 3 and Figure 18, spot No 3 and 8), which was also reported previously in the apoplast and cell walls of plants (Baluska et al. 2003, Watson et al. 2004).

Seven proteins were detectable in several different spots on the same 2D-gel. This suggests protein degradation as well as alternative posttranslational modifications like diverse glycosylation patterns. The proteins were mostly affected in both their molecular weight and in the isoelectric point. Protein carbamylation and protein oxidation could also be observed. For example, spot 3 or spot 8 in figure 18 show the same molecular weight but different isoelectric points for the same protein.

On the other hand spot 9 and spot 16 have the opposite combination of shifted molecular weights and fixed isoelectric points. More often a combination of shifts in both properties was observed, e.g., spots 1 and 2 in figure 18. Beside these, it could be observed that proteins spots were sometimes not clearly resolved, showing smearing around the spot.

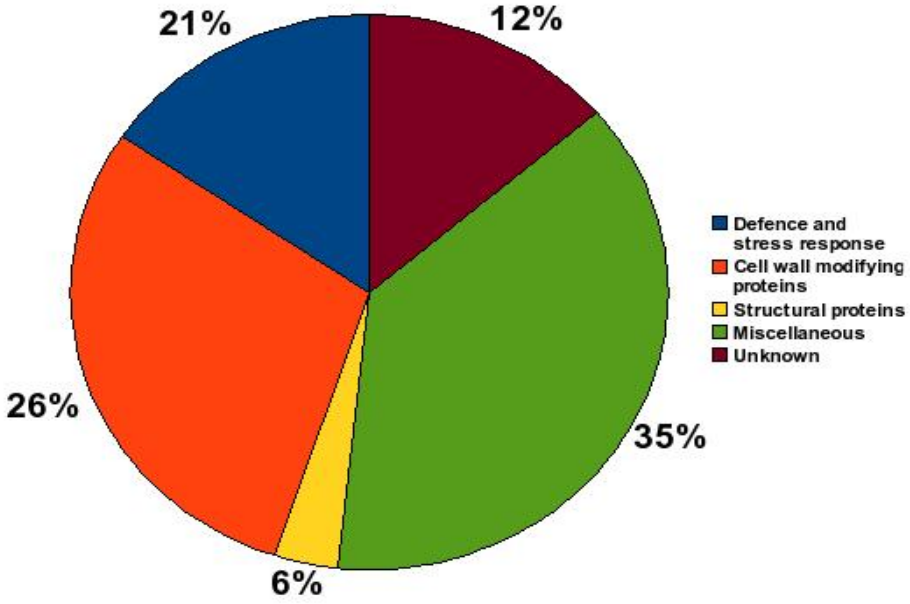


Figure 19: Graphical view of the classification of cell wall proteins separated by 2D gel electrophoresis

4.3.2. Analysis of CWPs extracted from cell wall fragments of *M. truncatula*

Characterisation of CWPs is challenging because of many reasons like;

- Isolation of low abundance cell wall proteins from soluble or membrane-associated proteins is difficult.
- Purity of the extracted protein is a significant concern.
- Also CWPs are known to be glycosylated, and some of the cell wall proteins are very basic (Borderies et al. 2003).
- Isoelectric focusing of proteins with these characteristics is often problematic.
- During extraction of cell wall proteins other materials like polysaccharides and polyphenolics are also co extracted and high concentrations of these materials result in streaking and background staining on 2-DE gels, making analyses of the proteins separated by 2-DE difficult or impossible.

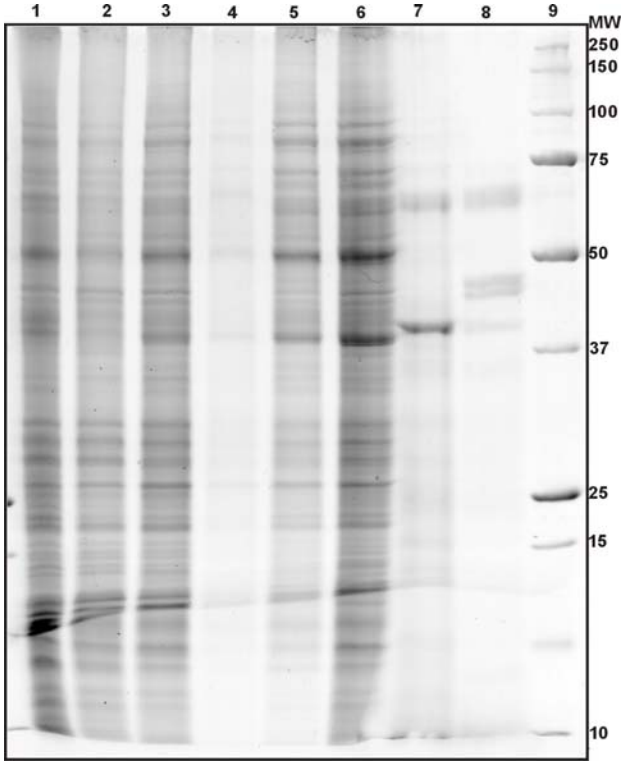


Figure 20: SDS–PAGE analysis showing concentration and complexity of the proteins removed from cell walls by the various washes. Lane 1- crude extract in grinding buffer, lane 2- 0.1M NaCl wash, lane 3- 1st H_2O wash, lane 4- acetone wash, lane 5- 2nd H_2O wash, lane 6- 10mM NaAcet wash, lane 7- 0.2M $CaCl_2$ extract, lane 8- 3.0M LiCl extract and lane 9, molecular weight marker.

Taking all this in considerations and also to have the purified cell wall fragments, the *M. truncatula* suspension cells were washed with numerous solutions

like salt buffer, water, organic solutions and buffers. In order to prove the effectiveness of these washings, aliquots from each sequential wash were analysed by SDS-PAGE which is shown in figure 20. From figure 20 it was obvious that most of the cytosolic proteins were extracted in the homogenization buffer and subsequent sequential washes were effective in removing the remaining proteins. Further, the CWPs extracted using $CaCl_2$ were separated by 2D-gel electrophoresis and analyzed by MALDI-TOF-MS as described before. For verification, three sets of protein extraction and 2D gel electrophoresis were performed as technical replicates. 750µg of CWPs were used for the 2D PAGE analysis. From CBB stained 2D gels of the $CaCl_2$ extracts (Figure 21), about 45 prominent and 20 less abundant protein spots could be resolved in a pI range of 4 to 9 showing a molecular weight of 15 to 150kD.

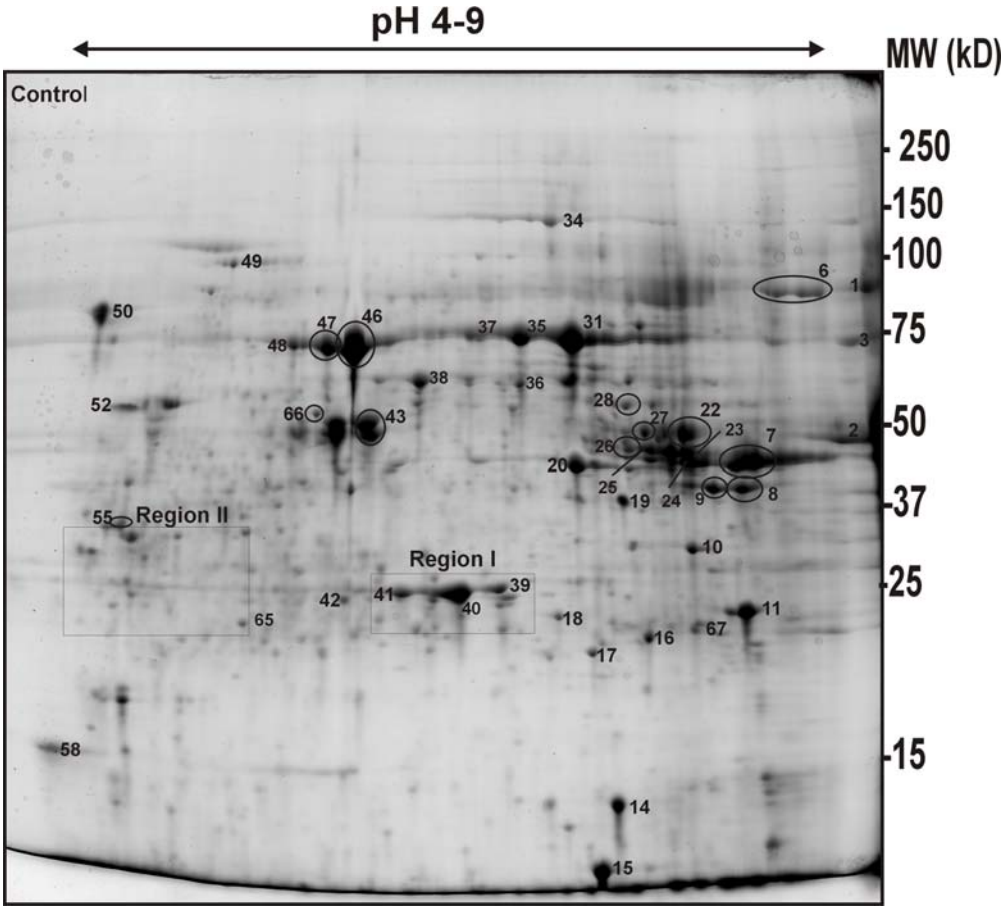


Figure 21: Two-dimensional map of the *M. truncatula* cell wall proteins extracted (from cell wall fragments) with 0.2M $CaCl_2$, after differential extraction and separated on IEF a 24cm Immobiline DryStrip (pH 3-10) and by SDS-PAGE (12.5%) as second-dimension. The gels were subsequently stained with CBB. Numbers indicate the protein spots selected for tryptic digestion and finger print MALDI-TOF analysis.

MALDI-TOF-MS and subsequent database searches using Mascot resulted in the identification of 46 protein spots (out of 67) in total, corresponding to 30 different proteins without redundancy. This discrepancy in numbers results from the co-migration of proteins in more than one visible protein spot with shifting in both isoelectric point and molecular weight.

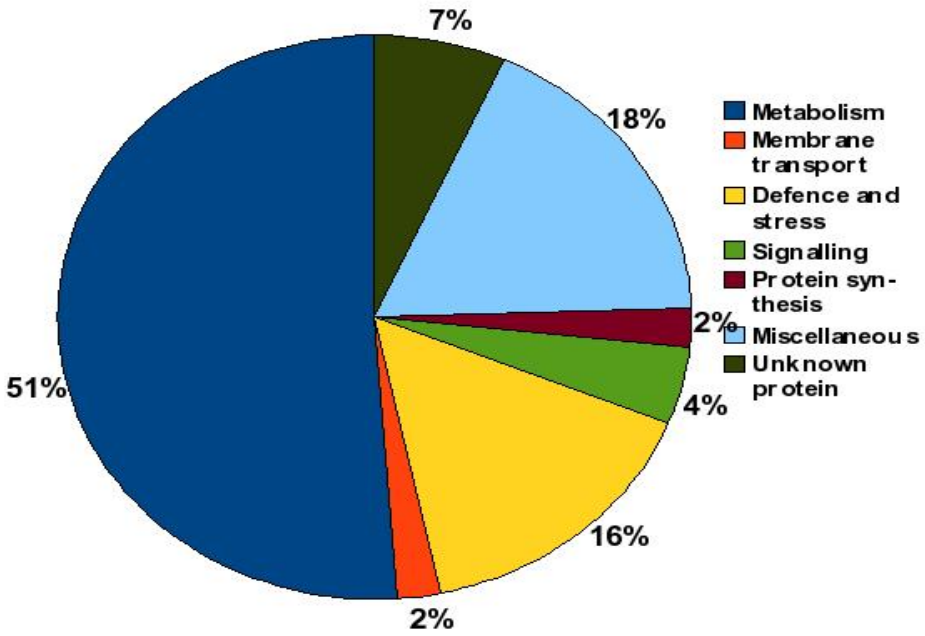


Figure 22: Graphical view of the classification of cell wall proteins separated by 2D gel electrophoresis.

Among the identified CWPs were proteins involved in metabolism, defence and stress related enzymes, membrane transport proteins, proteins involved in protein synthesis and processing, signaling proteins, misellaneous and unknown proteins (Figure 22). Examples of identified CWPs were cell wall modifying proteins like glycosyl hydrolase family protein, glyceraldehyde-3-phosphate dehydrogenase. High abundant proteins like enolase which was secreted to the cell wall (Edwards et al. 1999) and wall bound malate dehydrogenase were also identified. Members of the defence related proteins were L-ascorbate peroxidase, superoxide dismutase and glutathione S-transferase. Calreticulin and Nucleoside diphosphate kinase I, proteins involved in signal transduction were also identified. Membrane transport proteins like vacuolar ATP synthase subunit E and eukaryotic translation initiation factor 5A-2, protein involved in protein synthesis were also identified in this study. In addition, the proteins grouped under miscellaneous functions include alpha 1-4 glucan protein

synthase, cytochrome b5 reductase and grf2 14-3-3 like protein.

Few proteins were detectable in several different spots on the same 2D-gel. This suggests protein degradation as well as alternative posttranslational modifications like glycosylation patterns. The proteins were mostly affected in both their molecular weight and in the isoelectric point. Protein carbamylation and protein oxidation could also be observed. For example, spot 39 or spot 65 in figure 21 show the same molecular weight but different isoelectric points for the same protein. More often, a combination of shifts in both properties was observed, e.g., spots 3 and 31 in figure 21.

The absence of structural proteins may have following reasons: (1) they are low abundant components of the wall and are therefore not easy to detect. (2) some genes are only expressed in response to specific intrinsic or environmental influences

4.4. Identification of cell wall proteins by LC-MS/MS

In order to validate the diversity of the CWPs with different methods (2D-PAGE and LC-MS/MS) and to increase the coverage of the cell wall proteome, the CWPs extracted from cell wall fragments were digested with trypsin. Further, these protein samples were analysed by LC-MS/MS and this resulted in an identification of 65 proteins from MASCOT database query which were listed in table 6. The LC-MS/MS date obtained for one of the identified protein spot in the database query results and their MS spectrum was shown in figure 24. For protein revealing score, more than seventy or double the threshold for significancy calculated by the MASCOT database was supposed to represent as identified proteins. Several classical cell wall proteins were identified in the *M. truncatula* cell suspension culture and their classification was shown in figure 23. These include cell wall modifying proteins like alpha-glucosidase, glycosyl hydrolase and pectinacetylesterase. Defence related proteins include peroxidase 12, catalase and L-Ascorbate peroxidase. Some of the signalling proteins like nucleoside diphosphate kinase I and GTP binding proteins were identified. Alpha-expansin, Alpha 1-4 glucan protein synthase, prefoldin subunit 4 represents some of the proteins that were grouped

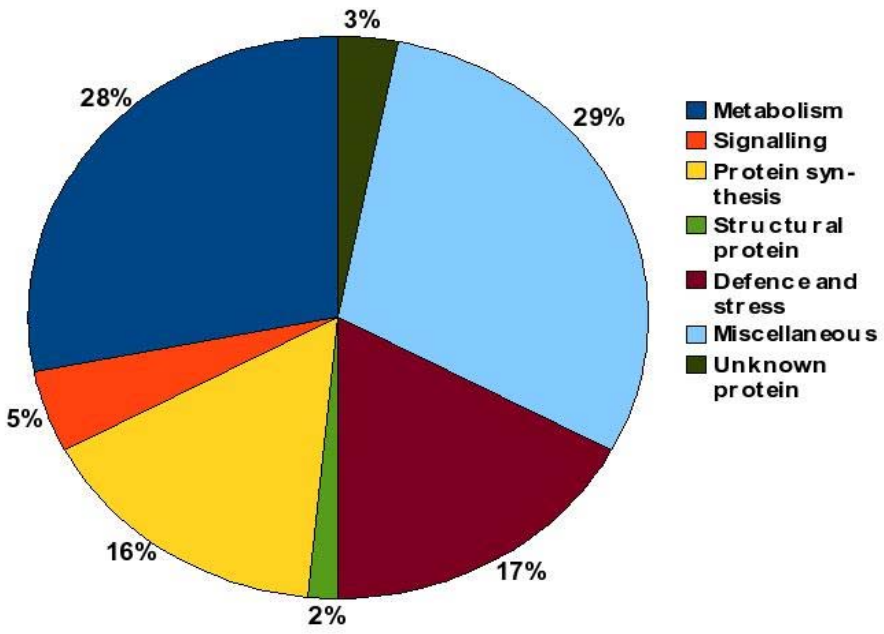
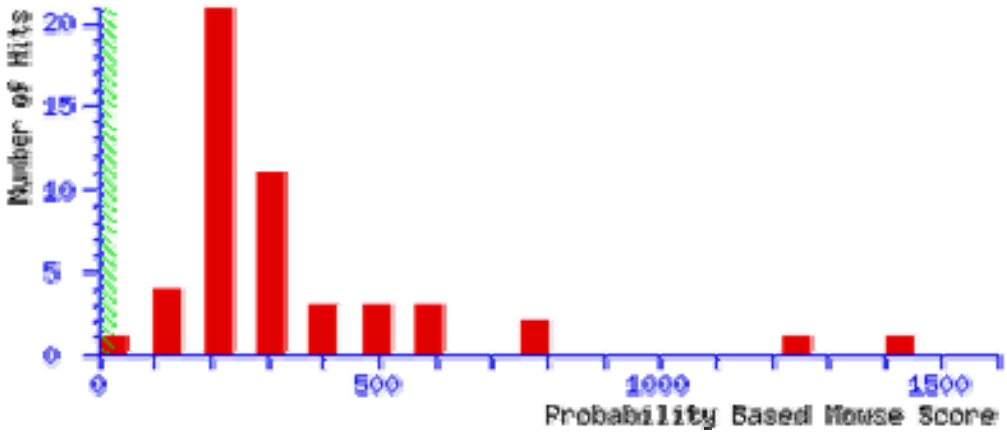


Figure 23: Graphical view of the classification of cell wall proteins separated by LC-MS/MS.

under proteins with miscellaneous function. One structural protein, pistil specific extensin like protein was also identified. Seven percent of the proteins identified exhibit unknown function. SignalP (Nielsen et al. 1997, 1999) was used to predict the presence of a secretory signal peptide. From this signal peptide analyses, only some of the proteins identified to have a secretory signal peptide (22%). The presence of a number of proteins known to be located in other compartments was observed. This was also the case in the proteome analysis of the *Arabidopsis* cell wall, where several proteins were found in cell wall extracts although they are normally not considered as being secreted (Chivasa et al. 2002). These include several mitochondrial enzymes and some cytoplasmic metabolic enzymes. In this study, the identified proteins show few similarities with defence related proteins and cell wall modifying proteins identified in *A. thaliana* (Chivasa et al. 2002, Borderies et al. 2003, Bayer et al. 2006) and *M. sativa* (Watson et al. 2004), could also identify some novel proteins which were grouped under unknown and miscellaneous function. In addition, iron, alpha 1-4-glucan synthase, grf2 14-3-3-like protein and other structural proteins were also identified, which were not found in Arabidopsis and/or *M. sativa.*

Probability Based Mowse Score

Ions score is-10*Log (P), where P is the probability that the observed match is a random event. Individual ions scores>27 indicate identity or extensive homology (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

1. lcl|TC100309_1 **Mass:** 52482 **Score:** 1286 **Queries matched:** 16 ENO2 **Enolase** 4.2.1.11 (GenDB-ID=1308)

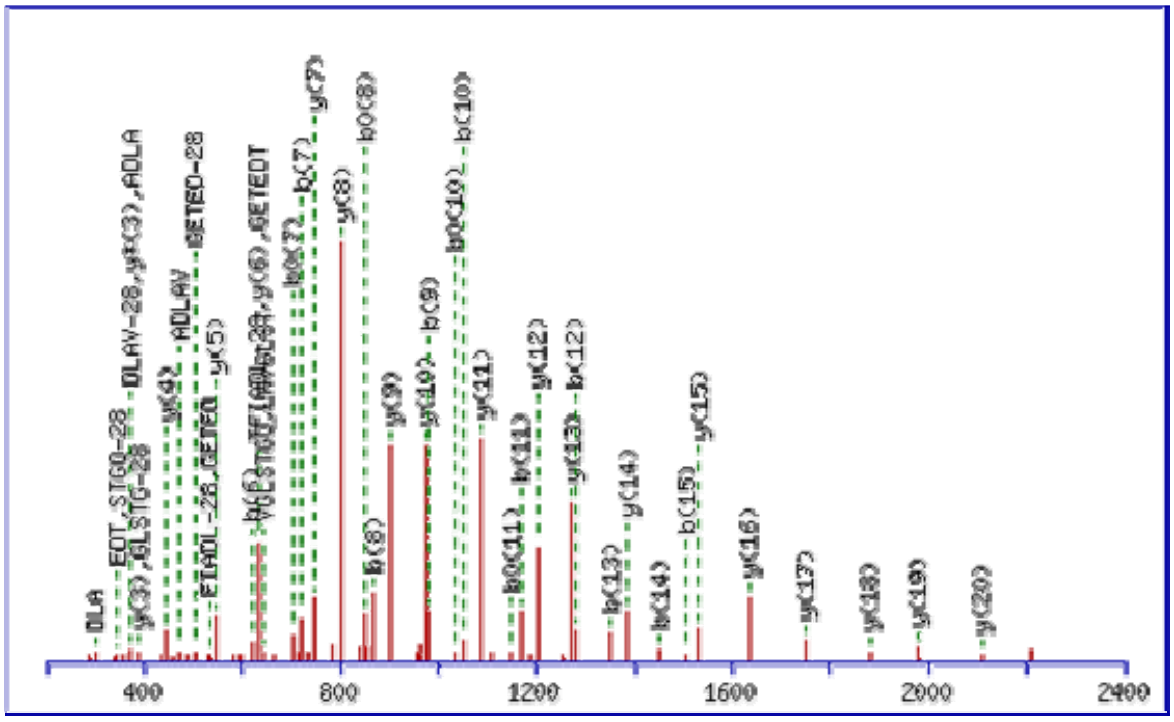


Figure 24: LC-MS/MS data obtained for one of the spot in the database query results, which was considered to be identified and the MS spectrum of eluted peptides (Top to Bottom)

5.0. The impact of the induced and suppressed plant defence responses on the cell wall proteome

5.1. Reaction of *M. truncatula* cell culture to elicitation and suppression with lipopolysaccharide (LPS)

M. truncatula suspension-cultured cells derived from roots were used as a test system for the perception of microbial signal molecules. To verify the responsiveness of the cell culture, invertase was used as a well-established elicitor of plant defence reactions. Cell suspension cultures of *M. truncatula* were prepared and diluted in preincubation medium as described in materials and methods. The time-dependent generation of H_2O_2 following the application of yeast elicitor (25µg/ml), *S. meliloti* lipopolysaccharide (20µg/ml) and of a combination of both substances to *M. truncatula* cell cultures was determined. The application of yeast elicitor caused an oxidative burst reaction. The maximum H_2O_2 generation reached 2.4µM after 5min and dropped thereafter (Figure 25). The application of *S. meliloti* LPS (20µg/ml) alone had no significant effect on H_2O_2 generation (Figure 25). When yeast elicitor and *S. meliloti* LPS were added simultaneously the oxidative burst reaction was suppressed (Figure 25).

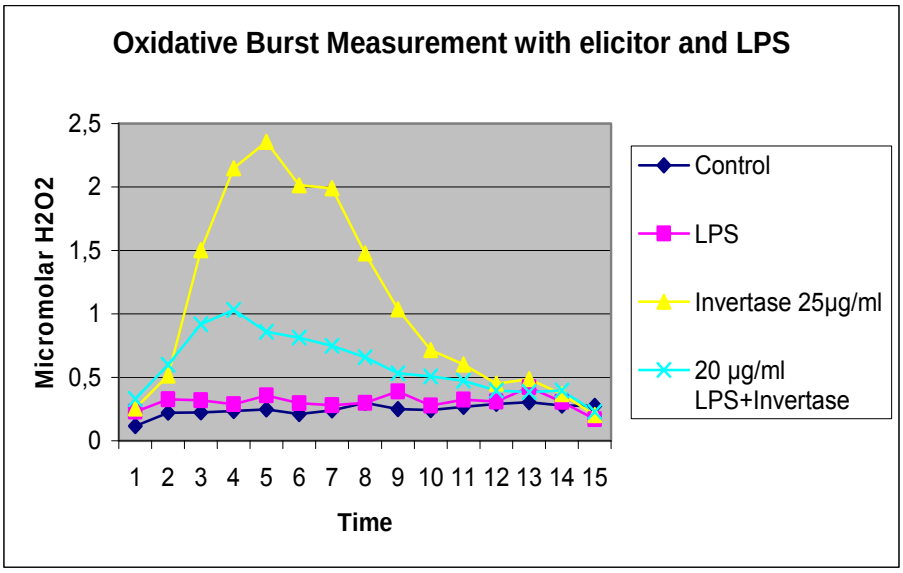


Figure 25: Oxidative burst measurement for the control (water), 25 µg/ ml yeast invertase (Sigma), 20 µg /ml *S. meliloti* LPS, or a combination of 25µg/ ml invertase and 20µg/ml *S. meliloti* LPS.

Therofore, lipopolysaccharides isolated from the wild-type *S. Meliloti* are able to suppress the elicitor induced oxidative burst in cell cultures of the host plant *M. truncatula*.

5.2. Changes in the cell wall proteome upon elicitation

A number of studies using microarray or chips have been done in order to analyse the changes in the transcriptome induced by pathogens in *Arabidopsis* (Ramonell et al. 2002, Maleck et al. 2004, Zimmerli et al. 2004) and in *M. truncatula*

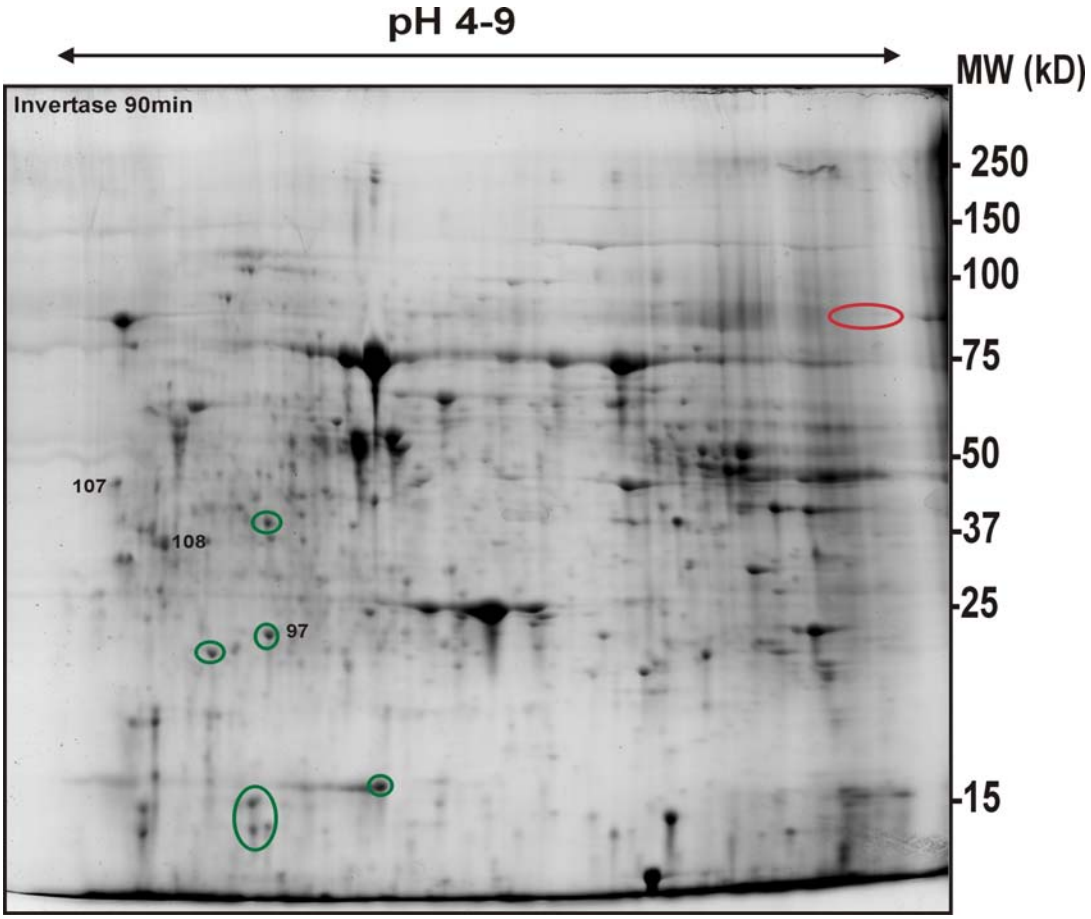


Figure 26: Two-dimensional proteome map of the *M. truncatula* cell wall proteins incubated with yeast elicitor for 90min and extracted (from cell wall fragments) with 0.2M $CaCl_2$, after differential extraction and separated on IEF a 24cm Immobiline DryStrip (pH 3-10) and by SDS-PAGE (12.5%) as second-dimension. The gels were subsequently stained with CBB. Numbers indicate the protein spots selected for tryptic digestion and finger print MALDI-TOF analysis.

(Tellström et al. 2007). But these transcriptional changes do not reflect the entire cellular regulatory events. Alternative approaches such as proteome expression

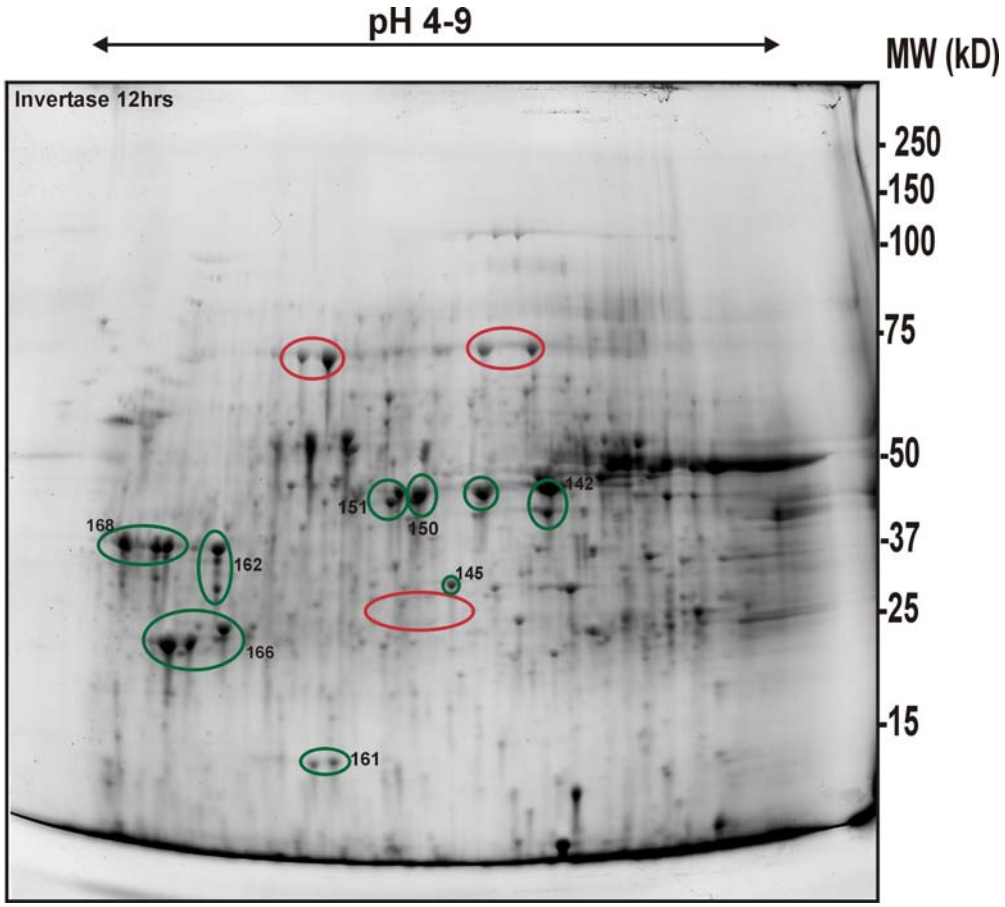


Figure 27: Two-dimensional proteome map of the *M. truncatula* cell wall proteins incubated with yeast elicitor for 12h and extracted (from cell wall fragments) with 0.2M $CaCl_2$, after differential extraction and separated on IEF a 24cm Immobiline DryStrip (pH 3-10) and by SDS-PAGE (12.5%) as second-dimension. The gels were subsequently stained with CBB. Numbers indicate the protein spots selected for tryptic digestion and finger print MALDI-TOF analysis.

analysis are essential to study the plant pathogen interaction.

In order to identify the proteins that are differentially expressed in response to defence, the *M. truncatula* cells were treated with yeast elicitors. The responsiveness of elicitors was checked for two different time points, 90min (Figure 26) and 12h (Figure 27). Further the CWPs extracted using $CaCl_2$ were concentrated and analysed by 2D PAGE. From CBB stained 2D gels of the $CaCl_2$ extracts (Figure 26 and 27) about 49 and 56 protein spots after 90 min and 12h elicitor treated cell wall proteins respectively were taken for trypic digestion. MALDI-TOF-MS and subsequent database searches using Mascot were performed the corresponding proteins are listed in table 5.

The same concentration (750µg) of cell wall proteins were used from the 90min and 12h elicitor treated cells. Elicitation of *M. truncatula* cell culture after 90min

did not shown any marked difference in the proteome map (Figure 26) when compared to the control, but after 12h of incubation with elicitor there was a significant reduction of the cell wall proteins like L-ascorbate peroxidase (Figure 27, Table 5, spot no 39-41), indicating the elicitor induced cross linking of some proteins in the cell wall, decreasing the solubility and extractability. But different isomers of the same proteins like enolase (Figure 27, Table5, spot no 151, 162, 168) and glyceraldehyde-3-phosphate dehydrogenase (Figure 27, Table 5, spot no 142, 145, 150) were seen in the proteome map. These different isomers of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) suggest association to specific induction of the pathogen defence response (Chivasa et al. 2005) Further SDS-PAGE analysis by Chivasa et al (2005) proved that the presence of GAPDH in cell wall proteome in response to elicitor was specifically targeted to the cell wall during a defensive reaction. Also L-ascorbate peroxidase (Figure 27, Table 5, spot no 166) and eukaryotic translation initiation factor 5A-1 (Figure 27, Table 5, spot no 161) were upregulated in acidic regions.

5.3. Adaptation of the cell wall proteome under elicitation and suppression

In order to analyse the defence and symbiotic properties of the *M truncatula*, cell culture were treated with the yeast elicitor and *S. meliloti* LPS respectively and incubated for 12h. The cell wall proteome of *M.truncatula* cell culture treated with LPS and elicitor or only LPS were shown figure 29 and 28 respectively. From the CBB stained 2D gels, about 67 proteins from the proteome map of LPS treated cells and 53 proteins from the LPS and elicitor treated were taken for tryptic digestion and MALDI TOF MS. In LPS treated cell wall proteome map there was no marked difference except proteins like L-ascorbate peroxidase and eukaryotic translation initiation factor 5A (Figure 28, table 5, spot 166 and 161 respectively). The elicitor and LPS treated cell wall proteome resembled the same as the proteome map of the elicitation with invertase alone with the upregulated proteins like eukaryotic translation initiation factor 5A-1 (Figure 29, table 5, spot 161), L ascorbic peroxidase (Figure 29, table 5, spot 166) and high abundant enolases (Figure 29, table 5, spot, 151,162 and 168) with two different isomers. But the presence of defence specific GAPDH (Figure 29,

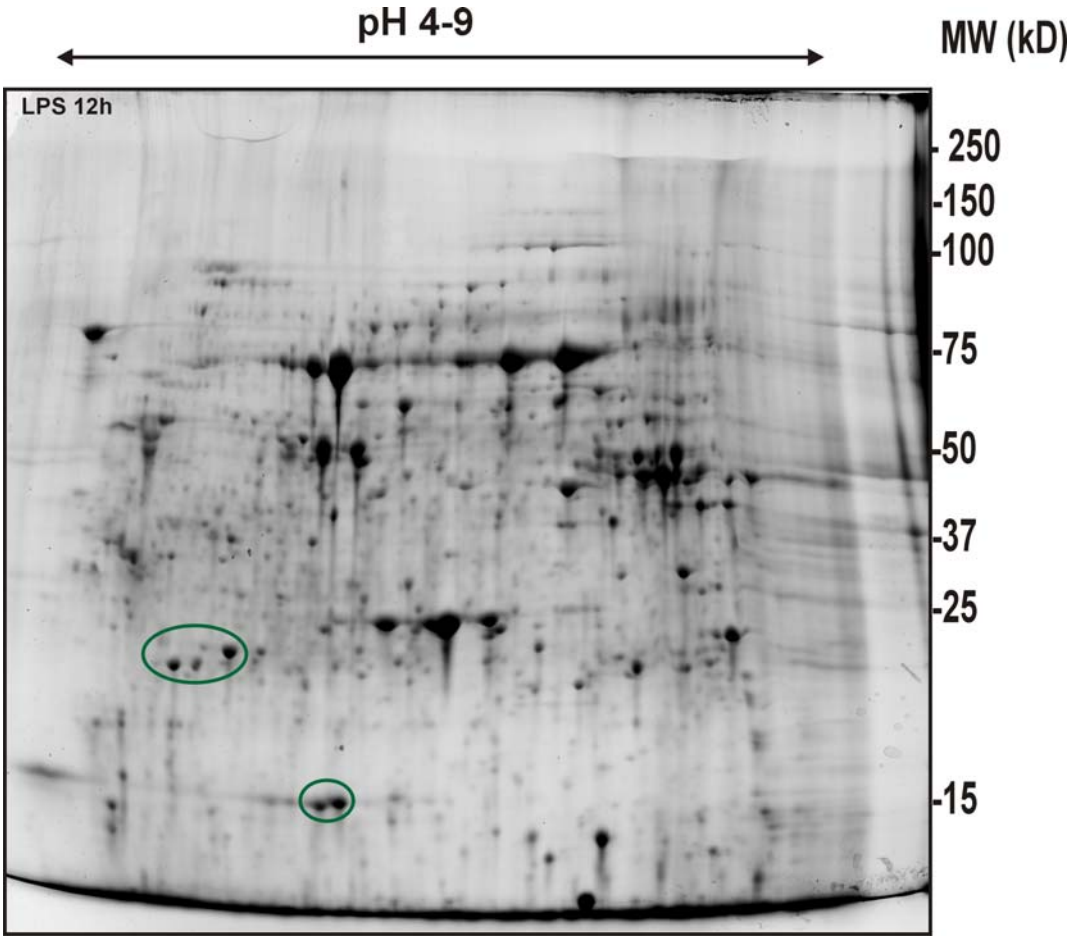


Figure 28: Two-dimensional proteome map of the *M. truncatula* cell wall proteins incubated with Sm LPS for 12h and extracted (from cell wall fragments) with 0.2M $CaCl_2$, after differential extraction and separated on IEF a 24cm Immobiline DryStrip (pH 3-10) and by SDS-PAGE (12.5%) as second-dimension. The gels were subsequently stained with CBB. Numbers indicate the protein spots selected for tryptic digestion and finger print MALDI-TOF analysis.

Table 5, spot no 7, 22) and ferredoxin-NADP(+) reductase (Figure 29, Table 5, spot no 8, 9) were downregulated. Nevertheless, this resembled nearly the same as the microarray analysis reported by Tellström et al (2007), where the rhizobial LPS has a moderate effect in transcriptional rearrangement when it was treated along with elicitors.

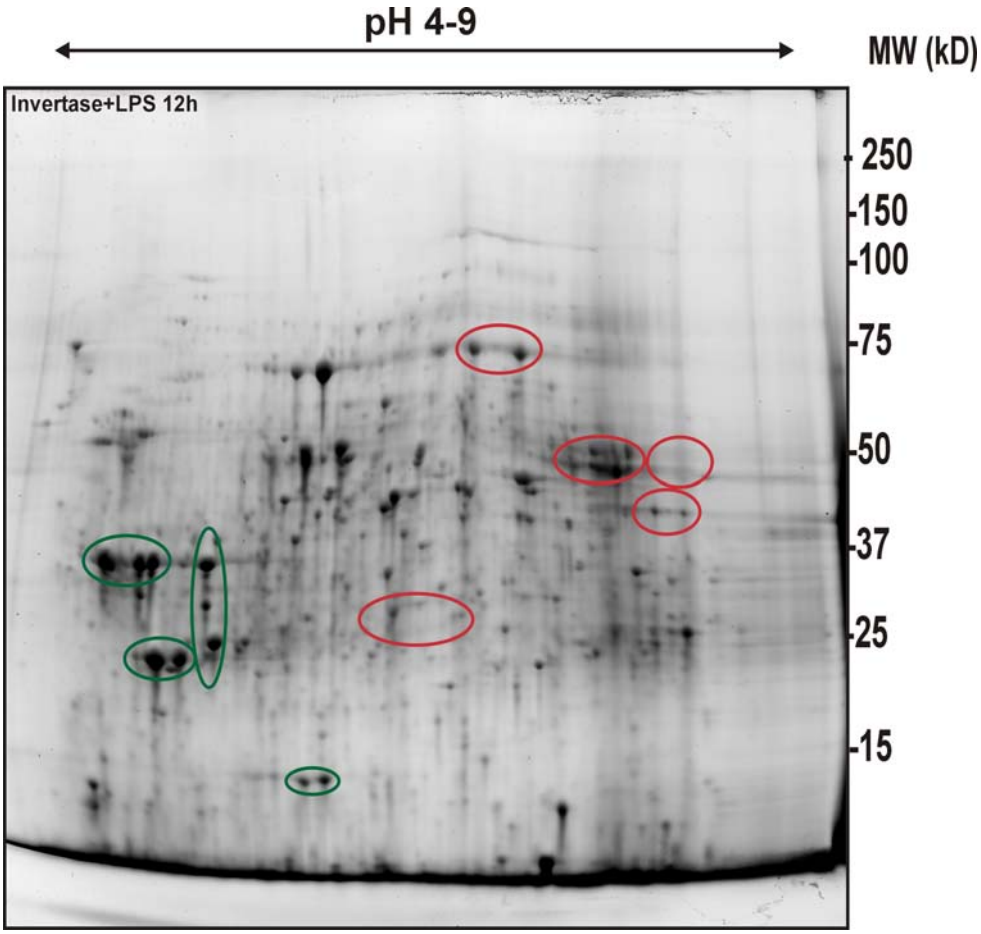


Figure 29: Two-dimensional proteome map of the *M. truncatula* cell wall proteins incubated with yeast elicitor and Sm LPS for 12h and extracted (from cell wall fragments) with 0.2M $CaCl_2$, after differential extraction and separated on IEF a 24cm Immobiline DryStrip (pH 3-10) and by SDS-PAGE (12.5%) as second-dimension. The gels were subsequently stained with CBB. Numbers indicate the protein spots selected for tryptic digestion and finger print MALDI-TOF analysis.

6.0. Discussion

6.1. Conceptional approach of this work

A lot of work has been done in both bacterial and plant proteomics but only few investigations were involved in the cell wall proteins of plants except recent efforts in *Arabidopsis thaliana* and in *Medicago sativa* (Bayer et al. 2006, Borderies et al. 2003, Chivasa et al. 2002, Chivasa et al. 2005, Watson et al. 2004). This is mainly due to the reason that appropriate sample preparation is one of the most critical steps in analysis of the cell wall proteome. Especially plant material does not provide a ready source of proteins for the proteomic analysis. Plant cell walls have generally low protein content and contain high concentrations of proteases, polysaccharides and eventually other interfering compounds (e.g. salts, phenols, lignins, terpenes). Precipitation of proteins is very efficient in removing interfering compounds and in concentrating proteins but may result in an uncontrolled loss of proteins. Furthermore, the nature of the cell wall proteins varies with many functions and they have a high degree of cross-links into the wall, show high glycosylation and many repetitive sequences (Cassab 1998, Showalter 2001).

Systematic transcriptomic approaches have been combined with genetic analyses (Somerville et al. 2004), but these do not address the occurrence of alternative splicing or the post-translational modifications (PTM) of proteins as the question whether a mRNA is translated at all. Also, proteins can move in and out of complexes, modifying their functionality. This problem cannot be overcome by transcriptomics alone (Peck 2005). Proteomics approaches not only a large scale study used to identify the proteins present in a particular organ at a given stage of development, but also can be used to solve some of these issues. Several recent reports on plant proteomics have described the methods in this area (Bertone and Snyder 2005, Peck 2005, Rose et al. 2004) and the application of proteomics to study the cell walls of plants (Lee et al. 2004). Proteomics approaches are essential to address the three other main questions: isoforms or PTMs, location, and activity of proteins. Proteomics is an increasingly ambiguous term used to almost all aspect of protein expression, structure or function. The proteome reflects the expression of the molecules that more directly influence and provide more accurate representation of the cellular state than analysing the expression of mRNAs which represents only information about the intermediates.

Although, several plant genomes are currently being sequenced and the number of

M. truncatula cell wall proteins in the protein databases is increasing. Since 2001 the proteome of total protein extracts from *M. truncatula* and of proteins extracted from different organs of this plant were analyzed and also some of the articles have been published on the *M. truncatula* six organs, tissues and embryogenic cell cultures (Chivasa et al. 2002, Imin et al. 2004, Mathesius et al. 2001). Also, the cell wall proteome in *Arabidopsis* (Bayer et al. 2006, Boudart et al. 2005, Chivasa et al. 2002, Borderies et al. 2003) and *M. sativa* (Watson et al. 2004) have been established in cell culture, apoplastic fluids and in the stem.

This work focused on the cell wall proteome of *M. truncatula* cell suspension culture in order to compare it with other proteomes and to establish a reference map for further experiments. The actively dividing suspension cell cultures were selected for this experiment because of their relative uniformity.

The quality of the protein extract is of supreme importance and most unsuccessful attempts to visualize large numbers of well-resolved spots with minimal streaking or smearing are the result of problems with extraction and subsequent preparation. In order to have an optimised protocol for the extraction of the cell wall proteins from *M. truncatula* cell suspension culture, five different approaches were made using different ionic or non-ionic substances: $CaCl_2$, NaCl, EDTA, LiCl, or DTT. Further the CWPs extracted using living cells and/or cell wall fragments were analysed using different techniques (Figure 30). PMF obtained by MALDI-TOF-MS was used for the identification of proteins. (Bestel-Corre et al. 2002, Corthals et al. 2000, Pappin et al. 1993, Yates 1998a, Yates 1998b). This study was the basis for a comparative proteome analysis of the cell wall proteins of *M. truncatula* during pathogenic or symbiotic condition.

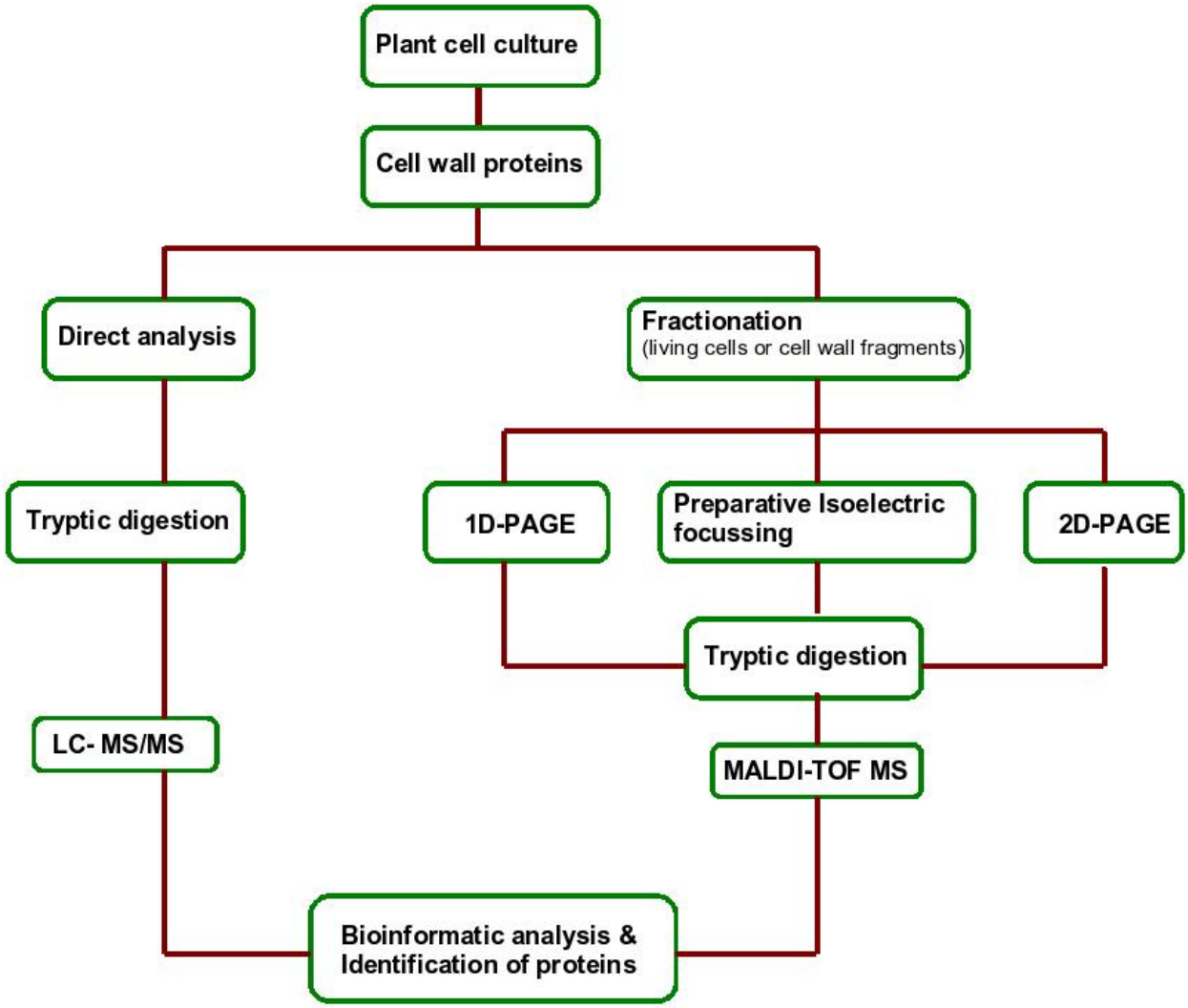


Figure 30: Strategy of cell wall proteomic analysis used in this study. Once extracted, CWPs were directly analysed by LC-MS/MS after tryptic digestion. Alternatively they were also fractionated and separated by 1D-PAGE, preparative isoelectric focussing or 2D-PAGE prior to in gel digestion and MALDI-TOF-MS analysis. In all the cases the last step of this procedure was bioinformatic analysis and identification of proteins.

6.1.1. Extraction of CWPs using different salt solutions

CWPs can be classified as three different types depending on their interactions with the cell wall components: (i) Loosely bound CWPs which move freely in the intercellular space or show no interaction with the cell wall polysaccharides (ii) CWPs that are weakly bound to the cell wall by hydrogen bonds, hydrophobic or ionic interactions and Van der Waals interactions. (iii) And CWPs strongly bound to the cell wall matrix and become resistant to salt extraction. For example, Extensins are cross-linked by covalent links

(Brady et al. 1996).

The analysis of *M. truncatula* CWPs was performed in parallel using solutions containing $CaCl_2$, NaCl, EDTA, LiCl, or DTT. In this context $CaCl_2$ was reported as a very efficient salt for the extraction of CWPs from purified cell walls while treatment with high salt (NaCl or LiCl) is expected to elute proteins associated through ionic interactions and Hydroxyproline-rich glycoproteins (HRGPs). EDTA as a calcium chelatant extracts proteins which are associated with pectins, and DTT may help to extract proteins that have a high content of disulphide bonds (Blee et al. 2001, Robertson et al. 1997, Smith et al. 1984, Voigt 1985).

The concentration of the proteins extracted varied particularly depending upon the applied reagent. Extracted protein samples showed very low protein content and high concentrations of interfering substances (salts, and polysaccharides). The highest yield could be obtained by extraction with $CaCl_2$ or NaCl (10mg and 12.5mg, extracted from 200 ml cell culture). LiCl seemed to be the same as NaCl in cell wall protein extraction (12.5 mg, extracted from 200ml cell culture). The lowest yield was observed after the incubation of the whole cells with DTT (6.5mg, extracted from 200ml cell culture). $CaCl_2$ was seemed to be more efficient salt for CWP extraction.

6.1.2. Identification of *M. truncatula* CWPs by SDS-PAGE

From 1D-PAGE, it was possible to identify some proteins within the same band, the patterns of proteins separated by SDS-PAGE were different. Also, large number of these proteins cannot be identified by data base search. The identified proteins include 15% of cell wall modifying enzymes e.g., ß-1.3-exoglucanase with O-glycosyl hydrolase activity, was also reported in *Arabidopsis* and in *M. sativa* (Borderies et al. 2003, Chivasa et al. 2002, Watson et al. 2004). Furthermore, 39% of the proteins were defence related proteins, which include pathogen related protein belonging to the secreted pathogenesis-related proteins (PRps) which were reported to be induced in the plant hypersensitive reaction leading to cell death and pathogen resistance (Kim et al. 2004), 23% of the proteins identified were grouped under miscellaneous and 23% were proteins of unknown function (Figure 14). 27 proteins did not show any significant similarity to any protein in the databases.

The absence of structural proteins may have following reasons: (1) some genes were

only expressed in response to specific intrinsic or environmental influences. (2) additionally, differentiation of the cells, which in part results in profound changes in wall structure, and the extent to which individual proteins were immobilized within these structures increases complexity. Thus, proteins like extensins get extensively cross linked in the wall (Showalter 2001) and insoluble resulting in the detection of fewer than the expected amounts of proteins. (3) these proteins were less amenable to tryptic digestion due to the high content of repeating sequences (Cassab 1998) containing amino acid residues like proline or hydroxyproline. (4) highly glycosylated proteins were problematic to be stained with Coomassie or silver. (5) they were not present in the EST data base. (6) salt extraction can solubilize some but not all proteins from the cell wall. This is probably due to the relative inaccessibility of cell wall proteins in the plant. For example, it is conceivable that PRPs interact ionically with other wall components such as acidic pectins. It is also likely that extensins interact ionically with pectins.

Inspite of the low resolution, still 1D-PAGE could identify some of the proteins which were hypothetically classified depending on their functions. This was a primary start to visualise the presence and to identify some classes of cell wall proteins.

6.1.3. Identified CWPs by Preparative IEF in Combination with SDS-PAGE

The analysis of CWPs can be done in different ways. The primary and direct way of analysis was by 2D-PAGE. Other methods like preparative isoelectric focussing and LC-MS/MS can also be used. But all these methods do not identify the same set of proteins but combination of methods can increase the number of identified CWPs.

The preparative IEF using the Rotofor system (Bio-Rad) separates proteins in free flow solution by their isoelectric points into 20 fractions. The fractions were separated by SDS-PAGE resulting in a pseudo 2D gel. Because the rotofor system identified CWPs using this preparative IEF include 29% of cell wall modifying enzymes, e.g., endo-beta-1, 4-glucanase reported to be induced in many other plants in response to various pathogens and during developmental events (Henning et al. 1993), peroxidase, cellulase and acid beta fructofuranosidase, which belongs to the glycosyl hydrolase 32 family and catalyses the hydrolysis of glycosidic bonds between carbohydrates. It contains a 38mer signal peptide and shows several potential N-glycosylation sites. The C-terminal ER retention motif is missing (Weber et al. 1995). 14% were defence related proteins like glutathione S

transferase and NADP dependent oxidoreductase which play a distinct role in plant antioxidant defence (Babiychuk et al. 1995). Hydroxyproline-rich glycoproteins (HRGPs) as a candidate of structural proteins (3%) represent a constituent of higher plant extra cellular matrix (ECM) and much work has been done to analyze the structure of these proteins. Involvement of HRGPs in remarkably rapid remodelling of the cell wall and the ECM was observed under the influence of pheromones and was also found to be inducible by wounding (Ender et al. 1999). 40% of the proteins identified were grouped under miscellaneous functions. These include phosphatase, which could also be involved in signal mediation (Watson et al. 2004) and elongation factor 1-alpha, a GTP-binding subunit of the translation elongation complex and also act as actin-binding protein (Yang et al. 1990). More than 14% were still unknown proteins. This method was used to cleanup the crude sample and to eliminate specific contaminants from proteins that might be difficult to remove by other means.

In conclusion, the identification of proteins using this method was high but, time consuming and more over this method was not suitable for comparative proteomics. So widely used 2-D gel electrophoresis, which has been the method of choice for separation of complex protein mixtures for several decades was used.

6.2. Detection of the glycosylated *M. truncatula* cell wall proteins using the DIG Glycan detection kit

Since CWPs go through the secretory pathway, most of them are supposed to be glycosylated. Among the post translational modifications (PTMs) of CWPs, glycosylations and hydroxylations of proline are the most abundant events because they occur during their transit through the ER and golgi. Glycosylation is the process or result of addition of saccharides to proteins and lipids. The process is one of four principal co-translational and Post translational modification steps in the synthesis of membrane and secreted proteins and the majority of proteins synthesized in the rough ER undergo glycosylation. It is an important post-translational modification associated with many proteins that have a regulatory function. Two types of glycosylation exist: *N*-linked glycosylation to the amide nitrogen of asparagine side chains and *O*-linked glycosylation to the hydroxy oxygen of serine and threonine side chains and the latter one is specific to plants. In plants, N-linked glycans have numerous roles such as the prevention of the proteolytic degradation or the induction of the correct folding and biological activity of the protein. Furthermore, N-linked oligosaccharides may contain targeting information, or may be directly involved in protein

recognition or cell–cell adhesion processes. Peroxidases are one of the structurally characterised cell wall N-glycoproteins (Gray et al. 1996). Thus protein glycosylation is widely recognized as a modulator of protein structure, localization, and cell-cell recognition in multicellular systems (Grogan et al. 2002).

Detection of glycoproteins is difficult because: (1) It is difficult to separate heavily glycosylated proteins since they cannot migrate. (2) Normal CBB staining method is insensitive for the detection of glycoproteins. (3) Possible steric hindrance of glycans near proteolytic sites during tryptic digestion. (4) Ionization of these glycopeptides are less efficient (5) MS analysis is not possible due to the lack of data for glycopeptide masses.

In order to visualise the presence of glycoprotein in the extracted cell wall proteome of *M. truncatula* suspension cell culture; Digoxigenin (DIG)/ Anti DIG alkaline phosphatase (AP) labelled, an extension of the periodic acid- Schiff method was used, because of their higher sensitivity (0.1µg glycoprotein). Glycoproteins were detected on a PVDF membrane. Vicinal (adjacent) hydroxy groups in sugars of glycoconjugates were oxidised to aldehyde groups by mild periodate treatment. The spacer-linked steriod hapten digoxigenin (DIG) was then covalently attached to these aldehydes via a hydrazide group. DIG labelled glycoconjugates were subsequently detected in an enzyme immunoassay using a DIG specific antibody conjugated with AP. Some minor proteins of *M. truncatula* revealed by DIG-glycan kit were glycosylated as shown in figure 15. Especially the higher molecular weight regions of the phenol extracted CWPs of *M. truncatula* were glycosylated (Lane 1, Figure 15). Indeed, the same as it was previously reported that the phenol-based extraction method showed enhanced extraction of glycoproteins (Saravanan and Rose 2004).

These results suggest that, the detected proteins were only mildly glycosylated. Also there was a possiblity that heavily glycosylated proteins may be washed away during the extraction or the extraction procedure used was not suitable for extracting these heavily glycosylated proteins.

6.3. 2D-PAGE analysis and identification of *M. truncatula* CWPs extracted from living cells

Protein separation by 2D-PAGE is one of the important approaches for large scale proteome analysis. Protein identification is done by MS analyses of excised and digested

protein spots. Till now, 2D-PAGE is one of a main method for protein separation, which allows the resolution of several hundreds of proteins simultaneously.

Among the identified CWPs, 26% were cell wall modifying enzymes, 21% defence related enzymes, 6% structural proteins and 12% unknown proteins. Examples of identified cell wall modifying enzymes were the peroxidase, normally secreted pectin esterase involved in the degradation of plant cell walls pectin and expansin which was known to be predominantly expressed in roots by wounding and water stress (Corbin et al. 1987). Members of the defence related proteins were, e.g., class II chitinase, which is able to degrade chitin in cell walls of fungal pathogens. However, the substrates and functions of most chitinase-like enzymes are not completely known. A mutation in the chitinase-like gene classified in the GH 19 family (AtCTL1/At1g05850) caused a cellulose deficiency as well as aberrant patterns of lignification with incomplete cell walls in the stem pith (Zhong et al. 2002, Rogers et al. 2005). Structural proteins include glycine-rich cell wall protein, and a proline rich protein. Also 35% of the identified proteins were grouped under miscellaneous function. The presence of phosphorylated proteins and phosphatases in plant cell walls has recently been reported in several works (Chivasa et al. 2002, Baluska et al. 2003, Kwon et al. 2005, Jamet et al. 2006, Watson et al. 2004). These include phosphorylated proteins like acid phosphatases which was also reported previously in the apoplast and cell walls of plants (Baluska et al. 2003, Watson et al. 2004). An acid phosphatase, which was identified in the present study, could be involved in such signal mediation (Watson et al. 2004) or in the regulation of cell wall proteins. The phosphorylation of cell wall proteins in muro would play an important role in regulating their localizations and enzyme activities, thereby allowing multiple locations and/or functions of the proteins encoded by a single gene. It might be possible that a post-translational modification may participate in the extra cellular localization of proteins without any conventional signal peptide via an unknown trafficking mechanism. Therefore, the protein patterns of these walls display considerable differences to those of other primary cell wall analysis (Borderies et al.2003, Chivasa et al. 2002, Watson et al. 2004).

In conclusion, the 2-D pattern for the CWPs extracted from living cells was not well reproducible and especially for low-abundant proteins the detection method was not sensitive enough to identify all CWPs sufficiently. Accordingly, this approach failed to identify the CWPs reliably, the more drastic extractions required to release proteins more strongly embedded in the extra cellular matrix cannot be done with living cells and also the

isoelectric focussing in the first step was limiting. Also several experimental conditions were tested (variation of focussing time and application of different protein amounts between 0.25mg and 1.5mg), but the data indicated that varying amounts of protein were lost during equilibration and focussing of the sample. A number of reasons for this observation are taken into account. First, CWPs were extracted with salt containing solutions ($CaCl_2$, NaCl), and hence, proteins are preferably soluble in solutions with high ionic strength. IEF is a method which is sensitive to ionic contaminations. The conductivity was lowered after reduction of the salt concentration either by dialysis or precipitation with phenol or acetone. Loss of proteins may have occurred due to the reduction of salt concentration which makes CWPs poorly soluble in rehydration solution. Second, the diluted CWPs after extraction must be concentrated before electrophoresis. Also increased amounts of sample applied to IEF led to the aggregation and precipitation of cell wall proteins stacking in the IPG strip.

6.4. 2D-PAGE analysis and identification of CWPs extracted from cell wall fragments

The increased popularity of 2D-PAGE has been mainly the result of significant improvements in resolution and reproducibility (Rabilloud 2002). A complete cell wall proteome cannot be obtained with a single extraction procedure. Using living cells extraction methods, loosely or weekly bound CWPs can be extracted. Also proteins can be extracted from purified cell walls using salt solutions. Either the living cells or purified cell wall is used, the extraction buffer was more critical and more important which type of proteins are extracted from the cell walls. Previous work reported that calcium chloride was very efficient since it allowed to recover 60% of the total number of CWPs identified in that study (Boudart et al. 2005).

Also studies on *A. thaliana* cell culture showed that membranes do not withstand extractions with ionic solutions and become leaky (Borderies et al. 2003). To limit potential contaminations with cytoplasmic proteins and to have a high number of CWPs, a new protocol was used for analyzing the cell wall proteome using cell wall fragments.
Extraction of CWPs using $CaCl_2$ and the analysis of cell wall proteome by 2D-PAGE and mass spectrometry combined with the bioinformatic analysis allowed the identification of 46 proteins and for some of the proteins the presence of a signal peptide were predicted using signal P software. Also it has been reported from previous cell wall proteome studies that proteins without signal peptide may be secreted (Chivasa et al. 2002).

Through MASCOT analysis of identified proteins, functions or putative functions could be deduced for most of them. Among the identified CWPs were proteins involved in metabolism, defense and stress related enzymes, membrane transport proteins, proteins involved in protein synthesis and processing, signaling proteins, miscellaneous and unknown proteins. Examples of identified CWPs were cell wall modifying proteins like glycosyl hydrolase family which might participate in rearrangement of polysaccharides during development (Rose and Bennett 1999), this was evident from previous cell wall proteome reports and glyceraldehydes-3-phosphate dehydrogenase which indicate that its presence in the cell wall was a result of authentic secretion to extracellular matrix in planta, also specifically targeted the cell wall during a defensive reaction (Chivasa et al. 2005). High abundant proteins like enolase which are secreted to the cell wall (Edwards et al. 1999) and wall bound malate dehydrogenase which is likely to regenerate NAD(P)H needed for the cell wall peroxidase for free radical generation (Gross 1977, Li et al. 1989) were also identified. Members of the defence related proteins were L-ascorbate peroxidase, superoxide dismutase and glutathione S-transferase. Calreticulin and Nucleoside diphosphate kinase I, proteins involved in signal transduction (Imin et al. 2004) were also identified. Membrane transport proteins like vacuolar ATP synthase subunit E and eukaryotic translation initiation factor 5A-2 proteins which is required for growth, development in several organisms and for cell proliferation (Feng et al. 2007) were identified. Though the presence of vacuolar ATP synthase is unusual it has been previously reported that it is recognised as an essential metabolite in cell wall (Chivasa 2005a). Also, the proteins grouped under miscellaneous function include alpha 1-4 glucan protein synthase, cytochrome b5 reductase and grf2 14-3-3 like protein, involved in covalent crosslinking of hydroxyproline-rich glycoproteins to the cell wall fraction (Voigt et al. 2003). Therefore, the protein patterns of these walls display considerable differences to those of other primary cell wall analysis (Chivasa et al. 2002, Borderies et al. 2003).

Few proteins were detectable in several different spots on the same 2D-gel. This suggests protein degradation as well as alternative posttranslational modifications like diverse glycosylation patterns. The proteins were mostly affected in both their molecular weight and in the isoelectric point. Protein carbamylation and protein oxidation could also be observed. For example, spot 39 or spot 65 in figure 21 showed the same molecular weight but different isoelectric points for the same protein. More often, a combination of shifts in both properties was observed, e.g., spots 5 and 23 in figure 21. The absence of

structural proteins is likely that they were removed during the the cell wall preparation because of the stringent washing steps or the glycosylation of polypeptides may reduce the chance for their identification by MS.

To summarise, the bioinformatic analysis identified 46 proteins that were either classical cell wall proteins or membrane proteins that were possibly associated with the cell wall, with a few exceptional noncanonical proteins. Thus this work demonstrated that purified cell wall can be a convenient source for extracting the cell wall proteins with reduced contamination.

6.5. Identification of cell wall proteins by LC-MS/MS

2D-PAGE is not the only platform for protein separation and there are inherent technical limitations, such as the limited ability to fractionate specific classes of proteins including hydrophobic proteins and glycoproteins, or to visualize low abundance proteins (Harry et al. 2000, Rabilloud, 2002). Some classes of proteins are very acidic or basic proteins, big or small proteins and membrane proteins are difficult to be seperated by 2-D gel electrophoresis (Rabilloud 2002). Also identification of glycosylated CWPs is very difficult using peptide mass mapping by MS. To overcome these limitations, proteome analysis based on the chromatographic techniques has been developed (Steen and Mann 2004). For this method, a complex protein mixture is digested first and the resulting digests are analysed by LC-MS/MS. Several hundreds of proteins can be identified in a single experiment.

Proteins identified by this study belong to the glycosyl hydrolase (GHs) family which plays important role in cell wall expansion, cell wall metabolism and modification during development, signalling and defence (Minic et al. 2006). These GHs were involved in cell wall reorganisation of the carbohydrate during growth and development (eg. Cellulose), defence against pathogen (eg. Chitinases and ß-1,3-Glucanases) reported to possess antifungal activity (Schlumbaum et al.1986), proteins involved in glycoprotein post translational modification (PTMs). Examples of these enzymes include chitinases, ß-D-galactosidases, alpha-D-mannosidases. The presence of heat shock protein is also confirmed as a bonafied cell wall protein in immunoflourescence study in yeast (López-Ribot and Chaffin 1996) and in *Candida albicans* (Swoboda et al. 1995).

Proteins without a secretory signal peptide have also been reported in the purified cell walls in previous report and their presence may be due to alternative pathway that

does not need transport through endomembrane system (Watson et al. 2004). Classical CWPs normally contains a cleavable signal peptide on the N terminus of the protein precursor, which is essential for their targeting to the ER. Then the protein supposed to be secreted leaves ER to Golgi to form complex where they are associated with the vesicles and subsequently, released in the extracellular membrane via exocytosis. Some of the CWPs extracted were predicted to contain a signal peptide. However non secreted or non-apoplastic proteins have also been reported in the cell walls of *Arabidopsis* cell cultures (Chivasa et al. 2002) and in yeast forms of *Candida albicans* (Pitarch et al. 2002).

It appears that most proteins without known secretory signal peptides are actually secreted. The conventional theory for protein trafficking cannot explain the mechanism by which proteins without any secretory signal peptide are exported into the extracellular spaces, several proteins that lack signal peptides are reportedly localized in the extra cellular matrix (Cleves 1997). For example, an elongation factor 1-α protein that has been identified in this present study was also reported to be localized in tobacco cell walls by immunogold labeling (Zhu et al. 1994), despite the lack of a secretory signal peptide.

In this study few antioxidative proteins were also identified in the cell wall supposed to protect the cells from oxidative damage. These include L-ascorbate peroxidase and glutathione *S*-transferases (Table 6). Plant tissues contain several ROS scavenging enzymes (like glutathione peroxidase) and detoxifying lipid peroxidation products (like glutathione S-transferases) (Chen et al. 2004), which protect cells from ROS and damage from stress conditions. Also the cell wall-bound peroxidase and a plasma membrane-bound NADPH oxidase are considered the major sources for ROS production as defence mechanisms during biotic stresses (Heath 2000, Kawano 2003, Melillo et al. 2006).

This study revealed that LC-MS/MS identified approximately 65 wall proteins with the identification of few novel proteins which were not identified previously by 2D-PAGE and MALDI-MS analysis.

6.5.1. Identified proteins from 2D and LC-MS/MS

The number of CWPs identified by 2D-PAGE and LC-MS/MS using cell wall fragments and the commonly identified CWPs are shown in figure 31. These include 25 proteins found to be common out of 46 and 65 CWPs identified by 2D-PAGE and by LC-MS/MS, respectively. Approximately two third of the proteins identified by LC-MS/MS

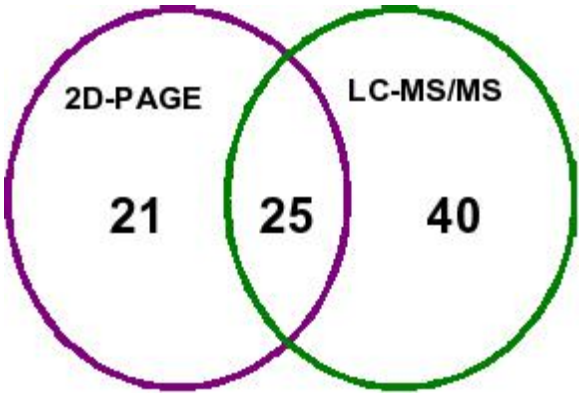


Figure 31: Venn diagram illustrating the overlap between the CWPs identified from 2D gel electrophoresis and LC-MS/MS.

analysis were found to be absent in the 2D-PAGE analysis.

Nevertheless, taking the comparative proteome as the significant concern, where 2D-PAGE is the best suitable method for visualising and quantification of proteins. Approximately 46% of the proteins identified were common to both the methods. Also few of the CWPs identified are involved in starch & sucrose metabolism, glycolysis and in ascorbate & aldarate metabolism (Red coloured EC, Figure 32 &33). Sucrose synthase (EC-2.4.1.13) is a key enzyme of sucrose metabolism in plant cells, providing carbon for respiration and for the synthesis of cell wall polymers and starch. Since sucrose synthase is important for plant cell growth, insights into its structure, localization and features is useful for defining the relationships between nutrients, growth and cell morphogenesis (Persia et al. 2008). The outer cell wall layer consists of pectins but the inner layer is composed of cellulose and callose; both polymers require metabolic precursors in the form of UDP-glucose, which is synthesised by sucrose synthase. Alpha-glucosidase (EC 3.2.1.20) is required for cellulose biosynthesis and morphogenesis in *Arabidopsis* (Gillmor et al. 2002).

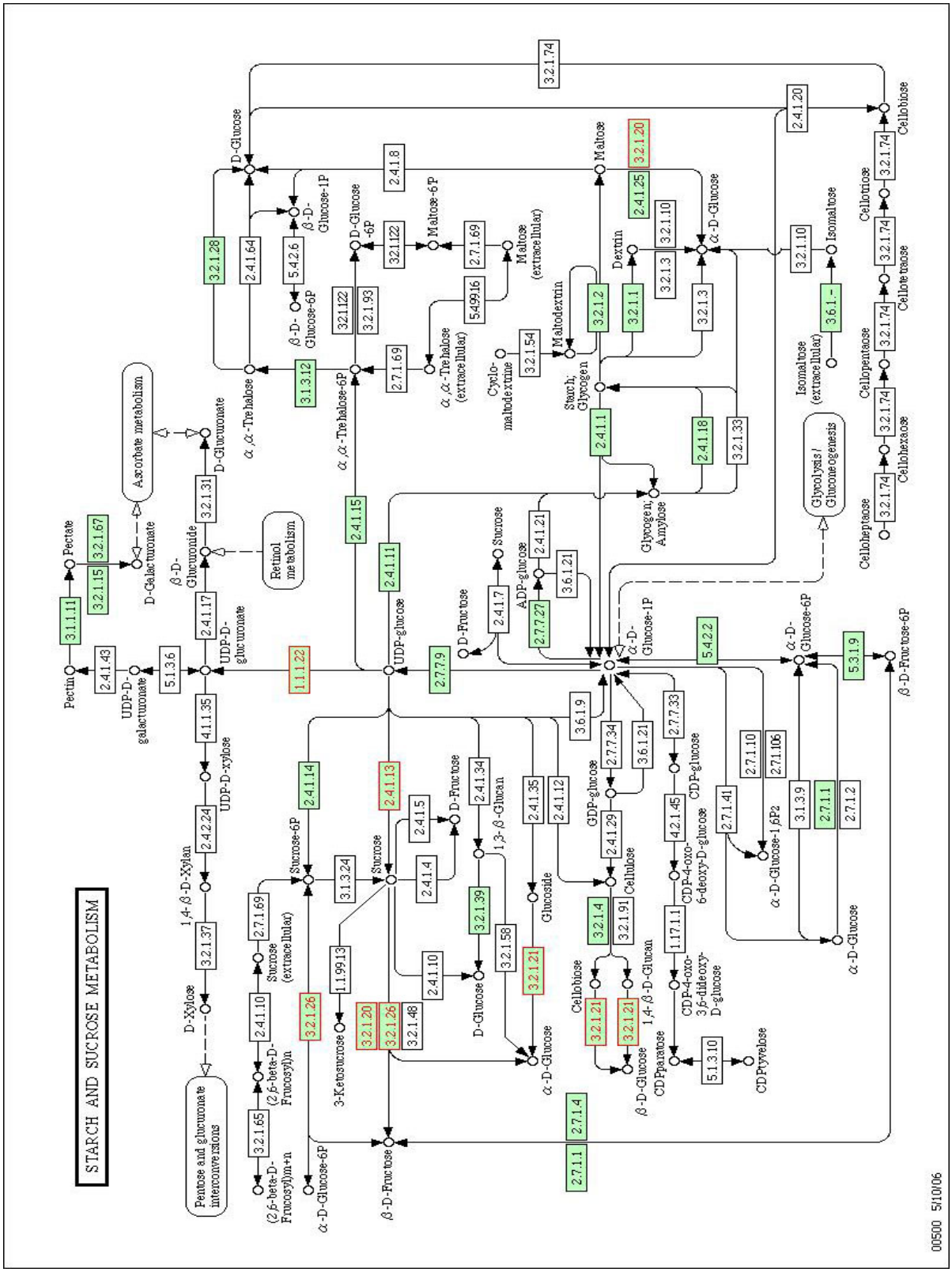


Figure 32: KEGG pathway, red colored EC indicating the identified CWPs of *M. truncatula* involved in starch and sucrose metabolism. EC 1.1.1.22-UDP-glucose 6-dehydrogenase; EC 2.4.1.13-sucrose synthase; EC 3.2.1.20-alpha-glucosidase; EC 3.2.1.21-beta-glucosidase; EC 3.2.1.26-beta-fructofuranosidase.

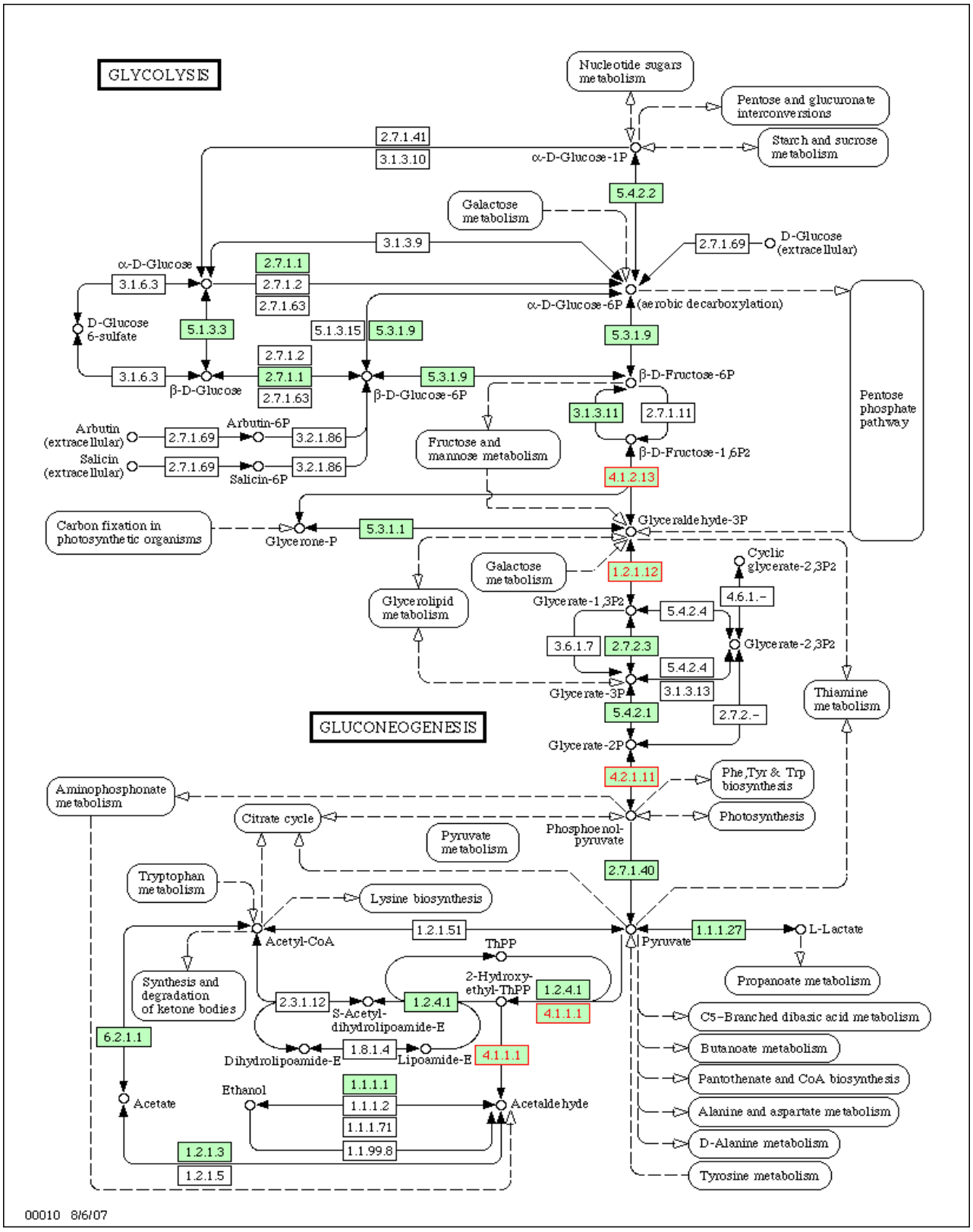


Figure 33: KEGG pathway scheme indicating (Red coloured EC) the identified CWPs of *M. truncatula* involved in glycolysis. EC 1.2.1.12-glyceraldehyde-3-phosphate dehydrogenase; EC 4.1.1.1-pyruvate decarboxylase; EC 4.1.2.13-fructose-bisphosphate aldolase; EC 4.2.1.11-phosphopyruvate hydratase (enolase).

L-ascorbic acid (EC 1.10.3.3) is characteristic of plant tissues, and ascorbate is recognized as one of the most important anti-oxidant molecules (Howard et al. 2000). Apart from playing an important role in plant growth and cell division and expansion as well as during maturity, fruit ripening and abscission phenomena, it plays an essential role to protect metabolic processes against H_2O_2 and other toxic derivatives of oxygen (Mittler et al. 1999).

6.6. Comparative Proteome analysis of CWPs during elicitation and suppression

For quantitative comparative proteomic analysis until now there are no widely available techniques that surpass 2D-PAGE, which is the method of choice for separating protein mixtures for several years. 2D-PAGE can provide an excellent means of comparing the expression of hundreds proteins between samples and of revealing which show quantitatively substantial differences in expression. In the context of comparative proteomics, where the goal is to identify quantitative and qualitative differences between protein samples, a 2D-PAGE approach is currently the method of choice, as it generates data in a form that allows far easier identification and quantitative comparisons (Rabilloud 2002).

The cell wall proteome of *M.truncatula* cell culture treated with water (control), elicitor, and elicitor with LPS are shown in figure 34 A, B, and C respectively. In this study defence response was stimulated using yeast elicitor and suppression effect by *S. meliloti* LPS. The defence response was obvious from the initial oxidative burst measurement where there was a maximum H_2O_2 generation after 5min (Figure 25) (Thordal- Christensen et al. 1997). Secondly, 2D Proteome map showed the disappearance of CWPs after elicitor treatment in accordance with previous report pathogen induced oxidative cross linking of the CWPs (Bradley et al. 1992). These changes or absence of CWPs suggest that this is probably because of covalent crosslinking in the cell wall matrix thereby defending against pathogen attack. Also our results showed some of the defence protein that was secreted upon elicitation. They include L-ascorbate peroxidase and eukaryotic translation initiation factor 5A. It has been reported that peroxidase expression correlates with resistance in many plant species like tobacco, rice and wheat. Generally pathogenesis-related peroxidases are involved in controlling the invasion of pathogen by the production of reactive oxygen species and by the reinforcement of physical barriers that prevent pathogen penetration of the cell walls (Kim et al. 2004). In addition to the protein classes discussed above, other proteins of three different isomers involved in

glycolysis for example glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase were also differentially expressed during pathogen elicitor treatment. Induction of cytosolic GAPDH in response to pathogen attack has been previously reported by Chivasa et al (2005) where it is specifically targeted to the cell wall. Though their presence is puzzling, it has been demonstrated to be secreted protein in many organisms like yeast and bacteria (Alloush et al. 1997, Winram and Lottenberg 1996). Several possibilities can suggest the presence of these proteins in the cell wall. Such possibilities are: i) Existence of several alternative PTMs that could affect the final destination of the protein. ii) Either GAPDH possess a signal peptide that has not yet been identified or there is an alternative secretory mechanism that does not require the canonical signal peptide as reported in yeast and animals (Cleves et al. 1996 and Muesch et al. 1990). iii) Presence of several translation sites giving rise to functional proteins that differ in the length of the N-terminus. For example, clusterin can be translated from two in frame ATG sites. Initiation from the 1st ATG encodes a secreted version of the protein while initiation from the second ATG, located 33 aminoacids downstream of the first and the lacking signal peptide, encodes a truncated intracellular clusterin (Reddy et al. 1996). iv) The different isomers could arise from one gene by expression of alternative transcripts as reported by Mezquita et al (1998). The alternative transcript could actually possess recognisable signal peptides.

Also few of the L-ascorbate peroxidase (Figure 35, region I, Inv 12h) was down-regulated by elicitor treatment. The role of ascorbate peroxidase in defence against oxidative stress was well established (Davletova et al. 2005). Differential expression of the L-ascorbate peroxidase proteins reveals that oxidant protection conferred by these proteins was regulated in *Arabidopsis* during elicitor treatment (Chivasa et al. 2006).

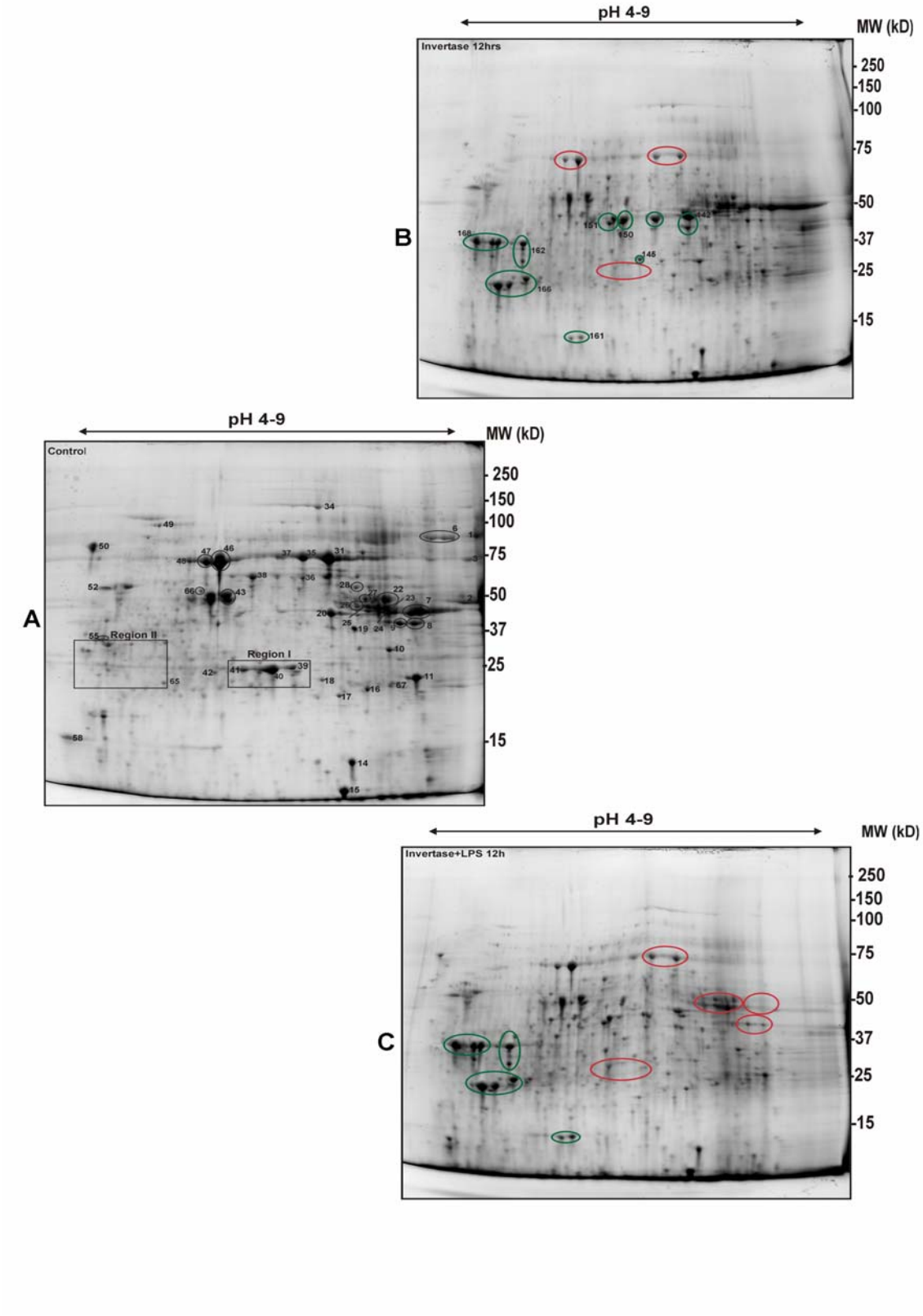


Figure 34: Comparative 2D gel electrophoresis proteome map for (a) control (b) elicitation with invertase and (c) invertase and *S meliloti* LPS.

In LPS treated cell wall proteome map there was no marked difference except proteins like L-ascorbate peroxidase and eukaryotic translation initiation factor 5A. This was evident from the earlier studies with white clover root hairs that the LPS induced changes in the protein composition but they play a significant role on successful development of infection threads in the Rhizobium-legume symbiosis (Dazzo et al. 1991). Also the infection-modulating activity of *R.trifolii* LPS displayed dosage and incubation time dependency (Dazzo et al. 1991). But the proteome map with invertase and rhizobial LPS was the same as the proteome map after invertase alone. Nevertheless the changes were less in the presence of *S. meliloti* LPS. This was also evident from transcriptome analysis by Tellstroem et al (2007) describing the moderating activity of the rhizobial LPS.

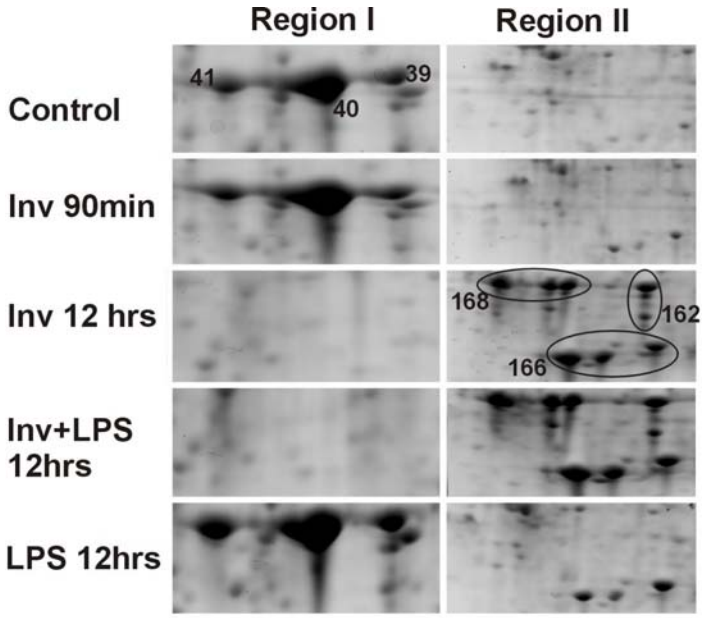


Figure 35: The regions I and II from figure 21 are highlighted to show the presence or absence of CWPs in control, elicitation with invertase (90min and 12h), invertase and LPS or only LPS. Numbers inside the panel indicate the identified CWPs which are listed under the table 5

These results suggest that the proteome of the wild type and after induction of elicitor with *S. meliloti* LPS, there was no marked difference between except induction of few defence related proteins. Since these LPS are dosage and incubation time dependent to perform their role (Dazzo et al. 1991), additional time course experiments need to be performed, which may support the evidence that LPS play a role and has a moderate effect in suppressing the defence related proteins during pathogenesis. Also in addition to the absence of several proteins during elicitation due to oxidative cross linking, other novel proteins were also upregulated. This may be due to the diversity of the biochemical

pathways responding to these elicitors that switch from primary metabolism to primed defence secondary metabolism. Probably the non essential pathways need to be downregulated leading to the production of defence proteins. Similarly, the essential pathways can be upregulated directly by increasing the protein components.

6.7. Functional classification of cell wall proteins

The tentative functional classification of CWPs identified using 2D-PAGE and LC-MS/MS were shown in figure 36. They comprise six main groups of CWPs–metabolic enzymes, signaling proteins, defence proteins, membrane transport protein, structural proteins and proteins involved in protein synthesis and processing. Some of the enzymes were proposed to be involved directly in cell wall modification, including peroxidases, alpha-glucosidase and pectin acetyl esterase. Other proteins in this group were involved in plant defence and stress reactions due to external influences.

A significant amount of the unidentified proteins were classified as hypothetical. Such proteins were annotated in databases as unknown, hypothetical or putative proteins due to the theoretical translation of open reading frame (ORFs) sequences. These findings demonstrate that the ORFs encoding these hypothetical proteins were expressed and secreted. The role of these proteins remains to be investigated.

Classical CWPs should exhibit expected features. Since these proteins were translocated from the cytoplasm to the periplasmic space, we expect to find 1) A signal peptide sequence–the analysis of these cleavable signal peptides was strongly recommended for the analysis of secreted CWPs in plants. They comprise the N-terminus of the amino acid chain, cause ER targeting and are cleaved off during translocation. The structure contains a positively charged n-region, followed by a hydrophobic h-region and a neutral C-region containing the cleavage site (Chivasa et al. 2002, Roberts 1994). 2) A canonical ER retention motif should not be present. These C-terminal tetrapeptide sequences prevent the transport of proteins located in the ER. 3) A hydrophobic transmembrane domain in the protein was absent which targets proteins secreted by the same pathway to the membrane.

Proteins from the same functional classes as in previous cell wall proteomic studies were found, but few proteins were not identified before (Chivasa et al. 2002, Borderies et al. 2003, Borner et al. 2003, Schultz et al. 2004, Boudart et al. 2005, Charmont et al. 2005,

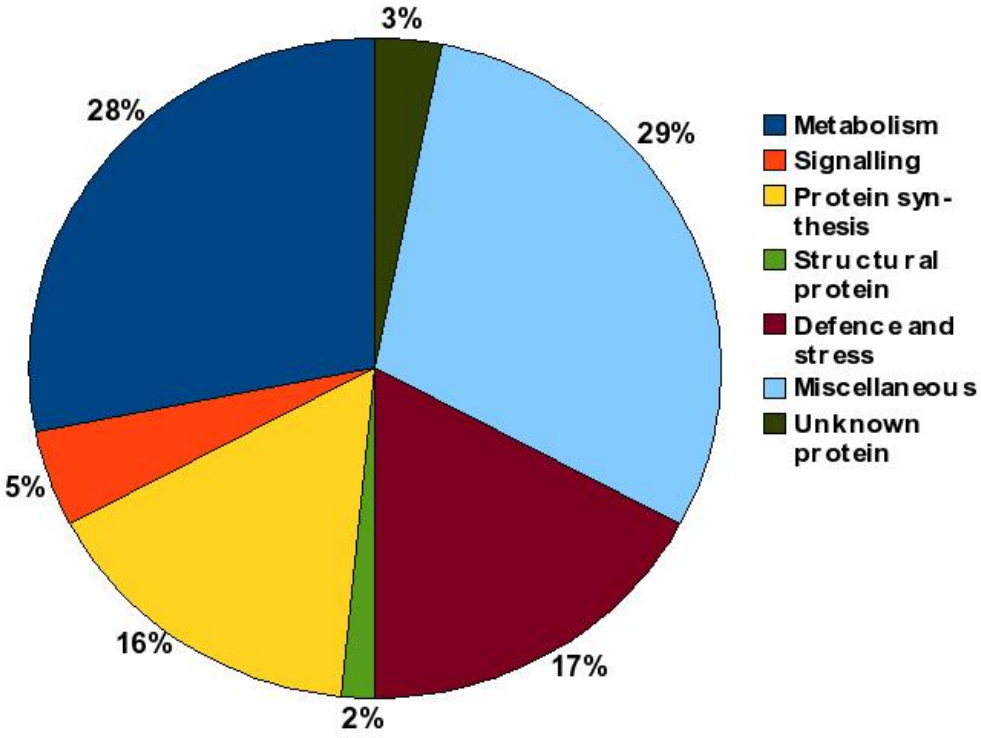


Figure 36: Pie chart diagram representating the identified cell wall proteins from 2D-PAGE and LC-MS/MS analysis.

Kwon et al. 2005, Bayer et al. 2006, Feiz et al. 2006).

The presence of a number of proteins known to be located in other compartments was observed. This was also the case in the proteome analysis of the *Arabidopsis* cell wall (Chivasa et al. 2002), where several proteins were found in cell wall extracts although they were normally not considered as being secreted. These included several mitochondrial enzymes and some cytoplasmic metabolic enzymes. In this study, the identified proteins showed few similarities with defence related proteins and cell wall modifying proteins identified in *Arabidopsis thaliana* (Chivasa et al. 2002, Borderies et al. 2003, Bayer et al. 2006) and *M. sativa* (Watson et al. 2004), we could also identify some novel proteins which were grouped under unknown and miscellaneous function. In addition, we identified iron, alpha 1-4-glucan synthase, grf2 14-3-3-like protein and structural proteins, which were not found in *Arabidopsis* and/or *M. sativa*

In conclusion, this study demonstrates the effectiveness of the purification procedure for extracting the CWPs. Taken together, this proteomic approach using the cell wall fragments and living cells *M. truncatula* suspension-cultured cells has disclosed a set of proteins required for cell wall modifications, signaling, defence mechanism, membrane

transport, protein synthesis and processing, as well as wall construction processes. Few of these proteins show a differing status of post-translational modifications such as phosphorylation and glycosylation. More over, the use of a cell wall fragments for the analysis of CWPs allowed us to reduce the contamination from cytosolic fractions, thus making it possible to identify a new class of CWPs that were predicted not to be localized in the cell wall spaces. Also comparative proteome analysis in response to elicitation and suppression and the identification of novel CWPs with dynamic changes, provides not only new insights into the proteome during pathogenic and symbiotic interaction but also a good starting point for further dissection of their functions. All these results strongly reflect the usefulness of a proteomic approach for cell wall dynamics. Functional analyses for each of these newly identified CWPs, together with their biochemical characterization and expression analyses, should extend our knowledge of understanding the whole picture of cell wall dynamism from a genomic level, and give an invaluable molecular basis for metabolomic approaches of this complex supermolecule.

Outlook

The reported work can be seen as a preliminary study to have detailed information about the presence/absence of the *M. truncatula* cell wall proteins during pathogenesis and symbiosis. Nevertheless to know the full biological functions of these proteins complementary approaches are needed. Future work will focus on the functions of the cell wall proteins by studying the patterns of the expression and immunocytochemistry. These studies can probably answer the question of unexpected proteins identified in the cell wall proteome.

Also the extracted protocol used in this study is for isolating loosely bound proteins. Covalently bound proteins are missing; enzymatic treatment can help to isolate such proteins. For example purified cell walls can be treated either by mild alkali conditions or by enzymatic treatment with glucanases and chitinases. CWPs can be released under mild alkali conditions (beta-elimination process), based on data from Kapteyn et al (1999) and Mrsa et al (1999). They are covalently linked to the beta-1, 3-glucan network either through their O-chains–these CWPs can be extracted by breakage of such chains–or by other uncharacterized linkages. Characteristic supramolecular complex of GPI-CWPs can be solubilized by beta -1,3-glucanases, according to data from Kapteyn et al (1996) and Kollar et al (1997). GPI-CWPs are covalently linked to beta-1,6-glucan through a phosphodiester bridge. Other CWPs attached to beta-1,3-glucan through other linkages can be extracted by this treatment. Beta-1,3-glucanase-resistant proteins can be released by exochitinase treatment, based on data from Kapteyn et al (1997). Beta-1,3-glucanase-resistant CWPs linked either through a beta-1,6-glucan moiety, such as some GPI-CWPs, or via some other uncharacterized linkage may be released by this procedure.

Also combination of this sequential fractionation and 2DPAGE followed by Western blotting using specific antibodies against known CWPs to characterize the incorporation mechanisms of such CWPs into the cell wall and their interactions with other wall components can be studied (Pitarch et al. 2002).The above suggested experiments would answer the question for finding some of the heavily glycosylated structural proteins which are covalently linked to the cell wall.

Since LPS are dosage and incubation time dependent to perform their role (Dazzo et al. 1991), additional time course experiments can be performed which may support the evidence in suppressing the defence related proteins during pathogenesis.

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Appendix

Table 2

Identification of the cell wall proteins of *M. truncatula* by SDS-PAGE and peptide mass fingerprinting. Each entry is indicated by a number which correspond to the protein spot numbers in figure 13, accession number, score, the number of matched peptides, the percentage sequence coverage (SC) of the PMF, signal peptide, the relative molecular weight M_r(cal) and pI calculated by the MASCOT software, the M_r(gel) values estimated from the spot positions in the SDS gel.

Spot no	M_r(gel)	Protein	Accession No	Score	Matched peptides	SC (%)	pI/Mr (cal)	Signal peptide
Defence and stress response								
33	40	Pathogenesis-related protein - chickpea	Q39450	68	5	20	10.1/35	no
33	40	Aspartate transaminase precursor-soybean	Q42803	56	4	14	7.7/36	no
46	31	Similar to receptor kinase {*Arabidopsis thaliana*}	Q9LRJ9	58	4	17	8.6/29	yes
17	82	Serine protease inhibitor {*Arabidopsis thaliana*}	Q9S7T8	64	5	15	5.7/42	no
29	66	NADPH oxidase {*Nicotiana tabacum*}	Q8W4X7	57	4	14	10.7/30	no
Cell wall modifying proteins								
11	55	Glucose-1-phosphate adenylyltransferase oriental melon	O22630	62	4	21	9.3/58	no
23	40	Beta-1 3 exoglucanase precursor-fungus {*Trichoderma harzianum*}	O14402	61	4	22	4.5/23	yes

Miscellaneous

9	67	Phosphoinositide-specific phospholipase C {*Medicago truncatula*}	Q93YX8	70	7	9	6.0/67	no
22	45	Trehalose-6-phosphate phosphatase {*Arabidopsis thaliana*}	Q9FJQ1	75	6	13	9.2/43	no
43	44	Trehalose-6-phosphate phosphatase {*Arabidopsis thaliana*}	Q9FJQ1	66	5	11	9.2/43	no

Unknown proteins

18	77	Unknown protein {*Arabidopsis thaliana*}	Q5RJC3	62	6	11	9.6/47	no
30	54	Similar to GP|9280661|gb|AAF86530.1 F21B7.20 {*Arabidopsis thaliana*}	Q9LR65	59	5	9	7.5/51	no
43	44	Similar to GP|15809950|gb|AAL06902.1 AT4g22740/T12H17_130 {*Arabidopsis thaliana*}	Q940N4	63	6	14	6.4/44	no

Table 3

Identification of the cell wall proteins of *M. truncatula* (extracted from living cells) by 2-DE and peptide mass fingerprinting. Each entry is indicated by a number which correspond to the protein spot numbers in figure 18, accession number, score, the number of matched peptides, the percentage sequence coverage (SC) of the PMF, signal peptide, the relative molecular weight pI/M_r (cal) calculated by the MASCOT software, and the pI/M_r (gel) values estimated from the spot positions in the 2-D gel.

Spot name	pI/Mr (gel	Protein	Accession No	Score	No of matched peptides	SC (%)	pI/Mr (cal)	Signal-peptide
Defence and stress response								
8	7.5/40	Probable dioxygenase {*Arabidopsis thaliana*}	E84648	100	8	19	6.1/40	no
9	7.5/36	Probable glutathione S-transferase	Q9FQF1	80	6	15	5.7/36	no
16	5.0/26	Glutathione S-transferase GST 9 {*Glycine max*}	Q9FQE9	58	4	19	5.6/26	no
33	5.5/35	Putative heme oxygenase 1 precursor {*Pisum sativum*}	Q93VB1	71	5	14	6.7/33	no
48	6.8/36	Receptor-like protein kinase-like protein {*Arabidopsis thaliana*}	Q8LBQ5	63	6	22	6.8/36	yes
49	6.0/20	Probable glutathione transferase 2 4-D inducible-soybean	O49235	58	4	18	6.4/26	no
25	4.5/38	Class II chitinase	Q9SBT5	57	4	25	8.6/27	yes

Cell wall modifying proteins

9	7.5/36	Peroxidase precursor {*Medicago truncatula*}	Q40372	93	6	32	9.6/36	no
18	5.5/28	Putative beta-1 3-glucanase	Q8H822	71	7	17	8.8/32	yes
13	5.6/22	UDP-glucosyltransferase {*Stevia rebaudiana*}	Q8LKG3	70	5	28	5.6/26	no
1	9.4/50	Peroxidase1A {*Medicago sativa*}	Q93XK6	77	5	10	9.4/42	yes
2	8.7/36	probable peroxidase ATP2a {*Arabidopsis thaliana*}	Q42580	66	4	10	6.8/37	yes
18	5.5/28	UDP-glucose 4-epimerase GEPI48	O65781	98	7	37	7.9/25	no
43	7.0/60	Pectinesterase precursor - garden pea	O24298	63	5	10	8.5/60	no
4	7.0/24	Expansin-like 1 precursor	Q8LC65	101	8	33	8.3/27	yes
5	6.8/23	NADPH oxidase {*Nicotiana tabacum*}	Q8LRN5	118	9	42	8.5/32	no

Structural proteins

14	5.8/23	Proline rich protein auxin-induced	O24072	62	5	18	9.1/20	yes
14	5.8/23	Glycine-rich RNA-binding protein GRP1	O24601	88	7	32	6.1/32	no

Miscellaneous

3	8.4/38	Acid phosphatase - soyabean	O49855	61	4	10	8.4/38	yes

8	7.5/40	Acid phosphatase-soybean	O49855	78	6	15	8.4/38	yes
1	9.4/50	Flavonoid 3' 5'-hydroxylase 2	P48419	89	6	18	8.9/54	no
2	8.7/36	Elongation factor 1-alpha {*Lycopersicon esculatum*}	P17786	77	5	11	8.7/44	no
7	7.7/63	Putative aminotransferase {*Arabidopsis thaliana*}	Q9SR86	103	8	15	7.7/63	no
12	6.0/24	MAP protein kinase MPKA {*Emericella nidulans*}	Q9Y7V6	105	7	31	8.6/24	no
15	6.0/24	Pentatricopeptide (PPR) repeat-containing protein {*Arabidopsis thaliana*}	Q69MJ7	60	4	13	9.7/21	no
28	5.0/45	Small GTPase Rab2 {*Nicotiana tabacum*}	Q946G3	64	5	24	9.3/33	no
32	4.5/67	Phosphoinositide-specific phospholipase C {*Medicago truncatula}*	Q93YX8	137	15	32	6.0/67	no
37	6.0/38	Putative protein phosphatase type 2C {*Arabidopsis thaliana*}	Q8S8Z0	73	6	22	5.1/38	no
40	6.2/55	Arginine methyltransferase-like protein {*Arabidopsis thaliana*}	Q9FI68	83	8	20	8.8/61	no
44	6.0/55	Pyrophosphate-dependent phosphofructo-1-kinase {*Arabidopsis thaliana*}	Q9C5J7	65	6	16	5.7/55	no

Unknown function

6	6.8/21	Unknown protein {*Arabidopsis thaliana*}	Q9FMC7	74	5	33	8.5/21	no
38	5.8/42	Hypothetical protein {*Arabidopsis thaliana*}	O23129	59	5	22	6.4/42	no
46	6.4/47	Unknown protein {*Arabidopsis thaliana*}	Q8LG71	57	5	16	6.4/47	no
47	6.8/43	Unknown protein (Na+/H+ antiporter) {*Arabidopsis thaliana*}	Q5XWR7	60	5	17	6.8/43	no

Table 4

Identification of the cell wall proteins of *M. truncatula* by preparative isoelectric focussing and peptide mass fingerprinting. Each entry is indicated by a number which correspond to the protein spot numbers in figure 16, accession number, score, the number of matched peptides, the percentage sequence coverage of the PMF (SC), signal peptide, the relative molecular weight pI/M_r(cal) calculated by the MASCOT software, and the pI/M_r(gel) values estimated from the spot positions in the SDS gel.

Spot no	pI/Mr (gel)	Protein	Accession No	Score	No of matched peptides	SC (%)	pI/Mr (Cal)	Signal peptide
Defence and stress response								
24	5.1/70	Monooxygenase 2 {*Arabidopsis thaliana*}	O81816	72	5	6	9.8/60	no
25	5.1/63	Putative receptor kinase	Q9LKY7	60	4	6	6.5/74	no
27	5.1/37	Ascorbate oxidase promoter-binding protein AOBP - winter squash	Q39540	66	4	15	5.2/37	no
45	6.0/30	Probable receptor protein kinase {*Arabidopsis thaliana*}	G96602	57	4	16	5.9/23	no
50	6.3/27	Probable glutathione transferase 2 4-D inducible - soybean	O49235	59	4	19	6.4/26	no
54	6.7/24	Glutathione S-transferase GST 8 {*Glycine max*}	Q9FQF0	57	4	16	5.4/29	no
61	7.1/25	Glutathione S-transferase {*Petunia x hybrida*}	O24261	61	4	22	5.5/28	no
81	8.8/45	Ascorbate oxidase promoter-binding protein	Q39540	87	5	23	10/42	no
12	3.7/38	Probable NADP-dependent oxidoreductase P2 {*Arabidopsis thaliana*}	Q39173	64	4	10	6.5/38	no

Cell wall modifying proteins

19	5.0/31	Endo-beta-1 4-glucanase {*Fragaria x ananassa*}	Q8L6S6	92	7	16	9.1/63	yes
60	7.1/37	1 3-beta-glucanase acidic - alfalfa	Q40314	63	4	9	9.4/41	yes
62	7.1/24	Peroxidase precursor {*Medicaga truncatula*}	Q40372	60	4	11	9.4/33	no
62	7.1/24	Peroxidase {*Arabidopsis thaliana*}	O49360	60	4	12	8.4/35	no
72	7.8/30	Peroxidase precursor {*Medicago truncatula*}	Q40372	60	4	19	9.5/33	no
100	10.9/30	Peroxidase precursor {*Medicago truncatula*}	Q40372	64	4	12	9.2/36	no
25	5.1/63	Glycosyltransferase-like protein {*Arabidopsis thaliana*}	Q9S7G2	58	4	8	6.3/54	yes
27	5.1/37	Pectinesterase PPE8B precursor {*Prunus persica*}	Q43062	65	4	18	6.0/35	yes
36	5.7/40	Ribulose 1 5-bisphosphate carboxylase small subunit {*Cicer arietinum*}	Q9ZP07	67	4	20	6.0/30	no
36	5.7/40	Acid beta-fructofuranosidase precursor	Q43857	63	4	18	5.3/40	no
39	5.7/28	Similar to Flavonol 3-O-Glucosyltransferase {*Arabidopsis thaliana*}	Q9XIQ4	64	4	10	6.7/35	no
65	7.4/40	Cellulase precursor	Q38890	70	4	20	9.5/27	no
72	7.8/30	Putative beta-mannosidase {*Gossypium hirsutum*}	Q93X75	65	5	14	10/35	no
75	8.1/30	Cellulose synthase homolog {*Arabidopsis thaliana*}	O80891	68	4	17	9.2/30	no

76	8.4/30	UDP-glucosyltransferase {*Stevia rebaudiana*}	Q8LKG3	81	5	15	5.7/35	no
85	8.8/30	Cellulose synthase homolog {*Arabidopsis thaliana*}	O80891	68	4	16	9.2/30	no
98	10.9/30	Cellulose synthase homolog {*Arabidopsis thaliana*}	O80891	68	4	16	9.2/30	no
Misellaneous								
41	5.7/23	Protein phosphatase 2C - alfalfa	O24078	72	4	19	6.0/23	no
3	3.1/60	Putative subtilisin-like protein {*Oryza sativa*}	Q8H8B6	87	6	19	9.6/53	no
6	3.1/37	Similar to auxin-independent growth promoter {*Arabidopsis thaliana*}	Q9SK88	78	5	19	9.5/43	-
9	3.1/25	Ubiquitin-fusion degradation protein	Q9LF41	72	4	14	11.8/25	no
10	3.1/22	1-aminocyclopropanecarboxylic acid synthase {*Medicago truncatula}*	Q8VZY4	86	6	14	6.7/55	no
13	3.7/29	Elongation factor 1-alpha {*Lycopersicon esculentum*}	P17786	78	6	8	8.8/44	no
19	5.0/31	Pyrroline-5-carboxylate synthetase 2 {*Medicago truncatula*}	Q8GUA7	64	4	17	6.3/28	no
27	5.1/37	Protein kinase-like protein {*Arabidopsis thaliana*}	Q94JZ6	68	4	13	11.8/33	no
33	5.7/60	Probable wall-associated kinase T4O24.5 {*Arabidopsis thaliana*}	Q9C617	92	6	18	5.5/42	yes
38	5.7/35	Caffeoyl-CoA O-methyltransferase 5-common tobacco	O04899	68	4	13	5.9/29	no

39	5.7/28	Cytochrome P450 71D10 {*Glycine max*}	O48923	70	4	22	7.8/26	no
42	5.8/35	Phosphoinositide-specific phospholipase C {*Medicago truncatula*}	Q93YX8	68	5	9	6.0/67	no
43	6.0/45	Kinesin-related protein katB {*Arabidopsis thaliana*}	P46864	61	4	12	5.4/45	no
45	6.0/30	Cysteine synthase (O-acetylserine sulfhydrylase)	O81154	58	4	18	5.8/34	no
68	7.4/30	ABC transporter {*Arabidopsis thaliana*}	O04323	68	4	12	9.0/30	no
81	8.8/45	NADP-dependent isocitrate dehydrogenase {Glycine max}	O82585	78	5	17	7.7/47	no
83	8.8/42	Trehalose-6-phosphate phosphatase {*Arabidopsis thaliana*}	Q9FJQ1	62	4	9	9.2/42	no
84	8.8/34	Putative protein kinase {*Arabidopsis thaliana*}	Q7XUF4	67	4	7	10.9/34	no
87	9.1/40	Cysteine proteinase 3 precursor - kidney bean	O24323	72	5	10	9.1/49	yes
89	9.1/30	Putative exostoses {Oryza sativa}	Q9AUK5	83	5	21	8.9/33	no
90	9.6/30	ABC transporter {*Arabidopsis thaliana*}	O04323	86	5	18	9.0/30	no
13	3.7/29	Beta-expansin/allergen protein {*Arabidopsis thaliana*}	Q9M0I2	68	4	19	8.4/29	yes
Structural proteins								
49	6.3/30	Hydroxyproline-rich glycoprotein 1 - garden pea (fragment)	P55844	64	5	23	10.5/17	no
65	7.4/40	Serine-rich protein - fission yeast	Q09788	61	4	8	5.6/35	yes

Unknown function

2	3.1/75	Unknown protein {*Arabidopsis thaliana*}	Q8L640	68	5	11	5.7/65	no
6	3.1/37	Unknown {*Arabidopsis thaliana*}	Q8L9C4	78	5	16	9.5/36	no
27	5.1/37	Unknown {*Arabidopsis thaliana*}	Q8GYT4	63	4	14	5.8/36	no
53	6.7/27	Similar to PIR|G86340|G86340 protein F2D10.35 {*Arabidopsis thaliana*}	Q8GWA7	70	4	22	11.1/27	no
62	7.1/24	Strong similarity to unknown protein	Q9FK89	74	4	22	9.4/20	no
76	8.4/30	Unknown protein {*Arabidopsis thaliana*}	Q94AI7	67	4	13	8.4/31	no
85	8.8/30	Unknown protein {*Arabidopsis thaliana*}	Q949V4	84	5	23	8.0/30	no
95	10.9/40	Hypothetical protein F4B14.210 {*Arabidopsis thaliana*}	O65631	62	4	12	9.2/40	no

Table 5

Identification of the cell wall proteins of *M. truncatula* (extracted from cell wall fragments) by 2D-PAGE and PMF. Each entry is indicated by a number which correspond to the protein spot numbers in figure 21, 26-29, accession number, score, the percentage sequence coverage (SC) of the PMF, signal peptide, the relative molecular weight pI/M_r(cal) calculated by the MASCOT software, and the pI/M_r(gel) values estimated from the spot positions in the 2-D gel.

Spot no	Identified proteins	Accession No	pI/M_r (gel)	pI/M_r (cal)	Score	SC (%)	Signal peptide
1	Glycosyl hydrolase family	P83344	84/8.9	50/8.9	150	12	No
18	Glutathione S-transferase, putative	O49235	26/6.4	26/7.2	80	15	No
17	Superoxide dismutase	P27084	29/8.6	26/7.2	100	17	No
2	Peroxidase 12 precursor	Q9XFI8	40/9.0	38/9.5	118	32	Yes
3, 36, 38, 47, 48	Enolase	Q6RIB7	53/5.4	48/9.0	112	15	No
6	Glycosyl hydrolase family	P83344	84/8.9	50/8.5	93	8	No
19	Vacuolar ATP synthase subunit E	Q84T14	32/8.6	26/7.8	151	26	No
20	Malate dehydrogenase	O48904	38/9.1	36/7.3	113	23	Yes
22, 24, 25, 26, 27	Fructose-bisphosphate aldolase	O65735	44/7.2	38/7.8	149	29	No
7	Glyceraldehyde-3-phosphate dehydrogenase	Q6RUQ2	40/6.1	37/8.2	78	20	No
28	Probable glutamate dehydrogenase	Q43314	45/6.2	45/7.2	108	26	No
31, 34, 35, 37	Enolase	Q42971	24/5.0	48/7.1	62	32	No

34	Methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	O50008	89/6.1	84/7.0	88	11	No
8, 9	Ferredoxin--NADP(+) reductase, putative / adrenodoxin reductase, putative	Q41014	52/9.0	42/8.3	78	13	No
10	Cytochrome-b5 reductase	P83291	34/8.3	36/7.9	81	26	No
11	Osmotin-like protein precursor	Q41350	29/7.0	27/8.2	70	31	Yes
14	Ubiquitin-conjugating enzyme	Q9CZY3	21/7.2	16/7.2	109	32	No
15	Nucleoside diphosphate kinase I	Q8GV24	19/6.0	16/7.1	66	34	No
39	L-ascorbate peroxidase	P48534	27/5.5	27/6.5	72	24	No
40, 41	L-ascorbate peroxidase	P48534	27/5.5	27/6.0	72	24	No
42	Proteasome subunit alpha type 2	O23708	26/5.5	26/5.5	65	19	No
43	Alpha-1,4-glucan-protein synthase	O04300	43/6.1	42/6.0	88	28	No
46	Enolase	Q6RIB7	53/5.4	48/5.4	112	15	No
49	70 kDa peptidylprolyl isomerase	Q7F1F2	62/5.1	64/5.0	61	11	No
50	Calreticulin precursor	P93508	49/4.4	48/4.4	80	17	Yes
52	Hypothetical protein	P32583	36/4.8	41/4.3	63	14	No
55	grf2 14-3-3-like protein	Q9XEW4	29/4.7	29/4.3	68	23	No
23	Glyceraldehyde-3-phosphate dehydrogenase	P34922	40/7.8	37/6.6	76	31	No
16	Ferritin	Q9ZP90	22/6.0	28/7.5	63	15	No
65, 97	L-ascorbate peroxidase	P48534	27/5.5	27/5.0	72	24	No
58	Eukaryotic translation initiation factor 5A-2	Q945F4	18/5.4	17/3.8	77	35	No

66	Probable protein disulfide-isomerase A6	P38661	40/5.4	40/5.4	88	17	Yes
67	Expressed protein	Q9LYG2	23/7.5	23/6.6	108	36	No
107	Cytochrome c oxidase subunit 6b, putative	Q8LCP1	21/4.5	21/4.0	157	44	No
108	Heat shock protein 2	Q8GSN3	71/5.1	71/4.5	58	9	No
142	Glyceraldehyde-3-phosphate dehydrogenase	Q6RUQ2	40/6.1	37/7.2	112	17	No
145	Glyceraldehyde-3-phosphate dehydrogenase,	P34922	40/7.2	37/6.8	66	23	No
161	Eukaryotic translation initiation factor 5A-1	P26564	18/5.5	18/5.5	89	26	No
162	Enolase	Q42971	24/5.0	48/5.0	62	32	No
166	L-ascorbate peroxidase	P48534	25/4.5	27/6.5	68	24	No
168	Enolase	Q6RIB7	37/4.3	48/5.4	79	22	No
151	Enolase	Q42971	24/6.0	48/7.1	66	30	No
150	Glyceraldehyde-3-phosphate dehydrogenase	P34922	42/6.5	37/6.8	66	28	No

Table 6

Identification of the cell wall proteins of *M. truncatula* (extracted from cell wall fragments) by LC-MS/MS. Each entry is indicated with the identified proteins, accession number, calculated molecular weight (M_r) and pI, score, the percentage sequence coverage (SC) and signal peptide.

Identification	Accession No	M_r/pI (Cal)	Score	SC (%)	Signal peptide
Enolase 4.2.1.11	Q42971	52/5.4	1145	43	No
Fructose-bisphosphate aldolase, cytoplasmic isozyme 2 4.1.2.13	O65735	44/7.2	662	31	No
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3 1.2.1.12	Q9XG67	40/6.1	576	27	No
Peroxidase 12 precursor 1.11.1.7	Q43854	40/9.0	566	22	Yes
Phospholipase D beta 2 3.1.4.4	Q9AWC0	93/5.5	539	17	No
Iron 3.1.3.2	Q38924	33/6.4	516	30	Yes
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 1.2.1.12	Q9XG67	40/7.2	450	37	No
Ctsd aspartyl protease family protein 3.4.23	Q39311	56/6.0	404	20	Yes
Nucleoside diphosphate kinase I 2.7.4.6	Q8GV24	19/6.0	399	45	No
hypothetical protein predicted by Glimmer/Critica	O04275	61/6.0	393	16	No
heat shock protein (hsc70)	Q9M4E8	76/5.1	392	18	Yes
Enolase 4.2.1.11	P42896	24/5.0	384	31	No
translation elongation factor 2 (EF-2) 3.6.5.3	Q9SGT4	95/5.9	373	12	No
Alpha-1,4-glucan-protein synthase 2.4.1.112	O04300	43/6.1	366	29	No
cysteine proteinase, putative 3.4.22	O24323	55/6.1	356	14	Yes

expressed protein	Q9C6U3	42/9.0	344	18	No
L-ascorbate peroxidase, cytosolic 1.11.1.11	P48534	27/5.5	335	24	No
EFT2 translation elongation factor 2 (EF-2) 3.6.5.3	Q9ASR1	95/5.8	322	15	No
grf2 14-3-3-like protein	Q9T0N0	29/4.7	301	23	No
Putative GTP-binding protein PTD004 homolog	Q9M7P3	30/5.9	295	27	No
pdc pyruvate decarboxylase-related protein 4.1.1.1	Q684K0	64/5.6	265	7	No
glycosyl hydrolase family 3 3.2.1.21	Q7XJH8	85/8.9	247	14	Yes
sh-1 Sucrose synthase 1 2.4.1.13	Q9T0M6	93/5.9	244	7	No
STI1 Heat shock protein STI	Q43468	59/5.8	242	8	No
Probable prefoldin subunit 4 (VIP3 protein)	Q9M4C4	15/4.5	233	26	No
fda Fructose-bisphosphate aldolase 1, chloroplast precursor 4.1.2.13	P07764	43/8.3	220	14	No
grf2 14-3-3-like protein A (VFA-1433A)	P42653	33/4.8	212	15	No
Gdi1 Rab GDP dissociation inhibitor alpha	Q706C9	56/6.2	210	8	No
Ubiquitin-conjugating enzyme spm2	Q69TB0	21/7.2	206	32	No
L-ascorbate oxidase homolog precursor 1.10.3.3	P24792	61/8.9	201	7	Yes
KAR2 heat shock protein 2	Q9M4E7	71/5.1	196	8	No
HYP2 Eukaryotic translation initiation factor 5A-2	Q71F50	18/5.6	190	32	No
Alpha-1,4-glucan-protein synthase 2.4.1.112	Q8RU27	42/5.8	181	17	No
sh-1 sucrose synthase, putative / sucrose-UDP glucosyltransferase, putative 2.4.1.13	P31926	93/5.9	179	3	No
bip luminal binding protein 1 precursor (BiP-1)	Q9LKR3	71/5.0	178	7	Yes

Mor1 malate dehydrogenase 1.1.1.37	O48904	38/9.1	169	12	Yes
Importin alpha-1 subunit	Q9SLX0	59/5.1	169	9	No
ef1a elongation factor 1-alpha / EF-1-alpha 3.6.5.3	O64937	59/9.2	169	4	No
Alpha-1,4-glucan-protein synthase 2.4.1.112	Q8RU27	42/5.7	166	15	No
cysteine synthase, mitochondrial, putative / O-acetylserine (thiol)-lyase, putative	Q43725	28/5.1	166	15	No
Fructose-bisphosphate aldolase, cytoplasmic isozyme 2 4.1.2.13	P07764	42/8.3	150	10	No
Mdh1 malate dehydrogenase 1, NAD (soluble) 1.1.1.37	O48904	40/7.2	147	14	Yes
petH ferredoxin--NADP(+) reductase, putative / adrenodoxin reductase, putative 1.18.1.2	Q41014	52/9.1	146	12	No
TIF6 Eukaryotic translation initiation factor 6	Q9M060	32/5.5	127	16	Yes
Alpha-expansin 4 precursor	Q6T5H7	29/9.6	124	10	Yes
Ugdh UDP-glucose 6-dehydrogenase 1.1.1.22	Q96558	57/7.2	119	8	Yes
Tpt1 Translationally controlled tumor protein homolog	P28014	22/4.9	118	11	No
calmodulin-7 (CAM7)	P06787	22/5.2	116	17	No
ferredoxin-sulfite reductase 1.8.7.1	Q9AWB2	77/9.2	106	9	No
Alpha-expansin 6 precursor	Q38865	29/9.4	103	9	Yes
Alpha-glucosidase precursor 3.2.1.20	Q9LEC9	33/6.6	100	9	Yes
Elongation factor 1-beta' (EF-1-beta')	O96827	29/4.9	100	11	No
cat2 catalase 1.11.1.6	P25890	57/6.7	95	5	No
pectinacetylesterase, putative	Q6YVK6	44/8.8	93	5	Yes
Glycine-rich RNA-binding protein 10	Q7XI13	20/8.5	86	12	No

Putative GTP-binding protein PTD004 (PRO2455)	Q8LDR5	16/7.8	85	23	No
Pistil-specific extensin-like protein precursor	Q41122	30/9.5	80	13	Yes
Eukaryotic initiation factor	Q8H179	86/6.0	68	4	No
Glycine-rich RNA-binding protein GRP1A	P49310	16/4.8	65	14	No
glutathione S-transferase, putative 2.5.1.18	O49235	26/6.4	91	5	No
ivr1 Beta-fructofuranosidase, cell wall isozyme precursor 3.2.1.26	Q43089	65/9.0	124	6	Yes
pfk Pyrophosphate--fructose 6-phosphate 1-phosphotransferase alpha subunit 2.7.1.90	Q9ZST2	68/6.6	92	5	No
Limonoid UDP-glucosyltransferase 2.4.1.210	Q9ZR25	57/5.5	86	5	No
Rhicadhesin receptor precursor	Q9S8P4	23/8.9	86	13	Yes
Methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 2.1.1.14	O50008	89/6.1	1379	37	No

Declaration

Herewith I affirm that I wrote the presented dissertation independently only using the cited references as a support.

Furthermore I affirm that the presented dissertation was not submitted at any other faculty either partly or complete to obtain an academic title.

Herewith I submit to obtain the title doctorate of science from the Bielefeld University for the first time.

Bielefeld, May 2008