Characterization of defense responses of susceptible and resistant tomato genotypes against bacterial wilt caused by *Ralstonia solanacearum*, a proteomic approach

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades eines

Doktors der Naturwissenschaften
-Dr. rer. nat.-
genehmigte Dissertation
von

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geboren am 4. Dezember 1974 in Nepal

2009

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Tag der Promotion: Dezember 01, 2009

DECLARATION BY CANDIDATE

I, Diwakar Dahal, hereby declare that this thesis, entitled **"Characterization of defense responses of susceptible and resistant tomato genotypes against bacterial wilt caused by *Ralstonia solanacearum*, a proteomic approach"** is an original work conducted by myself and has not been submitted for a degree in any other university.

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December 2009, Hannover

ZUSAMMENFASSUNG

Bakterielle Welke, verursacht durch *Ralstonia solanacearum,* ist eine der verheerendsten bakteriellen Erkrankungen an Kulturpflanzen in den Tropen und Subtropen. Der Anbau resistenter Sorten bleibt die praktikabelste Maßnahme, welche bei Eingliederung im Rahmen eines integrierten Pflanzenschutzkonzeptes, eine Bekämpfung in einigen Regionen ermöglicht. Dennoch, die Resistenz ist nicht stabil und der Mechanismus der Resistenz auf molekularer Ebene weitestgehend ungeklärt. Daher wurden zur Aufklärung von möglichen Resistenzmechanismen in diesen Arbeiten molekulare und biochemische Methoden. verwendet. Wir analysierten die Protein-Profile von anfälligen und resistenten Genotypen von *Solanum lycopersicum* bei Befall mit *R. solanacearum*.

Zunächst wurde das Proteom des gesamten mittleren Stängelabschnitts untersucht, in dem in vorangehenden Arbeiten der Arbeitsgruppe Resistenzmechanismen gegen Bakterielle Welke lokalisiert worden sind Nur die anfälligen Pflanzen reagierten auf die Inokulation des Pathogens mit unterschiedlicher Regulation der detektierbaren Proteine, welchen Funktionen in an der Pathogenabwehr, Stressantwort und im Metabolismus zugeschrieben wurden. Die Sensitivität der Methode wurde durch Analyse einer Subfraktion des Gewebes, des Zellwand-Proteoms des mittleren Stängelabschnitts, weiter erhöht. Ebenso konnten konstitutive, genotypische Unterschiede zwischen zwei *S. lycopersicum* Linien, die sich im Grad der Resistenz gegen *R. solanacearum* unterscheiden, erfolgreich identifiziert werden (Primärstoffwechsel- , Abwehr- und Stress-induzierte Proteine). Unterschiede in der Proteinregulierung wurden in *S. lycopersicum* Genotypen auch nach Inokulation mit dem Pathogen festgestellt: In dem anfälligen Genotyp waren nach Induktion durch Infektion Pathogenese-assoziierte (PR)-Proteine stärker exprimiert, wohingegen bei der resistenten Linie Proteasen und Signalproteine auftraten. Weiterhin konnte eine verminderte Expression von Stress-induzierten- und antioxidativ wirksamen Proteinen bei den resistenten Genotypen ermittelt werden, während bei der anfälligen Linie Proteine des Zellwandstoffwechsels in der Interaktion mit dem Pathogen herunterreguliert waren. Proteine des Primär- und Energiestoffwechsels zeigten unterschiedliche Expressionsstärken in beiden Genotypen.

Schließlich wurde das Proteom des Xylemsaftes, eine weitere wichtige Schnittstelle der Pflanze-Pathogeninteraktion, erstmals in diesem Zusammenhang analysiert. Dieses beinhaltet ein umfangreiches Netz von 208 Proteinen und ermöglicht einen Überblick der Funktionen des Xylemsaftes in einer Gefäßpflanze. Der Vergleich der Xylem-Proteome von gesunden Pflanzen zweier unterschiedlich resistenter Genotypen zeigte einen höheren Prozentsatz an Proteasen, Peroxidasen und anderen an der Verteidigung beteiligten Proteinen in den resistenten Pflanzen auf, wohingegen bei anfälligen Pflanzen der Anteil von Proteinen höher war, welche in Signalwege und Transkriptionsfaktoren involviert sind.

Zusammengefasst zeigt die vorliegende Arbeit konstitutive Unterschiede zwischen resistenten und anfälligen *S. lycopersicum* Genotypen auf Proteom-Ebene im Xylemsaft und in den Zellwänden des Stängels. Weiterhin wurden pathogen-induzierte Differenzen in der Proteinexpression sowohliam mittleren Stängelabschnitt als auch auf Zellwandebene festgestellt. Die Ergebnisse liefern einen wichtigen Beitrag für das Verständnis der Resistenzmechanismen der Tomate gegen bakterielle Welke und können in Züchtungsprogrammen verwendet werden.

Schlüsselwörter:

Bakterielle Welke von Tomaten / Proteomics und die Massenspektrometrie / *Ralstonia solanacearum* / Sekretorisch Proteine / Stängels, Zellwand und Xylemsaftes proteome

SUMMARY

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most devastating bacterial diseases in the tropics and subtropics. Use of resistant cultivars remains the most useful individual control measure which, after incorporation in a framework of integrated disease management, provides good control in some regions. However, the resistance is rather unstable and the mechanism of resistance at the molecular level is largely unclear. Therefore, we initiated investigations on the molecular level of resistance mechanism by analyzing the protein profiles that are specific to susceptible and resistant tomato genotypes against bacterial wilt caused by *R. solanacearum*.

The proteome was examined first from the whole mid-stem, where resistance mechanisms had previously been reported after root inoculation. Only the susceptible plants responded to pathogen challenge by differentially regulating their proteins, which were identified as pathogenesis as well as stress related and metabolic proteins. The sensitivity of the analysis was further increased by studying the cell wall proteome from mid-stems, and successfully revealed genotypic differences primarily metabolic, defence and stress related proteins between the two genotypes. Similarly, plants of both genotypes showed the differential regulation of proteins in response to pathogen inoculation. PR proteins in susceptible and protease as well as a signaling proteins in resistant plants were up regulated where as stress related proteins as well as an antioxidant in resistant and cell wall metabolic proteins in susceptible genotypes showed down regulation during the interaction. Proteins of primary and energy metabolism also displayed differential regulation in both genotypes. Finally, xylem sap, another key site for plant-pathogen interaction, were analyzed which for the first time demonstrated as many as 208 proteins. They included large networks of proteins providing an overview of the xylem sap functions in a vascular plant. The comparison of the xylem proteome of healthy plants of two genotypes also disclosed the higher percentage of protease, peroxidase and other defense related proteins in resistant plants, while susceptible plants contained mainly signaling and transcription related proteins.

In conclusion, the present study provided constitutive differences in tomato genotypes of variable degrees of resistance on proteome level in the xylem sap and stem cell walls. Additionally, pathogen-induced differences in whole stem as well as in stem cell wall proteome present an important contribution to understanding of bacterial wilt resistance in tomato. The results give valuable information for future breeding programmes and genetic improvement of tomato bacterial wilt resistance.

Key words:

Bacterial wilt of tomato / Proteomics and mass spectrometry / *Ralstonia solanacearum* / Secretory protein / Stem, cell wall and xylem sap proteome

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ACKNOWLEDGEMENT

I wish to express my sincere gratitude to Prof. Dr. Kerstin Wydra for considering me in her working group. I deeply appreciate her for providing invaluable suggestion, friendly discussion and a very nice working environment throughout the course of my PhD. I really like her readiness to discuss matters and solve problems promptly.

I am also grateful to Prof. Dr. Hans-Peter Braun, Institute of Plant Genetics, for providing cordial supervision and a pleasant working environment in his lab as well as all time encouragement.

I would also take a chance to grately thank Prof. Dr. Andreas Pich, Institute of Toxicology, Medical High School Hannover, for his proper guidance, valuable suggestions and kindness.

I am also thankful to all our lab members, specifically Birgit Milde, for their cooperation. Dagmar Lewejohann in Institue of Plant Genetics and Karin Agternkamp in Institute of Toxicology will be remembered for their friendly assistance in my lab work. Hendrik Führs from Plant Nutrition, Till Lesker from Plant Virology and Marika Muetzelburg from Toxicology were also very helpful.

Finally, I can not miss to grately acknowledge my parents Dadhiram Dahal and Sarita Dahal for their contribution to make me what I am now and my lovely wife Sweta Khanal who is always with me with her support and love.

ABBREVIATIONS

1-, 2- or 3-D	One-, two- or three-dimensional
A	Angström
ACC	1-aminocyclopropane-1-carboxylic acid
ACN	Acetonitril
AFLP	Amplified fragment length polymorphism
ATP	Adenosine triphosphate
AUDPC	Area under disease progress curve
avr	Avirulence
BIP	(Luminal)-binding protein
BLAST	Basic local alignment search tool
CBB	Coomassie brilliant blue
CCR	Cinnamoyl CoA reductase
cfu	Colony forming units
CHAP	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHCA	α-cyano-4-hydroxycinnamic acid
cm	Centimeter
CNGC	Cyclic-nucleotide-gated channel
CS	Chalcone synthase
CWP	Cell wall proteins
DI	Disease incidence
DNA	De-oxyribonucleic acid

dpi	Days post inoculation
DS	Disease severity
DTT	Dithiothritol
EDTA	Ethylene-diamine-tetra-acetic acid
eIF	Eukaryotic translation initiation factor
EPS	Exopolysaccharide
ER	Endoplasmic reticulum
ESI	Electrospray ionization
EST	Expressed sequence tag
F3H	Flavanone-3-hydroxylase
FKK	Frucktokinase
FNR	Ferrodoxin-NADP-reductase
g	Gram; Gravity (in case of centrifuge)
G or GTP protein	Guanine nucleotide-binding protein
GGT	γ-glutamyl transferase/transpeptidase
GH	Glyoside hydrolase
GLP	Germin like protein
GPI	Glycosylphosphatidylinositol
GRP	Glycine rich protein
GT	Glycosyltransferases
h	Hour
HGA	Homogalacturonan
HPLC	High performance liquid chromatography

HR	Hypersensitive response
hsp	Heat shock protein
IAA	Indole acetic acid/Auxin
IEF	Isoelectric focussing
IPG	Immobilized PH gradient
LC	Liquid chromatography
LRR	Leucine-rich repeat
mA	Milliampere
MALDI	Matrix assisted laser desorption and ionization
MAPK	Mitogen activated protein kinase
mg	Milligram
min	Minute
MIPs	Major intrinsic proteins
mL	Milliliter
µL	Microliter
µm	Micrometer
MOWSE	Molecular weight search
Mr	Molecular weight
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSDB	MS protein sequence database
NAC	Nascent polypeptide-associated complex
NADP	Nicotinamide dinucleotide phosphate

NBS	Nucleotide-binding site
NDPK	Nucleoside diphosphate kinase
NGA	Nutrient glucose agar
NH	Nucleoside hydrolases
NL	Non-linear
nL	Nanoliter
nm	Nanometer
OEE	Oxygen evolving enhancer
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed cell death
PDI	Protein disulphide isomerase
PGIP	Polygalacturonase like protein
pI	Isoelectric point
PIPs	Plasma membrane intrinsic proteins
PMF	Peptide mass fingerprinting
PMSF	Phenyl-methyl-sulfonyl fluoride
ppm	Parts per million
PR protein	Pathogenesis related protein
psi	Pound per square inch
PTM	Post translational modification
QTLs	Quantitative trait loci
Q-TOF	Quadrupole-time of flight
R	Resistance

R. solanacearum	*Ralstonia solanacearum*
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
RIL	Recombinant inbred line
RLKs	Receptor protein kinases
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
rpm	Rotations per minute
RT	Room temperature
s	Second
SBP	Selenium binding protein
SDS	Sodium dodecyl-sulphate
SOD	Superoxide dismutase
TCA	Tricarboxylic acid
TEPs	Transposable element proteins
TFA	Trifluoroacetic acid
TPI	Triose phosphate isomerase
TTC	2, 3, 5-triphenyl tetrazolium chloride
V-ATPase	Vacuolar ATPase
v/v	Volume by volume
w/v	Weight by volume
XTH	Xyloglucan endotransglucosylase-hydrolase

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GENERAL INTRODUCTION

Bacterial wilt and its causative agent

Tomato (*Solanum lycopersicum*) is one of the most consumed vegetables next to potato, grown in different cropping systems all over the world, and therefore, has a high economic importance. Production of tomato has been hampered severely by numerous biotic and abiotic stresses, albeit, the increasing use of cultivating land. Bacterial wilt is a collective term used for diseases caused by at least 15 bacterial species, however, the wilt caused by *Ralstonia solanacearum* is the most devastating systemic vascular disease of crop plants worldwide (Denny 2006). The pathogen does not behave as a single bacterium with a uniform biology and was therefore referred to as a 'species complex' (Fegan and Prior 2005). *R. solanacearum* as a species complex invades over 200 plant species in more than 50 families in the tropics, subtropics, and warm temperate regions. The host plant affected includes dicotyledones and monocotyledones, annual plants to trees and shrubs, and, more rigorously the Solanaceae plants such as tomato, potato, eggplant, and tobacco (Denny 2006). The high economic importance of the disease can be estimated from the destruction of 75% of potato and even up to 100% of the tomato harvest in some areas, and losses, are attributed to the fast lethality of the disease, and the persistence, extensive host range and broad geographical distribution of the pathogen (Elphinstone 2005).

R. solanacearum is an aerobic, Gram-negative rod of 0.5-1.5 μm length and a polar flagellum. This bacterium belongs to the non-fluorescent rRNA homology group II of the β-subdivision of Proteobacteria on the basis of 16S rRNA sequence analysis (Oepp/Eppo 2004). For this bacterium which exhibits a great degree of phenotypic and genotypic diversity with strains differing in host range, geographical distribution, pathogenicity, epidemiological relationship, and physiological properties, different classification system exist (Denny 2006). However, the race system based on the selectiveness in host range of the bacterium and the biovar system based on the ability to utilize and/or oxidize several hexose alcohols and disaccharides, categorize the strains into five races and six biovars, respectively, and are still often used (Denny 2006). The only agreement between the two systems is that biovar 2

strains belong to race 3. Further two classification schemes, one based on the restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and DNA sequence analysis of 16S rRNA, egl and pglA, and the other on genetic properties of the bacterial strains, grouped the pathogen into two major clusters (American and Asian) and other subdivisons including African strains due to its geographical origin and four phylotypes respectively (Villa et al. 2005, Prior and Fegan 2005). Phylotype I, II, III and IV correspond to cluster I (Asian), II (American), African and Indonesian strains respectively.

R. solanacearum is a soilborn saprophytic organism, able to survive extended periods in surface water and soil without the need of host plants. Even though the soil contains several toxic compounds and presents an oligotrophic environment (Williams 1985), the bacterium copes with such adverse environments, probably due to its association with asymptomatic hosts and weeds (Hayward 1994), its conversion to the 'viable but not culturable' dormant-like state (Grey and Steck 2001), or its shift between saprophytic type and the other virulent/wild type (Denny 2006). *R. solanacearum* spreads usually with surface water and within the soil system and enters the host plant through roots, and wounds produced mechanically by various agents such as nematodes, insects, and agricultural practices and/or naturally due to lateral root emergence (Denny 2006). The dissemination is further assisted by the flagella mediated swimming motility of the bacterium (Tans-Kersten et al. 2004). After entering the host plants, they colonize the intercellular spaces of the root cortex, and followed by the vascular parenchyma. They invade xylem vessels by disrupting the cell walls, and circulate rapidly through the vascular system of the plant (Grey and Steck 2001). In xylem vessels, the numbers as high as 10^{10} cells/cm was reached in tomato stems in later stages of infection (Dannon and Wydra 2004). When the pathogen attains such a high cell concentration, virulence genes are expressed and cells become non-motile and secrete acidic, high molecular mass (>10^6 Da) exopolysaccharides (EPS I) and pectin-degrading enzymes, leading to blockage of the vascular system (Clough et al. 1997, Saile et al. 1997). Such a vascular dysfunction is the major cause of typical wilting that appears within 4-6 days post inoculation (dpi) in susceptible plants, while they are still green, and mostly leads to fast death of the plants (Denny et al. 1990). The bacterial cells are then released into the soil from

the infected dead plant, build up an inoculum in the soil, and restart the infection cycle in other available host plants.

In susceptible tomato plants, the pathogen initially causes the wilting of youngest leaves within 4-5 dpi, followed by extension of wilt to other plant parts, and eventually death of the plant within 10-12 dpi under favorable conditions. Onset of wilt was correlated with bacterial density exceeding 4 x 10^7 cfu/g tissues at mid-stem level probably causing the brown discoloration in the vascular tissues of the stem which upon horizontal cut shows whitish or yellowish bacterial ooze (Denny 2006). Higher temperature (24-35°C), soil moisture, and periods of wet weather or rainy seasons are considered to accelerate the disease severity which however would be slowed down under less favorable conditions resulting in the adventitious roots formation at lower as well as mid-stem level, and stunting of the plants may occur.

Disease management and resistance mechanism

Bacterial wilt is among the most difficult diseases to control due to the extensive host range, broad geographical occurrence, variability, and saprophytic nature of the pathogen among others (Denny 2006). Several single control strategies including chemical, cultural and biological have been employed, but none provided complete and sustainable protection in areas where strains of wide host ranges were endemic (Saddler 2005). Therefore, the integration of several different disease management measures such as rotation with tolerant crops, intercropping, use of phytosanitary practices such as eradicating asymptomatic weeds and hosts, soil amendments, controlling disease promoting nematodes and insects are commonly applied as conventional measure of disease control (Saddler 2005, Denny 2006). Among individual control measures, the use of resistant cultivars has proved the most promising, economical and environment friendly method (Boshou 2005). Unfortunately, host plant resistance was generally confined to geographical locations and its stability and/or durability were frequently broken due to high genetic diversity of the strain as well as variable, local environmental conditions (Carmeille et al. 2006). Moreover, the quantitative trait loci (QTLs) determining the wilt resistance in tomato are also often linked to undesirable traits hampering the commercial production of resistant tomatoes with good agricultural traits

(Wang et al. 1998). Therefore, the use of disease resistant cultivars in combination with other control measures in the framework of an integrated disease management system seems effective to combat the bacterial wilt disease.

Considering the characteristics of the pathogen and the problems of its control, it becomes essential to understand the resistance mechanism at the biochemical and molecular level to develop cultivars of durable resistance with desirable agronomic traits. Two types of resistance are generally described in plants. The non-host resistance is the predominant form which is shown by all members of plant species against a specific pathogen, and is durable against the majority of potential microbes. A second form of resistance is the host resistance exhibited by a specific cultivar or accession which is often governed by single resistance (*R*) gene (Jones and Dangl 2006). Invading phytopathogens are obstructed first by the constitutive physical barriers provided from the cytoskeleton of the plant termed "passive or preformed" resistance. In addition to these physical barriers, there are two overlapping yet different forms of active plant defense. The first is known as the basal plant defense which includes pathogen associated molecular pattern-triggered immunity and is independent of *R* genes. They are activated around the sites of infection in susceptible plants limiting the disease severity, but are relatively weaker to prevent the disease compared to *R* gene mediated defense (Jones and Dangl 2006). The second is the inducible plant defense mechanism that involves specific recognition of the invading pathogen by plant resistance (*R*) genes called “gene-for-gene” interaction. Upon recognition of the invading pathogen, the products of *R* genes directly or indirectly interact with the specific elicitors produced by the avirulence (*avr*) genes of pathogens. The initiated incompatible interaction leads to resistant plants while the compatible interaction results in diseased plant. Although all plants possess active resistance mechanisms against pathogen attack, these mechanisms do not succeed in a compatible interaction due to slow and/or inefficient response of the plant to the pathogen, or the avoidance of triggering of defense responses and the suppression of the resistance reactions by the pathogen (Ton and Mauch-Mani 2004). The disease resistance gene *Pto* in tomato and the *avrPto* gene in *Pseudomonas syringae* pv. tomato is one example of a classical "gene-for-gene" interaction (Ronald et al. 1992) and was required for the activation

of disease resistance (Scofield et al. 1996). This kind of active resistance responses often involve reprogramming of the cellular metabolism leading to rapid necrosis of the localized cells called the hypersensitive response (HR), synthesis of defense related proteins such as pathogenesis related (PR) proteins, secondary metabolites and reinforcement of cell wall (Jones and Dangl 2006). A more comprehensive and global monitoring of the physiological and molecular phenomena mediating the pathogen-host plant interactions is pivotal in controlling plant disease, where the ‘‘omics’’ experimental approaches, particularly proteomics, are expected to significantly contribute to an increased understanding of plants reactions to pathogen attack.

Proteomics

Proteomics study the post genomic events which particularly consist of analyzing the proteome, i.e. protein complement expressed by the whole genome, in a cell, tissue, or organism under defined conditions. In fact, the proteome of a particular cell represents a subset of all gene products. Genes are considered as the construction code of the cellular system and are static while proteins are the effector molecules that realize and regulate them and are dynamic in nature. The proteome therefore offers a more accurate representation of the cellular state compared to genes and transcriptomes. Proteomes are physicochemically highly heterogeneous, structurally complex and are modified both spatially as well as temporally and during their biochemical interaction with the biotic and abiotic environments. Proteomics is used as an ideal tool for understanding how complex biological processes occur at a molecular level, how they differ in various cell types, and how they are altered during interactions with microbes. Benefited from the increasing genomic sequences, expressed sequence tag (ESTs) databases, and the advancement of the mass spectrometry for the protein analysis, comparative proteomics has become the common approach to study the complex molecular phenomena mediating the resistance reactions of the plant against pathogen invasion. Although, the gene microarrays provide the snapshot of all genes at one time point, the level of specific mRNAs does not necessarily predict the level of corresponding proteins. Also, the substantial regulation of cellular events occurring at the protein level with no apparent change in the transcriptome increases the importance of proteomics in deciphering

the molecular events undergoing in cells (Gygi et al. 1999). One of the common workflows of gel based comparative proteome analysis principally consists of the extraction of desired proteomes, separation by gel electrophoresis, comparison of their differential expression with respect to pathogen attack, and the identification of the desired protein with mass spectrometry (MS).

Gel electrophoresis and mass spectrometry

In proteomics, the protein separation should not only simplify the separation of the complex protein mixture into individual/small groups of proteins but also allow comparing the differences in the protein abundance level due to the disease state of the plant. Two-dimensional (2-D) gel electrophoresis serves both purposes and presents a most common method to resolve the complex mixture of protein in one gel providing a snap shot of the particular (sub-) proteome. It involves the separation of the protein complex according to the isoelectric points (*pI*) in the first dimension followed by the individual molecular mass (*MM*) in the second dimension. This is correspondingly achieved by isoelectric focussing (IEF) which focuses proteins until zero net charges are achieved, and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) that separates proteins by their molecular size. The 2-D separation of the protein complex encounters some limitations such as the small dynamic resolution of the resolved proteome, dominance of abundant and soluble proteins, and scarcity of the most basic and low abundant proteins (e.g. transcription factors, protein kinases etc) (Lopez et al. 2007). Other protein separation techniques such as various 1-D SDS-PAGE and chromatography provide good options for the inclusion of such proteins in the analysis and can be coupled with MS for the analysis of proteins. MS principally consists of an ion source to produce ions from the protein samples in the gaseous phase, a mass analyzer which separates ionized analytes based on their mass-to-charge ratio (m/z), and a detector that registers the number of resolved ions at each m/z value. Due to the introduction of soft ionization methods such as matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), MS has been widely used in the examination of large biomolecules such as proteins. These ion sources can be connected to one or several mass analyzers such as time of flight (TOF), ion trap as well as quadrupole (Q) depending

upon the sensitivity, resolution and mass accuracy of the instruments required for the protein analysis. Gel separated proteins are generally identified by two different but complementary methods: peptide-mass fingerprinting (PMF) by MALDI-TOF when the species' whole genome sequences are available, and by peptide sequencing by ESI tandem MS for the partially sequenced genome. The expansion of genome, EST and protein sequence databases such as MSDB, SwissProt, NCBInr and browser based database search tools like MASCOT and Sequest, which can match MS data with specific protein sequences in databases, are the integral part of MS based proteomics in order to derive the identity of the MS-analyzed proteins. PMF is based on the exact measure of the masses of unique proteolytic peptides generated by digesting the desired protein and subsequent matching of the experimental mass with corresponding theoretical peptide masses obtained from protein or nucleotide sequence databases. Therefore, such MS require only one mass analyzer, and the successful protein identification depends on the quality of the MS data obtained, accuracy of the database and the power of the search algorithms and software. The ESI tandem mass spectrometer (MS/MS), on the other hand, requires two mass analyzers in tandem where the second analyzer creates systematic fragmentation of the peptides selected by the first analyzer in order to deduce the peptide sequences. The algorithms are then used to match these identified peptide sequences with the peptides sequences present in the databases or, more accurately, to correlate the experimental MS/MS spectral data with theoretical MS/MS spectra to reveal the identity of the proteins.

Research framework

The management of bacterial wilt disease of tomato can be substantially improved by the use of the resistant cultivars. However, the aim is to achieve a durable resistance across the major tomato cultivating environments. Cultivars with stable resistance can then be grown within the framework of an integrated disease approach in order to obtain a sustainable disease management. Understanding of the resistance reactions at the molecular level should offer directives to develop long lasting disease resistant cultivars; but, only limited information is available on the molecular interactions in the tomato-*R. solanacearum* system. The ever expanding genetic and molecular tools as well as databases of the tomato and the

availability of the complete genomic sequences of some strains of the bacterium offer a promising model for investigations. The major purpose of this study is therefore to improve the resistance of the tomato cultivars by characterizing the molecular components involved in susceptibility as well as resistance of the plants against bacterial wilt, and effectively utilizing the genetic resources present within the species to create stable resistance against this disease. The study involves the understanding of biochemical and molecular characteristics of resistant and susceptible tomato genotypes that are activated in response to invasion by *R. solanacearum*. A comparative proteomics approach is used for this purpose to monitor the changes in the protein profiles that are directly influenced by the biochemical cellular pathways activated during host pathogen interactions and hence represents the more direct approach. Former studies located the presence of bacterial wilt resistance in the mid-stem of tomato revealing it as an important site for the proteome analysis of the proposed host pathogen interaction (Vasse et al. 2002, Wydra and Beri 2007, Dahal et al. 2009). Similarly, the 5 dpi correspond to the time needed by the bacterium which is applied to the soil, to reach and invade the root system and multiply heavily in the stem. The higher expression of mRNA of defence related genes observed in time-course analysis at three days after root inoculation of the pathogen and the begining of wilting symptoms at 5 dpi in susceptible plants led us to analyze the proteome at 5 dpi (H. Ghareeb 2007 master thesis, Dahal et al. 2009).

Hence, in order to gain a deeper insight into the reactions against the pathogen in the proteome of the plant, the research work was divided into 3 major sections that comprise (1) the analysis of plant proteome of the whole mid stem in a first phase, followed by the examination of the plant subcellular proteome, (2) the cell wall of the stem and (3) the xylem sap, aiming to increase of the sensitivity of the analysis. The following three chapters present the comparative analysis of the tomato proteome from healthy susceptible and resistant genotypes and the regulation of the proteins in response to pathogen attack. Both the whole stem and its cell wall proteome were separated and displayed by two-dimensional isoelectric focussing/sodium dodecyl-sulphate polyacrylamide gel electrophoresis (2-D IEF/SDS-PAGE), compared for their differential abundance in genotypes and treatments and finally analysed the desired spots with mass spectrometry to identify the proteins. In case of the

xylem sap, the whole protein profiling was performed from both susceptible and resistant plants in order to receive an overview of the proteins present in the sap and to achieve a comparative analysis of the two genotypes based on the differential abundance of the proteins in either of the genotypes. The screening of the xylem proteome also aimed to provide a platform for future comaparative proteome analyis of the sap proteins that are regulated in response to pathogen inoculation.

CHAPTER 1: Pathogenesis and stress related, as well as metabolic proteins are regulated in tomato stems infected with *Ralstonia solanacearum*.

Adapted from the publication, **Plant Physiology and Biochemistry 47:838-846.**

Abstract

A comparative proteome analysis was initiated to systematically investigate the physiological response of tomato to infection with *R. solanacearum,* causal agent of bacterial wilt. Plants of the susceptible tomato recombinant inbred line NHG3 and the resistant NHG13 were either infected or not infected with *R. solanacearum* and subsequently used for proteome analysis. 2-D IEF/SDS-PAGE allowed the separation of about 650–690 protein spots per analysis. Twelve proteins were of differential abundance in susceptible plants in response to bacterial infection, while no differences were observed in the resistant genotype. LC-MS/MS analysis of these spots revealed 12 proteins, six of which were annotated as plant and six as bacterial proteins. Among the plant proteins, two represent PR proteins, one stress response protein, one enzyme of carbohydrate and energy metabolism, and one hypothetical protein. A constitutive difference between resistant and susceptible lines was not found.

Keywords: 2-D gel electrophoresis, bacterial wilt, LC-MS/MS, PR protein, *Ralstonia solanacearum*, *Solanum lycopersicum*

1. 1 Introduction

Bacterial wilt caused by *R. solanacearum* is the most devastating, systemic vascular wilt disease of crop plants (Smith 1896, Denny 2006). *R. solanacearum* as a species complex has a host range of more than 200 plant species representing over 50 botanical families (Denny 2006). Among these, solanaceous plants including tomato are the most affected species which was damaged up to 75-100% in the lowland and highland tropics and subtropics (Smith 1896, Ram-Kishun and Kishun 1987).

Figure 1. Tomato infected with *R. solanacearum* showing wilt symptoms.

R. solanacearum is an aerobic, Gram-negative rod with a high degree of phenotypic and genotypic diversity (Denny 2006). The soilborne bacterium potentially requires only small wounds in the roots such as occur by lateral root emergence to establish a systemic infection (Vasse et al. 1995). Bacteria start multiplying in the intercellular spaces of the root cortex at the early phase of infection when the pathogen is still motile, and circulate throughout the vascular system of the plant (Vasse et al. 1995). Cell numbers as high as 10^{10} cells/cm of stem are reached in xylem vessels of tomato (Dannon and Wydra 2004), leading to blockage of the vascular system and thereby alteration of water fluxes (Saile et al. 1997). Such a

vascular dysfunction is the major cause of typical green-wilting and subsequent plant death (Denny et al. 1990).

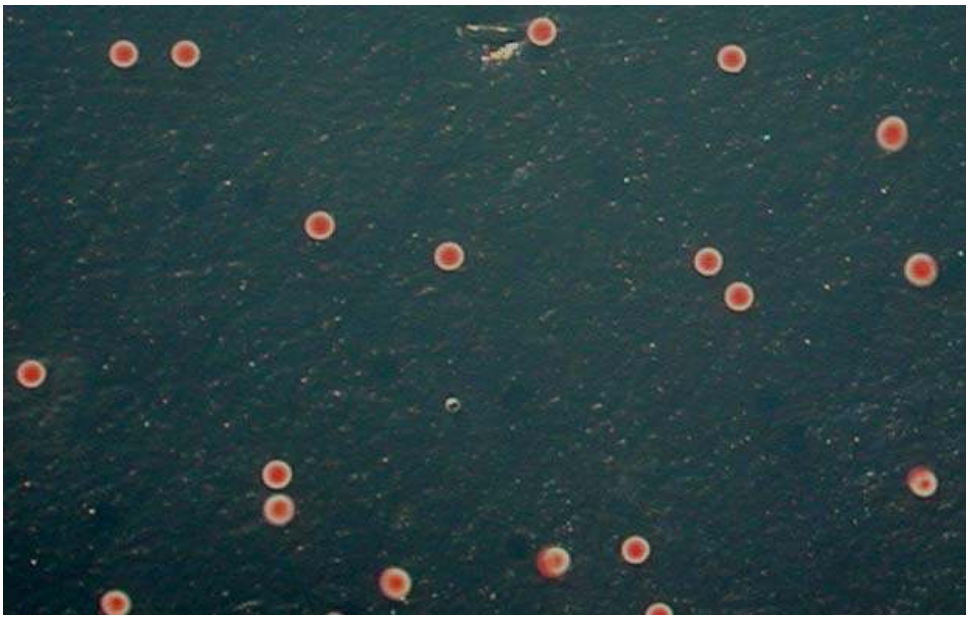

Figure 2. *R. solanacearum* seen on TTC medium after 48 h of incubation at 30°C. Colonies appear as large, elevated and fluidal mass with pink/red centers.

Bacterial wilt resistance, a polygenic trait in tomato, with QTLs often linked to undesirable characteristics, was generally found to be specific to geographical sites, and frequently broken due to high genotype x environment interactions (Wang et al. 1998). Therefore, understanding the resistance mechanisms is essential in developing a cultivar with stable resistance, to effectively control the disease.

The mechanisms of downstream signalling and induced responses were studied both in pathogens and plants in various host-pathogen interactions. When a plant comes into contact with a pathogen, close communication occurs between the two organisms (McDowell and Woffenden 2003). If the initial resistance provided by preformed plant barriers is passed successfully, the defence responses are activated by an interacting set of both exogenous and endogenous signalling molecules. The induced defence responses include localized cell death, production of antimicrobial secondary metabolites, further reinforcement of the cell

walls and the synthesis and accumulation of PR proteins (Walters et al. 2005). These complex series of cellular responses may lead to enhanced disease resistance against a broad spectrum of phytopathogens when expressed in a synchronized manner (McDowell and Woffenden 2003).

However, only limited studies exist on the biochemical and molecular background of the *R. solanacearum*-tomato interaction. The resistance of tomato genotypes against bacterial wilt did not result from a limitation of bacterial penetration into the roots, but from the ability of the plant to restrict the pathogen spread in the stem (Grimault and Prior 1993, Nakaho et al. 2004). The bacterial population was reduced significantly in mid-stems compared to the taproot and collar region after root inoculation (Vasse et al. 2002). Similarly, we also found comparably high numbers of bacteria in roots of both susceptible and resistant genotypes, while those in stems were significantly reduced in the resistant plants (Dannon and Wydra 2004, Diogo and Wydra 2007). The capacity of the plant to restrict pathogen spread in the stem occurred either by inhibiting the growth of the pathogen or limiting the effects of bacterial virulence factors (Prior et al. 1996, Dannon and Wydra 2004). Therefore, tomato stem was considered an important site for further analysis of plant-pathogen interaction. In our former studies, the roles of both constitutive resistance mechanisms and pathogen-induced changes on plant cell wall level were described, where modifications in the composition and structure of the galacturonan components of the pectic cell wall polysaccharides and the amount of arabinogalactan protein were related to the resistance of tomato genotypes (Wydra et al. 2005, Wydra and Beri 2006 and 2007, Diogo and Wydra 2007). Nevertheless, the exact picture of the multiple resistance reactions of the plant acting singly or in combination is not well understood.

The proteomic approach should reveal whether changes on protein level play a role in the pathogen-plant interactions, since it represents more directly the cellular status of the cell (Lopez 2007). The tomato-*R. solanacearum* system is a suitable model for investigating the molecular basis of plant disease reactions towards *R. solanacearum*, since extended genetic and molecular tools for both tomato and the pathogen are available (Salanoubat et al. 2002, Pedley and Martin 2003). For tomato, a dense molecular marker linkage map

(www.sgn.cornell.edu) and for the relatively small genome (950 Mb) extensive databases of expressed sequence tags (www.tigr.org) exist. When combined with information from other *Solanaceae* and related species, the databases provide useful information for the identification of proteins in tomato. Similarly, the availability of the complete genomic sequence of *R. solanacearum* strain GMI1000 (Salanoubat et al. 2002) and the in-depth study of the type III secretion system and related pathogenicity and effector proteins allowed the identification of *in planta* expressed proteins (Alfano and Collmer 2004).

Since the reaction to *R. solanacearum* infection on tomato stem-proteome level has not been studied, susceptible and resistant tomato recombinant inbred lines, derived from the cross between the resistant Hawaii7996 (*Solanum lycopersicum*) and the susceptible Wva700 (*Solanum pimpinellifolium*) parental lines, were chosen for identification of differential protein expression.

1.2 Materials and methods

1.2.1 Plant material and bacterial strain

The tomato recombinant inbred lines NHG3 and NHG13, susceptible and resistant to bacterial wilt, respectively, were received from the Genetic Resources and Seeds Unit of the Asian Vegetable Research and Development Centre (AVRDC, Taiwan). The recombinant inbred lines (RILs) were developed by eight generations of single seed descents from the interspecific cross between two parental tomato lines: the highly resistant line Hawaii7996 and the highly susceptible line WVa700.

The highly virulent *R. solanacearum* strain ToUdk2 (race 1, biovar 3) obtained from Thailand (N. Thaveechai, Kasetsart University, Bangkok) was used for inoculation of the plants. A suspension of a fresh re-isolate of the strain was streaked on nutrient growth agar (NGA) medium (0.3% beef extract, 0.5% Bacto peptone, 0.25% D-glucose, and 1.5% agar) and incubated at 30°C for 48 h. Bacterial colonies were harvested with sterile distilled water and the inoculum was prepared by adjusting the concentration of bacterial cells to an optical density of 0.06 at 620 nm wavelength (Spectrotonic 20, Bausch and Lomb) corresponding to about 7.8×10^7 colony-forming units per millilitre (cfu/mL).

1.2.2 Plant growth conditions and inoculation

Tomato seeds were sown in the greenhouse [20°C, 14 h photoperiod per day, 30K lux and 70% relative humidity (RH)], transplanted after 4 weeks to individual pots with approximately 330 g of soil (Fruhstorfer Erde, type P: 150 mg/L N, 150 mg/L P_2O_5, and 250 mg/L K_2O) and transferred to a climate chamber (30/28°C day/night temperature, 14 h photoperiod, 30 K lux, and 85% RH). Soon after transplanting, plants were inoculated by pouring 25 mL of bacterial suspension per pot around the base of the plant to obtain a final inoculum concentration of approximately 10^7 cfu/g of soil, followed by watering the soil up to soil field capacity.

1.2.3 Bacterial quantification

The bacterial multiplication was determined at 5 dpi in the same tomato plants that were used for proteome analysis. Approximately 0.5-0.7 g of the lower stem part was surface-disinfected with 70% ethanol for 15 s, rinsed and macerated in 2 mL of sterile water. After 20 min, the macerate was filtered through cotton to remove plant debris and pelleted by centrifugation (7000 x g, 10 °C for 10 min). The pellet was re-suspended in 1 mL sterile water and serially diluted 10 fold at least four times. Then 100 µL of each dilution were plated in two replicates on triphenyl tetrazolium chloride (TTC) medium: 20 g Bacto peptone, 5 g glucose, 1 g casamino acids, 15 g Bacto agar and 1 L H_2O; after autoclaving, 10 mL of filter-sterilized 0.5% (w/v) 2, 3, 5-TTC (SERVA, Germany) solution as an redox indicator was mixed with sterile medium before pouring into Petri plates. Bacterial colonies after 48 h of incubation at 30°C appeared as large, elevated, fluidal colonies with red centers due to consumption of TTC dye by the pathogen and were counted to calculate bacterial population as cfu per gram of fresh weight (cfu/g).

1.2.4 Monitoring and evaluation of disease symptoms

The typical symptoms of bacterial wilt were monitored daily in six disease severity scores from 0–5, where 0 = no symptoms, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three

leaves wilted, 4 = all leaves wilted without tip, and 5 = whole plant wilted, plant death. The symptoms were evaluated for 4 weeks from the day of first symptom appearance.

The wilt incidence (WI) was calculated as the percentage of dead plants (disease score 5) to the total number of plants in the treatment at the evaluation date. Additionally, disease severity (DS) was calculated as the mean of disease scores at the evaluation date. The area under disease progress curve (AUDPC) for each plant in each treatment and experiment was calculated on the basis of disease severity and of wilt incidence using the trapezoid integration of the disease progress curve over time using the following equation (Jeger and Viljanen-Rollinson 2001).

$$AUDPC = \sum [(x_i + x_{i-1})/2](t_i - t_{i-1})$$

Where, x_i and x_{i-1} are disease severity or wilt incidence at time t_i - t_{i-1}, respectively, and t_i and t_{i-1} are consecutive evaluation dates, with t_i - t_{i-1} equal to 1. The total AUDPC represents the sum of AUDPC for all plants in each treatment.

1.2.5 Stem proteome analysis

The proteome was analysed in both healthy and infected stems of genotypes NHG3 and NHG13 at 5 dpi. The tomato mid-stem, approximately 8-10 cm above the root level was used for proteome analysis, with more than three individual plants per genotype and treatment to obtain at least three reproducible results. About 1 g of stem was cut, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis.

1.2.5.1 Protein extraction and sample preparation

Total protein extraction from the tomato stem was carried out according to the protocol of Mihr and Braun (2003). The plant cells were disrupted by pulverizing the frozen stem to fine powder in a swing mill after chilling the required tools with liquid nitrogen. Approximately 0.5 g of tissue powder was well mixed with 750 µL of extraction buffer pH 8.0 (700 mM saccharose, 500 mM Tris, 50 mM EDTA, 100 mM KCl, 2% v/v β-mercapto-ethanol, and 2 mM PMSF). After incubating for 10 min on ice, an equal volume of water-saturated phenol (Amresco Biotech Chemicals, Germany) was added, vortexed and shaken at 300 rpm, at

room temperature (RT) for 30 min (Mixer 5432, Eppendorf). The mixture was centrifuged at 1100 x g, 4°C for 10 min and the upper phenolic phase containing solubilized proteins was taken. The same centrifugation step was repeated after mixing the recovered phenolic phase with an equal volume of extraction buffer. The proteins extracted in the resulting phenolic phase were precipitated at -20°C by adding 100 mM ammonium acetate in methanol with five times the volume of the recovered phenol phase, for at least 4 h. The protein pellet was obtained by centrifuging (17000 x g, 4°C for 3 min) and washed by resuspending the pellet in 1 mL of 100 mM ammonium acetate in methanol before re-centrifugation. The pellet was rinsed once more with 80% (v/v) ice-cold acetone as before and air-dried at RT for 5-10 min.

An approximately 0.5 mg protein pellet was solubilized in 350 µL of "rehydration buffer" (8M urea, 2% w/v CHAPS, 0.5% v/v carrier ampholyte mixture (IPG buffer 3-11 non-linear, GE Healthcare, Germany), 30 mM dithiothreitol, DTT, and 2-4 mg Bromophenol Blue)). The suspension was well vortexed and centrifuged (17000 x g, 4°C for 5 min). The supernatant containing soluble protein mixtures was flash frozen in liquid nitrogen before isoelectric focusing (IEF).

1.2.5.2 Two-dimensional gel electrophoresis

The complex mixtures of protein were separated in one direction by their charges (IEF) and in the perpendicular direction by their relative molecular masses (SDS-PAGE) using the 2-D gel electrophoresis approach.

IEF of protein mixtures was carried out using 18 cm immobiline dry gel strips (IPG strips, pH 3-11 non-linear (NL), GE Healthcare, Munich, Germany). In-gel rehydration of the dry gel stripes was combined with loading of 0.5 mg protein resolved in "rehydration buffer". IEF was carried out for 24 h using the IPGphor system (GE Healthcare, Germany) according to Werhahn and Braun (2002) as follows: (1) rehydration for 12 h at 30 V (step and hold); (2) initial focussing for 1 h at 500 V (step and hold); (3) further focussing for 1 h at 1000 (gradient), 4 h at 8000 V (gradient), and 6 h at 8000 V (step and hold); T 67610Vh.

Prior to SDS-PAGE, gel stripes of the IEF dimension were incubated for 15 min with "equilibration solution I" (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v)

SDS, 1% (w/v) DTT and 2-4 mg Bromophenol Blue) to denature proteins as well as to reduce their thiol groups and for 15 min with "equilibration solution II" (same as equilibration solution I except that DTT was substituted by 2.5% (w/v) iodoacetamide) to alkylate free thiol groups of the proteins.

Second-dimension electrophoresis was performed on a vertical SDS gel according to Schägger and Von Jagow (1987) using the Protean II electrophoresis unit (BioRad, Hercules, USA). Equilibrated IPG stripes were placed horizontally onto the second gel dimension and fixed in place with 0.5% agarose solution tricine (0.5% agarose, and 2-4 mg Bromophenol Blue in 100 mL tricine gel buffer pH 8.45 (3M Tris, and 0.3% SDS)) at a temperature below 60°C. The gel was run at constant current (35 mA per mm gel thickness) for 18-20 h.

1.2.5.3 Protein staining, gel scanning and image analysis

After completion of SDS-PAGE, gels were fixed by incubation with "fixing solution" (100 mL/two gels; 40% (v/v) methanol, and 10% (v/v) acetic acid) for 2 h. Proteins were visualized by staining overnight with colloidal Coomassie staining (0.1% w/v CBB-G250, 10% w/v ammonium sulphate, 2% ortho-phosphoric acid in 20% methanol) as described by Neuhoff et al. (1985, 1990). To remove background staining, gels were washed with bidest water and finally scanned using a UMAX Power Look III scanner (UMAX Technologies, Fremont, USA). Protein spots were compared for differential abundance between genotypes (NHG3 and NHG13) and treatments (pathogen infected and healthy plants) by visual inspection.

1.2.5.4 Mass spectrometric analysis and data interpretation

Protein in-gel digestion, peptide extraction, and mass spectrometry analysis were performed as described by Führs et al. (2008).

Briefly, each SDS-PAGE gel spot was dried under vacuum. In-gel digestion was performed with an automated protein digestion system, MassPREP Station (Micromass, Manchester, UK). The gel slices were washed three times. The cysteine residues were reduced and alkylated. After dehydration, the proteins were cleaved inside the gel with 40 µL of 12.5 ng/mL modified porcine trypsin (Promega, Madison, USA) in 25 mM NH_4HCO_3 at RT

for 14 h. After extraction, the resulting tryptic peptides were analysed by Nano-liquid chromatography (LC) MS/MS on a capillary LC (CapLC) system (Micromass) coupled to a hybrid quadrupole orthogonal acceleration TOF tandem mass spectrometer (Q-TOF II, Micromass). Protein identification was performed by classical protein database searches performed on a local Mascot (Matrix Science, London, UK) server. To be accepted for the identification, an error of less than 100 ppm on the parent ion mass was tolerated and the sequences of the peptides were manually checked. One missed cleavage per peptide was allowed and some modifications were taken into account: carbamidomethylation for cysteine and oxidation for methionine. In addition, the searches were performed without constraining proteins *Mr* and *pI*, and without any taxonomic specifications. These searches did not always lead to a positive identification since the tomato genome has not yet been sequenced. In such cases, the use of a *de novo* sequencing approach was necessary for a successful identification. For this purpose, the interpretation of the MS/MS spectra was performed with the PepSeq tool from the MassLynx 4 (Micromass) as well as the PEAKS studio softwares (Bioinformatics Solutions, Waterloo, Canada v.3). The resulting peptide sequences were submitted to the BLAST program provided at the EMBL site (http://dove.embl-heidelberg.de/Blast2/msblast.html) in order to identify them by homology with proteins present in the databases as described by Führs et al. (2008).

1.3 Results

1.3.1 Symptom development and bacterial populations in stems

Plants of the susceptible genotype NHG3 started wilting at 4-5 dpi, and progressed to plant death within 11 days. The mean WI and DS were calculated from three biological replications (Fig. 3). The AUDPC of WI and DS were 316.62±10.61 SE and 22.56±1.64 SE, respectively, indicating a highly susceptible reaction. However, no symptoms were observed in the resistant genotype NHG13 until 30 dpi. The bacterial populations in the stems used for proteome analysis were 2.58 $x10^9$, 3.06 $x10^9$, and 2.55 $x10^9$ cfu/g fresh weight of stem in genotype NHG3 compared to 2.99 $x10^5$, 4.91 $x10^4$, and 2.49 $x10^5$ cfu/g fresh weight of stem in genotype NHG13 at 5 dpi.

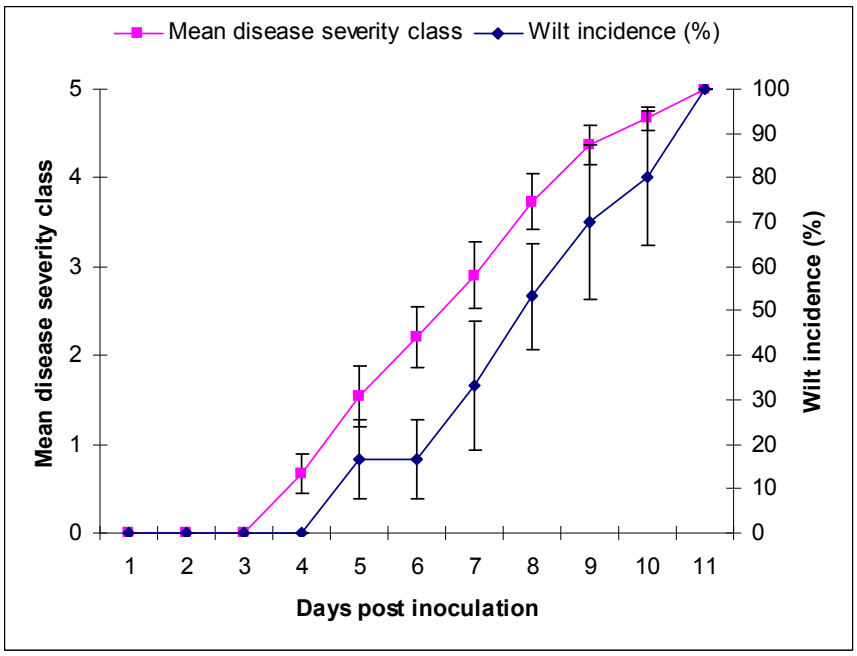

Figure 3. Development of wilt incidence (WI) and disease severity (DS) of tomato genotype NHG3 inoculated with *R. solanacearum* strain ToUdk2.

The mean WI and DS with corresponding standard errors were calculated from three biological replications of NHG3 plants. Line NHG13 did not show symptoms.

1.3.2 Analysis of the stem proteome

The total soluble protein extract of the mid-stem from an individual plant of each genotype, NHG3 and NHG13, inoculated and non-inoculated with *R. solanacearum* at 5 dpi, was resolved on 2-D gels. The 2-D gels were prepared more than three times from each genotype and treatment so that each protein spot difference was identified on at least on three individual gels. Approximately 650-690 protein spots, separated in the molecular mass range of 10-100 kDa and a pH range of 3-11, were visible in all replicate gels. Some further spots representing proteins with extreme *pI* or size were not clearly resolved. One representative 2-D gel is shown (Fig. 4), with proteins of differential abundance in genotype NHG3 after bacterial challenge being circled and numbered in two gel regions.

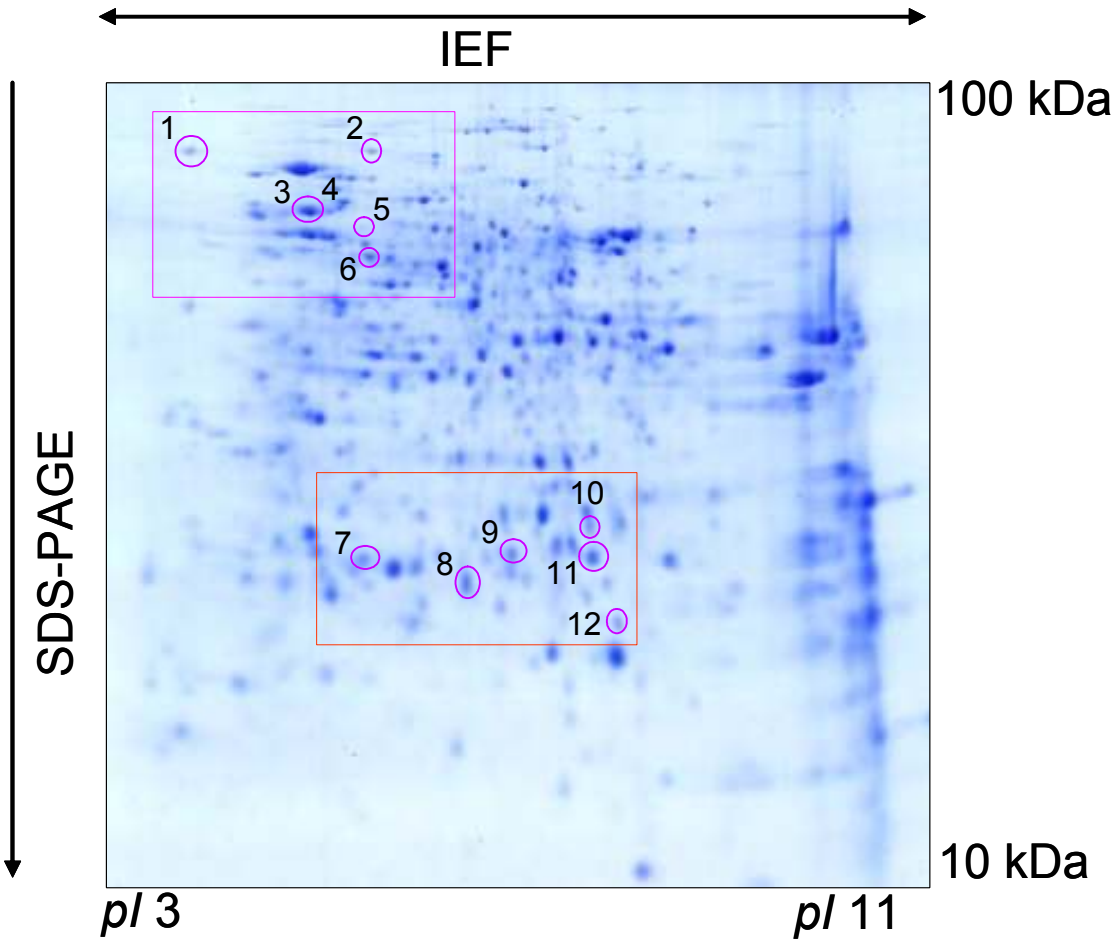

Figure 4. Overview of 2-D IEF/SDS-PAGE analyses of mid-stem proteomes isolated from tomato.

Protein separations were based on IEF using non-linear gel stripes in the range of pH 3 to 11 (horizontal separation) and on SDS-PAGE in the size range between 100 and 10 kDa (vertical separation). Protein spots differing in abundance in NHG3 genotype before and after pathogen inoculation were circled and numbered (for protein designation see Table 1). The two boxes indicate regions shown as "Zoom-in" in Fig. 5.

1.3.3 Characterization of tomato proteins induced after inoculation with *R. solanacearum*

Analysis of the tomato stem proteome of genotype NHG3 revealed 12 protein spots of changed abundance in response to *R. solanacearum* inoculation (Fig. 5). Among them, 10 proteins were newly induced after inoculation (spot numbers 1, 2, 3, 4, 7, 8, 9, 10, 11 and 12), one protein had higher abundance (spot 6), and another was of lower abundance (spot 5).

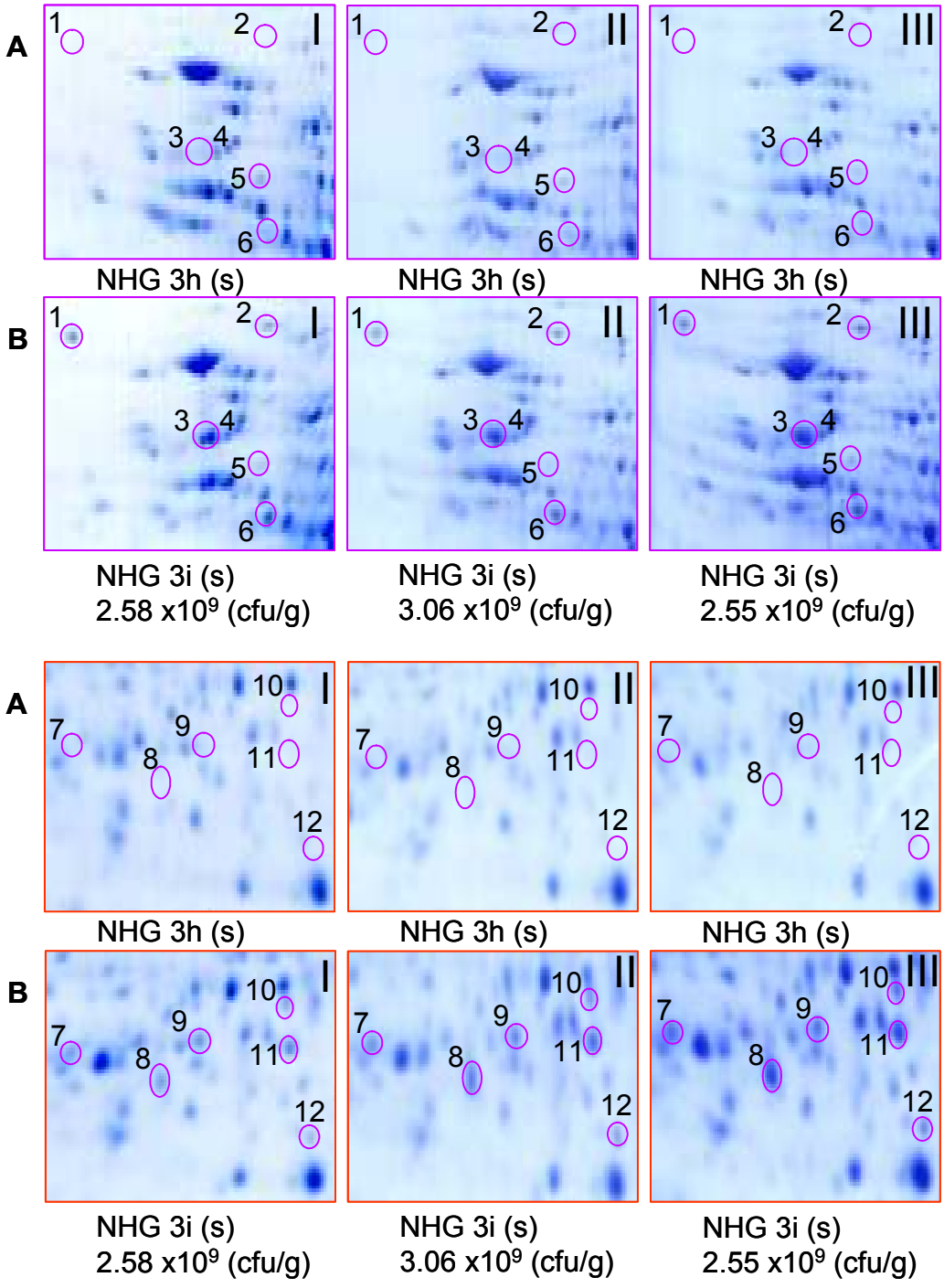

Figure 5. "Zoom-in" of the two regions selected in Fig. 4 showing the differential regulation of 12 protein spots in response to *R. solanacearum* inoculation in three biologically replicated samples (I, II and III).

Gel regions shown in "A" row are from the susceptible healthy genotype (NHG 3). Gel regions shown in "B" rows are susceptible infected genotype (NHG3). Proteins of different abundances are circled and numbered consecutively in correspondence with Fig. 4. Protein identity of all 12 gel spots are given in Table 1. The degree of infection in the inoculated plant samples was calculated as colony forming units per gram of stem (cfu/g).

The analysis of the 12 gel spots by nano-LC MS/MS revealed the identity of proteins (Table 1), where four spots included a single protein type (spots number 2, 8, 9 and 10). Analysis of seven further spots (1, 3, 4, 5, 7, 11 and 12) revealed one major type of protein but additionally traces of other proteins (with comparatively low MOWSE (molecular weight search) identification score and peptide sequence coverage, both of which are conventional validity measures of protein/peptide database identification). In the case of spot 6, two equally abundant proteins with high MOWSE scores and coverage were identified. Six proteins were identified as plant proteins including a hypothetical protein, while six others were annotated as *R. solanacearum* proteins. Since the tomato genome sequence is not yet fully sequenced we combined classical protein identification based on protein database interrogation using MS/MS spectra with the peptide *de novo* sequencing strategy as described by Winkelmann et al. (2006) . Using this approach, all plant proteins were found to represent known tomato proteins or to be highly similar to known proteins from other organisms (other *Solanum* species, *Brassica*, *Boquila,* and *Citrus species*).

Table 1. Proteins identified by LC-MS/MS analysis and peptide *de novo* sequencing.
The proteins of differential abundance in the susceptible tomato genotype NHG3 not infected and infected with *R. solanacearum*. Each peptide sequence is separated by a dash.

Gel spot no[a]	Peptides sequence[b]	Protein identity (organism)[c]	Primary accession number[d]	Calculated mol mass (kDa) / IEP	MOWSE score[e]	Percent coverage (peptides)[f]	Reaction to pathogen inoculation
1	YPSFEADHMGGLSK-FNRDE FGLDYGK-DAPT EAEGQLTLH -CMQHPMLK-DSLDFGFAK-SNG VVTLDR-EVCGADAEFK-GPDLFDVAK-LELDS FK	Probable signal peptide protein (*Ralstonia solanacearum*)	Q8XVW0	21.84 /6.97	633	45	Induced
1	ASFVLNPEGVV-AAQYVA-GGVCPAK-SWNDG SD-YEELQ	Peroxiredoxin (*Vibrio vulnificus*)	Q7MDI6	21.80 / 5.20	215	15	
1	SFVLNPEGVVK-AAQYVA-VCPAK-EVYNC	Alkyl hydroperoxide reductase C (*Porphyromonas gingivalis*)	Q7MWJ2	21.07 / 5.37	184	15.4	

Table 1 continued

2	VALVYGQMNEPPGAR-FVAEVFTGSPGRYVGL-FVQAGSEVSALLGR-PATTFAHLDATTVLSR-IVGEEHYETAQR-EGNDLYMEMK-VLGEPVDNLG-LGLDELSEEDR-PNIYNALVVKGR-VVDLLAPYR-APLSVPVGG-LSIFETGIK-LLFELLNNLAK-QQLLGNNR-VGLTALTFAE-AVAFSATEGLTR-DSTSTML-FGGVGER-EVQQLLGN-VLNTGSPLTV-AMNLEFES-LVGNIDEA-PTTSGP-SAPAFI-VEGSTLGR-QLDTK-PLTVPTGAA-ESNLK 260	ATP synthase beta subunit (*Boquila trifoliolata*)	Q31931	n. d.	1704	50	Induced
3	WHAVEHAVMTVEQR-HADVWGTFSQLR-PVLTLSLLVNTPAK-PPHQPVMPM-PFYAVSLQQAK-LDKLHTELAK-ASGDLGQMK-LADAPQLK-TGQASYR-ALASLAEK-VFHADV	Hypothetical protein RSc1727 (*Ralstonia solanacearum*)	Q8XYN1	20.77 / 6.14	771	56	Induced
3	VYFDLSLGNPVGK-GLYGDDV-DLQSK	Similar to *Arabidopsis thaliana* peptidyl-prolyl cis-trans isomerise (*Arabidopsis thaliana*)	At5g13120*	28.30 / 9.81	176	14.5	
3	MAIGVLEAIQQAK-IRGLPIPIDQ-SDTDPLV	ABC transporter substrate binding protein (*Agrobacterium tumefaciens*)	Q8UB19	36.45 / 5.67	151	9	
4	YSFLEGDVLGDKLESLSYDLK-VTSYTHETTTPVAPTR-GDYVLKDEEHNEGK-NLEAEGDG SLK-MNFVEGSPLK-LHVVDRSNLVTK-GGGCVCK-EAPADGSLKK	Pathogenesis related protein STH-21 (*Solanum tubersom*)	P17641	17.20 / 5.73	622	64	Induced
4	VEYNPGVSAVALK-FPFLLVDR	Putative 3-keto-acyl-ACP dehydratase (*Brassica napus*)	Q94F93	24.63 / 9.19	126	8.5	
5	GDHVVSEEEHNVGR-NTYTYESTTTLS-QMNFVEGGPLK-FEANDNGGSVYK-YSLLEGDVLG-LESLTYDLK-VEGDGG AGSLK-ALVLDFDRAVPK	TSI-1 protein (*Solanum lycopersicum*)	O49881	20.22 / 5.61	594	50.5	Lower abundance
5	VLQLSGER-NGVLEATVPK	HSP 20.0 protein (*Solanum peruvianum*)	O82012	17.57 / 5.22	174	10	
6	YAQIAIGTDDVYK-DPDGYLFELLQR-FYTECFGMK-YDIGTGFG-TIAMMGYAPE-SAEVVKIVNQEL-VVKIVNQELGGK-SVIAFVK-TPEPLC-FLHAVYR-LTSFLDPD-PTPEPL-ALATPDV-QVMLR-SFLDPGGGSLPGLNTK-YGVTE	Putative lactoylglutathione lyase (*Brassica oleracea*)	Q39366	31.64 / 5.19	886	48.5	Higher abundance
6	DPDGYIFELIQR-YAQLALGTDDVYK-FYTECFGMK-PGSIPGLNTK-SAEVVNLALQEL-FALATPDVYK-VVNLALQELGGK-YDIGTGFG-FLHAVYR-LTSFLDPD-TPEPLC-PTPEPL-TSFLDPGG-QVMLR-YGVTE-IAFVK-TVLVD	Hypothetical protein (*Citrus paradisi*)	O04428	32.64 / 5.46	994	46	
7	NSQGAWSLTK-SAGGQGGNSQGA-MKDLYVK-TTFGEVTV-FDSPAIK-DMVFSK-SLLQPR-AAEGIP-YLEVK-DGEGN-IEVNS	Hypothetical protein RS01963 (*Ralstonia solanacearum*)	Q8XRT6	18.45 / 5.80	535	45.5	Induced
7	EDVVLQFVNPK	BTF-3 like transcription factor (*Nicotiana plumbaginifolia*)	O24121	17.85 / 8.86	77	16.5	
7	VYFDEALGNPVGK	Cyclophilin-like protein (*Triticum aestivum*)	Q6XPZ4	25.89 / 9.59	76	5	
8	AAVEEGLVAGGGVALLR-YVAAGMNPMDLK-MVEGVNLLAN AVK-AQLEEATSDYDR-ENTTIIDGAGDAR-TTDCAVAELPK-LQNMGAQMVK-EDELDVVEGM-DNPFVLLFDK-DLLPVLEQVAK-GANADQDAGIK-AVA AAVEELKK-VGAATEVEMK-LDNPFVLLFD-DVVEGMQFDR-GDNVEFGVLDPTK-DVV FGDAAR-EGVLTLQDGK-VANVIAGK-EDALHA-ALISGLK-SFGGVVVTK-ARIAEA-EEPLR	60 kDa Chaperone GroEL (*Ralstonia solanacearum*)	Q8Y1P8	57.40 / 5.09	1762	44.4	Induced
9	AAVEEGLVAGGGVALLR-VQLDNPFVLLFDKK-GDGTTTATVLAQSIVR-AQIEEATSDYDR-MLTTDCAVAELPK-LSPYFINNPEK-VEGVNILANAVK-YVAAGMNPFDLK-LQNMGAQMVK-DVVFGDAAR-ENTTLLDGAGD-LPVLEQVAK-ASVVVANVLAGK-GANADQDAGLK-VEFGVLDPTK-VGAATEVEFK-FGGPTVTK-LSANSDESLGAR-VITVEDGK-LAGGVAVIK-EEIGLTLEK-APGFGDR-AAVEELK-EDALHA-DELDVVGG-RAAVESG-VVLER-LHATR-VEDAL	Molecular chaperone (*Ralstonia pickettii*)	Q75T66	57.32 / 5.07	1796	53	Induced
10	TTTYYSAGGVLVR-DAGGGDNTIYAGR-YYQGIFGVLPG-DSNATGHAVVK-ESLMPVIASSWK-ALVQTSLEFFR-FFADGSSIR-NVVTVGDG-QTAVQDR-ILGLGHDVVD-TQVLGFR-LRGPA	Putative hemolysin-type protein (*Ralstonia solanacearum*)	Q8XT20	70.18 / 4.37	752	16.6	Induced

Table 1 continued

11	GNAYAQLALGTDDVYK-DPDGYIFELIQR-TPEP LCQVMLR-FYTECFGMK-ITSFLDPDGWK-PGSI PGLNTK-YTLAMMGYAPE-VVNLALQELGGK-Y DIGTGFG-ALATPDVYK-LHAVYR-PTPEPL-GG SSVIAFVK	Hypothetical protein (*Citrus paradasi*)	O04428	32.64 / 5.46	854	43	Induced
11	LGDDEFGHMLAGILK-APGGAPANVAIAVTR-FSCANSSLTTTK	Fructokinase (*Solanum lycopersicum*)	Q42896	34.76 / 5.76	255	12.8	
12	TVDTTGAGDSFVGALLTK-LGDDEFGHMLAGIL K-VSDVELEFLTGSNK-IPALPTASEALTLLK-TN GVQAEGINFDK-FSCACGAITTTK-IVDDQTILED EAR-IDDESAMSL-TVGGFHVK-EFMFYR- WPS AEEA-DSADVIK-IVEPCR-LPLWPSAE-TALAFV-PSADM 143	Fructokinase -2 (*Solanum lycopersicum*)	Q42896	34.76 / 5.76	1218	43.6	Induced
12	VYPLDAVFDSPEDV-VLPDGSLMEIAK-NYSLE NAPLQK-ASSYSFISLL-SASSYSFIS-WTVSEV AEDAK-LAFEAGR-VNTISAG-ANGLLVSKHEP-YGGGVGTAK-SLANGLLVSK	Enoyl-ACP reductase precursor (*Petunia x hybrida*)	O24258	41.79 / 7.76	650	27.5	
12	TDEEVQELTVR-NAGTEVVEAK-LFNINANIVK-ALDALKPELK-LYDIANVK	NAD-malate dehydrogenase precursor (*Nicotiana tabacum*)	Q9XQP4	43.30 / 8.03	585	12.1	

a Gel spot numbers correspond to the numbers given in the gels shown in Fig. 4 and 5

b Peptide sequences as revealed by *de novo* sequencing

c Identified protein / most similar protein (species)

d Corresponding protein accession number (SwissProt and TAIR accessions)

e MOWSE (molecular weight search) score

f Percent sequence coverage of the identified peptides

n.d. Not determined (partial sequence)

Functions were assigned to the identified proteins based on published studies. Among the six plant proteins, two belonged to PR proteins, and one was identified as an oxidative stress protein. One was an enzyme of carbohydrate metabolism and another was of energy metabolism. The remaining identified protein was hypothetical with unknown function.

Comparison of protein profiles of healthy plants of genotypes NHG3 and NHG13 did not reveal visible differences at the proteome level. Similarly, analysis of the stem proteome of the resistant genotype NHG13 exhibited no proteins with differential regulation after pathogen inoculation.

1.4 Discussion

The mid-stem was considered for comparative proteome analysis on the basis of the hypothesis that resistance mechanisms against bacterial wilt are present in the mid-stem of tomato plants. In our experiment, the resistant plants showed latent infection in stems without

visible symptoms pointing to the existence of some degree of pathogen tolerance by the plants. More interestingly, the occurrence of a relatively lower bacterial population compared to the susceptible lines when plants were root inoculated with the same inoculum pressure would indicate the presence of resistance mechanisms limiting pathogen multiplication. Since the gene expression analysis at mRNA level at different time points after root inoculation showed high expression after 3 dpi (unpublished data), and the susceptible plants started wilting around 5 dpi, mid-stem proteome were analysed at 5 dpi. Additionally, our histochemical and biochemical analyses also revealed a stronger reaction of resistance mechanisms on stem and xylem cell wall levels only at 5 dpi or later (Diogo and Wydra 2007, Wydra and Beri 2007). Among 12 differentially regulated proteins in genotype NHG3, six belonged to proteins of *R. solanacearum,* in contrast to other proteomic studies on plant-pathogen interaction, where the identification of pathogen proteins was not described (Colditz et al. 2004, Kim et al. 2004). The identification of pathogen proteins in inoculated, susceptible plants signifies the presence of relatively higher bacterial density in mid-stems at 5 dpi when *R. solanacearum* cells had already multiplied to about 10^9 cfu/g of stem. Out of six bacterial proteins, two were molecular chaperones, one hemolysin-type protein, and a signal peptide protein, and a further two were proteins of unknown function. Even though protein identification by MS is facilitated by the availability of the complete genomic sequence information of *R. solanacearum*, the molecular basis of the pathogenicity of the bacterium remains obscure due to the lack of detailed information on the function of some of these bacterial proteins.

Contrary to the general expectation, the comparison of the stem proteome resolved in 2-D SDS gels did not show clear visible differences in the protein patterns in any of the replicate gels from the resistant genotype before and after pathogen inoculation nor in the gels for genotypic comparison. This result probably indicates that there are no major changes in the expression of, at least, the abundant proteins in the resistant tomato line NHG13 due to pathogen challenge. Tao et al. (2003) reported much less biological variation in an incompatible interaction compared to the compatible interaction. It was discussed that the response reactions of resistant lines are more robust to input signals than susceptible lines and

the differences are largely quantitative and kinetic. On the other hand, some major limitations of the classical 2-D SDS-PAGE approach in the separation and visualization of proteins can also not be underestimated (Lopez 2007). For example, proteins expressed in low copy number that include receptors, transcription factors, regulatory and other key proteins involved in plant-pathogen interactions would not be resolved and/or visualized. Additionally, the small dynamic range of Coomassie staining hinders the detection of all and weakly expressed proteins at the same time. Therefore, the use of an integrative approach by complementing gel free comparative and quantitative methods with differential labelling of proteins and peptides followed by MS analysis, at additional time points after inoculation, is suggested for elucidation of more subtle plant-pathogen interactions. The analysis of sub-cellular proteome including stem cell wall and xylem sap would further shed light on the projected plant-pathogen interactions. Moreover, the identification of physiological roles of each of the identified proteins in the context of given interactions would be recommended.

1.4.1 Proteins involved in plant defence

In genotype NHG3, two PR proteins of low molecular weight were identified, of which STH-21 (Fig. 4 and 5; spot 4) was induced, while TSI-1 (Fig. 4 and 5; spot 5) was slightly down-regulated upon infection. PR gene expression is activated by a number of biotic or abiotic stresses, including pathogen infection (Van Loon et al. 2006). The enzymatic functions of some of these PR proteins indicate their role in plant defence against pathogens. However, the accumulation of PRs is not a prerequisite for the induction of resistance, since they make only a small contribution to the protective state of the plant (Van Loon et al. 2006).

STH-21 was initially identified as a member of a small multigene family accumulated in potato upon infection (Constabel and Brisson 1992). The up-regulation of PR proteins in the susceptible genotype compared to its resistant counterpart was also shown in *Medicago truncatula* (Colditz et al. 2005). TSI-1(tomato stress induced-1) protein is an intracellular PR protein (IPR) organized as a multigene family in the tomato genome (Sree Vidya et al. 1999). It is highly homologous to the potato STH-2 and STH-21 proteins. Like other IPR proteins, TSI-1 proteins are generally induced upon pathogen colonization and act as defence proteins by degrading the invading pathogenic RNA (Park et al. 2004). Surprisingly, the reduced

abundance of TSI-1 protein upon pathogen challenge was observed in the study. This could be due to the degradation of protein or inhibition of further protein synthesis in the plant as a result of increased activities of the pathogen, since the average number of bacterial colonies in the analysed stem was already to about 10^9 cfu/g of stem. The overlapping of gene expression, and the activation or suppression of the corresponding genes was also possible in response to biotic and abiotic stress (Zhu et al. 1995). A similar finding was reported by Constabel and Brisson (1992), where potato STH-2 protein disappeared completely at 4-5 dpi with a high concentration of compatible *Phytophthora infestans* spores.

1.4.2 Proteins involved in plant stress

An enzyme involved in oxidative stress, the putative lactoylglutathione lyase (spot 6 in (Fig. 2 and 3), was increased in abundance upon infection. This is the enzyme which participates, together with glyoxalase I and II, in the glutathione-based detoxification of methylglyoxal and other detrimental compounds formed primarily as a by-product of carbohydrate and lipid metabolism (Singla-Pareek et al. 2003). Actually, the physiological significance of such a glyoxalase system is still unclear in plants, however, it is often considered as a ‘‘marker for cell growth and division’’ and also considered to maintain cellular homeostasis (Yadav et al. 2008). The identification of a hypothetical protein in spot 6 with similar score and sequence coverage is not uncommon in the 2-D gel approach. A single spot may contain multiple proteins which could be due to co-migration of the proteins (Baltz et al. 2004). Since both the theoretical molecular weight (31.64 and 32.64) and isoelectric point (5.19 and 5.46) of both lactoylglutathione lyase and a hypothetical protein in spot 6 were close to equal, it could be a co-migration of both proteins during 2-D separation. However, there is also the possibility of diffusion of proteins present nearby on the 2-D gel before being excised for the analysis.

1.4.3 Proteins involved in carbohydrate metabolism

Spot 12 (Fig. 4 and 5), which was identified as fructokinase (FRK), was newly induced upon infection. FRK is a member of the hexose kinase family and catalyzes the phosphorylation of fructose to fructose-6-phosphate by utilizing primarily ATP *in vivo*. FRK occurs in cytosol or plastid and is one of the key enzymes in metabolization of sucrose, the major form of

transportable carbohydrate in vascular plants, through glycolysis, pentose-phosphate, or starch synthesis pathways (German et al. 2004). However, FRK2 seems to play a greater role in sugar sensing or signalling than as a metabolic enzyme (Pego and Smeekens 2000). Even though the biological role of FRK in plant defence is yet to be fully elucidated, it was observed that PR genes were expressed in photosynthetically active plant tissues with elevated sugar levels (Herbers et al. 1996). In potato, an increased level of sugar metabolism correlated to enhanced susceptibility to a root rot pathogen was reported (Otazu and Secor 1981).

1.4.4 Proteins involved in energy production

The ATP synthase beta subunit (Fig. 4 and 5; spot 2), newly induced in the susceptible genotype upon pathogen challenge, is well known for its role in the energy production system. The accumulation of proteins associated with energy production in infected plants is required for cellular activities including the activation of defence responses (Seo et al. 2007). In plants, the terminal step in the energy production system i.e. the oxidative phosphorylation of ADP into ATP, is catalyzed by the ATP synthase complex (F0F1) located in mitochondrial or chloroplast membranes. Among five non-identical subunits, the ß subunit is one of the two largest subunits of the soluble part (F1) of the enzyme complex that plays a central role in ATP synthesis (Van Lis et al. 2007).

The regulation of primary metabolic enzymes such as those of carbohydrate and energy metabolisms in the above plant-pathogen interaction shows an increasingly important role of primary metabolism in relation to the disease susceptibility or resistance of the plant as reported earlier (Castillejo et al. 2004).

1.5 Conclusion

In conclusion, this study provides information on differentially expressed proteins in tomato stems after pathogen challenge in the compatible interaction. The finding of PR proteins, stress and metabolic proteins in susceptible plants suggests their direct or indirect involvement in the reaction of the plant to pathogen infection. Plant susceptibility or tolerance to *R. solanacearum* is suggested to result from complex interactions in vascular

tissues, and timing and magnitude of several defence responses may be more important than the number and type of proteins. The static nature of the resolved proteome, at least the most abundant proteins, from the resistant genotype on 2-D gels after bacterial challenge indicates a higher constitutive resistance, making the plant more robust in reactions to the pathogen ingress. These mechanisms could be morphological and physical barriers of a polysaccharide nature or toxic metabolites contributing to resistance to bacterial wilt of tomato. For further studies, the use of more sensitive gel free methods such as differential labelling of proteins or peptides followed by MS analysis is suggested for the quantitation and comparison of less abundant, unsolubilizable and membrane proteins, which could be key elements in the plant-pathogen interaction.

CHAPTER 2: Analysis of cell wall proteins regulated in stem of susceptible and resistant tomato genotypes after inoculation *with Ralstonia solanacearum*, a proteomic approach

Adapted from the manuscript prepared for submission

Abstract

Proteomics approach was used to elucidate the molecular interactions taking place at the stem cell wall level when tomato genotypes were inoculated with *R. solanacearum*, the causative agent of bacterial wilt. Cell wall proteins from both resistant and susceptible plants before and after the bacterial inoculation were extracted from purified cell wall with salt buffers and separated with 2-D IEF/SDS-PAGE and with 3-D IEF/SDS/SDS-PAGE for basic proteins. The gels stained with colloidal Coomassie were analysed with Image master v6.0 revealed reproducible and statistically significant regulation of protein spots among genotypes and treatments comparisons. Combination of MALDI-TOF/TOF MS and LC-ESI-IonTrap MS/MS successfully lead to the identification of proteins differential in either genotypes (eight proteins in higher abundance to resistant and six other to susceptible genotypes) and those exclusively regulated in response to bacterial inoculation in resistant (seven proteins up regulated and eight other down regulated) as well as in susceptible plants (five proteins elevated and eight other suppressed). Plants responded to pathogen inoculation by increasing the expression of PR, other defense related and glycolytic proteins in both genotypes. However, cell wall metabolic proteins in susceptible, and antioxidant, stress related as well as energy metabolism proteins in resistant lines were suppressed. Most of the proteins of the comparative analysis and other randomly picked spots were predicted to have secretion signals except some classical cytosolic proteins.

Key words:

Defense and metabolic proteins, *R. solanacearum*, Secretion signals, Stem cell wall proteome, Tomato genotypes, 3-D PAGE

2.1 Introduction

The cell wall of the plant is one of the most important distinguishing features and a dynamic structure that consists predominantly of polysaccharides but also proteins along with minor amounts of polyphenols in specialized cells. The primary cell wall is formed during cell growth and elongation which is accompanied by the inwardly deposition of the secondary cell wall after the cessation of cell growth. The secondary walls of xylem fibers, tracheids, and sclereids are further strengthened by the incorporation of lignin (Cassab and Varner 1988). A common cell wall model for the structure and architecture of the primary cell wall describes the existence of interwoven networks of polysaccharides and proteins (Cosgrove 2005). Relative to about 90% polysaccharides, the primary cell wall of dicotyledon plants possess less than 10% proteins however several hundreds in number, of the cell wall mass (Jamet et al. 2008a).

Terrestrial plants are subjected to many biotic and abiotic stresses during their lifetime and therefore, evolved a wide range of defence mechanisms to protect themselves, consisting of the resistance mechanisms at the constitutive level and induced defence systems. The interactions between plants and microbes lead either to disease resistance or plant disease depending on the type of interactions, however, the latter case could results in a huge economic loss. Bacterial wilt caused by *R. solanacearum* is one of the most devastating, systemic vascular wilt diseases causing up to 100% tomato yield loss in the lowland and highland tropics and subtropics (Denny 2006). Due to the wide host range and the variability of the pathogen, control measures based on the use of resistant cultivars remain the most effective, economical and environment friendly method (Denny 2006). The bacterial wilt resistance in tomato is a polygenic trait and was reported to be present in the mid-stem when plants were root inoculated with *R. solanacearum* (Dahal et al. 2009). Our previous analysis of the tomato stem proteome revealed the regulation of pathogenesis, stress related and metabolic proteins in susceptible genotype but not in resistant plants (Dahal et al. 2009). To further elucidate the interactions, sub cellular fractions such as the stem cell wall were analysed to increase the sensitivity of the performed analysis. The cell wall acts as a site of both constitutive and induced resistance through structural changes and modification during

the interactions with pathogens (Carpita and McCann 2000, Jamet et al. 2008a). Histochemical analysis showed various differences on the constitutive level and changes after pathogen interaction on the level of pectic polysaccharide, though the differences could not conclusively explain the entire background of the resistance reactions (Wydra and Beri 2006). Since proteins are the vital molecules which perform the enzymatic, regulatory, and structural functions in a biological system, the role of proteins secreted into the plant cell wall by the plants and pathogen during the host-pathogen interactions can be important in establishing and determining the outcome of plant-microbe interactions. The roles of plant cell wall proteins as structural, antimicrobial and enzyme molecules has been established, however, cell wall proteins associated both with susceptible and resistance tomato lines in the interaction with *R. solanacearum* were not characterized. Proteomics approach was therefore, undertaken to simultaneously analyze the broad spectrum of the cell wall protein (CWP) profiles that could be decisive for the susceptibility or resistance of the plants. The sub cellular proteome analysis should also increase the sensitivity of our earlier results from whole stem analysis. Extraction of wide range of cell wall proteins from mature tomato stems to a substantial purity is a challenging task due to the structural complexities of the cell wall and the nature of CWP (Jamet et al. 2008b). Therefore, disruptive and two steps salt extraction method was used to enrich cell wall proteins after purification of stem cell walls by rigorous washing with both aqueous and organic buffers to remove cytoplasmic contaminants (Watson et al. 2004).

2.2 Materials and Methods

2.2.1 Plant material and inoculum preparation

Tomato plants were grown from parental lines, Hawaii7996 and WVa700 as highly resistant and susceptible genotypes against bacterial wilt respectively. The seeds from both genotypes were obtained from AVRDC, Taiwan. The inoculum from the highly virulent *R. solanacearum* strain ToUdk2, race 1, biovar 3 was prepared by adjusting the bacterial cell concentration to about 7.8×10^7 cfu/mL as described by Dahal et al. 2009.

2.2.2 Plant growth conditions and inoculation

Tomato plants were grown in the greenhouse for 4-6 weeks (20°C, 14 h photoperiod per day, 30K lux and 70% RH). Each plant was then transferred to an individual pot with approximately 330 g of soil (Fruhstorfer Erde, type P: 150 mg/L N, 150 mg/L P_2O_5, and 250 mg/L K_2O) and grown in climate chamber (30/28°C day/night temperature, 14 h photoperiod, 30 K lux, and 85% RH). Some plants were root inoculated after transplantation by pouring 25 mL of the prepared inoculum per pot, to reach a final concentration of approximately 10^7 cfu/g of soil and the soil was watered up to soil field capacity.

2.2.3 Bacterial quantification

The number of bacteria residing in the stem was determined at 5 dpi considering the time the bacteria need to reach and multiply in the stem. The pathogen quantification was done as explained by Dahal et al. 2009. The pathogen developed as an elevated fluidal colony with red centre after 48 h/30°C incubation on TTC medium were quantified as cfu/g.

2.2.4 Disease symptoms evaluation

Ten pathogen inoculated plants from each susceptible and resistant genotype were observed for symptoms assessment. The symptoms were evaluated over the period of four weeks in six disease severity classes from 0-5 (Dahal et al. 2009). The WI and DS were calculated as the percentage of dead plants (class 5) to the total number of plants at the evaluation date and as the mean of disease scores at the evaluation date respectively.

2.2.5 Protein extraction from cell walls of tomato stems

The stem cell wall proteins from both Hawaii7996 and WVa700 genotypes were extracted before and after pathogen inoculation at 5 dpi. Individual samples were prepared by combining mid stems of three individual plants to approximately 8 g. The samples were stored at -80°C until the extraction was performed from purified cell walls with salt solutions (Watson et al. 2004).

Approximately 8 g of mature tomato stem was powdered in liquid nitrogen followed by several washes and filtration through 47 μm^2 nylon mesh membrane (SEFAR Nitex,

Germany) to purify cell wall material for the protein extraction. The washing started with 100 mL of grinding buffer (50 mM Sodium acetate pH 5.5, 50 mM NaCl, and 30 mM Ascorbic acid) followed successively by 50 mL wash buffer 1 (100 mM NaCl), 100 mL bidest, 250 mL ice-cold acetone, and finally with 50 mL wash buffer 2 (10 mM Sodium acetate pH 5.5). Wash extract from each of the washings was preserved to asses the purity of the extracted cell wall protein. The extraction of protein from the purified cell wall was performed in two sequential steps and finally combined to 30 mL: first, by shaking the debris with 8 mL of extraction buffer 1 (200 mM $CaCl_2$, and 50 mM sodium acetate pH 5.5) twice each for 1 h and then with 15 mL of extraction buffer 2 (3 M LiCl, and 50 mM sodium acetate pH 5.5) overnight. The protein extract was concentrated in a centrifugal concentrator with a molecular mass cut off at 5 kDa (Vivaspin 6, Vivascience, Germany) at 5000 rpm x 4°C x 2-3 h until a final volume of 100 µL was obtained. The concentrate was washed with double volume of bidest water and precipitated with a commercial 2-D clean up kit (Bio-Rad, Germany).

2.2.6 Protein separation with 2-D and 3-D SDS-PAGE

The complexity of the proteome extracted from the stem cell wall was resolved by 2-D IEF/SDS-PAGE and for basic proteins by 3-D IEF/SDS/SDS-PAGE. In case of 2-D gels, three replicate gels were prepared for each genotype and treatment from separate biological samples where as only 2 replicates were prepared for 3-D gels.

Approximately 700 µg of protein quantified by Bradford assay (Coomassie protein assay reagent, Fluka biochemical) was dissolved in 350 µL of "rehydration buffer" [8M urea, 2% w/v CHAPS, 0.5% v/v carrier ampholyte mixture (IPG buffer 3-11 NL, GE Healthcare, Munich, Germany), 30 mM DTT, and 2-4 mg Bromophenol Blue] with the addition of 4.2 µL of DeStreak reagent (GE healthcare, Munich, Germany) to facilitate the basic proteins separation. The protein complex was then separated in first dimension by using IEF and in second dimension by vertical SDS-PAGE using Protean II electrophoresis unit (Biorad, Hercules, CA, USA) as explained in Dahal et al. 2009.

In order to separate proteins with very basic *pI*, that were repetitively unresolved in 2-D IEF/SDS-PAGE, a three-dimensional PAGE system was established, which is based on the transfer of the most basic gel region of a 2-D PAGE gel horizontally onto another SDS-PAGE. For this approach, new samples were required to be resolved by 2-D PAGE, because these non-fixed 2-D gels served as the starting-samples for the third gel dimension separation.

2.2.7 Protein staining, gel scanning and image analysis

After completion of SDS-PAGE, proteins were first fixed and stained with colloidal Commassie solution as described in Dahal et al 2009. The stained 2-D gels were washed with bidest water, and 3-D gels additionally with 20% methanol to clean background staining. The gels were scanned with a UMAX Power Look III Scanner (UMAX Technologies, Fremont, USA) and analysed by using the ImageMaster™ 2-D Platinum Software 6.0 (GE Healthcare, Germany) in order to accomplish the spots detection and calculate the quantitative values of all the differentially regulated spots. The comparison was performed in all three well reproducible replicate gels prepared for each genotypes and treatments. The abundance of each protein spot was estimated by the percentage volume (% vol). The Student's t-test with ≥ 0.05 probability (p-) value of differences in abundance was used to show statistical significance and reproducibility of each spot. In order to select the up/down regulated proteins, quantitative differences of ±30% variance in spot ratio (< 1.3 or > 0.7), were considered as biologically significant based on the previously determined threshold value of 20% for the analytical variance in comparative proteomic studies (Asirvatham et al. 2002).

2.2.8 Tryptic digestion

All except few big and intense gel spots were hand picked from all three gel replicates to increase the protein amount for digestion. Proteins were destained by gently shaking in 20 mM NH_4HCO_3, 50% acetonitrile (ACN) for 30 min at 37°C. This step was repeated until spots became clear. The gel was first dehydrated in 100% ACN for 5 min followed by drying in a speed vac system (Eppendorf, Germany). Trypsin (4 ng/µL) in 20 mM NH_4HCO_3/10% ACN was added, incubated on ice for 1 h and the remaining trypsin solution was removed. Digestion was carried out at 37°C over night after adding 20 mM NH_4HCO_3/10% ACN to

cover gel pieces. The supernatant containing peptides was collected and the gel pieces were re-extracted using 0.2% trifluoroacetic acid (TFA), containing increasing amounts of ACN (10-50%). All peptide containing solutions of each spots were combined, dried in a speed vac and stored at 4°C until further analysis.

2.2.9 Matrix assisted laser desorption and ionization-time of flight tandem mass spectrometry (MALDI-TOF MS/MS)

Peptides were dissolved in 10 µL of 5% ACN conatining 0.2% TFA. A saturated solution of alpha-cyano-4-hydroxycinnamic acid, CHCA (4 mg/mL) in 50% ACN and 0.2% TFA was diluted 1:10 with ethanol and 0.8 µL of the matrix was spotted on each spot of a MALDI Anchor Chip 800/384 target plate (Bruker Daltonik GmbH). After mixing 0.5 µL of each sample with the applied matrix, air-dried samples were recrystallized with 0.2 µL of ethanol containing 0.1% TFA. For MS calibration 0.5 µL of peptide calibration standard (Bruker Daltonik GmbH) were spotted on the target with 0.8 µL of CHCA matrix and recrystallized too. Samples were analyzed in an MALDI-TOF/TOF mass spectrometer (Ultraflex, Bruker Daltonics) in reflectron mode. Peptides with a signal to noise ratio above 100 were MS/MS analyzed by using the LIFT technology that is embedded in the Ultraflex MS. Data analysis was performed by using the FlexAnalysis 2.4 and BioTools 3.0 software. Databases search was carried out with the matrix science search tool (MASCOT) algorithm version 2.2 (Matrix Science, UK) and taking MS protein sequence database (MSDB). Searches were performed using the following parameters: trypsin as the proteolytic enzyme, allowing for one missed cleavage, carbamidomethylation of cysteine and oxidation of methionine.

2.2.10 Electrospray inonization (ESI) ion trap MS

Peptides were dissolved in 5% ACN with 0.1% formic acid and were applied to reversed phase chromatography-high performance liquid chromatography (HPLC) system (Agilent Technologies, Germany) that was directly mounted to the ion source of the ion trap MS. The HPLC system consists of an auto sampler and a gradient pump. The sample was dissolved in eluent A (5% ACN and 0.1% formic acid) and an aliquot was injected onto a C18 column (Zorbax SB-C18, particle size 5 µm, 300 A, 0.5 mm inner diameter, length 150 mm) at a flow

rate of 5 µL/min. After loading, the column was washed for 15 min with buffer A and the peptides were eluted using a gradient of eluent A and eluent B (70% ACN in 0.1% formic acid) from 0-53.9% eluent B in 60 min. Then buffer B was increased to 100% for 10 min and subsequently, the column was equilibrated with buffer A for 20 min. The HPLC outlet was directly connected to the agilent coaxial sheath-liquid sprayer (Agilent Technologies). The outlet capillary was hold by a surrounding steel needle and locked 0.1-0.2 mm out of it. The spray was stabilized by N_2 as nebulizer gas (5 l/min). Ionization voltage was set to 4500 V and dry gas was applied at 5 psi and 250°C. Spectra were collected with an Esquire3000$^+$ ion trap mass spectrometer (Bruker Daltonik) at a scan speed of 13000 m/z per second. Using ESI in positive mode, mass spectra were acquired from m/z 50 to 1600 in scanning mode and data dependent switching between MS and MS/MS analysis. To increase the quality of MS/MS spectra only two precursor ions from one spectrum were selected for MS/MS analysis and active exclusion was set to 2 min to exclude precursor ions that had already been measured. Data processing was performed with the Data Analysis (version 3.0) and BioTools (version 3.0) software packages (Bruker Daltonik GmbH). Protein identification was done using MACOT software (version 2.1) and MSDB data base (Matrix Science, UK). Search parameters for mass tolerance were set to 0.7 Da for precursor ions and 0.9 Da for fragment ions with 2 allowed missed trypsin cleavage, and 1^+, 2^+ and 3^+ charged state. Data base hits were taken if the peptide ion score was above 25 and proteins were identified if at least two peptides could be identified.

2.3 Results

2.3.1 Symptom development and bacterial populations in stems

Plants of the susceptible genotype WVa700 started showing symptoms at 4 dpi with both the WI and DS gradually increased until all plants had died at 10 dpi. The mean wilt incidence and disease severity were calculated from three biological replications (Fig. 6). The resistant genotype Hawaii7996, on the other hand, did not show any level of symptoms until 30 dpi.

Since each biological protein sample was prepared by combining two to three individual plants, the number of bacteria present in the sample was calculated by taking the average of

the bacterial population quantified in each plant. The pathogen population in three replicate samples was calculated as 25.6 x 10^7, 16.6 x 10^7 and 26.1 x 10^7 cfu/g fresh weight of stem for susceptible genotypes and 45.8 x 10^6, 33.3 x 10^6 and 22.2 x 10^6 cfu/g fresh weight of stem for resistant genotypes.

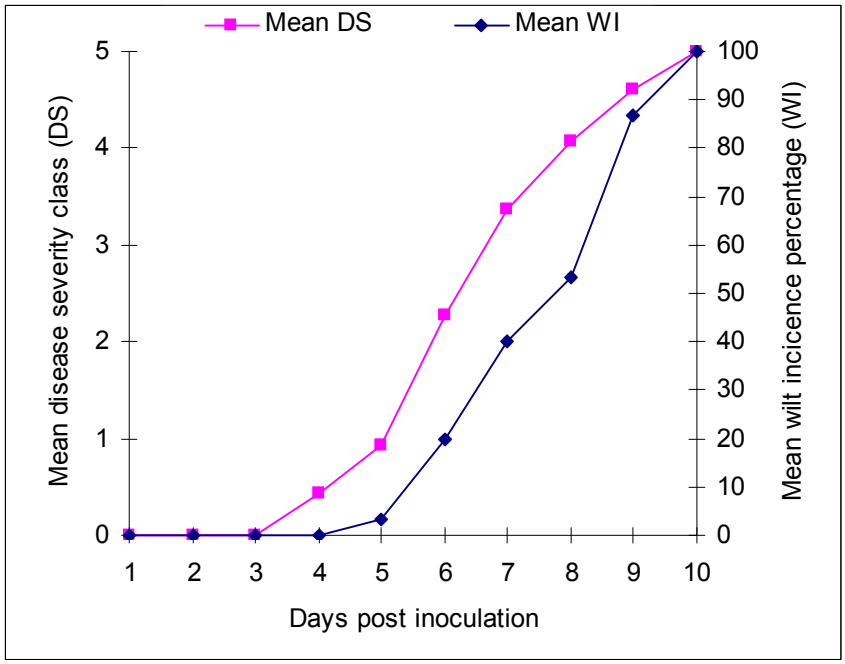

Figure 6. Mean DS and WI in susceptible tomato plants (WVa700) on the days following *R. solanacearum* strain To-udk2 inoculation.

The mean value was calculated from three independent biological replications. Both DS and WI increased continuously to maximum at 10 dpi.

2.3.2 Cell wall protein analysis

About 600-800 µg protein was obtained from 8 g of mid-stem sample. The resolution of approximately 700 µg protein loaded on each gel displayed an average of 370-470 protein spots in addition to several "poorly separated" spots on basic *pI* region of the gel (Fig. 7A). These unresolved spots upon further separation by 3-D SDS-PAGE revealed 25-35 spots detected with Image master (Fig 7: C and D). The comparison of the 2-D gels was performed in between genotypes as well as in treatments each with three replicated biological samples. The differentially expressed spots were first analysed by MALDI-TOF MS/MS, which gave

70% (29 out of 42 spots) successful identifications. The remaining 13 unidentified spots were successfully analyzed with liquid chromatography-electrospray inonization-ion trap tandem MS (LC-ESI-ion trap MS/MS). ESI MS/MS analyses of each spot allowed identifying more than one homology proteins originating from other plant species. In this case, only the proteins derived from the tomato were considered positive identification.

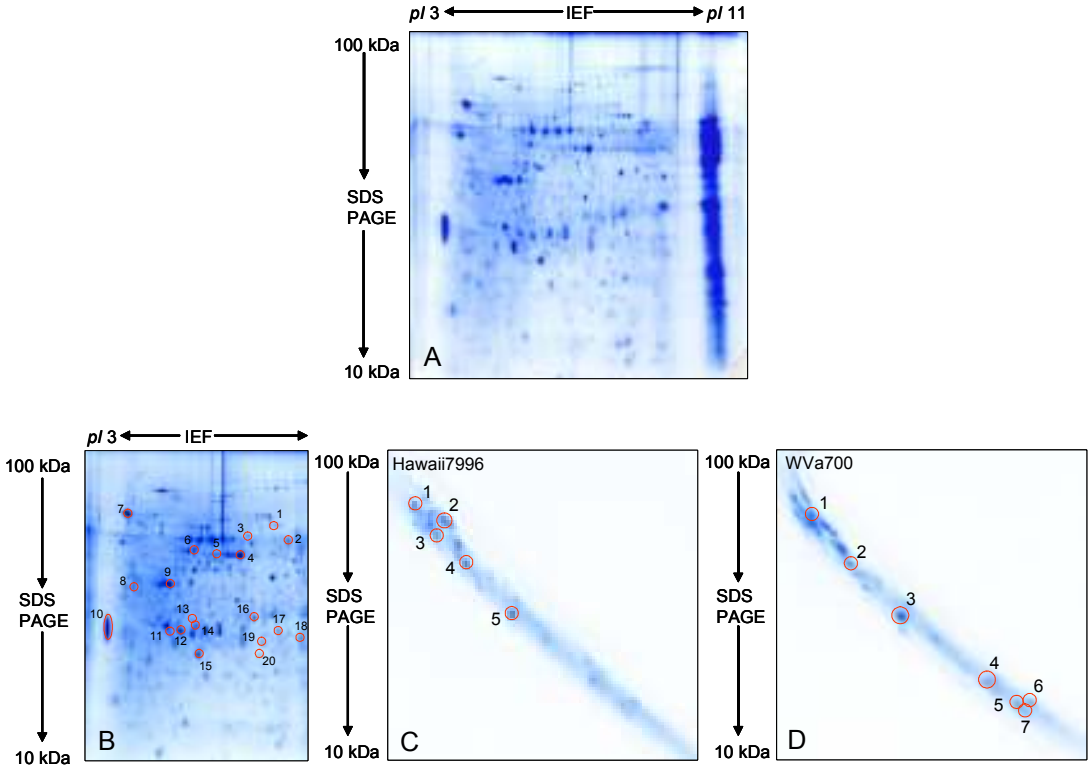

Figure 7. Overview of the cell wall proteome analyzed from the mature tomato stem and separated in two as well as in three gel dimensions.

A: The resolution of proteome with 2-D IEF/SDS-PAGE in *pI* 3-11 non-linear and 100-10 kDa molecular mass. The proteins on the basic *pI* range are poorly resolved.

B: The 2-D IEF/SDS gels resolved in the same *pI* 3-11 non-linear and 100-10 kDa molecular mass range but stained after cutting out the gel region with the poorly resolved vertical streak. Twenty spots were randomly picked out to check the extracellular nature of cell wall

proteome extracted with the method applied. The identity of the encircled spots is given in table 6 with the spot numbers used accordingly.

C and D: Separation of the unresolved vertical streak by SDS in the 3rd gel dimension. The streaked gel piece was cut out and separated again by SDS-PAGE. The encircled spots were differentially expressed after pathogen inoculation and their identity was given in table 5 with the spot numbers corresponding in this figure and table 5.

2.3.2.1 Protein regulation in resistant genotype

The proteomic reactions of resistant plants to pathogen invasion were evaluated by comparing triplicate gels developed before and after pathogen inoculation which revealed 15 spots of differential abundance (Fig. 8: A, B and C). Seven spots identified as subtilase, peroxidase, hypothetical protein, luminal binding protein (BIP), fructokinase-2, nucleoside diphosphate kinase (NDPK) and PII like protein (spots 3, 6-9, 14 and 15 respectively) were up regulated. Eight other spots annotated as BIP, stress induced protein, catalase, enolase, vacuolar H^+ ATPase (V-ATPase), oxygen evolving enhancer protein (OEE) 2, eukaryotic translation initiation factor 5A (eTIF 5A) -3 and eTIF 5A-4 (spots 1, 2, 4, 5, and 10-13 respectively) were down regulated (Table 2).

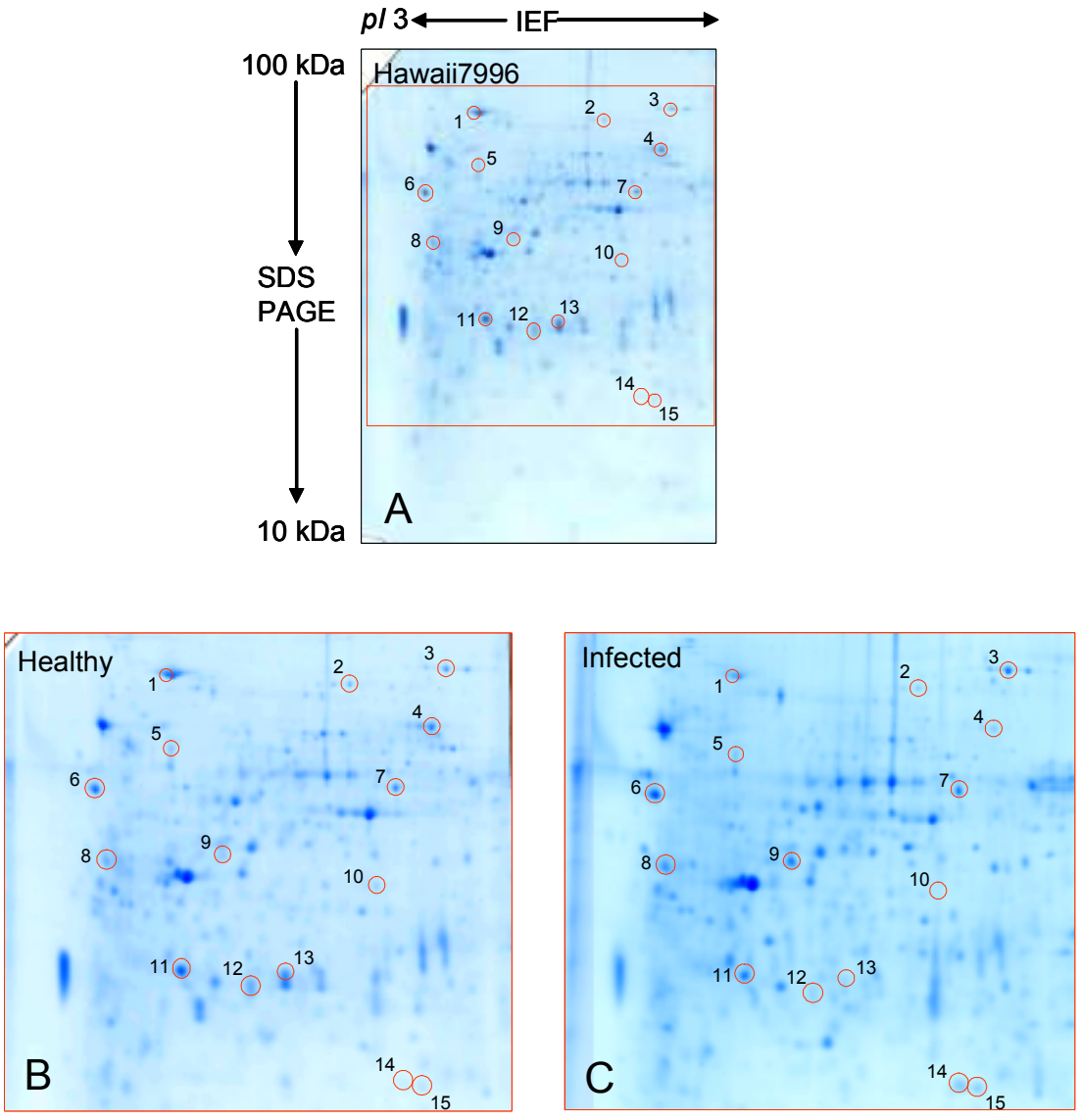

Figure 8. Overview of the cell wall proteome analysed from the mature tomato stem of Hawaii7996 (resistant genotype) and the differential regulation of the plant proteins in response to *R. solanacearum* inoculation.

A: A representative 2-D gel from the tomato stem cell wall proteome of the resistant genotype. Protein separation took place between *pH* 3-11 (non-linear IPG stripes) and in the molecular mass range between 100-10 kDa. However, the basic *pI* side of the 2-D gel containing poorly separated proteins was removed before fixing and staining. The red

rectangle indicates the area which displayed the protein spots differentially expressed in three biological replications due to pathogen invasion (Student's t test, $p \geq 0.5$).

B and C: A comparison of the area in healthy and infected plant proteome is correspondingly shown in images B and C in "zoom-in" view. The spot number is in accordance with the number used in table 2.

Table 2. List of stem cell walls proteins which are differentially regulated in tomato genotypes Hawaii7996 (resistant) after *R. solanacearum* challenge.

The listed proteins are those consistently reproduced in three biological replications and are statistically significant (Student's t test, $p \geq 0.5$).

* Notation (a-j) are given in foot note at the end of Table 6

Spot[a]	Identity[b]	Accession[c]	Organism[d]	Score[e]	*Mr*/*pI*[f]	Peptides[g]		Coverage[h]		Regulation[i]	SiP-SeP[j]
Resistant genotype (Hawaii7996)						MS	MS/MS	MS	MS/MS		
1	Luminal-binding protein	P49118	*Solanum lycopersicum*	260	73.23/5.10	14	3	21.6	6	0.30	Y
2*	Stress induced protein	Q6H660	*Oryza sativa*	185	64.19/6.03		7		8.5	0.44	0.55
3*	Subtilase	O82777	*Solanum lycopersicum*	136	82.22/8.22		5		3	1.67	Y
4	Catalase	P30265	*Solanum lycopersicum*	493	56.50/6.57	27	9	56.9	22	0.38	0.41
5*	Enolase	P26300	*Solanum lycopersicum*	92	47.79/5.68		3		6	0.54	0.52
6	Peroxidase	Q9LWA2	*Solanum lycopersicum*	144	34.94/4.56	9	2	34.5	5.5	1.88	Y
7*	Hypothetical protein	Q9C6U3	*Arabidopsis thaliana*	88	34.70/7.17		5		3	1.62	0.61
8*	Luminal-binding protein	P49118	*Solanum lycopersicum*	219	73.23/5.10		7		9.9	1.72	Y
9*	Fructokinase-2	Q42896	*Solanum lycopersicum*	510	34.76/5.76		9		33	1.91	0.68
10	Vacuolar proton ATPase subunit E	Q9LKG0	*Solanum lycopersicum*	46	27.13/6.63	7	1	22.4	4.2	0.51	0.27
11	Oxygen-evolving complex protein 2	P29795	*Solanum lycopersicum*	362	27.79/8. 27	4	5	42.6	27	0.54	0.8
12	Translation initiation factor 5A-3	Q9AXQ4	*Solanum lycopersicum*	93	17.37/5.47	6	1	45.9	6.3	0.65	0.24
13	Translation initiation factor 5A-4	Q9AXQ3	*Solanum lycopersicum*	255	17.51/5.6	8	3	61.9	28	0.23	0.23
14	Nucleoside diphosphate kinase	P47921	*Solanum lycopersicum*	165	15.67/7.04	3	2	33.8	12	1.75	0.40
15 *	PII like protein	Q6T2D2	*Solanum lycopersicum*	201	21.73/9.33		5		22	1.75	0.88

2.3.2.2 Protein regulation in susceptible genotype

The proteomic reactions of the susceptible plants were also investigated in response to pathogen inoculation where 13 spots turned out to be differential abundance (Fig. 9: A, B and C). Among them, five proteins (spot 3, 4 and 6-8) were up regulated while eight proteins (spot 1, 2, 5 and 9-13) were down regulated upon pathogen inoculation. The identity of the up regulated spots was correspondingly shown as peroxidase, peroxidase cevi16, basic 30 kDa endochitinase, triose phosphate isomerise (TPI) and PR-5 like protein and of down regulated as α-galactosidase, disulphide isomerase like protein (PDIs), xyloglucan endotransglucosylase-hydrolase7 (XTH7), two eTIF 5A-4, one eTIF 5A-1 and two glycine rich proteins (GRP) (Table 3).

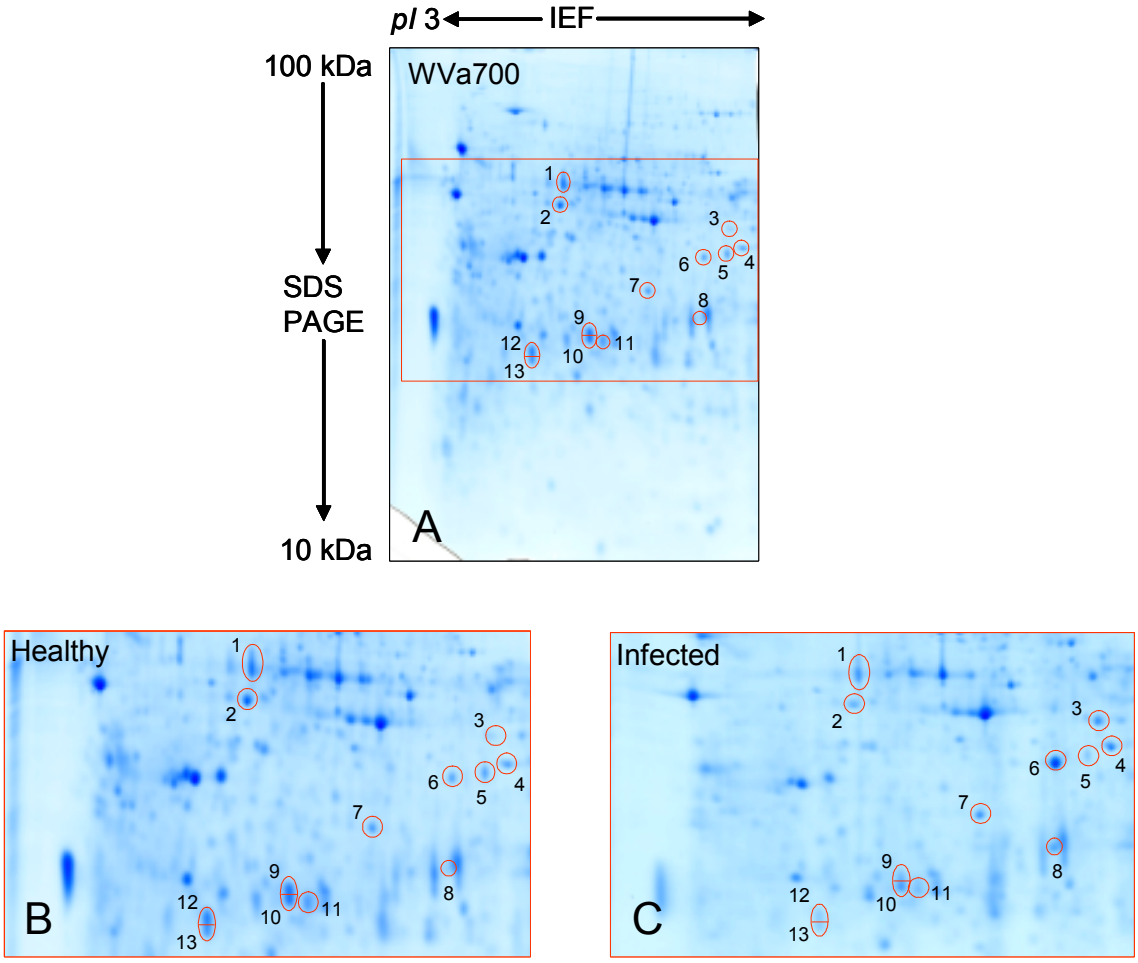

Figure 9. Overview of the cell wall proteome analyzed from the mature tomato stem of WVa700 (susceptible genotype) and the differential regulation of the plant proteins in response to *R. solanacearum* inoculation.

A: A representative 2-D gel from the tomato stem cell wall proteome of the susceptible genotype. Protein separation took place between *pH* 3-11 (non-linear IPG stripes) and in the molecular mass range between 100-10 kDa. However, the basic *pI* side of the 2-D gel containing poorly separated proteins was removed before fixing and staining. The red rectangle indicates the area which displayed the protein spots differentially expressed in three biological replications due to pathogen invasion (Student's t test, $p \geq 0.5$).

B and C: A comparison of the area in healthy and infected plant proteome is correspondingly shown in images B and C in "zoom-in" view. The spot number is in accordance with the number used in table 3.

Table 3. List of stem cell walls proteins which are differentially regulated in tomato genotypes WVa700 (susceptible) after *R. solanacearum* challenge.
The listed proteins are those consistently reproduced in three biological replications and are statistically significant (Student's t test, p ≥ 0.5).

* Notation (a-j) are given in foot note at the end of Table 6

Spot[a]	Identity[b]	Accession[c]	Organism[d]	Score[e]	*Mr*/*pI*[f]	Peptides[g]		Coverage[h]		Regulation[i]	SiP-SeP[j]
WVa700 (Susceptible genotype)						MS	MS/MS	MS	MS/MS		
1	α-galactosidase, putative	Q9FWV8	*Oryza sativa*	137	44.66/5.47	2	2	9.4	5.6	0.58	Y
2	Disulfide isomerase like protein	Q38JJ2	*Solanum tuberosum*	153	39.49/5.62	7	2	17.5	8.1	0.61	Y
3*	Peroxidase	Q07446	*Solanum lycopersicum*	273	35.99/7.52		5		17	2.99	Y
4	Peroxidase cevi16	Q4A3Y6	*Solanum lycopersicum*	170	31.74/7.71	11	3	46.1	15	1.86	0.44
5	Xyloglucan endotransglucosylase-hydrolase XTH7	Q6RHX8	*Solanum lycopersicum*	184	33.46/7.57	13	2	35.6	11	0.59	Y
6	Basic 30 kDa endochitinase	Q05538	*Solanum lycopersicum*	266	34.34/6.19	13	4	37.3	18	3.56	Y
7	Triose phosphate isomerase	Q6T379	*Solanum chacoense*	411	27.04/5.73	10	8	37	36	1.3	0.67
8	PR 5-like protein	Q7Y1P9	*Solanum lycopersicum*	144	27.52/5.76	7	2	45.6	14	2.88	Y
9	Translation initiation factor 5A-4	Q9AXQ3	*Solanum lycopersicum*	255	17.51/5.60	8	3	61.9	28	0.3	0.23
10	Translation initiation factor 5A-4	Q9AXQ3	*Solanum lycopersicum*	255	17.51/5.60	8	3	61.9	28	0.37	0.23
11	Translation initiation factor 5A-1	Q9AXQ6	*Solanum lycopersicum*	139	17.30/5.71	3	2	40.3	18	0.66	0.23
12	Glycine-rich protein	Q04130	*Solanum lycopersicum*	272	73.31/9.98	2	4	58.5	43	0.52	0.47
13	Glycine-rich protein	Q04130	*Solanum lycopersicum*	210	73.31/9.98	2	4	78	61	0.63	0.47

2.3.2.3 Protein variation in genotypic comparison

Fourteen genotypic differences were found comparing protein profiles between susceptible and resistant plants (Fig. 10: A, B and C). Eight spots which were identified as BIP, three enolase, a hypothetical protein, fructokinase-2, nascent polypeptide-associated complex (NAC)-α-like protein 3 and OEE2 (spots 1, 2, 9, 12, 7, 8, 11 and 13 respectively) were of higher abundance in resistant genotype. Similarly, six further spots namely α-galactosidase, peroxidase, a hypothetical protein, ferredoxin-NADP reductase (FNR), OEE1, and eIF-5A-1 (spot 3, 4, 5, 6, 10 and 14 respectively) occurred in higher level in susceptible genotype (Table 4).

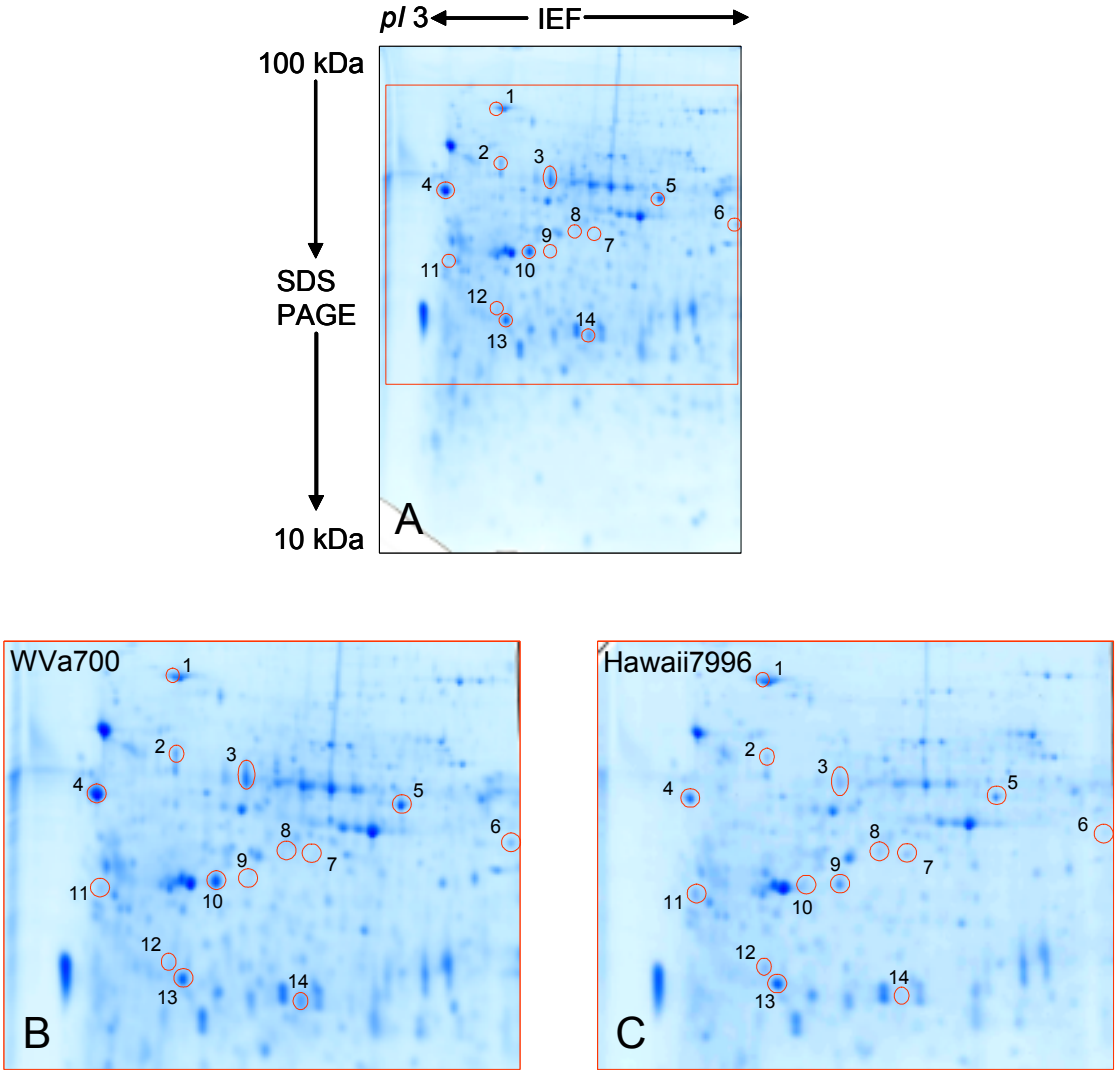

Figure 10. Overview of the cell wall proteome extracted from the healthy mature tomato stem of Hawaii7996 (resistant) and WVa700 (susceptible) and the proteome level differences between the two genotypes.

A: A representative 2-D gel from the tomato stem cell wall proteome of the susceptible genotype. Protein separation took place between *pH* 3-11 (non-linear IPG stripes) and in the molecular mass range between 100-10 kDa. However, the basic *pI* side of the 2-D gel including poorly separated proteins was removed before fixing and staining. The red rectangle indicates the area which displayed the protein spots of varied abundance in between

the two genotypes differing on the resistance to bacterial wilt disease. The spots were consistently varied in three biological replications (Student's t test, $p \geq 0.5$).

B and C: A comparison of the area in healthy and infected plant proteome is correspondingly shown in images B and C in "zoom-in" view. The spot number is in accordance with the number used in table 4.

Table 4. List of differential stem cell wall proteins in between healthy tomato plants of genotypes Hawaii7996 (resistant) and WVa700 (susceptible).

The listed proteins were those consistently reproduced in three biological replications and are statistically significant (Student's t test, $p \geq 0.5$).

* Notation (a-j) are given in foot note at the end of Table 6

Spot[a]	Identity[b]	Accession[c]	Organism[d]	Score[e]	*Mr*/*pI*[f]	Peptides[g]		Coverage[h]		Variation[i]	SiP-SeP[j]
Hawaii7996 Vs WVa700						MS	MS/MS	MS	MS/MS		
1	Luminal-binding protein	P49118	*Solanum lycopersicum*	260	73.23/5.10	14	3	21.6	6	0.53	Y
2*	Enolase	P26300	*Solanum lycopersicum*	92	47.79/5.68		3		6	0.60	0.52
3	α-galactosidase, putative	Q9FWV8	*Oryza sativa*	137	44.66/5.47	2	2	9.4	5.6	1.79	Y
4	Peroxidase	Q9LWA2	*Solanum lycopersicum*	144	34.94/4.56	9	2	34.5	5.5	2.92	Y
5*	Hypothetical protein T8G24.2	Q9C6U3	*Arabidopsis thaliana*	88	34.70/7.17		5		3	1.69	0.61
6	Ferredoxin-NADP reductase	O04397	*Nicotiana tabacum*	92	41.95/8.67	4	2	18.9	5.9	1.38	0.73
7	Hypothetical protein	O04428	*Citrus paradisi*	92.8	32.64/5.46	4	1	13.7	4.1	0.38	0.50
8	Fructokinase-2	Q42896	*Solanum lycopersicum*	208	34.76/5.76	6	3	28	11	0.36	0.68
9*	Enolase	P26300	*Solanum lycopersicum*	291	47.79/5.68		5		14	0.50	0.52
10*	Oxygen-evolving enhancer protein 1	P23322	*Solanum lycopersicum*	423	34.98/5.91		12		34	2.00	0.45
11	NAC-alpha-like protein 3	Q6ICZ8	*Arabidopsis thaliana*	151	22.10/4.41	4	2	22.5	16	0.44	0.6
12*	Enolase	P26300	*Solanum lycopersicum*	181	47.79/5.68		4		15	0.41	0.52
13	Oxygen-evolving complex protein 2	P29795	*Solanum lycopersicum*	362	27.79/8.27	4	5	42.6	27	0.69	0.8
14	Translation initiation factor 5A-1	Q9AXQ6	*Solanum lycopersicum*	139	17.30/5.71	3	2	40.3	18	4.12	0.23

2.3.3 Resolution of cell wall proteins at basic *pI* range

2-D IEF/SDS-PAGE of cell wall proteins resulted in repetitive vertical streaking and poor focussing/resolution in the basic *pI* region even though the DeStreak reagent (GE Healthcare, Germany) was used to facilitate basic protein separation (Fig. 7: A). The problem of poor resolution was improved considerably by separating them further with SDS-PAGE in the 3rd dimension (Fig. 7: B, C and D). The 3-D gels were prepared for both genotypes and treatments in order to check the reproducibility. This simple method provided reproducible gels for comparative proteomic analysis. The visual comparison of the proteome of both resistant and susceptible genotypes in response to bacterial invasion revealed five and seven proteins of differential abundance (Fig. 7: C and D). They were identified by MS analysis as peroxidase (PR-9), β-1, 3-endoglucanase (PR-2) and osmotin like protein (PR-5) among others (Table 5). All of these PR proteins constitute important components of the disease resistance response. However, they were not considered for discussion due to lack of three biological replications.

Table 5. List of major basic stem cell wall proteins that were poorly resolved in 2-D IEF/SDS-PAGE but well separated in 3rd dimension SDS-PAGE.

*Notation (a-j) are given in foot note at the end of Table 6

Spot[a]	Identity[b]	Accession[c]	Organism[d]	Score[e]	*Mr*/*pI*[f]	Peptides[g]		Coverage[h]		SiP-SeP[j]
Hawaii7996 (resistant genotype)						MS	MS/MS	MS	MS/MS	
1	Peroxidase	Q94IQ1	*Nicotiana tabacum*	480	39.06/5.99	5	8	35.3	27	Y
2	Peroxidase prx14	Q9M4Z3	*Spinacia oleracea*	139	37.22/9.29	3	2	16	7.7	Y
3*	Peroxidase	Q07446	*Solanum lycopersicum*	299	35.99/7.52		5		17	Y
3*	Pectinesterase	Q43143	*Solanum lycopersicum*	280	64.10/8.97		6		9	Y
4	Glucan endo-1,3- β-glucosidase B	Q01413	*Solanum lycopersicum*	244	39.71/7.84	14	5	53.9	17	y
5	Osmotin-like protein precursor	Q41350	*Solanum lycopersicum*	404	27.26/8.15	8	6	55.2	29	Y

Table 5 continued

WVa700 (susceptible genotype)						MS	MS/MS	MS	MS/MS	
1*	Peroxidase	Q94IQ1	*Nicotiana tabacum*	391	39.06/5.99		6		14	Y
2	Glucan endo-1,3-β-glucosidase B	Q01413	*Solanum lycopersicum*	172	39.71/7.84	13	2	35.3	6.7	Y
3	Osmotin-like protein	Q41350	*Solanum lycopersicum*	284	27.26/8.15	15	2	55.2	10	Y
4	Photosystem I reaction center subunit IV B	Q41229	*Nicotiana sylvestris*	131	15.22/9.74	2	2	25.9	19	0.82
5	Oxygen-evolving complex protein 3	Q672Q6	*Solanum lycopersicum*	201	24.57/9.64	7	3	25.7	11	0.65
6	Oxygen-evolving complex protein 3	Q672Q6	*Solanum lycopersicum*	73	24.57/9.64	7	1	36.5	4.3	0.65
7	Photosystem I reaction center subunit II	P12372	*Solanum lycopersicum*	125	22.91/9.71	5	3	24	12	0.80

2.3.4 Prediction of secretion signals

Since the secretory pathway is involved in the biosynthesis of cell wall proteins and their transport to the cell wall, the presence of secretion signals in the above identified proteins was evaluated with SignalP and SecretomeP programs. Most of the proteins were predicted to have secretion signals (Table 2, 3, 4 and 5). The extracellular nature of cell wall proteins were further tested by random picking of 20 spots (Fig. 7B), 90% of which showed signal peptides (Table 6). However, some glycolytic and other metabolic proteins that are conventionally not considered as extracellular proteins were also found in the cell wall.

Table 6. Overview of stem cell wall proteins randomly picked from the 2-D gels.

Spot[a]	Identity[b]	Accession[c]	Organism[d]	Score[e]	*Mr*/*pI*[f]	Peptides[g]		Coverage[h]		SiP-SeP[j]
						MS	MS/MS	MS	MS/MS	
1	Catalase isozyme	P30264	*Solanum lycopersicum*	211	56.50/6.60	18	2	35.6	5.3	0.34
2	Peroxidase	Q07446	*Solanum lycopersicum*	149	35.99/7.52	6	5	29.6	16	Y
3	NADH-glutamate dehydrogenase	Q8W1X4	*Solanum lycopersicum*	257	44.68/6.28	20	3	52.3	12	0.56

Table 6 continued

4*	Peroxidase	Q42964	*Nicotiana tabacum*	124	34.52/ 4.65		4		4	Y
5	Glyceraldehyde 3-phosphate dehydrogenase	O04891	*Solanum lycopersicum*	42	31.94/ 5.93	4	1	17	4.8	0.39
6	Hypothetical protein	O24329	*Ricinus communis*	80	40.00/ 7.56	7	1	18.1	4.4	Y
7	Calreticulin	Q40401	*Nicotiana plumbaginifolia*	167	47.48/ 4.45	8	4	32.9	9.9	Y
8	Ripening regulated protein	Q9FR30	*Solanum lycopersicum*	152	22.20/ 4.72	17	2	78	15	0.73
9	Oxygen-evolving complex protein 1	P23322	*Solanum lycopersicum*	205	34.94/ 5.91	13	2	69.9	8.2	0.44
10*	Calmodulin	P84339	*Agaricus bisporus*	277	16.78/ 4.15		6			0.70
11	ATP synthase D chain	Q6L460	*Solanum demissum*	191	19.80/ 5.34	18	2	81	8.9	0.61
12	Oxygen-evolving complex protein 2	P29795	*Solanum lycopersicum*	292	27.79/ 8.28	6	4	52.7	19	0.80
13	Soluble inorganic pyrophosphatase	Q43187	*Solanum tuberosum*	70	24.26/ 5.59	7	2	25.1	12	0.79
14	Temperature-induced lipocalin	Q38JE1	*Solanum lycopersicum*	171	21.25/ 5.96	8	3	38.4	17	0.51
15	Superoxide dismutase (Cu-Zn) 1	P14830	*Solanum lycopersicum*	67	15.30/ 5.83	3	1	39.1	8.6	0.68
16	Dehydroascorbate reductase	Q4VDN8	*Solanum lycopersicum*	200	23.53/ 6.32	12	2	45.2	13	0.36
17	Hypothetical protein	Q5XEP2	*Arabidopsis thaliana*	67	64.52/ 5.85	4	3	8.8	7.5	0.58
18	Tomato invertase inhibitor	O82001	*Solanum lycopersicum*	62	18.76/ 8.30	7	2	38.6	15	Y
19	Translation initiation factor 5A-4	Q9AXQ3	*Solanum lycopersicum*	102	17.51/ 5.60	7	1	41.3	6.3	0.23
20	Nucleoside diphosphate kinase	Q2KK37	*Thlaspi caerulescens*	121	25.79/ 9.34	6	1	23.1	5	0.89

Notations given below are used in tables 2-6

[a] Assigned spot number corresponding to the number used in the respective figures.

[b] Identity of the proteins annotated by MALDI-TOF MS/MS

[*] Identity of the proteins revealed by LC-ESI-Ion Trap MS/MS

[c] Protein database accession number (UniProt)

[d] Plant species from which the protein was annotated

[e] Mowse (Molecular weight search) score

[f] Theoretical molecular mass and isoelectric point computed from ExPASy *Mr/pI* calculation tool

[g] Number of matched peptides with the corresponding protein in MSDB database

MS: by peptide mass fingerprinting method

MS/MS: by tandem mass spectrometer

[h] Percentage of peptide sequences coverage for the identified protein

MS: in peptide mass fingerprinting method

MS/MS: in tandem mass spectrometer

[i] Regulation: The fold increase or decrease in % spot volume of each spot after the *R. solanacearum* inoculation.

Variation: The ratio in the abundance of spot % spot volume between the genotypes

[j] The results of SignalP (SiP) and SecretomP (SeP) analysis.

Y: presence of signal peptide evaluated from SignalP

Secretom NN score calculated from SecretomP, in case no signal peptides were identified, and the NN score > 0.5 was considered as secretory protein as suggested by the author

2.4 Discussion

Various roles of CWPs, particularly their involvement in the regulation of growth and development, defence against biotic or abiotic stresses, and contribution to wall architecture are increasingly studied (Jamet et al. 2008a). Regulation of plant cell wall proteins following pathogen invasion have sparingly been reported (Bradley et al. 1992; Brisson et al. 1994). However, the comprehensive analysis of a broad range of CWPs expressed in response to bacterial inoculation is lacking (Chivasa et al. 2005). Therefore, the current study was initiated firstly to find out the differences at the proteome level between susceptible and resistant genotypes and followed by the simultaneous characterization of mid-stem cell wall protein profiles that are regulated in susceptible and resistant tomato plants due to *R. solanacearum* ingress. Due to the reported expression of bacterial wilt resistance in the mid-stem of tomato and the time needed by *R. solanacearum* to reach and grow extensively in the stem after soil inoculation, the cell wall proteome was analyzed from the mid-stem and at 5 dpi (Dahal et al. 2009). The specificity of proteins to one of the genotypes and subsequent up or down regulation of the identified protein profiles after pathogen inoculation further elucidates the resistant and susceptible reactions of the genotypes.

2.4.1 Expression of plant defense mechanisms

We observed the up regulation of several defense related proteins both in susceptible and resistant genotypes at 5 dpi after pathogen inoculation. The susceptible plants responded by

increasing the expression of endochitinase (PR-3) and PR-5 family proteins whereas the resistant plants showed the up regulation of NDPK and subtilase. The abundance of peroxidase (PR-9) was elevated in both genotypes. When plants are challenged with pathogens, the general defense responses are generally inducted by the synthesis of PR proteins and fortification of plant cell walls among others responses.

PR proteins can be constitutively presents in different plant species at low level, however, they are increased dramatically upon challenge with pathogens and abiotic stresses. Plant chitinase, the majority of which are of the endo type, are believed to mediate defence responses because of their potential to degrade fungal cell walls. Many endochitinase also displayed a lysozyme activity enabling the hydrolysis of bacterial cell walls (Brunner et al. 1998). Acidic endochitinase was also implicated in the modulation of mechanical properties of the cell wall in addition to their defense roles (Yokoyama and Nishitani 2004). PR-5 family proteins contain several unique proteins with diverse functions. Some of them were reported to have antifungal capacity (Ibeas et al. 2000) as well as β-glucanase activity (Grenier et al. 1999), and were involved in the signal transduction pathway (Yun et al. 1998). Many PR-5 protein isoforms (PR-5a to PR-5d) were accumulated in the extracellular space of tobacco plant cells (Koiwa et al. 1994). PR proteins are considered as the general markers for basal defense response induction and hence increased in susceptible reactions too. Therefore, the timing and kinetics of their expression may be decisive for the outcome of host-pathogen interactions.

NDPK not only performs a house keeping functions of regulating nucleotide pools but also involved in signal transduction in plants. *NDPK1* expression, induced by a variety of stresses including bacterial infection, reported to enhance multiple stress tolerance in transgenic plants by activating the MAPK cascade (Tang et al. 2008). The *NDPK1* gene has also been associated with cell growth and division in potato (Dorion et al. 2006). Subtilisin-like serine proteases or subtilases are endoproteases secreted into the extracellular space of the plant. Plants subtilases have been associated with cellular defense and stress responses by mediating a restructuring and reinforcement of plant cell walls in order to arrest pathogen spread (Dixon

and Lamb 1990). They are also proposed to be involved in the maturation of CWPs, generation of active peptides in the cell wall and signaling cascades (Boudart el al. 2005).

Plant peroxidases are class III secretory peroxidases with a large number of isoforms performing a wide range of functions. In many plant species, increase in peroxidases expression was correlated with resistance due to their involvement in the production of reactive oxygen species (ROS), the fortification of the cell wall structure, and synthesis of secondary metabolites, and consequently controlling the penetration and cellular spread of the pathogen (Passardi et al. 2005). Cationic peroxidises are involved in cell wall biosynthesis except monolignols polymerization (Hiraga et al. 2001).

The up regulation of these PR and other defense related proteins in plant stem cell wall in response to bacterial invasion could support their so far known physiological roles in plant defense. Both the tolerant and susceptible plants showed the uprising of the defense related proteins however with different functional roles suggesting their involvement in a generalized resistance response to *R. solanacearum*.

Interestingly, three other defense and/or stress related proteins, namely BIP, stress induced protein1 and catalase, were down regulated in resistant plants after pathogen invasion. In fact, BIP was observed in two spots (Fig. 8: spot 1 and 8) and regulated in opposite ways. BIP is a member of the hsp70 family protein which contains signal peptides for their translocation through the endoplasmic reticulum (ER) membrane. In addition to their roles as molecular chaperones in protein processing, import and subsequent maturation in ER, they have been implicated in disease resistance. The up regulation of this protein after interaction with a pathogen is in support of this hypothesis. However, Shen et al. (2003) observed the down regulation of calreticulin, which has molecular chaperone function, in the rice leaf sheath after wounding. Therefore, it is still unclear how the BIP can be related to the defense response. Stress induced protein STI1 may play a role in mediating the heat shock response of some hsp70 genes (Nicolet and Craig 1989). Catalase has been demonstrated to be present in the plant cell wall and probably detoxifies H_2O_2 produced therein (Olson and Varner 1993). The down regulation of catalase, the primary antioxidant, in the plants can be expected following pathogen inoculation if the elevation of H_2O_2 and ROS level serves as second

messengers to further trigger the downstream defense responses (Foyer and Noctor 2005). Furthermore, increased level of ROS, but not excessive, can act directly to suppress the pathogens. An increase in H_2O_2 with consequent reduction of catalase and superoxide dismutase activities was observed in jute under water stress (Roy Chowdhuri and Choudhuri 1985).

Both up and down regulation of these defense related proteins indicate the reactions of the plants to pathogen infection by regulating the expression of their resistance proteins. Nonetheless, the temporal and spatial regulation of resistance responses and hence the efficacy are the most decisive factors determining the susceptibility or resistance to the given pathogen.

2.4.2 Change in cell wall metabolism

The analysis of the cell wall proteome revealed cell wall metabolism proteins such as XTH7, α-galactosidase, and GRP in susceptible plants, all of which were down regulated in response to pathogen invasion. Both XTH and α-galactosidase are carbohydrate modifying proteins and belong to the glycoside hydrolases (GHs) family which are generally involved in reorganization/reconstruction of cell wall polysaccharides during active development, defense, signaling, and mobilization of storage reserves (Minic 2008). XTHs can exhibit both endo-glycanase and endo-transglycosylase activities and have a potential to modify architecturally complex plant cell wall, by allowing cell expansion and incorporating xyloglucans into the wall, both during wall synthesis and disease responsive fortification processes (Fry 2004). The enzyme α-galactosidase, generally acidic forms, is common in plants and is also suggested to have transglycosylase actions (Soh et al. 2006). Down-regulating α-galactosidase was shown to enhance freezing tolerance in transgenic Petunia at the whole plant level (Pennycooke et al. 2003). GRPs are plant cell wall structural proteins characterized with high content of glycine (20-70%) and are localized in lignified cell walls. Members of a group of GRPs have a signal peptide and were suggested to play a role in cell wall reinforcement or in signal transduction of pathogen-induced defense responses in addition to their roles in the development of vascular tissues, wound healing, and dead xylem wall repairing (Ryser et al. 1997, Park et al. 2001, Lin et al. 2005). It has been suggested that

the GRP is possibly involved in enhancement of wall flexibility and mechanical strength in specific physiological processes (Chen et al. 2007). Down regulation of GRP due to water stress was reported earlier where it has been argued that remodeling of the cell wall as part of the plant defense response not only requires accumulation of pathogen restricting proteins, but also the reduction of some proteins that are more suitable for cell wall function during normal conditions (Harrak et al. 1999). Additionally, oxidative cross linking of GRP occurring during pathogen invasion can lead to their suppression (Bradley et al. 1992). The observance of GRP in two different spots and reduction of both isoforms indicates the significance of a possible post translation modification (PTM) which could be crucial in the outcome of reactions. The lowered abundance of both cell wall hydrolases and GRP due to bacterial inoculation in our study may reflect the decline of cell wall polysaccharide metabolism and mechanical stability in susceptible plants during disease expression.

2.4.3 Metabolic activities alteration

2.4.3.1 Variation in primary metabolism

Proteins associated with glycolysis such as fructokinase, TPI and enolase were differentially regulated in the two genotypes. Fructokinase and TPI were correspondingly up regulated in resistant and susceptible lines while enolase was down regulated in resistant plants. The glycolytic pathway not only supplies carbohydrates for respiratory and biosynthetic pathways during plant growth (Dorion et al. 2005) but also replenishes the increased demands of carbohydrate fuels arisen as a result of stress conditions such as pathogen invasion. The activated defense responses caused by pathogen attack require extra energy, reducing powers, and metabolites and the increased abundance of fructokinase and TPI during bacterial infection can be the consequences of increased metabolism to compensate for the cost of resistance reactions (Curto et al. 2006). Additionally, soluble carbohydrates are known to control the expression of various metabolic and defense-related genes via sugar sensing (Rolland et al. 2006). The abundance of PII like protein which is considered to participate in metabolic regulatory mechanism and in signaling the status of carbon and nitrogen was also increased in resistant varieties (Hsieh et al. 1998). On the other hand, the expression of enolase was suppressed in resistant plants in response to bacterial challenge. Enolase

catalyzes the penultimate reversible reaction of glycolysis, however, their exact function in the cell wall is still unclear. It was identified as a major glucan-associated cell wall protein in *Candida albicans*, as well as in Arabidopsis, and Medicago cell walls (Angiolella et al. 2002, Lee et al 2004, Watson et al. 2004) even though it lacks signal peptides. The reduction of glycolytic enzymes in response to pathogen attack could be to suppress the growth of the pathogen by limiting the supply of sugars. In fact, the multiplication of bacteria was repressed in the resistant plant compared to susceptible one. Glycolysis inhibition would also be a mechanism for accumulating sugars as an energy source for recovery during or after pathogen attack. In overall, regulation of these metabolic proteins in both genotypes after pathogen challenge supports the observation that the plant defense responses are associated with active metabolic changes in host plants.

2.4.3.2 Suppression of energy metabolism

Both OEE 2 and V-ATPases sub unit E were down regulated in resistant reactions. OEE is associated with the photosystem II complex, (PSII) and is composed of three proteins namely OEE1 (PsbO), OEE2 (PsbP) and OEE3 (PsbQ) (Raymond and Blankenship 2004). They are believed to be important for efficient water splitting required for photosynthesis and overall PSII stability. Any fluctuation in the activity of PSII will affect photosynthesis (Ruban et al. 2003), photodamage (Ohnishi et al. 2005), and photoinhibition (Silva et al. 2003) and therefore, any damage or inhibition of PSII due to stresses could lead to suppression of OEE proteins. Down regulation of OEC proteins was reported earlier due to water stress and tobacco mosaic virus infections (Lehto et al. 2003, Echevarría-Zomeño et al. 2009). It has been proposed that a reduction in photosynthetic activity could modulate defense responses by dissipating excessive ROS and mitigating photooxidative damage (Zeier et al. 2004, Moreno et al. 2005). Nevertheless, their precise mechanism of regulation is unknown. OEE2 has been implicated in photosynthetic oxygen evolution required in plant respiration. It was demonstrated that OEE2 interacts with and acts as a substrate for WAK1, cell wall-associated kinases in the AtGRP-3/WAK1 signalling pathway (Yang et al. 2003).

V-ATPases are multimeric enzymes composed of peripheral V_1 and integral V_0 domain containing at least eight (A-H) and five (a-d) different subunits respectively. They can be

associated with various membranes of the secretory system and functions in the acidification of endomembrane compartments and energization of many solute transport processes including reactions required for osmoregulation, homeostasis, storage, and plant defense. They also promote cell growth or expansion and secretion of cell wall components at the plasma membrane (Cipriano et al. 2008).

Reduction of energy metabolism proteins in our analysis is in line with previous reports (Castillejo et al. 2004). It is assumed that root inoculation of tomato plant with *R. solanacearum* could inhibit the root carbohydrate oxidation pathways leading to the decrease in the overall energy production.

2.4.4 Variation in other proteins

The abundance of PDIs was also reduced after pathogen invasion in susceptible plants. PDIs are oxidoreductases are involved in the folding, assembling and sorting of plant secretory or plasma membrane proteins via ER which is essential for the stability and activity of extracellular proteins (Wilkinson and Gilbert 2004). PDIs were secreted to the cell surface of animal cells, by an unknown mechanism (Turano et al. 2002). The induction of PDI against fungal infection was reported in resistant lines of wheat (Ray et al. 2003). Therefore, the down regulation of PDI may contribute to the colonization of bacteria and establishment of disease in the susceptible plants rather than to the defensive state.

Four spots identified as eukaryotic translation initiation factor 5A (eIF-5A) were repressed in both susceptible (eIF-5A-1 and eIF-5A-4) and resistant (eIF-5A-3 and eIF-5A-4) interactions. Eukaryotic translation initiation factor is a multigene family protein and functions not only as a conventional translation initiation factor but also as a nucleo-cytoplasmic shuttle protein (Jao and Chen 2005). It is known to be activated posttranslationally and involved in RNA metabolism and trafficking, thereby regulating cell division, cell wall expansion and cell death (Thompson et al. 2004). In Arabidopsis, plant eIF5A was involved in the development of disease symptoms induced by bacterial phytopathogen and the down-regulation of AteIF5A-2 suppressed bacterial growth and disease symptoms in susceptible interactions

(Marianne et al. 2008). Both isoforms of eIF-5A were repressed in both genotypes suggesting the possible importance of their PTMs during host-pathogen interaction.

2.4.5 Constitutive differences in tomato genotypes

Proteomic variations between the two genotypes differing in the degree of bacterial wilt tolerance followed by their expression to bacterial inoculation could provide important information regarding the resistance mechanisms. Interestingly, many of the proteins were constitutively more specific to either genotype. BIP, enolase, fructokinase-2, OEE2, and NAC-α-like protein 3 occurred in higher abundance in resistant plants while α-galactosidase, peroxidase, FNR, OEE1, and eIF-5A-1 in susceptible plants.

The resistant genotype displayed the higher abundance of glycolytic proteins such as enolase observed in three different spots (Fig. 8: spot 2, 9 and 12) and fructokinase-2 than the susceptible one. It was suggested that the maintenance of metabolic proteins such as glycolytic enzymes is important to fulfill the increased carbohydrate "fuel" demand required during pathogen invasion, and for the recovery, too. Moreover, resistant plant showed the elevation of NAC-α-like protein 3 which could assist in the prevention of disordered metabolism because the decreased NAC-α-like protein leads to mistargeting, mistranslation, and proteolysis of proteins by affecting overall NAC function (Yan et al. 2005). Defense and stress related proteins also showed constitutive differences among the genotypes. BiP that functions as molecular chaperones was in higher abundance in resistant varieties where as multifunctional peroxidase and α-galactosidase in susceptible plants. The peroxidase has been associated with the reinforcement of cell wall, while α-galactosidase in the modifications of cell wall both during development and defense. It should also be noted that an increase or decrease in the abundance of protein spots can also occur by selective decay of either protein in addition to synthesis or suppression of the protein. The susceptible genotypes also differed from the resistant one in the expression of FNR, which is considered as detoxifying agents due to its free radical scavenging ability and also participates in the $NADP^+$ photoreduction and nitrate assimilation pathways. Both genotypes showed the abundance of OEE but were differed in the variation only in their isoforms. The increased abundance of OEE proteins is considered to improve the photosynthetic efficiency which could contribute to fight off the

pathogen infection upon challenge. Several proteins were identified in multiple spots such as enolase, OEE, BIP, GRP, and eIF-5A and their isoforms were regulated in the same way (Fig. 8, 9 and 10; Table 2, 3 and 4). However, the differences in the protein isoforms may indicate that the protein modifications could be a crucial phenomenon in determining the genetic status of the plants. The correlation between the elevation of eIF-5A to the development of disease symptoms leads to the assumption that susceptible plant are more prone to bacterial attack due to the higher expression of eIF-5A-1 (Marianne et al. 2008). Based on the above finding, it can be argued that the metabolic proteins in addition to defense and stress related proteins, play crucial roles in determining the resistance or susceptibility of the plants. They are abundant and soluble proteins too and hence often appeared in 2-D gels.

2.4.6 Nature of cell wall proteins

The presence of secretion signals that are characteristic to all extracellular proteins were predicted with SignalP and SecretomP. Among 42 differentially expressed proteins, 29 were identified to have signal peptides and ten more were predicted to posseses non-classical secretion signals (Fig. 8, 9 and 10; Table 2, 3 and 4). Further 20 proteins from 2-D gel (Fig. 7B) and 12 proteins from the comparative 3-D gels (Fig. 7: C and D) were tested for their secretory nature and 30 of them contain secretion signals (Table 5 and 6). The overall results showed that most of the extracted cell wall proteins contain signals for extracellular localization. However, some proteins generally predicted to have other than extracellular locations, such as glycolytic proteins, V-ATPase, OEE, eIF-5A, BIP, enolase, catalase and others were detected in the cell wall fraction. Growing evidences suggest the possibility of them being “moonlighting” proteins due to the evidence of their localization in the extracellular space. These so called “moonlighting” proteins perform more than one function in the cell as a consequence of changing their cellular localization, oligomeric state, or ligand concentration (Copley 2003). For example, enolase is also supposed to have additional non-glycolytic functions such as mitochondrial targeting of tRNA (Entelis et al. 2006). Therefore, further validations with immunolocalization or other methods would be suggested.

2.4.7 Resolution of basic proteins

We showed the use of simple 3rd dimension SDS-PAGE to separate the basic *pI* range proteins, that are often components of cell wall proteins, in a resolution enough for the comparative gel based proteomic analysis. The 3-D gels from both genotypes and treatments consistently resolved the basic range of cell wall proteins (Fig. 7: C and D). The identification of 12 major spots from 3-D gels showed the presence of proteins with theoretical *pI* higher than 7 (Table 5).

2.5 Conclusion

To conclude, the current study provides for the first time the broad spectrum analysis of the stem cell wall proteome of tomato genotypes followed by their specific regulation after bacterial challenge. It unveiled constitutive proteomic differences between tomato genotypes differing in resistance to bacterial wilt, and the differential regulation of their respective protein profiles in response to *R. solanacearum* invasion which further extends the understanding of the molecular basis of the host-pathogen interactions. The selective differential expression of defense/stress related and metabolic proteins in both resistant and susceptible genotypes triggered by the pathogen support their pivotal roles in the make up of generalized defense mechanism. Though, clear statements on the role of the proteins can only be made after their functional analysis and the demonstration of their role in susceptible and resistant genotypes. The work further supports the hypothesis that the resistance mechanism is a complex interplay of several proteins where their activation kinetics might play more pivotal role than their number and type in the outcome of the host-pathogen interaction. Further experiments determining the physiological roles of each of the regulated proteins will help elucidating the resistance or susceptibility mechanisms.

CHAPTER 3: High-throughput expression profiling of xylem sap proteome of susceptible and resistant tomato genotypes revealed networks of metabolic, defense as well as cell wall related and signalling proteins

Adapted from the manuscript prepared for submission

Abstract

Xylem sap has been considered as the primary conduit for water and minerals translocation from roots to aerial parts of the plant. Though, evidences of presence of proteins are already established, the comprehensive proteome profile of xylem sap is still at infancy. Herein, we described xylem protein profiles of healthy adult tomato plants collected under root pressure exudate. The complete gel lane obtained after protein separation in 1-D polyacrylamide gradient gel was divided into small gel pieces. The peptides were separated with nano-HPLC before identifying the corresponding proteins with MALDI-TOF/TOF MS. Large number of proteins were identified which comprises several physiologically important groups such as cell wall metabolism proteins; proteases; groups of defense related proteins including peroxidase, PR and resistance proteins; detoxifying proteins; signalling and transport proteins; transcription factors; and various metabolic enzymes. The presence of peroxidase, cell wall associated proteins, proteases, and defense related proteins, that were reportedly conserved in many plants, indicates their involvement in xylem growth, development, and differentiation essential for the functional xylem conduit formation. The occurrence of many signalling and transport proteins is expected for root to shoot communication. Numerous proteins of unknown functions may provide candidates with novel physiological functions. The xylem sap not only contained secretory proteins but also proteins without secretion signal. The comparison of xylem proteins between the susceptible and resistant plants demonstrated a relatively high number of proteins in susceptible genotype including signalling and transcription related proteins but lower percentage of defense related proteins, peroxidase, proteins degradation/modification enzymes and metabolic proteins.

Key words: Tomato/ Xylem sap proteome/ LC MS/MS/ Secretory proteins/ Defense related proteins/Cell wall metabolic proteins.

3.1 Introduction

Terrestrial plants grow in diverse environmental conditions, and the growth and development of functionally specialized plant organs is dependant on internal communications and the balanced allocation of water and nutrients throughout the plant (Kolek and Kozinka 1991). In vascular plants, the function of translocation is principally mediated by two specialized long-distance transport systems, the xylem and phloem which interconnect all organs. The phloem transports predominantly the photosynthates from the site of synthesis to the consumption site, whereas the xylem sap carries mainly water and dissolved minerals from the soil system to aboveground plant parts (De Boer and Volkov 2003). The ability of the aerial plant parts to photosynthesize and function is primarily influenced by the supply of dissolved materials through the soil system (Gibson 2004, Alabadí et al. 2008). Therefore, the conduction and composition of xylem sap is considered the key determinant of the physiological states and activities of aerial organs.

Xylem and phloem are heterogeneous tissues comprising the vascular bundles but are grown on opposite sides of the cambium layer. Among four different tracheary elements of xylem tissue, only xylem tracheid and tracheae (also called vessel element) are primarily involved in water-nutrient translocation (Carlsbecker and Helariutta 2005). The tracheids are relatively more primitive and are elongated dead cells with pointed end-plates that connect cell to cell, whereas vessels are comparatively shorter and wider, and lack end plates. During functional specialization, both tracheids and vessels become hollow, non-living, water-conducting pipelines due to the disintegration of cells and their contents (Fukuda 2000). The unidirectional xylem transport in terrestrial plants is driven by the pressure gradient where a transpiration pull contributes to the bulk flow but also by root pressure during high water potentials and low transpiration conditions (Tyree and Zimmermann 2002). The mineral-containing water in soil enters the plant through the apical part of the roots, moves via the apoplast, transmembrane and symplast pathways until the endodermis, and then through the xylem vessels to aerial organs.

Xylem sap was initially considered to carry only inorganic ions, however growing evidences showed that it also contains and transports organic compounds such as amino acids, sugars,

and organic acids (Satoh 2006). Moreover, the identification of information molecules that possibly play an important role in root to shoot communication such as proteins, and mRNAs together with secondary metabolites and hormones increased the interest (Alvarez et al. 2008). The occurrence of proteins in xylem sap has been reported in many plants such as watermelon, apple, peach, pear, cucumber, squash, rice and tomato (Alvarez et al. 2006). Xylem sap composition was modified during its transport from the roots and throughout the plant (Djordjevic et al. 2007). Since xylem conducting elements loose nuclei, functioning ribosomes, and cytoplasmic contents during functional maturation, they are not equipped for transcription and translation necessary to synthesize proteins by themselves (Fukuda 2000). The identification of proteins in the sap, therefore, draws the attention regarding their origin, function, and fate. Apart from few reports, most of the studies demonstrated only few major proteins such as peroxidases, chitinases, a glycine-rich protein, a cysteine-rich protein, and a 30 kDa lectin in the sap (Kehr et al. 2005). Therefore, the aim of the present study was to provide a comprehensive overview of the proteins that are present in the xylem sap of healthy tomato plants differing in resistance to bacterial wilt in order to understand the underlying cellular processes taking place within the xylem conduit system. The study also provides a basis for the comparative analysis of the xylem proteome regulated in tomato genotypes in response to invasion with xylem colonizing bacteria, *R. solanacearum* which in turn offer a broader overview of our proteome level knowledge of the tomato-*R. solanacearum* interaction that had been studied earlier at whole mid-stem as well as its cell wall level (Dahal et al. 2009). The proteome profiling was performed by separating total soluble sap proteins in 1-D SDS polyacrylamide gradient gel followed by in-gel tryptic digestion of whole protein, separation of the peptides through nano-HPLC and protein identification by MALDI-TOF/TOF MS.

3.2 Materials and Methods

3.2.1 Plant materials and growth conditions

Tomato plants were grown from the two parental lines Hawaii7996 and WVa700, highly disease resistant and susceptible genotypes against bacterial wilt, respectively. Seeds were obtained from AVRDC, Taiwan.

Tomato plants were grown in the greenhouse for 4 weeks (20°C, 14 h photoperiod per day, 30K lux and 70% RH). Each plant was then transferred to an individual pot with approximately 330 g of soil (Fruhstorfer Erde, type P: 150 mg/L N, 150 mg/L P_2O_5, and 250 mg/L K_2O) and grown in the climate chamber (30/28°C day/night temperature, 14 h photoperiod, 30 K lux, and 85% RH).

3.2.2 Xylem sap collection, concentration and precipitation

Xylem sap was collected from stems of 6 week-old tomato plants under root pressure exudates system. Tomato stems of both susceptible and resistant genotypes were cut perpendicular to the stem axis at about 5 cm above the root. The decapitated stumps on the root side were washed with distilled water and blotted dry with filter paper. The first drop of xylem sap was discarded to remove possible contaminations from wounded cells and its contents (Buhtz et al. 2004). The detopped stems were fitted with silicon tubes and sap continuously oozing out from the cut surface inside tubes was regularly pipetted out. Xylem sap was collected for the period of 4 h over ice and stored at -20°C until further analysis.

Due to negligible amount of protein, the xylem sap was concentrated in a centrifugal concentrator with a molecular mass cut off at 5 kDa at 5000 rpm x 4°C x 2-3 h (Vivaspin 6, Vivascience, Germany). The amount of protein in the concentrated sap was measured with Bradford assay by mixing 5 µL of sample with 795 µL of H_2O and 200 µL of Bradford reagent (Coomassie protein assay reagent, Fluka biochemical) and measured after 5 min at 595 nm. A standard calibration curve was prepared with bovine serum albumin from 0 to 20 µg protein. The concentrate was precipitated using the chloroform-methanol-water method (Wessel and Flügge 1984). The volume of sample was mixed with 4:1:3 volumes of methanol, chloroform, and water respectively, and the solution was vortexed and centrifuged

at 13000 x g for 2 min at 4°C. Three volume of methanol was added after discarding the upper water phase and centrifuged at 13000 x g for 3 min at 4°C. The supernatant was removed and the pellet was air-dried.

3.2.3 Polyacrylamide gradient gel electrophoresis

A discontinuous mini gel of 10 cm x 8 cm x 1 mm was prepared by first pouring a 8.5–18% linear gradient resolving gel (pH 8.8) with help of a gradient maker overlaid by a 5% stacking gel (pH 6.8). Approximately 80-100 µg of protein was denatured and solubilised in 40 µL of 1X SDS sample buffer at 95°C for 5 min, followed by incubation with 5 µL of 40 % acrylamide at RT for 30 min. The sample was then loaded into the gel along with the protein size standard and run at 65 V, 0.01 A/10 min; 100V/20 min and at 200 V until the tracking dye reaches the bottom of the gel. The protein bands were visualized by Coomassie staining (0.1% CoomassieR-250, 40% methanol, and 10% acetic acid) at 47°C for 15 min. De-staining of the gel was done at 47°C with 30% methanol containing 10% acetic acid until a clear background appeared.

3.2.4 In-gel digestion with trypsin

The whole protein lane was divided into 8-10 pieces along the axis of the band containing approximately an equal amount of protein. Protein destaining was done with 250 µL of 50% v/v ACN in 50 mM NH_4HCO_3, pH 7.8 (1:1 v/v) at 37°C and shaking at 400 rpm for 30 min. The gel slices were then dehydrated in 250 µL of 100% ACN and dried in a speed vac system (Eppendorf, Germany). Trypsin was added to the dry gel pieces in a concentration of 4 ng/µL in 25 mM NH_4HCO_3, pH 7.8 containing 10% ACN and samples were incubated on ice for 60 min. Excess trypsin solution was removed and 15 µL of 25 mM NH_4HCO_3, pH 7.8 containing 10% ACN was added. The protein was digested overnight at 37°C. The proteolytic reaction was stopped by adding 100 µL of 0.2% TFA in 10% ACN at RT for 10 min and corresponding peptides were extracted. Peptides were re-extracted two more times, combined and dried in speed vac.

3.2.5 LC-MS/MS analysis

Peptide samples were separated in a nano-HPLC system by reversed phase chromatography using a C18 trap column (PepMap 300 µm x 5 mm, 3 µm, 100 Å, Dionex) with a flow rate of 30 µL/min and a C18 separation column (PepMap, C18 reversed phase material, 75 µm x 150 mm, 3 µm, 100 Å, Dionex) with a flow rate of 250 nL/min. Peptides were separated using eluent A (5% ACN in 0.1% TFA) and eluent B (80% ACN in 0.1% TFA) with a gradient from 16-18% eluent B in 27 minutes, 18-24% in 40 min, 24-40% in 64 min and 40-80% in 10 min. Fractions of 90 nL were spotted using a microfraction collector (Probot) directly onto a pre-spotted MALDI target plate with a collinear sheath flow of 2.5 µL/min 5% ACN, 0.1% TFA aqueous solution. To prepare the MALDI target plate 0.6 µL of a matrix solution (4 mg/mL CHCA in 50% ACN, 0.1% TFA and 10 mM $NH_4H_2PO_4$, diluted 1:4 with ethanol) had been spotted on each spot of an Anchor target plate (600/384 Bruker Daltonik). 0.3 µL of peptide calibration standard (Bruker Daltonik) was used for external calibration. Re-crystallization was done with 0.2 µL of 0.1% TFA in ethanol. MS and MS/MS spectra were generated in an Ultraflex TOF/TOF I (Bruker Daltonik) mass spectrometer.

3.2.6 Data analysis

For protein identification a MACOT search (version 2.1) was performed. Experimental MS/MS-spectra were matched with the MSDB database (Matrix Science, London, UK) released in April 27th, 2007. Search parameters for mass tolerance were set to 100 ppm for precursor ions and 0.7 Da for fragment ions with one allowed missed trypsin cleavage. Data base hits were taken, if the peptide ion score was above 25 and proteins were identified if at least two peptides could be identified.

Due to the incomplete genome sequence of *Solanum lycopersicum*, a lot of excellent MS/MS data generated could not be related to tomato proteins and were not included. However, several MS/MS spectra fit exactly to proteins from other Solanacaeae or plants. Probably those database hits are due to sequence similarities between the identified (foreign) proteins and the so far unsequenced tomato proteins and are also listed.

3.2.7 Prediction of secretory proteins

The presence of N-terminal signal peptides that anchor the protein for extracellular location was determined with SignalP 3.0 (Bendtsen et al. 2004b), whereas, non-classical secretory signal sequences were evaluated with SecretomP 2.0 (Bendtsen et al. 2004a).

3.3 Results and Discussion

For the first time, a comprehensive proteome screening analysis has been carried out for tomato xylem sap revealing as many as 208 proteins (Table 7). Owing to the several limitations of 2-D SDS-PAGE (López 2007), the xylem sap proteins were analyzed by a combined approach consisting of protein separation by 1-D gradient SDS-PAGE followed by reversed phase separation on peptide level and protein identification using tandem mass spectrometry (Figure 11: A and B).

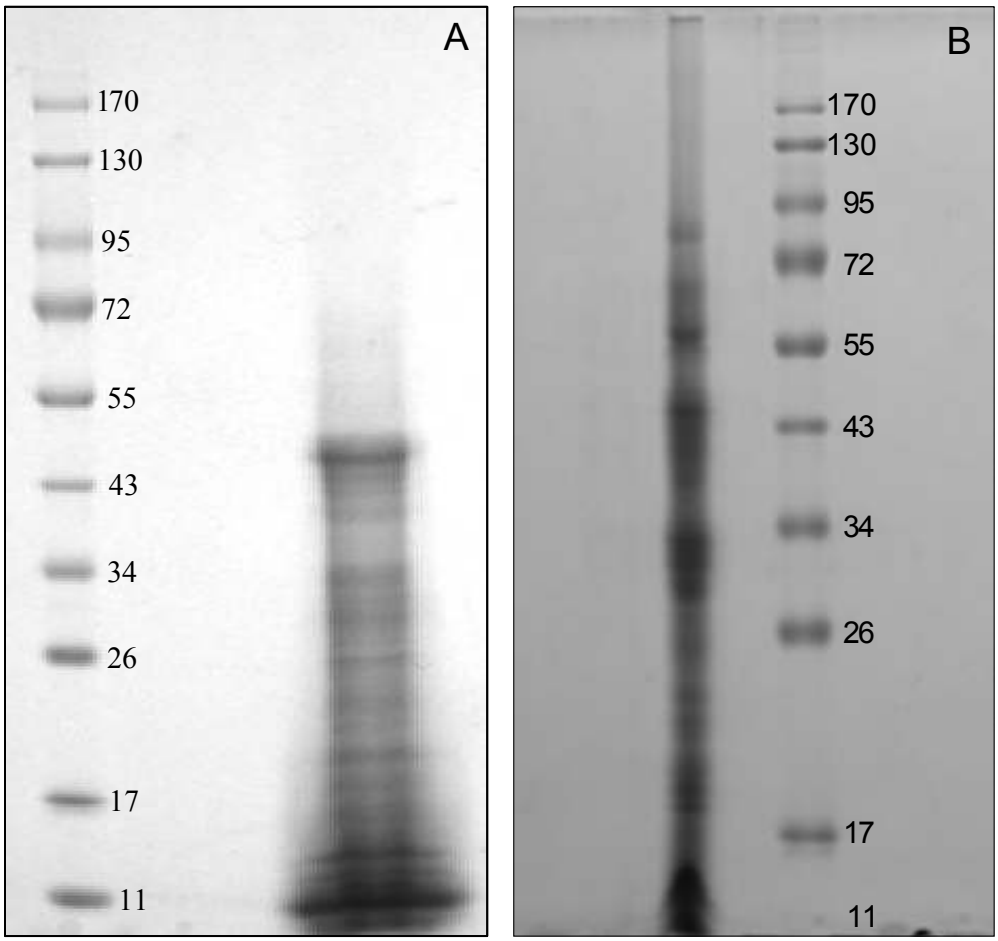

Figure 11. Overview of the xylem sap protein extracted from healthy tomato plants and separated in 1-D polyacrylamide gradient gels with 8.5-18% resolving gel over laid by 5% stacking gel.

About 80-100 µg of protein was loaded in each gel and were visualized with Coomassie-R staining. The molecular mass was represented by numbers in kDa besides the gel lane. The complete gel lane of each genotype were divided into 8-10 sections and sequentially analyzed with LC-MS/MS

A Xylem sap proteins in WVa700 (susceptible genotype) healthy plants and identified protein lists is presented in table 7.

B Xylem sap proteins in Hawaii7996 (resistant genotype) healthy plants and identified protein lists is presented in table 8.

Due to the low sequence coverage of the tomato genome, only few tomato proteins could be identified with the classical data base dependent protein identification tools like mascot and sequest. Several high quality spectra could not be related to tomato proteins. However, many MS/MS spectra were related to peptides and proteins from other plant species of the Solanaceae family or of plants like *Oryza sativa* or *Arabidopsis thaliana* which genomes have already been completely sequenced. Thus, it is tempting to assume that the identification of foreign proteins identified by tomato MS/MS data sets is due to the presence of orthologues in both organisms that share sequence identity. The majority of the identified proteins were correlated with *Oryza sativa* (32%) and *Arabidopsis thaliana* (16%), and 9% with tomato (list of proteins in Table 7 and 8). Based on the physiological role of the identified proteins, they were categorized into several putative functional groups (Fig 12; Table 7 and 8). The major groups appeared correspondingly in susceptible and resistant genotypes were peroxidase (20% and 27%), cell wall related proteins (9% and 9%), metabolic enzymes (8% and 9%), proteases (5% and 13%), defense related proteins (7% and 21%), transcription (8% and 7%), signalling proteins (6% and 4%), transport proteins (2% and 0%), transposable element proteins (4% and 0%), and hypothetical proteins (26% and 4%). The comparison of the xylem proteins between the resistant and susceptible genotypes disclosed fairly high number of proteins in susceptible plants. Even though, 80-100 µg protein was analysed in each genotype, the susceptible plants showed the presence of 208 proteins compared to 84 in resistant lines. The further look on both protein lists revealed higher percentage of defense related proteins that includes several PR proteins, peroxidase, detoxifying enzymes and resistance proteins in resistant plants. Enzymes belong to protein degradation and modifications were also in higher proportion. On the other hand, the susceptible plants clearly showed the higher occurrence of proteins involved in signal transduction and cellular communication as well as transcription related proteins. Even though metabolic proteins were in higher proportion in resistant plants, transport proteins, photosynthetic, secondary metabolic and retroelements proteins were not identified in these plants.

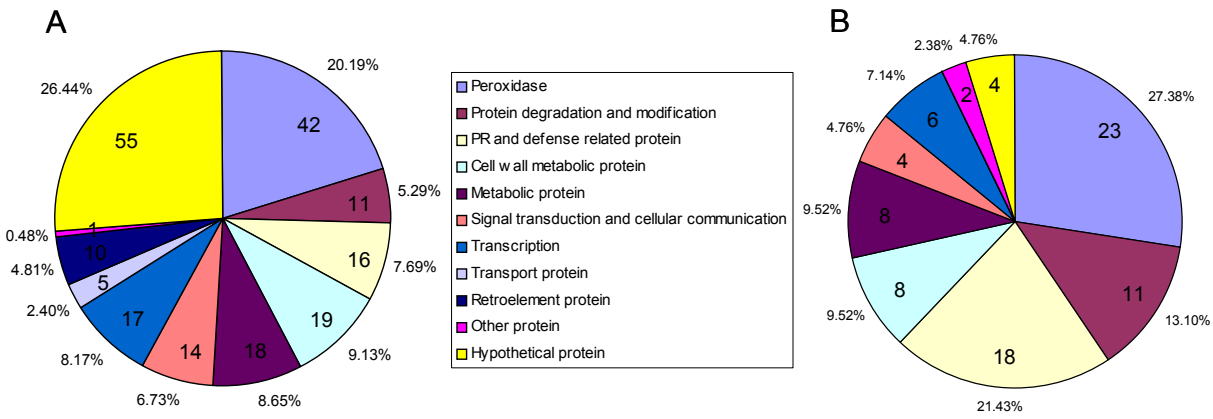

Figure 12. Comparative pie diagrams showing 11 putative functional classes of the xylem sap proteins identified in tomato genotypes.

The actual number of proteins identified is shown inside whereas their respective percentage value outside of the pie chart. The transport protein and retroelement protein groups were not identified in resistant plants. The comparative percentage value of the remaining protein groups showed higher occurrence of peroxidase, protein degradation and modification protein, PR and defense related protein and metabolic proteins in resistant genotype while proteins of signal transduction and cellular communication as well as transcription related proteins were in susceptible genotype

A: Xylem sap proteins in WVa700 (susceptible genotype) and the list of proteins are given in table 7.

B: Xylem sap proteins in Hawaii7996 (resistant genotype) and the list of proteins are given in table 8.

Table 7. List of proteins identified in the xylem sap of Wva700 (susceptible genotype) healthy plants (Fig. 11A).

They were separated by 1-D SDS-PAGE and analyzed with LC-MS/MS as described in materials-methods part. Proteins are categorized based on their putative functions.

Protein name[a]	Accession[b]	Organism[c]	*Mr/pI*[d]	Score[e]	Peptide[f]	SiP[g]	SeP[h]
Peroxidase							
Peroxidase precursor	Q6T1C8	*Quercus suber*	35.78/9.55	46	1	Yes	
Anionic peroxidase swpb3	Q5JBR1	*Ipomoea batatas*	34.27/9.50	102	1	Yes	
Peroxidase 68	Q9LVL1	*Arabidopsis thaliana*	35.62/9.49	93	4	Yes	
Peroxidase	Q5W5I3	*Picea abies*	37.31/9.47	40	1	Yes	
Peroxidase	Q9XIV9	*Nicotiana tabacum*	34.34/9.40	116	1	Yes	
Class III peroxidase	Q7XYR7	*Gossypium hirsutum*	35.42/9.33	43	1	Yes	
Peroxidase 3	Q9XFL4	*Phaseolus vulgaris*	35.01/9.32	94	1	Yes	
Anionic peroxidase swpb2	Q5JBR2	*Ipomoea batatas*	36.84/9.31	172	2	Yes	
Peroxidase prx14	Q9M4Z3	*Spinacia oleracea*	37.22/9.29	46	1	Yes	
Peroxidase	Q84ZT6	*Asparagus officinalis*	32.07/9.23	43	1	No	0.658
Peroxidase	Q9ZRG5	*Glycine max*	35.23/9.19	43	1	Yes	
Peroxidase	Q27U89	*Eucalyptus globulus*	28.89/9.18	129	2	No	0.864
Peroxidase prx15	Q9M4Z2	*Spinacia oleracea*	36.94/9.14	79	1	Yes	
Peroxidase P7	P00434	*Brassica rapa*	31.08/9.10	49	1	No	0.713
Class III peroxidase 49	Q5U1P4	*Oryza sativa*	37.95/9.04	44	1	Yes	
Peroxidase	Q4W2V2	*Picea abies*	34.06/9.00	41	1	Yes	
Peroxidase	Q58GF4	*Populus alba*	33.38/8.89	42	1	Yes	
Peroxidase	Q84ZT7	*Asparagus officinalis*	33.93/8.80	43	1	Yes	
Putative peroxidase	Q2LGJ7	*Musa accuminata*	19.41/8.67	43	1	No	0.762
Cationic peroxidase	Q41324	*Stylosanthes humilis*	33.84/8.65	43	1	Yes	
Peroxidase PX3	Q52QY2	*Manihot esculenta*	39.35/8.53	48	1	Yes	
Peroxidase POA1	Q4A3Y5	*Capsicum annuum*	31.87/8.43	51	1	No	0.418
Class III peroxidase 3	Q9LI45	*Oryza sativa*	36.36/8.28	43	1	Yes	
Peroxidase 2	Q9SSZ8	*Scutellaria baicalensis*	34.58/8.09	42	1	Yes	
Peroxidase	Q43782	*Linum usitatissimum*	38.19/8.07	51	1	Yes	
Peroxidase	P93548	*Spinacia oleracea*	35.28/7.57	44	1	Yes	
Cationic peroxidase	Q2WEC9	*Solanum lycopersicum*	35.85/7.55	163	5	Yes	

Table 7 continued

Peroxidase 25	O80822	*Arabidopsis thaliana*	35.88/7.52	43	1	Yes	
Peroxidase	Q07446	*Solanum lycopersicum*	35.99/7.52	317	6	Yes	
Peroxidase	P93551	*Spinacea oleracea*	33.18/7.13	45	1	No	0.735
Peroxidase	Q96512	*Arabidopsis thaliana*	37.74/6.94	40	1	Yes	
Peroxidase	Q50LG4	*Nicotiana tabacum*	38.52/6.10	42	1	Yes	
Putative peroxidase	Q8LMR6	*Oryza sativa*	33.47/5.71	46	2	Yes	
Putative peroxidase	Q6YZD5	*Oryza sativa*	35.73/5.60	43	1	Yes	
Putative peroxidase	Q5QQS8	*Zinnia elegans*	14.22/5.41	44	1	No	0.399
Anionic peroxidase	Q5GMM6	*Capsicum chinense*	30.97/5.37	171	3	Yes	
Putative peroxidase	Q8GVP1	*Oryza sativa*	35.90/5.18	40	1	Yes	
Peroxidase	Q6V2C9	*Orobanche cernua*	26.09/5.14	44	1	No	0.625
Suberization-associated anionic peroxidase 1	P15003	*Solanum lycopersicum*	38.74/4.91	353	6	Yes	
P17	Q40878	*Petunia hybrida*	21.97/4.90	42	1	No	0.8
Peroxidase	Q9LWA2	*Solanum lycopersicum*	34.94/4.56	42	1	Yes	
Peroxidase	Q43055	*Populus Kitakamiensis*	34.17/4.44	67	3	Yes	
Protein degradation and modification							
Carboxypeptidase type III	Q8L6A7	*Theobroma cacao*	56.52/5.10	57	1	Yes	
Serine carboxypeptidase-like	P52712	*Oriza sativa*	47.78/5.12	77	1	No	0.711
Serine carboxypeptidase II-2 chain B	P55748	*Hordeum vulgare*	48.95/6.01	46	1	No	0.64
Subtilisin-like protease	O82777	*Solanum lycopersicum*	82.22/8.22	114	4	Yes	
Subtilisin-like protease1	P93204	*Solanum lycopersicum*	81.35/6.17	69	2	Yes	
Ulp1 protease	Q5Z7R1	*Oryza sativa*	30.18/5.47	45	1	No	0.923
Ulp1 protease	Q5ZCW2	*Oryza sativa*	21.28/5.07	45	1	Yes	
Chloroplast nucleoid DNA-binding protein	Q94K53 *At1g09750	*Arabidopsis thaliana*	39.77/8.44	62	1	No	0.725
Lipid transfer family protein	Q8LBY9 *At5g05960	*Arabidopsis thaliana*	12.44/9.30	106	2	Yes	
Early responsive to dehydration 1	Q94C10 *At5g51070	*Arabidopsis thaliana*	69.61/6.21	50	1	No	0.488
Lipid transfer family protein	Q7XTF6	*Oryza sativa*	11.68/9.00	53	1	Yes	
PR and defense related protein							
Putative basal resistance related chitinase	Q4ZFU8	*Nicotiana tabacum*	10.66/4.82	77	1	No	0.715
Putative RGH1A	Q6Z021	*Oryza sativa*	111.62/6.10	41	1	No	0.283

Table 7 continued

Putative endochitinase B	Q5W1I6	*Nicotiana glauca*	15.50/8.70	61	1	No	0.754
Class I chitinase	O81144	*Solanum tuberosum*	35.41/6.57	72	1	Yes	
Class I chitinase	O81145	*Solanum tuberosum*	35.33/5.66	54	2	Yes	
β-1,3-glucanase-like protein	Q8H0I0	*Nicotiana tabacum*	38.50/5.38	47	1	Yes	
Germin-like protein	Q5VJG4	*Nicotiana attenuata*	23.29/9.08	158	2	Yes	
Germin-like protein	O65358	*Solanum tuberosum*	23.23/8.79	161	2	Yes	
Germin-like protein 2	Q0MYQ7	*Vitis vinifera*	22.73/8.53	118	1	Yes	
Germin-like protein	Q5DT23	*Capsicum annuum*	23.19/8.54	154	3	Yes	
Superoxide dismutase (Cu-Zn)	P27082	*Nicotiana plumbaginifolia*	15.23/5.47	70	1	Yes	
Superoxide dismutase (Cu-Zn)	Q58ZE5	*Manihot esculenta*	15.11/5.42	50	1	No	0.647
Putative tocopherol polyprenyltransferase	Q6ZLA8	*Oryza sativa*	41.45/9.99	48	1	Yes	0.554
Gamma-glutamyltransferase	Q8VYW6 *At4g39640	*Arabidopsis thaliana*	61.18/9.53	41	1	Yes	
Selenium-binding protein	Q8GSH3	*Oryza sativa*	77.82/6.21	40	1	No	0.543
Putative CDR1	Q5VRD5	*Oryza sativa*	45.90/4.88	47	1	Yes	
Cell wall metabolic protein							
Polygalacturonase-like protein	Q84LI7	*Fragaria ananassa*	51.53/7.52	74	1	Yes	
Polygalacturonase	Q153G1	*Eucalyptus globulus*	26.54/5.40	74	1	No	0.444
Putative hydroxyproline-rich glycoprotein	Q69S58	*Oryza sativa*	65.92/9.33	40	1	No	0.233
Cellulose synthase 2	Q7XB33	*Gossypium barbadense*	64.76/9.11	46	1	No	0.155
Expansin-like protein	Q7XHJ2	*Quercus robur*	28.80/7.62	42	1	Yes	
Erwinia induced protein 1	Q84XG7	*Solanum tuberosum*	38.29/5.61	55	1	Yes	
Pectinesterase	O04870	*Solanum lycopersicum*	56.24/8.59	131	2	No	0.551
Putative β-galactosidase	Q9LLT0	*Solanum lycopersicum*	93.24/6.80	107	3	Yes	
β-galactosidase 14	Q7XFK2	*Oriza sativa*	90.07/5.90	45	1	Yes	
β-galactosidase	Q4QYX3	*Mangifera indica*	92.13/5.07	44	1	Yes	
Lipase-like protein	Q8GS76	*Oriza sativa*	42.01/8.68	51	1	Yes	
UDP-glucuronic acid decarboxylase	Q6I683	*Oryza sativa*	48.70/9.23	41	1	SA	
Glycosyltransferase protein 2-like	Q6K8F2	*Oryza sativa*	104.29/9.35	44	1	SA	
Galacturonosyltransferase 11	Q949Q1 *At1g18580	*Arabidopsis thaliana*	61.87/8.10	46	1	SA	

Table 7 continued

Putative phragmoplast-associated kinesin-related protein 1	Q6K765	*Oryza sativa*	122.89/5.45	42	1	No	0.249
Microtubule-binding protein TANGLED1	Q9FUH9	*Zea mays*	40.94/12.03	49	2	SP	
MAP kinase kinase	Q94EV7	*Zea mays*	44.66/10.60	41	1	No	0.322
Putative inosine-uridine preferring nucleoside hydrolase	Q6L553	*Oryza sativa*	100.13/6.40	42	1	No	0.507
Rcd1-like cell differentiation family protein	Q8L8C5 *At2g32550	*Arabidopsis thaliana*	32.33/6.20	41	1	No	0.626
Metabolic protein							
Carbohydrate metabolism							
Ribose-phosphate pyrophosphokinase 2	Q69XQ6	*Oryza sativa*	43.08/8.77	46	1	SP	
Soluble starch synthase II-2	Q6Z2T8	*Oryza sativa*	75.62/6.04	45	1	SP	
Energy and nitrogen metabolism							
ATP synthase subunit alpha	Q3ZML0	*Cantua buxifolia*	42.57/7.09	42	1	No	0.485
FMO family protein	Q9FWW6 *At1g12160	*Arabidopsis thaliana*	53.27/6.23	45	1	SA	
Arginine decarboxylase	O24549	*Vitis vinifera*	68.31/5.31	41	1	No	0.626
3-phosphoshikimate 1-carboxyvinyltransferase	Q30CZ8	*Fagus sylvatica*	55.49/7.52	50	1	No	0.443
Methionine synthase	Q4H1G2	*Beta vulgaris*	87.80/6.05	46	2	No	0.455
Methionine synthase	Q9LM03	*Solanum tubersom*	84.66/5.93	127	3	No	0.45
Copper amine oxidase	Q8H1H9 *At1g62810	*Arabidopsis thaliana*	80.13/5.98	42	1	Yes	
Delta 1-pyrroline-5-carboxylate synthetase B	Q9AXN3	*Brassica napus*	78.73/6.85	46	1	No	0.328
Photosynthesis							
Oxygen-evolving enhancer protein 2	P29795	*Solanum lycopersicum*	27.79/8.27	56	1	No	0.802
Geranylgeranyl hydrogenase	O81335	*Mesembryanthemum crystallinum*	51.41/8.95	42	1	No	0.693
Photosystem II subunit T	Q67HZ1	*Iris missouriensis*	4.04/10.00	40	1	SA	
PAC3 protein	Q39175	*Arabidopsis thaliana*	36.14/4.88	42	1	SP	
Secondary metabolism							
Flavanone-3-hydroxylase	Q6R3N2	*Gypsophila paniculata*	40.94/5.30	41	1	No	0.326
Chalcone synthase	Q9FW79	*Oryza sativa*	49.90/8.99	42	1	No	0.222
Putative cinnamoyl CoA reductase	Q6L5E8	*Oryza sativa*	41.25/7.56	42	1	SA	
Putative cytochrome p450	Q6Z9D5	*Oryza sativa*	59.79/9.21	42	1	SA	

Table 7 continued

Signal transduction and cellular communication							
Putative β- transducin-like protein	Q7XA22	*Solanum bulbocastanum*	68.33/6.48	42	1	No	0.434
Calmodulin	P93087	*Capsicum annuum*	16.83/4.10	41	1	No	0.636
Phototropin	Q401Q4	*Mougeotia scalaris*	90.83/5.72	41	1	No	0.338
Leucine rich repeat family protein	Q53PD8	*Oryza sativa*	111.67/7.56	41	1	No	0.306
Leucine-rich repeat protein	Q96477	*Solanum lycopersicum*	24.18/5.71	78	2	Yes	
Receptor protein kinase like protein	O49445 *At4g28350	*Arabidopsis thaliana*	72.06/5.90	48	1	Yes	
Putative receptor protein kinsase PERK1	Q6K6B7	*Oryza sativa*	75.07/6.95	43	1	No	0.802
S-locus receptor kinase	Q5QH07	*Raphanus sativus*	32.64/5.46	43	1	No	0.354
Putative lipid transfer protein	Q8H9B7	*Solanum tuberosum*	9.87/9.44	61	1	Yes	
Armadillo repeat-containing protein-like	Q8GSZ9	*Oryza sativa*	72.69/6.57	45	1	No	0.329
ACC oxidase	O65378 *At1g12010	*Arabidopsis thaliana*	36.53/5.09	44	1	No	0.275
Transducin family protein	Q3E9H4 *At5g15550.2	*Arabidopsis thaliana*	44.16/5.83	44	1	No	0.553
Phosphatidylinositol-glycan class N	Q9SGH9 *At3g01380	*Arabidopsis thaliana*	103.95/6.93	44	1	SA	
GPI-anchored protein	Q9SUC9 *At4g28100	*Arabidopsis thaliana*	33.09/8.93	42	1	Yes	
Transcription							
DNAJ heat shock N-terminal domain-containing protein	Q84TH2 *At4g19570	*Arabidopsis thaliana*	62.94/9.09	43	1	No	0.231
DNA-directed RNA polymerase	Q1L6V7	*Sphagnum spec.*	73.25/8.53	44	1	No	0.358
DNA-directed RNA polymerase subunit alpha	P60315	*Physcomitrella patens*	49.46/5.47	48	1	No	0.43
Transcription factor-like	Q6EPG4	*Oryza sativa*	86.75/5.11	41	1	SP	
Putative transcriptional regulator	Q9SFG5	*Arabidopsis thaliana*	129.93/6.11	41	1	No	0.316
Heat shock factor 1b	Q4L0F7	*Medicago sativa*	55.35/4.73	41	1	No	0.335
Methyl-CPG-binding domain 9	Q9SGH2 *At3g01460	*Arabidopsis thaliana*	240.43/5.34	51	1	No	0.222
Pentatricopeptide repeat containing protein	Q9SAK0 *At1g79490	*Arabidopsis thaliana*	94.17/9.18	41	1	No	0.35
Probable integrase	Q1S5K2	*Medicago truncatula*	34.17/9.29	41	1	No	0.433
Zinc finger family protein	Q10RY0	*Oryza sativa*	75.80/6.41	48	1	No	0.361
Zinc finger-like	Q69TX4	*Oryza sativa*	35.23/4.73	41	1	No	0.393
Putative F-box protein	Q9LRZ2 *At3g16820	*Arabidopsis thaliana*	34.21/7.09	49	3	No	0.264

Table 7 continued

Auxin-responsive protein IAA5	P33078	*Arabidopsis thaliana*	18.35/6.37	40	1	No	0.58
Phytochrome B	A2XFW2	*Oryza sativa*	128.45/5.97	41	1	No	0.471
OSJNBa0043L24.18 protein	Q7XM99	*Oryza sativa*	38.54/8.54	41	2	No	0.36
OSJNBa0042L16.12 protein	Q7XUT8	*Oryza sativa*	37.29/8.46	41	1	No	0.26
OSJNBa0058K23.9 protein	Q7XTU3	*Oryza sativa*	35.76/8.74	42	1	No	0.263
Transport protein							
Major intrinsic protein PIPc	O23772	*Craterostigma plantagineum*	20.95/9.84	44	1	SP	
Putative cyclic nucleotide binding transporter 1	Q6ZHE3	*Oryza sativa*	88.35/9.08	43	1	No	0.806
Putative P-type II calcium ATPase	Q70TF1	*Physcomitrella patens*	121.62/6.24	40	1	No	0.606
MtN20 protein	O24098	*Medicago truncatula*	26.30/5.75	48	1	No	0.346
Putative vesicle transfer ATPase	O81459 *At4g04180	*Arabidopsis thaliana*	58.96/4.98	45	1	Yes	
Retroelement protein							
Transposon protein,CACTA, En/Spm sub-class	Q2QVP6	*Oryza sativa*	17.98/9.27	43	1	No	0.809
Transposon protein, unclassified	Q2QZM0	*Oryza sativa*	99.20/8.62	42	1	No	0.43
Retrotransposon protein, Ty3-gypsy subclass	Q53KK1	*Oriza sativa*	25.23/11.51	42	1	No	0.673
Retrotransposon protein,Ty3-gypsy subclass	Q2QPA0	*Oryza sativa*	32.54/8.27	44	1	No	0.532
Putative retroelement pol polyprotein	Q9SKF9 *At2g12920	*Arabidopsis thaliana*	98.67/9.84	62	2	No	0.168
Retrotransposon protein, Ty1-copia sub-class	Q7XBV6	*Oryza sativa*	39.75/7.19	40	1	No	0.673
Retrotransposon protein, unclassified	Q53N41	*Oryza sativa*	102.10/5.36	42	1	No	0.368
Expressed protein	Q2QT26	*Oryza sativa*	59.24/11.16	46	1	No	0.266
Transposable element	Q9T0D8 *At4g11710	*Arabidopsis thaliana*	55.50/9.48	42	1	No	0.406
Transposable element	Q6NMD3 *At2g06190	*Arabidopsis thaliana*	16.69/9.64	40	1	No	0.806
Other protein							
Phaseolin	P80463	*Phaseolus lunatus*	47.95/5.40	44	1	Yes	
Hypothetical protein							
Hypothetical protein	Q10P78	*Oryza sativa*	38.75/6.37	41	1	No	0.744
Hypothetical protein	Q53L56	*Oryza sativa*	14.08/10.52	42	1	No	0.67
Hypothetical protein	Q2R1Y3	*Oryza sativa*	86.31/8.14	46	1	No	0.41
Hypothetical protein	Q8LIW2	*Oryza sativa*	32.00/11.10	41	1	No	0.436

Table 7 continued

Hypothetical protein	Q69T50	*Oryza sativa*	47.90/11.57	46	1	No	0.84
Hypothetical protein	Q7XM53	*Oryza sativa*	19.44/10.61	42	1	No	0.401
Hypothetical protein	Q7XPT6	*Oryza sativa*	25.26/6.00	44	1	SP	
Hypothetical protein	Q53MH3	*Oryza sativa*	16.06/10.28	41	1	No	0.635
Hypothetical protein	Q5JLY6	*Oryza sativa*	19.35/5.93	45	1	No	0.32
Hypothetical protein	Q5N769	*Oryza sativa*	58.50/8.85	40	1	No	0.582
Hypothetical protein	Q5N7R0	*Oryza sativa*	12.43/12.27	45	1	No	0.592
Hypothetical protein	Q5Z648	*Oryza sativa*	34.61/12.16	49	1	No	0.404
Hypothetical protein	Q5Z6H9	*Oryza sativa*	15.69/11.71	42	1	No	0.476
Hypothetical protein	Q5Z6V0	*Oryza sativa*	11.11/11.36	40	1	No	0.871
Hypothetical protein	Q5Z8T0	*Oryza sativa*	8.49/10.74	45	1	No	0.737
Hypothetical protein	Q65X91	*Oriza sativa*	29.86/9.31	55	2	SA	
Hypothetical protein	Q65XH3	*Oryza sativa*	14.51/11.61	41	1	No	0.359
Hypothetical protein	Q69KG8	*Oriza sativa*	36.29/9.24	48	2	No	0.432
Hypothetical protein	Q6EPK5	*Oryza sativa*	16.74/12.36	46	1	No	0.492
Hypothetical protein	Q6H6P6	*Oryza sativa*	24.40/5.15	42	1	No	0.144
Hypothetical protein	Q6K242	*Oryza sativa*	24.50/11.67	42	1	No	0.451
Hypothetical protein	Q6K607	*Oryza sativa*	12.77/11.44	51	1	No	0.706
Hypothetical protein	Q6K8C9	*Oryza sativa*	19.95/11.47	44	1	No	0.592
Hypothetical protein	Q6YY27	*Oryza sativa*	34.35/12.11	41	1	No	0.502
Hypothetical protein	Q6ZKP4	*Oryza sativa*	37.26/11.69	41	1	SP	
Hypothetical protein	Q6ZKS8	*Oryza sativa*	10.19/12.18	48	1	No	0.711
Hypothetical protein	Q7XQE1	*Oriza sativa*	41.57/11.35	48	1	No	0.379
Hypothetical protein	Q8RZX3	*Oryza sativa*	28.34/8.75	45	1	No	0.265
Hypothetical protein	Q8W5H3	*Oryza sativa*	17.31/9.58	50	1	No	0.392
Hypothetical protein	Q9SNL4	*Oryza sativa*	17.12/11.65	48	1	No	0.74
Hypothetical protein	Q5N7C1	*Oryza sativa*	38.27/11.65	42	1	No	0.328
Hypothetical protein	Q5SNE3	*Oryza sativa*	35.72/11.54	41	1	No	0.422
Hypothetical protein	Q5VP73	*Oryza sativa*	28.35/10.53	40	1	No	0.306
Hypothetical protein	Q6K2X5	*Oriza sativa*	12.23/4.72	42	1	No	0.64
Hypothetical protein	Q6YXI2	*Oryza sativa*	27.63/4.67	66	2	No	0.661
Hypothetical protein	Q8SAV2	*Oryza sativa*	19.27/10.22	46	1	No	0.232
Hypothetical protein	Q9AV28	*Oryza sativa*	14.21/9.50	42	1	No	0.702
Hypothetical protein	Q6ZLH1	*Oryza sativa*	25.53/10.76	41	1	SP	
Hypothetical protein	Q7XKB8	*Oryza sativa*	52.24/9.58	40	1	Yes	
Hypothetical protein	Q7XPX5	*Oryza sativa*	43.99/5.03	41	1	No	0.585
Hypothetical protein	Q7XSW0	*Oryza sativa*	97.37/5.48	63	2	No	0.288
Hypothetical protein	Q5NA76	*Oryza sativa*	54.03/11.43	43	1	Yes	
Hypothetical protein	Q7XPF0	*Oryza sativa*	97.34/5.74	41	1	No	0.336
Unknown protein	Q9C9V2 *At1g67860	*Arabidopsis thaliana*	8.30/9.50	41	1	No	0.397
Unknown protein	Q3EC39 *At2g09388	*Arabidopsis thaliana*	17.06/6.29	42	1	No	0.493
F6D8.1 protein	Q9SSS8	*Arabidopsis thaliana*	4.51/10.01	42	1	No	0.82
Hypothetical protein	Q1EPB8	*Musa accuminata*	14.87/9.55	40	1	No	0.476
Hypothetical protein	Q2HSH5	*Medicago truncatula*	11.37/10.30	52	1	No	0.865

Table 7 continued

Hypothetical protein	Q9XEP3	*Sorghum bicolor*	157.21/8.48	42	1	No	0.137
Hypothetical protein	Q657J8	*Oryza sativa*	22.58/4.47	41	1	No	0.877
Hypothetical protein	Q9LYC4	*Arabidopsis thaliana*	30.86/6.22	42	1	No	0.517
Dentin sialophospho protein	Q9FGR1 *At5g52530	*Arabidopsis thaliana*	92.41/6.5	42	1	No	0.651
Similar to F-box family protein	Q58G02 *At2g30615	*Arabidopsis thaliana*	22.63/9.05	45	1	No	0.572
F-box domain containing protein	Q10KX1	*Oryza sativa*	48.04/9.45	45	1	No	0.629
Hypothetical protein	Q8S5G4	*Oryza sativa*	19.44/11.65	61	2	Yes	

Table 8. List of proteins identified in the xylem sap of Hawaii7996 (resistant genotype) healthy plants (Fig. 12B).

They were separated by 1-D SDS-PAGE and analyzed with LC-MS/MS as described in materials and methods part. Proteins are categorized based on their putative functions.

Protein name[a]	**Accession**[b]	**Organism**[c]	***Mr/pI***[d]	**Score**[e]	**Peptide**[f]	**SiP**[g]	**SeP**[h]
Peroxidase							
Peroxidase 68	Q9LVL1	*Arabidopsis thaliana*	35.62/9.49	114.12	2	Yes	
Peroxidase	Q5W5I3	*Picea abies*	37.31/9.47	44.13	1	Yes	
Peroxidase 73	Q43873	*Arabidopsis thaliana*	35.92/9.44	55.04	2	Yes	
Peroxidase	Q9XIV9	*Nicotiana tabacum*	34.37/9.40	135.86	4	Yes	
Peroxidase 3	Q9XFL4	*Phaseolus vulgaris*	35.01/9.32	72.72	2	Yes	
Anionic peroxidase swpb2	Q5JBR2	*Ipomoea batatas*	36.84/9.31	62.60	2	Yes	
Peroxidase	Q27U89	*Eucalyptus globulus*	28.89/9.18	51.67	2	No	0.864
Peroxidase	Q4W2V2	*Picea abies*	34.06/9.00	62.03	1	Yes	
Peroxidase 3	Q9SSZ7	*Scutellaria baicalensis*	33.90/8.87	40.58	2	Yes	
Putative peroxidase	Q948Z3	*Solanum tuberosum*	35.84/8.53	365.16	6	Yes	
Peroxidase	Q4KXC3	*Helianthus annuus*	10.34/8.06	40.58	2	No	0.723
Peroxidase cevi16	Q4A3Y6	*Solanum lycopersicum*	31.74/7.71	372.41	7	No	0.439
Bacterial-induced class III peroxidase	Q8RVP4	*Gossypium hirsutum*	35.14/7.55	49.20	2	Yes	
Cationic peroxidase	Q2WEC9	*Solanum lycopersicum*	35.85/7.55	397.94	8	SA	

Table 8 continued

Peroxidase	Q07446	*Solanum lycopersicum*	35.99/7.52	638.13	13	Yes	
Peroxidase	P93551	*Spinacia oleracea*	33.18/7.13	56.81	1	No	0.735
Peroxidase	Q94IQ1	*Nicotiana tabacum*	39.06/5.99	93.18	3	Yes	
Peroxidase1C	Q43791	*Medicago sativa*	38.27/5.80	49.61	3	Yes	
Anionic peroxidase	Q5GMM6	*Capsicum chinense*	30.97/5.37	107.16	3	Yes	
Suberization-associated anionic peroxidase 1	P15003	*Solanum lycopersicum*	38.75/4.91	363.11	6	Yes	
Peroxidase Cevi-1	Q9LWA2	*Solanum lycopersicum*	34.94/4.56	77.08	1	Yes	
Peroxidase	Q43055	*Populus kitakamiensis*	34.17/4.44	41.84	3	Yes	
Peroxidase ATP29a	Q53YQ3	*Arabidopsis thaliana*	37.23/4.41	61.20	1	Yes	
Protein degradation and modification							
Serine carboxypeptidase	P52712	*Oryza sativa*	47.79/5.12	48.63	1	No	0.711
Serine carboxypeptidase 3	P21529	*Hordeum vulgare*	56.36/5.85	42.39	1	Yes	
Serine carboxypeptidase-like 47	Q9FFB0	*Arabidopsis thaliana*	56.54/7.06	41.05	1	Yes	
Ubiquitin I2	Q3E7K8 *At1g55060.1	*Arabidopsis thaliana*	25.84/6.24	110.92	3	No	0.496
Ubiquitin monomer protein	Q2VJ43	*Morus mongolica*	17.03/6.75	76.42	2	No	0.526
Putative polyubiquitin	Q6KFR8	*Arabidopsis thaliana*	28.17/9.10	74.19	2	No	0.433
Polyubiquitin	P93135	*Fragaria ananassa*	42.68/7.00	69.92	2	No	0.398
Chloroplast nucleotide DNA binding protein	Q94K53 *At1g09750	*Arabidopsis thaliana*	39.77/8.44	60.86	1	No	0.725
Lipid Transfer family protein	Q8LBY9 *At5g05960	*Arabidopsis thaliana*	12.44/9.30	141.39	3	Yes	
Lipid transfer family protein	Q8LBY9 *At5g05960	*Arabidopsis thaliana*	12.44/9.30	110.69	5	Yes	
PR and defense related protein							
PR2 protein	P32045	*Solanum lycopersicum*	16.02/8.53	275.34	3	Yes	
Resistance protein RPP5	O49470	*Arabidopsis thaliana*	193.48/8.63	43.50	1	No	0.358
Calmodulin	P93087	*Capsicum annuum*	16.83/4.10	130.45	1	No	0.636
Polygalacturonase inhibitor protein	Q2P9N7	*Capsicum annuum*	28.81/7.10	61.09	2	No	0.676
β-1,3-glucanase-like protein	Q9FUN5	*Capsicum annuum*	24.48/8.61	52.06	1	No	0.488
Glucan endo-1,3-β-D-glucosidase	Q42890	*Solanum lycopersicum*	37.86/9.68	98.62	2	Yes	

Table 8 continued

Putative basal resistance related chitinase	Q4ZFU8	*Nicotina tabacum*	10.64/4.82	105.51	1	No	0.715
Chitinase	Q7Y0S1	*Solanum lycopersicum*	27.66/5.93	62.31	1	Yes	
Class I chitinase	O81145	*Solanum tuberosum*	35.42/6.57	98.19	2	Yes	
Endochitinase protein	CAA02125	*Solanum lycopersicum*	32.56/8.95	97.93	2	Yes	
Basic endochitinase	Q05537	*Solanum lycopersicum*	26.68/8.46	71.35	1	No	0.72
Germin-like protein	Q5DT23	*Capsicum annuum*	23.19/8.54	51.93	1	Yes	
Nectarin-1	Q9SPV5	*Nicotiana plumbaginifolia*	24.76/7.71	114.67	1	Yes	
Superoxide dismutase (Cu-Zn) 1	P14830	*Solanum lycopersicum*	15.30/5.83	169.72	3	No	0.686
Superoxide dismutase (Cu-Zn)	Q58ZE5	*Manihot esculenta*	15.11/5.42	82.22	2	No	0.647
Superoxide dismutase (Cu-Zn)	P27082	*Nicotiana plumbaginifolia*	15.23/5.47	72.64	1	Yes	
Syringolide-induced protein 14-1-1	Q8S901	*Glycine max*	28.73/9.92	42.40	1	No	0.337
NBS-LRR-like protein cD7	Q9ZSN3	*Phaseolus vulgaris*	93.40/5.94	40.94	1	No	0.459
Cell wall metabolic protein							
Acid invertase	Q1KL65	*Solanum tuberosum*	70.68/5.69	82.17	1	SA	
6G-fructosyltransferase	Q5FC15	*Asparagus officinalis*	68.31/5.48	82.17	1	Yes	
Putative beta-galactosidase	Q9LLT0	*Solanum lycopersicum*	93.24/6.80	81.73	1	SA	
Glucan endo-1,3-β-D-glucosidase	Q42890	*Solanum lycopersicum*	37.86/9.68	372.48	6	Yes	
α-L-arabinofuranosidase	Q76LU4	*Solanum lycopersicum*	74.15/5.30	54.66	2	Yes	
Expansin-like protein	Q7XHJ2	*Quercus robur*	28.8/7.62	44.59	1	Yes	
Kinesin-like protein NACK2	Q8S949	*Nicotiana tabacum*	107.22/8.46	41.44	2	No	0.315
Metabolic protein							
Carbohydrate metabolism							
Fructose-bisphosphate aldolase	Q38HV4	*Solanum tuberosum*	38.43/8.52	172.57	3	No	0.421
Fructose-bisphosphate aldolase	Q2PYX3	*Solanum tuberosum*	38.61/7.51	160.65	3	No	0.383
Enolase	P26300	*Solanum lycopersicum*	47.79/5.68	93.72	2	No	0.515
Energy and nitrogen metabolism							
Malate dehydrogenase	Q645N1	*Solanum lycopersicum*	36.15/8.87	96.08	1	No	0.664
Methionine synthase	Q42662	*Solenostemon scutellarioides*	84.59/6.09	43.68	1	No	0.487

Table 8 continued

Methionine synthase	Q9LM03	*Solanum tuberosum*	84.66/5.93	239.11	5	No	0.45
Oxygen-evolving complex protein 2	P29795	*Solanum lycopersicum*	27.79/8.27	80.76	2	No	0.802
Cytochrome c	P00059	*Abutilon theophrasti*	12.03/9.70	55.1	1	No	0.737
Cytochrome b5	P49098	*Nicotiana tabacum*	14.97/4.89	74.24	1	No	0.654
Signal transduction							
Putative MEK kinase	Q6ZI89	*Oryza sativa*	80.03/9.23	45.59	1	No	0.643
MAP kinase WNK2	Q8S8Y9	*Arabidopsis thaliana*	65.32/5.16	40.37	2	No	0.241
Putative GTP-binding protein	Q56YJ4	*Arabidopsis thaliana*	72.63/6.29	50.71	1	Yes	
Calmodulin binding protein	Q8L7V5 *At3g52870	*Arabidopsis thaliana*	51.28/8.69	43.94	1	No	0.347
Trnscription							
DNA topoisomerase II	Q2L363	*Malus domestica*	164.87/7.56	41.71	1	No	0.132
DNA topoisomerase II	Q8GSC4	*Nicotiana tabacum*	166.42/6.36	41.71	1	No	0.111
DNA-directed RNA polymerase subunit beta	Q7YJY0	*Calycanthus fertilis*	156.09/9.36	43.10	2	No	0.335
Maturase K	Q7YIX9	*Panax stipuleanatus*	59.35/9.58	41.35	1	No	0.358
Modifier of rudimentary protein	Q10QR4	*Oryza sativa*	26.65/8.54	48.28	1	No	0.703
Pentatricopeptide	Q10N26	*Oryza sativa*	91.51/9.43	40.29	1	No	0.372
Other protein							
Patatin-like protein 3	Q9FZ08	*Nicotiana tabacum*	45.12/7.70	106.15	3	Yes	
Patatin-like protein 2	Q9FZ07	*Nicotiana tabacum*	22.41/9.36	71.60	2	No	0.682
Hypothetical protein							
Hypothetical protein	Q55BS4	*Dictyostelium discoideum*	106.90/9.31	44.68	1	No	0.088
F2J6.12 protein	Q9MA69	*Arabidopsis thaliana*	70.20/5.82	44.63	1	No	0.448
T26F17.7	Q9SFF1	*Arabidopsis thaliana*	60.37/60.37	60.37	2	Yes	
Hypothetical protein	Q7XTF5	*Oryza sativa*	12.61/9.78	43.84	1	Yes	
Hypothetical protein	Q8L7V5 *At3g52870	*Arabidopsis thaliana*	51.28/8.69	61.85	2	No	0.347

[a] Name of the protein derived from SwissProt and TAIR database (marked with an asterisk in accession number).
[b] SwissProt protein accession number except the accession number with an asterisk was taken from TAIR database.
[c] Plant species from which the protein was annotated
[d] Theoretical molecular mass (*Mr*) and isoelcctric point (*pI*) calculated from ExPaSy server

[e] Mowse score derived from Mascot database
[f] Number of matched peptides
[g] Prediction of signal peptides: Yes - predicted, No - not predicted and SA- signal anchor predicted
[h] Value of SecretomP_NN score, where score ≥ 0.5 was considered as secretary (Bendtsen et al. 2004a)

Several groups of proteins such as cell wall related proteins, proteases and defense related proteins were found similar to those previously identified in the xylem sap of different plants supporting that xylem protein composition tends to be conserved in higher plants (Buhtz et al. 2004). The conservation could be due to a high degree of structural similarity of functionally matured tracheary elements and suggesting the role of these proteins in maintaining xylem development, differentiation, and function (Buhtz et al. 2004).

3.3.1 Origin of xylem sap protein

Since xylem elements lack protein synthesis machinery, the protein should be synthesized in other tissues before being imported to xylem sap. Almost 40% in WVa700 and 37% in Hawaii7996 xylem sap proteins were predicted to contain N-terminal signal peptides with SignalP (Table 7 and 8), which are considered to mediate the secretion of these proteins into the xylem sap (Rep et al. 2003). Remaining proteins did not show the possession of signal peptides. Evidences of several proteins lacking signal peptides but localized in the extracellular matrix and cell wall were reported previously (Slabas et al. 2004). Thus, the existence of unconventional and unknown non-classical secretory signal sequences and pathways are widely believed and already reported in yeast, bacteria, and mammals (Bendtsen et al. 2004a). The remaining proteins were, therefore, analyzed with SecretomeP yielding 27% in WVa700 and 19% in Hawaii7996 more non-classical secretory proteins (Table 7 and 8). The presence of secretion signals proteins, either N-terminal signal peptide or non-classical secretion signals, suggested that these proteins could be targeted to the xylem sap after their synthesis in several living cells; the xylem parenchyma cells and contact cells that are in close contact with dead xylem conduits (Tyree and Zimmermann 2002). However, 32% proteins in WVa700 and 44% in Hawaii7996 (Table 7 and 8) lacked both signal peptides and non classical secretion signals were also present in the sap. It is noteworthy in

case of xylem sap proteins that the formation of the functional xylem conduit occurs as a result of developmental programmed cell death (PCD) during the terminal tracheary differentiation, and many of the xylem proteins could be those released after the degradation and lysis of xylem tracheary elements or other contact cells (Alvarez et al. 2006). Several other arguments are available for the extracellular localization of proteins that lack secretion signals. Same proteins could be present in more than one cellular compartment for different biological functions (Slabas et al. 2004, Millar et al. 2006) or web based prediction software may incorrectly assign location (Lee et al. 2004).

Several important groups of proteins that are reportedly involved in biochemical and cellular processes occurring in the xylem sap are discussed in the following sections.

3.3.2 Xylem development and differentiation

The majority of xylem sap proteins such as cell wall metabolic proteins, peroxidase, and proteases (Table 7 and 8) consist of those involved in growth, development, and differentiation of xylem elements that lead to formation of functional conducting tubes. Development of xylem involves several fundamental processes of plant growth and development such as cell division, cell expansion, secondary cell wall formation, lignification, and PCD (Mellerowicz et al. 2001).

3.3.2.1 Cell wall metabolism, modifications, and remodelling

The identification of cell wall related proteins (Table 7 and 8) in xylem sap is reasonable because the xylem elements are continually accompanied by cell walls throughout their growth and development, however, with different composition and concentration. In fact, mature tracheids and vessel elements are dead cell wall skeletons surrounded by living xylem parenchyma cells.

The cell wall related proteins identified in the sap comprise proteins involved in synthesis and remodelling/restructuring of plant cell walls such as cellulose synthase, TANGLED1, kinesin, glycosyltransferase, galacturonosyltransferase, UDP-D-glucuronic acid decarboxylase, expansin, glcosyl hydrolase, and chitinase. The plant cell wall is a dynamic structure and is developed by the biosynthesis of cell wall components by the actions of

membrane-bound enzymes followed by the assembly and rearrangement of cell wall structures by the actions of extracellular proteins (Cosgrove 2005). The synthesis of major cell wall polysaccharides are carried out primarily by two enzymes; catalysing the biosynthesis of cellulose microfibrils by cellulose synthase-complex (Doblin et al. 2002), and of hemicellulose, pectic polysachharides, and various glycoproteins by glycosyltransferases (GTs) (Egelund et al. 2004). The identification of both of these enzymes shows the importance of these polysachharides for the xylogenesis. Other identified proteins that contribute to the orientation of cellulose microfibrils were microtubule binding protein (TANLED1) and kinesin. Galacturonosyltransferase is a glycosyltransferase involved in the formation of homogalacturonan (HGA), the backbone of the plant cell wall pectin. UDP-D-glucuronic acid decarboxylase catalyzes the biosynthesis of UDP-xylose, which is an important sugar donor for the synthesis of hemicellulose xyloglucan, glycoproteins, and other glycoconjugates in cell (Harper and Bar-Peled 2002).

The identified enzymes responsible for cell wall remodelling and restructuring were expansin and hydrolase. Expansin is proposed to play a key role to control the cell wall extension required for cell and tissue growth by causing expansion of the cellulose/xyloglucan framework (Cosgrove et al. 2002) and in vascular cell differentiation (Cho and Kende 1998). Several members of hydrolases namely ß-galactosidases, ß-1, 3-glucanase, chitinase, lipases, pectin esterase, acid invertase, 6, G-fructosyltransferase, α-L-arabinofuranosidase and polygalacturonase were identified. ß-galactosidases, ß-1, 3-glucanase, chitinase, and lipases could be involved in the degradation of the primary cell wall which is coupled with the secondary cell wall formation, and is necessary for the development of functional xylem elements (Fukuda 2004). Cell wall hydrolase may also play a role in cell wall perforations largely present in the functional tracheary elements. Pectin methylesterases are implicated in cell wall extension, rigidification, xylem cell differentiation, and cell growth and was essential for pectin modification during secondary cell wall deposition in xylem cells (Pelloux et al. 2007). A polygalacturonase hydrolyses α-1, 4-linkage in homogalacturonan backbone of pectic polysaccharides and act co-ordinately with other cell wall-modifying enzymes to increase cell wall extensibility (Hadfield and Bennet 1998). Lipase in xylem sap

could be involved in the early stages of cell content degradation during PCD (Fukuda 2000). Hydroxyproline rich glycoproteins including extension which are the most abundant structural protein in the plant cell wall were also identified in xylem sap. They are involved in strengthening of the plant cell wall as well as repairing of differentiated xylem walls especially in growing organs or after injury (Sakuta and Satoh 2000). Nucleoside hydrolases (NH) are well known nucleoside-modifying enzymes that play key roles in the purine salvage pathway of many pathogenic organisms which are unable to synthesize purines de novo but their roles in plants are unclear (Porcelli et al.2008).

3.3.2.2 Protease and PCD

Both exo and endo-peptidases belonging to serine protease (subtilisin like serine protease and serine carboxypeptidase), aspartic protease (CDR1), cysteine proteases (Ulp1) family, lipase, and ubiquitine were identified in tomato xylem sap (Table 7 and 8). The presence of serine proteases, cysteine proteases, and lipases in the xylem sap could be related to the regulation of various processes of plant development such as xylem differentiation and PCD (Beers et al. 2000). Xylem proteases may be involved in PCD process as mediators of signal transduction or as effectors of PCD (Beers et al. 2000). The terminal stage of xylem elements differentiation involves the developmental PCD, leading to the complete degradation of the primary cell wall and protoplast along with vacuole (Fukuda 2000). The cell's digestion releases several proteases and nucleases into a hollow tracheary element. Xylem protease could be involved in endogenous signalling, maturation and turnover of cell wall proteins and in the generation of active peptides in the cell wall (Jamet et al. 2006). Ubiquitine is involved in ubiquitination and thus targeting of proteins for intracellular proteolysis by ubiquitin proteosome pathway. The ubiquitination contributes significantly to plant development by affecting a wide range of processes, including embryogenesis, hormone signalling, and senescence.

3.3.3 Defence protein

Xylem sap analysis revealed networks of defense related proteins including peroxidase, antioxidants, detoxification proteins, resistance and PR proteins (Table 7 and 8). The

presence of constitutive defense proteins had previously been identified in xylem sap from different plant species (Buhtz et al. 2004).

3.3.3.1 Peroxidase

The xylem sap protein was characterised by the presence of high number of different peroxidases. Plant peroxidases are PR-9 class, large multigene family proteins (e.g. 138 genes in *Oriza sativa*) and one of the major compounds detected in xylem sap of a various plants (Buhtz et al. 2004). The functional contribution of xylem sap peroxidase could be the enforcement of the cell wall structure by cross-linking and polymerization of cell wall structural proteins and polysaccharides or by catalyzing polymerization and deposition of lignin, and suberin in the xylem tissues (Passardi et al. 2005). Since xylem transport takes place under negative pressure in all vascular plants, the walls of the tracheary elements must be reinforced to withstand compression and collapse. Peroxidase catalysed lignin impregnation provides protection to cellulose and hemicellulose from enzymatic attack and makes the xylem elements waterproof to facilitate water transport. Xylem peroxidases can also regulate and participate in reactive oxygen intermediates (ROI) production during PCD and pathogen defense, and also in operation of antioxidant defense mechanisms (Dat et al. 2000, Grant and Loake 2000). Plant peroxidase can be anionic, neutral, and cationic according to their isoelectric point. The xylem sap revealed 31 peroxidases of basic *pI* and 11 of acidic *pI* in susceptible plants. The presence of such a high number of peroxidase isozymes/isoforms indicates their involvement in multitudes of physiological and developmental processes. Anionic peroxidases were reported in ligninification (Rodriguez-Lopez et al. 2000), vascular plugging (Biles and Abeles 1991) and after wounding or pathogen invasion (Robb et al. 1991), whereas, cationic peroxidises were involved in cell wall biosynthesis except monolignols polymerization (Hiraga et al. 2001).

3.3.3.2 Antioxidant and detoxification

Several enzymes with antioxidative and detoxification properties were found in xylem sap which includes superoxide dismutases (SOD), tocopherol polyprenyltransferase, germin like proteins (GLP), nectarin-1, selenium binding protein (SBP) and γ-glutamyl

transferase/transpeptidase (GGT). Reduced O_2 species (ROS) including ROI are produced in all cellular compartments of plants as a by-product of aerobic metabolisms such as photosynthesis and respiration and are compounded during a reactions to various aboitic and biotic stress (Matamoros et al. 2003). Excess ROS are toxic and can lead to the oxidative stress damage of various cellular components (Moller et al. 2007). Antioxidant and detoxifying proteins are essential to avoid such damage to maintain cellular functions especially for the long living xylem tracheary elements which specifically can generate H_2O_2 during lignification (Olson and Varner 1993). The occurrence of such detoxifying enzymes may also contribute to the delay of PCD and thereby promote the duration of cell wall thickening imparting longer life to xylem cells.

Cu/Zn-SOD and tocopherol polyprenyltransferase are primary ROS scavenger. Tocopherol polyprenyltransferase has also been suggested to participate in intracellular signalling and in cyclic electron transport around photosystem II affecting the plant development and stress responses (Krieger-Liszkay and Trebst 2006). GLP functions primarily as SODs and associated with cell wall formation, expansion, extension, and re-enforcement (Christensen et al. 2004). Nectarin-1 is a soluble GLP that could acts as defense proteins (Carter and Thornburg 2000). SBP was involved in detoxification of and/or tolerance to excess selenium which has deleterious effects on normal cell development and enhances tolerance to different pathogens (Sawada et al. 2004). GGT are assumed to be involved in the utilization and maintenance of glutathione homeostasis and thereby keeping the cell redox balance (Noctor et al. 2002).

3.3.3.3 PR proteins

Chitinase, β-1, 3-glucanase-like protein, and pathogenesis-related protein P2 are PR proteins identified in healthy xylem sap. Chitinases (PR3) and β-1, 3-glucanases (PR2) are multigene family proteins which are constitutively expressed in plants, however induced significantly after pathogen invasion (Ferreira et al. 2007). Chitinase and β-1, 3-glucanases in xylem sap is considered to protect plants from many xylem invading fungi by degrading fungal cell walls that consists of chitin and β-1, 3-glucan and can act synergistically (Theis and Stahl 2004). Xylem sap showed the presence of multiple chitinase isozymes including class I chitinase

having both acidic and basic *pI* which could be to allow plants to respond in a tissue specific and stimulus specific manner or each providing different functions. Acidic endochitinase are usually extracellular and was implicated in the modulation of mechanical properties of the cell wall, and signal generation and transfer during infection where as basic vacuolar chitinases take part in repressing pathogen growth (Collinge et al. 1993, Yokoyama and Nishitani 2004). Similarly, both acidic and basic glucanase were identified in the sap. In plants, extracellular β-1, 3-glucanases are generally acidic and involved in cell division besides in defense mechanism, while those of vacuolar organs are basic (Van Den Bulcke 1989).

3.3.3.4 Resistance protein

Several disease resistance proteins namely NBS-LRR cD7, RPP5, RGH1, polygalacturonase like protein (PGIP), and syringolide induced protein were identified in the sap. Most of plant disease resistance (R) proteins that function in gene-for-gene manner are characterized by the presence of a series of leucine-rich repeats (LRRs), a nucleotide-binding site (NBS), and a putative amino-terminal signalling domain and thus termed as NBS-LRR proteins. Disease resistance proteins are known for their role in the recognition of invading pathogens and the activation of defense responses that confine pathogen growth and spread (De Young and Innes 2006). Both RPP5 and RGH1 belong to the NBS-LRR family proteins. PGIP is a LRR-family glycoprotein that binds to the plant cell wall and is induced by pathogen infection and stress related signals (Di et al. 2006). Syringolides induced protein is considered to induce syringolides which are water-soluble, low-molecular-weight glycolipid elicitors that trigger defense responses in plants (Ji et al. 1998).

3.3.4 Signalling proteins/signal transduction

Several proteins involved in cellular communications or signalling pathways were identified in xylem sap such as MAP kinase, MEK, receptor protein kinase, LRR proteins, lipid transfer protein (LTP), G-protein, β-transducin, phosphatidylinositol glycan, calmodulin, phototropin, phytochrome B, armadillo repeat containing protein, and 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase. The identification of these proteins support the hypothesis that xylem

sap consists of proteins involved in both signal production and transduction, which ensures root to shoot communication (Sakuta and Satoh 2000, Maldonado et al. 2002, Rep et al. 2003).

The mitogen-activated protein kinases (MAPKs) including MEKs are evolutionary conserved enzymes involved in the signal transduction cascade regulating a variety of physiological processes including cell proliferation, differentiation, movement and death (Mishra et al. 2006). Plant MAPK cascades are activated in response to abiotic and biotic challenges, and during developmental processes and the initiation of the cascades often involves membrane-located receptors proteins and G-proteins. Several members of several receptor protein kinases (RLKs) family such as S-domain class, LRR class, and PERK class were identified in the sap. The presence of several RLKs and G-proteins in the xylem sap suggests that they are capable of responding to a wide array of signals. RLKs have regulatory or signalling functions and play roles in regulating plant developments and defense. Guanine nucleotide-binding protein (GTP-binding/G-proteins) mediate signalling is another widespread pathway existing in plants. G-proteins including β-transducin (G_β) participate in signal transduction, development regulation, ion channel regulation, vesicular traffic, and cytoskeleton assembly including cell wall formation (Temple and Jones 2007). Phosphatidylinositol glycan is an enzyme that participates in reactions to produce a glycosylphosphatidylinositol (GPI) anchor molecule. GPI anchoring is used to target a specific subset of proteins to the cell surface where most of them take part in cell wall synthesis and remodelling (Gillmor et al. 2005). Plant calmodulins are primary intracellular receptor for Ca^{2+} signals which activates wide ranges of downstream signalling pathways and thus regulating many plant growth and developmental processes including PCD (Bouche et al. 2005). Xylem LTP could be a candidate for long distance signal carrier and are known to interact with cell surface receptors (Buhot et al. 2001, Maldonado et al. 2002) besides having antimicrobial activity (Wang et al. 2004). ACC oxidase present in xylem sap is involved in the synthesis of ethylene that was reported to control xylem differentiation (Pesquet and Tuominen 2007). Ethylene was also reported to induce β-1, 3-glucanase as well as chitinase in plants, and both of these PR proteins were detected in xylem sap (Wu and Bradford 2003). Plant Arm (armadillo)-repeat

proteins have known roles in several signalling pathways such as light, abscisic acid and receptor-kinase signalling, regulating plant developmental process, cytoskeleton regulation and protein degradation pathways (Samuel et al. 2006). Two classes of photoreceptors namely phototropin and phytochrome B were identified. These photoreceptors absorb light to regulate a wide range of developmental and physiological responses. Phototropin is a plasma membrane blue light receptor kinase that optimises photosynthesis and minimizes photodamage in plants where as phytochromes are red and far-red light photoreceptor proteins that control photomorphogenesis in plants (Chen et al. 2004).

3.3.5 Transcription and transcription factors

Several proteins associated with DNA replication, transcription and translation such as DNA topoisomerase, RNA polymerase, maturase, pentatricopeptide, methyl CpG-binding proteins, and integrase were identified in xylem sap which might be related to a high rate of cell divisions taking place in growing and dividing cells during xylogenesis. Various transcription factors like zinc finger protein, F-box protein, Rcd1, and IAA5 were identified in the sap. Plant growth and developments are regulated by the action of transcription factors which activate or repress transcription in response to endogenous and exogenous stimuli (Yanagisawa 2006). IAA5 plays a significant role in auxin signalling influencing the vascular development and an ubiquitin-dependent protein degradation process (Mockaitis and Estelle 2008). IAA is a well-known promoter of tracheid production (Little and Pharis 1995).

3.3.6 Nutrient transport

Several groups of ion and water carriers such as cyclic-nucleotide-gated channel (CNGC), several plasma membrane ATPase, and plasma membrane intrinsic proteins (PIPs) were identified in the sap. Since xylem sap is the primary means of transporting mineral nutrients and water throughout the plant, the presence of cations transport pathway such as ion channels and ion carriers are reasonable. Such processes contribute to plant nutrition, but also to cell signalling and toxic ions homeostatis. CNGC is one of the non-selective ion channels that participate in the uptake and/or translocation of several ions such as sodium, potassium, or calcium (Kaplan et al. 2007). Plant plasma membrane ATPase such as Ca^{2+} ATPases and

vesicle transfer ATPase plays key roles in the transport of ions and solutes through plasma membrane or vesicle intermediate. It was reported that AAA-ATPase that includes vesicle transfer ATPase are involved in sorting and translocation of ubiquitinated endosomal membrane proteins (Babst et al. 2002). The identification of ubiquitin in the present study also supports this hypothesis. PIP is a type of major intrinsic proteins (MIPs) in plants that are known to regulate plant cell turgor and/or transcellular water transport in growing tissues in addition to membrane permeability (Forrest and Bhave 2007).

3.3.7 Enzymes of primary and secondary metabolism

Several enzymes of central metabolic processes involved in the housekeeping of the cell such as those participating in glycolysis, tricarboxylic acid (TCA) cycle, respiration/energy production, photosynthetic reactions and nitrogen metabolism were identified in xylem sap (Table 7 and 8). Growth and development of plants are principally affected by highly interconnected primary metabolic processes. The identification of these basic metabolic enzymes may indicate an increased level of the primary metabolism during the xylem formation process. Cell division and expansion during the xylem formation are more energy and metabolic-needs demanding process.

Starch synthase, fructose-bisphosphate aldolase, and enolase were the glycolytic enzymes identified in xylem sap. Glycolysis is the main pathway for carbohydrate catabolism and is a key metabolic component of the respiratory process in non-photosynthetic cells of mature plants such as xylem elements. The glycolytic pathway also supplies several unoxidised carbon sources used in the biosynthesis of secondary metabolites, isoprenoids, amino acids, nucleic acids and fatty acids. Also identified ribose-phosphate pyrophosphokinase is the enzyme of the oxidative pentose phosphate pathway and is necessary for the biosynthesis of purine and pyrimidine nucleotides and nucleic acids. Proteins involved in amino acid synthesis such as methionine synthase, arginine decarboxylase, 3-phosphoshikimate 1-carboxyvinyltransferase, copper amine oxidase, and delta 1-pyrroline-5-carboxylate synthetase were also detected. Amino acids serve as precursors for proteins, vitamins, phytohormones, and nucleotides. The abundance of methionine synthase in xylem sap may reflect the higher demand for methyl transfer reactions required for the monolignols and

pectin synthesis (Moffatt and Weretilnyk 2001). Arginine decarboxylase appears to be the primary enzyme for cell extension, secondary metabolic processes, and stress responses. Copper amine oxidase in developing tracheary elements assists lignifications and occurs in cells destined to undergo PCD (Moller and McPherson 1998).

Identification of proteins involved in energy metabolism such as photosynthesis, TCA cycle and electron transport in the xylem sap could be related to the high energy demand for tracheids elongation and growth. In fact, the proteins participating in glycolysis, the pentose phosphate pathway, and respiration can also be grouped into energy metabolism proteins. Several proteins associated with photosynthesis such as subunit T of photosystem II, OEE protein 2, cytochrome complex (c and b5), PAC protein, plant geranylgeranyl hydrogenase, ATPase/synthase complex and malate dehydrogenase were present in xylem. The net result of photosynthesis is the generation of a proton gradient which leads to ATP synthesis in addition to the production of molecular oxygen. The four complexes namely the photosystem I, photosystem II, ATP synthase and cytochrome complex are involved in the photosynthetic electron transfer chain and ATP synthesis. PAC protein and plant geranylgeranyl hydrogenase are regarded as photosynthesis regulating proteins where PAC protein functions in plastid mRNA maturation and accumulation (Meurer et al. 1998) and plant geranylgeranyl hydrogenase provides the side chain to chlorophylls, tocopherols, and plastoquinones to assist their synthesis (Keller et al. 1998). The mitochondrial ATPase/synthase complexes and malate dehydrogenase are essential enzymes for energy production. Chalcone synthase (CS), flavanone-3–hydroxylase (F3H), cinnamoyl CoA reductase (CCR) and cytochrome P450, which are involved in the production of secondary metabolites, were identified in xylem sap. Flavonoids and lignin are important secondary phenolic compounds present in the cell wall. Both CS and F3H participate in flavonoids biosynthesis whereas CCR is involved in monolignol biosynthesis. CCR plays a key regulatory role in lignin biosynthesis, by their involvement in monolignols biosynthesis from phenylpropanoid metabolites (Boerjan et al. 2003). Cytochrome P-450 is involved in later hydroxylation reactions of the flavonoid and isoflavonoid metabolism leading to the production of several secondary metabolites including phytoalexin and lignin (Ayabe and Akashi 2006). Some well known storage proteins such as

patatin, starch synthase, and phaseolin were also present in the xylem sap. Vegetative storage proteins generally act as a temporary storage for nutrients buffering.

3.3.8 Transposable element proteins

Several transposable element proteins (TEPs) especially retroelements such as Ty1/copia, Ty3/gypsy, and CACTA transposon derived proteins were identified in xylem sap. TEPs play important roles in the plant genome structure, variation, and evolution in response to diverse environmental stress including microbial invasion (Deragon et al. 2008). They can alter gene expression by preventing expression, producing splicing products, or providing new regulatory signals. Class I retrotransposon (retroelement) transpose through reverse transcription of RNA intermediate where as class II transposon moves via a DNA intermediate. Ty1/copia and Ty3/gypsy are long terminal repeats (LTR) containing retrotransposon where as CACTA transposon (also called En/Spm family) is a class II transposon.

3.3.9 Hypothetical proteins

Due to incomplete genomic sequence information, the protein identification and functional categorization still remains difficult in tomato. This becomes evident in the presence of almost 20% hypothetical proteins that do not have any similarity to known proteins in other organisms. The identification of large number of functionally unknown proteins might indicate the presence of yet unidentified cellular process that are specific for the functional xylem as well as for the whole plant physiology. Therefore, elucidation of their biological and physiological role is a suggested future task.

3.4 Conclusion

Analysis of healthy xylem sap from tomato revealed large groups of proteins from cell wall metabolisms to networks of defense related proteins and several signaling and transport proteins which are crucial to the development and function of xylem sap in the plant. The xylem proteins from resistance plants showed the presence of higher percent of defense related proteins including peroxidase, and protein modifying enzymes compared to susceptible genotypes. The present study will definitely severs as a platform for a future

comparative analysis of the xylem proteome which are differentially regulated in response to pathogen inoculation especially for the xylem colonizing *R. solanacearum*. In addition to secretory proteins, several non-secretion signal proteins were also identified, the location of which can be confirmed with genetic experiments complemented with immunological detection. Similarly, further experiments using knockout lines and genetic, biochemical and cell biological data could elucidate the cellular functions of large number of hypothetical proteins present in the sap.

GENERAL DISCUSSION AND CONCLUSION

Use of resistant tomato cultivars provides a valuable individual control measure for bacterial wilt. Nevertheless, the instability of resistance mechanisms in some geographic areas as a result of variable environmental conditions provides a major problem in tomato cultivation and makes investigations necessary leading to a more thorough understanding of resistance mechanisms at the molecular level. The time point of analysis of the mid-stem tissue at 5 dpi was selected, based on the previous observations of the time needed by the bacteria to reach the stem and multiply heavily in the vascular system. The stem tissue was chosen for analyis due to the described presence of resistance mechanism at mid stem level, with a suggested role of cell wall components in the resistance reaction. The tomato-*R. solanacearum* system offers a useful model to investigate the interactions between bacteria and tomato stem components on a molecular level. To date, these resistance responses were reported only on histochemical background, but the broad spectrum proteome level responses at mid stem and in detail on its cell wall have not been analyzed before. The study systematically evaluated the physiological responses of the tomato plant at proteome level that are activated after the inoculation with *R. solanacearum* by a proteomic approach. 2-D gel based proteomics was primarily used for the comparative analysis of protein profiles of two genotypes followed by the comparison of the protein regulation with respect to pathogen inoculation for whole stem extracts and the stem cell wall proteome.

In the first experiment, the proteome of the whole mid stem was extracted from two RIL genotypes differing in resistance to bacterial wilt, and compared for the differential abundance of the resolved proteins on 2-D gels which did not disclose differences. The 2-D analyses were continued by evaluating the differences in the abundance of proteins of susceptible and resistant genotypes regulated in response to pathogen inoculation. The compatible reaction showed a stronger response to the pathogen by displaying consistently reproducible 12 proteins of differential abundance, of which six were mainly annotated from plant origin and related to metabolism, pathogenesis, and stress. Pathogenesis as well as stress related proteins and metabolic proteins are among the key proteins involved in the

resistance or susceptibility of the host plant. The identification of further six proteins from bacterial origin could be due the presence of a heavy bacterial colonization of the stem (10^9 cfu/g of stem). No visible differences were detected in 2-D gels of the resistant plant after pathogen inoculation, which may indicate the static nature of at least the most abundant and soluble proteins in the resistant reaction. It was discussed that the defense reactions are more robust to input signals and express less biological variation compared to susceptible ones. The resistance could be influenced decisively by the kinetics of the reaction and their quantitative differences rather than the number and type of proteins (Tao et al. 2003). However, the technical limitations of the 2-D technique could also be the reason for non-visibility of reactions on protein level in the resistant plant, since e.g. transcription factors, receptors and regulatory proteins are hardly identifiable on 2-D gels, but are vital in mediating defense responses in plants.

The results from the first experiment encouraged us to increase the sensitivity of the analysis and investigation by studying the sub cellular proteome, which offers a good option by decreasing the complexities of the proteome and thereby enhancing the possibility to detect more subtle interaction. The cell wall of the stem was considered for this purpose in the subsequent experiment owing also to several other potential roles of cell wall in host pathogen interactions. Cell walls provide the first line of barrier to a pathogen and their reinforcement is a well known defense response of the plant towards pathogen ingress. This was suggested by our earlier report on the roles of the cell wall in strengthening the resistance (Wydra and Beri 2006, 2007). Supporting our assumption, 2-D SDS-PAGE analysis of the cell wall proteome from the stem of healthy susceptible and resistant parental inbred lines showed 14 proteins of differential abundance due to genotype differences that had not been identified in the whole stem proteome. The two genotypes that differ in the degree of resistance to bacterial wilt exhibited metabolic as well as defense and stress related proteins as the major difference between them. Again, these proteins are well known for their active roles in influencing the selection of the susceptibility and resistance of the plant. The investigation of the cell wall proteome was extended further by analyzing the proteome of susceptible and resistant plants regulated differentially in response to pathogen attack. The

result clearly demonstrated the differences not only in the susceptible genotype but also in the resistant genotype which did not show any visible differences in the whole stem analysis. PR and other defense related proteins were differentially displayed in both plant varieties supporting the proposition that each genotype regulates the expression of its resistance proteins when attacked by the pathogen. The efficiency and kinetics of the regulation may be decisive for the outcome of the interaction. The cell wall proteome analysis also led us to develop a simple 3-D SDS-PAGE that reproducibly resolved the basic range proteins in the 3rd dimension SDS-PAGE, which are otherwise poorly resolved on 2-D gels. Additionally, most of the cell wall proteins were found secretory supporting to their extracellular location.

The third and last experiment deals with analysis of the proteome of another sub cellular fraction i.e. the xylem sap which acts as an important site for host pathogen interaction especially for the xylem colonizing *R. solanacearum*. The high throughput expression profiling of the xylem proteome was performed to present the basic understanding of the physiological process carried out by the xylem sap and to build the platform which will be useful for the comparative analysis of the xylem proteome that are regulated in response to pathogen invasion. With the purpose of overcoming the limitations of 2-D SDS-PAGE, the xylem sap proteome was separated with 1-D gradient SDS-PAGE and the whole protein bands were analyzed with LC MS/MS. The complete protein screening revealed more than 200 proteins in the xylem sap with diverse functional roles. Peroxidase, cell wall metabolic protein, proteases, and other defense related proteins were reportedly conserved in many plant species indicating their potential roles in the functional xylem conduit development. Identification of several signaling and transport proteins supports the important physiological roles of the xylem sap as carrier of signals and nutrients providing the needed root to shoot communication. The detection of several receptor kinases, transcription factors, and other signaling proteins clearly showed the advantages of this technique over 2-D gel approach in capturing the low abundance proteins. Coverage of a higher number of proteins in relatively less amount of protein sample is another plus point compared to 2-D gels. Several hypothetical proteins were found in the xylem sap signifying the possible novel functions which are yet to be determined. Xylem sap proteome possessed a high number of secretory

proteins that guides them to the extracellular location however, a large number of proteins with no secretion signals were also found in the xylem sap. The unorthodox presence of non-secretory protein in xylem sap supports the existence of moonlighting proteins which are being increasingly discovered in other extracellular locations. The comparison of healthy plant xylem sap between susceptible and resistant plants disclosed the occurrence of a higher percentage of defense related, and metabolic proteins as well as proteases in resistant plants, where as susceptible plants had a higher percentage of signaling and transcription related proteins. The susceptible plants showed the presence of a higher number of proteins than the resistant genotype which could indicate that the susceptible plants are more reactive and sensitive.

In overall, the present study revolves around understanding of the resistance mechanism of the tomato plant towards *R. solanacearum* inoculation. The study highlighted the major proteome which was selective to either genotypes differing in the degree of bacterial wilt resistance, and, consequently, their regulation in both susceptible and resistant reactions in response to pathogen attack. The sensitivity of the whole stem analysis was increased by integrating the cell wall and xylem sap proteome examination in order to provide a deeper insight into the molecular interactions. The differential expression of PR, defense and stress related as well as metabolic proteins in both compatible and incompatible interactions strongly supports the hypothesis that both types of plants regulate their proteins during the interaction as a part of general defense mechanism however, the kinetic and magnitudes of the interactions would be more influential for the outcome. Measurement of reactions at different time points and the integration of gel based and gel free chromatographic methods would provide complementary information and, therefore, shed more light on the proposed resistance responses. Further experiments determining the physiological roles of each of the regulated proteins would provide more accurate information. The screening of the xylem proteome provided an overview of the xylem functions in whole plant physiology, and also a good platform for the further investigations of xylem protein regulated after pathogen invasion.

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Internet resources used in the thesis

UniProtKB server: www.uniprot.org/

SignalP server: www.cbs.dtu.dk/services/SignalP/

SecretomP server: www.cbs.dtu.dk/services/SecretomeP/

ExPASy proteomic server: www.expasy.ch/tools/pi_tool.html

TIGR Arabidopsis database: www.tigr.org

Solanaceae genomic database: www.sgn.cornell.edu

Publication

1. Dahal D, Heintz D, Van Dorsselaer A, Braun HP and Wydra K (2009) Pathogenesis and stress related, as well as metabolic proteins are regulated in tomato stems infected with *Ralstonia solanacearum*. *Plant Physiology and Biochemistry* **47**: 838-846.

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Contact Address	Email: dahal@ipp.uni-hannover.de diwakardahal@gmail.com
Language	Nepali (Mother tongue), English (fluent), Hindi (fluent), German (basic)

Academic background

- PhD (May, 2006-December, 2009) Characterization of resistance responses of susceptible and resistant tomato genotypes against bacterial wilt disease caused by Ralstonia solanacearum, a **proteomic** approach, Leibniz Universität Hannover, Germany**.**
- Master of Science (M.Sc.) in ARTS program (March, 2004-May, 2006) Specialization in **Molecular plant genetics**, University of Bonn, Germany.
- Bachelor of Science (B. Tec. Food) in **Food technology** (1994-1998) Majors in **Biochemistry, Food and General Microbiology**, Tribhuwan University, Nepal.
- Certificate Level in Science (I.Sc.) (1992-1993) Major in **Biology**, Tribhuwan University, Nepal.
- School Leaving Certificate (S.L.C.) Degree (1991) HMG Board of Nepal.

Thesis and Publications

- **Dahal, Diwakar**; Heintz, Dimitri; Van Dorsselaer, Alain; Braun, Hans-Peter and Wydra, Kerstin (2009) Pathogenesis and stress related, as well as metabolic proteins are regulated in tomato stems infected with *Ralstonia solanacearum.* ***Plant Physiology and Biochemistry*** **47: 838-846.**
- **Dahal, Diwakar**; Pich, Andreas and Wydra, Kerstin (2009) High-throughput expression profiling of xylem sap proteome of susceptible and resistant tomato genotypes revealed

networks of metabolic, defense as well as cell wall related and signaling proteins. ***Submitted.***

- **Dahal, Diwakar**; Pich, Andreas; Braun, Hans-Peter and Wydra, Kerstin (2009) Analysis of cell wall proteins regulated in stem of susceptible and resistant tomato genotypes after inoculation *with Ralstonia solanacearum*: A proteomics approach. ***Submitted.***
- **Dahal, Diwakar**; Blank, Birgit and Leon Jens (2005) Fine mapping of QTL region responsible for high ß-D-Glucan content in barley chromosome 1H in the grain. **Master thesis** from University of Bonn, Germany.
- **Dahal, Diwakar** and Ray, Subhajit (1999) Comparative study of physico-chemical and bacteriological parameters of water due to its types and sources. **Bachelor thesis** from Central Campus of Technology, Tribhuwan University, Nepal.

Conference

Oral presentation

- **Dahal, Diwakar**; Braun, Hans-Peter and Wydra, Kerstin (2008) Proteomic approach to characterize the reaction of tomato to infection with *Ralstonia solanacearum* on 29th Annual meeting of the working group of Phytobacteriology of the German Phytomedicine Society. September 4, 2008 in Erfurt Germany.
- **Dahal, Diwakar**; Pich, Andreas; Braun, Hans-Peter and Wydra, Kerstin (2008) Analysis of tomato stem and xylem proteome in response to infection with *Ralstonia solanacearum o*n 56th German plant protection workshop (Deutsche Planzenschutztagung). September 25, 2008 in Kiel, Germany.

Poster presentation

- **Dahal, Diwakar**; Pich, Andreas; Wydra, Kerstin (2009) High throughput expression profiling of xylem sap proteome from both susceptible and resistant tomato genotypes with LC MS/MS. Deutscher Tropentag, Hamburg, Germany.
- **Dahal, Diwakar**; Braun, Hans-Peter and Wydra, Kerstin (2007) Investigations on resistance mechanisms of tomato genotypes against bacterial wilt disease: a proteomics approach. German Phytomedical Society e.V.- Workshop on Mycology/Host-pathogen interaction.15-16 March 2007, Halle Germany.

- Blank, Birgit; **Dahal, Diwakar**; Binder Andrea and Leon, Jens (2006) The inheritance of β–D-glucan contents in barley (Die Vererbung des β-D-Glukangehaltes in Gerste) on 57. Working meeting of the association of plant breeders and seed merchants. Austria, 21-23 November 2006.

Participation

17-20 July, 2006	The 4th International Bacterial Wilt Symposium. Central Science Laboratory, York, United Kingdom
Oct 11-13, 2005	The Global Food and Product Chain– Dynamics, Innovations, Conflicts, and Strategies. Deutscher Tropentag, Hohenheim, Germany
Oct 05 -07, 2004	Rural Poverty Reduction through Research for Development and Transformation, Deutscher Tropentag, Berlin, Germany

Short term course and training

February 2008	4-weeks intensive practical course on **Plant Biotechnology** Leibniz Universität Hannover, Hannover, Germany
August 2007	4-weeks intensive practical course on **Molecular Cell Biology** Leibniz Universität Hannover, Hannover, Germany
March 3, 2005	Crash course on GIS and Database training, Institut für Landwirtschaftliche Zoologie und Bienenkunde, Bonn, Germany

Scholarship

- PhD scholarship (May 2006- Sept 2008 and from July 2009 – October 2009) Institute of Plant Disease and Plant Protection, Leibniz Universität Hannover, in a project funded by German Ministry of Collaboration (BMZ) with Asian Vegetables and Research Development Centre (AVRDC)
- DAAD scholarship for international Doctoral Student (Oct 2008-June 2009)

Membership

- Student member (since Feb, 2009) International Society for Molecular Plant-Microbe Interactions, Canada

- Life Member of ARTS Club (since 2004) Agricultural Sciences and Resource Management in the Tropics and Subtropics (ARTS), Agricultural Faculty, University of Bonn.

Laboratory experience

- DNA/RNA/Protein extraction
- Western blotting
- Agarose gel electrophoresis and staining
- PCR, AFLP, and SSR marker analysis
- LiCor sequencing gel
- 1-D, gradient gel, mini gel, 2-D SDS and BN-PAGE
- Protein gel staining- Silver and Coomassie colloidal
- Image master platinum v6.0
- Tryptic digestion, LC MALDI-TOF/TOF MS and Quantitative proteomics with ICPL labeling (in collaboration with MS group-MHH)
- Bacterial inoculation and quantification