Plant Mol Biol (2010) 73:643–658
DOI 10.1007/s11103-010-9646-z

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

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Received: 30 October 2009 / Accepted: 1 May 2010 / Published online: 23 May 2010

Abstract Proteomics approach was used to elucidate the molecular interactions taking place at the stem cell wall level when tomato species were inoculated with *Ralstonia solanacearum*, a 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 revealed varied abundance of protein spots between two species (eight proteins in higher abundance in resistant and six other in susceptible). Moreover, proteins were regulated differentially in response to bacterial inoculation in resistant (seven proteins increased and eight other decreased) as well as in susceptible plants (five proteins elevated and eight other suppressed). Combination of MALDI-TOF/TOF MS and LC-ESI-IonTrap MS/MS lead to the identification of those proteins. Plants responded to pathogen inoculation by elevating the expression of pathogenesis related, other defense related and glycolytic proteins in both species. 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.

Keywords Defense and metabolic proteins · *R. solanacearum* · Secretory proteins · Stem cell wall proteome · Tomato species · 3-D PAGE

Abbreviations

2-, 3-D IEF/SDS–PAGE	2 or 3 dimensional isoelectric focusing/sodium dodecyl sulphate–protein gel electrophoresis
MALDI-TOF/TOF MS	Matrix assisted laser desorption and ionization-time of flight tandem mass spectrometry
LC-ESI-IonTrap MS/MS	Liquid chromatography-electrospray ionization-ion trap tandem mass spectrometry
PR protein	Pathogenesis related proteins

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Introduction

The cell wall of the plant is one of the distinguishing features and a dynamic structure that consists predominantly of polysaccharides. Primary cell wall of dicotyledon

plants possess less than 10% proteins of the total cell wall mass relative to 90% polysaccharides but still constitute several hundred in number (Jamet et al. 2008a). The primary cell wall is formed during cell growth and elongation where as the secondary cell wall is deposited after the cessation of cell growth inwards to the primary wall. 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).

Terrestrial plants are subjected to many biotic and abiotic stresses during their lifetime and correspondingly evolved a wide range of defence mechanisms to protect themselves. The plant defense is provided both at the constitutive as well as at induced level when they come into contact with invading pathogen. 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 cause a huge economic loss. Bacterial wilt caused by *Ralstonia solanacearum* is one of the most devastating, systemic vascular wilt diseases causing up to 75–100% 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 species 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 is deeply involved in both constitutive and inducible defences 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 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 play vital roles in establishing 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 provide further insight to understand the defense responses of the plant and also the outcome of the host-pathogen interaction. The sub-cellular proteome analysis should also present additional information and increase the usefulness 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).

Materials and methods

Plant material and inoculum preparation

Tomato plants were grown from highly resistant Hawaii7996 (*Solanum lycopersicum)* and susceptible WVa700 (*Solanum pimpinellifolium*) tomato lines against bacterial wilt. The seeds from both species were obtained from Asian Vegetables Research and Development Centre (AVRDC, Taiwan). The inoculum from the highly virulent *R. solanacearum* strain ToUdk2, race 1, biovar 3 (kindly provided by N. Thaveechai, Kasetsart University, Bangkok) was prepared by adjusting the bacterial cell concentration to about 7.8×10^7 colony-forming units per millilitre (cfu/mL) as described by Dahal et al. 2009.

Plant growth conditions and inoculation

Tomato plants were grown in the greenhouse for 4–6 weeks (20°C, 14 h photoperiod per day, 30 K lux and 70% relative humidity, 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.

Bacterial quantification

The number of bacteria residing in the stem was determined at 5 days post inoculation (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 triphenyl tetrazolium chloride (TTC) medium were quantified as colony-forming units per gram of fresh weight of plant stem (cfu/g).

Disease symptoms evaluation

Ten pathogen inoculated plants from each susceptible and resistant species were observed for symptoms assessment. The symptoms were evaluated over the period of 4 weeks in six disease severity classes from 0 to 5 (Dahal et al. 2009). The wilt incidence (WI) and disease severity (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.

Protein extraction from cell walls of tomato stems

The stem cell wall proteins from both Hawaii7996 and WVa700 species were extracted before and after pathogen inoculation at 5 dpi. Individual samples were prepared by combining mid stems of two to 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 100% acetone and finally with 50 mL wash buffer 2 (10 mM sodium acetate pH 5.5). The extraction of protein from the purified cell wall was performed in two sequential steps: 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 total volume of protein extract recovered from each sample was adjusted to 30 mL owing to some losses during filtration. The protein solution was concentrated in a centrifugal concentrator with a molecular mass cut off at 5 kDa (Vivaspin 6, Vivascience, Germany) at 5,080 g × 4°C × 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).

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

The complexity of the proteome extracted from the stem cell wall was resolved by two- dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (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 species and treatment from separate biological samples where as only 2 replicates were prepared for 3-D gels.

Approximately 700 µg of total protein quantified by Bradford assay (Coomassie protein assay reagent, Fluka biochemical) was dissolved in 350 µL of "rehydration buffer" [8 M urea, 2% w/v CHAPS, 0.5% v/v carrier ampholyte mixture (IPG buffer 3–11 non linear (NL), GE Healthcare, Germany), 30 mM DTT, and 0.002% w/v (7 µg/350 µL) Bromophenol Blue] with the addition of 4.2 µL of DeStreak reagent (GE healthcare, Munich, Germany) to facilitate the basic proteins separation. The protein was then separated in first dimension by IEF using 18 cm NL IPG strip pH 3–11 (GE HealthCare, Munich, Germany) and in second dimension by vertical 12% SDS–PAGE (20 cm × 20 cm × 1 mm) using Protean II electrophoresis unit (Biorad, Hercules, CA, USA) as described by Werhahn and Braun 2002 and Schagger and Von Jagow 1987, respectively.

2-D IEF/SDS–PAGE of the cell wall proteome of all samples resulted in repetitive vertical streaking in very basic side of the gel and making 2-D gels inappropriate for comparative analysis of the basic protein spots. Therefore, a three-dimensional PAGE system was established, which is based on the transfer of the very basic gel region of a 2-D PAGE gel, before fixing and staining them, horizontally onto 3rd SDS–PAGE. For this approach, extra new samples were first required to be resolved and visualised by 2-D PAGE to check the exact position of the streaking on the gel so that these regions, without fixing and visualising, can be transferred correctly onto the 3rd gel.

Protein staining, gel scanning and image analysis

After completion of SDS–PAGE, proteins were first fixed and stained with colloidal Commassie solution (Neuhoff et al. 1985, 1990). 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™ 2D platinum software 6.0 (GE Healthcare, Germany) in

order to accomplish the spots detection and calculate the quantitative values of the differentially regulated spots. The abundance of each protein spot was estimated by the percentage volume (% vol), which represents the contribution of each protein spot (volume) to the total protein spots (volume) on the whole gel, and thus minimizes inter-gel loading differences. Master gels of each treatment and species (each comprising at least three biological replications) were created to allow statistical comparison and only those spots present in all triplicate gels were included in the statistical analysis. Only statistically significant difference in the abundance of protein spot with Student's t test $P \leq 0.05$ (probability value) was considered. Moreover, quantitative differences of ±30% variance in a spot ratio (>1.3 or <0.7), were considered as biologically significant based on previous study (Asirvatham et al. 2002).

Tryptic digestion

All except few big and intense gel spots were handpicked 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). The dried gel piece was directly treated with trypsin (4 ng/μL) in 20 mM NH_4HCO_3/10% ACN on ice with 1 h incubation because proteins were incubated with DTT followed by iodoacetamide during the equilibration steps before SDS–PAGE. The remaining trypsin solution was removed and 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% 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.

Protein analysis by MALDI-TOF/TOF MS

Peptides were dissolved in 10 μL of 5% ACN containing 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 CHCA matrix was spotted on each spot of a matrix assisted laser desorption and ionization (MALDI) Anchor Chip 800/384 target plate (Bruker Daltonic 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 a MALDI-time of flight/time of flight (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; on average ten MS/MS spectra were measured for each protein digest leading to 2–10 identified peptides. Data processing was done using the flex analysis and biotools software packages (Bruker Daltonik).

Protein analysis by electrospray (ESI) ion trap MS

Peptides were dissolved in 5% ACN, 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 to 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 ion source of the ion trap MS. The spray was stabilized by N_2 as nebulizer gas (5 l/min). Ionization voltage was set to 4,500 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 13,000 m/z per second. Using ESI in positive mode, mass spectra were acquired from m/z 50 to 1,600 in scanning mode and data dependent switching between MS and MS/MS analysis. After each MS scan, two precursor ions from one spectrum were selected for subsequent 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 software (V. 3.0, Bruker Daltonik GmbH, Germany).

MS data analysis

Data analysis was performed using BioTools 3.0 software and the MASCOT search engine (Matrix Sciences, UK). Searches were performed using the following parameters: mass tolerance was set to 100 ppm (MALDI-TOF/TOF) and 200 ppm (ESI-ion trap) for precursor ions and 0.7 Da for fragment ions. Trypsin was set as proteolytic enzyme with 2 allowed missed cleavages. Charge state 1^+, was used for MALDI-TOF/TOF and charge states 1^+, 2^+ and

3^+ for ESI–MS. Carbamidomethylation of cysteine residues and oxidation of methionine residues was set as variable modification.

MSDB, swissprot and NCBInr databases were used to identify tomato proteins either with an in house MASCOT server or online available MASCOT server (Matrix Science, UK). Proteins were termed identified, if at least two peptides were identified with a MASCOT peptide ion score higher than 25. The threshold of 25 for the peptide ion score was obtained searching a reverse data base (MSDB) with all generated MS/MS spectra. All hits obtained from this reverse database search showed a peptide ion score significantly below 25. Additionally, if high peptide ion scores (>46) were obtained for single peptides and a *y*-ion series of nearly all ions was obtained, the corresponding proteins were termed identified even if only one peptide was found for the protein. The databases used in house were released in August 2006 (MSDB), in October 2007 (NCBInr) and May 2009 (Uniprot/Swissprot, release 15.2). The same database releases were used at Matrix Science.

Results

Symptom development and bacterial populations in stems

Plants of the susceptible species WVa700 started showing symptoms at 4 dpi and both the WI and DS gradually increased until all plants had died at 10 dpi. The mean WI and DS were calculated from three biological replications (Fig. 1). The resistant species Hawaii7996, on the other hand, did not show any level of symptoms until 30 dpi.

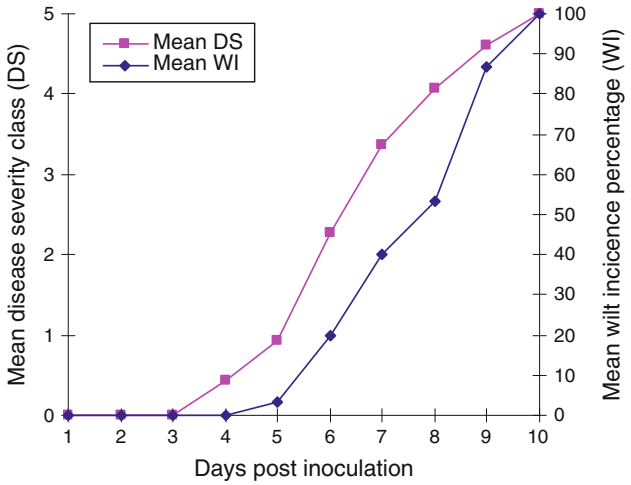

Fig. 1 Mean disease severity (DS) and wilt incidence (WI) in susceptible tomato plants (WVa700) on the days following *R. solanacearum* strain To-udk2 inoculation. The mean value was calculated from three independent biological replications. Both DS and WI increased continuously to maximum by 10 dpi

Since each biological protein sample was prepared by combining two to three individual plants, the number of bacteria present in each sample was calculated by taking the mean of the bacterial population from the individual plants that were combined to make that sample. The pathogen population in three biological samples at 5 dpi was calculated as 25.6×10^7, 16.6×10^7 and 26.1×10^7 cfu/g of stem in susceptible species and 45.8×10^6, 33.3×10^6 and 22.2×10^6 cfu/g of stem in resistant species. All the susceptible plants considered for the sample preparation showed clear wilt symptoms of 1–3 degree of disease severity.

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. 2A, B). These unresolved spots upon further separation by 3-D SDS–PAGE revealed 25–35 spots detected with Image master (Fig. 2C, D). The comparison of the 2-D gels was performed within species and treatments. The differentially expressed spots were first analysed by MALDI-TOF tandem mass spectrometer (MS/MS), which gave 70% (29 out of 42 spots) successful identifications. The remaining 13 unidentified spots were unambiguously identified with liquid chromatography (LC)-ESI-Ion trap MS/MS which in one case (Spot 3, Table 4), correlated to two proteins.

Protein regulation in resistant species

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. 3A, B). 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 increased. Eight other spots annotated as BIP, stress induced protein, catalase, enolase (2-phospho-D-glycerate hydrolase), vacuolar H^+ ATPase (V-ATPase), oxygen evolving 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 decreased (Table 1).

Protein regulation in susceptible species

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. 4A, B).

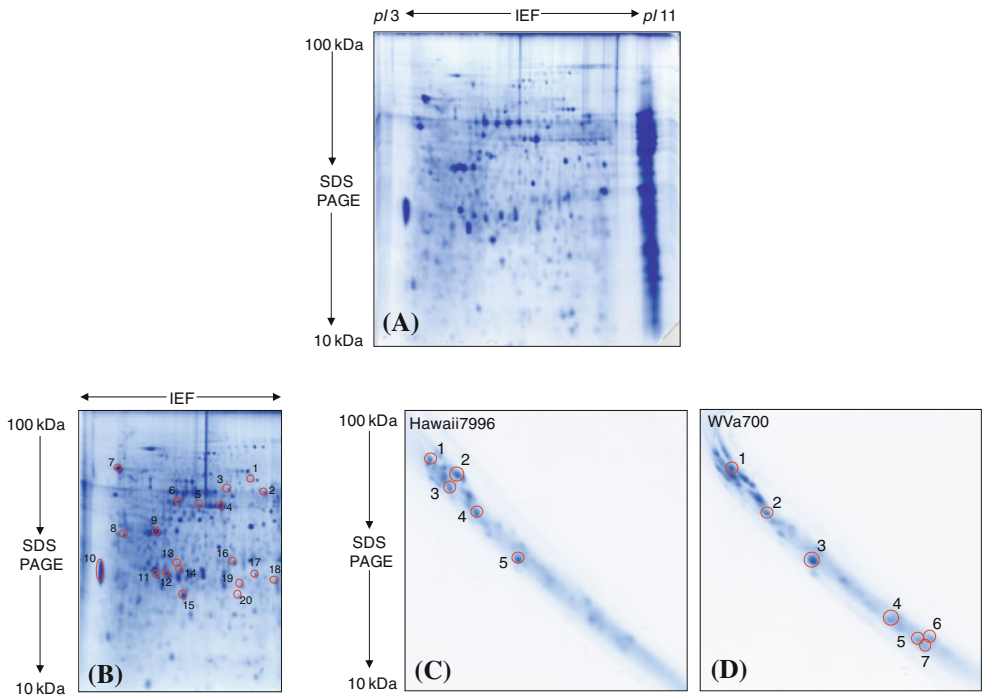

Fig. 2 Overview of the cell wall proteome analyzed from the mature tomato stem and separated in two as well as in three gel dimensions. (**A**) The resolution of proteome with 2-D IEF/SDS–PAGE in *pI* 3–11 non linear and 100–10 kDa molecular mass. The poorly resolved proteins on the basic *pI* range were seen as vertical streaking. (**B**) A representative 2-D IEF/SDS gel 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. The poorly resolved basic proteins without fixing and staining served as the starting material for the 3rd SDS–PAGE. Twenty spots were randomly picked out to check the presence of secretion signals of the cell wall proteins extracted with the method applied. The identity of each encircled spots and the information of secretion signals are given in Table 5. (**C**) and (**D**) 3rd dimension SDS gels showing the resolution of the unresolved vertical streak. The streaked gel piece was cut out before fixing as well as staining and separated again by SDS–PAGE. The encircled spots in (**C**) and (**D**) were differentially expressed in resistant (Hawaii7996) and susceptible (WVa700) species after pathogen inoculation, however in two replicates. The identity of all proteins was given in Table 4 with the spot numbers corresponds to each other

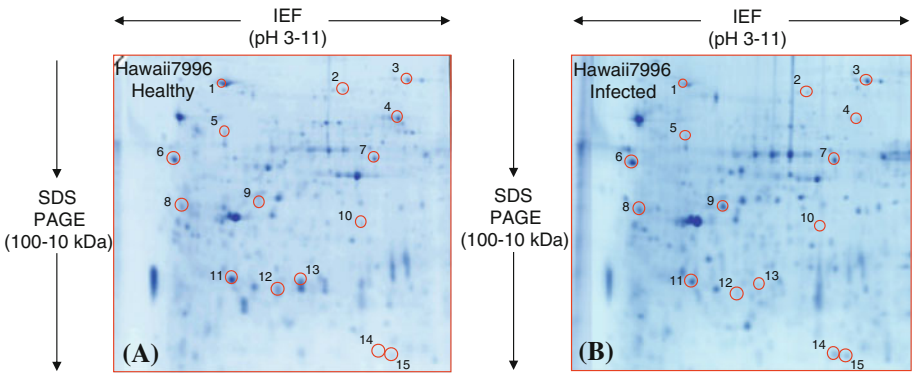

Fig. 3 "Zoom-in" view of the only area of the 2-D gels that shows differentially accumulated protein spots when the healthy plant of the resistant species Hawaii7996 (panel **A**) was inoculated with the pathogen *R. solanacearum* (panel **B**). The spot number is in accordance with the number used in Table 1. The tomato stem cell wall proteome was separated between pH 3–11 (non linear IPG stripes) and in the molecular mass range between 100 and 10 kDa. However, the basic *pI* side of the 2-D gel containing poorly separated proteins was removed before fixing and staining. All the shown spots regulation was consistently reproduced in three biological replications and is statistically significant (Student's *t* test, $P \leq 0.05$)

Among them, five proteins (spot 3, 4 and 6–8) were increased while eight proteins (spot 1, 2, 5 and 9–13) were decreased upon pathogen inoculation. The identity of the increased spots was correspondingly shown as peroxidase, peroxidase cevi16, basic 30 kDa endochitinase, triose phosphate isomerise (TPI) and PR-5 like protein and of

Table 1 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, $P \leq 0.05$)

Spot[a]	Identity[b]	Accession[c]	Organism[d]	Score[e]	*Mr/pI*[f]	Peptides[g]	Coverage[h]	Regulation[i]	SiP-SeP[j]
1	Luminal-binding protein	P49118	*Solanum lycopersicum*	260	73.23/5.10	3	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	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	2	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	1	4.2	0.51	0.27
11	Oxygen-evolving complex protein 2	P29795	*Solanum lycopersicum*	362	27.79/8. 27	5	27	0.54	0.8
12	Translation initiation factor 5A-3	Q9AXQ4	*Solanum lycopersicum*	93	17.37/5.47	1	6.3	0.65	0.24
13	Translation initiation factor 5A-4	Q9AXQ3	*Solanum lycopersicum*	255	17.51/5.6	3	28	0.23	0.23
14	Nucleoside diphosphate kinase	P47921	*Solanum lycopersicum*	165	15.67/7.04	2	12	1.75	0.40
15*	PII like protein	Q6T2D2	*Solanum lycopersicum*	201	21.73/9.33	5	22	1.75	0.88

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

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

[c] Protein database accession number (UniProt)

[d] Plant species from which the protein was annotated

[e] MASCOT score

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

[g] Number of matched peptides of MSMS data with the corresponding protein in MSDB, SwissProt and NCBInr databases

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

[i] Regulation: The fold increase or decrese in % spot volume of each spot after the *R. solanacearum* inoculation. Variation: The ratio in the abundance of spot % spot volume between the species

[j] The results of SignalP (SiP) and SecretomP (SeP) analysis. *Y* presence of signal peptide evaluated from SignalP. The numbers are 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

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

decreased as α-galactosidase (α-D-galactoside galactohydrolase), disulphide isomerase like protein (PDIs), xyloglucan endotransglucosylase-hydrolase7 (XTH7), two eTIF 5A-4, one eTIF 5A-1 and two glycine rich proteins (GRP) (Table 2).

Protein variation in genotypic comparison

Fourteen genotypic differences were found comparing protein profiles between susceptible and resistant plants (Fig. 5A, B). 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 species. 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 species (Table 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. 2A). The problem of poor resolution was improved considerably by separating them further with SDS–PAGE in the 3rd dimension (Fig. 2C, D). Two

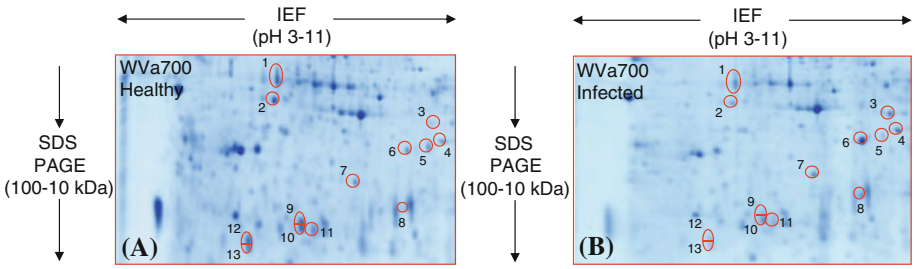

Fig. 4 "Zoom-in" view of the only area of the 2-D gels that shows differentially accumulated protein spots when the healthy plant of the susceptible species WVa700 (panel **A**) was inoculated with the pathogen *R. solanacearum* (panel **B**). The spot number is in accordance with the number used in Table 2. The tomato stem cell wall proteome was separated between pH 3–11 (non linear IPG stripes) and in the molecular mass range between 100 and 10 kDa. However, the basic *pI* side of the 2-D gel containing poorly separated proteins was removed before fixing and staining. All the shown spots regulation was consistently reproduced in three biological replications and is statistically significant (Student's *t* test, $P \leq 0.05$)

3-D gels were prepared from all species 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 species in response to bacterial invasion revealed five and seven protein spots of differential abundance (Fig. 2C and D). They were identified by MS analysis as peroxidase [pathogenesis related (PR)-9], β-1, 3-endoglucanase (PR-2) and osmotin like protein (PR-5) among others (Table 4). All of these PR proteins constitute important components of the disease resistance response. However, they were not considered for discussion due to only two biological replications.

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 (V. 3.0, www.cbs.dtu.dk/services/SignalP) and SecretomeP (V. 2.0, www.cbs.dtu.dk/services/SecretomeP) programs. Most of the proteins were predicted to have secretion signals (Tables 1, 2, 3, 4). In order to check the extracellular nature of cell wall proteins extracted by this particular method, 20 additional spots were picked randomly from the gel (Fig. 2B) and 90% of them showed signal peptides (Table 5). However, some glycolytic and other metabolic proteins that are conventionally not considered as extracellular proteins were also found in the cell wall.

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. Therefore, the current study was initiated firstly to find out the differences at the proteome level in mid-stem cell wall between susceptible and resistant species against bacterial wilt and followed by the simultaneous characterization of the 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 species and subsequent up or down regulation of the identified protein profiles after pathogen inoculation further elucidates the resistant and susceptible reactions of the species.

Expression of plant defense mechanisms

We observed the up regulation of several defense related proteins both in susceptible and resistant species 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 species. When plants are challenged with pathogens, the general defense responses are generally inducted by the synthesis of PR proteins and fortification of plant of cell walls among others responses.

PR proteins can be constitutively present 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

Table 2 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, $P \leq 0.05$)

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

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

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

[c] Protein database accession number (UniProt)

[d] Plant species from which the protein was annotated

[e] MASCOT score

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

[g] Number of matched peptides of MSMS data with the corresponding protein in MSDB, SwissProt and NCBInr databases

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

[i] Regulation: The fold increase or decrese in % spot volume of each spot after the *R. solanacearum* inoculation. Variation: The ratio in the abundance of spot % spot volume between the species

[j] The results of SignalP (SiP) and SecretomP (SeP) analysis. *Y* presence of signal peptide evaluated from SignalP. The numbers are 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

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

endo type, are believed to mediate defence responses because of their potential to degrade fungal cell walls. Many endochitinases also displayed a lysozyme activity enabling the hydrolysis of bacterial cell walls (Brunner et al. 1998). PR-5 family proteins contain several unique proteins with diverse functions including antifungal (Ibeas et al. 2000) as well as β-glucanase activity (Grenier et al. 1999). 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 protein induction is considered a marker of the activation of the basal defense, thus increased also in susceptible hosts.

NDPK not only performs a house keeping functions of regulating nucleotide pools but also involved in signal transduction in plants. *NDPK1* expression was reported to enhance multiple stress tolerance in transgenic plants by activating the MAPK cascade (Tang et al. 2008). Subtilisin-like serine proteases or subtilases are endoproteases secreted into the extracellular space of the plant. They have been associated with cellular defense and stress responses

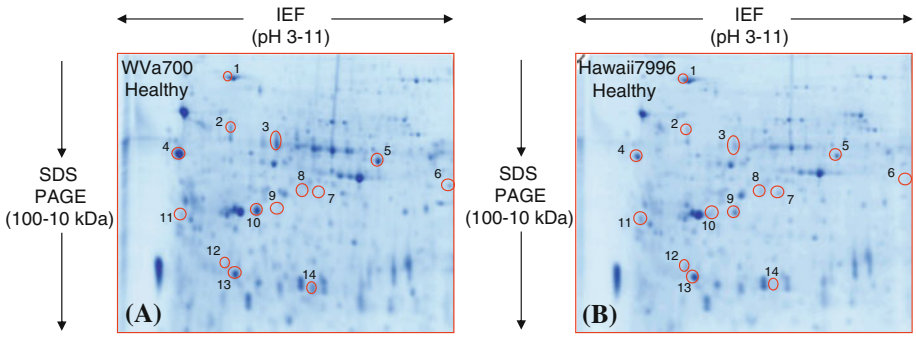

Fig. 5 "Zoom-in" view of the only area of the 2-D gels that shows differentially accumulated protein spots with differential abundance between WVa700 (panel **A**) and Hawaii7996 (panel **B**) healthy plants. The spot number is in accordance with the number used in Table 3. The tomato stem cell wall proteome was separated between pH 3–11 (non linear IPG stripes) and in the molecular mass range between 100 and 10 kDa. However, the basic *pI* side of the 2-D gel containing poorly separated proteins was removed before fixing and staining. All the shown spots regulation was consistently reproduced in three biological replications and is statistically significant (Student's *t* test, $P \leq 0.05$)

Table 3 List of differential 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, $P \leq 0.05$)

Spot[a]	Identity[b]	Accession[c]	Organism[d]	Score[e]	*Mr/pI*[f]	Peptides[g]	Coverage[h]	Variation[i]	SiP-SeP[j]
1	Luminal-binding protein	P49118	*Solanum lycopersicum*	260	73.23/5.10	3	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	5.6	1.79	Y
4	Peroxidase	Q9LWA2	*Solanum lycopersicum*	144	34.94/4.56	2	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	2	5.9	1.38	0.73
7	Hypothetical protein	O04428	*Citrus paradisi*	92.8	32.64/5.46	1	4.1	0.38	0.50
8	Fructokinase-2	Q42896	*Solanum lycopersicum*	208	34.76/5.76	3	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	2	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	5	27	0.69	0.8
14	Translation initiation factor 5A-1	Q9AXQ6	*Solanum lycopersicum*	139	17.30/5.71	2	18	4.12	0.23

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

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

[c] Protein database accession number (UniProt)

[d] Plant species from which the protein was annotated

[e] MASCOT score

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

[g] Number of matched peptides of MSMS data with the corresponding protein in MSDB, SwissProt and NCBInr databases

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

[i] Regulation: The fold increase or decrese in % spot volume of each spot after the *R. solanacearum* inoculation. Variation: The ratio in the abundance of spot % spot volume between the species

[j] The results of SignalP (SiP) and SecretomP (SeP) analysis. *Y* presence of signal peptide evaluated from SignalP. The numbers are 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

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

Table 4 List of major basic proteins that were poorly resolved in 2-D IEF/SDS–PAGE but well separated in 3rd dimension SDS–PAGE

Spot[a]	Identity[b]	Accession[c]	Organism[d]	Score[e]	*Mr/pI*[f]	Peptides[g] MS/MS	Coverage[h] MS/MS	SiP-SeP[i]
Hawaii7996 (resistant genotype)								
1	Peroxidase	Q94IQ1	*Nicotiana tabacum*	480	39.06/5.99	8	27	Y
2	Peroxidase prx14	Q9M4Z3	*Spinacia oleracea*	139	37.22/9.29	2	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	5	17	Y
5	Osmotin-like protein precursor	Q41350	*Solanum lycopersicum*	404	27.26/8.15	6	29	Y
WVa700 (susceptible genotype)								
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	2	6.7	Y
3	Osmotin-like protein	Q41350	*Solanum lycopersicum*	284	27.26/8.15	2	10	Y
4	Photosystem I reaction center subunit IV B	Q41229	*Nicotiana sylvestris*	131	15.22/9.74	2	19	0.82
5	Oxygen-evolving complex protein 3	Q672Q6	*Solanum lycopersicum*	201	24.57/9.64	3	11	0.65
6	Oxygen-evolving complex protein 3	Q672Q6	*Solanum lycopersicum*	73	24.57/9.64	1	4.3	0.65
7	Photosystem I reaction center subunit II	P12372	*Solanum lycopersicum*	125	22.91/9.71	3	12	0.80

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

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

[c] Protein database accession number (UniProt)

[d] Plant species from which the protein was annotated

[e] MASCOT score

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

[g] Number of matched peptides of MSMS data with the corresponding protein in MSDB, SwissProt and NCBInr databases

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

[i] The results of SignalP (SiP) and SecretomP (SeP) analysis. *Y* presence of signal peptide evaluated from SignalP. The numbers are 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

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

by mediating a restructuring and reinforcement of plant cell walls in order to arrest pathogen spread (Dixon and Lamb 1990).

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).

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 elevation of the defense related proteins, however, with different functional roles suggesting their involvements in resistance response to *R. solanacearum*.

Interestingly, three other defense and/or stress related proteins namely BIP, stress induced protein1 and catalase were decreased in resistant plants after pathogen invasion. In fact, BIP was observed in two spots (spot 1 and 8) and accumulated in opposite ways. BIP is a member of the hsp70 family protein and functions as a molecular chaperone. They have also been implicated in disease resistance but still unclear how they 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 is a primary antioxidant and has been demonstrated to be present in the plant cell wall. The down regulation can be expected following pathogen inoculation if the elevation of H_2O_2 and ROS level serves as second messengers to further trigger the

Table 5 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] MS/MS	Coverage[h] MS/MS	SiP-SeP[i]
1	Catalase isozyme	P30264	*Solanum lycopersicum*	211	56.50/6.60	2	5.3	0.34
2	Peroxidase	Q07446	*Solanum lycopersicum*	149	35.99/7.52	5	16	Y
3	NADH-glutamate dehydrogenase	Q8W1X4	*Solanum lycopersicum*	257	44.68/6.28	3	12	0.56
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	1	4.8	0.39
6	Hypothetical protein	O24329	*Ricinus communis*	80	40.00/7.56	1	4.4	Y
7	Calreticulin	Q40401	*Nicotiana plumbaginifolia*	167	47.48/4.45	4	9.9	Y
8	Ripening regulated protein	Q9FR30	*Solanum lycopersicum*	152	22.20/4.72	2	15	0.73
9	Oxygen-evolving complex protein 1	P23322	*Solanum lycopersicum*	205	34.94/5.91	2	8.2	0.44
10*	Calmodulin	P84339	*Agaricus bisporus*	277	16.78/4.15	6	18	0.70
11	ATP synthase D chain	Q6L460	*Solanum demissum*	191	19.80/5.34	2	8.9	0.61
12	Oxygen-evolving complex protein 2	P29795	*Solanum lycopersicum*	292	27.79/8.28	4	19	0.80
13	Soluble inorganic pyrophosphatase	Q43187	*Solanum tuberosum*	70	24.26/5.59	2	12	0.79
14	Temperature-induced lipocalin	Q38JE1	*Solanum lycopersicum*	171	21.25/5.96	3	17	0.51
15	Superoxide dismutase (Cu–Zn) 1	P14830	*Solanum lycopersicum*	67	15.30/5.83	1	8.6	0.68
16	Dehydroascorbate reductase	Q4VDN8	*Solanum lycopersicum*	200	23.53/6.32	2	13	0.36
17	Hypothetical protein	Q5XEP2	*Arabidopsis thaliana*	67	64.52/5.85	3	7.5	0.58
18	Tomato invertase inhibitor	O82001	*Solanum lycopersicum*	62	18.76/8.30	2	15	Y
19	Translation initiation factor 5A-4	Q9AXQ3	*Solanum lycopersicum*	102	17.51/5.60	1	6.3	0.23
20	Nucleoside diphosphate kinase	Q2KK37	*Thlaspi caerulescens*	121	25.79/9.34	1	5	0.89

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

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

[c] Protein database accession number (UniProt)

[d] Plant species from which the protein was annotated

[e] MASCOT score

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

[g] Number of matched peptides of MSMS data with the corresponding protein in MSDB, SwissProt and NCBInr databases

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

[i] The results of SignalP (SiP) and SecretomP (SeP) analysis. *Y* presence of signal peptide evaluated from SignalP. The numbers are 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

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

downstream defense responses (Foyer and Noctor 2005). Furthermore, increased ROS level, however, under a threshold not triggering a cell death, can inhibit pathogen growth. An increase in H_2O_2 with consequent reduction of catalase and superoxide dismutase activities was observed in jute under water stress (Chowdhuri and Chowdhuri 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. However, the outcome of susceptibility or resistance to the given pathogen could be determined by the temporal and spatial regulation of these responses and hence their efficacy.

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 decreased 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 plant cell wall during synthesis and also disease responsive fortification processes (Fry 2004). The enzyme α-galactosidase, generally acidic forms, is common in plants and is 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 in addition to their roles in the development as well as repair of vascular tissues and wound healing (Park et al. 2001; Ryser et al. 1997). Down regulation of GRP due to water stress was reported with the argument 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 presence 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.

Metabolic activities alteration

Proteins that are known to have active roles in plant metabolism were identified in the cell wall fraction of our analysis, though more specific experiments such as immuno-cytochemistry has not been performed to confirm their presence. The occurrence of cytosolic proteins in the extracellular space of the plants was reported in several previous studies. The possibility of moonlighting functions of these intracellular proteins was also increasingly revealed. Therefore, the association of the following metabolic proteins with defense responses of the plants are discussed in more generalized concept rather than within the boundary of extracellular proteins.

Variation in primary metabolism

Proteins associated with glycolysis such as fructokinase, TPI and enolase were differentially accumulated in the two species. Fructokinase and TPI were correspondingly increased in resistant and susceptible lines while enolase was decreased in resistant plants. The increased abundance of fructokinase and TPI during bacterial infection can be assumed as consequences of increased metabolism to compensate for the cost of defences (Curto et al. 2006). PII like protein is considered to participate in metabolic regulatory mechanism and was increased in resistant varieties (Hsieh et al. 1998). On the other hand, the expression of enolase was suppressed in resistant plants. Enolase is known to catalyze the penultimate reversible reaction of glycolysis but also identified as a major glucan-associated cell wall protein in Arabidopsis and Medicago cell walls (Lee et al. 2004; Watson et al. 2004). Suppression of enolase in resistant plants could be correlated to inhibition of the pathogen by limiting the supply of sugars.

In overall, regulation of these metabolic proteins in both species after pathogen challenge supports the observation that the plant defense responses are associated with active metabolic changes in host plants.

Inhibition of energy metabolism

Both OEE 2 and V-ATPases sub unit E were decreased in resistant reactions. OEE is associated with the photosystem II complex and are believed to be important for photosynthesis and overall PSII stability (Raymond and Blankenship 2004). Therefore, any damage or inhibition of PSII due to stresses could lead to suppression of OEE proteins. Down regulation of OEE proteins was reported earlier due to water stress and tobacco mosaic virus infections (Echevarría-Zomeño et al. 2009; Lehto et al. 2003). V-ATPases are multimeric enzymes and can be associated with various membranes of the secretory system to promote cell growth or expansion and secretion of cell wall components (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.

Variation in other proteins

The abundance of PDIs was reduced after pathogen invasion in susceptible plants. PDIs are involved in the folding, assembling and sorting of plant secretory or plasma membrane proteins, which are essential for the stability and

activity of extracellular proteins (Wilkinson and Gilbert 2004). 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. eIF-5A is a nucleo-cytoplasmic shuttle protein (Jao and Chen 2005) and were also known to regulate 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 (Hopkins et al. 2008). Both isoforms of eIF-5A were repressed in both species suggesting the possible importance of their PTMs during host-pathogen interaction.

Constitutive differences in tomato species

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. Again, the identification of these cytosolic proteins in our cell wall fraction does not validate their localization in the apoplast without additional experiments but their roles in the make-up of the defense reactions of the plant can be discussed.

The resistant species displayed the higher abundance of glycolytic proteins such as enolase observed in three different spots (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 (Yan et al. 2005). Defense and stress related proteins also showed constitutive differences among the species. BIP was in higher abundance in resistant variety where as multifunctional peroxidase and α-galactosidase in susceptible plants. The susceptible species also differed from the resistant one in the expression of FNR, which is considered as detoxifying agents due to its free radical scavenging ability. Both species 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 ward off the pathogen infection upon challenge. 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 the synthesis or suppression of the protein.

Several proteins identified in multiple spots such as enolase, OEE, BIP, GRP as well as eIF-5A and their isoforms were regulated in the same way (Tables 1, 2 and 3). 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.

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 and hence often appeared in 2-D gels.

Nature of cell wall proteins

The presence of secretion signals that are characteristic to 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 posses non-classical secretion signals (Tables 1, 2 and 3). Further 20 proteins from 2-D gels and 12 proteins from the comparative 3-D gels were tested for their secretory nature and 30 of them contain secretion signals. 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 required to confirm their presence in cell wall.

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 species and treatments consistently resolved the basic range of cell wall proteins. The identification of 12 major spots from 3-D gels showed the presence of proteins with theoretical *pI* higher than 7 (Table 4).

Conclusion

To conclude, the current study provides for the first time the broad spectrum analysis of the stem cell wall proteome

of tomato species followed by their specific regulation after bacterial challenge. It unveiled constitutive proteomic differences between tomato species 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 species triggered by the pathogen support their pivotal roles in the make-up of 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 species. 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.

Acknowledgments This study was funded by the Federal Ministry for Economic Cooperation and Development (BMZ), Germany, in the framework of the collaborative project number 03.7860.4–001.00 with the Asian Vegetable Research and Development Center (AVRDC), Taiwan.

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