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Research article

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

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ABSTRACT

A comparative proteome analysis was initiated to systematically investigate the physiological response of tomato (*Solanum lycopersicum*) to infection with *Ralstonia 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. Two-dimensional isoelectric focussing/sodium dodecyl-sulphate polyacrylamide gel electrophoresis (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 pathogenesis related (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.

1. Introduction

Bacterial wilt is a collective term for wilting diseases caused by at least 15 bacterial species, however, the wilt caused by *Ralstonia solanacearum* [9,34] is the most devastating, systemic vascular wilt disease of crop plants. *R. solanacearum* as a species complex has a host range of more than 200 plant species representing over 50 botanical families including dicotyledones and monocotyledones, extending from annual plants to trees and shrubs [9]. Among these, solanaceous plants including economically significant hosts of global importance such as tomato, potato, tobacco and eggplants are the most affected species [34]. In tomato (renamed as *Solanum lycopersicum*), losses up to 75–100% were reported in the lowland and highland tropics and subtropics [28].

Ralstonia solanacearum is an aerobic, Gram-negative rod with a high degree of phenotypic and genotypic diversity, including strains differing largely in host range, geographical distribution, pathogenicity, epidemiological relationship and physiological properties [9]. The soilborne bacterium potentially requires only small wounds in the roots such as occur by lateral root emergence to establish a systemic infection [41]. 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 [41]. Cell numbers as high as 10^{10} cells/cm of stem are reached in xylem vessels of tomato [7], leading to blockage of the vascular system and thereby alteration of water fluxes [29]. Such a vascular dysfunction is the major cause of typical green-wilting and subsequent plant death [8].

Bacterial wilt resistance, a polygenic trait in tomato, with quantitative trait loci (QTLs) often linked to undesirable characteristics, was generally found to be specific to geographical sites, and frequently broken due to high genotype × environment interactions [43]. 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 [18]. 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

Abbreviations: DTT, dithiothreitol; PR protein, pathogenesis related protein; MS/MS, tandem mass spectrometry.

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death, production of antimicrobial secondary metabolites, further reinforcement of the cell walls and the synthesis and accumulation of pathogenesis related proteins [42]. These complex series of cellular responses may lead to enhanced disease resistance against a broad spectrum of phytopathogens when expressed in a synchronized manner [18].

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 [13,20]. The bacterial population was reduced significantly in mid-stems compared to the taproot and collar region after root inoculation [40]. 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 [7,10]. 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 [7,27]. 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 [10,46–48]. 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 [17]. 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 [25,30]. For tomato, a dense molecular marker linkage map (http://www.sgn.cornell.edu) and for the relatively small genome (950 Mb) an extensive database of over 184,000 expressed sequence tags ([37], http://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 [30] and the in-depth study of the type III secretion system (T3SS) and related pathogenicity and effector proteins allowed the identification of *in planta* expressed proteins [1].

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 and the susceptible Wva700 parental lines, were chosen for identification of differential protein expression.

2. Results

2.1. Symptom development and bacterial populations in stems

Plants of the susceptible genotype NHG3 started wilting 4–5 days post inoculation (dpi), progressing to plant death within 11 days. The mean wilt incidence and disease severity were calculated from three biological replications (Fig. 1). The area under disease progress curve (AUDPC) of wilt incidence and disease severity 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

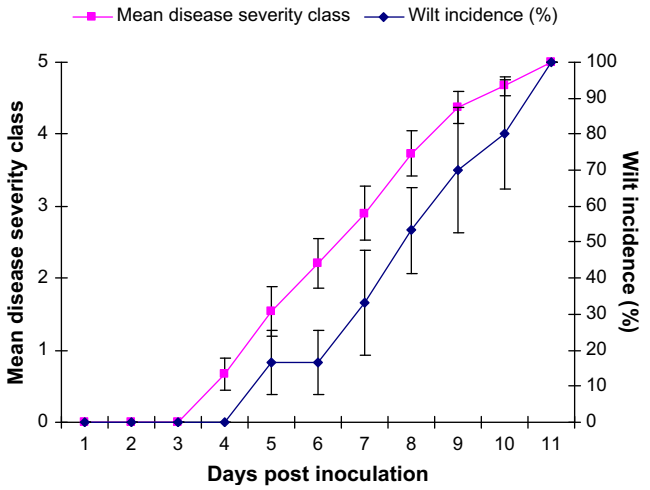

Fig 1. Development of wilt incidence and disease severity of tomato genotype NHG3 inoculated with *R. solanacearum* strain ToUdk2. The mean wilt incidence and disease severity class with corresponding standard errors were calculated from three biological replications of NHG3 plants. Line NHG13 did not show symptoms.

2.58×10^9, 3.06×10^9 and 2.55×10^9 CFU/g fresh weight of stem in genotype NHG3 compared to 2.99×10^5, 4.91×10^4 and 2.49×10^5 CFU/g fresh weight of stem in genotype NHG13 at 5 dpi.

2.2. 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 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. 2),

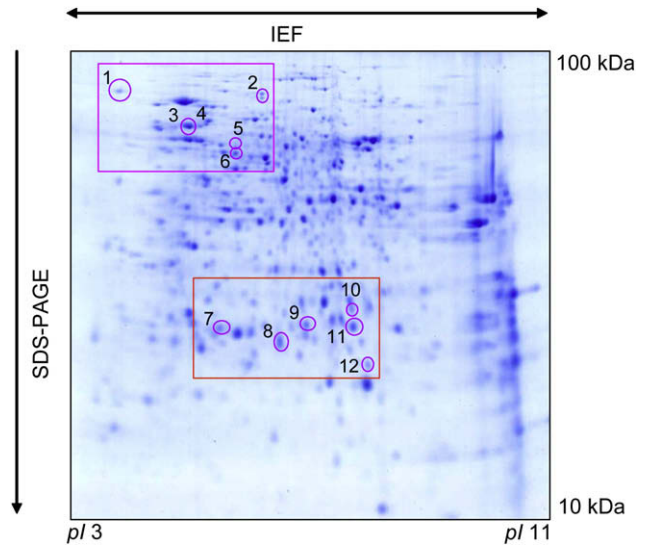

Fig. 2. Overview of 2-D IEF/SDS-PAGE analyses of mid-stem proteomes isolated from tomato. Protein separations were based on IEF using non-linear gel stripes in the range of pH 3–11 (horizontal separation) and on SDS-PAGE in the size range between 100 and 10 kDa (vertical separation). Protein spots differing in abundance in NHG3 genotype before and after pathogen inoculation were circled and numbered (for protein designation see Table 1). The purple and red boxes indicate regions shown as "Close ups" in Fig. 3.

with proteins of differential abundance in genotype NHG3 after bacterial challenge being circled and numbered in three gel regions.

2.3. 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. 3). 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).

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

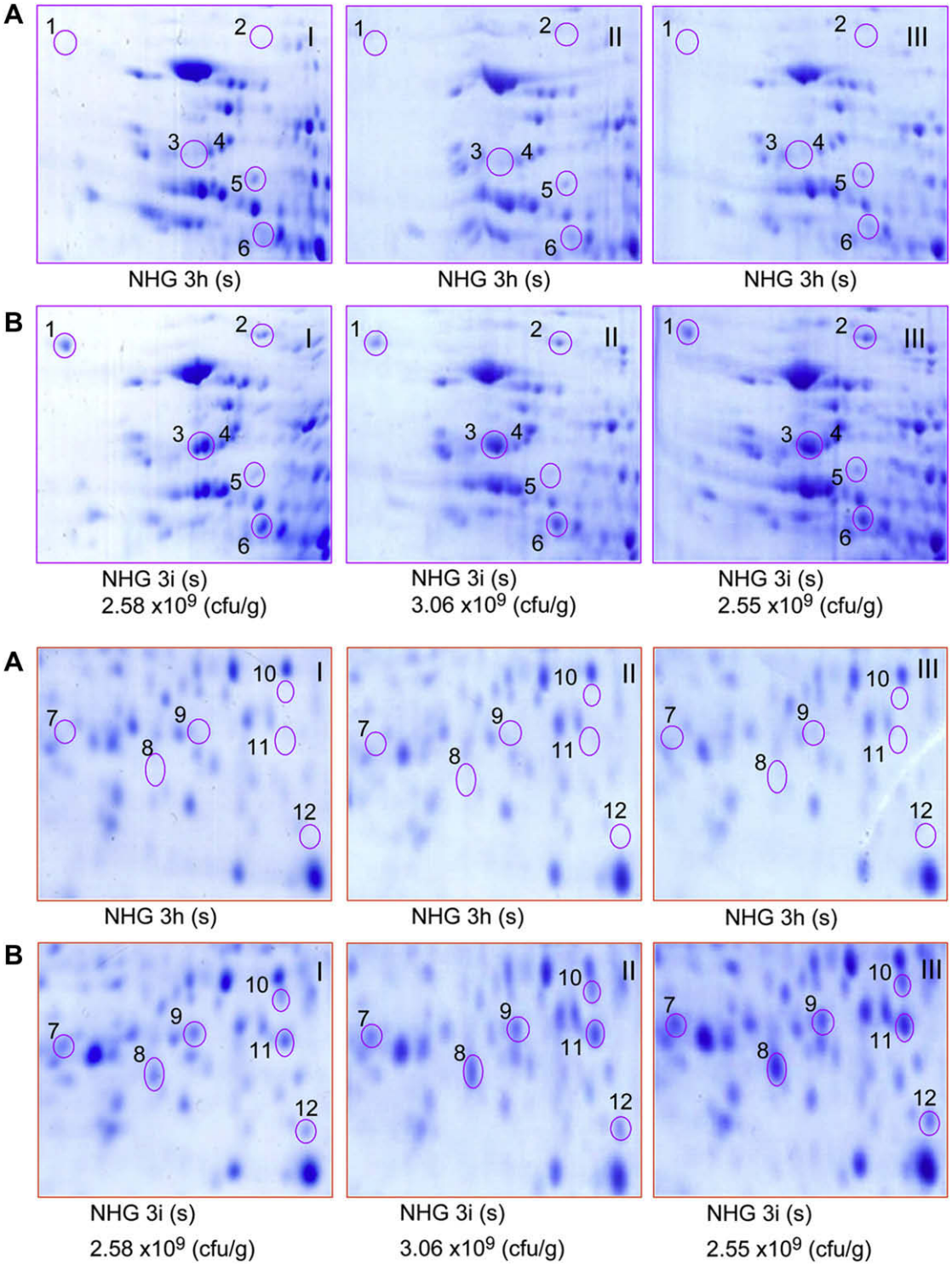

Fig. 3. "Close ups" of two gel regions indicated on the 2-D gels in Fig. 2. Gels of three independent experiments (I, II, III) are shown. Gel regions shown in "A" rows: susceptible genotype (NHG 3), healthy. Gel regions shown in "B" rows: genotype NHG3, infected. Proteins of different abundances are circled and numbered consecutively in correspondence with Fig. 2. 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).

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/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	
2	VALVYGQMNEPPGAR-FVAEVFTGSPGRYVGL - FVQAGSEVSALLGR-PATTFAHLDATTVLSR-I VGEEHYETAQR-EGNDLYMEMK-VLGEPVDNL G-LGLDELSEEDR-PNIYNALVVKGR-VVDLLAP YR-APLSVPVGG-LSIFETGIK-LLFELLNNLAK-Q QLLGNNR-VGLTALTFAE-AVAFSATEGLTR-DS TSTML-FGGVGER-EVQQLLGN-VLNTGSPLTV -AMNLEFES-LVGNIDEA-PTTSGP-SAPAFI-VE GSTLGR-QLDTK-PLTVPTGAA-ESNLK 260	ATP synthase beta subunit (*Boquila trifoliolata*)	Q31931	n.d.	1704	50	Induced
3	WHAVEHAVMTVEQR-HADVWGTFSQLR-PVLT LSLLVNTPAK-PPHQPVMPM-PFYAVSLQQAK- LDKLHTELAK-ASGDLGQMK-LADAPQLK-TGQ ASYR-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-VTSYTHETTTP VAPTR-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-QMNF VEGGPLK-FEANDNGGSVYK-YSLLEGDVLG-L ESLTYDLK-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-FYTECFG MK-YDIGTGFG-TIAMMGYAPE-SAEVVKIVNQE L-VVKIVNQELGGK-SVIAFVK-TPEPLC-FLHAV YR-LTSFLDPD-PTPEPL-ALATPDV-QVMLR-SF LDPGGGSLPGLNTK-YGVTE	Putative lactoylglutathione lyase (*Brassica oleracea*)	Q39366	31.64/5.19	886	48.5	Higher abundance
6	DPDGYIFELIQR-YAQLALGTDDVYK-FYTECFG MK-PGSIPGLNTK-SAEVVNLALQEL-FALATPD VYK-VVNLALQELGGK-YDIGTGFG-FLHAVYR-LTSFLDPD-TPEPLC-PTPEPL-TSFLDPGG-QV MLR-YGVTE-IAFVK-TVLVD	Hypothetical protein (*Citrus paradisi*)	O04428	32.64/5.46	994	46	
7	NSQGAWSLTK-SAGGQGGNSQGA-MKDLYVK-TTFGEVTV-FDSPAIK-DMVFSK-SLLQPR-AAE GIP-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-M VEGVNLLAN AVK-AQLEEATSDYDR-ENTTIID GAGDAR-TTDCAVAELPK-LQNMGAQMVK-ED ELDVVEGM-DNPFVLLFDK-DLLPVLEQVAK-GA NADQDAGIK-AVA AAVEELKK-VGAATEVEMK-LDNPFVLLFD-DVVEGMQFDR-GDNVEFGVLD PTK-DVV FGDAAR-EGVLTLQDGK-VANVIAGK-EDALHA-ALISGLK-SFGGVVVTK-ARIAEA-EEP LR	60 kDa chaperone GroEL (*Ralstonia solanacearum*)	Q8Y1P8	57.40/5.09	1762	44.4	Induced
9	AAVEEGLVAGGGVALLR-VQLDNPFVLLFDKK-GDGTTTATVLAQSIVR-AQIEEATSDYDR-MLT TDCAVAELPK-LSPYFINNPEK-VEGVNILANAV K-YVAAGMNPFDLK-LQNMGAQMVK-DVVFGD AAR-ENTTLLDGAGD-LPVLEQVAK-ASVVVAN VLAGK-GANADQDAGLK-VEFGVLDPTK-VGAA TEVEFK-FGGPTVTK-LSANSDESLGAR-VITVE DGK-LAGGVAVIK-EEIGLTLEK-APGFGDR-AA VEELK-EDALHA-DELDVVGG-RAAVESG-VVL ER-LHATR-VEDAL	Molecular chaperone (*Ralstonia pickettii*)	Q75T66	57.32/5.07	1796	53	Induced
10	TTTYYSAGGVLVR-DAGGGDNTIYAGR-YYQG IFGVLPG-DSNATGHAVVK-ESLMPVIASSWK-ALVQTSLEFFR-FFADGSSIR-NVVTVGDG-QTA VQDR-ILGLGHDVVD-TQVLGFR-LRGPA	Putative hemolysin-type protein (*Ralstonia solanacearum*)	Q8XT20	70.18/4.37	752	16.6	Induced
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

(*continued on next page*)

Table 1 (*continued*)

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
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	

n.d., not determined (partial sequence).
[a] Gel spot numbers correspond to the numbers given in the gels shown in Figs. 2 and 3.
[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.

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. [45]. 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*).

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.

3. 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 5 days after inoculation. 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 [10,46]. 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 [5,16]. 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, 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. [36] 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 [17]. 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 is recommended.

3.1. Proteins involved in plant defence

In genotype NHG3, two pathogenesis related (PR) proteins of low molecular weight were identified, of which STH-21 (spot 4) was induced, while TSI-1 (spot 5) was slightly down-regulated upon infection. PR gene expression is activated by a number of biotic or abiotic stresses, including pathogen infection [39]. 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 [39].

STH-21 was initially identified as a member of a small multigene family accumulated in potato upon infection [6]. The up-regulation of PR proteins in the susceptible genotype compared to its resistant counterpart was also shown in *Medicago truncatula* [4]. TSI-1 (tomato stress induced-1) protein is an intracellular PR protein (IPR) organized as a multigene family in the tomato genome [35]. 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 [24]. 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 [50]. A similar finding was reported by Constable and Brisson [6], where potato STH-2 protein disappeared completely at 4–5 days after inoculation with a high concentration of compatible *Phytophthora infestans* spores.

3.2. Proteins involved in plant stress

An enzyme involved in oxidative stress, the putative lactoylglutathione lyase (spot 6), was increased in abundance upon infection. This is the enzyme which participates, together with glyoxalase I and II, in the glutathione-based (GHS) detoxification of methylglyoxal (MG) and other detrimental compounds formed primarily as a by-product of carbohydrate and lipid metabolism [33]. 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 [49]. 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 [2]. 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.

3.3. Proteins involved in carbohydrate metabolism

Spot 12, 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 [12]. However, FRK2 seems to play a greater role in sugar sensing or signalling than as a metabolic enzyme [26]. 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 [14]. In potato, an increased level of sugar metabolism correlated to enhanced susceptibility to a root rot pathogen was reported [23].

3.4. Proteins involved in energy production

The ATP synthase beta subunit (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 [32]. 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 [38].

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 [3].

4. 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.

5. Materials and methods

5.1. 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 (*Solanum lycopersicum*) Hawaii7996 and the highly susceptible line (*Solanum pimpinellifolium*) 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 NGA agar 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).

5.2. Plant growth conditions and inoculation

Tomato seeds were sown in the greenhouse (20 °C, 14 h photoperiod per day, 30 K lux and 70% 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.

5.3. Bacterial quantification

The bacterial multiplication was determined at 5 days post inoculation (dpi) in the same tomato plants that were used for proteome analysis. Approximately 5–7 g of the lower stem part were 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 × *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 centres due to consumption of TTC dye by the pathogen and were counted to calculate bacterial population as colony-forming units per gram of fresh weight (CFU/g).

5.4. Monitoring and evaluation of disease symptoms

The typical symptoms of bacterial wilt were monitored daily in six disease severity scores from 0 to 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 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 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 [15].

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

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.

5.5. 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.

5.5.1. Protein extraction and sample preparation

Total protein extraction from the tomato stem was carried out according to the protocol of Mihr and Braun [19]. 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) was added, vortexed and shaken at 300 rpm, at room temperature (RT) for 30 min (Mixer 5432, Eppendorf). The mixture was centrifuged at 1100 × *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 (17,000 × *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" [8 M 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]. The suspension was well vortexed and centrifuged (17,000 × *g*, 4 °C for 5 min). The supernatant containing soluble protein mixtures was flash frozen in liquid nitrogen before isoelectric focussing.

5.5.2. Two-dimensional (2-D) gel electrophoresis

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

5.5.2.1. Isoelectric focussing (IEF). Isoelectric focussing of protein mixtures was carried out using 18 cm immobiline dry gel strips (IPG strips, pH 3–11 non-linear, 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, Munich, Germany) according to Werhahn and Braun [44] 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.

5.5.2.2. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 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 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 [31] using the Protean II electrophoresis unit (BioRad, Hercules, CA, 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 (3 M 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.

5.5.3. 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, and 2% ortho-phosphoric acid in 20% methanol) as described by Neuhoff et al. [21,22]. 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.

5.5.4. Mass spectrometric analysis and data interpretation

Protein in-gel digestion, peptide extraction and mass spectrometry analysis were performed as described by Führs et al. [11].

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, WI, USA) in 25 mM NH_4HCO_3 at room temperature for 14 h. After extraction, the resulting tryptic peptides were analysed by Nano-LC-MS/MS on a CapLC capillary LC 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) software, as well as the PEAKS Studio software (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. [11].

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