Proteome Analysis of Disease Resistance against *Ralstonia solanacearum* in Potato Cultivar CT206-10

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Potato is one of the most important crops worldwide. Its commercial cultivars are highly susceptible to many fungal and bacterial diseases. Among these, bacterial wilt caused by *Ralstonia solanacearum* causes significant yield loss. In the present study, integrated proteomics and genomics approaches were used in order to identify bacterial wilt resistant genes from *Rs* resistance potato cultivar CT-206-10. 2-DE and MALDI-TOF/TOF-MS analysis identified eight differentially abundant proteins including glycine-rich RNA binding protein (GRP), tomato stress induced-1 (TSI-1) protein, pathogenesis-related (STH-2) protein and pentatricopeptide repeat containing (PPR) protein in response to *Rs* infection. Further, semi-quantitative RT-PCR identified up-regulation in transcript levels of all these genes upon *Rs* infection. Taken together, our results showed the involvement of the identified proteins in the *Rs* stress tolerance in potato. In the future, it would be interesting to raise the transgenic plants to further validate their involvement in resistance against *Rs* in potato.

**Keywords**: genomics, MALDI-TOF-MS, potato, proteomics, *Ralstonia solanacearum*

Potato (*Solanum tuberosum* L.) is third most important crops worldwide following maize and rice (FAO Crops statistics database: http://faostat.fao.org/). The productivity and quality of potatoes are affected by a number of constraints including biotic and abiotic stresses. *Ralstonia solanacearum* (*Rs*) is one of soil borne pathogen which causes bacterial wilt in numbers of solanaceae and non-solanaceae plants, resulting in huge loss of their productivity (Wenneker et al., 1999). *Rs* is a heterogeneous species, subdivided into five races and five biovars based on the host range and ability to utilize carbohydrates respectively (Hayward, 1991). *R3bv2*, a subgroup of *Rs* which especially causes bacterial wilt in potato in tropical regions of Asia, Africa and Latin America (Champoiseau et al., 2009). However, the outbreaks of this disease has been noticed sometimes even in the temperate regions (Elphinstone et al., 1996; Laferriere et al., 1999). The global damage estimated with this disease is more than $ 1 billion/ year (Elphinstone et al., 2005). There is no any effective means developed so far to protect the potato plants from this devastating disease.

Plants respond to pathogens by exhibiting various changes at molecular and physiological levels. Various studies have been conducted in potato to investigate differential transcripts expression upon different pathogenic infections. *Phytophthora infestans* which causes late blight disease in potato showed up-regulation of 2,344 transcripts upon infection which belongs to stress inducible protein, zinc finger transcription factor, heat stress transcription factor, defense related protease and pathogenesis related proteins (Siddappa et al., 2014). In other reports, transcripts level of osmotin like protein (El-Komy et al., 2010) and Lipoxygenase (Kolomiets et al., 2000) were shown to be up-regulated upon *P. infestans* infection. Potato plant infected with *Fusarium solani* f. sp. *eumartii* showed up-regulation of gene coding for heat shock and ribosomal proteins (D’Ippolito et al., 2011).
There are only few reports on differential proteins expression in potato under pathogen infections. Quantitative transcriptomics and proteomics of potato in response to *P. infestans* showed change in abundance of more than 17,000 transcripts and 1,000 secreted proteins. Major identified proteins were related to subtilase, peroxidase and protease inhibitor families (Ali et al., 2014). Aspartic protease content and its activity have been reported to increase in potato upon *P. infestans* infection (Guevara et al., 2004). Inductions of metallocarboxypeptidase protein from potato cultivar bintje, and β-glucosidase and transcription activator PTI6 from late bright resistant genotype of potato have also been reported upon *P. infestans* infection (Taoutaou et al., 2011).

To the best of our knowledge, there is no report on differential protein expression in potato upon *Rs* infection. Therefore, the present study was carried out with the aim to identify *Rs* resistance genes and proteins from *Rs* resistance potato cultivar CT206-10 which could be used further to raise *Rs* resistance transgenic potato plants in future. For this, disease resistance assay and trypan blue staining were carried out to investigate the resistance in the Superior and CT206-10 cultivars. To analyze the *Rs* induced changes at the molecular level, these two cultivars were subjected to a proteomic analysis using a 2-DE-MS approach which were further validated by semi-quantitative RT-PCR.

**Materials and Methods**

**Growth conditions of plant and *Rs***. Potatoes, superior and resistance cultivar CT206-10 (Kim-Lee et al., 2005), were propagated in sterile containers containing Murashige and Skoog, 1962 (MS) medium supplemented with 3% sucrose and 0.8% agar. The pH of the medium was adjusted to 5.8 prior to autoclaving. All cultures were maintained at 20 ± 1°C under 16 h light/8 h dark photoperiod. Seedlings were transferred to greenhouse after 4–5 weeks and allowed to grow in natural light condition. *Rs* strain KACC10722 (race 3, biovar 2) was grown on CPG medium (0.1% casamino acid, 1% peptone, 0.5% glucose, pH 6.5), cultured at 28°C for 24–48 h. The bacterial concentration was diluted to 10⁷ cfu/ml prior to the plant infection.

**Rs infection.** For infiltration of pathogen *Rs*, the bacterial solutions at 10⁷ cfu/ml in an approximately 1 ml volume, measured according to indicator syringes no needle, were infiltrated into potato leaves. *Rs* infection to roots was carried out as described earlier (Deslandes et al., 1998) with slight modifications. Approximately 2 cm of roots from the bottom of five to six weeks old potato seedling were cut and infected with *Rs* for 1 hr. The infected potato plants were then transferred to Jippy pots and bacterial wilt disease caused by *Rs* was observed for 14 days. Whole potato tissues from ten plants, each of superior and resistance cultivar CT206-10 were harvested after 7, 14 and 21 days of post-infection and stored at −80°C until used.

**Trypan-blue staining.** Trypan-blue staining was performed as described earlier (Peterhansel et al., 1997). In brief, potato leaves were boiled in trypan-blue solution [0.033% (w/v) trypan-blue, 8% (v/v) lactate, 8% (v/v) glycerol, 8% (v/v) phenol, 8% (v/v) water, and 67% (v/v) ethanol] until the green color disappeared. The leaves were then washed with water and transferred to the saturated chloral hydrate solution (2.5 mg/ml) to remove non-specific staining. The leaves were then immersed in the 50% (v/v) glycerol solution until analysis.

**Protein preparation and 2-DE analysis.** Total protein from ten grams of potato root was extracted using Mg/NP-40 extraction buffer [0.5 M Tris-HCl, pH 8.3; 2% (v/v) NP-40; 20 mM MgCl₂; 2% (v/v) β-mercaptoethanol] followed by phenol extraction method as described earlier (Kim et al., 2001). Approximately, 600 μg protein was dissolved in the rehydration buffer [7 M (w/v) urea, 2 thiourea, 4% (w/v) CHAPS, 0.002% (w/v) bromophenol blue, 20 mM DTT, 0.5% (v/v) IPG buffer (pH 4–7)] and loaded on 24 cm IPG strips, pH 4–7 by rehydration loading overnight. First dimensional separation was carried out using following protocol: 50 V 4 h, 100 V 1 h, 500 V 1 h, 1,000 V 1 h, 2,000 V 1 h, 4,000 V 2 h, 8,000 V 5 h, and 8,000 V 9 h by IPG-Phore2 platform (GE healthcare). Focused IPG strips were equilibrated using equilibration buffer containing 1% DTT as first step which was replaced by 2.5% iodoacetamide in the second step. Second dimensional separation was carried out using 12% SDS-PAGE after which the gels were stained with colloidal Coomassie brilliant blue G-250 (CBB).

**MALDI-TOF/TOF MS identification of differential protein spots.** Differential protein spots were excised from the 2D gels and were subjected to in-gel digestion as described previously (Kim et al., 2013). Prepared samples of tryptic peptides were subjected to MALDI-TOF/TOF MS using ABI 4800 Plus TOF-TOF Mass Spectrometer (Applied Biosystems, Framingham, MA, USA). The ten most and least intense ions per MALDI spot with signal/noise ratios >25 were selected for subsequent MS/MS analysis in 1 kV mode using 800–1,000 consecutive laser shots. Data were subjected to a Mass Standard Kit for the 4700
Proteomics Analyzer (calibration Mixture 1). MS/MS spectra were searched against the Uniprot/Swiss-Prot (14926175 sequences; 5299740401 residues) by Protein Pilot v.3.0 software (AB Sciex, Framingham, MA, USA) using MASCOT as search engine (ver. 2.3.0, Matrix Science, London, UK). The search parameters were as follows: fixed modifications-carbamidomethylation of cysteines, variable modification-methionine oxidation, peptide and fragment ion mass tolerances- 50 ppm, maximum trypsin missed cleavage- 1 and instrument type- MALDI-TOF/TOF. Only significant hits, as identified by the MASCOT probability analysis ($p<0.05$) were accepted.

**Semi-quantitative RT-PCR.** First-strand cDNA was synthesized from 1 μg of total RNA from the roots of CT206-10 potato cultivar infected with *Rs* in 20 μl of reaction mixture using Superscript III reverse transcriptase (Life Technologies, NY, USA). The semi-quantitative RT-PCR was performed using Actin and Tubulin genes as internal control. The PCR reaction conditions were initial denaturation at 94°C for 5 min, 22–35 cycles of 94°C for 30 s, 52–58°C for 30 s, 72°C for 30 s and then final extension at 72°C for 5 min. The PCR amplified products were electrophoresed on 1.5% agarose gel and transcript levels of genes were estimated.

**Results and Discussion**

**Evaluation of potato cultivar CT206-10 against *Rs* infection.** At first, we compared the resistance of “CT206-10” with that of wild type “superior” against *Rs* infection (Kim-Lee et al., 2005). Leaf wilting is one of the early symptoms of *Rs* infection in plants therefore; we observed the leaf wilting on both the cultivars after infection. Leaves of both the cultivars showed varied degree of wilting after infection with *Rs*, suggesting successful colonization of *Rs* in both the cultivars (Fig. 1). Although both the cultivars showed wilting of leaves, wilting percentage was far less in leaves of CT206-10 cultivar as compared to superior, suggesting increased resistance of former cultivar to *Rs* than later (Fig. 1). To further cross-check this observation, leaves

![Fig. 1. Representative plants showing symptoms produced on wild type ‘Superior’ and resistant CT206-10 potato plants after 14 days of *Rs* (KACC10722) infection.](image1)

![Fig. 2. Trypan blue staining of leaves in Superior and CT206-10 after 3 or 4 days treatment with *Rs*. Superior potato leaves showed higher cell death as compared to CT206-10.](image2)
of both the plants were subjected to trypan blue staining. Trypan blue staining is a measure of cell viability as it stains selectively the dead cells (Peterhansel et al., 1997). Results of trypan blue staining showed stronger blue color in the green leaves of superior plants in comparison with the CT206-10 leaves, indicating that cell death was weakly induced in CT206-10 cultivar (Fig. 2). Taken together, the results of disease resistance assay and trypan blue staining

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein annotation</th>
<th>Accession no.</th>
<th>Mr/pl(T)</th>
<th>Mr/pl(E)</th>
<th>SC(%)</th>
<th>Score</th>
<th>Expect</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Actin-5 partial protein of Solanum lycopersicum</td>
<td>gi</td>
<td>3219772</td>
<td>37.3/5.3</td>
<td>67.4/6.0</td>
<td>57%</td>
<td>404</td>
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<tr>
<td>4</td>
<td>Hypothetical protein OsJ_20312 [Oryza sativa Japonica Group]</td>
<td>gi</td>
<td>222635062</td>
<td>78.2/9.0</td>
<td>78.2/9.0</td>
<td>40%</td>
<td>97</td>
</tr>
<tr>
<td>6</td>
<td>Putative glycine-rich RNA binding protein-like [Solanum tuberosum]</td>
<td>gi</td>
<td>82623423</td>
<td>17.6/5.6</td>
<td>17.6/5.6</td>
<td>59%</td>
<td>261</td>
</tr>
<tr>
<td>7</td>
<td>TSI-1 protein [Solanum lycopersicum]</td>
<td>gi</td>
<td>2887310</td>
<td>20.4/5.6</td>
<td>20.8/8.1</td>
<td>25%</td>
<td>152</td>
</tr>
<tr>
<td>8</td>
<td>Hypothetical protein POPTR-0002s02160g (Populus trichocarpa)</td>
<td>gi</td>
<td>224065795</td>
<td>28.2/9.9</td>
<td>18.0/8.2</td>
<td>32%</td>
<td>64</td>
</tr>
<tr>
<td>9</td>
<td>Pathogenesis-related protein STH-2 (Solanum tuberosum)</td>
<td>gi</td>
<td>131026</td>
<td>17.4/5.7</td>
<td>18.4/9.3</td>
<td>52%</td>
<td>193</td>
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<tr>
<td>10</td>
<td>Putative pathogenesis related protein [Capsicum chinense]</td>
<td>gi</td>
<td>58531054</td>
<td>17.3/5.2</td>
<td>19.1/9.6</td>
<td>7%</td>
<td>70</td>
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<td>12</td>
<td>Pentatricopeptide repeat containing protein [Arabidopsis thaliana]</td>
<td>gi</td>
<td>22331104</td>
<td>69.7/8.5</td>
<td>70.2/4.3</td>
<td>21%</td>
<td>56</td>
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</tbody>
</table>

Mr/pl(T); Theological molecular weight and pI, Mr/pl(E); Experimental molecular weight and pI, SC; Sequence coverage
indicate that CT206-10 cultivar is relatively more resistant to Rs in comparison with the superior cultivar.

2-DE analysis of CT206-10 cultivar roots infected with Rs. Rs is a soil borne pathogen which invade susceptible plant through root. Therefore, proteins were extracted from the potato roots infected with Rs and used for 2-DE analysis. More than 500 reproducible spots were observed on the high-resolution 2-DE gels using Image Master 2D Platinum software (Fig. 3). Among these, 12 spots showed differential expression in Rs infected samples, of which 8 spots were identified by MALDI-TOF-MS (Table 1). The identified proteins were Actin 51 partial protein (Spot 2), Hypothetical protein of *Oryza sativa* Japonica group (Spot 4), Glycine-rich RNA binding protein (GRP; Spot 6), Tomato stress induced-1 protein (TSI-1; Spot 7), Hypothetical protein POPTR, *Populus trichocarpa* (Spot 8), Pathogenesis-related protein STH-2 (Spot 9), Putative pathogenesis protein (Spot 10) and Pentatricopeptide repeat containing protein (PPR; Spot 12).

In the present study, we observed that expression of glycine rich RNA binding protein in potato up-regulated upon Rs infection. Up-regulation of GRP in maize and carrot has also been reported in response to wounding and abscisic acid (ABA) (Gomez et al., 1988; Sturm, 1992). Homologs of GRP gene were also identified in different plants like pea, bean and parsley upon infection with different pathogens or elicitation (Carpenter et al., 1994). The mutant plant of GRP7 (glycine rich protein 7) showed higher susceptibility for *Pseudomonas syringae* infection as compared to wild plant (Fu et al., 2007).

TSI-1 protein was found to up-regulated upon Rs infection. It belongs to intracellular pathogenesis related protein which is characterized in tomato, tobacco and pea during wounding, osmotic stress and pathogen colonization (Chiang and Hadwiger, 1990; Iturriaga et al., 1994; Vidya et al., 1999). TSI-1 protein also showed 71% homology with potato’s STH2 protein at nucleotide level (Vidya et al., 1999). This protein also has RNase activity against biotic stresses in some plants (Moisseyev et al., 1994).

Pathogenesis related protein (STH2, spot 9) was also found to be upregulated upon Rs infection. Similar result has also been reported earlier in potato in response to *Phytophthora infestans* infection (Constabel and Brisson, 1992). Homologs of STH2 gene have also been identified in different plants like pea, bean and parsley upon infection with different pathogens or elicitation (Chiang and Hadwiger, 1990; Somssich et al., 1988; Walter et al., 1990).

In contrast to the above mentioned proteins, the expression of PPR protein was down-regulated upon Rs infection. PPR protein is a RNA binding protein which control family of post-transcriptional regulation like RNA editing, splicing, stability, polyadenylation and translation of organelle genes (Schmitz-Limneweer and Small, 2008; Tan et al., 2014). It was reported that WSL, a PPR protein targets to the chloroplast and *wsl* mutant plant showed defect in splicing of *rpl2* transcript, resulted in transcript accumulation and reduction in its protein synthesis. The *wsl* mutant showed higher sensitivity to abiotic stresses like ABA, salinity and sugar (Tan et al., 2014). It has also been reported that in absence of PPR protein, Arabidopsis plant became susceptible to necrotrophic fungal pathogen and hypersensitive to abiotic stresses like ABA, glucose and salinity.

### Table 2. Sequences of primers used for semi-quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number*</th>
<th>Primer sequence (5’-3’)</th>
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</thead>
<tbody>
<tr>
<td>StGRP (spot 6)</td>
<td>DQ207875</td>
<td>Forward: ATGTTTTTTT TCA GATCTGCTTGTGTTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CTAACT CTTCCATGTCCCATCGGA</td>
</tr>
<tr>
<td>StTSI-1 (spot 7)</td>
<td>NM_001247423</td>
<td>Forward: ATGGGGTGAATACCTATCTTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TTAAGCGTAGGCAGAAGGATTCG</td>
</tr>
<tr>
<td>StPOTPR (spot 8)</td>
<td>XM_006354748</td>
<td>Forward: GCACAAATTCAGTACCCATATTCTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: GGACCACCAAGAATGGGACC</td>
</tr>
<tr>
<td>StSTH-2 (spot 9)</td>
<td>M29041</td>
<td>Forward: ATGGGGTGCCTAGCTACACATAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TTAAGCAGTAGAGGATGGG</td>
</tr>
<tr>
<td>StPPR (spot 12)</td>
<td>XM_006349566</td>
<td>Forward: ATGATTGGCGGCGACATGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CACCCATGTAAAGGCTGACCA</td>
</tr>
<tr>
<td>StTUB</td>
<td>Z33402</td>
<td>Forward: ATATCTCTAAACAGTGCCAGCTACTCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TCTGCAACGGGTCATCCAT</td>
</tr>
<tr>
<td>StACT</td>
<td>X55749</td>
<td>Forward: GCTTCCCCATGTCAAGTCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TGGACGATGGGACGGACCTT</td>
</tr>
</tbody>
</table>

*Accession numbers are from NCBI (www.ncbi.nlm.nih.gov/).
Spot nos. 4 and 8 were identified as hypothetical proteins due to their unknown functions. Taken together, our results have shown up-regulation of both the proteins after Rs infection, suggesting their roles in plant defense. However, it needs further characterization of these identified proteins. Based on our observations and previous reports, it can be speculated that induction of these proteins under Rs infection may be involved in increasing Rs tolerance in potato.

Expression profiling of the corresponding genes regulated under Rs infection. MALDI-TOF-MS analysis identified eight differentially expressed proteins upon Rs infection, among them, five genes (StGRP, StTSI-1, StSTH-2, Hypothetical protein POPTR of Populus trichocarpa and StPPR) were selected to quantify their transcript levels by semi-quantitative RT-PCR. The present study identified up-regulation of all five genes after 7 days of Rs infection (Fig. 5). Interestingly, StTSI-1 was not expressed in control, whereas others were induced after Rs infection, suggesting it may play a role in defense response against Rs infection.

GRP protein expression increased 7 days after Rs infection and almost constant throughout all the investigated stages. The GRP transcript levels correspondingly up-regulated after Rs infection. TSI-1, Hypothetical protein POPTR and STH-2 proteins were not detected in control plant but induced 7 days after Rs infection, and consistently decreased at later stages (7 to 21 days). Similar to the trend observed in TSI-1 protein, its transcripts were also not detected in control sample. However, the transcript levels of TSI-1 were increased consistently from 7 days to 21 days of post-Rs-infection.

Overall, not 1:1 correlations were found in most of the examined transcripts level and their proteins level. This discrepancy can be due to various factors including post-transcriptional and post-translational modifications. Besides, functioning of some other regulatory mechanism cannot be neglected (Griffin et al., 2002; Gry et al., 2009; Velez-Bermudez and Schmidt, 2014).

Conclusion

*R. solanacearum* is one of the major pathogenic bacteria causing significant yield loss of potato. In the present study, we used an integrated proteomics and transcriptomics approach in order to find out the Rs responsive proteins in potato. Among the identified proteins, GRP, TSI-1, STH-2, hypothetical protein POPTR of *Populus trichocarpa* and PPR were further validated by semi-quantitative RT-PCR. Results of the present study showed that proteins regulated by Rs infection are also regulated at transcript level, suggesting roles of these proteins in resistance against Rs. Taken together, our results indicate the involvement of these 5 proteins in the Rs stress tolerance in potato.

Fig. 4. Enlarged view of selected protein spots showing differential protein expression in 2-DE after Rs infection the roots of CT-206-10 potato cultivar.

Fig. 5. Expression pattern of five different expressed genes in the roots of CT-206-10 potato cultivar after 0, 7 and 21 days of Rs infection by RT-PCR. GRP, Glycine-rich RNA binding protein; TSI-1, Tomato stress induced-1 protein TSI-1; STH-2, Pathogenesis-related protein; PPR, Pentatricopeptide repeat containing protein. The semi-quantitative RT-PCR was performed using Actin (StACT) and Tubulin (StTUB) genes as internal control.
future, it would be interesting to raise the transgenic plants to further validate their involvement in resistance against Rs in potato.

Acknowledgments
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References


parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. *Planta* 193:470–472.


