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ITRAQ-Based Proteomic Analysis of The Response to *Ralstonia solanacearum* in Potato

Jinlin Feng, Lixia Yao, Minghui Qin and Gang Gao

College of Life Science, Shanxi Normal University, Linfen, 041004, Shanxi, China
*Corresponding author's e-mail: ggsxnu@126.com

Bacterial wilt is a serious disease of potato (*Solanum tuberosum* L.) caused by the soil-borne pathogenic bacterium *Ralstonia solanacearum*. Detecting changes in protein abundance in potato plants in response to *R. solanacearum* is a pivotal step in uncovering the molecular interactions of plant pathogens. In this study, using the disease-resistant cultivar 'Zhongshu 3', we analyzed protein expression in potato seedlings inoculated with *R. solanacearum* every 12 h for a total of 72 h using isobaric tags for relative and absolute quantitation-based proteomics. Our results indicate that pathogenesis-related proteins, stress-related proteins, non-specific lipid transfer proteins, small heat shock proteins, and osmotin-like proteins were up-regulated in response to pathogen infection at different time points. The accumulation of these proteins in response to biotic stress suggests that these proteins play an important role in pathogen resistance. Our findings will provide an important basis for characterizing the role of these proteins in increasing plant resistance to pathogens and in breeding bacterial wilt-resistant plants.
Keywords: Bacterial wilt; iTRAQ; Potato; *Ralstonia solanacearum*, Proteomic.

INTRODUCTION

The potato plant (family Solanaceae) is a key player in securing the global food supply; in fact, it is the third most important food crop (Gerbens-Leenes *et al*., 2009). However, potato cultivation and production are seriously affected by bacterial wilt caused by the soil-borne bacterium *Ralstonia solanacearum* (Bae *et al*., 2012; Barrell *et al*., 2013). Bacterial wilt, the second most severe bacterial disease in potato, can decrease production by 80–100% (Wei *et al*., 2017). Thus, it is vital to breed bacterial wilt-resistant potato cultivars to ensure the global food supply. Detecting changes in protein abundance in potato in response to *R. solanacearum* is a critical step in uncovering the molecular interactions of plant pathogens and will provide a theoretical basis for the breeding of disease-resistant varieties.

A few proteins have been reported to confer resistance to bacterial wilt. For example, transformation of the gene encoding APl, an endogenous anti-microbial protein isolated from the bacterial wilt-resistant potato variety MS42.3, was shown to confer increased resistance in transgenic potato plants to bacterial wilt caused by *R. solanacearum* (Liang, 2002). Meanwhile, susceptible *Arabidopsis* ecotype *Ler* plants transformed with the wild-type *ERECTA* gene showed increased resistance to *R. solanacearum*, including reduced wilt symptoms and reduced bacterial growth (Godiard *et al*., 2003). Similarly, expression of the cationic peptide cecropin B from a synthesized gene in transgenic tomato plants increased their resistance to bacterial wilt and bacterial spot (Jan *et al*., 2010). In *Arabidopsis thaliana*, binding of the bacterial pathogen-associated molecular pattern elongation factor (EF)-Tu by the pattern recognition receptor EF-Tu receptor (EFR) promoted anti-bacterial immunity (Kunze *et al*., 2004). Further, the overexpression of *A. thaliana* EFR (AtEFR) in tomato increased plant resistance to *R. solanacearum* (Lacombe *et al*., 2010), while AtEFR expression from a transgene increased bacterial wilt resistance in a commercial potato line (INIA Iporá) and a breeding potato line (09509.6) (Boschi *et al*., 2017).

In this study, using the disease-resistant cultivar 'Zhongshu 3', we analyzed changes in protein abundance in potato seedlings inoculated with *R. solanacearum* every 12 h for a total of 72 h using isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomics. Pathogenesis-related proteins, stress-related proteins, non-specific lipid transfer proteins (nsLTPs), small heat shock proteins (HSPs), and osmotin-like proteins were up-regulated in response to pathogen infection at different time points. The accumulation of these proteins in response to a biotic stressor suggests that they play a crucial role in pathogen resistance.

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MATERIALS AND METHODS

***Materials*:** The disease-resistant potato cultivar 'Zhongshu 3' was provided by the Institute of Vegetables and Flowers of the Chinese Academy of Agricultural Sciences. *Ralstonia solanacearum* PO41 (biovar 2) was used in this study.

***Ralstonia solanacearum treatment*:** Potato seedlings with 7–8 leaves were infected with *R. solanacearum*. Subsequently, the leaves and stem were collected at 0 h (represented by p0), 12 h (represented by p1), 24 h (represented by p3), 36 h (represented by p4), 48 h (represented by p6), 60 h (represented by p7), or 72 h (represented by p8) after inoculation.

***Total protein extraction*:** Each sample was minced in liquid nitrogen and lysed in a buffer containing 100 mM ammonium bicarbonate (pH 8.0), 0.2% sodium dodecyl sulfate, and 6 M urea, followed by 5 min of ultrasonication on ice. The lysate was centrifuged for 15 min at 12,000 x *g* and the supernatant was transferred to a clean tube. Extracts from each sample were reduced with 2 mM dithiothreitol for 1 h at 56°C and subsequently alkylated with iodoacetamide for 1 h at room temperature in the dark. Next, four times the volume of precooled acetone was mixed with each sample by vortexing and the tubes were incubated at -20°C for at least 2 h. The samples were then centrifuged and the precipitate was collected. After washing three times with cold acetone, the pellet was dissolved in a buffer containing 0.1 M triethylammonium bicarbonate (pH 8.5) and 6 M urea. The protein concentration in each tube was determined using the Bradford protein assay.

***Peptide preparation and iTRAQ labeling*:** A total of 0.12 mg of protein from each sample was digested with Trypsin Gold (Promega, Madison, WI, USA) at a 1:50 enzyme-to-substrate ratio. After 16 h of digestion at 37°C, the peptide was desalted with a C18 cartridge to remove urea, and the desalted peptides were dried by vacuum centrifugation. The desalted peptides were then labeled with iTRAQ reagents (iTRAQ® Reagent-8PLEX Multiplex Kit; Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions.

***Analysis by liquid chromatography with tandem mass spectrometry and the identification and quantitation of each protein*:** Shotgun proteomics analyses were performed using an EASY-nLCTM 1200 UHPLC System (Thermo Fisher Scientific, Waltham, MA, USA) coupled with an Orbitrap Q Exactive HF-X Mass Spectrometer (Thermo Fisher Scientific) operating in data-dependent acquisition mode. The resulting spectra from each fraction were searched separately against the P101SC18091068-01-Solanum-tuberosum-GCF_000226075.1 database using the search engine Proteome Discoverer 2.2 (Thermo Fisher Scientific). For protein identification, proteins with at least one unique peptide were identified based on a false discovery rate <1.0% at the peptide level and protein level, respectively. The protein quantitation results were statistically analyzed using the Mann-Whitney U test; significant ratios, defined as a ratio >1.5 or a ratio <0.67 (fold change), were used to screen for differentially expressed proteins.

RESULTS

***Identification of differentially expressed proteins upon pathogen infection*:** To explore the response of potato plants to infection by *R. solanacearum*, we examined the changes in abundance of proteins collected from the aerial parts of plants inoculated with *R. solanacearum* at different time points. We examined the protein levels in the plants every 12 h for a total of 72 h. Protein expression at each time point was compared with that at p0. In total, 4,813 proteins were identified, with 618 proteins showing changes in abundance by iTRAQ-based proteomic analysis. These 618 proteins are shown in Supplemental Table 1. The number of down-regulated proteins exceeded the number of up-regulated proteins at each time point (Figure 1A). Overall, 4 proteins were all up-regulated and 34 proteins were all down-regulated at each time point (Figure 1B and C). The 618 proteins showing changes in abundance were then clustered into six profiles (Figure 2). The proteins in clusters 1 and 3 were found to be down-regulated at 24 h after inoculation but showed improved expression at 36 h after inoculation. The proteins in cluster 2 were down-regulated at 12 h after inoculation and continued to show a low level of expression after that time point. The proteins in cluster 4 showed peak expression at 24 h. The proteins in cluster 5 were obviously up-regulated at 24 h and 48 h. The proteins in cluster 6 were only up-regulated at 72 h.

***Stress-related proteins were up-regulated in response to pathogen infection*:** Six pathogenesis-related proteins were up-regulated in response to pathogen infection, indicating that the plants responded normally to the pathogen (Figure 3A and Table 1). In addition, our proteomic results indicate that four additional stress-related proteins, cold and drought-regulated protein CORA-like, defensin-like protein, desiccation protectant protein Lea14 homolog, and low-temperature-induced 65 kDa protein-like isoform X2, were up-regulated following pathogen infection at different time points (Figure 3B and Table 1). Cold and drought-regulated protein CORA-like participates in the control of root adaptation to water and salinity stress (Merchan *et al*., 2007). Constitutive overexpression of the defensin protein AtPDF1.1 in *A. thaliana* results in a decrease in symptoms caused by the nonhost *Cercospora beticola*, leading to non-spreading spots on *A. thaliana* leaves (De Coninck *et al*., 2010). Tolerance to the necrotrophic bacterium *Pectobacterium carotovorum subsp. carotovorum* was positively correlated with AtPDF1.1 expression in transgenic *A. thaliana* plants, suggesting an important defensive role for the protein against this bacterium (Hsiao *et al*., 2017). LEA proteins are induced under conditions of stress, including desiccation, heat,

osmotic stress, and cold (Singh *et al*., 2005). These results suggest that stress-related proteins respond to different stressors and that the pathways underlying plant stress responses exhibit crosstalk.

Table 1. Description of the proteins in Figure 3, which were up-regulated in response to pathogen infection

Protein cluster	Protein	Description
A	XP_006340889.1	Pathogenesis-related protein STH-2-like
	NP_001274822.1	Pathogenesis-related protein STH-21
	XP_006364121.2	Pathogenesis-related protein R major form-like
	XP_006364119.1	Pathogenesis-related protein R major form
	NP_001275608.1	Pathogenesis-related protein P2-like precursor
	NP_001275095.1	Pathogenesis-related protein 1b precursor
B	XP_015169598.1	Cold and drought-regulated protein CORA-like
	XP_006365640.1	Defensin-like protein
	XP_006339634.1	Desiccation protectant protein Lea14 homolog
	XP_006353392.1	Low-temperature-induced 65 kDa protein-like isoform X2
C	NP_001275625.1	Non-specific lipid transfer protein AKCS9-like precursor
	XP_006367394.1	Non-specific lipid transfer protein 2-like
	XP_006362360.1	Non-specific lipid transfer protein 2-like
	XP_015160886.1	Non-specific lipid transfer protein 2-like
	XP_006367396.1	Non-specific lipid transfer protein 2
	XP_006367904.1	Non-specific lipid transfer protein 1-like
	XP_006367397.1	Non-specific lipid transfer protein 1
D	XP_006360820.1	18.2 kDa class I heat shock protein-like
	XP_006350800.1	17.8 kDa class I heat shock protein
	NP_001275610.1	17.6 kDa class I heat shock protein-like
	XP_006355460.1	14.7 kDa heat shock protein-like
	XP_006345019.1	22.7 kDa class IV heat shock protein-like
E	XP_006358890.1	Osmotin-like protein OSML81
	XP_006358888.1	Osmotin-like protein OSML81
	XP_006358891.1	Osmotin-like protein OSML15
	XP_006368018.1	Osmotin-like protein OSML13
F	XP_006356334.1	2-Oxoisovalerate dehydrogenase subunit alpha 1, mitochondrial-like isoform X1
	XP_006358286.2	Umecyanin-like
	XP_006367397.1	Non-specific lipid transfer protein 1
	XP_015169620.1	Glycine-rich cell wall structural protein-like isoform X2

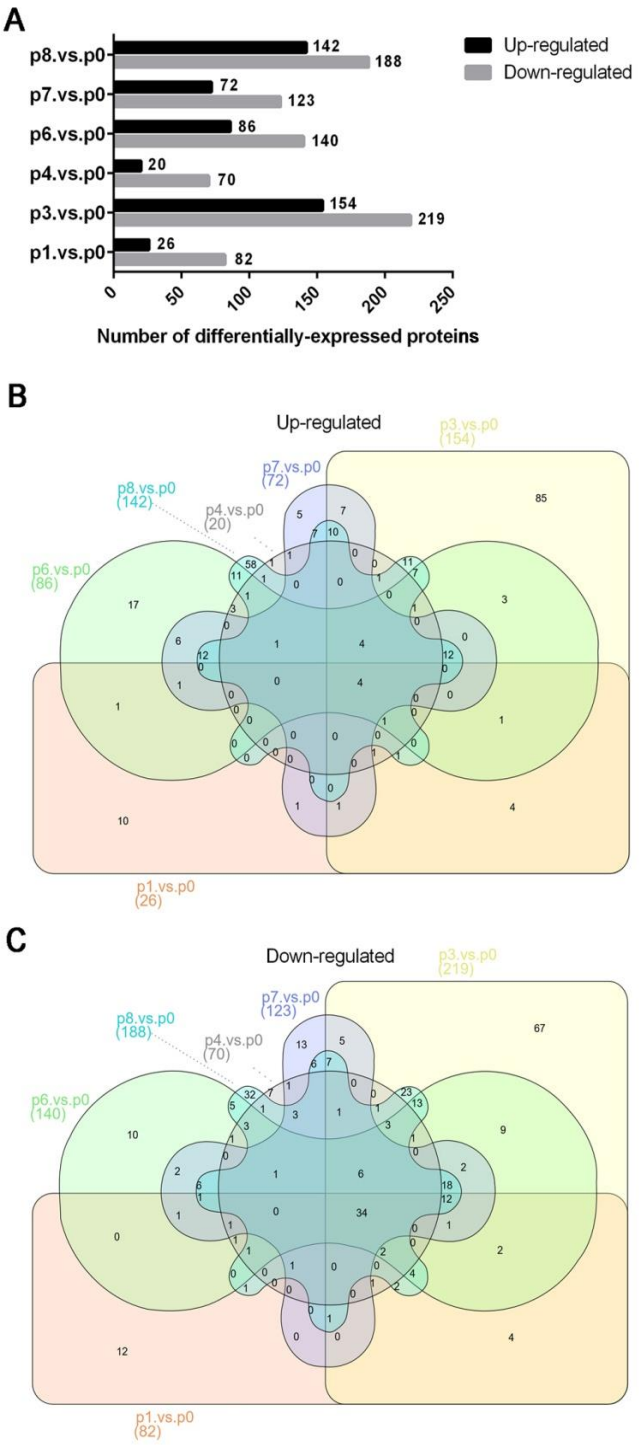

Figure 1. Changes in protein abundance in potato in response to *Ralstonia solanacearum* at different time points after infection. A: The numbers of differentially expressed proteins at each time point. B: A Venn diagram showing the up-regulated proteins. C: A Venn diagram showing the down-regulated proteins.

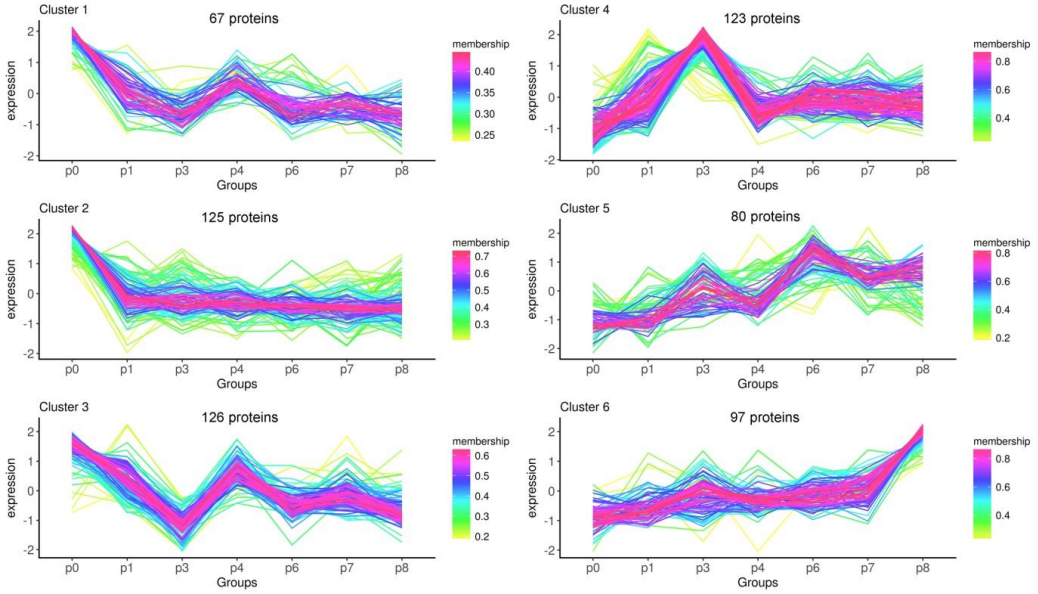

Figure 2. Expression profiles of the differentially expressed proteins in the six main clusters.

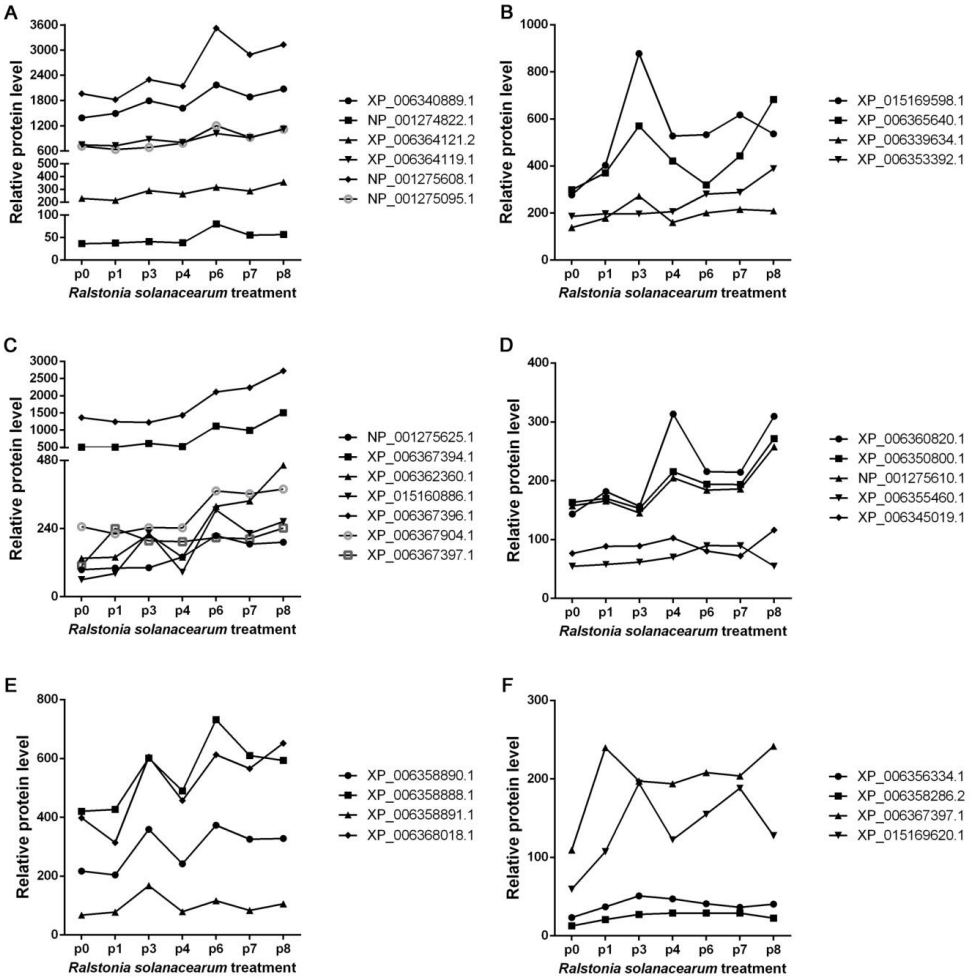

Figure 3. Proteins that were up-regulated in response to pathogen infection at various time poin

***NsLTPs were up-regulated in response to pathogen infection*:** Plant nsLTPs are small, basic proteins that are abundant in higher plants. Plant nsLTPs contain a conserved eight-cysteine residue motif linked via four disulfide bonds and an internal hydrophobic cavity, which comprises the lipid-binding site. This structure confers stability and increases the ability of the protein to bind and/or carry hydrophobic molecules (Liu *et al*., 2015). Overexpression of nsLTP genes, including those encoding LTP in barley (Molina and Garcia-Olmedo, 1997), CALTP1 and CALTP2 in pepper (Jung *et al*., 2005), *Ace*-AMP1 in onion (Roy-Barman *et al*., 2006), and LJAMP1 and LJAMP2 in motherwort (Jia *et al*., 2010), have been found to significantly enhance resistance to fungal and bacterial pathogens; indeed, the *Arabidopsis ltpg1* mutant was more susceptible to infection by the fungus *Alternaria brassicicola* than wild type (Lee *et al*., 2009). The potato *StLTPA7* gene exhibits a complex Ca^{2+}-associated expression pattern during the early stages of the potato–*R. solanacearum* interaction (Gao *et al*., 2009). Our proteomic results indicate that seven nsLTPs were up-regulated in response to pathogen infection at different time points (Figure 3C and Table 1). The accumulation of nsLTPs in response to pathogen stress suggests that these proteins play an important role in stress tolerance.

***Small HSPs were up-regulated in response to pathogen infection*:** Plants respond to high temperatures and other abiotic stresses by accumulating HSPs (Jacob *et al*., 2017). HSPs act in general as molecular chaperones, regulating the folding, accumulation, localization, and degradation of proteins in plants and animals (Al-Whaibi, 2011). Small HSPs (12–40 kDa), which exhibit a highly conserved α-crystallin domain at their C-terminal end, are the most abundant stress-induced proteins (Sun *et al*., 2002). Overexpression of the rice small HSP gene *sHSP17.7* increased the drought tolerance of transgenic rice seedlings (Sato and Yokoya, 2008), while expression of a small HSP encoded by *LimHSP16.45* from the David Lily (*Lilium davidii*) was induced by various abiotic stressors in lily and transgenic *Arabidopsis.* Heterologous expression of *LimHSP16.45* can enhance plant resistance to abiotic stress by preventing irreversible protein aggregation and by scavenging cellular reactive oxygen species (Mu *et al*., 2013). Our proteomic data show that four small HSPs were up-regulated in response to pathogen infection at different time points (Figure 3D and Table 1). The accumulation of small HSPs in response to pathogen stress suggests that these proteins play an important role in pathogen resistance.

***Osmotin-like proteins were up-regulated in response to pathogen infection*:** Osmotins and osmotin-like proteins are found in the model plant *A. thaliana*, tobacco, most crops, vegetables, and fruits. The expression of these proteins is induced by salt stress; thus, osmotin is considered a stress response protein. Under conditions of salt stress, osmotin increases the accumulation of proline and quenches reactive oxygen species and free radicals (Wan *et al*., 2017). Our proteomic analysis revealed the up-regulation of five osmotin-like proteins in response to pathogen infection at different time points (Figure 3E and Table 1). Bacterial wilt can cause drought stress and elevate the level of reactive oxygen species; therefore, pathogen-resistant potato cultivars may promote an immune response via the overexpression of osmotins and osmotin-like proteins.

DISCUSSION

Detecting changes in plant protein abundance in response to *R. solanacearum* is a key step in uncovering the molecular interactions of plant pathogens. (Park *et al*., 2016) identified eight differentially abundant proteins upon *R. solanacearum* infection by 2-DE. In this study, we found that pathogenesis-related proteins, stress-related proteins, nsLTPs, small HSPs, and osmotin-like proteins were up-regulated in response to pathogen infection at different time points. The accumulation of these proteins in response to pathogen stress suggests that they play an important role in pathogen resistance.

We also found that four proteins were up-regulated at each time point: an nsLTP, a glycine-rich cell wall structural protein, 2-oxoisovalerate dehydrogenase subunit alpha 1, and umecyanin (Figure 3F and Table 1). Glycine-rich proteins have been found in the cell walls of many higher plants, and they reportedly accumulate in vascular tissues; their synthesis is thought to be part of a defensive mechanism (Mousavi and Hotta, 2005). Meanwhile, 2-oxoisovalerate dehydrogenase transcription was significantly induced by water stress in drought-susceptible and drought-tolerant wheat (Li, 2012), and suppression of the cotton umecyanin-like gene *GhUMC1* weakens the jasmonate signaling pathway and down-regulates lignin synthesis, increasing seedling susceptibility to verticillium wilt (Zhu *et al*., 2018). Given these findings, these four up-regulated proteins may play an important role in pathogen resistance.

In this study, we identified several potential pathogen resistance-related proteins. Our findings will provide an important basis for understanding how changes in protein abundance can enhance plant resistance to biotic stress and breeding bacterial wilt-resistant plants to ensure the security of the global food supply.

***Disclosure of potential conflicts of interest*:** No potential conflicts of interest were disclosed.

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