Proteomic Analysis of Potato Responding to the Invasion of *Ralstonia solanacearum* UW551 and Its Type III Secretion System Mutant

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The infection of potato with *Ralstonia solanacearum* UW551 gives rise to bacterial wilt disease via colonization of roots. The type III secretion system (T3SS) is a determinant factor for the pathogenicity of *R. solanacearum*. To fully understand perturbations in potato by *R. solanacearum* type III effectors (T3Es), we used proteomics to measure differences in potato root protein abundance after inoculation with *R. solanacearum* UW551 and the T3SS mutant (UW551 Δ HrcV). We identified 21 differentially accumulated proteins. Compared with inoculation with UW551 Δ HrcV, 10 proteins showed significantly lower abundance in potato roots after inoculation with UW551, indicating that those proteins were significantly downregulated by T3Es during the invasion. To identify their functions in immunity, we silenced those genes in *Nicotiana benthamiana* and tested the resistance of the silenced plants to the pathogen. Results showed that miraculin, HBP2, and TOM20 contribute to immunity to *R. solanacearum*. In contrast, PP1 contributes to susceptibility. Notably, none of four downregulated proteins (HBP2, PP1, HSP22, and TOM20) were downregulated at the transcriptional level, suggesting that they were significantly downregulated at the posttranscriptional level. We further coexpressed those four proteins with 33 core T3Es. To our surprise, multiple effectors were able to significantly decrease the studied protein abundances. In conclusion, our data showed that T3Es of *R. solanacearum* could subvert potato root immune-related proteins in a redundant manner.

Keywords: genomics, metabolomics, potato, proteomics, *Ralstonia solanacearum*, resistance, T3Es, type 3 secretion

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Potato (*Solanum tuberosum* L.) is one of the most important food and economic crops worldwide. Potato bacterial wilt, caused by *Ralstonia solanacearum*, is one of the most destructive bacterial diseases (Mansfield et al. 2012) and limits potato production worldwide (Champoiseau et al. 2009; Elphinstone 2005). *R. solanacearum* is a soilborne pathogen that invades its host by entering the roots (Genin 2010), and rapidly colonizes the xylem vessels (Dignonnet et al. 2012). Large amounts of exopolysaccharides (EPS) are produced in the xylem vessels that stop the flow of water, which leads to wilting and, eventually, the death of plants (Garg et al. 2000; Genin and Denny 2012). In roots, EPS distribution in vascular tissues of susceptible varieties is more extensive than that of resistant varieties at 5 days after inoculation (McGarvey et al. 1999), suggesting that the root system may limit bacterial colonization.

Bacterial wilt is difficult to control due to the pathogen aggressiveness and lack of resistance in commercial potato varieties (Coll and Valls 2013; Puigvert et al. 2017). Resistant plants can delay colonization by the pathogens in the root vascular system (Caldwell et al. 2017); however, the mechanism underlying this phenomenon is not clear.

As with most Gram-negative pathogens, the type III secretion system (T3SS) is the main determinant of *R. solanacearum* pathogenesis (Boucher et al. 1985, 1987), because it enables the pathogen to inject virulence factors, called type III effectors (T3Es), directly into host cells, thereby disabling host defenses and causing disease (Brito et al. 2002; Büttner 2016). *hrcV* is one of the T3SS structural genes, and its mutant is not toxic to plants (Lonjon et al. 2016). *hrcV* mutant strains can infect plant roots naturally but the bacteria can only colonize the roots and cannot spread (Vasse et al. 2000).

When approaching plant cells, conserved bacterial molecules (pathogen-related molecular model) may be sensed by receptors on the surface of plant cells, triggering activation of the plant immune system (Darvill and Albersheim 1984). This is called pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), which can limit bacterial growth and prevents the development of disease (Chisholm et al. 2006). There have been many reports about the inhibition of plant PTI by pathogen effectors. For example, 16 *R. solanacearum* T3Es have been reported as inhibitors of flg22-induced reactive oxygen species (ROS) production, which is a typical PTI-related marker (Landry et al. 2020). Studies have shown that ROS are involved in the interaction between roots and pathogens (Jamet et al. 2003). When plants are invaded by pathogens, they produce ROS (Imlay 2008; Joo et al. 2001; Mori and Schroeder

2004) by a process known as oxidative burst (Cao et al. 2012). During colonization, *R. solanacearum* produces an oxidative stress response as an adaptation to the ROS produced by the host (Brown and Allen 2004; Flores-Cruz and Allen 2009). ROS are mainly produced by cell-wall-bound peroxidases and NADPH oxidases (Torres and Dangl 2005), and they represent the main method used by plants to initially resist pathogen invasion (Bolwell 1999; Miller and Britigan 1997). Effector-triggered immunity (ETI) is an accelerated and amplified PTI response that leads to disease resistance and usually produces a hypersensitive cell death reaction at the infected site (Jones and Dangl 2006). Resistant plants have evolved a mechanism for detecting such bacterial perniciousness through a family of polymorphic intracellular nucleotide-binding leucine-rich-repeat (NB-LRR) receptors (Cui et al. 2015; Jacob et al. 2013). During infection, a given effector is “specifically recognized” by one of the NB-LRR proteins, causing ETI, which effectively prevents pathogen multiplication (Chiang and Coaker 2015). Natural selection drives pathogens to avoid ETI by shedding or diversifying the identified effector, or by acquiring more effectors to suppress ETI (Macho 2016). Although the suppression of PTI by T3Es has been widely documented (Landry et al. 2020), reports about T3Es that suppress ETI might vary substantially among different host species, as well as between host species with unknown targets.

Because the root is the first line of defense against invasion by *R. solanacearum*, exploring root colonization by pathogens is key to understanding plant resistance against infection (McAvoy et al. 2012). Proteomics is a powerful tool with which to study host–pathogen interactions and consider the underlying mechanisms of disease, basic defense, and gene resistance as a whole. Isobaric tags for relative and absolute quantitation (iTRAQ) is an isobaric labeling method used in quantitative proteomics (Ross et al. 2004). This is one of the newest techniques used for the quantitative study of gene expression at the proteome level and it can detect low-abundance proteins in complex samples (Gaffken and Lampe 2006).

In this work, we aimed to screen differentially accumulated proteins after inoculation with *R. solanacearum* UW551 and the T3SS mutant by iTRAQ and examine their relationship with plant immunity. We found that bacterial colonization and multiplication differed among roots inoculated with the wild-type (WT) *R. solanacearum* strain and those inoculated with its T3SS mutant strain. This led us to confirm that the presence or absence of effectors would make a difference in responses. We then performed a proteomic analysis of plant roots inoculated with *R. solanacearum* using the iTRAQ approach and identified 21 differentially accumulated proteins (DAPs). Using the virus-induced gene silencing (VIGS) system, four proteomics-identified proteins were preliminarily confirmed to be involved in plant defense. Four downregulated proteins had no change on the transcriptional level. Then, we coexpressed the four proteins with 33 core T3Es and found that the abundance of these proteins significantly decreased when expressed with multiple effectors. Our data suggest that the downregulation of proteins is directly related to the presence of T3Es, and the existence of multiple downregulated proteins is also related to plant defense response.

RESULTS

Roots colonization of the *R. solanacearum* UW551 T3SS mutant is limited in the susceptible potato C9701.

Pathogenicity assays of the *R. solanacearum* UW551 WT and *hrcV* deletion mutant were performed on a susceptible potato accession, C9701 (Chen et al. 2013). A functional T3SS was required to cause disease in the susceptible potato (Figs.

1A; Supplementary Fig. S1). To observe the infection process, in vitro potato plants were root inoculated with the *R. solanacearum* reporter strains UW551::GUS and UW551Δ*HrcV*::GUS. Bacteria were detected in plants from 2 to 7 days post-inoculation (Fig. 1B). Three experiments were performed using three to five replicate plants that were arranged in a completely randomized design. The UW551 strain displayed a stronger *R. solanacearum* β-glucuronidase (GUS) signal in the roots than the *hrcV* mutant did (Fig. 1B). The GUS signal of the *hrcV* mutant strain was only present in the roots, and its range of colonization was smaller than that of the UW551 strain in the plant as a whole.

To further confirm the differences in root bacterial colonization levels, root samples from plants inoculated with either strain were used for quantitative analysis of pathogen colonization by plate counting. The results showed that the *R. solanacearum* WT proliferated abundantly in the roots, rapidly increasing to 1×10^6 CFU/g on the third day of inoculation and reaching more than 1×10^9 CFU/g after 7 days of inoculation (Fig. 1C). In contrast, the concentration of the *hrcV* mutant showed no apparent increase after infection. These data showed that the potato roots could limit the T3SS mutant strain colonization of the whole plants but not that of the WT strain.

iTRAQ protein profiling.

Because of the phenotypic differences observed in the differentially infected roots, we compared the root tissue proteomes of potato plants infected with the *R. solanacearum* WT strain and those infected with the T3SS mutant strain. Because the bacterial quantification in the root showed that the *R. solanacearum* UW551 population was significantly higher than the T3SS mutant strain at day 7 after inoculation, root samples were collected at 6 days postinoculation and proteomic analysis was performed using iTRAQ. In total, 2,830 original proteins and 2,424 trusted proteins were obtained (at least one unique peptide with high confidence) and identified by iTRAQ analysis against the Uniprot database *Solanum tuberosum*.

All proteins were annotated to 38 gene ontology (GO) terms by GO analysis (Fig. 2; Supplementary Table S4). In terms of biological process categories, most proteins were categorized into the metabolic process (671, 28.7%), cellular process (802, 34.3%), and response to stimulus (169, 7.2%). The major cellular components were cell (999, 42.7%), cell part (999, 42.7%), and organelle (611, 26.1%). The largest molecular functions of proteins obtained by GO analysis were catalytic activity (709, 30.3%) and binding (441, 18.1%).

Comparative potato root proteomic analysis identified 21 proteins with significant differential abundance between infection with the *R. solanacearum* WT and the T3SS mutant.

Proteins displaying a fold change >1.40 or <0.71 in relative abundance and a *P* value < 0.05 were considered DAPs. We identified 10 DAPs with significantly lower abundance in potato inoculated with the WT UW551 strain and 11 proteins with significantly higher abundance in potato inoculated with the WT UW551 strain (Table 1). A pie chart of a PANTHER classification analysis with associated GO terms for the 21 DAPs is shown in Figure 3. In terms of biological process categories, most proteins were categorized into the metabolic process (1, 16.7%), cellular process (3, 50%), and response to stimulus (2, 33.3%). The major cellular components were cell (5, 41.7%), cell part (5, 41.7%), organelle (1, 8.3%), and protein-containing complex (1, 8.3%). The largest molecular functions of proteins obtained by GO analysis were catalytic activity (4, 50%), translation regulator activity (1, 12.5%), and binding (3, 37.5%).

Fig. 1. The type III secretion system (T3SS) is necessary for full virulence of *Ralstonia solanacearum* in potato. **A**, Potato plants were inoculated by soil drench with the wild-type (WT) UW551 strain and its T3SS mutant. Cultured *R. solanacearum* was prepared to an optical density at 600 nm = 0.1 and inoculated with 5.0 ml of *R. solanacearum* suspension in each potato pot. Disease severity was rated from 0 to 21 days using a disease index scale. Data correspond to one representative experiment and each experiment included five plants per treatment. Error bars represent standard errors and DPI = days postinoculation. **B**, Visualization of the tissue expression of *R. solanacearum*. Potato plants were inoculated with the WT UW551 strain or its T3SS mutant harboring the β -glucuronidase (GUS) protein under the control of the constitutive *psbA* promoter. In vitro potato plants (*Solanum chacoense* accession C9701) were harvested from 0 to 8 days postinoculation (dpi) and stained with GUS. **C**, In vitro bacterial populations of potato roots 3, 5, and 7 dpi inoculated with the WT UW551 strain or the T3SS mutant strain. Data correspond to one representative experiment and each experiment included four plants per treatment. Error bars represent standard errors and an asterisk (*) = $P < 0.05$ with the Holm-Sidak method.

The silencing of genes encoding the downregulated proteins by VIGS altered the susceptibility of *Nicotiana benthamiana* to *R. solanacearum*.

To verify whether the 10 downregulated proteins identified in potato were associated with the susceptibility of the plants to *R. solanacearum* infection, we employed VIGS, an approach frequently used in functional genomic studies in the family Solanaceae. Because it is difficult to silence these genes in potato, we instead silenced the homologous genes in the model plant *Nicotiana benthamiana* (Table 2).

The relative gene expression for the *N. benthamiana* homologous genes before silencing is listed in Table 2. The homologous genes were selected based on the highest sequence similarity to potato genes identified but considering the existence of other homologous genes with high sequence similarity (>80%). The selected silenced regions are mainly conserved regions; thus, multiple homologous genes can be silenced at the same time (Supplementary Table S2). Fragments of 200 to 300 bp specific to these homologous genes in *N. benthamiana* were used to construct tobacco rattle virus (TRV)2 VIGS vectors (primers are shown in Supplementary Table S1). Real-time quantitative reverse-transcription PCR (qRT-PCR) was used to measure the silencing efficiency of these constructs (primers are shown in Supplementary Table S2). Transcript levels of these genes were reduced at least 30% compared with the levels in control plants (Fig. 4).

Compared with control plants, silencing of the *MEE32*, *MGPI*, and *PPR* genes induced phenotypic changes. Gene-silenced plants showed yellowing, lethality, and rolled leaves, respectively (Supplementary Fig. S2). The NbMGPI-silenced plants could not survive normally but the NbPPR- and NbMEE32-silenced plants could be efficiently infected by

R. solanacearum and showed wilting symptoms comparable with the control TRV:00. In all, 24 tobacco plants were silenced for each gene used for silencing. Subsequently, we performed soil-drenching inoculation of gene-silenced plants with the *R. solanacearum* K60 strain, and the survival of the tobacco plants was monitored, except for the NbMGPI-silenced plants that showed the lethal phenotype (Fig. 5A). Strain UW551 is classified as phylotype IIB, and these phylotype strains have strong pathogenicity to potato but not to tobacco, whereas the strain K60 is classified as phylotype IIA, and these phylotype strains have strong pathogenicity to tobacco. We found that the susceptibility of the silenced lines to *R. solanacearum* changed to varying degrees. Compared with that in the control plants, tobacco plants with silenced *PPR*, *MEE32*, *LRX1*, *HSP22*, and *EIF4G* genes showed no change in susceptibility, whereas those with silenced *miraculin*, *HBP2*, and *TOM20* genes showed more severe symptoms and a reduced survival rate, and PPI-silenced lines exhibited increased resistance in response to *R. solanacearum* (Fig. 5B). The results of three repeated experiments are shown in Figure 5 and Supplementary Figures S3 and S4.

Transcriptional expression of the identified proteins in potato roots.

To examine whether the observed up- and downregulation of protein abundance was due to changes at transcriptional level, we measured the mRNA levels of the 21 genes corresponding to the DAP identified (Fig. 6). A part of the root samples prepared for proteomic analysis was used for total RNA isolation, to ensure the reliability of the proteins and transcripts. Total RNA together with primers designed for the genes were used for qRT-PCR analysis (Supplementary Table S3). The transcriptional levels of *HSP22*, *HBP2*, *TOM20*, *PPI*, oxidoreductase, and ZKT did not

Fig. 2. Gene ontology (GO) analysis by PANTHER for all iTRAQ proteins. In addition to each category, the percentage of gene frequency was reported. The number of assigned genes may be greater than the number of recognized genes because the same gene can be included in different categories.

change significantly in potato roots following inoculation with the *R. solanacearum* WT and *hrcV* mutant strains, wherein the HSP22, HBP2, TOM20, and PP1 were posttranscriptionally downregulated upon *R. solanacearum* infection.

Presence of T3Es decreased abundance without significantly affecting the transcriptional level in four downregulated proteins.

Bacteria cannot inject effectors into host cells to perform their functions when the T3SS is inactivated, suggesting that DAPs are likely the result of the influence of T3Es. The downregulation of four proteins with no change at the transcriptional level can be attributed to the existence of T3Es.

To examine whether the downregulation of HBP2, PP1, HSP22, and TOM20 proteins was due to T3Es, we transiently coexpressed the downregulated proteins and T3Es in *N. benthamiana*. In a previous study, 33 core effector proteins were identified by analyzing the effector repertoire of 13 sequenced phylotype IIB strains (Table 3) (Zheng et al. 2019). In all, 33 FLAG-tagged T3Es and four HA-tagged expression vectors corresponding to the downregulated proteins were constructed for this work. Tobacco leaves were infiltrated with coexpressing FLAG-tagged effectors and HA-tagged downregulated proteins. Coexpression of FLAG-tagged green fluorescent protein and HA-tagged downregulated proteins were infiltrated as a negative control on the other side of tobacco leaves (Supplementary Fig. S5). Analysis of the abundance of these four proteins (Supplementary Fig. S6) in the two samples showed that, when these four proteins were coexpressed with T3Es, the protein abundance decreased significantly (Fig. 7). The protein abundance of TOM20 was decreased significantly when it was coexpressed with RipAE, RipBH, RipC1, RipE1, and RipG7 effectors. A significant decrease in the protein abundance of HSP22 was observed following coexpression with the RipAC, RipAD, RipAE, RipAP, RipAR, RipAT,

RipAV, RipB, RipC1, RipE1, RipF2, RipG2, RipG6, RipG7, RipH1, RipN, RipTPS, and RipV1 effectors. Similarly, the abundance of the HBP2 protein showed a significant decrease when it was coexpressed with the RipAD, RipAP, RipAV, RipB, RipBH, RipE1, RipG6, RipG7, and RipH1 effectors, and the abundance of the PP1 protein showed a significant decrease when it was coexpressed with the RipAC, RipAE, RipAM, RipAP, RipAJ, RipAR, RipAT, RipB, RipE1, RipG2, RipG6, and RipH1 effectors. The results of the effector and potato protein coexpression were summarized into a network (Fig. 8). The network shows effectors that decrease potato proteins abundance connected to potato protein by lines of the same color.

DISCUSSION

In vitro pathosystems have become a very effective technique to study the early infection of vascular pathogen *R. solanacearum* (Lu et al. 2018; Vasse et al. 2000; Zolobowska and Van Gijsegem 2006). In our study, we have used in vitro-grown potato for GUS staining, colony count, and protein sample preparation. T3Es represent the key weapon used by *R. solanacearum* to counteract host immunity (Van Gijsegem et al. 1993; Zheng et al. 2019). The T3SS knockout mutant cannot deliver T3Es into plant cells, resulting in the loss of pathogenicity (Etchebar et al. 1998; Frey et al. 1994). Microbial localization and visualization can help determine the interactions between pathogens and their host plants (Ferreira et al. 2017). GUS staining is one means of detecting the localization of strains in plants. To identify the localization and colonization differences between a WT *R. solanacearum* strain and T3SS mutant strains inoculated in potato plants, we detected the localization of both strains by GUS staining and tracked the bacterial populations in the plant roots. The UW551::GUS strains were readily detected in potato plants by their lethal effects and spreading over the entire plant soon after

Table 1. Root proteins with significant differential abundance following inoculation of potato with wild-type (WT) *Ralstonia solanacearum* or its type III secretion system mutant strain, identified by their best hit protein or gene

<i>Solanum tuberosum</i> ^a	Best matching gene product ^b	MW (kDa)	FC (WT or <i>hrcV</i>) ^c	P value ^c
M1AGQ5	Heat shock protein 60 (HSP60)	60.3	4.418604651	7.21E-05
M1CSI8	Heat shock protein (HSP70)	70.8	1.836534358	0.000183745
M1B7H0	FKBP type peptidyl-prolyl cis-trans isomerase (FKBP16-3)	25.5	1.807782466	0.006929319
M0ZYM1	Midasin-like (MDN1)	43.9	1.748399247	6.80E-05
M0ZKQ8	Chloroplastic lipocalin (CHL)	30.4	1.565553746	0.013781156
M1AZP7	Threonine dehydratase biosynthetic (OMR1)	64.7	1.501545595	8.82E-06
M1D527	Protein binding protein (ZKT)	36.4	1.476069462	0.022483707
M1AZT7	Germin-like protein (GER3(a))	22	1.470679012	0.000730884
M1B3M1	Germin-like protein (GER3(b))	21.5	1.445501097	0.00668519
M1BF20	Oxidoreductase	39.4	1.431206764	0.002113528
M1AJD8	Low psii accumulation 3 (LPA3)	27.7	1.426017497	0.028994609
M1A0Z7	Mitochondrial small heat shock protein (HSP22)	23.9	0.705955956	0.000634315
M1CER0	Dehydroquinase dehydratase/shikimate dehydrogenase (MEE32)	24	0.705530217	0.003383769
M1CKA5	Miraculin	22.1	0.701395573	0.000247865
M1A616	Leucine-rich repeat/extension (LRX1)	79.1	0.689750693	0.000275945
M1BT09	Mitochondrial import receptor subunit TOM20 (TOM20)	22.7	0.67679558	0.005436501
M1BTL1	Pentatricopeptide repeat-containing protein (PPR)	82.6	0.673297003	0.000749548
M1ARE5	Soul heme-binding family protein (HBP2)	22.2	0.647744946	5.47E-07
M0ZN99	Eukaryotic translation initiation factor 4G (EIF4G)	195.8	0.577300931	0.000792221
P80496	ATP synthase 24 kDa subunit (MGPI)	27.7	0.572877736	0.001338535
M1C5B7	Serine/threonine-protein phosphatase (PP1)	47	0.487826472	9.40E-05

^a Accession numbers were acquired from the liquid chromatography tandem mass spectrometry data.

^b Best matches were based on accession number searches against the *Solanaceae* Genomics Resource and NCBI protein databanks.

^c Differentially accumulated proteins were considered as those displaying a fold change (FC) > 1.40 or < 0.71 in relative abundance and a P value < 0.05.

inoculation. In contrast, the GUS signal of the T3SS mutant strain was detected only in the roots and with a low population density. The results confirmed that the pathogenicity of *R. solanacearum* was related to the T3SS and the injection of T3E effector proteins into plant cells and that the T3Es play a key role in pathogen infection. Due to the lack of T3SS, effectors cannot inhibit plant immune response, resulting in restrictions on the proliferation and pathogenicity of pathogens. We infer that effectors may act on certain proteins in the root to help pathogens infect plants.

Based on the results of the bacterial quantification in the roots, we infer that the pathogen–plant interaction peaks at 6 days postinoculation. Proteomic analysis was performed based on the difference in bacterial density between the WT and the T3SS mutant strains infecting potato roots, leading to the identification of 21 DAPs. Given that the mechanisms involved in the upregulation of protein expression may be complex, we concentrated on determining whether the downregulated proteins were related to plant resistance in tobacco by VIGS. Because there is no mature VIGS system applying in potato to quickly test those DAPs functions thus far, we silenced homologous genes in the model plant *N. benthamiana*. With this system, we could only test the function of the homolog genes in *N. benthamiana*. The silencing of *TOM20*, *PP1*, *HBP2*, and *miraculin* in tobacco plants significantly changed the resistance of plants to *R. solanacearum*. *TOM20* is a marker of oxidative phosphorylation, and increased oxidative phosphorylation can lead to increased ROS production (Urvalek et al. 2015). ROS are considered a representative cellular immune response triggered by PAMP (Sang and Macho 2017) and an important line of defense against pathogen invasion (Aver'yanov et al. 2001). In tomato, increased ROS generation can improve resistance to

bacterial wilt (Mandal et al. 2011), and the evidence suggests that *R. solanacearum* infection can induce ROS production in tolerant plants (Ferreira et al. 2017). The results of our study suggest that *R. solanacearum* can inhibit ROS production in the process of infection because the *TOM20* protein was downregulated in plants inoculated with the WT strain compared with that in plants inoculated with the T3SS mutant strain, and *TOM20* silencing increased plant susceptibility to infection. *HBP2* is a heme-binding protein, and the homologous *Arabidopsis* gene *AtHBP5* is reported to be involved in the antioxidant pathway. Overexpression of *AtHBP5* in plants can reduce H_2O_2 content (Lee et al. 2012). ROS production in plants increases significantly in the early resistance response against pathogens (Mehdy 1994); however, excessive ROS production can cause damage to plants, including enzyme inactivation and cell death (Greene 2002). *HBP2*-silenced plants cannot defend against ROS damage, which further aggravates plant damage. The Miraculin protein has a Kunitz inhibitor domain and can act as a moderate trypsin protease inhibitor (Ohkura et al. 2018). Plant Kunitz-type inhibitors are considered to play an important role in defending against pathogen invasion (Bendre et al. 2019), and Kunitz proteinases are reported to be activated by jasmonate (Yamchi et al. 2018). The silencing of Miraculin indicated that the gene is associated with plant resistance, which merits further investigation. The PP1 protein is a protein phosphatase and plays an important role in plant immune regulation (Liu et al. 2020) and is reported to be a negative regulator of plant defenses, consistent with the results of this study. We will investigate this discrepancy in future work using potato transgenics. In this study, four of the downregulated genes were related to plant resistance to pathogen attack, which shows the reliability of our experimental design.

Fig. 3. Gene ontology (GO) annotation pie chart of 21 differentially expressed genes via PANTHER classification analysis. GO hits for these genes were assigned to molecular functions, cellular components, and biological process. In all, 6 genes were annotated in biological processes, 8 genes in molecular functions, and 12 genes in cellular components categories. The percentage of function hits is indicated in parentheses.

The expression of DAPs at the transcription level shows no difference after inoculation with the *R. solanacearum* WT and *hrcV* mutant strains (Fig. 6), which is consistent with the transcriptome data of wild potato *S. commersonii* in potato roots upon *R. solanacearum* infection (Zuluaga et al. 2015). We found that 4 of the 10 downregulated proteins showed unaltered transcriptional expression, suggesting that changes in the abundance of these proteins are likely to occur at the protein level. In plants inoculated with T3SS mutant strains, effector proteins cannot enter into plant cells (Coll and Valls 2013). We believe that the downregulation of the four proteins was caused by the T3Es; therefore, we aimed to identify effectors that influence each one of potato proteins. To verify this, we employed the *N. benthamiana* transient expression system, an efficient and high-level model system for transient protein expression (Xu et al. 2015), to directly coexpress the selected proteins with the effectors. The results indicated that the downregulation of these four proteins was likely to have resulted from the combined action of multiple effector proteins. Interestingly, the abundance of HSP22 was decreased by 18 effectors when coexpressed in tobacco; however, the result of VIGS showed that it did not have an effect on potato immunity. This circumstance may be due to VIGS experimental systems. The VIGS systems have some limitations in potato gene function verification. In our cases, HSP22 could be downregulated by 18 effectors, which is unusual. Two reasons may explain this: one is the limitation of the VIGS system and the other is the redundancy of T3Es. Due to the redundancy of T3Es, multiple effectors may play some role in the perturbation of plant immunity.

Identifying the toxicity associated with a single effector has been reported to be difficult owing to functional redundancy (Poueymiro et al. 2014), and our results also confirmed this. Studies have shown that the effectors of *R. solanacearum* can inhibit plant defense mechanisms, regulate host metabolism, or avoid bacterial recognition through a variety of molecular mechanisms. It has been shown that *R. solanacearum* effectors can suppress plant defense mechanisms, modulate the host

metabolism, or avoid bacterial recognition through a wide variety of molecular mechanisms (Landry et al. 2020). Although the inhibitory effect of T3Es on PTI has been widely reported, there are still few reports on the T3E and its biochemical properties in inhibiting ETI (Yu et al. 2020). After the T3SS was knocked out, the pathogen could not inject the effectors to plant cells and lost the ability to suppress regulated plant immunity. Therefore, the decreases of DAPs were likely to be caused by the presence of T3Es, and we believe that the screened genes may be directly or indirectly involved in plant immune regulation.

This study aimed to screen effector-related plant proteins by proteomics. In our study, we showed that T3Es play a key role in potato colonization and multiplication. In particular, there were four genes that affect plant resistance mentioned in the VIGS experiment and four other genes that are inhibited by

Fig. 4. Relative gene expression in the gene-silenced plants. Targeted genes exhibit a 70 to 99% reduction in expression in silenced plants compared with that of mock-treated TRV:00 plants, which were set to '1'. Experiments were repeated three times, with three independent biological repetitions each time. Error bars indicate the standard error. Relative transcript levels of analyzed tobacco genes were normalized to the transcript levels of *NbActin*.

Table 2. *Nicotiana benthamiana* homologs of the 10 genes downregulated in potato roots inoculated with wild-type *Ralstonia solanacearum* UW551, and its relative gene expression

Gene name	<i>Solanum tuberosum</i> (accession number)	<i>N. benthamiana</i> sequence ID ^a	Relative gene expression ^b
EIF4G	M0ZN99	Niben101Scf10210g01004.1	0.029
	...	Niben101Scf06902g02007.1	0.046
MEE32	M1CER0	Niben101Scf04146g01023.1	0.049
	...	Niben101Scf02709g04001.1	0.030
TOM20	M1BT09	Niben101Scf04216g05005.1	0.009
	...	Niben101Scf00109g10030.1	0.172
PPR	M1BTL1	Niben101Scf03766g01008.1	0.001
	...	Niben101Scf05095g01004.1	0.036
MGPI	P80496	Niben101Scf14939g01027.1	0.057
	...	Niben101Scf05649g05013.1	0.028
PPI	M1C5B7	Niben101Scf00751g04001.1	0.011
	...	Niben101Scf05057g02005.1	0.344
HSP22	M1A0Z7	Niben101Scf08308g04016.1	0.118
	...	Niben101Scf01942g05024.1	0.240
LRX1	M1A616	Niben101Scf02724g05005.1	0.021
	...	Niben101Scf11953g03005.1	0.004
HBP2	M1ARE5	Niben101Scf03064g08007.1	0.026
	...	Niben101Scf00317g09001.1	0.028
Miraculin	M1CKA5	Niben101Scf04091g00013.1	0.007
	...	Niben101Scf27793g02008.1	0.042
		Niben101Scf16542g01003.1	0.022
		Niben101Scf18771g00012.1	0.054
		Niben101Scf06825g00018.1	0.010
		Niben101Scf06424g02024.1	0.009

^a These genes were searched against the Solanaceae Genomics Network.

^b Relative expression of target genes is expressed in comparison with a reference gene (*NbActin*).

multiple effectors at the protein level in the coexpression experiment, and, interestingly, three of the four genes (*PPI*, *TOM20*, and *HBP2*) in the results of the two experiments are the same. These three genes have the value of further research on their relationship with plant immunity.

MATERIALS AND METHODS

Plant materials.

Potato plants (*S. chacoense* accession C9701) were grown at 18 to 25°C in a glasshouse with cycles of 16 h of light and 8 h of

Fig. 5. Virus-induced gene silencing of target genes changed the susceptibility of tobacco to *Ralstonia solanacearum* strain K60. **A**, Survival analysis of gene-silenced plants (named as TRV:target gene) inoculated with *R. solanacearum* strain K60. Statistical analysis was performed via the log-rank test and the Gehan-Breslow-Wilcoxon test (* indicates $P < 0.05$). Each point represents the survival rates of one independent experiment, comprising 24 silenced plants per treatment. **B**, Disease symptoms of the empty vector control plants inoculated with *R. solanacearum* K60 by root irrigation at 14 or 21 days postinoculation (dpi).

darkness. After 3 weeks, plants were sown in plastic boxes with soil mix (Tref Substrates BV, Moerdijk, The Netherlands) and used as the susceptible WT species for virulence identification.

Four C9701 potato plants were micropropagated in a sterile culture box from a node with 30 ml of Murashige and Skoog medium with sucrose at 30 g/liter and kept at 22°C with cycles of 16 h of light and 8 h of darkness. After 2 weeks, plants were used for the GUS assay.

N. benthamiana plants were grown in a soil mix in plastic pots under long-day (16 h of light) conditions at 22°C and relative humidity of 70% in a glasshouse.

Bacterial materials.

Strain UW551 is classified as phylotype IIB, and it has a strong pathogenicity to potato but not to tobacco. Strain K60 is classified as phylotype IIA, and it has a strong pathogenicity to tobacco. *R. solanacearum* strains was grown on BG agar (bactopeptone at 10 g/liter, casamino acids at 1 g/liter, and glucose at 2.5 g/liter) plated at 28°C for 48 h.

Construction of *R. solanacearum* *hrcV* mutant and GUS-producing strains. To generate the *R. solanacearum* T3SS mutant strain, the whole *hrcV* gene was replaced with *Km^r*. Each sequence contains the target gene, and its flanking region (500 to 1,000 bp) was amplified from the genome of strain UW551 and inserted into a pEASY-blunt vector. Reverse amplification of the target gene was used to delete the target gene that produces only linear vectors with flanking regions. Using the ClonExpress II one-step cloning kit (Vazyme Biotech), the *Km^r* cassette that contained the promoter and terminator was inserted into the linear vector to create the pEASY-flanking region-*Km^r* vector. The *Km^r* cassette fragment harboring the flanking regions of the mutant gene was then amplified and purified for the transformation of the *R. solanacearum* strain (Jacobs et al. 2013).

The GUS-producing strains were constructed via homologous recombination, and the gene GUS with a *psbA* promoter and a *Km^r* cassette were inserted into a permissive chromosomal site between RRSL_04294 and RRSL_04295, just like the construction of the mutant.

GUS assay.

A whole plant inoculated with GUS-producing strains was vacuum infiltrated with a GUS staining solution (1 mM sodium phosphate buffer [pH 7] 10 mM EDTA, 0.1% Triton-X, chloramphenicol at 100 µg/ml, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and X-glucuronide at 0.5 mg/ml) for 15 min, incubated overnight at 37°C, then destained using 70% ethanol (Pecinka et al. 2009). Selected plants were observed from day 0 to 7 after inoculation for infection by GUS-producing strains of *R. solanacearum*.

VIGS assay.

The TRV-based VIGS vector is the most efficient VIGS vector thus far (Dong et al. 2007). To construct 10 vectors for VIGS, parts of the 3-untranslated region of the 10 target genes were cloned into the destination vector pTRV2. All of the primers used in VIGS vector construction are listed in Supplementary Table S1. For the VIGS assay, TRV vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation (Holsters et al. 1980). Bacteria containing TRV1 or TRV2 plasmids were incubated overnight at 28°C with shaking and then resuspended in infiltration buffer (10 mM morpholineethanesulfonic acid, 10 mM MgCl₂, 200 µM acetosyringone, pH 5.6). *Agrobacterium* cultures at an optical density at 600 nm (OD₆₀₀) = 0.5 containing TRV1 or TRV2 derivative plasmids were mixed in a 1:1 ratio. After 3 h of incubation, the mixed *Agrobacterium* cultures were injected into the leaves of four-leaf-stage *N. benthamiana* plants. A gene-

silenced phenotype appeared in the new leaves 2 weeks post-injection (Wang et al. 2013).

qRT-PCR.

For gene expression analysis, total RNA was extracted using a Plant RNA kit (Bio-tek, Winooski, VT, U.S.A.) in accordance

Fig. 6. Relative gene expression of the differentially accumulated proteins. Data represent means ± standard deviation, *n* = 3; an asterisk (*) indicates *P* < 0.05, multiple *t* tests, corrected for multiple comparisons using the Holm-Sidak method.

Table 3. Core type III effectors (T3Es) (*n* = 33) identified in *Ralstonia solanacearum* comparative analysis of 13 sequenced phylotype IIB strains^a

Effector name	Description ^b
RipA2	AWR2
RipA5-2	AWR5
RipAB	NLS harboring protein
RipAC	LRR domain
RipAD	...
RipAE	Putative acetyltransferase
RipAI	...
RipAM	...
RipAJ	...
RipAN	...
RipAP	...
RipAR	Ubiquitin ligase domain
RipAT	...
RipAV	...
RipAY	Glutamyl cyclotransferase
RipB	Nucleoside Nribohydrolase
RipBH	...
RipC1	HAD-like phosphatase
RipD	...
RipE1	...
RipE2	...
RipF1	T3SS translocator
RipF2	T3SS translocator
RipG2	F-box LRR GALA2
RipG6	F-box LRR GALA6
RipG7	F-box LRR GALA7
RipH1	HLK
RipN	Nudix hydrolase
RipO1	...
RipR	...
RipTPS	Trehalose-phosphate synthase
RipU	...
RipV1	Ubiquitin ligase domain

^a Effector name and functional description were obtained from the T3E database (Peeters et al. 2013).

^b LRR = leucine-rich repeat and T3SS = type III secretion system.

with the manufacturer's instructions. First-strand cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, U.S.A.). qRT-PCR was performed using SYBR Green Super mix (Bio-Rad) and a CFX96 Real-Time system (Bio-Rad), and the qRT-PCR data were analyzed as described previously (Livak and Schmittgen 2001). Three independent

biological replicates of each treatment were performed, and three technical replicates were performed for each biological replicate. Primer pairs were designed outside the region of cDNA targeted for selected genes following the manufacturer's guidelines (Supplementary Tables S2 and S3). In each case, PCR conditions were 95°C for 15 min, followed by 40 cycles of

Fig. 7. Abundance of the potato proteins PP1, HSP22, HBP2, and TOM20 decreased when coexpressed with type III effectors. Detection of the abundance of HA-tagged potato protein PP1, HSP22, HBP2, and TOM20. An effector (RipU) was selected as a control that has no effect on the plant proteins. Proteins were extracted at 48 h postinfection (hpi). The experiment was conducted in triplicate. The protein loading is indicated by Coomassie Brilliant Blue (CBB) staining. GFP = green fluorescent protein.

95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Expressions in silenced plants were compared with that in the control TRV:00 plants. The potato gene *Stef1α* (XM_006343394) and tobacco gene *Ntactin* (XM_016658880) were used as internal reference genes (Nicot et al. 2005). All primers used in qRT-PCR are listed in Supplementary Tables S2 and S3.

Pathogenicity assays.

In vitro potato tests were used for GUS assays and bacterial quantification and inoculated with 5.0 ml of *R. solanacearum* suspension ($OD_{600} = 0.1$). Three independent tests, with eight potato plants per experiment, were carried out. Plant wilting symptoms were recorded every day and expressed according to a disease index scale (0 = no wilting; 1 = 25%, 2 = 50%, and

3 = 75% wilted leaves; and 4 = death). To measure root colonization by *R. solanacearum* strains, from 0 to 9 days after inoculation, we sampled the middle of potato roots every day, and four inoculated plants per strain per time point were harvested in a sterile environment. The root segment was weighed, ground in 5 ml of sterile water, and dilution plated onto BG medium; colonies were counted after 48 h of incubation at 28°C. (Colburn-Clifford et al. 2010).

The soil drench is a method of pouring bacterial liquid directly into the soil and allowing pathogens to infect plants naturally, which was used for the VIGS lines and virulence assays. With respect to the infection of the VIGS lines, the surviving plants were recorded daily after inoculation. In total, 24 (4 × 6) silenced plants inoculated with 500 ml of *R. solanacearum* suspension

Fig. 8. A summary network of the effector/potato protein coexpression. Lines of the same color connect each of the four potato proteins (PP1 [green], HBP2 [yellow], HSP22 [blue], and TOM20 [orange]) to the effectors that led to their decreased abundance when coexpressed.

(OD₆₀₀ = 0.1) were used for the analysis of the survival rates and three independent experiments were evaluated. The number of surviving plants was recorded daily after inoculation.

Transient expression in *N. benthamiana*.

Four- to six-week-old plants were used for transient expression assays. *A. tumefaciens* cultures (strain GV3101) carrying target constructs were cultured overnight in the presence of the appropriate antibiotics at 28°C. Bacteria were pelleted, centrifuged, and resuspended in infiltration buffer, after which their concentration was adjusted to the required OD₆₀₀ of 0.3 for immunoblots. After 3 h of incubation, the cultures were infiltrated into leaves of *N. benthamiana* plants.

Immunoblot analysis.

After 48 h of *Agrobacterium*-mediated transformation, tobacco leaves were ground in liquid nitrogen and extracted in extraction buffer (100 mM Tris [pH = 8], 150 mM NaCl, 10% glycerol, 5 mM EDTA, 2 mM dithiothreitol [DTT], 1% protease inhibitor cocktail, and 2 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 15,000 × g for 20 min. (Kadota et al. 2016). A 5× sodium dodecyl sulfate (SDS) loading buffer (250 mM Tris-HCL [pH 6.8], 10% [wt/vol] SDS, 0.5% [wt/vol] bromophenol blue, 50% [wt/vol] glycerol, and 5% [wt/vol] β-mercaptoethanol) was added to the supernatant and heated at 95°C for 10 min on a heating block. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membranes (Bio-Rad), and the chemiluminescence bands were detected with ECL Plus reagent.

Sample preparation and proteomic analyses.

In this study, the root samples of 12 potato plants per treatment were harvested at 6 days postinoculation and partial root mixtures samples of 12 potato plants were sent for proteomic analyses. For each sample, 100 µg of protein was mixed with 120 µl of reducing buffer (10 mM DTT, 8 M urea, and 100 mM triethylammonium bicarbonate, pH 8.0) in an Amicon Ultra 0.5 Centrifugal Filter (10 kDa) and was incubated at 60°C for 1 h. Then, iodoacetamide was added to the solution with a final concentration of 50 mM and incubated for 40 min at room temperature in the dark. The iTRAQ labeling proteomic and mass spectrometric data analyses were carried out by Shanghai LuMing Biological Technology Co., Ltd. (Elias and Gygi 2007; Wiśniewski et al. 2009). The raw mass spectrometry proteomics data have been deposited at iProX (Ma et al. 2019) with the data set identifier IPX0001659000.

Bioinformatics.

In this study, the biological and functional properties of all of the identified proteins were mapped with GO terms by PANTHER.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

iProX: <https://iprox.org>
 PANTHER: <http://www.pantherdb.org/>
Ralstonia solanacearum GMI1000, MOLK2 and IPO1609:
<https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi>

Solanaceae Genomics Network:

https://solgenomics.net/organism/nicotiana_benthamiana/genome

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