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Proteomic analysis provides integrated insight into mechanisms of *Turnip mosaic virus* long distance movement in *Brassica rapa*

C. LIU¹, G.-S. SUN², R.-J. ZHANG¹, S.-W. LV¹, L. GAO¹, L.-W. GAO¹, T.-K. LIU¹, D. XIAO¹, X.-L. HOU¹, and C.-W. ZHANG^{1*}

State Key Laboratory of Crop Genetics and Germplasm Enhancement, College of Horticulture, Nanjing Agricultural University, Nanjing, Jiangsu, 210095, P.R. China¹
Zhenjiang Institute of Agricultural Sciences in Hilly Area of Jiangsu Province, Jurong, Jiangsu, 212400, P.R. China²

Abstract

In non-heading Chinese cabbage, the yield relies mostly on the health of leaves, which can be heavily impacted by *turnip mosaic virus* (TuMV). The virions or viral ribonucleoprotein complexes are transported through the phloem and xylem. Plasmodesmata are indispensable because they traverse cell walls and connect companion cells, allowing virus particles long distance movement. However, which complexes and genes participate in this process is still unknown. Plants can activate defense mechanisms and apply disease resistance genes to respond to pathogen attacks. In this study, we collected the stems and petioles infected by TuMV for 7 d (TuMV-7), 14 d (TuMV-14), and 21 d (TuMV-21). Using isobaric tags for relative and absolute quantification-based proteomic technology, 6 043 distinct proteins were identified and 323, 240, 285, 203, 253, and 363 differentially expressed proteins were found in the comparable pairs of TuMV-7/control, TuMV-14/TuMV-7, TuMV-14/control, TuMV-21/TuMV-7, TuMV-21/TuMV-14, and TuMV-21/control, respectively. We performed a functional annotation analysis of all identified proteins and a functional enrichment analysis of all differentially expressed proteins. The results indicated that the long distance movement of TuMV involved many complex regulatory pathways. The respective proteins were related to those occurring in plasmodesmata and to Ca²⁺ transporters. Further, we also found proteins related to heat shock proteins, pathogenesis-related proteins, and proteins scavenging reactive oxygen species.

Additional key words: disease resistance, iTRAQ, non-heading Chinese cabbage, pathogenesis-related proteins, plasmodesmata.

Introduction

Brassica rapa crops (AA, 2n = 20), such as Chinese cabbage (*B. rapa* ssp. *pekinensis*), non-heading Chinese cabbage (*B. rapa* ssp. *chinensis*), and turnip (*B. rapa* ssp. *rapa*) are well-known for their rich nutrients, short growth periods, and adaptability.

Potyviruses make up the largest genus of plant viruses and affect more than 50 % of crops. *Turnip mosaic virus* (TuMV) contains an approximately 10-kb RNA genome.

Electron microscopy shows that the viral particles are nonenveloped, approximately 680 - 900 nm in length and 11 - 15 nm in diameter, and form flexuous rods (Wan *et al.* 2015a). TuMV has become the second most prevalent virus in fields, after cucumber mosaic virus.

One priority in breeding has been the development of disease-resistant agricultural cultivars. Many genes related to disease resistance have been identified since Jenner

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Abbreviations: APX - ascorbate peroxidase; DREPP - developmentally-regulated PM polypeptide; ER - endoplasmic reticulum; HSPs - heat shock proteins; iTRAQ - isobaric tags for relative and absolute quantification; MP - movement protein; PATL - patellin; PCaP - plasma membrane-associated cation binding protein; PD - plasmodesmata; PGIPs - PG-inhibiting proteins; PGs - endopolygalacturonases; PM - plasma membrane; PME - pectin methylesterase; PRs - pathogenesis-related proteins; ROS - reactive oxygen species; RT-PCR - reverse transcription PCR; sHSPs - small heat shock proteins; TuMV - *turnip mosaic virus*.

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* Corresponding author; fax: (+86) 25 84395266, e-mail: changweizh@njau.edu.cn

et al. (2002) observed the 12 TuMV groups, for which some resistance loci exist in *Brassica* crops. Walsh *et al.* (1999) identified *TuRB01* and *TuRB02* and Jenner *et al.* (2002) identified *TuRB04* and *TuRB05*. Other identified genes include *TuRB01b*, *ConTR01* and *retr01* (Rusholme *et al.* 2007), *Rnt1* (Fujiwara *et al.* 2011), and *retr02* (Qian *et al.* 2013) in *B. napus*. These genes play significant roles in virus-plant interactions. The largest group is the nucleotide-binding site (NBS)-leucine-rich repeat (LRR) family including resistant (*R*) genes against viruses (*HRT*, *N*, *RCY1*, *Rx1*, *Rx2*, and *Sw5*) (Kang *et al.* 2005). In addition, host defense mechanisms, with the support of chemical compounds, function to protect themselves from external invasions.

Normally, movement of viruses between cells occurs on the 3rd day after inoculation and the systemic infection starts on the 5th day after inoculation in the TuMV-agroinfiltrated plants (Wan *et al.* 2015b). Cell walls protect cells from external invaders, which makes it challenging to exchange information throughout the surroundings. Plasmodesmata (PD) play an irreplaceable role in breaking the restrictions of cell walls and allowing the movement of different molecules in the xylem and phloem in which the

long distance movement of loading and unloading occurs (Wan and Laliberte 2015). The desmotubule tightly compresses the endoplasmic reticulum (ER), creating a space between the plasma membrane (PM) and the desmotubule, called the cytoplasmic sleeve, through which various molecules, such as transcription factors, RNAs, and phytohormones, can move. The PD are used to connect sieve elements with companion cells. The movement of molecules upward and downward in the phloem may occur through these pore plasmodesmal units, which function more ubiquitously than PDs (Wan *et al.* 2015b). PD connect different replication complexes with viral movement and demonstrate the interaction and cooperation between protein complexes produced by viruses and plants (Lucas 2006). Coat proteins were found in the xylem, so people believed that cellular movement of these complexes is mediated by virus-encoded movement proteins (MPs), and viruses move in the form of virions (Deng *et al.* 2015).

In this study, we chose the isobaric tags for relative and absolute quantification (iTRAQ) method to investigate the underlying mechanisms of the long distance movement of TuMV in non-heading Chinese cabbage.

Materials and methods

Plants and TuMV-inoculation: Non-heading Chinese cabbage (*Brassica rapa* L. ssp. *chinensis*) cv. 49caixin as well as *Nicotiana benthamiana* Domin plants were grown in a cultivation chamber with a 16-h photoperiod, an irradiance of 225 $\mu\text{mol m}^{-2} \text{s}^{-1}$, day/night temperatures of 22/17 °C, and a relative humidity of 60 - 65 %. Six-week-old *N. benthamiana* plants were inoculated with TuMV-GFP. For this, TuMV-GFP was first introduced into *Agrobacterium tumefaciens* strain GV3101. The *Agrobacterium* was incubated in 50 cm³ of Luria-Bertani medium containing 50 mg dm⁻³ rifampicin and 50 mg dm⁻³ kanamycin and grown in a shaker at 28 °C overnight. Then, the *Agrobacterium* was resuspended in infiltration buffer (0.25 g of D-glucose, 5 cm³ of 500 mM MES stock solution, 5 cm³ of 20 mM Na₃PO₄, 12 H₂O stock solution, 5 cm³ of 1 M acetosyringone stock solution, and distilled water to 50 cm³). The infiltration buffer was adjusted to an absorbance A₆₀₀ of 0.6 - 0.8, incubated at room temperature for 3 to 4 h before infiltration and then infiltrated into leaves using a 1-cm³ syringe. The non-heading Chinese cabbage was infected mechanically in the six-leaf stage. We collected 2 g of infected stems and petioles before inoculation and on the 7th, 14th, and 21st day after inoculation (named control, TuMV-7, TuMV-14, and TuMV-21, respectively). Then, the samples were frozen in liquid nitrogen and stored them at -70 °C for protein separation.

Reverse transcription PCR: Total RNA was extracted

from samples using the total RNA extraction kit (Tiangen, Beijing, China). RT-PCR was performed using gene-specific primers and *SYBR Green* (TaKaRa, Kyoto, Japan) on an *ABI PrismR 7900HT* system (Applied Biosystems, Carlsbad, CA, USA) according to the instructions. The amplification procedure was as follows: pre-denaturation at 94 °C for 10 s, followed by 40 cycles at 94 °C for 30 s and at 60 °C for 30 s, and finally a melting curve was performed (61 cycles at 65 °C for 10 s). The expression of β -actin was used to standardize the RNA sample for each analysis. The RT-PCR assay was performed at least three times for each experimental line. Primers were designed according to the gene sequences using *Becon Designer v. 7.9* and they are listed in Table 3 Suppl. The expression is presented with the heatmap (<http://www.omicsshare.com/tools/index.php/Home/Soft/heatmap>) and the gene sequences information can be found in our database (<http://brassicadb.org/brad/>).

Transmission electron microscopy: The petioles were sampled from the young leaves which were close to growing tips and the stems were sampled below the node which were close to the root. Then they were fixed with 2.5 % (m/v) glutaraldehyde at 4 °C for 24 h. A series of single-axis-tilt images were collected with a transmission electron microscopy (*Tecnai Hitachi HT7700*, Tokyo, Japan), operated at an accelerating voltage of 80 kV, and critical sample features were identified in the viewing screen of the CCD camera operating in ambient light.

Protein extraction, digestion, and desalting: Tissue samples (2 g) were dissolved in 1 M triethylammonium bicarbonate (TEAB) dissolution buffer with 0.2% (m/v) sodium dodecyl sulphate, broken by ultrasonic waves for 15 min, centrifuged at 15 000 g for 20 min, then a four-fold volume of cold acetone containing 10 mM dithiotreitol (DTT) was added. After ~2 h, the precipitate was collected by centrifugation at 15 000 g and 4 °C for 20 min, mixed with 800 mm³ of cold acetone and warmed to 56 °C to break protein disulfide bonds, centrifuged again at 15 000 g and 4 °C for 20 min and dried. Samples were then dissolved with 100 mm³ of TEAB buffer and finally stored at -80 °C for later use.

The total protein content was measured using the Bradford method. For each sample, 100 µg of protein was dissolved in 100 mm³ of TEAB buffer, and then diluted with 500 mm³ of 50 mM NH₄HCO₃. After reduction and alkylation, 2 µg of trypsin was added, and then, samples were incubated at 37 °C overnight for protein digestion. After protein digestion, an equal volume of 0.1 % (v/v) formic acid (FA) was added for acidization. Peptides were purified on *Strata-X* (Torrance, USA) C18 columns that had been activated with methanol and then balanced by adding 1 cm³ of 0.1 % FA three times, washed with 0.1 % FA + 5 % (v/v) acetonitrile (ACN) twice, and eluted with 1 cm³ of 0.1 % FA + 80 % ACN. Eluted peptides were dried using a vacuum concentration pump. The dried peptide powders were redissolved with 20 mm³ of 0.5 M TEAB for peptide labelling.

iTRAQ labeling and fractionation: Samples were labelled using an *iTRAQ Reagent-8 plex Multiplex* kit (AB Sciex, Framingham, USA) according to the manufacturer's instructions. TuMV-7, TuMV-14, TuMV-21, and control were labelled with reagents 116, 117, 118, and 121 respectively. All of the labelled samples were mixed in equal amounts. Next, the labelled samples were fractionated using a high-performance liquid chromatography (*Thermo DINOEX Ultimate 3000 BioRS*, Waltham, USA) with a *Durashell* C18 column (5 µm, 100 Å, 4.6 × 250 mm). Finally, 12 fractions were collected.

The liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis was performed on an *AB Sciex nanoLC-MS/MS (Triple TOF 5600 plus)* system. Samples were chromatographed using a 90 min gradient ranging from 2 to 30 % buffer A [0.1 % (v/v) formic acid, 5 % (v/v) acetonitrile] and buffer B [0.1 % formic acid, 95 % acetonitrile], injected directly onto a 20-µm *PicoFrit* emitter (*New Objective*, Ringoes, USA) and packed with 12 cm of *Magic C18AQ* (3 µm, 120 Å) stationary phase. The MS1 spectra were collected in the range from 350 - 1500 m/z for 250 ms. The most intense 20 precursors with charge state 2 - 5 were selected for fragmentation. The MS2 spectra were collected in the

range from 50 - 2 000 m/z for 100 ms, and precursor ions were excluded from reselection for 15 s.

Data analysis: The original MS/MS file data were submitted to *ProteinPilot* v.4.5 (<https://sciex.com/products/software/protein-pilot-software>) for analysis. The protein identification was employed against *Uniprot Chlorella B. rapa* (41 720 items, updated in Aug. 2016) for a database search. The parameters were set as follows: the instrument was a *TripleTOF 5600*; iTRAQ quantification and cysteine were modified with iodoacetamide; biological modifications were selected as ID foci; trypsin digestion; and the quantitation, bias correction and background correction were checked for protein quantification and normalization. The false discovery rate calculation used an automatic decoy database search strategy with the *Proteomics System Performance Evaluation Pipeline* software (integrated into the *ProteinPilot* software) algorithm. Only proteins with at least one unique peptide and an unused value more than 1.3 were considered for further analysis. Some identified peptides were excluded from the quantitative analysis for one of the following reasons: 1) peaks corresponding to the iTRAQ labels were not detected; 2) the identified peptide had a low identification confidence values; 3) the peptide was claimed by more than one protein; 4) the signal-to-noise ratio for the peptide was too low; or 5) the combined feature probability for the peptide was less than 30 %, because it was a semitryptic peptide, missing an iTRAQ reagent label, had a low probability modification, or had a large delta mass. For protein abundance ratios measured using iTRAQ after normalization, a 1.5-fold change and a *P*-value less than 0.05 were used as the thresholds to identify significant changes.

Bioinformatics: To determine the biological and functional properties of the identified proteins, their protein sequences were mapped using gene ontology (*GO*) terms (<http://geneontology.org/>). A homology search was first performed for all of the identified sequences using a localized *NCBI BLASTP* program against the *NCBI nr* animal database. The *e*-value was set to less than 1e-5, and the best hit for each query sequence was used for matching *GO* terms. The *GO* term matching was performed with *blast2go v4.5 pipeline5*. Clusters of orthologous groups of proteins (<http://www.ncbi.nlm.nih.gov/COG/>) were employed for the functional annotation of genes from new genomes and for research into genome evolution. To identify candidate biomarkers, we employed a hypergeometric test to perform *GO* enrichment and *KEGG* pathway enrichment (<http://www.genome.jp/kegg/>), and each protein sequence, as well as functional information, was provided by *UniProt* databases (<http://www.uniprot.org/>). All other figures in this paper were drawn using *R language* (<http://www.r-project.org/>).

Results

To ensure that the plants were infected with TuMV, we used transmission electron microscopy to investigate the ultrastructural particles of TuMV (Fig. 1). Chloroplasts with starch grains were spread around the cell walls (Fig. 1A,B). The viral replication complexes in plants have different forms. We found that round-like viral structures, containing an axis and radial arms, only exist in the infected samples (Fig. 1C,D). The structures in the petioles indicated that viral replication and transport occurred in the phloem and xylem. To complete the long distance movement, the virus must distribute the virions to new distant sites through phloem cells and sieve elements, thus invading the whole plant (Hipper *et al.* 2014).

Fig. 1. Ultrastructure of Chinese cabbage cells. *A* and *B* are the control plants *C* and *D* are the TuMV-infected plants. *A* and *C* are the stems, *B* and *D* are petioles. The scale bars in *A*, *B*, *C*, and *D* are 50, 20, 20, and 2.5 µm, respectively.

After summarizing the iTRAQ results, 415 772 spectra were obtained and approximately 164 219 MS spectra were identified, which was in accordance with the known spectra and the identification rate was 39.50 %. *ProteinPilot* software was employed to characterize the number of identified peptides (see the identification criteria under Materials and methods). In total, 40 478 peptides were screened, from which 6 043 proteins were identified and 5 322 (88.07 %) proteins contained at least two unique peptides. The identified proteins coverage from 0 to 10 % was 28.62 %, the coverage ≥ 20 % was 50.07 % and the average coverage was 25.64 % (Fig. 2A). The peptide lengths of identified proteins ranged mainly from 8 aa to 15 aa, with the most common length being 11 aa, and the average length being 14.46 (Fig. 2B). The confidence percentage of matching amino acids was ≥ 95 %. According to the criteria for defining differentially expressed proteins (fold change ratio ≥ 1.5 and $P \leq 0.05$), the number of down-regulated and up-regulated proteins

are shown in Table 1. This indicates that the TuMV infection changed the proteins to a certain degree, and that the iTRAQ results were reliable and worthy of being investigated further.

Fig. 2. Protein identification and function classification of identified proteins. *A* - Pie chart of protein coverage (peptides with confidence ≥ 95 %). *B* - Peptides length distribution.

The identified proteins were sub-categorized into 54 hierarchically-structured *GO* classifications, including 28 in biological process, 11 in cellular component, and 13 in molecular function (Fig. 3). Specifically, 'metabolic processes' (14.68 %) and 'cellular processes' (15.18 %) (biological process), 'cell part' (25.42 %) and 'cell' (24.53 %) (cellular component), 'catalytic activity' (40.24 %) and 'binding' (43.67 %) (molecular function) were highly represented. All of the proteins were also sub-categorized into 24 *COG* classifications, and the *R* (general function prediction only), *O* (posttranslational modification, protein turnover, chaperones) were highly represented (Fig. 4). All 4 419 proteins were sub-categorized into 257 *KEGG* classifications. The top 10 pathway annotations for the identified proteins are shown in Table 1 Suppl.

Most differentially expressed proteins, both up- and down-regulated, were enriched in 'metabolic process and cellular process' (biological process), 'cell and cell part'

Table 1. The number of differentially expressed proteins identified in our experiments.

Sample pairs	TuMV-7/ control	TuMV-14/ TuMV-7	TuMV-14/ control	TuMV-21/ TuMV-7	TuMV-21/ TuMV-14	TuMV-21/ control
Quantified	5941	5942	5934	5942	5940	5939
Up-regulated	180	127	161	83	104	186
Down-regulated	143	113	124	120	149	176
Total different	323	240	285	203	253	362

BIOLOGICAL PROCESS

CELLULAR COMPONENT

MOLECULAR FUNCTION

Fig. 3. Gene ontology classification of all identified proteins.

(cellular component) and ‘binding and catalytic activity’ (molecular function) (Fig. 1 Suppl. - Fig. 6 Suppl.). *KEGG* pathway enrichment analyses were used to uncover the enriched pathways of the significantly altered proteins. Significantly enriched pathways were identified using a *P*-value based on a hypergeometric distribution (Fig. 1 Suppl. - Fig. 6 Suppl.). In summary, we found that

‘Metabolic pathway’ and ‘Biosynthesis of secondary metabolites’ had the greatest percentage in all of the different pairwise comparisons. There were 41.3 % (102), 46.67 % (84), 44.66 % (92), 37.33 % (56), 42.16 % (78), 44.15 % (117) proteins in ‘metabolic pathway’ and 28.74 % (71), 27.78 % (50), 28.16 % (58), 21.33 % (32), 27.57 % (51), 30.57 % (81) in ‘biosynthesis of secondary

metabolites' in TuMV-7/control, TuMV-14/TuMV-7, TuMV-14/control, TuMV-21/TuMV-7, TuMV-21/

TuMV-14, and TuMV-21/control, respectively.

Fig. 4. COG classification of all identified proteins.

We investigated the proteins significantly expressed in TuMV long distance movement and resistance, such as connected with PD, plasma membrane (PM; Lee 2015), Ca^{2+} signalling (Vosolsobě *et al.* 2017), chitinase (Patel and Goyal 2015), and ROS (Agrawal *et al.* 2003, Zhang *et al.* 2008). In this study, 7 proteins (4 up- and 3 down-regulated) were found in ' Ca^{2+} signalling pathway', 130 proteins in 'oxidative phosphorylation', 88 proteins in 'glutathione metabolism' and 56 proteins in 'ascorbate and aldarate metabolism' in TuMV-7/control, TuMV-14/

TuMV-7, TuMV-14/control, TuMV-21/TuMV-7, TuMV-21/TuMV-14 and TuMV-21/control, respectively. Moreover, 96, 75, 74, 74, 86, 93 PD proteins and 98, 77, 75, 78, 88, 96 PM proteins were enriched, respectively, in these comparisons. Additionally, plenty of proteins were found in the vacuole, symplast, cell wall, and apoplast. For 'molecular function', most proteins were distributed among 'peroxidase activity', 'oxidoreductase activity', 'hydrolase activity', 'glutathione transferase activity', and 'chitin binding'. Additionally, 'response to stimulus', 'response to stress', 'hydrogen peroxide metabolic process' and 'oxygen and reactive oxygen species metabolic processes' were highly represented within the major category 'biological process'.

In Fig. 5, the symptoms on plants 21 d after inoculation are shown. In this study, we chose some differentially expressed genes related to virus resistance and long distance movement (Zhang *et al.* 2008, Patel and Goyal 2017, Vosolsobě *et al.* 2017) and ran RT-PCR (<http://brassicadb.org/brad/>)(Table 2 Suppl.). The relative expression (Fig. 6) showed that, in general, the up and down regulation of genes was consistent with the iTRAQ results, further confirming the resistance effect.

Fig. 5. Symptoms of infected non-heading Chinese cabbage on the 21th day after infection (A - non-infected, B - infected).

Discussion

Plasmodesmata serve as the gateways for most plant viruses that pirate and modify the channel to complete the virions spread using their movement proteins. The movement protein (MP) of TMV moves between cells and influences the size-based exclusion limit of PD (Ueki and Citovsky 2011). We found that some host proteins, including pectin methylesterase (PME) (*Bra001547*, PME3) and *Arabidopsis* patellin (PATL) family proteins (*Bra008012*, PATL1), interact with MPs, having certain effects on the long distance movement (Chen *et al.* 2000, Peiro *et al.* 2014). PMEs are cell wall-associated proteins which regulate viral unloading from the phloem (Chen and Citovsky 2003). PMEs can interact with the MP of TMV during the virus long distance movement (Chen *et al.* 2000). Reduced content of PME can cause significant delay of TMV systemic infection (Chen and Citovsky 2003), which fits our RT-PCR results. PATL1 is a member of the phosphatidylinositol transfer proteins family and plays key role in vesicle/membrane trafficking during cell expansion or maturation (Peterman *et al.* 2004, 2006). The expression of PATL1 increased after inoculation in our study. PATLs can restrain movements of alfalfa mosaic virus (AMV) through cells by targeting MPs to PD, resulting in a decrease in the infection's foci sizes (Deom *et al.* 1987). Meanwhile, viral RNA expression increase in PATL knockouts (Peiro *et al.* 2014). Though the

expressions of host proteins changed in our study, it was unsure whether the host proteins were involved in the long distance movement.

Developmentally-regulated PM polypeptide (DREPP) proteins are specific proteins that bind the PM and calcium (Yamada *et al.* 2015). DREPP proteins are specific to plants, with no specific functions identified until now. We found *Bra013450*, which is a DREPP, *Bra020155*, *Bra018970*, *Bra021847* and *Bra027209*, which bound calcium ions, and *Bra026877*, which bound calmodulin. *Arabidopsis* plasma membrane-associated cation binding protein 1 (PCaP1) and protein 2 (PCaP2) can be regulated by Ca^{2+} /calmodulin, performing the function when the proteins are released from the PM (Vosolsobě *et al.* 2017). In this study, the expression of *Bra013450* increased, indicating that the DREPP might be involved in the defense process. Ca^{2+} is considered as the second messenger and calmodulin is a Ca^{2+} -binding protein that can be regulated in response to different stimuli. The up-regulated and down-regulated expression of *Bra020155*, *Bra018970*, *Bra021847*, and *Bra027209* showed that the Ca^{2+} pump balance was upset. Ca^{2+} regulates PM H^+ -ATPase (*Bra011172*, PMA2), which is sensitive to cell expansion and movement, as well as to pH changes induced by stress (Piette *et al.* 2011). Ca^{2+} act as a regulator during the interaction between PM-localized ATP-binding cassette transporter PEN3 (*Bra003527*, PEN3) and myrosinase (*Bra037954*, PEN2). The two interacting proteins are responsible for exporting glucosinolates, tryptophan-derived secondary metabolites (Campe *et al.* 2016). The PEN2 and PEN3 proteins also take part in microbe-associated molecular pattern-triggered callose deposition (Clay *et al.* 2009). The changes in expressions of *Bra011172*, *Bra003527*, and *Bra037954* further confirmed the participation of Ca^{2+} in the immune system.

Callose, a β -glucan polysaccharide, is comprised of β -1,3-linked glucose residues and it is located at the neck region of PD during pathogen attack (Zavaliev *et al.* 2011). We found four genes that had β -1,3-glucanase activities (*Bra007315*, *Bra008667*, *Bra011537*, and *Bra028526*) and two (*Bra003527* and *Bra032004*) that could respond to callose deposition in cell walls. Their expressions were very different, the expressions of *Bra007315*, *Bra008667*, *Bra011537*, and *Bra003527* increased and the expressions of *Bra028526* and *Bra032004* decreased, but their functions remain unknown. The function of PD may be regulated by callose deposition (Xu and Jackson 2010), which forms a barrier blocking the movement and thus restricts viral transmission.

Endopolygalacturonases (PGs) are cell wall-degrading enzymes (Ziaei *et al.* 2016). To counteract PGs activity, plants have produced PG-inhibiting proteins (PGIPs) (*Bra005917*). PGIPs are important defence proteins and

Fig. 6. Heatmap of relative expression level of genes related to TuMV resistance. The red colour represents up-regulated genes and the green represents down-regulated genes.

belong to *R* gene members of the extracellular LRRs, and they are located in cell walls (Di Matteo *et al.* 2003). It is said that overexpressing PGIPs increases resistance and reduce symptoms (Janni *et al.* 2008, Ferrari *et al.* 2003), but the expression of PGIPs decreased in our study.

Chitin serves as the energy source for some bacteria. Chitinases (*Bra004771*) are the enzymes to hydrolyze chitin. The genes *Trichoderma* chitinases confer much higher antifungal capacities than those of other chitinases (Zarinpanjeh *et al.* 2016). Pathogenesis-related proteins (PRs) are one of the strong weapons to fight against stresses. Chitinase (PR-3) and thaumatin-like protein (PR-5) can enhance the resistance capability (Aghazadeh *et al.* 2016). In this work, we identified *Bra001453* (chitinase, PR-3), *Bra004771* (chitinase), *Bra003774* (thaumatin-like protein), and *Bra025730* (pathogenesis-related thaumatin family protein). Thaumatin-like proteins are active in destroying cell membranes and affect glucanase activity (Liu *et al.* 2010). PR5 proteins destroy the lipid bi-layer, creating trans-membrane pores that increase the permeability of the pathogen's PM (Vigers *et al.* 1992). PR-2 proteins (β -1,3-glucanases, *Bra007315*) hydrolyze β -1,3-glucans which form fungal cell walls (Singh *et al.* 2014). PR-4 (*Bra001122*) contains ribonuclease activity and the expression increases under pathogen invasion and causes programmed cell death (Sugawara *et al.* 2016). The genes associated with PRs and chitinase showed up-regulated trend after inoculation except *Bra001453*, indicating TuMV-induced infection will up-regulate the resistance related genes.

Phytoalexins are the important secondary metabolites occurring under biotic stress (Hull *et al.* 2000). CYP83A1 (*Bra032734*), belonging to the glucosinolate pathway, reinforces resistance to the powdery mildew by accumulating the camalexin (Weis *et al.* 2013). CYP83A1 affects the biosynthesis of aliphatic and indole glucosinolates (Liu *et al.* 2016) which are actively released by glucohydrolases when plants facing biotic stress (Schlaeppli and Mauch 2010). The glucosidases (*Bra018969* and *Bra038094*) and myrosinases (*Bra037954*) form an important self-defense system in cruciferous plants. The gene expression difference of

Bra032734, *Bra018969*, *Bra038094*, and *Bra037954* as compared to control showed their involvement in the resistance.

Pathogen attack can induce the hypersensitive response, reactive oxygen species (ROS), cell death, and systemic acquired resistance (Van Breusegem and Dat 2006). ROS accumulation can result in increased callose deposits and decreased cell-to-cell transport (Benitez-Alfonso and Jackson 2009). Peroxidase and ascorbate peroxidase (*Bra034076*, APX2) can turn H_2O_2 into water and O_2 (Asada 1999) to reduce the oxidative damage. The expression of APX1 is low in many plant tissues normally but will be significantly up-regulated when responding to stresses (Zimmermann *et al.* 2004). However, APX2, a second cytosolic APX isozyme, was barely detected in many tissues which fits the expression level in this study. But APX1 and APX2 in *Arabidopsis* will work together to increase the plants resistance ability (Zimmermann *et al.* 2004, Frank *et al.* 2009). Furthermore, the increasing expression of glutathione S-transferase (*Bra000875*) protects against oxidative damage caused by excessive ROS (Sappl *et al.* 2009).

Heat shock proteins (Hsps) are found to be an important host factor in viral infection. HSP90s (*Bra013774*) are ubiquitous molecular chaperones that are involved in cell signalling (Pratt *et al.* 2006), protein degradation (Imai *et al.* 2003), hormone reception (Raman *et al.* 2015), as transcription factors and danger signals (Zhu *et al.* 2016). Small heat shock proteins (sHSPs) (*Bra000703*) are a major family within HSPs with functions resembling that of HSPs (Uversky *et al.* 2005), and sHSPs act as the type of molecular chaperone that contributes to the development of thermotolerance (Waters *et al.* 1996). The sHSP proteins can lead, jointly with HSP27, to cell death when defending the stress attack. We found that the expression of HSP90s (*Bra013774*) and sHSPs (*Bra000703*) in this study decreased. Blocking their function will make the cells less resistant. Some HSP90 inhibitors were proved to be helpful in virus infection (Raman *et al.* 2015). Phosphorylation can regulate HSPs and induce the pathogenesis-related protein genes.

Conclusions

In this project, we determined proteins associated with TuMV long distance movement and TuMV resistance with iTRAQ analysis. The results showed that proteins were enriched significantly in 'metabolic and secondary metabolites pathway', 'plant-pathogen interaction' and 'oxidative phosphorylation' groups. We found that many

proteins were connected with PD and PM, and also many proteins were involved in calcium signalling pathway. We screened 29 genes and explored their functions with RT-PCR. They all showed significantly difference in expression after TuMV inoculation. Our proteomics findings will facilitate the further study of TuMV resistance.

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