Integrated Phloem Sap mRNA and Protein Expression Analysis Reveals Phytoplasma-infection Responses in Mulberry*§

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To gain insight into the response of mulberry to phytoplasma-infection, the expression profiles of mRNAs and proteins in mulberry phloem sap were examined. A total of 955 unigenes and 136 proteins were found to be differentially expressed between the healthy and infected phloem sap. These differentially expressed mRNAs and proteins are involved in signaling, hormone metabolism, stress responses, etc. Interestingly, we found that both the mRNA and protein levels of the major latex protein-like 329 (MuMLPL329) gene were increased in the infected phloem saps. Expression of the MuMLPL329 gene was induced by pathogen inoculation and was responsive to jasmonic acid. Ectopic expression of MuMLPL329 in Arabidopsis enhances transgenic plant resistance to Botrytis cinerea, Pseudomonas syringae pv tomato DC3000 (Pst. DC3000) and phytoplasma. Further analysis revealed that MuMLPL329 can enhance the expression of some defense genes and might be involved in altering flavonoid content resulting in increased resistance of plants to pathogen infection. Finally, the roles of the differentially expressed mRNAs and proteins and the potential molecular mechanisms of their changes were discussed. It was likely that the phytoplasma-responsive mRNAs and proteins in the phloem saps were involved in multiple pathways of mulberry responses to phytoplasma-infection, and their changes may be partially responsible for some symptoms in the phytoplasma-infected plants. Molecular & Cellular Proteomics 17: 1702–1719, 2018. DOI: 10.1074/mcp.RA118.000670.

Information exchange between cells and tissues to coordinate responses to environmental changes, development, and pathogen defense is required for plant viability (1), and higher plants have evolved different strategies to allow efficient intercellular exchange of information (2). The phloem network represents a well-developed long-distance translocation system for both nutrients and systemic signals (3). Despite the importance of phloem, the mechanisms involved in phloem transport and signaling remain unknown, and there is little information reported about the way in which its function is regulated (4–5). It has been established that there are many mRNA transcripts in the phloem translocation stream, and plants can utilize the mobile RNAs to regulate development and responses to biotic and abiotic stresses at the whole-plant level (6–11). Compared with the number of mRNA transcripts identified, a limited number of proteins have been identified because phloem sap sample is difficult to obtain in sufficient quantities in most plant species (12–13). Interestingly, a large proportion of RNAs and proteins identified in plant phloem sap were predicted to be associated with stress and defense responses, although their exact physiological functions were unclear (14–16). However, a comprehensive understanding of the mechanisms involved in the translocation of RNAs and proteins and the underlying cellular processes taking place within the phloem sap is still lacking. Therefore, it is important to identify and characterize a more comprehensive set of phloem sap RNAs and proteins that may be candidates that have significance regarding functional phloem and whole plant physiology.

Mulberry yellow dwarf disease is one of the most devastating diseases of mulberry (Morus spp.) caused by phytoplasma (17). Because phytoplasmas are difficult to culture in vitro, the underlying molecular mechanisms of their pathogenicity are still poorly understood (18). In the process of plant-pathogen interaction, plants not only can perceive the pathogen invasion and initiate local defense responses but also can transmit the signal of pathogen infection over long distances and activate a multicomponent response at the whole-plant level (19). Because phytoplasmas are strictly confined to the phloem compartment and contact with the sieve element (SE) contents directly (20), the influence of phytoplasma infection on phloem sap protein and RNA composition is easily conceivable. Identification and characterization of the responsive RNAs and proteins present in the phloem sap are a prerequisite to understanding the molecular mechanisms involved.
in the disease symptom development. In recent years, genomic and proteomic strategies have been successfully used to analyze plant-phytoplasma interactions, and many genes and proteins regulated following infection of the phytoplasma have been recognized in several plant species (21–25). However, as far as we know, very little information is available on phloem sap RNAs and proteins associated with phytoplasma infection.

In the present study, high-throughput transcriptomic and iTRAQ1 proteomic approaches were combined to profile the phloem sap mRNAs and proteins involved in the response of mulberry to phytoplasma infection, and the differentially expressed genes and proteins were identified and their functions were discussed. Moreover, one of the differentially expressed gene, major latex protein-like 329 (MuMLPL329) was cloned and its functions were analyzed. Our results demonstrate that MuMLPL329 acts as a positive regulator participating in plant defense response. The information provided will help better understand the plant-phytoplasma interactions and shed light on the underlying molecular mechanisms of phytoplasma pathogenicity.

EXPERIMENTAL PROCEDURES

Plant Materials—Dormant hardwood stem branches of the same tree, Husang 32 (M. multicaulis Perr.), were cut into 17-cm cuttings and incubated in a growth chamber at 26 °C, humidity 90% and under 12 h of light. In the next summer, the cutting seedlings were inoculated with phytoplasma by being grafted with the scions collected from phytoplasma-infected mulberry trees (Husang 32), and the seedlings grafted with the scions collected from healthy mulberry trees were used as controls. Six weeks after inoculation, the plants showing typical symptoms as yellowing of the leaves, stunting, and witches'-broom were used to detect phytoplasma by the PCR assay with an amplified fragment of the 16S rRNA gene of phytoplasma (GenBank Accession No. EF532410) as described previously (21). The plants showing positive symptoms and the controls were used to collect phloem saps.

Phloem Sap Sampling—Phloem sap was collected from infected and healthy mulberry plants using the shoot exudation method (26) with modifications. Briefly, the shoot was cut with a sterile razor blade between the fourth and sixth leaves from the top of the grafted shoots, and the first droplet was discarded, and the cut surface was blotted with sterile filter paper (3 mm; Whatman, Maidstone, UK) several times to avoid contamination. Exuding phloem saps thereafter were collected using sterile micropipette tips (200 μl) and stored immediately at −80 °C.

Library Construction and Sequencing—The gene expression libraries were prepared using the Illumina Gene Expression Sample Preparation Kit (Illumina San Diego, CA) according to the manufacturer’s instructions. Briefly, total RNA was isolated from phloem sap samples using TRIzol® reagent (Invitrogen, CA) following the manufacturer’s instructions, and mRNA was isolated from the total RNA using magnetic oligo (dT) beads, and the ds-cDNAs were digested by the restriction enzymes Nla III and Mme I. After ligation with sequencing adaptors, PCR was performed, and the products were purified by 6% polyacrylamide Tris borate-EDTA gel. The tag libraries constructed were deep-sequenced on the Illumina sequencing platform (GAII) (Illumina).

Differential Expression Analyses of mRNAs—The raw sequence output data were processed using the Illumina pipeline, and “Clean Tags” were obtained by filtering and removing the 3’ adapter sequences, adaptor-only tags, and low-quality tags. The clean reads obtained were then aligned to our in-house mulberry transcriptome dataset, and the ambiguous tags with multiple hits were excluded. The number of annotated clean tags for each gene was then normalized by RESM based algorithm using perl scripts in the Trinity package (v2013–02–25) (27) to obtain the number of transcripts per million clean tags (TPM). The fold change and p value were calculated from the normalized expression, and the false discovery rate (FDR) was applied to determine the threshold of the p value in multiple tests and analyses. An “FDR < 0.01 and the absolute value of log2 Ratio > 1” was used as the threshold to judge the significance of gene expression difference.

Protein Preparation and Isobaric Labeling—Aliquots of 300 μl of phloem sap were collected from diseased and healthy plants, and were expelled into 700 μl of cold trichloroacetic acid/acetone and precipitated overnight at −20 °C. The precipitated proteins were collected by centrifugation for 30 min at 40,000 × g at 4 °C and washed with acetone, after which they were air dried. The dried powder was resuspended in lysis buffer at 25 °C for 1.5 h, and then was centrifuged at 25,000 × g for 1 h at 15 °C. Protein was purified using the Clean Up Kit (GE Healthcare, Uppsala, Sweden) following the manufacturer’s instructions. The cleaned samples were dissolved in 0.1% sodium dodecyl sulfate containing 500 mM triethylammonium bicarbonate and then reduced, alkylated and trypsin-digested before labeling with an 8-plex iTRAQ Reagents Kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions.

LC-MS/MS Analysis—After being labeled, the samples were mixed, dried, resuspended, and then loaded on a MacroSpin Vydac C18 reverse-phase minicolumn (Nestgroup Inc., Southborough, MA, USA). After washing and elution, the samples were dried down and fractionated using a strong cation-exchange (SCX) column. Mobile phase A (25% ACN, 10 mmol/L KH2PO4) and mobile phase B (25% ACN, 2 M KCl, 10 mmol/L KH2PO4) were selected. Peptides were eluted at a flow rate of 200 μl/min with a linear gradient of 0–5% solvent B for 30 min and then 30–50% solvent B for 30 min, maintained for 10 min, and then ramped up to 100% solvent B in 5 min, after which they were held for 10 min. The absorbance at 280 nm was monitored: a total of 20 fractions were collected and pooled into 10 fractions. Each SCX fraction was lyophilized and redissolved in solvent [5% (v/v) acetonitrile, 0.1% (v/v) acetic acid] plus 0.01% trifluoroacetic acid. The peptides were loaded onto a C18 capillary trap cartridge (LC Packings) and then separated on a 15-cm nanoflow C18 column at a flow rate of 200 nL/min with a Proxeon EASY-nLC system (Odense, Denmark). Mobile phase A (0.1% formic acid in water) and mobile phase B [100% acetonitrile, 0.1% formic acid (v/v)] were selected. The peptides were eluted from the HPLC column by a linear gradient as follows: 5% solvent B for 10 min, 5–30% solvent B for 30 min, 30–60% solvent B for 5 min, 60–80% solvent B for 5 min and holding for 5 min, and 80–5% solvent B for 5 min, after which they were maintained at 5% solvent B for 5 min. Flow from the column was directed to a Q-Exactive quadrupole Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) operating in positive ion mode.

1 The abbreviations used are: iTRAQ, isobaric tags for relative and absolute quantification; ACN, acetonitrile; FDR, false discovery rate; CFU, colony-forming units; EGFP, enhanced green fluorescent protein; GO, gene ontology; LC-MS/MS, liquid chromatography tandem mass spectrometry; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time PCR; RT-PCR, reverse transcription PCR; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis. TAIL-PCR, thermal asymmetric interlaced PCR; TAIR, the Arabidopsis information resource.
Precursors with a mass range of 300–2000 m/z and a calculated charge of +2 to +4 were selected for fragmentation. For each MS spectrum, a maximum of three of the most abundant peptides above 5-count threshold were selected for MS/MS. Each selected precursor ion was dynamically excluded for 30 s with a mass tolerance of 0.03 Da. The fragment intensity multiplier was set to 20, and the maximum accumulation time was 2 s.

**MS Spectra and Differential Expression Analysis of Proteins**—The MS/MS data were analyzed by a thorough search considering biological modifications against the protein database deduced using the GENScan software from our mulberry transcriptome using the Paragon algorithm in ProteinPilot software version 2.0.1. Briefly, fixed modification of methionine and cysteine, iTRAQ modification of free amine at the N terminus and lysine, variable modification of oxidation on methionine, and two misscleavages of trypsin digestion were considered. The mass tolerance for both MS and MS/MS was 0.2 Da. A concatenated target-decoy database search strategy was also employed to estimate the FDR. FDR was calculated as the 2-fold percentage of decoy matches divided by the total matches, and FDR of the reported iTRAQ data set was <1%. The ProteinPilot software employed the peak area of iTRAQ reporters to quantify the abundance ratio. The list of proteins with significant expression changes were calculated using Pro Group (Applied Biosystems). The mass spectrometry proteomics raw data and identifying information have been deposited to PeptideAtlas (http://www.peptideatlas.org/) with the data set identifier PASS00997.

**Bioinformatics**—BlastN searches against the reference Arabidopsis thaliana database downloaded from TAIR (http://www.arabidopsis.org/; release 10) were used to provide gene ontologies for the genes and proteins identified. GO analysis was performed for BLAST-matched Arabidopsis accession entries of the target genes based on their TAIR GO categories, and the assignment of functional terms was supported by Blast2GO. MapMan software (http://gabi.rzpd.de/projects/MapMan) was used to provide a graphical overview of the metabolic and regulatory pathways for the detected genes as described by Gao et al. (28).

**RNA Gel Blotting**—Total RNA extracted was separated on 1% (v/v) formaldehyde denaturing agarose gel and then was blotted onto a nylon Hybond N membrane. The blots were hybridized with digoxigenin-labeled RNA probes which were complementary to the genes and prepared using the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany). The probes used are given in the supplemental Table S3. Prehybridization, hybridization, membrane washing, and detection were performed according to the procedure described by Umezawa et al. (29).

**Protein Gel Blot Analysis**—Prepared protein was mixed with 5 × SDS-PAGE sample buffer. Samples were heated at 95 °C for 3 min and loaded on 12% (v/v) SDS-polyacrylamide gels. After electrophoresis, proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes. Western blot analysis was performed according to a previously described method (30) using the horseradish peroxidase (HRP)-conjugated polyclonal anti-Rubisco or anti-MuMLPL329 protein polyclonal antibody which were generated by immunizing rabbits with the purified proteins.

**qRT-PCR Analysis**—RNA was extracted using the TRIzol® reagent (Invitrogen) and digested with DNase I. qRT-PCR was performed using the CFX96TM Real-time System (Bio-Rad, CA) with the SYBR Premix Ex Taq™ kit from TaKaRa according to the manufacturer’s protocol. The EF1-α or actin gene was amplified as a reference gene. The relative gene expression was evaluated using the comparative cycle threshold (Ct) method (31). All samples were assayed in triplicate. The primers used for qRT-PCR are given in the supplemental Table S2.

**Gene Cloning**—RNA was isolated from leaves of *M. multicaulis* using TRIzol® reagent (Invitrogen) and digested with DNase I and used to synthesize cDNA with 100 units of reverse transcriptase M-MLV (Promega, Madison, WI) in 20 ml reactions. The specific oligonucleotide primers were designed based on our available mulberry transcriptome data for PCR amplifications, and the DNA fragment obtained from RT-PCR was subcloned individually into the pMD18-T vector (Invitrogen). After transformation, positive clones were selected and further sequenced. The primers used for RT-PCR are given in the supplemental Table S3.

**Sequence and Phylogenetic Analysis**—The deduced amino acid sequences were aligned using DNAMAN software (version 6.0). The theoretical isoelectric point (pI) was calculated using pi/Mw tool within the ExPaSy Proteomics Server (http://www.ca.expasy.org/tools/pi_tool.html), and structural prediction was performed with SWISS-MODEL tools (http://www.swissmodel.expasy.org/). The neighboring joining method was used to produce the phylogenetic tree using the MEGA program. Bootstraping was performed 1000 times to obtain support values for each branch.

**Production of Transgenic Plant Lines**—The coding region of the MuMLPL329 gene was amplified from pMD18-MuMLPL329 plasmid DNA with the sense primer containing an XbaI sequence at the 5′ end, and the antisense primers containing a SacI sequence at the 3′ end, and the amplified DNA was integrated into pMD18-T vector. The primers used for RT-PCR are given in the supplemental Table S3. The positive plasmid was digested with XbaI and SacI, and the products were analyzed by agarose gel electrophoresis. The DNA fragment of ~450 bp was recovered and subcloned into binary plasmid vector pBI121 (digested with XbaI and SacI) under the control of the 35S promoter. Then, the construct was introduced into wild-type Arabidopsis plants through an Agrobacterium tumefaciens-mediated (strain GV3101) T-DNA transformation with the floral dip method. After transformation, the T1 seeds were sterilized and plated on kanamycin selection plates (MS media supplemented with 50 mg/ml kanamycin) to select transformed plants.

**Determination of MuMLPL329 Subcellular Localization**—The cDNA fragment of MuMLPL329 was cloned into the binary plasmid vector pROKII-EGFP under the control of 35S to produce 35S::MuMLPL329-EGFP expression vector. Mesophyll protoplasts from Arabidopsis thaliana were isolated according to the method described previously (32) and transformed with the 35S::MuMLPL329-EGFP construct. After 12 h of incubation at 28 °C, fluorescence images were acquired with a Bio-Rad MRC1024 confocal laser scanning microscope (Bio-Rad Microscience).

**Plant Treatment**—Mulberry and Nicotiana benthamiana seedlings used in the experiment were planted in a growth chamber at 26 °C, 90% RH, and 12 h of light. The salicylic acid (SA) and jasmonate (JA) treatments were achieved by spraying the leaves with 5 mmol/L SA or 100 mmol/L JA solution. The control plants were sprayed with distilled water. Mulberry seedlings were inoculated with *Pseudomonas syringae* pv. *mori* by brushing the bacterial suspension (106 CFU/ml) onto the abaxial surfaces of young leaves. For *Colletotrichum dematium* inoculation, a filter paper disc (8 mm in diameter) soaked in conidial suspension (2.5 × 107 conidia/ml) of *C. dematium* was placed on the adaxial surface of the young leaves. Inoculated mulberry plants were covered with polyethylene bags for 48 h after inoculation. *N. benthamiana* seedlings were inoculated with *Pst. DC3000* by injecting 50 μl of *Pst. DC3000* (107 CFU/ml) bacterial suspensions into a leaf with a syringe, and inoculated with *Botrytis cinerea* by placing 5-μl droplets of a spore suspension (2 × 106 conidia/ml) in 24/2 mL potato dextrose broth on the leaves. Inoculated plants were covered with a transparent plastic lid to maintain high humidity.

**Detection of Resistance Against Pathogens**—The MuMLPL329 gene was ligated to pBI121 vector and then introduced into GV3101
under the control of the \(^{35}\text{S}\) promoter. The wild type Arabidopsis plants were transformed, and 4-week-old transgenic Arabidopsis plants were used for the resistance analysis. Inoculation with \(Pst.\) DC3000 was conducted by injecting 50 \(\mu\)l of \(Pst.\) DC3000 (10\(^{5}\) CFU/ml) bacterial suspensions or 10 mmol/L MgCl\(_2\) (mock) into the rosette leaves with a syringe. To quantify the bacterial growth within the leaves, the inoculated leaves were ground in 1000 \(\mu\)l of sterile water, serially diluted 1/10 with sterile water and plated on King’s B agar medium. Plates were placed at 28 °C for 2 days, after which the colony-forming units were counted. Each treatment was conducted independently at least three times. Inoculation with \(B.\) cinerea was performed as described above. Inoculated plants were covered with a transparent plastic lid to maintain high humidity and incubated at 22 °C with a 12-h photoperiod. The disease incidence and disease severity were examined daily after inoculation.

Inoculation of Arabidopsis with phytoplasma was performed following the method described before (20). Simply, to obtain phytoplasma-infected leafhoppers, leafhoppers (\(Hishmonus\) sellatus) were transferred to phytoplasma-infected mulberry for 2 weeks to allow oviposition and the hatched nymphs were kept on the infected plants until adulthood. Five adult leafhoppers were transferred to Arabidopsis seedlings which were at the stage of 4 to 5 rosette leaves and covered with clear tubes and then removed after 5 days. Plants infected with uninfected leafhoppers were used as controls. The presence of phytoplasma in the Arabidopsis samples was determined using qRT-PCR described previously (33). The primers and TaqMan probes (Forward primer 5' CGTACGGGAATGTAGAATTAAAGAA; Reverse primer 5' TCTTGGATTAACACGATGTTCA 3'; Probe 5' TGACGGGACTCCGCCACAAGCG 3') were used to amplify the 16S rRNA gene of phytoplasma. The primers and TaqMan probes (Forward primer 5' GACTAGCTCCCGCCGAGAGG 3'; Reverse primer 5' AACACTTACCGCAGCAGCACA 3'; Probe 5' ACGACCGCCGCTCGCTTC 3') were used to amplify the 18S rRNA gene of Arabidopsis. Normalization of phytoplasma quantities was performed by assays for plant 18S rDNA from each sample, and the number of phytoplasma cells per microgram of plant DNA was obtained by dividing the phytoplasma amount by the amount of DNA in each sample. In each qRT-PCR plate, water control was also included. All the samples were run in triplicate.

Promoter Analysis—To obtain the promoter sequence of \(MuM\) LPL329, chromosome walking was performed using the TAIL-PCR method. Three specific primers SP1, SP2 and SP3 were designed based on the \(MuM\) LPL329 sequence, and four arbitrary primers LAD-1, LAD-2, LAD-3, and LAD-4 and the AC1 primer complementary to the adaptor sequence within the LAD primers were designed according to the method described before (34). All the primers used are given in the supplemental Table S3. Mulberry genomic DNA was isolated using CTAB (cetyltrimethyl-ammonium bromide) method (35) and used to preamplification using LAD and SP1 primers. In the primary TAIL-PCR, the amplification product was used as the template and the primer pairs AC1 and SP2 were used. In the secondary TAIL-PCR, the primary TAIL-PCR product was used as the template and the primer pairs AC1 and SP3 were used. The secondary TAIL-PCR amplified products were fractionated on an agarose gel, and the interest bands were isolated and sequenced. The potential cis-regulatory elements within the sequence were analyzed with the PlantCARE software (http://bioinformatica.psb.ugent.be/webtools/plantcare/html/). The promoter was cloned into the vector pBl121 to replace the \(^{35}\text{S}\) promoter and fused to the \(\beta\)-glucuronidase (GUS) reporter gene to create the promoter expression vector pMLP329::GUS which was then introduced into GV3101. For transient expression, tobacco leaves were infiltrated with transformed \(Agrobacterium\) according to the method described by Arpat et al. (36). For stable expression, Arabidopsis was transformed using the floral dipping method. Histochemical staining for GUS activity was performed as described by Jefferson et al. (37).

Determination of Flavonoid Content—Leaves sampled were dried to constant weight and then were ground to a powder. Leaf powder was extracted with 70% (v/v) ethanol and the extract was filtered. Total flavonoid content was measured with a NaNO\(_2\)-Al(NO\(_3\))\(_3\) method as described by Zhang et al. (38).

Experimental Design and Statistical Rationale—For the proteomic analysis, we obtained 3 biological replicates with healthy and infected phloem sap used for iTRAQ-based mass spectrometry analysis. For each of the 3 biological replicates, we performed three LC-MS/MS experiments as technical replicate. For gene expression and other biochemical, functional and quantitative assays, all the experiments were performed with at least three biological replicates, and the significance of the results was analyzed afterward via analysis of variance. Differences were considered significant when \(p\) values were less than 0.05.

RESULTS

Purity Assessing of Phloem Sap—To assess the purity of the sampled phloem sap, the Rubisco subunit which is found in other cell types but not in the SEs, and CmPP16, which is found in the SEs but not in other cell types, were used as specific cellular protein and mRNA markers, respectively, and their frequency in the samples was determined. The Western blotting results showed that the large subunit of Rubisco was not detected in the phloem sap sampled from either the healthy or the infected tissues, but it was clearly present in leaf tissue samples free of major veins (Fig. 1A). The purity of the collected phloem sap samples was further confirmed by Northern blotting experiments using a Rubisco large subunit RNA specific probe and a phloem-specific CmPP16 RNA probe. The results showed that Rubisco large subunit RNA were present in the leaf tissue samples but absent in the phloem sap samples (Fig. 1B). In contrast, the phloem-specific CmPP16 RNA was detected in the phloem sap samples but barely detectable in the leaf tissue samples (Fig. 1C). Therefore, the contamination from surrounding tissues in the phloem sap samples was very low.

Gene Differential Expression Analysis Between Phytoplasma-infected and Healthy Mulberry Phloem Sap Libraries—After discarding low-quality and single-copy tags, totals of 3392 293 and 3421 516 clean tags remained were obtained in the infected phloem sap (IPS) and healthy phloem sap (HPS) libraries, respectively. Differences between the tag frequencies that appeared in the IPS and HPS libraries were significantly in the IPS library (\(p < 0.05\), fold-change > 2.0). The top 20 up- and downregulated genes were given in Table I, and the unigenes detected with at least 2-fold-change differences in the two libraries are shown in supplemental Table S4.

To validate the Solexa expression profiles, qRT-PCR analysis for 14 individual unigenes covering different expression
patterns were performed (Fig. 2). Even though, the different scales of these unigenes detected by qRT-PCR analysis not in accordance with those detected by the Solexa sequencing, there was a very strong correlation between the PCR results and the Solexa-sequencing results, indicating that the profiles of these unigenes detected by Illumina sequencing are reliable.

To better understand the functions of these differentially expressed unigenes, the comprehensive tool MapMan was used to visualize the pathways affected by phytoplasma-infection in mulberry phloem saps. In the category “metabolism,” primary metabolic pathways like TCA, lipid metabolism, carbohydrate (CHO) metabolism, as well as cell wall was changed. In addition secondary metabolisms in the terpene, flavonoid, phenylpropanoid, and amino acid metabolism pathways were also changed (Fig. 3A). This indicated that the metabolism in mulberry phloem saps was affected by phytoplasma-infection. Next to the category “regulation,” differentially expressed unigenes are attributed to the bins for protein degradation and modification, redox, regulation of transcription, receptor kinases, G-proteins, MAP kinases, and phosphoinositides. Specifically it was showed that some unigenes are attributed to the bins for the metabolism of hormone such as abscisic acid (ABA), indole acetic acid (IAA), cytokinins (CK), gibberellic acid (GA), 6-benzyladenine (6-BA), JA, SA, etc. This suggested that the regulation pathways and hormone-related crosstalk in mulberry phloem saps were profoundly affected by phytoplasma-infection (Fig. 3B). When focusing on the category “biotic stress,” it was revealed that the unigenes involved in hormone metabolism, and some transcription factors and defense stress-related genes were significant changes in the phytoplasma-infected phloem saps (Fig. 3C). Therefore, these differentially expressed unigenes were related to a variety of biologic processes, and the regulatory networks of the sap mRNAs involved in the response to phytoplasma-infection are intricate.

Identification and Quantification of Differentially Expressed Proteins Between Phytoplasma-infected and Healthy Mulberry Phloem Saps—The protein database deduced from our mulberry transcriptome was used to identify phloem sap proteins, and there were 739 proteins were identified and quantified, and 136 proteins were differentially expressed proteins (p < 0.05, fold-change > 2.0) between infected and healthy phloem saps. A total of 96 proteins increased, whereas 40 proteins decreased in the infected phloem sap compared with the healthy sap (supplemental Table S5). The top 20 up- and down-regulated proteins were given in Table II.

The GO analysis of the differentially expressed proteins was performed, and these proteins were classified into 14 functional categories (Fig. 4). The first category of proteins was involved in carbohydrate transport and metabolism (15%), and the second category included proteins associated with protein posttranslational modification and chaperones. The proteins associated with stress-related and defense mechanisms belong to the third category, and all the proteins, except for three in this category, were upregulated in the infected phloem sap. The proteins whose functions are unknown were numerically equal to the third-category proteins. Further analyses of these proteins will likely yield new scientific insights into the phytoplasma-mulberry interactions and reveal the new biological functions of phloem sap. The other differentially expressed proteins belong to categories such as energy production and conversion, transcription and regulation of transcription, secondary metabolite biosynthesis, transport and catabolism, and signal transcription mechanisms, among others. Therefore, the differentially expressed proteins in the phloem sap may play important roles in diverse biologic processes, and the regulatory networks of proteins involved in the response of mulberry to phytoplasma infection are also intricate.

Correlation of mRNA and Protein Profiles in Response to Phytoplasma Infection—In contrast to the proteome data, which showed that 18.4% of the proteins measured, were differentially expressed, the transcriptomic analysis showed that only 14.1% of transcripts measured were differentially expressed. Among all the differentially expressed genes, only 14 were regulated both at the mRNA and protein levels in the response to phytoplasma infection; moreover, 11 of the 14
TABLE I

Expression profiling of the top 20 up- and downregulated mRNAs in the phloem-saps of infected and healthy mulberry plants

TPM: the number of transcripts per million clean tags. IPS and HPS indicate infected phloem sap and healthy phloem sap, respectively.

| Accession number | Normalized value | Fold-change | P-value | FDR | NCBI reference sequence | Gene |
|-------------------|------------------|-------------|---------|-----|-------------------------|------|
|                   | TPM-IPS          | TPM-HPS     | log2(IPS/HPS) |     |                         |      |
| Unigene20785      | 106.97           | 5.95        | 4.168172767   | 1.36E-79 | XM_010933032.2 | Ethylene-responsive transcription factor |
| Unigene29906      | 11.98            | 0.63        | 4.249132269    | 4.1E-10  | XM_01091438.1 | Hypothetical protein |
| Unigene42851      | 12.28            | 0.63        | 4.284814922    | 2.3E-10  | XM_01012005.1 | E3 ubiquitin-protein ligase |
| Unigene32585      | 160.75           | 7.52        | 4.417942265    | 1.7E-123 | XM_01008443.1 | MLP-like protein 329 |
| Unigene49396      | 15.78            | 0.63        | 4.646601567    | 4.0E-13  | XM_010098707.1 | Protein SRG1 |
| Unigene10088      | 19.29            | 0.63        | 4.936357505    | 7.4E-17  | XM_01008935.1 | Oligopeptidase transporter 3 |
| Unigene12161      | 1150.07          | 35.08       | 5.034929117    | 0         | XM_010112895.1 | Hypothetical protein |
| Unigene8367       | 21.34            | 0.63        | 5.082064337    | 9.0E-19  | XM_010111783.1 | Hypothetical protein |
| Unigene10165      | 9569.73          | 234.44      | 5.351142345    | 0         | XM_010103987.1 | Class I pathogenesis-related protein 1 |
| CL3672.Contig1    | 32.15            | 0.63        | 5.673323099    | 5.0E-29  | 4.06E-27          | Beta-1,4-xylosidase 1 |
| Unigene24644      | 4.09             | 0.01        | 8.675957033    | 0.0000175 | 0.000854239 | ABC transporter A family member 2 |
| CL1323.Contig1    | 4.97             | 0.01        | 8.957102042    | 1.4E-05  | 0.00014982 | G-type lectin S-receptor-like serine/threonine-protein kinase |
| CL5315.Contig1    | 4.97             | 0.01        | 8.957102042    | 1.4E-05  | 0.00014942 | Hypothetical protein |
| Unigene13539      | 4.97             | 0.01        | 8.957102042    | 1.4E-05  | 0.00014897 | Hypothetical protein |
| CL1106.Contig1    | 5.26             | 0.01        | 9.038918989    | 7.2E-06  | 8.20E-05 | Beta-1,4-xylosidase 1 |
| CL5409.Contig1    | 5.85             | 0.01        | 9.19229814     | 9.5E-06  | 2.52E-05 | Beta-1,4-xylosidase 1 |
| CL5043.Contig1    | 6.14             | 0.01        | 9.26209485     | 1.0E-06  | 1.40E-05 | Beta-1,4-xylosidase 1 |
| CL248.Contig1     | 9.06             | 0.01        | 9.82336724     | 1.3E-09  | 3.04E-08 | Beta-1,4-xylosidase 1 |
| Unigene11174      | 16.95            | 0.01        | 10.72709656    | 2.59E-17 | 1.17E-15 | Beta-1,4-xylosidase 1 |
| Unigene14964      | 24.26            | 0.01        | 11.24436384    | 1.8E-24  | 1.23E-22 | Beta-1,4-xylosidase 1 |
| CL6965.Contig1    | 1.17             | 10.02       | -3.098302074   | 3.85E-07 | 5.85E-06 | Beta-1,4-xylosidase 1 |
| Unigene19789      | 11.69            | 106.19      | -3.183030708   | 5.0E-65  | 9.00E-63 | Beta-1,4-xylosidase 1 |
| Unigene46648      | 0.58             | 5.64        | -3.281570357   | 0.00012075 | 0.000990202 | Putative kinase-like protein TMKL1 |
| Unigene47372      | 0.58             | 5.64        | -3.281570357   | 0.00012075 | 0.000990678 | Hypothetical protein |
| Unigene24720      | 0.58             | 5.64        | -3.281570357   | 0.00012075 | 0.00099251 | Hypothetical protein |
| Unigene20112      | 0.58             | 5.64        | -3.281570357   | 0.00012075 | 0.000991155 | Hypothetical protein |
| Unigene7637       | 4.09             | 41.04       | -3.326876783   | 4.5E-27  | 3.4E-25 | Hypothetical protein |
| Unigene7215       | 0.58             | 5.95        | -3.358764863   | 6.3E-05  | 0.000564692 | Hypothetical protein |
| Unigene13877      | 1.46             | 15.98       | -3.452227134   | 1.2E-11  | 3.42E-10 | Hypothetical protein |
| Unigene88874      | 0.58             | 6.89        | -3.57037178    | 9.1E-06  | 0.000109392 | Hypothetical protein |
| Unigene39990      | 1.17             | 15.04       | -3.68424132     | 1.4E-11  | 4.02E-10 | Hypothetical protein |
| Unigene30729      | 0.58             | 7.83        | -3.75488752     | 1.2E-06  | 1.74E-05 | Hypothetical protein |
| Unigene46083      | 1.75             | 24.43       | -3.803227036    | 1.8E-18  | 8.99E-17 | Hypothetical protein |
| Unigene49859      | 0.58             | 10.02       | -4.11068798     | 1.22E-08 | 2.33E-07 | Hypothetical protein |
| Unigene39016      | 0.58             | 11.9        | -4.358764863    | 2.1E-10  | 5.17E-09 | Hypothetical protein |
| Unigene62753      | 0.58             | 12.22       | -4.397047575    | 1.0E-10  | 2.72E-09 | Hypothetical protein |
| CL6342.Contig1    | 1.17             | 26.31       | -4.49103084     | 4.7E-22  | 2.84E-20 | Hypothetical protein |
| Unigene25713      | 0.58             | 16.6        | -4.83896531     | 7.0E-15  | 2.69E-13 | Hypothetical protein |
| Unigene20379      | 0.01             | 5.01        | -8.96666679     | 8.3E-06  | 9.27E-05 | Hypothetical protein |
| Unigene21125      | 0.01             | 15.98       | -10.64205169    | 7.0E-17  | 3.10E-15 | Hypothetical protein |

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genes were changed with the same trend. However, there were three genes, CL6661.Contig1 (heat shock 70-kDa protein), Unigene20103 (ATP synthase subunit delta) and Unigene8723 (GDSL esterase/lipase) that were regulated with the opposite trend at the protein and mRNA levels (supplemental Table S4 and supplemental Table S5). Therefore, integrative transcriptomic and proteomic analysis of the phloem sap would provide more information for the identification of genes involved in the biological response of mulberry to phytoplasma infection.

Identification and Characterization of the Phytoplasma-responsive MLP-like Protein 329 Gene—Integrated analysis showed that the Unigene32585, which was annotated as MLP-like protein 329 gene, was increased at the protein and mRNA levels in the phytoplasma-infected phloem sap, and this result was confirmed by Northern blotting and Western blotting analyses (Fig. 5). This indicates that the Unigene32585 gene may have important roles in the response of mulberry to phytoplasma infection.

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Identification and Characterization of the Phytoplasma-responsive MLP-like Protein 329 Gene—Integrated analysis showed that the Unigene32585, which was annotated as MLP-like protein 329 gene, was increased at the protein and mRNA levels in the phytoplasma-infected phloem sap, and this result was confirmed by Northern blotting and Western blotting analyses (Fig. 5). This indicates that the Unigene32585 gene may have important roles in the response of mulberry to phytoplasma infection. However, MLP-like protein 329 gene was previously unrecognized related with phytoplasma infection. To examine the potential role of the gene in mulberry response to phytoplasma infection, the gene was cloned and a full length encoding cDNA of 456 bp was obtained, which encoded a protein 151 AA residues in length with a predicted size of 1.73 kDa and pI of 5.50 (GenBank accession MG871460). Putative conserved domain of the protein was detected and the results showed that it has a Bet v1-like domain which is primarily found in major latex protein-like (MLP-like) proteins. However, its homology to other subfamily proteins from the Bet v1 family is low (Fig. 6). SWISS-MODEL predictions showed that the structural properties of Unigene32585 gene were similar to other Bet v1 family proteins, which contain a Y-shaped hydrophobic cavity formed by seven antiparallel β-sheets and three α-helices (Fig. 7A). Phylogenetic analysis of this gene and those from other plants was conducted, and the result showed that it was closest to the MLP-like protein 329 gene from M. notabilis (Fig. 7B). Therefore, the Unigene32585 gene was named as MuMLPL329. N-terminal extension prediction suggested that MuMLPL329 contained neither obvious signal peptide (http://www.cbs.dtu.dk/services/SignalP/) nor obvious sublocalization sequence (http://www.cbs.dtu.dk/services/targetP). To clarify its subcellular localization, the coding region of the MuMLPL329 gene was fused in frame to an EGFP at its C terminus and introduced into Arabidopsis mesophyll protoplasts. Fig. 8 shows that MuMLPL329-EGFP fusion proteins were accumulated in both the nucleus and cytoplasm of mesophyll protoplasts.

Expression Profile of MuMLPL329 Gene—To examine the potential role of MuMLPL329 gene in plant defense, the ex-
expression pattern of the gene in various organs of mulberry was examined by qRT-PCR analysis. As shown in Fig. 9A, the gene was ubiquitously expressed in all organs investigated. In stem bark where the phytoplasma are mainly restricted, the abundance of MuMLPL329 transcript was higher than in other organs of mulberry. At the same time, the mulberry seedlings...
### TABLE II
Expression profiling of the top 20 up- and downregulated proteins in the phloem-saps of infected and healthy mulberry plants

IPS and HPS indicate infected phloem sap and healthy phloem sap, respectively.

| Accession number | NCBI reference sequence | Protein                                      | Unique peptides | Coverage | Ratio (IPS/HPS) | Score  |
|------------------|-------------------------|----------------------------------------------|-----------------|----------|----------------|--------|
| Unigene21659     | XP_010090235.1          | Glucan endo-1,3-beta-glucosidase, basic isof | 2               | 8.93     | 4.52           | 150.94 |
| Unigene32585     | XP_010086735.1          | MLP-like protein 329                         | 3               | 12.65    | 4.67           | 141.57 |
| Unigene21662     | XP_018860176.1          | Major allergen Pru ar 1-like                 | 2               | 32.77    | 4.69           | 775.93 |
| Unigene8504      | XP_010061850.1          | Hypothetical protein L484_003561             | 4               | 16.22    | 4.95           | 196.91 |
| Unigene8789      | XP_010113258.1          | Osmotin-like protein OSM34                   | 3               | 10.44    | 4.98           | 168.08 |
| Unigene8544      | XP_010111407.1          | Polygalacturonase inhibitor                  | 2               | 12.62    | 5.62           | 143.73 |
| CL4903.Contig1   | XP_015875040.1          | Cyanate hydratase                            | 2               | 11.59    | 6.17           | 149.17 |
| Unigene13369     | XP_010108295.1          | Aspartic proteinase nepenthesin-2            | 3               | 6.99     | 6.67           | 129.29 |
| Unigene20170     | XP_010093996.1          | Peroxiredoxin-2F                             | 7               | 43.28    | 6.67           | 442.99 |
| Unigene25584     | XP_010112068.1          | Hypothetical protein L484_012654             | 3               | 28.7     | 7.04           | 142.68 |
| Unigene9251      | XP_010091501.1          | Protein SET                                  | 3               | 8.13     | 7.04           | 152.25 |
| Unigene19947     | XP_010092082.1          | Reversibly glycosylated polypeptide          | 4               | 15.20    | 7.46           | 145.48 |
| Unigene20031     | XP_010094136.1          | Hypothetical protein L484_017174             | 2               | 9.76     | 8.33           | 159.19 |
| Unigene20600     | XP_010101135.1          | Hypothetical protein L484_016699             | 3               | 19.11    | 8.40           | 353.73 |
| CL791.Contig1    | XP_010099272.1          | Putative serine/threonine-protein kinase     | 3               | 12.72    | 8.77           | 135.42 |
| Unigene10165     | XP_010102289.1          | Class I pathogensis-related protein 1        | 3               | 18.57    | 13.08          | 116.46 |
| Unigene24812     | XP_010112042.1          | DNA-damage-repair/tolerance protein          | 3               | 36.30    | 13.33          | 143.18 |
| Unigene10295     | XP_010111995.1          | Subtilisin-like protease                     | 2               | 10.73    | 18.52          | 88.96  |
| Unigene9416      | XP_010091162.1          | Translation initiation factor IF-3           | 3               | 8.64     | 27.03          | 199.43 |
| Unigene29599     | XP_010085507.1          | Lysine-specific histone demethylase 1-1-like protein | 3   | 18.48    | 40.00        | 200.21 |
| Unigene19796     | XP_010100818.1          | Hypothetical protein L484_015849             | 4               | 54.88    | 0.39           | 539.75 |
| CL5636.Contig1   | XP_010090797.1          | Glutathione S-transferase                    | 2               | 23.47    | 0.39           | 166.78 |
| Unigene19678     | XP_010096098.1          | Protein gmpE                                 | 5               | 12.39    | 0.39           | 168.55 |
| Unigene20435     | XP_010097229.1          | Methyl-CpG-binding domain-containing protein 10 | 4   | 25.42    | 0.37          | 218.69 |
| Unigene19797     | XP_010103758.1          | Hypothetical protein L484_014647             | 10              | 45.3     | 0.37           | 739.91 |
| Unigene2636      | XP_010096321.1          | Hypothetical protein L484_021067             | 3               | 15.72    | 0.36           | 203.56 |
| Unigene20727     | XP_010110465.1          | Hypothetical protein L484_001864             | 2               | 11.49    | 0.36           | 153.68 |
| Unigene20445     | XP_010090161.1          | Thiol protease aleurain-like protein          | 2               | 9.34     | 0.36           | 164.81 |
| Unigene44735     | XP_010100567.1          | Polyphenol oxidase                           | 2               | 10.94    | 0.36           | 104.22 |
| Unigene21642     | XP_010109780.1          | Hypothetical protein L484_003140             | 2               | 9.04     | 0.36           | 99.46  |
| Unigene20954     | XP_010104628.1          | Peptidyl-prolyl cis-trans isomerase CYP19-4  | 9               | 43.17    | 0.35           | 610.34 |
| Unigene9973      | XP_010100791.1          | FK506-binding protein 2                      | 2               | 15.89    | 0.35           | 220.74 |
| Unigene20573     | XP_010103258.1          | HMG1/2-like protein                          | 2               | 8.46     | 0.35           | 59.73  |
| Unigene21190     | XP_010094556.1          | High mobility group B protein 2              | 2               | 13.7     | 0.34           | 165.65 |
| Unigene7884      | XP_010105917.1          | Serine/arginine-rich splicing factor 2       | 3               | 11.02    | 0.33           | 123.3  |
| CL3901.Contig1   | XP_008365661.1          | Predicted: elongation factor 1-alpha         | 6               | 17.59    | 0.31           | 144.48 |
| Unigene20825     | XP_010105174.1          | Glyceraldehyde-3-phosphate dehydrogenase     | 10              | 38.02    | 0.29           | 235.31 |
| Unigene45922     | XP_010106006.1          | Deoxyuridine 5'-triphosphate nucleotidohydrolase | 3   | 23.95    | 0.28           | 129.55 |
| Unigene18925     | XP_010101971.1          | HMG-Y-related protein A                      | 3               | 10.67    | 0.25           | 153.01 |
| CL4112.Contig1   | XP_010108267.1          | Malate dehydrogenase                        | 7               | 26.65    | 0.11           | 443.79 |
were challenged with \textit{P. syringae} pv. \textit{mori} and \textit{C. dematium}, and treated with JA and SA, respectively (Fig. 9B). The induced expression of \textit{MuMLPL329} was verified by qRT-PCR analysis, and the results showed that the expression of \textit{MuMLPL329} was induced after \textit{P. syringae} pv. \textit{mori} and \textit{C. dematium} challenge. Moreover, the expression of \textit{MuMLPL329} was also enhanced by exogenous application of JA. Whereas, there was no remarkable change in the expression level of \textit{MuMLPL329} gene after SA treatment. To further investigate whether \textit{MuMLPL329} was involved in the disease responses, the putative promoter, 2000 bp DNA upstream of the \textit{MuMLPL329} coding region sequence was cloned (designed as \textit{pMuMLPL329}; GenBank accession MH370165) and fused to the reporter gene encoding GUS and transient expression of the GUS gene in tobacco leaves was performed. Staining results showed that GUS driven by \textit{pMuMLPL329} was induced after inoculation of \textit{B. cinerea} or \textit{Pst. DC3000}, meanwhile, GUS activity was enhanced by exogenous application of JA, but not by application of SA (Fig. 10). These results described above indicated that \textit{MuMLPL329} was involved in defense against pathogen infection and its expression may be modulated by different plant hormone signaling in mulberry.

\textbf{Overexpression of \textit{MuMLPL329} Gene Enhances the Disease Tolerance of Transgenic Arabidopsis Plants—Because the efficient genetic transformation system has not been established in mulberry trees, to examine the role of \textit{MuMLPL329} in the defense response against pathogens, transgenic Arabidopsis plants overexpressing \textit{MuMLPL329} were generated. The wild-type plants and transgenic Arabi-}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Distribution percentage of the differentially expressed proteins in various categories.}
\end{figure}
FIG. 6. Multiple sequence alignment between MuMLPL329 and other MLP proteins. Amino acid residues conserved in all proteins were black shaded and similar amino acids were red or blue shaded. The aligned sequences included those MLP-like protein 328 from Arabidopsis (NP 565264.1), MLP-like protein 329 from Arabidopsis (NP 565265.1), MLP-like protein 329 from Prunus persica (XP 007225991.1), MLP-like protein 328 from Malus domestica (XP 009892000.1), MLP-like protein 28 from Malus domestica (XP 009892001.1), MLP-like protein 329 from M. notabilis (XP 010086735.1), MLP-like protein 328 from M. notabilis (XP 010086736.1), MLP-like protein 28 from Juglans regia (XP 018824600.1), MLP-like protein 328 from Manihot esculenta (XP 021593664.1), MLP-like protein 328 from Prunus avium (XP 021823275.1), and MLP-like protein 329 from Cucurbita moschata (XP 022942726.1).

FIG. 7. Proposed 3-D structures of the MuMLPL329 proteins established by homology-based modeling and phylogenetic relationship between MuMLPL329 and MLPs in other plants. A. Three-dimensional model of the MuMLPL329 protein was constructed by SWISS-MODEL. The Y-shaped hydrophobic cavity is formed by a seven-stranded-sheet wrapped around a long C-terminal helix, and is closed at one end by two short helices. B. Phylogenetic analysis was performed using the neighbor-joining method. The scale indicates branch lengths. Bootstrap values below 80 are indicative of low confidence. The accession numbers of the proteins are indicated.
dopsis plants ectopic expression of MuMLPL329 were incubated with the bacterial pathogen Pst. DC3000, respectively. The results showed that there were severe disease symptoms showing gray-brown lesion with chlorosis in the wild-type plants. In contrast, the disease symptoms were not evident in the leaves of transgenic plants, although mild chlorosis or necrosis was occasionally observed (Fig. 11A). Bacterial growth in the leaves was monitored to ascertain whether the lack of symptom development reflected the restriction of bacterial growth and multiplication inside the leaves. The result of detection showed that the CFU of Pst. DC3000 in the leaves of wild-type plants was significantly higher than that in the leaves of transgenic plants (Fig. 11B). Similar experiments were performed with transgenic Arabidopsis plants following inoculation with B. cinerea to examine the role of MuMLPL329 in the defense response to fungal pathogens. Four days after inoculation (dai), dark necrotic lesions and fungal hyphae were observed at the inoculation sites on the leaf surface of wild-type plants, and the beginning of chlorosis was also observed around the inoculation site. However, no disease sign was observed on the leaves of MuMLPL329-overexpressing plants at 4 dai (Fig. 11C). Therefore, the results presented above indicate that MuMLPL329-overexpressing in Arabidopsis enhances plant resistance to B. cinerea and Pst. DC3000.

Because phytoplasmas are difficult to culture in vitro, to explore whether plant resistance to phytoplasma can also be enhanced by MuMLPL329, wild-type and MuMLPL329-overexpressing plants inoculated with phytoplasma via sap-feeding of phytoplasma-infected insect vectors, leafhoppers. Three weeks post infection, transgenic lines that expressed MuMLPL329 did not develop dwarfism though showed some symptoms of witches’ broom showing improved resistance to phytoplasma (Fig. 12A). In contrast, wild-type plants inoculated with phytoplasma via sap-feeding of phytoplasma-infected leafhoppers exhibited severe developmental abnormalities, including symptoms of witches’ broom and dwarfism (Fig. 12B). Therefore, the expression of MuMLPL329 gene alleviates the phytoplasma-associated disease symptoms and partially reduces the growth inhibition of phytoplasma in transgenic Arabidopsis. Phytoplasma growth in the leaves was also monitored using qRT-PCR to ascertain whether the
lack of symptom development reflected the restriction of phytoplasma growth and multiplication inside the leaves. The results showed that many phytoplasma was detected in the wild type plants inoculated with phytoplasma viaphytoplasma-infected leafhoppers. Though phytoplasma was also found in the \textit{MuMLPL329}-overexpressing plants inoculated with phytoplasma, the number of phytoplasma was lower than that in the wild type plants. There was no phytoplasma detected in the wild type and transgenic Arabidopsis plants infected by healthy leafhoppers (Fig. 12). Therefore, \textit{MuMLPL329}-overexpressing in Arabidopsis restrains phytoplasma growth and multiplication in some extent and alleviates the symptoms of phytoplasma diseased.

Ectopic Expression of \textit{MuMLPL329} Affects Defense-related Gene Expression—To explore whether the disease resistance observed in the \textit{MuMLPL329}-overexpressing transgenic plants was resulted from the expression of defense genes, the expressions of some defense-related genes were monitored in the transgenic lines. Fig. 13 shows that the defense-related gene \textit{PR-1}, \textit{PR-5}, \textit{β}-1,3-glucanase and \textit{PDF1.2} were expressed at very low levels in the wild type plants. However, the \textit{PR-5}, \textit{β}-1,3-glucanase and \textit{PDF1.2} genes were highly expressed in the \textit{MuMLPL329}-overexpressing transgenic plants. These results suggest that the enhanced disease resistance in the transgenic plants may be because of the constitutively high-level expression of some defense-related genes.
DISCUSSION

Transcripts and Proteins Identified as Responding to Phytoplasma Infection—Thus far, the composition of phloem proteins and mRNAs has been characterized in many plant species and many transcripts and proteins present in the phloem of mulberry were identified in this study. When the transcripts identified were compared with previously published collections of mobile RNAs in Arabidopsis thaliana (39) through BLAST analyses, 1124 mobile RNAs were found in the present study. Based on this approach, we compared the phloem proteins identified in this study to the previously published sequences of the pumpkin phloem proteome (12), and 214 pumpkin phloem proteins were also found in our data. Many pumpkin proteins have also been found in rice, rape, and castor bean. Therefore, the phloem proteins and mRNAs in species may have a high degree of conservation in higher land plants (12, 14). However, to our knowledge, this study is the first large-scale analysis of phloem proteins and mRNAs involved in the response to phytoplasma in mulberry. The data obtained from transcriptomic and proteomic analyses showed a poor correlation, suggesting there are selectively import mechanisms for maintaining proper levels of transcripts and proteins in the phloem during the response to phytoplasma infection.

Recognition is the initial event in the response of plants to pathogens, and it can occur through adhesins, fimbriae, flagella, and Type III and Type IV secretion systems (TTSS) (40). Though phytoplasma has no flagella and TTSS, it resides within the plant cell and can secrete proteins into plant hosts via the bacterial Sec translocation system, and the proteins secreted may function in the infected-plant cytoplasm like TTSS virulence factors (41). Mulberry may recognize these proteins and activate a signal transduction cascade and elicit defense responses. Our data showed that there were 40 mRNAs and 5 proteins involved in signaling were found to be differentially expressed between healthy and infected phloem sap (supplemental Table S4 and supplemental Table S5). These differentially expressed mRNAs and proteins probably serve as signaling molecules in response to phytoplasma-infection when they are released from the phloem, and they likely activate their signaling cascades in the non-infected tis-

Fig. 12. Phenotypes of the phytoplasma-infected Arabidopsis and detection of phytoplasma. A, Phenotypes of phytoplasma-infected MuMLPL329 transgenic Arabidopsis. B, Phenotypes of phytoplasma-infected wild type Arabidopsis. C, qRT-PCR amplification of the 16S rDNA of phytoplasma to detect phytoplasma. Error bars indicate the S.D. of three technical replicates within one biological experiment. Three biological repeats were performed.

Fig. 13. Expression of defense-related genes in MuMLPL329-overexpressing plants. Error bars indicate the S.D. of three technical replicates within one biological experiment. Three biological repeats were performed. OE1, OE2 and OE3: Transgenic Arabidopsis lines overexpressing MulMLPL329.

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issues and play important roles in modulating the response to phytoplasma infection at the whole-plant level.

Phytohormone signaling is crucial for plant growth and development, as well as plant response to environmental stresses (42). It was reported that multiple auxin responsive genes and auxin efflux carrier genes of the infected plants can be down regulated by the phytoplasma effector TENGU (2), and the effector SAP11 can reduced JA synthesis (20). Our transcriptomic analysis showed that 23 mRNAs associated with the signaling and metabolism of diverse phytohormones, including IAA, ABA, 6-BA, JA, CK, were differentially expressed in the infected phloem sap. These differentially expressed mRNAs partially accounted for the disturbance of hormonal signaling and metabolism in the infected plants, and this is in accordance with previous reports that phytoplasma infection disrupts the phytohormone balances in the host plants, resulting in reprogramming of growth and developmental patterns and in other symptoms in infected plants (18, 43–47).

As intracellular parasites, phytoplasmas lack many genes related to amino acid and fatty acid biosynthesis, the tricarboxylic acid cycle and oxidative phosphorylation, suggesting that they must obtain essential metabolites from their hosts and this will have a great impact on the metabolome of infected plants (48). Our results showed that phytoplasma infection alters the expression of several genes and proteins correlated with metabolic processes of TCA, lipid, carbohydrate metabolism, as well as secondary metabolism (supplemental Table S4 and supplemental Table S5). The differential expression of the genes and proteins might meet the requirements of phytoplasma for energy, growth and spread. From a different perspective, the changes of these genes or proteins may alter the activity of specific enzymes involved in some metabolic processes and disturb the normal metabolic processes in the infected plants, and this may be partly responsible for some of the mulberry yellow dwarf symptoms.

In addition, our data showed that 38 mRNAs and 16 proteins involved in various aspects of biotic and abiotic stress were differentially expressed (supplemental Table S4 and supplemental Table S5). However, the expressions of these mRNAs and proteins do not all increased, the defense responses are induced but not completely or sufficiently to kill the phytoplasmas in the diseased plants. Another possible explanation is that phytoplasmas may have achieved a strategy to overcome plant defenses at this level. Interestingly, although the mulberry plants were planted in well-watered conditions and suffered no temperature stress, some mRNAs and proteins involved in the response to temperature and dehydration stress were differentially expressed in the infected phloem sap. For example, HSP70, known to be involved in response to a variety of abiotic stresses, was also found to be differentially expressed between the healthy and infected phloem sap. HSP70 is known to perform chaperone functions by stabilizing new proteins to ensure correct folding or by helping refold proteins that were damaged by cellular stress (49). Therefore, the increased expression of HSP70 may reflect an adaptive response in the infected plants to stabilize proteins or to help refold proteins that were damaged by phytoplasma infection.

Moreover, it was reported that the effector SAP11 of phytoplasma targets the nuclei of plant cells and interacts with transcription factors and changes plant gene transcription levels (20). Our results showed that some transcription factors (TFs), such as transcription factor VIP1, WRKY transcription factor 11 and squamosa promoter-binding-like protein 1, were also differentially expressed in the phloem sap under phytoplasma infection (supplemental Table S4 and supplemental Table S5). These TFs are known to be involved in responses to both biotic and abiotic stresses and play crucial roles in the regulation of defense genes, signaling, growth and development, and metabolism. Therefore, phytoplasma infection may be more than a biotic stress; the infection may also result in some abiotic stresses in the infected plants, and these differentially expressed genes may mediate the cross-talk between the abiotic and biotic stress responses. Furthermore, the differential expression of their TFs may result in up or downregulation some genes associated with plant growth and development and disturb the plant development process and be responsible for some mulberry yellow dwarf symptoms, which may aid phytoplasma colonization and increase plant attractiveness to insect vectors.

MuMLPL329 Is a Defense-related Protein in Mulberry—Though lots of genes involved in the response to phytoplasma infection have been reported in different plants, there are few reports to confirm the roles of these genes in resistance against phytoplasma diseases. As far as we know, this is the first report that MLP-like protein 329 gene was involved in the response to phytoplasma infection. Major latex protein (MLP) was first identified from the latex of opium poppy (Papaver somniferum) (50). Its orthologs, named MLP-Like proteins (MLPL), were later found in Arabidopsis as well as in other plants. Based on modest sequence similarity, they have been characterized as members of the Bet v 1 protein superfamily which contains proteins with low sequence similarity, but a similar three-dimensional (3D) structure (51). Although the MuMLPL329 protein has low similarity to other MLP proteins, it has a similar 3D structure to the Bet v 1 family proteins (Fig. 7A). In addition, as other MLP subfamily proteins, MuMLPL329 has no signal peptide, and subcellular localization analysis showed it was accumulated in both nucleus and cytoplasm (Fig. 8). In this study, the MuMLPL329 protein was detected in the phloem sap of mulberry, and this is consistent with the role of Bet v 1 family protein as a type of receptor taking part in binding or transporting molecules like plant hormones and secondary metabolites (52).

It has been reported that the function of MLPs is related to fruit and flower development in peach (53) and kiwifruit (54). Our data showed that MuMLPL329 was also expressed in various organs of mulberry plants indicating that the gene
may also related to fruit development in mulberry. Meanwhile, several studies have reported that the expression of MLP genes is responsive to pathogen invasion, but the biological function of this protein family in defense responses is poorly understood (55), and there was little report about the function of MLPL329 proteins. In this study, MuMLPL329 was identified as a phytoplasma-responsive gene, and the induced expression of MuMLPL329 in mulberry in response to P. syringae pv. mori and C. dematium was also detected (Fig. 9B). This indicated that MuMLPL329 may play a role in the defense response in mulberry. Our results demonstrate that ectopic overexpression of the MuMLPL329 gene confers the transgenic Arabidopsis plants enhanced disease tolerance to B. cinerea and Pst. DC3000 (Fig. 11). Moreover, the transgenic Arabidopsis plants showed more tolerance to phytoplasma infection than wild type plants (Fig. 12). These results showed that the function of MuMLPL329 was tightly associated with plant defense response, and the expression of MuMLPL329 has been a strategy to overcome phytoplasmas in the diseased mulberry plant.

The role of Bet v 1 family protein in resistance to biotic and abiotic stresses is thought to be a type of receptor taking part in binding or transporting molecules like plant hormones and secondary metabolites (51). It was reported that Gh-MLP can binding or transport of ligand in transgenic Arabidopsis, thereby directly or indirectly affect flavonoid biosynthesis and change transgenic plant resistance to stress (52). Interestingly, it was found that some genes involved in flavonoid synthesis were changed in the grapevines (Vitis vinifera L.) plants infected by Flavescence dorée phytoplasma (54). In our data, the gene involved in flavonoid synthesis, CL773.Contig1 (flavonoid 3’-monooxygenase), was also found to be induced at both mRNA and protein levels in the phloem sap of phytoplasma infected mulberry plants (supplemental Table S4 and supplemental Table S5). The total flavonoid content in the leaves of infected mulberry was significantly higher than that in the leaves of healthy mulberry (Fig. 14). In addition, the total flavonoid of the leaves from transgenic Arabidopsis plants were also determined, and the results showed that the average flavonoid content of transgenic Arabidopsis plants was significantly higher than that of wild type plants (Fig. 14). It was reported that many flavonoid compounds function as passive or inducible barriers against herbivores or microbial pathogens, and the flavonoid content can increase response to pathogen attack (57). These results suggest that MuMLPL329 is possible changing transgenic Arabidopsis plants resistance to pathogens infection by changing the flavonoid levels. However, the mechanism of MuMLPL329 involved in the variation of flavonoid content is not clear. Previous studies have shown that phytoplasma infections resulted in some phytohormones and metabolites being accumulated in the infected phloem saps and an inhibition of phloem transport, and this may be partly responsible for some of the mulberry yellow dwarf symptoms (18). Because MLPs were thought to take part in binding or transporting molecules like plant hormones and secondary metabolites, the increased expression of MuMLPL329 may make for transportation of metabolites and alleviating the symptoms.

It has been demonstrated that one of the MLPs of upland cotton, GhMLP28, can interact with ethylene response factor 6 (GhERF6) and enhance its transcription factor activity, which facilitate the expression of some GCC-box genes, such as PDF1.2 and PR-5, and led to an enhanced disease tolerance of the transgenic tobacco plants (55). In our data, the expression levels of PR-5, β-1,3-glucanase and PDF1.2 genes were also found to be increased in the MuMLPL329 transgenic Arabidopsis plants (Fig. 13). Because the Bet v 1 family proteins have similar 3D structure (Fig. 7A), MuM-
LPL329 protein may as well interact with ethylene response factor 6 and facilitate the expression of PDF1.2 and PR-5 genes, and enhance disease tolerance of the transgenic Arabidopsis plants. In addition, our data showed that MuMLPL329 gene can be induced by JA treatment (Fig. 9B, Fig. 10). So, one possibility is that MuMLPL329 acts as a receptor, binding plant hormone and activating the transduction signal, and then affecting the expressions of some defense genes. It is to be regretted that the PDF1.2 and PR-5 genes were not found to be induced in the infected mulberry phloem saps (supplemental Table S4), though the expression level of MuMLPL329 protein was increased in the infected phloem saps. Therefore, the regulatory networks of genes involved in the response of mulberry to phytoplasma-infection is complex, and further studies are required to clear the molecular basis of the function of MuMLPL329.

In conclusion, interpretation of the transcriptomic and proteomic data has uncovered several phytoplasma-responsive candidate genes/proteins and provides a global picture of the gene/protein expression changes in the phloem sap of mulberry under phytoplasma infection. Our results provide a critical line of evidence showing MuMLPL329 acts as a positive regulator participating in plant defense response. The information provided here is particularly useful to understand the function of genes/proteins in the phloem sap of mulberry under phytoplasma infection. The information provided here is particularly useful to understand the molecular basis of the function of MuMLPL329.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to PeptideAtlas (http://www.peptideatlas.org/) with the dataset identifier PASS00997.

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REFERENCES

1. Ortiz-Castro, R., Contreras-Comelio, H. A., Macias-Rodriguez, L., and Lopez-Bucio, J. (2009) The role of microbial signals in plant growth and development. Plant Signal. Behav. 4, 701–712.
2. Kehr, J., and Buhz, A. (2008) Long distance transport and movement of RNA through the phloem. J. Exp. Bot. 59, 85–92.
3. Notaguchi, M., and Okamoto, S. (2015) Dynamics of long-distance signal- ing via plant vascular tissues. Front. Plant Sci. 6, 161.
4. Crawford, K. M., and Zambryski, P. C. (1999) Phloem transport: are you chaperoned? Curr. Biol. 9, R281–R285.
5. Heo, J. O., Roszak, P., Furuta, K. M., and Helariutta, Y. (2014) Phloem development: Current knowledge and future perspectives. Annu. Bot. 101, 1393–1402.
6. Jansen, R. P. (2001) mRNA localization: message on the move. Nat. Rev. Mol. Cell Biol. 2, 247–256.
7. Lough, T. J., and Lucas, W. J. (2006) Integrative plant biology: role of phloem long-distance macromolecular trafficking. Annu. Rev. Plant Biol. 57, 203–232.
8. Deeken, R., Ache, P., Kajahan, I., Klinkenberg, J., Bringmann, G., and Hedrich, R. (2008) Identification of Arabidopsis thaliana phloem RNAs provides a search criterion for phloem-based transcripts hidden in complex datasets of microarray experiments. Plant J. 55, 746–759.
9. Kehr, J. (2006) Phloem sap proteins: their identities and potential roles in the interaction between plants and phloem-feeding insects. J. Exp. Bot. 57, 767–774.
10. Atkins, C. S., Smith, P. M., and Rodriguez-Medina, C. (2011) Macromolecules in phloem exudates—a review. Proteomics 11, 165–172.
11. Yang, Y., Mao, L., Jittayakosoth, Y., Kang, Y., Jiao, C., Fei, Z., and Zhong, G. Y. (2015) Messenger RNA exchange via scions and rootstocks in grafted grapevines. BMC Plant Biol. 15, 25.
12. Lough, T. J., Lee, Y. J., Lo, S.-J., T. J., Pinney, B. S., and Lucas, W. J. (2009) Analysis of the pumpkin phloem proteome provides insights into angiosperm sieve tube function. Mol. Cell. Proteomics 8, 343–356.
13. Cho, W. K., Chen, X. Y., Rim, Y., Chu, H., Kim, S., Kim, S. W., and Park, Z. Y. (2010) Proteome study of the phloem sap of pumpkin using multidimensional protein identification technology. J. Plant Physiol. 167, 771–778.
14. Kehr, J. (2006) Phloem sap proteins: their identities and potential roles in the interaction between plants and phloem-feeding insects. J. Exp. Bot. 57, 767–774.
15. Li, P., Chen, L., Zhou, Y., Xia, X., Shi, K., Chen, Z., and Yu, J. (2013) Brassinosteroids-induced systemic stress tolerance was associated with increased transcripts of several defence-related genes in the phloem in Cucuris sativus. PLoS ONE 8, e66582.
16. Serra-Soriano, M., Navarro, J. A., Genoves, A., and Pàllàs, V. (2015) Comparative proteomic analysis of melon phloem exudates in response to viral infection. J. Proteomics 124, 11–24.
17. Maejima, K., and Oshima, K. (2014), and Namba, S. Exploring the phytoplasmas, plant pathogenic bacteria. J. Gen. Plant Pathol. 80, 210–221.
18. Gai, Y. P., Han, X. J., Li, Y. Q., Yuan, C. Z., Mo, Y. Y., Guo, F. Y., Liu, Q. X., and Ji, X. L. (2014) Metabolomic analysis reveals the potential metabolites and pathogenesis involved in mulberry yellow dwarf disease. Plant Cell Environ. 37, 1474–1490.
19. Henry, E., Yadeta, K. A., and Coaker, G. (2013) Recognition of bacterial plant pathogens: local, systemic and transgene rational immunity. New Phytol. 199, 908–915.
20. Sugio, A., Kingdom, H. N., MacLean, A. M., Grieve, V. M., and Hogenhout, S. A. (2011) Phloemplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 108, 1254–1263.
21. Ji, X., Gai, Y., Zheng, C., and Mu, Z. (2009) Comparative proteomic analysis provides new insights into mulberry dwarf responses in mulberry (Morus alba L.). Proteomics 9, 5328–5339.
22. Margaria, P., and Palmano, S. (2011) Response of the Vitis vinifera L. cv. ‘Nebbiolo’ proteome to Flavescence dorée phytoplasma infection. Proteomics 11, 212–224.
23. Margaria, P., Abbà, S., and Palmano, S. (2013) Novel aspects of grapevine response to phytoplasma infection investigated by a proteomic and phospho-proteomic approach with data integration into functional networks. BMC Genomics 14, 38.
24. Abbà, S., Galetto, L., Carle, P., Carrère, S., Delledonne, M., Foissac, X., Palmano, S., Veratti, F., and Marzachi, C. (2014) RNA-Seq profile of flavescence dorée phytoplasma in grapevine. BMC Genomics 15, 1088.
25. Sievert, C., Luge, T., Duduk, B., Seemüller, E., Bütter, C., Sauer, S., and Kubé, M. (2014) Expression of expressed genes of the bacterium ‘Candidatus phytoplasma Mali’ highlights key features of virulence and metabolism. PLoS ONE 9, e94391.
26. Zhang, S., Sun, L., and Krägerl, F. (2009) The phloem-delivered RNA pool contains small noncoding RNAs and interferes with translation. Plant Physiol. 150, 378–387.
27. Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Nikrle, A., Rhind, N., di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., and Regev, A. (2011) Full-length transcriptome assembly from RNA-seq data without a reference genome. Nat. Biotechnol. 29, 64–652

28. Gao, Y., Xu, H., Shen, Y. Y., and Wang, J. (2013) Transcriptomic analysis of rice (Oryza sativa) endosperm using the RNA-Seq technique. Plant Mol. Biol. 81, 363–378

29. Umezawa, T., Okamoto, M., Kishiro, T., Nambara, E., Oono, Y., Seki, M., Kobayashi, M., Koshiba, T., Kamiya, Y., and Shinozaki, K. (2006) CYP707A3, a major ABA 8'-hydroxylase involved in dehydroxylation and rehydration response in Arabidopsis thaliana. Plant J. 46, 71–82

30. Poppenberger, B., Rozhon, W., Khan, M., Husar, S., Adam, G., Luschnig, M., Giovannoni, S., and Sieberer, T. (2011) CESTA, a positive regulator of brassinosteroid biosynthesis. EMBO J. 30, 1149–1161

31. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta Ct) Method. Methods 25, 402–408

32. Zhang, Y., Su, J., Duan, S., Ao, Y., Dai, J., Liu, J., Wang, P., Li, Y., Liu, B., Feng, D., Wang, J., and Wang, H. (2011) A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast related processes. Plant Methods 7, 30–43

33. Christensen, N. M., Nicolaisen, M., Hansen, M., and Schulz, A. (2004) Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. Mol. Plant Interact. 17, 1175–1184

34. Liu, Y., G. Chen, Y. (2007) High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. Biotechniques 43, 649–650, 652, 654

35. Sato, M., Mitsuhashi, W., Watanabe, K., and Kawakita, H. (1996) PCR detection of mulberry dwarf disease-phytoplasmas in mulberry tissues, phloem sap collected by laser stylectomy and insect vector Hishimonus serratellus. J. Seric. Sci. Jpn. 65, 352–358

36. Arpat, A. B., Maglione, P., Wege, S., Rouach, H., Stefanovic, A., and Poirier, Y. (2012) Functional expression of PHO1 to the Golgi and trans-Golgi network and its role in export of inorganic phosphate. Plant J. 71, 479–491

37. Jefferson, R., Kavanagh, T., and Bevan, M. (1987) GUS fusions: a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901–3907

38. Zhang, S., Liu, C., Bi, H., and Wang, C. (2008) Extraction of flavonoids from Rhodiola sachlinesis A. Bor by UPE and the antioxidant activity of its extract. Nat. Prod. Res. 22, 178–187

39. Thieme, C. J., Rojas-Triana, M., Stecyk, E., Schudoma, C., Zhang, W., Yang, L., Mirhambes, M., Wather, D., Schulte, W. X., Paz-Ares, J., Scheible, W. R., and Kragler, F. (2015) Endogenous Arabidopsis messenger RNAs transported to distant tissues. Nat. Plants 1, 794–803

40. Lutgenberg, B. J., Chin Woeng, A. T.-F., and Bloemberg, G. V. (2002) Microbe-plant interactions: principles and mechanisms. Antonie Van Leeuwenhoek 81, 373–383

41. Hoshi, A., Oshima, K., Kakizawa, S., Iashl, Y., Ozeki, J., Hashimoto, M., Komatsu, K., Kagiwada, S., Yamaji, Y., and Namba, S. (2009) A unique virulence factor for proliferation and dwarfism in plants identified from a phytopathogenic bacterium. Proc. Natl. Acad. Sci. U.S.A. 106, 6416–6421

42. Pozo, M. J., López-Raže, J. A., Azcón-Aguilar, C., and Garcia-Garrido, J. M. (2015) Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses. New Phytoi. 205, 1431–1436

43. Davey, J. E., Van Staden, J., and De Leeuw, G. T. N. (1981) Endogenous cytokinin levels and development of flower virecence in Catharanthus roseus infected with mycoplasmas. Physiol. Plant Pathol. 19, 193–200

44. Pertot, I., Musetti, R., Pressacco, L., and Odier, R. (1998) Changes in indole-3-acetic acid level in micropropagated tissues of Catharanthus roseus L. infected by the agent of the clorphyll phyology and effect of exogenous auxins on phytoplasma morphology. Cytobios 95, 13–23

45. Lee, I. M., Davis, R. E., and Gundersen-Rindal, D. E. (2000) Phytoplasma: Phytopathogenic mollicutes. Annu. Rev. Microbiol. 54, 221–255

46. Tan, P. Y., and Whitlow, T. (2001) Physiological response of Catharanthus roseus (perwinkle) to ash yellows phytoplasmal infection. New Phytoi. 150, 757–769

47. Gai, Y. P., Li, Y. Q., Guo, F. Y., Yuan, C. Z., Mo, Y. Y., Zhang, H. L., and Ji, X. L. (2014) Analysis of phytoplasma-responsive sRNAs provide insight into the pathogenic mechanisms of mulberry yellow dwarf disease. Sci. Rep. 4, 5378

48. Bai, X., Zhang, J., Ewing, A., Miller, S. A., Jancso Radek, A., Shevchenko, D. V., Tsuikerman, K., Walunas, T., Lapidus, A., Campbell, J. W., and Hogenhout, S. A. (2006) Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. J. Bacteriol. 188, 3682–3696

49. Sarkan, N. K., Kundnani, P., and Grover, A. (2013) Functional analysis of Hsp70 superfamily proteins of rice (Oryza sativa). Cell Stress Chaperones 18, 427–437

50. Nessier, C. L., and Burnett, R. J. (1992) Organization of Hsp70 superfamily proteins of rice (Oryza sativa). Plant Mol. Biol. 20, 749–752

51. Radauer, C., Lackner, P., and Breiteneder, H. (2008) The bet v 1 field: an ancient, versatile scaffold for binding of large, hydrophobic ligands. BMC Evol. Biol. 8, 286

52. Chen, Y., and Dai, X. F. (2010) Cloning and characterization of the Gossypium hirsutum major latex protein gene and functional analysis in Arabidopsis thaliana. Planta 231, 861–875

53. Ruperti, B., Bonghi, C., Ziliotto, F., Pagni, S., Rasori, A., Varotto, S., Tonutti, P., Giovannoni, J. J., and Ramina, A. (2002) Characterization of a major latex protein (MLP) gene down-regulated by ethylene during peach fruit-let abscission. Plant Sci. 163, 265–272

54. Chruszcz, M., Ciardiello, M. A., Osinski, T., Majorek, K. A., Gliangreico, L., Font, J., Breiteneder, H., Thalassinos, K., and Minor, W. (2013) Structural and bioinformatics analysis of the kiwifruit allergen Act d11, a member of the family of ripening-related proteins. Mol. Immunol. 56, 794–803

55. Yang, C. L., Lian, S., Wang, H. Y., Han, B. L., Wang, F. X., Cheng, H. Q., Wu, X. M., Qu, Z. L., Wu, J. H., and Xia, G. X. (2015) Cotton major latex protein 28 functions as a positive regulator of the ethylene responsive factor 6 in defense against Verticillium dahliae. Mol Plant. 8, 399–411

56. Margaria, P., Ferrandino, A., Caciagli, P., Kedrina, O., Schubert, A., and Palmano, S. (2014) Metabolic and transcript analysis of the flavonoid pathway in diseased and recovered Nebbiolo and Barbera grapevines (Vitis vinifera L) following infection by Flavescence dorée phytoplasms. Plant Cell Environ. 37, 2183–2200

57. Sandra, C., Calsenlinge, K., and Fornægaard, S. (2008) Biologically active secondary metabolites in white clover (Trifolium repens L.) – a review focusing on contents in the plant, plant–pest interactions and transformation. Chemoecology 18, 129–170