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Proteomics-Based Analysis of *Phalaenopsis amabilis* in Response toward *Cymbidium* Mosaic Virus and/or *Odontoglossum* Ringspot Virus Infection

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ABSTRACT

Stress response at the protein level to viral infection in orchid plants has not been extensively investigated to date. To understand the proteomic basis of *Phalaenopsis amabilis*'s responses to *Cymbidium* Mosaic virus (CymMV), and/or *Odontoglossum* ring spot virus (ORSV), the total proteins were extracted from *Phalaenopsis amabilis* leaves infected with CymMV, ORSV, or both respectively. Differentially expressed proteins were identified by two-dimensional electrophoresis, and 27 of these proteins that had significant changes were further examined by mass spectrometry. Comparing CymMV-infected leaves with mock-inoculated ones, 2 proteins were significantly up-regulated, 9 were significantly down-regulated and 1 previously undetected protein was identified. 10 proteins were significantly up-regulated, 3 significantly down-regulated and 1 previously undetected protein was identified in ORSV-infected leaves. 6 proteins were significantly up-regulated and 9 significantly down-regulated proteins were found in co-infected leaves. These identified proteins are involved in disease resistance, stress response, transcriptional regulation, energy metabolism, protein modification and the previously unknown proteins were not involved with known protein pathways. Proteins significantly up-regulated were ATP sulfurylase, down-regulated proteins included glutamate decarboxylase isozyme 2, RNA polymerase alpha subunit and chloroplastic peptide deformylase 1A were proteins with similar alteration trend after all infection treatments. Significantly up-regulated were Thioredoxin H-type and down-regulated Cytosolic phosphoglycerate kinase I which were proteins that have been shown to be specifically regulated by the infection with CymMV. Significantly up-regulated were proteins like Rubisco large subunit, Triosephosphate isomerase, NADP-specific isocitrate dehydrogenase and Cinnamoyl CoA reductase CCR2 by the infection of ORSV. Protein regulation in co-infected leaves followed a pattern similar to that of any of the single virus infection results. These experiments demonstrated that an exogenous pathogen infection in plants, regardless of viral type or pattern, induced a protein defense response system and a number of metabolism changes in *P. amabilis* with detectable protein alterations. These cellular alterations appeared to be specific responses to different pathogens. We suggest that altered proteins above might play important roles in pathogenesis and metabolic interactions between *P. amabilis* and viruses that infect them.

Keywords: *Phalaenopsis amabilis*; Two-Dimensional Electrophoresis; Mass Spectrometry; *Cymbidium* Mosaic Virus; *Odontoglossum* Ringspot Virus

1. Introduction

Phalaenopsis amabilis belongs to the genus *Phalaenopsis* of the family Orchidaceae. It has a single short stem, succulent, fleshy leaves, beautiful shape, colorful flowers, long lasting flowering period and a high ornamental value. It is one of the four most famous Orchidaceae along with *Cattleya hybrida*, *Vanda* spp. and *Dendrobium nobile* in the world [1]. However, *P. amabilis* is very susceptible to infection by *Cymbidium* mosaic virus (CymMV) and *Odontoglossum* ringspot virus (ORSV). CymMV-infected *P. amabilis* grows poorly and appears to have leaves with chlorotic stripes and sunken gray-white or necrotic ring spots as well as smaller and fewer flo-

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wers with a shortened flowering period. ORSV-infected *P. amabilis* is not easily distinguished from the uninfected healthy plants. Therefore, the virus-carrying plants being sold in commercial and local markets appear frequently and jeopardize and reduce their ornamental and economic value [2,3]. Molecular detection is the most effective and best way to identify ORSV and CymMV infected plants, which will also often combine together to infect *P. amabilis.*

At present, there have been some reports on the adverse effects of viral infection in *P. amabilis*. Liao *et al.* made a CymMV capsid protein gene that silenced the virus in *P. amabilis* and improved its resistance to CymMV [4]. Qin *et al.* found that expression of exogenous lipid transfer protein encoding gene could enhance the plants frost resistance [5]; Specified Tyrosine phosphatase *PaPTP*1 gene is related to flower development and external mechanical damage [6]. However, there are few studies on pathogenesis to *P. amabilis* and none of these reports used proteomics-based analysis to examine the mechanisms altered by infection with the exception of recent investigations with soybean [7] and cucumber [8]. Proteomics is currently a powerful tool to systematically analyze the cellular protein expression profiles and post-translational modifications under virus invasion in the host of rice [9-11], tobacco [12], soybean [13], tomato [14] and Arabidopsis thaliana [15] respectively.

In this study, we utilized the two-dimensional electrophoresis (2-DE) and mass spectrometry analysis to compare 2-DE gel profiles of the total proteins from CymMV and/or ORSV infected leaves in an attempt to find proteins that may be responsive to viral infection or proteins related to disease resistance. The results may provide new clues to the understanding of the interactive responses in protein expression of *P. amabilis* when infected with viruses.

2. Materials and Methods

2.1. Inoculations and Molecular Detections

Healthy *P. amabilis* seedlings with 5 - 6 leaves were cultured in a greenhouse at 25˚C and infected with CymMV or ORSV separately, or with both CymMV and ORSV, through sap inoculation. Four weeks post-inoculation, immune-captured reverse-transcript polymerase chain reaction (IC-RT-PCR) [16] and sequencing techniques were utilized to detect the presence of infection. Self-made high-titer polyclonal antibodies [17] and two virus specific primer pairs (5'-TCCGAATTCAGTCTTACACTATTACTGAC-3' and 5'-AAGTTCGAATTATTCAGTAGGGGGTGC-3' for CymMV; 5'-AATGGTGTTAGTGATATTCG-3' and 5'-CCACTATGCATTATCGTATG-3' for ORSV) were applied in the diagnosis.

2.2. Protein Extraction

Total leaf proteins were extracted using tris-saturated phenol method from *P. amabilis* plantlets 4 weeks after virus(es)- or mock-inoculation. In detail, 2 g leaf samples were collected and mixed using mortar and pestle with a small amount of quartz sand, polyvinylpyrrolidone (PVP) and 4 ml of extraction buffer (0.1 M Tris, 0.05 M vitamin C, 0.09 M sodium tetra-borate, 0.1 M EDTA-Na_2, 2% β-mercaptoethanol (v/v), 1% Triton-100 (v/v) and 30% sucrose (w/v)). This mixture was ground on ice and then transferred to a 50 mL centrifuge tube. 5 mL of the extraction was mixed with 9 mL of tris-saturated phenol (pH 8.0), shaken with a shaker for 15 min and centrifuged at 15,000 × g for 20 min at 4˚C. The lower phenol phase was then collected and precipitated by mixing with a 5-fold volume of ice-cold ammonium sulfate and methanol. After being stored at −20˚C for 12 h, the precipitated proteins were collected by centrifugation at 5000 × g for 15 min at 4˚C, and washed with ice-cold methanol and acetone for 2 - 3 times. After drying at room temperature for 5 min, the proteins were dissolved in 200 μL lysis buffer (7 M urea, 2 M thiourea, 1% dithiothreitol (w/v), 4% CHAPS (w/v), 1% ampholytes (v/v), pH 3 - 10) at room temperature for 2 - 3 h. The extraction was carried out three times with 3 days interval. All proteins from the three extractions for each sample were mixed together for further analysis. Total protein concentration was determined using Bradford method [18].

2.3. Two-Dimensional Gel Electrophoresis Analysis (2-DE)

Protein hydration and isoelectric focusing electrophoresis: Isoelectric focusing electrophoresis was carried out on an Ettan™ IPGphor II™ system (Amersham, USA). 150 μg protein lysate was mixed with 125 μL rehydration solution (8 M urea, 2% CHAPS (w/v), 0.2% bromo phenol blue (w/v), 0.5% ampholytes (v/v), pH 3 - 10) and incubated at 20˚C for 14 h to prepare 7 cm of pH 4 - 7 IPG strips. The prepared IPG strips were then transferred to the Ettan™ IPGphorII™ system and electrophoresed for 0.5 h at 300 V, 300 V/hr at Grad 1000 V, 5000 V/hr at Grad 5000 V, 5000 V/hr at 5000 V, and 1 h at 300 V.

Gel strip balance: After isoelectric focusing electrophoresis, gel strips were equilibrated with salt solution I (6 M urea, 0.075 M Tris-HCl, pH 8.8, 2% SDS (w/v), 0.4% bromo phenol blue (w/v) and 1% DTT (w/v)) and salt solution II (6 M urea, 0.075 M Tris-HCl pH 8.8, 2% SDS w/v), 0.4% bromo phenol blue (w/v), and 2.5% iodoacetamide (w/v)) by shaking the strips at room temperature for 15 min each.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was carried out on 12.5% separating gel and 5% stacking gel using a DYCZ-23A small vertical electrophoresis apparatus (Liuyi Company, Beijing). After gel polymerization, the pre-balanced IEF gels were placed on the top of the stacking gel. Proteins were further separated in electrode solution (25 mM Tris, 90 mM glycine and 0.1% SDS (w/v)) at 120 V for 1.5 h and stained with coomassie brilliant blue G-250.

Electrophoresis gel imaging analysis: Electrophoresis images were acquired using a gel scanner (GS800 Calibrated Densitometer, Bio-Rad) at transmission scanning mode and 300 dpi resolution, and saved as TIF files. Protein bands were analyzed using Image Master™ 2D Platinum software (Amersham Pharmacia Biotech, Uppsala, Sweden). To account for experimental variation, three biological replicate gels resulting from three independent experiments were analyzed for control and treatment. The amount of a protein spots was calculated based on the volume of that spot which was normalized against total volume of all valid spots. The normalized intensity of spots on three replicate 2-DE gels was averaged and analyzed using two-tailed, non-paired Student's *t*-test to determine whether the relative change was statistically significant between samples using SPSS software (SPSS Inc., Chicago, IL, USA). The differentially expressed protein spots whose expression level was more than 1.5 times higher or lower were manually excised for protein identification.

2.4. Protein Enzymatic Digestion and Identification by Mass Spectrometry

Enzymatic digestion: The target protein spots were destained using 100 μL of destaining solution (25 mM ammonium bicarbonate and 50% acetonitrile (v/v)) for 40 min and reduced by incubating with 100 μL reducing solution (10 mM DTT and 25 mM ammonium bicarbonate) at 56˚C for 1 h. After cooled to room temperature, the reducing solution was discarded and proteins were incubated with equal volume of 25 mM ammonium bicarbonate solution containing 55 mM iodoacetamide at room temperature for 45 min in dark. The supernatant was discarded and the proteins were dehydrated with 100% acetonitrile (v/v) and dried under a vacuum. The proteins were digested with 10 μL of trypsin solution for 1 h on ice, and incubated with 5 μL of 5 mM ammonium bicarbonate solution at 37˚C for 16 h. The mixtures were then extracted with 6 - 10 μL of 5% trifluoroacetic acid (v/v) at 40˚C for 1 h. After two successive extractions, the supernatants were collected and freeze-dried for future analysis.

Protein identification by mass spectrometry: Proteins were identified using Reflective Tandem Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS, 4800 MALDI TOF/TOF Analyzer, Applied Biosystems, USA) with MS Reflector Positive mode. The parameters were set as Laser Intensity 3880; Light Intensity 34; Mass Range 900 to 4000 Da; Focus Mass 2000 Da; Shots/Sub Spectrum 50, Total Shots/Spectrum 1000; Local Noise Window Width (m/z) 20; Min Peak Width at Full Width Half Max (bins) 2.9. Mass spectrometric data were processed using MassLynx™ software to generate peak list files and blasted in public NCBInr and SWISS-PROT protein databases using Mascot MS/MS Ion Search program on the Matrix Science public website (www.matrixscience.com). Search parameters were set as taxonomy, green plant; proteolytic enzyme, trypsin; max missed cleavages 1; fixed modifications, carbamidomethyl (c); variable modifications, oxidation; peptide mass tolerance, 150 ppm. In addition, "mono-isotopic mass" was chosen. Only significant hits as defined by Mascot probability analysis were considered. Mascot uses a probability-based Mowse score to evaluate data obtained from mass spectra. Mowse scores were reported as $-10 \times \text{Log10 (P)}$ where P is the probability that the observed match between the experimental data and the database sequence is a random event. Protein spots with a Mascot score greater than threshold (40) and C.I.% value were considered as validated ones and used to perform protein function analysis in UniprotKB/SWISS-PROT and UniprotKB/TrEMBL databases (www.uniprot.org).

Data analysis: The experimental data were collected from independent triplicates and significant differences were analyzed using SPSS 11.5 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered as significance.

3. Results

3.1. Producing CymMV and/or ORSV Infected Plants

IC-RT-PCR and sequencing successfully diagnosed the amplified band of approx. 600 bp for ORSV-cp (**Figure 1(a)**) and approx. 780 bp for CymMV-cp (**Figure 1(b)**) from the inoculated leaves respectively. The high homologies (98% for ORSV-cp and 99% for CymMV-cp) with the corresponding gene sequences in GenBank were shown for the consensus sequences. CymMV and/or ORSV infected plants of *Phalaenopsis amabilis* were confirmed by these results.

3.2. Analysis of 2-DE Images of Different Virus Infected *P. amabilis* Leaves

About 6 μg/μL extracted protein solution and total about 1.2 mg proteins were identified from 2 g leaf sample.

2-DE analyses of mock-inoculated, CymMV-infected, ORSV-infected, and CymMV and ORSV co-infected *P. amabilis* leaves showed a highly reproducible protein expression profiles. Most protein spots were concentrated in the range of pH 5 - 7 with a molecular weights of 16 - 105 kDa (**Figures 2(a)-(d)**). A total of 278, 263, 230 and 242 protein spots were detected, respectively, in mock-inoculated *P. amabilis* leaves (**Figure 2(a)**), CymMV infected *P. amabilis* leave (**Figure 2(b)**), ORSV infected *P. amabilis* leaves (**Figure 2(c)**) and co-infected *P. amabilis* leaves (**Figure 2(d)**).

The expression of 27 proteins changed significantly after virus infection (**Figure 3**). In addition, compared to mock-inoculated leaves, 2 up-regulated protein spots (Protein ID 144 and 157), 9 down-regulated protein spots (Protein ID 22, 23, 55, 64, 81, 125, 136, 146 and 186), 1 undetectable protein spot (Protein ID 59) and 15 unchanged protein spots were found in CymMV infected P. amabilis leaves, 10 up-regulated proteins (Protein ID 70, 100, 121, 144, 149, 150, 153, 154, 159 and 162); 3 down-regulated protein spots (Protein ID 59, 64 and 136), 1 undetectable protein spot (protein ID 186) and 13 unchanged protein spots were found in ORSV infected *P. amabilis* leaves, and 6 up-regulated proteins (Protein ID 100, 144, 149, 150, 159 and 162), 9 down-regulated protein spots (Protein ID 22, 23, 55, 59, 64, 125, 136, 146, and 186) and 12 unchanged protein spots (**Figure 3**) were found in co-infected *P. amabilis* leaves. Among them, 4 protein spots (Protein ID 64, 136, 144 and 186) showed the same regulation tendency in all three treatments compared with mock-inoculated leaves. Spots 64, 136 and 186 were down-regulated and spot 144 was up-

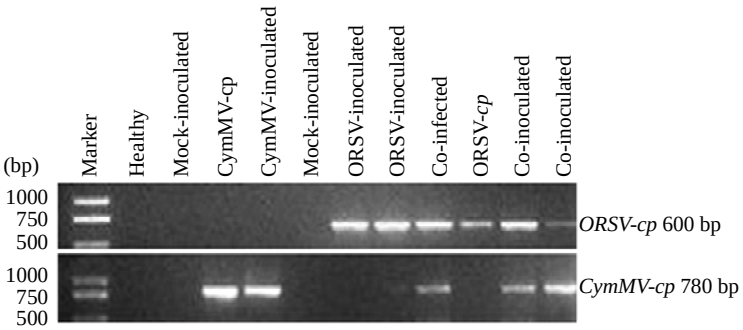

Figure 1. Agrose-gel electrophoresis of immune-captured reverse-transcript polymerase chain reaction (IC-RT-PCR) products from CymMV and/or ORSV inoculated leaves of *Phalaenopsis amabilis*. Panel A shows the amplified band of approx. 600 bp for ORSV-*cp*. Panel B shows the amplified band of approx. 780 bp for CymMV-*cp*.

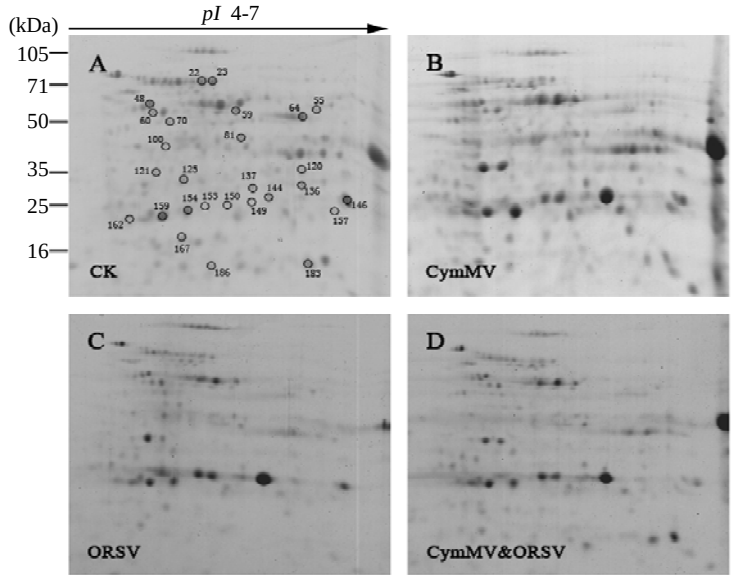

Figure 2. Two-dimensional patterns of total proteins in *Phalaenopsis amabilis* from mock-inoculated leaves (Panel A), CymMV-infected leaves (Panel B), ORSV-infected leaves (Panel C) and co-infected leaves (Panel D). Protein extraction and 2D electrophoresis were performed as described in Materials and Methods. Circles with black line indicate the differentially expressed proteins that have been identified by MADLI-TOF-MS. The spots are numbered, corresponding to those in Tables 1. Electrophoresis strip: 7 cm long, IPG made, pH 4 - 7.

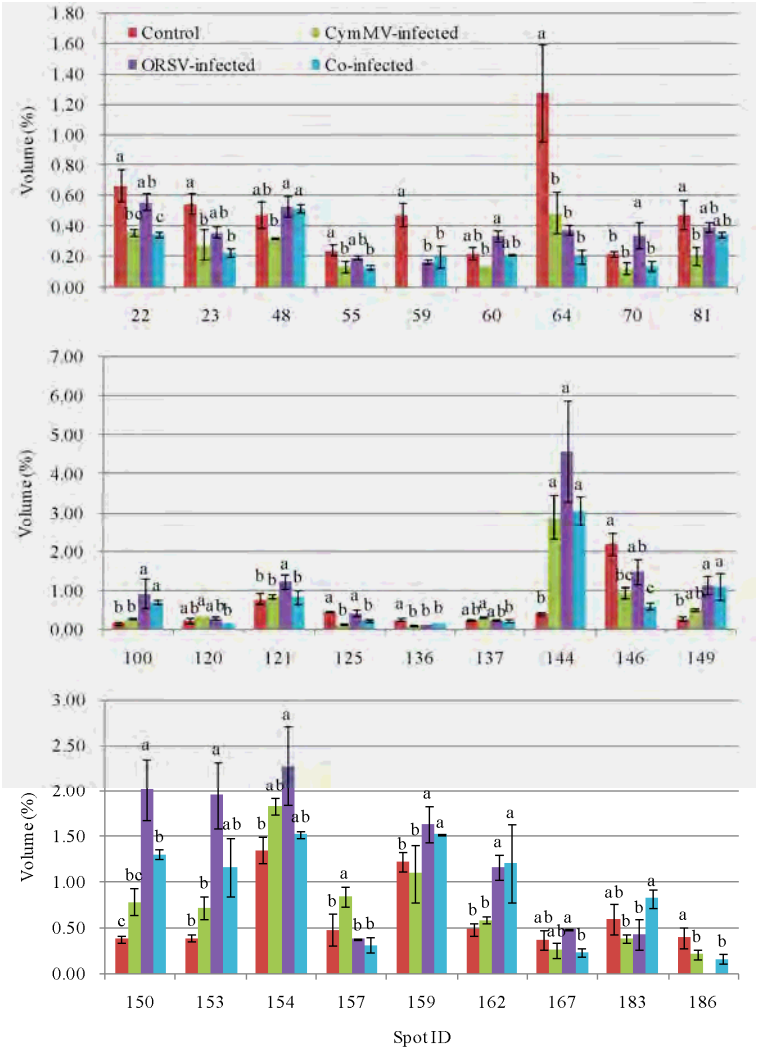

Figure 3. Abundance variance of the expressed proteins in CymMV and/or ORSV-infected leaves of *Phalaenopsis amabilis*. The spot volume was normalized as a percentage of the total volume of all spots on the corresponding gel. The graph represents an average of three biological replicates. Bars represent ± a standard error of the mean. Significance was calculated by Duncan's multiple range test. Columns with different lower case letters (a, b or c) are significantly different from each other by the least significant difference test ($P < 0.05$).

regulated.

3.3. Identification of Differentially Expressed Proteins by Mass Spectrometry

27 differentially expressed proteins were analyzed and identified by MALDI-TOF-MS (**Table 1**). These proteins can be classified into six categories including disease resistance-related proteins, stress response proteins, transcriptional regulatory proteins, energy metabolism-related proteins, protein modifiers and unclassified proteins. Among these categories, disease resistance-related proteins such as resistance protein RGC2, *β*-cyanoalanine synthase, Flavanone-3-hydroxylase and metalloendopeptidase were all up-regulated after ORSV- and co-infection, while Thioredoxin H-type 2 was up-regulated specifically in CymMV-infected leaves. 4 of the proteins in this study with a similar response after infection are stress associated proteins such as glutamate decarboxylase (GAD) isozyme 2 and ATP sulfurylase, transcriptional regulatory protein such as RNA polymerase alpha subunit and protein modifier such as chloroplastic peptide deformylase 1A.

12 differentially expressed proteins are related with CymMV infection, while 14 are related with ORSV infection and 15 proteins are found with co-infection as listed in **Table 2**. There were two proteins which are specific to the infection of CymMV. One such protein is disease resistant related Thioredoxin H-type (TRX*h*), another is energy metabolism related Cytosolic phos-

Table 1. Differentially expressed proteins in *Phalaenopsis amabilis* leaves after infection of CymMV and/or ORSV.

Proteins ID	Homologous Protein	Theo. Mr (Da)/pI	NCBI Accession	Organism	Mascot Score/Threshold*	Sequences Matched
Disease resistant proteins						
149	Resistance protein RGC2	47,007/5.95	gi|34485415	*Lactuca saligna*	42/40	9
150	Beta-cyanoalanine synthase	38176.3/6.86	gi|11559260	*Solanum tuberosum*	44/40	6
157	Thioredoxin H-type 2	13038.7/5.56	gi|586099	*Nicotiana tabacum*	46/40	4
159	Flavanone-3-hydroxylase	20922.5/5.09	gi|20513407	*Sequoia sempervirens*	51/40	6
162	Metalloendopeptidase	93138.8/6.78	gi|42568277	*Arabidopsis thaliana*	68/40	19
Stress response proteins						
22	Vacuolar proton-ATPase	68409.5/5.23	gi|11527563	*Hordeum vugare* subsp. *vugare*	82/40	15
23	V-type proton ATPase catalytic subunit A	68791.9/5.29	gi|137460	*Daucus carota*	105/40	20
64	Glutamate decarboxylase isozyme 2	55895.4/5.58	gi|3252854	*Nicotiana tabacum*	41/40	7
121	Cinnamoyl CoA reductase CCR2	36606.8/6.11	gi|12407990	*Arabidopsis thaliana*	56/40	8
144	ATP sulfurylase	52262.9/8.73	gi|4033353	*Brassica juncea*	60/40	12
167	Heat shock protein 17a.11	12046.1/5.67	gi|7768333	*Triticum aestivum*	50/40	5
Proteins related to transcriptional regulation						
136	RNA polymerase alpha subunit	38922.4/6.36	gi|11467223	*Zea mays*	50/40	7
183	RNA recognition motif-containing protein	60758.7/9.2	gi|30682379	*Arabidopsis thaliana*	48/40	11
Proteins related to energy metabolism						
55	ATP synthase subunit alpha,mitochondrial	55229.6/5.7	gi|114419	*Arabidopsis thaliana*	43/40	7
59	ATP synthase beta subunit	53445.9/5.22	gi|14718074	*Hippeastrum papilio*	109/40	12
60	Rubisco large subunit	48780.6/6.57	gi|1518390	*Solaria atropurpurea*	195/40	16
70	Rubisco large subunit	51850.2/6.34	gi|16973388	*Tetrastigma hookeri*	41/40	7
81	Cytosolic phosphoglycerate kinase 1	42642.6/5.7	gi|3738259	*Populus nigra*	51/40	10
125	Ribulose-1,5-bisphosphate carboxylase	51539.5/5.91	gi|4176743	*Lliigera luzonensis*	122/40	12
153	Triosephosphate isomerase	27588.3/6.6	gi|553107	*Oryza sativa*	47/40	7
154	NADP-specific isocitrate dehydrogenase	45703.1/6.13	gi|20260384	*Arabidopsis thaliana*	50/40	8
Proteins related to protein modification						
48	Chaperoning 60 beta subunit	63769.6/6.21	gi|15222729	*Arabidopsis thaliana*	70/40	14
120	Cycling-dependent protein kinase	38105.4/5.07	gi|15220145	*Arabidopsis thaliana*	42/40	6
186	Peptide deformylase 1A, chloroplast	28895.3/8.82	gi|17433051	*Arabidopsis thaliana*	51/40	7
Others proteins						
100	Oxygen-evolving enhancer protein	34718.7/8.73	gi|131388	*Triticum aestivum*	52/40	9
137	Unkown protein	36053.7/6.25	gi|15220679	*Arabidopsis thaliana*	44/40	7
146	Hypothetical protein	27335.5/5.55	gi|2244768	*Arabidopsis thaliana*	46/40	3

*: specified for non-model plant—*Phalaenopsis amabilis.*

Table 2. Proteins potentially related with *Phalaenopsis amabilis*—CymMV and/or ORSV interactions.

CymMV-infected leaves	ORSV-infected leaves	CymMV and ORSV-infected leaves
Vacuolar proton-ATPase (22[*])	ATP synthase beta subunit (59)	Vacuolar proton-ATPase (22)
V-type proton ATPase catalytic subunit A (23)	Glutamate decarboxylase isozyme 2 (64)	V-type proton ATPase catalytic subunit A (23)
ATP synthase subunit alpha, mitochondrial (55)	[i]Rubisco large subunit (70)	ATP synthase subunit alpha,mitochondrial (55)
ATP synthase beta subunit (59)	Oxygen-envolving enhancer protein (100)	ATP synthase beta subunit (59)
Glutamate decarboxylase isozyme 2 (64)	[i]Cinnamoyl CoA reductase CCR2 (121)	Glutamate decarboxylase isozyme 2 (64)
[§]Cytosolic phosphoglycerate kinase 1 (81)	RNA polymerase alpha subunit (136)	Oxygen-envolving enhancer protein (100)
Ribulose-1,5-bisphosphate carboxylase (125)	ATP sulfurylase (144)	Ribulose-1,5-bisphosphate carboxylase (125)
RNA polymerase alpha subunit (136)	Resistance protein RGC2 (149)	RNA polymerase alpha subunit (136)
ATP sulfurylase (144)	Beta-cyanoalanine synthase (150)	ATP sulfurylase (144)
Hypothetical protein (146)	[i]Triosephosphate isomerase (153)	Hypothetical protein (146)
[§]Thioredoxin H-type 2 (157)	[i]NADP-specific isocitrate dehydrogenase (154)	Resistance protein RGC2 (149)
Peptide deformylase 1A, chloroplastic (186)	Flavanone-3-hydroxylase (159)	Beta-cyanoalanine synthase (150)
	Metalloendopeptidase (162)	Flavanone-3-hydroxylase (159)
	Peptide deformylase 1A, chloroplastic (186)	Metalloendopeptidase (162)
		Peptide deformylase 1A, chloroplastic (186)

[*]: protein ID no.; [§]: proteins specific to CymMV-infection; [i]: proteins specific to ORSV-infection.

phoglycerate kinase I (PGK). The previously undetected protein is an energy metabolism related ATP synthase beta subunit. There were four proteins which were specifically regulated when infected with ORSV. These are proteins, an energy metabolism related Rubisco large subunit, a Triosephosphate isomerase (TPI) and a NADP-specific isocitrate dehydrogenase (IDH), and stress response related Cinnamoyl CoA reductase 2 (CCR2). The previously undetected protein is a protein modification related Peptide deformylase 1A and is Chloroplastic. There were no proteins uniquely regulated with co-infection, and no protein was altered more significantly than that found in any single infection. There were also no detectable novel proteins detected in co-infected leaves. The pattern of expressed proteins in co-infected leaves followed that of any of the single virus infection results (**Figure 3** and **Table 2**).

4. Discussion

Although proteomics has been widely used in examining protein function and expression profiles, it is seldomly used for analyzing interactions between virus and its host horticulture plant. Ventelon-Debout *et al*. (2004) applied 2-DE and mass spectrometry and found 19 and 13 up- or down-regulated proteins, respectively, in rice yellow mottle virus (RYMV) inoculated rice variety IR64 and virus-resistance variety Azucena [10]. These proteins are related to metabolism, stress resistance and translation. Abiotic stress response pathway is also activated by RYMV in both cultivars through up-regulating salt-induced protein (SalT), heat shock proteins (HSPs) and superoxide dismutase (SOD). Brizard *et al*. (2006) identified that rice-RYMV interaction protein complex is involved in plant defense, metabolism, translation, protein synthesis and transport, and demonstrated that virus is able to recruit many proteins from its hosts to ensure its development [9]. Casado-Vela *et al*. (2006) revealed differential expression of peptidases, endoglucanase, chitinase and proteins in the ascorbate-glutathione cycle in TMV-infected, asymptomatic tomato fruits, and suggested that pathogenesis-related proteins and antioxidant enzymes may play a role in the protection against TMV infection [14]. Zhao and Liu *et al*. (2013) recently reported that the rubisco small subunit was involved in the tobamovirus movement and silencing of NbRbCS was able to compromise Tm-2^2-dependent resistance [12].

4.1. Proteins Specifically Regulated by the Infection with CymMV

TRX*h* is an intracellular small acidic protein (12 kDa) with redox activity and has multiple functions in plants. In arabidopsis, TRXh5 is involved in oxidative stress response and anti-fungal response [19]. In tobacco plants, tobacco mosaic virus could strongly induce the expression of NtTRX*h* [20]. In this study, CymMV infection also dramatically induces TRX*h* expression in *P. amabilis* leaves, in consistence with the previous result.

PGK is involved in glycolysis and tricarboxylic acid (TCA) cycle and plays important roles in energy production [21]. PGK expression was significantly decreased only in CymMV-infected *P. amabilis* leaves, indicating that the energy metabolism of the plant host was damaged severely after CymMV infection.

4.2. Proteins Specifically Regulated by ORSV Infection

It has been reported that sclerotinia virus infection could reduce Rubisco gene expression in sunflower even before its induced defense responses [22]. Similarly, our results showed that ORSV infection enhanced Rubisco expression in leaves, implying that the modified Rubisco struc-

ture is associated with different viral pathogens.

TPI is involved in glycolysis and TCA cycle and plays important roles in energy production [21]. IDH is a rate-limiting enzyme in TCA cycle and plays important roles in energy metabolism, amino acid synthesis and vitamin synthesis [23,24]. TPI and IDH expression was significantly increased in ORSV-infected *P. amabilis* leaves, indicating that the host accelerates the transformation of energy and increases carbohydrate intermediates mainly through TCA cycle to protect itself from the harm caused by virus infection.

CCR2 is the entry point for the lignin-specific branch of the phenylpropanoid pathway and one of the proteins related to plant physical defenses. CCR expression regulates lignin content. In this study, CCR2 expression was strongly up-regulated only in ORSV infected leaves, which may be related to the induction of specific pathogen. The detailed mechanism of CCR2 up-regulation is unclear.

4.3. Proteins Regulated by All Infection Treatment with Similar Alteration Trend

ATP sulfurylase is involved in cysteine and methionine biosynthesis [25]. Its expression was significantly up-regulated in all infected *P. amabilis* leaves, indicating that viruses could greatly induce ATP sulfurylase expression, thus promoting thiol generation to participate in the formation of sulfur-containing amino acids to meet the demand of *P. amabilis* for cysteine, methionine and other sulfur-containing amino acids, which have been found to be abundant in plant in response to diseases.

GAD isozyme 2 is a 5-pyridoxal phosphate dependent enzyme. Its expression and activity are increased in maize under stress from abscisic acid, methyl jasmonate, NaCl or cold. This up-regulation might occur at both transcriptional or post-transcriptional levels [26]. Ginseng GAD (PgGAD) gene expression is up-regulated under stress of non-biological factors such as temperature, osmotic pressure, hypoxia, and mechanical damage, and down-regulated under oxidative stress (H_2O_2) [27]. In this study, we found that GAD2 expression in infected *P. amabilis* leaves was significantly decreased, possibly due to virus infection-induced host allergic reactions, which could increase local accumulation of active oxygen, thus reducing GAD expression.

The α subunit of RNA polymerase is the core component for gene transcription, and regulates transcription initiation [28]. RNA polymerase expression was significantly decreased in all infected *P. amabilis* leaves, indicating that viral infection reduces protein metabolism and regeneration, and disrupts normal physiological activities of plants.

Peptide deformylase is a metalloprotease involved in the posttranslational deformylation of N-terminal methionine residue of newly synthesized proteins [29]. The expression of chloroplast peptide deformylase 1A was significantly reduced in all virus infected *P. Amabilis* leaves, especially in ORSV-infected leaves, suggesting that viral infection has significant impact on protein modification in *P. amabilis*, and the symptom of chlorotic stripes in infected leaves probably resulted from the dramatically reduced expression of peptide deformylase 1A in chloroplasts. The underlying mechanism needs to be further studied.

These results demonstrated that viral infection regardless of type and pattern could induce dramatic changes in defense response system and metabolism in *P. amabilis*. Nevertheless, there are apparent specific responses to different pathogens. For CymMV infection, we observed differentially expressed proteins including stress response proteins, altered transcriptional regulation and changes in energy metabolism proteins, all of which, particularly down-regulation of proteins, reflect the severe damage to the host and the susceptibility of *P. amabilis* to CymMV. In terms of ORSV infection, up-regulation of most proteins shown above reflects the obvious disease-resistance characteristics of host *P. amabilis* to this viral pathogen. This result actually explains why ORSV symptoms, which usually appear faintly in *P. amabilis*, can be detected only by molecular techniques. It also illustrates a good symbiotic relationship between ORSV and its host *P. amabilis*. Transcript levels for those specifically regulated proteins in CymMV and/or ORSV infected leaves of *P. amabilis* need to be investigated further.

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