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Proteomics of *Papaya ringspot virus*-Infected Papaya Leaves

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ABSTRACT

The *Papaya ringspot virus* (PRSV) causes severe economic losses in both papaya and cucurbits throughout the tropical and subtropical regions. An understanding of the interaction between the papaya plant and PRSV can help to improve papaya production. The protein profiles of virus-infected and healthy papaya leaves were compared by two-dimensional polyacrylamide gel electrophoresis. Among the observed 490 protein spots, 227 were identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry and liquid chromatography-mass spectrometry. Forty-three proteins were found to be similar to those identified in the Papaya EST and NCBI nr databases. They play roles in the areas of: photosynthesis (14%), photorespiration (5%), metabolism (20%), gene and biosynthesis (10%), defence related (7%), stress response (5%), signal transduction (10%), and unknown processes (29%). Spot intensity and transcription levels determined by real-time polymerase chain reaction showed ribulose-1, 5-bisphosphate carboxylase, Rieske protein ubiquinol cytochome C and chlorophyll A/B binding were down-regulated in infected plants. On the other hand, ubiquitin-like modifiers, vascular processing enzyme and germin-like protein were up-regulated in infected plants at transcription and translation levels. The results showed the novel virus-responding mechanism of the papaya plant that might be essential for developing viral-tolerant papaya in the agricultural industries.

Keywords: *Carica papaya*, proteomic, two-dimensional polyacrylamide gel electrophoresis (2DE), mass spectrometry, *Papaya ringspot virus* (PRSV), transcription and translation levels

INTRODUCTION

Papaya (*Carica papaya*) is one of the most widely grown fruits in the tropical and subtropical regions. It is delicious in taste and rich in essential vitamins such as vitamin A, and is an excellent source of enzymes such as papain and chymopapain which are of benefit as pharmaceuticals and in the food industry in terms of food processing (Gonsalves, 1998). A decrease in the annual papaya production was first reported in northeastern Thailand, which was attributed to large scale infection in the papaya plant with the *Papaya ringspot virus* (PRSV) (Sirithorn *et al*.,

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1989). The virus is a member of the *Potyviridae* in the genus *Potyvirus* and causes a range of symptoms including mosaicing and chlorosis in the lamina, water-soaked oily streaks on the petiole and upper part of the trunk, distortion of young leaves and ringspot on the fruit (Gonsalves, 1998). Several species of aphids transmit the virus in a non-persistent manner to a limited host range of cucumber and papaya plants. The genomic RNA consists of 10,326 nucleotides and has the typical array of genes found in potyviruses (Shukla *et al.*, 1989). The PRSV genome encodes a single large polyprotein that is subsequently processed by viral-encoded proteinases P1, HC-Pro and NIa.

Most viral plant diseases induce different shades of chlorosis and stunting which can considerably reduce the rate of photosynthesis of infected plants to not more than one-quarter of the normal rate, in the last stages of disease (Agrios, 2005). However, plants also have built-in defence mechanisms that respond and react to various stresses and phytopathogens. An early mechanism to prevent infection is the hypersensitive response (HR) which induces rapid death of cells in the local surrounding region of infection, is analogous to the innate immune system and ultimately leads to systemic acquired resistance; the function of HR depends on active metabolism and protein synthesis (Mur *et al.*, 2008). The HR of plants resistant to pathogens involves a complex form of programmed cell death (Heath, 2000). Plants have to develop an immune system to resist pathogen attack. Therefore, the basis of plant defence response is induced by pathogen-associated molecular patterns (Hématy *et al.*, 2009) and elicitors (Zhao *et al.*, 2005). Phytopathogens secrete a wide range of elicitors such as polypeptides, proteins and oligosaccharides, which penetrate into the plant cell wall, cell or extracellular space, as elicitins (Lascombe *et al.*, 2007), harpins (Wei *et al.*, 1992) and necrosis- and ethylene-inducing peptide 1 (Motteram *et al.*, 2009). Up-to-date research has not yet revealed or clarified how the signaling pathways lead to a local HR, whole-plant cell death and how death occurs (Zhang *et al.*, 2010).

Proteome analysis is becoming a powerful tool for the functional characterization of plants. One of the standard methods for proteome analysis employs two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS). Proteins are separated by 2DE and identified using MS after digestion into peptide fragments. The method is supported by robust and automated instrumentation that perform specific steps in the process; for example gel imaging, spot picking, protein digestion, peptide mass spectrometry and sequence database searching (Aebersold and Goodlett, 2001; Smolka *et al.*, 2002). The current study aimed to compare protein profiles produced by PRSV-infected and healthy papaya plants. The proteomic analysis was performed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS) and liquid chromatography-mass spectrometry (LC-MS/MS). real-time polymerase chain reaction assay (RT-PCR) was carried out to confirm specific responses of the protein production at the transcription level.

MATERIALS AND METHODS

Plant materials and virus inoculation

Papaya seedlings were grown in a containment greenhouse under controlled conditions of 24 ºC, 16 hr light and 60% humidity to prepare the sixth leaf stage plant for PRSV inoculation. Papaya seedlings were inoculated mechanically by rubbing fresh extract of PRSV infected leaf (1 g in 5 mL of 0.1M sodium phosphate buffer, pH 7) on to the top and second leaves dusted with carborundum. The sample was collected one month after inoculation. Later, 1 g of infected PRSV leave was frozen in liquid nitrogen (N_2) and then ground to powder. A similar procedure was followed using 10 mL of 0.1 M phosphate buffer pH 7 as the experimental control.

Virus isolation and detection of *Papaya ringspot virus* using specific primer (coat protein of *Papaya ringspot virus*)

Inoculated and non-infected plant samples were kept at -80 ºC and other samples were used for total RNA extraction using an RNeasy Plant Mini Kit (QIAGEN GmbH; Hilden, Germany) in accordance with the manufacturer's protocol. Total RNA was reverse-transcribed using the PRSV coat protein specific primers [SC501, 5' AAA GTG GTA TGA GGG AGT GAG GAA 3'] and [SC104, 5' ATT GCG CAT ACC TAG GAG AGA GTG 3'].

Protein extraction for two-dimensional gel electrophoresis

Total proteins were extracted from non-infected and PRSV-infected papaya leaves, with slight modification and application of the trichloroacetic acid (TCA)-acetone precipitation procedure (Rodrigues *et al.*, 2009). Fifteen grams of tissue powder was precipitated with 15 mL acetone, 10% TCA and 0.07% 2-mercaptoethanol at 20 ºC overnight. The supernatant was discarded after centrifugation at 16,000 rpm for 30 min at 4 ºC. Afterward, the pellets were washed three times in ice-cold acetone and 0.07% 2-mercaptoethanol. The pellet was sonicated for 30 min at 4 ºC and mixed in a vortex every 10 min to remove papaya pigments and other compounds. The samples were centrifuged at 16,000 rpm for 30 min at 4 ºC; then, the pellets were washed three times. The vacuum-dried pellets were resuspended in 2 mL of resolubilization buffer made up of 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 1% dithiothreitol (DTT). The samples were then sonicated for 30 min at 4 ºC and mixed in a vortex every 10 min. The supernatant was collected after centrifugation at 16,000 rpm for 30 min at 4 ºC. The protein concentration of the protein sample was measured using a Bio-Rad Protein Assay (Bio-RAD Laboratories; Hercules, CA, USA) according to the manufacturer's instructions and based on the method of Bradford (Kruger, 2002).

Separation by 2DE and Analysis of Protein Spots

Protein samples were isoelectrically focused in parallel with an IPGphor system (GE Healthcare Bio Sciences AB; Uppsala, Sweden). For the first dimensional separation, 18-cm IPG strips p*I* 4-7 and p*I* 6-11 were used to separate the total protein. Isoelectric Focusing (IEF) was carried out on the IPGphor system according to the manufacturer's instructions. After IEF, strips were equilibrated with an equilibrium solution containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a trace of bromophenol blue and DTT (1% w/v). The strips were loaded onto precast 11.0% homogenous polyacrylamide (slab) gels (20 cm × 20 cm). The proteins were then separated by 24 mA per gel. The gels were stained with SYPRO Ruby (BioRad Laboratories; Hercules, CA, USA) according to the manufacturer's instructions. Gel images were detected using a ChemiDoc XRS system (BioRad Laboratories; Hercules, CA, USA) and then loaded into the Proteomeweaver™ 2-D analysis software (version 4.0; BioRad Laboratories; Hercules, CA, USA) for processing, spot detection, quantitation, gel matching and warping in accordance with the manufacturer's instructions. The volumes or identity of the protein spots were normalized by dividing the volume of each spot by the sum of the total spot volume. The proteomic display experiments were repeated three times. The mean values of the normalized volumes from the three experiments determined the expression level of each protein and were used for statistical testing (*t*-test). The level of significance was established at $P < 0.05$.

Protein digestion for mass spectrometric analysis

The excised gel plugs were washed with 40% 1-propanol. The solvent was removed and

the plugs were then dehydrated in ammonium bicarbonate in 50% acetonitrile. The dehydrated cubes were treated with 30 μL of 20 ng.μL^{-1} trypsin gold (Promega Corp.; Madison, WI, USA) in ammonium bicarbonate for rehydration at 37 ºC overnight. The supernatant was collected and condensed to approximately 10 μL using a speedVac centrifugal evaporator (Shimizu *et al.*, 2009).

Spectrometry analysis of protein spots

Peptide mixtures were desalted using ZipTipsC_{18} (Millipore Corp.; Billerica, MA, USA). Subsequently, samples were loaded onto a target plate for MALDI-TOF-MS analysis. The solution was mixed with α-Cyano-4-hydroxycinnamic acid. Mass spectra were obtained using an AXIMA mass spectrometer equipped with 337 nm N_2 laser in the positive ion reflectron mode (Shimadzu Corp.; Kyoto, Japan). One part of each supernatant was detected by LC-MS/MS analysis.

Identification of peptides

Proteins were identified by MASCOT (Matrix Science; Boston, MA, USA) search engines using the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) protein database and an in-house protein sequence library. The Swiss-Prot/TrEMBL (Bairoch and Apweiler, 2000) and Gene Ontology (Ashburner *et al.*, 2000) databases were used for protein classification.

Real-time polymerase chain reaction

The total RNA was extracted from 1 g of non-inoculated and PRSV-infected papaya leaves. Total RNA was converted to cDNA using MMLV Reverse Transcriptase (Fermentas, Thermo Fisher Scientific; Vilnius, Lithuania) for RT-PCR according to the manufacturer's instructions. Primers were designed from peptide sequences obtained after MALDI-TOF and LC-MS/MS analyses. The sequences of each primer are shown in Table 1. RT-PCR was performed using a MiNiOpticon^{TM} (BioRad Laboratories; Hercules, CA, USA) in accordance with the manufacturer's instructions.

RESULTS

Virus challenge and *Papaya ringspot virus* detection

Papaya plants were inoculated with PRSV sap at the top and second leaf stages and periodically monitored for viral symptoms. Inoculated leaves showed mosaicing and chlorotic symptoms 7 d post-inoculation (dpi). No symptom was observed in non-inoculated leaves. Symptoms gradually progressed and all inoculated plants showed severe systemic symptoms including leaf deformation and stunting after 30 dpi. Total RNA prepared from leaves from the virus-infected papaya plants, and reverse-transcription PCR revealed a specific 550-bp DNA fragment corresponding to the PRSV coat protein transcript which confirmed viral infection.

Two-dimensional analysis of proteins

The proteomic profiles of infected and mock-inoculated control plants were compared. Two ranges of p*I* (p*I* 4–7 and p*I* 6–11) were selected for the first dimension electrophoresis for better separation (Figure 1). Three replicates of the 2DE gels were prepared for each analysis. The results produced 60 (p*I* 4–7 gel) and 130 (p*I* 6–11) protein spots with molecular masses between 20 and 100 kDa. The gel images were processed and the spots were characterized for their quantitation.

The identification of protein spots by LC-MS/MS and MALDI-TOF techniques and the MASCOT program was completed whilst ensuring that the identity and extensive homology at $P < 0.05$ was maintained. The reliability of these results was reinforced with the findings that 27 proteins were found in both the papaya expressed sequences tag and the NCBI nr databases (Table 2). The intensity of protein was used to show the quantity of protein expression that was measured

by the Proteomeweaver software. Based on the intensity values, the 27 protein spots were separated into three groups. The first group was up-regulated in the infected leaves as malate dehydrogenase mitrochondrial (13100064), hydroxylase (CB303437), 3-isopropylmalate dehydratase large subunit (GW748687), α glycosyl hydrolase family protein (DR257981), amino acid binding protein (EY661568), ubiquitin-like modifiers (SUMOs) (DT049035), vacuolar processing enzyme (EY056528), germin-like protein (2800051), protein disulfide isomerise (6400097), heat shock protein (11300033) and arrestin (or S-antigen) domain protein (BU040603). The second group of infected leaf proteins were down-regulated—namely, ribulose-1,5-bisphosphate carboxylase (gi349421), Rieske protein ubiquinol cytochome C (EX919347), phosphoglycerate synthease (ES330365), chloroplast-targeted copper chaperone (9000195), chlorophyll A/B binding protein (170087), carbonic anhydrase (2150009), glycolate oxidase (BU023646), ceramidase (GW219301), metalloprotease domain protein (ES820618), putative adenosine triphosphate (ATP)-dependent RNA helicase (DY273786), ATP-dependent DNA helicase (BP136793), protein kinase (CA698577), calcium ion binding protein (AU231319) and metalloprotease domain protein (ES820618). The third group of proteins were equally regulated between infected and normal leaves, specifically iron-sulfur cluster-binding (CJ548546) and glycoprotein glucosyltransferase (AW042912) proteins.

The proteins were grouped into areas of: photosynthesis (14%), photorespiration (5%), metabolism (20%), gene and biosynthesis

Table 1 Primers used for real-time polymerase chain reaction.

Gene	Sequence (5'→3')
Ubiquitin-like modifiers	
SUMOs/F	5' GAA AGC AGG TAC TGA TTG GGA AGG TGG 3'
SUMOs/R	5' GAT AAC CAT CTG TCT GTG CAG GAT CAG C 3'
Germin-like protein	
GLP/F	5' TGT ACC TGA CCC AAC ACA ACC ACA TGG 3'
GLP/R	5' ACG TTG ATG GAC TCA GAG TAC CAG GC 3'
Vacuolar processing enzyme	
VPG/F	5' ATG TAT GGT CAT GCC TAT CAA CTG CTG AGG 3'
VPG/R	5' ATC CCA AGA ACC CCA GGA CCA CC 3'
Ribulose-1, 5-bisphosphate carboxylase	
Rbcl/F	5' GCA GCA TTC CGA GTA ACT CCT CAA CC 3'
Rbcl/R	5' TTC GCA GAT CTT CCA GAC GTA GAG CG 3'
Rieske protein ubiquinol cytochome C	
Rie/F	5' CTT GCG TCT CTT GAG GTG GAT CTT TCC 3'
Rie/R	5' CAT GGC ATG GGC AAA ACC AAC CAC C 3'
Chlorophyll A/B binding	
Chl/F	5' AGG TGA CTA CGG GTG GGA CAC G 3'
Chl/R	5' ATC TGG GCT CCG GCC TTG AAC C 3'
5- enolpyruvyl-3-phosphoshikimate synthase*	
EPSPS/F	5' TGA TGG TCT TAA GCA GCT TGG CGC A 3'
EPSPS/R	5' TCG CTC CAT CAA CTT CAG TGT CAT TTC AAC 3'

*Housekeeping gene for reference of real-time polymerase chain reaction.

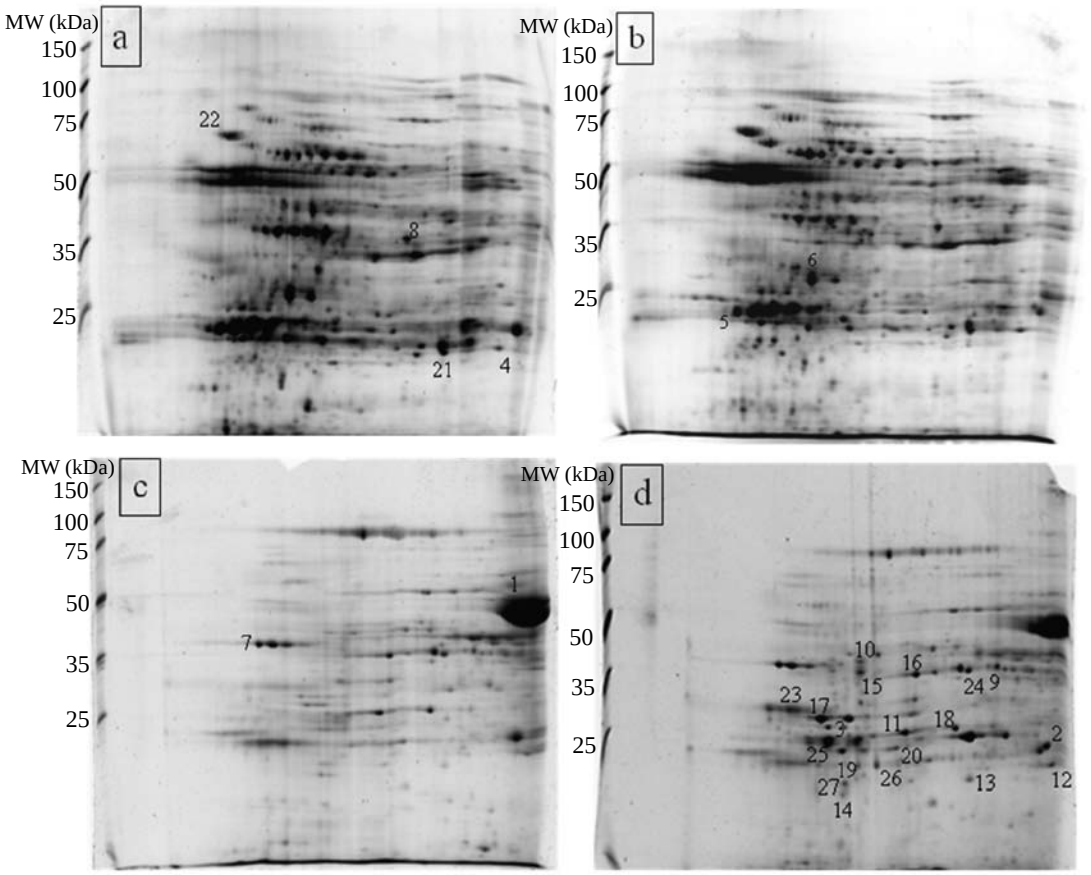

Figure 1 Protein profiling and separation by IPG pI 4-7 for: (a) healthy and (b) infected plants, with separation by IPG gel pI 6-11 for (c) healthy and (d) infected plants. The numbers in the image printouts correspond to identified proteins (Table 2).MW (kDa) = molecular weight (kDa)

(10%), defence related (7%), stress (5%), and signal transduction (10%). The functions of the remaining 29% of the proteins remained unknown (Figure 2). The results of the protein classification indicated that most proteins were involved in photosynthesis, gene and biosynthesis and signal transduction and down-regulated in virus-infected plants. On the other hand, other functions of the plant mechanism such as metabolism, defence related and stress mechanisms were found up-regulated after plants were virally infected.

The protein spot intensity and transcription level determined by the proteome weaver software and RT-PCR showed that expression of ubiquitin-like modifiers, a vacuolar processing enzyme and a germin-like protein in the PRSV-infected plants were more highly expressed than in the non-infected plants. On the other hand, there was lower expression of ribulose-1, 5-bisphosphate carboxylase, Rieske protein ubiquinol cytochome C and chlorophyll A/B binding in the PRSV-infected plants (Figure 3).

Table 2 Protein identification and comparison of healthy papaya plants and *Papaya ringspot virus*-infected papaya plants.

No. of protein spots	Name of protein	Accession number	Fold change	Score	Th PI/MW	Exp PI/MW	Sequence	Species
Up-regulated proteins under infected papaya plant								
Photosynthesis								
8	Malate dehydrogenase mitochondrial	13100064*	1.5	59	7.59/35.3	6.0/37	LALYDLANTPGVAAD VGHVNTR	*Carica papaya*
Metabolic								
9	Hydroxylase	CB303437	1.3	32	9.15/19	10.45/37.5	R.SCTPNHSCCPERMGP GTWGSVTPK.L	*Juglans regia*
12	3-isopropylmalate dehydratase large subunit	GW748687	2.3	28	9.78/28.7	10.80/23.2	-.DSSHYILLQR.L	*Pinus banksiana*
13	α glycosyl hydrolase family protein	DR257981	1.6	26	10.74/13.4	10.25/22	-.FPHSSITSKPKR.I	*Arabidopsis thaliana*
Gene and protein expression								
17	Amino acid binding protein	EY661568	1.3	24	10.15/34	8.42/30	K.HPGAGGGQPK.K	*Citrus sinens*
Defense mechanism								
19	Ubiquitin-like modifiers (SUMOs)	DT049035	3.0	27	9.32/18.5	8.65/19.5	.QVNLMVWHCTIPW	*Gossypium hirsutum*
20	Vacuolar processing enzyme	EY056528	1.9	28	9.06/25.7	9.28/25	R.MHLDSSVELIGRILFGLK. K	*Artemisia annua*
21	Germin-like protein	2800051*	1.4	88	5.84/21.8	6.10/21	VTENDFAFYLGK	*Carica papaya*
Stress								
22	Protein disulfide isomerase	6400097*	2.6	112	4.75/55.4	4.8/70		*Carica papaya*
23	Heat shock protein	11300033*	1.7	67	5.36/76.2	8.20/32		*Carica papaya*
Signal transduction								
26	Arrestin (or S-antigen) domain protein	BU040603	3.7	26	6.82/22.8	8.85/21.5	R.MVHHLXQEQLPDTQTSSQ EKGK.T	*Prunus persica*
Down-regulated proteins under infected papaya plant								
Photosynthesis								
1	Ribulose-1,5-bisphosphate carboxylase	gi349421	0.6	67	9.23/11.4	10.8/48.5	K.EIKFEFPAVDTI.-	*Carica papaya*
2	Rieske protein ubiquinol cytochome C	EX919347	0.4	31	9.12/26.6	10.75/26.5	K.CEFSPGSSGRLR.E	*Cycas rumphii*
3	Phosphoglycerate synthease	ES330365	0.6	27	8.65/43.4	8.55/28.5	R.HQENVRPPGVIR.S	*Ostreococcus lucimarinus*

Table 2 Continued.

No. of protein spots	Name of protein	Accession number	Fold change	Score	Th PI/MW	Exp PI/MW	Sequence	Species
4	Chloroplast-targeted copper chaperone	9000195*	0.8	44	6.06/24	6.5/23		*Carica papaya*
5	Chlorophyll A/B binding protein	170087*	0.8	55	4.5/22.6	4.8/23	FGEAVWFK	*Carica papaya*
6	Carbonic anhydrase	2150009*	0.8	57	6.96/37	5.6/31	EAVNVSLGNLLTYPFVR	*Carica papaya*
7	Glycolate oxidase	BU023646	0.6	55	9.69/23.6	7.95/38.5	R.VPVFLDGGVR.R	*Helianthus annuus*
Metabolic								
10	Ceramidase	GW219301	0.3	33	8.35/23	9.20/39.5	R.SCTCARQPDNSI.-	*Coccomyxa* sp.
14	Metalloprotease domain protein	ES820618	0.6	27	10.03/27	8.63/22.7	-.ILTSASIPSFFK.R	*Gossypium hirsutum*
16	Putative ATP-dependent RNA helicase	DY273786	0.6	31	10.99/46	9.45/36.8	R.GWSCPRSAAAACPSGCCP PSSSAPER.A	*Citrus clementina*
Gene and protein expression								
18	ATP-dependent DNA helicase	BP136793	0.8	28	10.78/20	9.78/28	K.FHRFIEQVTIPAAMGIQK.N	*Nicotiana tabacum*
Signal transduction								
24	Protein kinase	CA698577	0.5	27	10.72/18	10.15/36.5	K.NGTSAETQASIDQVLR.G	*Triticum aestivum*
25	Calcium ion binding protein	AU231319	0.8	35	10.51/15.4	8.45/25.5	K.LDDVTMITGIVSPPPAMA AKR.A	*Arabidopsis thaliana*
27	Metalloprotease domain protein	ES820618	0.6	27	10.03/27	8.63/22.7	-.ILTSASIPSFFK.R	*Gossypium hirsutum*
Constantly produced proteins								
Metabolic								
11	Iron-sulfur cluster-binding protein	CJ548546	1.0	27	9.64/24.8	9.35/27	R.QGLNFFAIIPASTSTMPIPH PRR.R	*Triticum aestivum*
15	Glycoprotein glucosyltransferase	AW042912	1.0	27	9.87/21	9.20/36.5	R.MALCTDGCGCHHR.I	*Pinus taeda*

Fold change = Fold changes for protein intensity up-regulated versus down-regulated of infected plants; Th PI/MW = Theoretical pI/Mass;
Exp PI/MW = Experimental pI/Mass.
* = Protein spots analyzed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

DISCUSSION

Mass spectrometry identified seven proteins: 1) ribulose-1,5-bisphosphate carboxylase, 2) Rieske protein ubiquinol cytochome C, 3) phosphoglycerate synthease, 4) chloroplast-targeted copper chaperone, 5) chlorophyll A/B binding protein, 6) carbonic anhydrase and 7) glycolate oxidase which are related to the process of photosynthesis; the protein intensity of these proteins was down-regulated in infected plants. Ribulose-1, 5-bisphosphate carboxylase, Rieske protein ubiquinol cytochome C and chlorophyll A/B binding protein were chosen to detect gene expression by RT-PCR. The results of the gene expression indicated that three genes showed down-expression in the infected plants (Figure 3). Photosynthesis is an important process in plants that uses solar energy to produce carbon compounds; this energy drives the synthesis of carbohydrates and the generation of oxygen from carbon dioxide and water (Taiz and Zeiger, 2006). After the plants had been infected by the virus, they showed symptoms such as mosaicing, chlorosis and yellowing of the leaves. This has been attributed to the irregularity in chloroplasts, as the pathogens in infected plants distort and reduce the photosynthesis process, especially in the last developing stages of the photosynthesis process and the toxics produced by the pathogens affect the chloroplasts (Pineda *et al.*, 2010).

Previous reports (Andaluz *et al.*, 2006; Rodriguez *et al.*, 2007) have examined the *pepper mild mottle virus* strain of Spanish-infected *Nicotiana benthamiana* which induced the down-regulation of several chloroplastidic proteins by using two-dimensional gel electrophoresis and mass spectrometry, where the chloroplastidic proteins involved in the photosynthetic electron-transport chain and the Benson–Calvin cycle can assemble into stable multi-enzyme complexes bound to the stromal face of thylakoid membranes in different plant species. This kind of association has been proposed to grant direct access to required

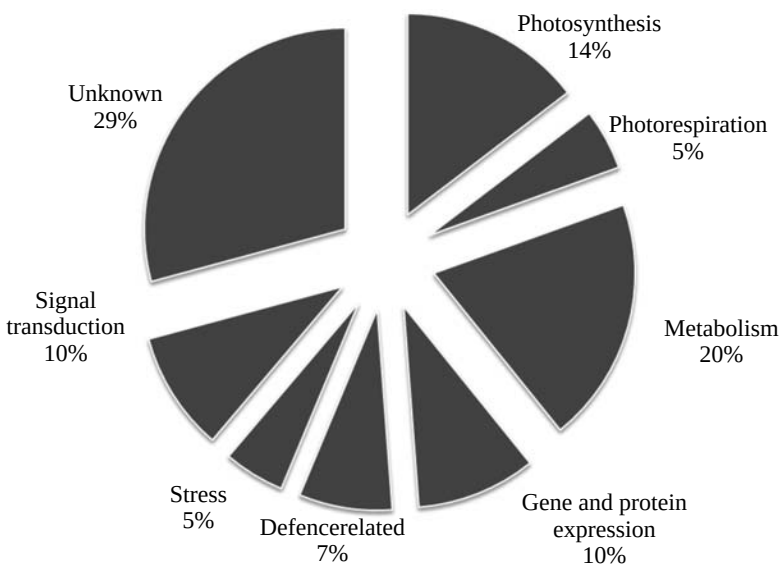

Figure 2 Functional distribution of the identified proteins in the healthy and infected papaya plants. Proteins were classified on the basis of data available in the literature and using the information available in the Swiss-Prot/TrEMBL (Bairoch and Apweiler, 2000 and Gene Ontology (Ashburner *et al*., 2000) databases.

cofactors such as ATP and nicotinamide adenine dinucleotide phosphate (NADPH), and to prevent interferences by other metabolic pathways (Suss *et al.*, 1993; Anderson. *et al.*, 1996). The results correspond with the report on the leaves of *Spinacia oleracea* inoculated with *Tobacco mosaic virus* (TMV) where the TMV-infected *S. oleracea* indicated inhibition of photosynthetic electron transport through PSII which was inhibited by 50% (Hodgson *et al.*, 1989).

A virus does not disturb only the photosynthetic pathway but also interferes with the growth regulator system of the plant, for example with gibberellins, auxins, cytokinins and ethylene (Pennazio and Roggero, 1996; Denancé *et al.*, 2013). Most viral diseases induce different shades of chlorosis and stunting. The rate of photosynthesis in a viral-infected plant is reduced considerably, with the rate being not more than one-quarter of the normal rate in the last stages of disease (Agrios, 2005).

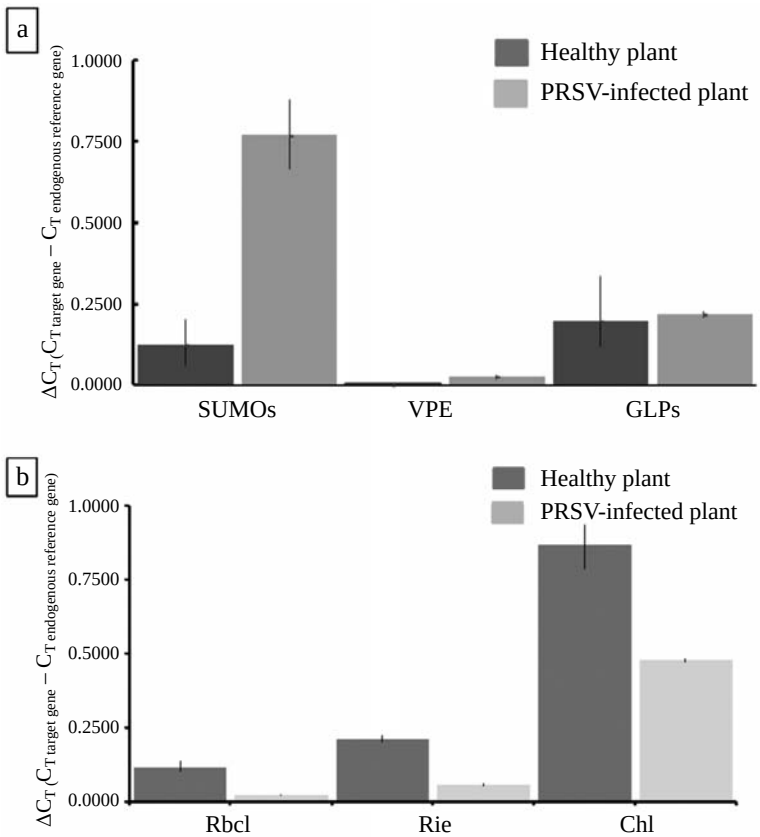

Figure 3 Real-time polymerase chain reaction analyses of mRNA expression levels of ubiquitin-like modifiers (SUMOs), vacuolar processing enzyme (VPE), and germin-like proteins (GLPs) and (b) Ribulose-1, 5-bisphosphate carboxylase (Rbcl), Rieske protein ubiquinol cytochome C (Rie) and chlorophyll A/B binding (Chl). The vertical bars show the standard error. Threshold cycle (C_T) is the cycle at which the amplification plot crosses the threshold; it serves as a tool for calculation of the starting template amount in each sample. The ΔC_T value describes the difference between the C_T value of the target gene and the C_T value of the corresponding endogenous reference gene, such as a housekeeping gene: $\Delta C_{T =} C_{T\ (target\ gene)} - CT_{\ (endogenous\ reference\ gene)}$.

The current study also investigated SUMO, VPE and GLP proteins up-regulated by the PRSV infection. Various approaches have implied their role in stress responses. This study is the first to identify them by using proteomics. To date, little is known of their roles in the viral response of plants.

The results of SUMO peptide sequences in papaya demonstrated that there was a high similarity to the SUMOs of *Arabidopsis thaliana* (Van den Burgan den Burg *et al.*, 2010). Recently, (Kurepa *et al.*, 2003) showed that the level of SUMO conjugation is increased by various stresses caused by hydrogen peroxide (H_2O_2), *N*-ethylmaleimide, osmotic stress and DNA-damaging agents in *Arabidopsis thaliana*. The yeast two-hybrid screen technique found that SUMO interacted with xylanase elicitors in tomato plants infected by the fungus *Trichoderma viride*. (Kurepa *et al.*,, 2003) also found that the xylanase elicitors in infected tomato plants were quantitatively higher than those found in healthy plants and that xylanase also acted as a strong elicitor in the rapid defence response mechanism of the tomato plants. SUMO protease also enhances the activation of the plant's defense mechanism against pathogenic bacteria (Hanania *et al.*, 1999; Hotson *et al.*, 2003; Roden *et al.*, 2004). The current findings of the up-regulated SUMO protein suggested the PRSV infection induced the stress response mechanisms of papaya as well, which means probably that the SUMO protein should act as an elicitor that involves the plant defense mechanism. In the current results, the SUMO protein up-regulated in the virus-infected plants. Plant pathogenesis-related (PR) proteins are induced and expressed by pathogens and abiotics to protect themselves. Furthermore, elicitors can be used to activate defensive systems in a desired plant; however the number of times they are used for the expression of an elicitor is also important. Based on the spot intensity and the quantitative analysis by RT-PCR, the expression of a protein was only studied 1 mth after virus infection of the plants. Expansion of the sampling period will help to discover the level of protein expression and protein signaling of SUMOs proteins. This is in addition to its effect on the environment, which is also an important factor in the study of protein expression.

However, in the plant protective mechanism, there is not a sole protein that can function in the protection from pathogens as the vacuolar processing enzyme (VPE) and germin-like protein (GLP) also function in the defense mechanism. The role of VPE in viral resistance remains obscure. It was also reported that virus-induced HR mediates in regulating the cell membrane collapse (Hatsugai and Hara-Nishimura, 2010). *Arabidopsis thaliana* VPE is also suggested to be involved in both mycotoxin-induced cell and developmental cell death (Kuroyanagi *et al.*, 2005). Moreover, the expression levels of PR genes were reduced by the silencing of VPE in the elicitor signalling (Zhang *et al.*, 2010). This implies that the papaya VPE may contribute to the elicitor-triggered immunity and/or play an important role in the plant immunity response and various types of cell death in the plant. Germin-like protein (GLP) gene expression and enzyme activity directly relate to plant defense responses against a wide variety of plant viruses. The germin gene is expressed in most plant tissues and is induced by biotic and abiotic stresses or both (Park *et al.*, 2004; Wang *et al.*, 2013) The viral response of OXO protein was reported in many plants as well; hot pepper (*Capsicum annuum* L. cv. Bugang) induced gene expression of GLP upon infection by *Tobacco mosaic virus* pathotype P_0 (TMV-P_0) and was related to the HR response by the plant and thus, the GLP is classified as a PR family protein (Park *et al.*, 2004).

CONCLUSION

The basics of proteomic information were examined using 2DE gel and mass spectrometric techniques to understand the level of protein

expression in PRSV-infected papaya plants. In total, 27 proteins influenced photosynthesis, photorespiration, metabolism, gene and protein expression, stress and signal transduction. Furthermore, symptoms such as mosaicing and chlorosis were present in papaya leaves which related to the expression levels of the proteins that were involved in the plant physiology. However, further research is needed to gain a better understanding of the plant physiology by addressing post viral reactions to plant infection.

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