Study of interaction between *Papaya ringspot virus* coat protein and infected *Carica papaya* proteins

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**ABSTRACT**

*Papaya ringspot virus* (PRSV) causes the Papaya ringspot disease. Virus–host interactions appear to play a significant role in the replication, pathogenesis, and infection caused by the PRSV. PRSV coat protein is likely to be involved in the processes of RNA replication, aphid transmission, and cell-to-cell movement, which is closely related to the host cell proteins resulting in a PRSV infection. To identify the host proteins that interact with the CP in vitro, immune precipitation, in-solution trypsin digestion, and LC–MS/MS were performed. Twenty-three identified proteins that interacted with the CP are involved in cellular metabolism, transcription, signal, translation, carbohydrate metabolism, protein metabolism, stress response, photosynthesis, nucleotide metabolism, respiration, and lipid metabolism processes. The search tool for interactions of chemicals (STITCH) results show that SWP (an RNA polymerase II transcription mediator) and MPPBETA (Mitochondrial processing peptidase) are involved in known plant defense mechanisms including transcription factors, cell division, hormones, stress, mitochondrial electron transferase, respiration, and proteasome. Therefore, analyzing virus–host protein interactions at a molecular level is important to build a better understanding of the virus replication mechanism and cellular responses mounted against viruses by the host defense system.

**Introduction**

Papaya ringspot disease caused by the *Papaya ringspot virus* (PRSV), which is a member of the *Potyviridae* genus. *Potyvirus* is recognized as the largest genus by the International Committee on Taxonomy of Viruses (ICTV 2020). More than 200 species of viruses have been reported to cause significant crop losses around the world (Shukla et al. 1994). *Potato virus Y* (PVY), *papaya ring spot virus* (PRSV), *sugarcane mosaic virus* (SCMV), *pepper mottle virus* (PepMoV), and *zucchini yellow mosaic virus* (ZYMV) are the most serious disease-causing viruses in Thailand (Bateson et al. 2002; Gemechu et al. 2006). Thailand’s Papaya production is also hampered by the papaya ringspot disease, affecting farmer income, healthy papaya production, as well as crop sustainability. More than 200 species of aphid are virus vectors (subfamily *Aphidinae*).

PRSV is the main pathogen affecting the papaya crop (*Carica papaya* L.). The PRSV translated protein is a polyprotein, cleaved by three proteases and separated into ten proteins, including a genome-linked protein (VPg), P1 serine proteinase (P1), helper component-protein (HC-Pro), P3 protein, 6K1 protein, cylindrical inclusion protein (CI), 6K2 protein, nuclear inclusion protein (NIa), large nuclear inclusion protein (Nlb), and coat protein (CP) (Shukla et al. 1994). CP is an essential protein for the survival of a virus and responsible for various viral infection mechanisms and steps, the study of the interaction between CP and papaya proteins has gained attraction amongst the community. Moreover, CP is also a multifunctional protein and is involved in the intercellular movement (Dolja et al. 1995), virus assembly (Jagadish et al. 1991; Varellmann and Maiss 2020), aphid transmission (Atreya et al. 1990; Shukla et al. 1994; Silva-Rosales et al. 2000), and plasmodesmatal gating (Rojas et al. 1997). CP structure and functions have been known since the earliest PRSV outbreaks in the West and Asia. Seo et al. (2010) have demonstrated that the potyvirus CP interacts with viral proteins such as HC-Pro protein, affecting virus accumulation, aphid transmission, and long-distance movement of the *soybean mosaic virus*.

It is important to study the protein–protein interactions, as they form a vital step in identifying the functions of a viral protein and the interaction mechanism between the virus and host cells. Currently, the interaction networks between virus and host are still poorly understood due to a lack of research data in this field. Yeast two-hybrid system (YTHS) is an extensive tool to study such protein–protein interactions and is a well-established classical biochemical and genetic in vivo approach. More than 5600 protein interactions have been reported using this method, including the establishment of general protein interaction networks (Uetz and Hughes 2000; Uetz et al. 2000; Ito et al. 2001). However, some groups of protein–protein interactions can be lost in YTHS. For example, the interactions related to membrane proteins, self-activating proteins, and post-translational protein modifications (Brückner et al. 2009). Another method also used to screen protein–protein interactions is...
affinity purification mass spectrometry (AP-MS). AP-MS has become a highly efficient tool to discover protein–protein interactions in various creatures and to identify the dynamics of protein–protein interactions in *in vivo* analysis to probe the protein interactions under certain conditions. This approach can capture higher-order components of a larger complex, with the information being crucial to determine the function of a protein in plant cellular (Zhang et al. 2010; Morris et al. 2014). Previously, we analyzed the protein–protein interactions between 23 papaya (*Carica papaya*) proteins and PRSV HC–Pro protein using the AP-MS approach (Siriwan et al. 2014). Recently, AP-MS has also been applied to explore the interaction of non-structure proteins of the virus into the plant, which was left for 10 min and then incubated at 37°C for 150 min. The PCR reaction mixture contained 20 ng of first stand cDNA, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 pmole primers (CP_Forward; 5’GGG GAT CCT CC A AAA CTG AAG CTG TGG ATG CTG G 3’ and CP_Reverse; 5’GGG CGG CCC CTG AAG ACT TGA CAT TGC GCA TAC CCA GGA GAG AGT 3’), 0.5 U Taq enzyme (Promega, Madison, WI, USA) in a final total volume of 20 µl. Thirty five PCR cycles at 95°C for 1 min, at 60°C for 1 min, and at 72°C for 1 min were performed. PCR products were separated by 1.5% (w/v) agarose gels electrophoresis in a 1×TBE buffer and visualized under UV light after staining with ethidium bromide.

The PCR products were digested with BamHI and NotI restriction enzymes and ligated into pET28a (+) (Novagen, Madison, WI, USA) to generate recombinant plasmids 6xHisCtPEt28α(+) in *Escherichia coli* DH5α. The PRSV-CP gene fragment was 1,070 bp. The reading frames of inserted genes were confirmed by a DNA sequence analysis (Capillary Electrophoresis on the ABI Prism 310) and SoftBerry-Alignments (Yu et al. 2002).

**Expression of recombinant PRSV-CP**

The PRSV-CP fragment was inserted into the pET28α(+) expression vector and transformed into *E.coli* JM109 (DE3). The recombinant colony was cultured in 50 ml of Luria–Bertani medium (LB) with Kanamycin (50 µg/mL) at a temperature of 30°C by shaking at 120 rpm. A starter culture (OD₆₀₀ 0.55–0.6) was added into 5,000 mL of fresh LB with Kanamycin (50 µg/mL), and the shaking was continued at 120 rpm, maintained at a temperature of 30°C. The recombinant protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.8 mM and when the OD₆₀₀ culture had reached 0.6. After gentle shaking for 6 h, bacterial cells were then collected by centrifugation (5500 rpm for 10 min at 4°C). The cell pellet was collected, re-suspended in 5 ml of binding buffer (made of 20 mM, sodium phosphate, 500 mM NaCl, 10 mM imidazole at a pH of 7.4, and 5 mM DTT) and sonicated for 15 min. The soluble protein fraction was loaded onto a 1 ml HisTrap FF crude column (GE Healthcare, Piscataway, NJ, USA), which was connected to a Fast Flow Liquid Chromatograph (FPLC, GE Healthcare, Piscatawy, NJ, USA). The recombinant protein was eluted with 0.8 M Imidazole. The recombinant PRSV CP fraction was separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and stained with 0.2% (w/v) Coomassie blue R250. The purified protein concentration was measured by using Bio-Rad Protein Assay (Bio-RAD, Hercules, CA, USA), which is based on the Bradford method. The purified recombinant proteins were used in pull down protein assay.

**Total papaya protein extraction**

The total proteins were extracted from PRSV infected papaya leaves with a slightly modified form of Trichloroacetic acid (TCA)-acetone precipitation procedure (Rodrigues et al. 2009). Fifteen grams of PRSV infected leaves sample was homogenized in liquid nitrogen (N₂) and the total proteins were precipitated in 15 ml acetone, 10% (w/v) TCA, and 0.07% (v/v) β-mercaptoethanol overnight at 20°C. The supernatant was discarded after centrifugation at 8000 rpm for 30 min at a temperature of 4°C. Next, the protein pellets were washed three times in cold acetone pulsed 0.07% (v/v) β-mercaptoethanol and dried by a
speed vacuum concentrator. The dried pellets were re-suspended in 2 ml of resolubilisation buffer (7 M urea, 2 M thiourea, 4% (w/v) 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) DTT), and sonicated for 30 min at 4°C and vortexed for 10 min. The supernatant was collected after centrifugation at 10,000 rpm for 30 min at 4°C. The total protein concentration was measured using a Bio-Rad Protein Assay (Bio-RAD, Hercules, CA, USA).

**In vitro pull-down assay**

The pull-down assay composition comprised of a recombinant CP (bait) and total plant protein (prey). Two milliliter of nickel beads were prewashed with H2O and a native binding buffer (ProBond nickel-chelating resin beads, Invitrogen). The experiments were performed on three replicates. The mixtures included 0.25 mg/ml of bait and pray protein each and the nickel beads were incubated at 7°C and gently rolled for 2 h. After washing thrice with a native washing buffer, the beads were collected. The bait-prey protein complex was eluted with a native elution buffer (250 mM imidazole in a native binding buffer) and collected for the LC–MS/MS analysis.

**Liquid chromatography–tandem MS (LC–MS/MS) analysis**

The interacting protein complex fractions obtained from the in vitro pull-down assay were directly applied to ZebaTM Spin Desalting columns (Thermo Scientific Inc, Beverly, MA, USA) and the protein concentration in each column was desalted and determined by Lowry assay using BSA as the standard protein. During the solution digestion, each fraction was hydrolyzed by trypsin at an enzyme to protein ratio of 1:50 and incubated at 37°C for 24 h. The peptides were then dried in a vacuum centrifuge. The extracted peptides were dissolved in 15 µl of 0.1% (v/v) formic acid, centrifuged at 10,000 rpm for 10 min and separated using an Ultimate 3000 LC System (Dionex Ltd., Leeds, UK) on a nanocolumn PepSwift monolithic column 100 mm i.d.650 mm, with a flow rate of 300 nl/min using a multi-step gradient of linear concentration increase from 10% to 90% of 80% (v/v) acetonitrile in 0.1% (v/v) formic acid within a duration of 20 min. The nanoLC system was connected with an electrospray interface with an ESI-Ion Trap MS/MS (Bruker Daltonik GmbH, Bremen, Germany). The collected LC–MS raw data were directly used for the quantification based on the individual MS signal intensities determined by the DeCyder MS differential analysis software (DeCyderMS, GE Healthcare). All the differential peptide data were searched against the NCBI Arabidopsis thaliana database using the MASCOT software version 2.2 (Matrix Science, London, UK). The search parameters used were as follows: trypsin was selected as the enzyme, with three potential missed cleavages, carbamido-methylated cysteine as a fixed modification, and oxidation of methionine residues as a variable modification. Peptide mass tolerance was kept at 1.2 Da, while fragment mass tolerance was kept at 0.6 Da, and the ESI ion trap was selected as the instrument type. Proteins were selected with a statistical significance of P < .05 as shown in the maps of protein levels using the Multi Experiment Viewer (MeV, version 4.6.1) software.

**Bioinformatics analysis**

The biological interpretation and function of protein identification were analyzed through GeneOntology (Go Cat (http://eagl.unige.ch/GOCat/)) and Uniprot (http://www.uniprot.org) sequence databases. Protein–protein interaction was determined according to the search tool for interactions of chemicals (STITCH) 4.0 database (http://stitch.embl.de/). STITCH is a database containing protein–chemical interactions and includes predicted interactions based on text mining and other prediction methods.

**Results**

**The recombinant PRSV CP**

PRSV was inoculated into the papaya plants on the first and second leaves of healthy papaya plants. The mosaic symptoms presented at day 7 after inoculation. The inoculated PRSV plants were diagnosed by RT–PCR with primers specific for CP. RT–PCR result of PRSV infected sample indicated to 450-bp DNA fragment which was similar to positive control. The DNA sequence of amplicon resulted in a 100% match with PRSV CP.

To construct a recombinant CP, amplified CP fragments were ligated into pET28a(+) and named 6xHisCPpET28a(+). The recombinant protein plasmids were transformed to E. coli strain JM109 (DE3) and then cell cultures were induced by 0.8 M IPTG for 6 hrs to produce the proteins. The result of SDS-PAGE analysis demonstrated that the recombinant coat protein yielded a 36 kDa soluble protein.

**Pull down assay and LC–MS/MS analysis**

The pull-down protein complexes were identified by LC–MS/MS analysis. Two samples of protein complexes from an infected and non-infected plant interacted with CP were digested in-solution through the trypsin enzyme. Three replications were obtained for each sample. The peptide samples were subsequently processed and labeled through the free shotgun proteomic analysis by LC–MS/MS. The DeCyder MS differential analysis software was used to align and quantify the raw MS data. The MASCOT search engine was used to identify the differentially expressed proteins against the green plant proteins database. The peptide ion intensities of these proteins were normalized against each control sample and any proteins with an average ratio above 1.5 were selected from the overall data. Through the criteria listed above, 33 proteins were identified as potential host proteins that could have interacted with the PRSV CP. However, 12 plant proteins were unnamed and hypothetical proteins. The identified proteins are listed in Table 1. The biological functions of the identified proteins were classified into 10 groups which consist of transcription (22%), signal (17%), translation (13%), carbohydrate metabolism (9%), protein metabolism (9%), stress response (9%), photosynthesis (9%), nucleotide metabolism (4%), respiration (4%), and lipid metabolism (4%) (Figure 1).

The possible functional interactions of the identified proteins were predicted using STITCH 5 software based on the following analysis parameters: species (Arabidopsis thaliana); medium confidence score (0.04); and active prediction methods (all and no more than 10). RNA polymerase II transcription mediator (SWP) and mitochondrial processing peptidase (MPPBETA) were predicted to determine cell
division, transcription factors, electron transferase, respiration, proteasome subunit, translation initiation, hormones, and stress. On the other hand, Calmodulin-domain protein kinase (CDPK), Pentatricopeptide repeat-containing protein (OTP51), and Beta galactosidase 1 (BGAL) are presented in the STITCH diagram, but they were not interact with those proteins (Figure 2).

Table 1. PRSV coat protein interacting host proteins identified in PRSV infected papaya plant.

| No. | Protein name                        | GI number | UniProt accession number | Peptides        | Intensity ratio of PRSV-CP to PRSV infected plant | Intensity ratio of PRSV-CP to Native buffer |
|-----|------------------------------------|-----------|--------------------------|-----------------|-----------------------------------------------|-------------------------------------------|
| 1   | Beta galactosidase 1                | gi|15231354 | Q9SCW1                      | GHAMH           | 118.14                                     | 0                                          |
| 2   | Calmodulin-domain protein kinase (CDPK) | gi|15228830 | Q38870                      | IMGGPV          | 122.72                                     | 0                                          |
| 3   | Carbamoyl-phosphate synthase        | gi|255075411| C1E3Y9                      | GVTIPSMK        | 122.72                                     | 0                                          |
| 4   | Cysteine synthase like protein      | gi|141351505| Q767A2                      | PGNPYTH         | 122.56                                     | 0                                          |
| 5   | Doc                                 | gi|46401725 | Q06259                      | GLPGMSDPQ       | 100.67                                     | 0                                          |
| 6   | E2 polyprotein                     | gi|151367859| A7KYB7                      | VSEVLGGAGLPG    | 100.67                                     | 0                                          |
| 7   | Envelope glycoprotein              | gi|154361337| A7L258                      | AXGB1           | 119.74                                     | 0                                          |
| 8   | GIF1                                | gi|293651114| D5LST9                      | DAAAXX          | 100.67                                     | 0                                          |
| 9   | Glycosylphosphatidylinositol anchor attachment protein GAA1 (ISS) | gi|308807647 | -                           | SGDPAGHRS       | 100.67                                     | 0                                          |
| 10  | Heat shock protein 101              | gi|82408817 | Q2VDS9                      | KAHPA           | 100.67                                     | 0                                          |
| 11  | Hydroxyphenylpyruvate dioxygenase   | gi|154240639| A7BG64                      | AIVGVPN         | 118.91                                     | 0                                          |
| 12  | LHC II Type III chlorophyll a/b binding protein | gi|405615 | Q39340                      | SSSFPN          | 100.67                                     | 0                                          |
| 13  | Mitochondrial processing peptidase  | gi|15232845 | Q42290                      | AIQGPI          | 119.20                                     | 0                                          |
| 14  | Myb-like protein                    | gi|27261073 | Q8GVH9                      | TGGSAANGMD      | 118.22                                     | 0                                          |
| 15  | NADH dehydrogenase, partial (chloroplast) | gi|12656882 | O46939                      | KXXGP           | 119.52                                     | 0                                          |
| 16  | Nephrocystin-4-like protein         | gi|159469538| A8IVX5                      | VVRGA1S         | 118.50                                     | 0                                          |
| 17  | Pentatricopeptide repeat-containing protein | gi|240254468| Q9XIL3                      | KVGDFAKA        | 100.67                                     | 0                                          |
| 18  | Putative lipoygenase               | gi|27436755 | Q8H016                      | THIAG           | 120.96                                     | 0                                          |
| 19  | Ribosomal protein L12 homolog      | gi|2331135 | O22386                      | ARIAS           | 100.67                                     | 0                                          |
| 20  | Ribosomal protein S2               | gi|11465943 | P06355                      | DAIS1           | 118.31                                     | 0                                          |
| 21  | Ribulose-1,5-bisphosphate carboxylase | gi|10179652 | Q9GE62                      | QPGGKX          | 119.41                                     | 0                                          |
| 22  | RNA polymerase II transcription mediator | gi|15229344 | Q5R0R2                      | DSGSKP          | 117.09                                     | 0                                          |
| 23  | RNA-dependent RNA-polymerase        | gi|57540396 | Q3ZN03                      | AAFMA           | 117.92                                     | 0                                          |

**Discussion**

The interplay between viral and host proteins is particularly important to understand the mechanisms and functions of the PRSV infection and host cellular response. The functions of most interacting proteins were classified and were related to signaling, transcription, and translation processes. Some interacting protein partners were involved in stress response and plant physiology. However, some interacting proteins produced results similar to those previously reported, such as in *alfalfa mosaic virus*, with the CP binding to 3'-terminal RNA-dependent RNA-polymerase (RdRp) at the initiation site resulting in an in vitro antisense strand synthesis (Reichert et al. 2007). In addition, RdRp was reported to be induced by viruses, which belong to post-transcriptional gene silencing (PTGS) (Ratcliff et al. 1997; Al-Kaff et al. 1998). Xie
et al. (2001) found that the activity of a tobacco RdRp was increased at the time of virus infection. This indicated that RdRp played an important role in the infection cycle or for suppression of immunity defense, while being targeted by antiviral responses (Shen et al. 2020). It also indicated that CP Potato virus X (PVX) activated a membrane bound calcium/calmodulin dependent kinase (CDPK), in addition to playing a key role in the activation of an early defense response Arabidopsis thaliana, which was related to phosphorylation targets in abiotic stress signaling (Perraki et al. 2017).

To quantify the correlation between the identified proteins, we used the STITCH (Search Tool for Interacting Chemicals) network program to predict the protein interaction networks as well as the interacting proteins and small molecules. The STITCH results showed four large groups of interacting proteins were related to each other. The four groups were divided into group 1: transcription factors and cell division; group 2: hormones and stress; group 3: mitochondrial electron transferase and respiration; and group 4: proteasome. We found that 5 out of 23 interacting proteins were detected in the STITCH network. SWP (RNA polymerase II transcription mediator) and MPPBETA (Mitochondrial processing peptidase) are involved in all these four groups. SWP is a component of the Mediator complex, a co-activator involved in the regulating transcription of nearly all RNA polymerase II-dependent genes. This Mediator functions as a bridge to convey information from gene-specific regulatory proteins to the basal RNA polymerase II transcription machinery. The Mediator complex, having a compact conformation in its free form, is recruited to promote direct interactions with regulatory proteins and serves in the assembly of a functional pre-initiation complex with RNA polymerase II. MPPBETA, whose probable function was identified as mitochondrial processing of peptidase subunit beta, mitochondrial; cleaving pre-sequences (transit peptides) from mitochondrial protein precursors, belongs to the peptidase M16 family.

Interestingly, we found that CPK2, OTP51 (Pentatricopeptide repeat-containing protein), and BGAL1 interacted candidates protein do not play role in the interaction map. Modes of action are shown in different color lines. Blue circles indicate identified proteins in this study.

![Diagram of protein interactions](image_url)

**Figure 2.** The involvement of 23 candidate proteins in the intrinsic and extrinsic cellular pathway predicted by STITCH. Based on the online STITCH 4.0 database, an association of 23 candidate proteins. SWP (RNA polymerase II transcription mediator) and MPPBETA (Mitochondrial processing peptidase) are involve were predicted to involve in the biological processes. CPK2 (Calmodulin-domain protein kinase), OTP51 (Pentatricopeptide repeat-containing protein) and BGAL1 interacted candidates protein do not play role in the interaction map. Modes of action are shown in different color lines. Blue circles indicate identified proteins in this study.
E2 polyprotein, envelop glycoprotein, NADH dehydrogenase, and RNA polymerase II transcription mediator interacting proteins were predicted to mediate in the plant-viral interaction and host immune response. Interestingly, RDRs (a member of the RNA polymerase II transcription Mediator) was found to play an important role in a plant’s antiviral defense mechanism via silencing-mediated regulation of the cellular mRNA encoding resistance factors. On the other hand, the level of RDR expression was induced by salicylic acid (SA) and jasmonic acid (JA) in *Nicotiana attenuate* when challenged by the TMV. However, the RDR gene regulation is still not well understood (Xie et al. 2001; Yu et al. 2003; Schwach et al. 2005; Wassenegger and Krczal 2006; Qin et al. 2017).

Viral infection damages a plant’s physiological systems. A consequence of deformed pathway shows up as mosaic or yellowing in plants. The infection interferes with the process of photosynthesis. A range of symptoms can be exhibited, based on whether or not the host–virus combination is incompatible or compatible (Almási et al. 2001). In our study, the PRSV CP interacted with LHC II type III chlorophyll a/b binding protein and ribulose-1, 5-bisphosphate carboxylase. It is a major protein involved in photosynthesis and is located in the cytoplasm and chloroplast. Dietzgen and Zaitlin (1986) reported that the function of TMV coat protein was to reduce the amount of the most abundant host proteins-ribulose bisphosphate carboxylase-oxygenase (rbcS or rubisco) and is one of the most common damages caused by viruses which causes mosaic and yellowing disease. In tobacco plants, *cucumber mosaic virus* (CMV) variants containing different CP mutations result in chlorosis symptoms at varying levels of severity, which is correlated with down regulation of photosynthesis-related genes (CPRGs) (Mochizuki et al. 2014).

Our findings confirm that networks of coat protein of potyvirus and host protein interaction are highly connected, with some proteins playing the role of hubs. However, interacted proteins results of this study did not directly connect with HC-Pro interacted protein that Siriwan et al. (2014) reported. Bosque et al. (2014) represented HC-Pro protein might interact with CP through CI, VPG and NlaPro or intermediary plant proteins. It might require a systems approach such as BiFC method and the pool of reliable intraviral interaction tested and detected increases and topology analysis to be fully described on data set of this study and previously reported. More detail and systematic understanding of how viral protein interact with each other, and with host protein might allow to developing new drugs and treatment that block viral replication.

With regards to a simplistic plant pathological and plant management viewpoint, functional proteomics via viral and plant protein interaction may help to understand how a virus enters a plant cell and how the plant mounts a defense. Additionally, investigating plant virus infectious cycles and host-potyvirus interactions is essential for the development of anti-viral strategies and resistant cultivars.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This research was supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education (AG-BIO/PERDO-CHE).

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