Investigation of the Effects of P1 on Hc-Pro-Mediated Gene Silencing Suppression Through Genetics and Omics Approaches

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Abstract

Background: Viral suppressor negatively controls posttranscriptional gene silencing (PTGS) by inhibiting microRNA (miRNA) and short-interfering RNA (siRNA) regulation in plants. The first identified viral suppressor P1/HC-Pro is a fusion protein. Upon infecting plants, the P1 protein itself gets released from HC-Pro by the self-cleaving activity of P1. P1 has an unknown function in enhancing HC-Pro-mediated PTGS suppression. We performed proteomics to identify P1-interacting proteins and observed whole gene correlations in P1/HC-Pro-mediated PTGS suppression through transcriptomic studied comparative networks.

Results: First, we demonstrated that P1 enhances HC-Pro function and that the mechanism might work through the P1 binding to VERNALIZATION INDEPENDENCE 3/SUPERKILLER8 (VIP3/SKI8), a subunit of the exosome, to interferewith the 5'-fragment of the PTGS-cleaved RNA degradation product. Second, specifically the AGO1 waspseudotranslational degraded in transgenic Arabidopsis expressing P1/HC-Pro of turnip mosaic virus (TuMV) (P1/HC<sup>Tu</sup> plant). Third, transcriptomic comparative network highlighted critical genes in PTGS, including miRNA targets, calcium signaling, hormone (JA, ET, and ABA) signaling, and defense response.

Conclusion: Through these transgenic and omics approaches, we revealed an overall perspective and new findings in our understanding of the mechanism of P1/HC-Pro-mediated PTGS suppression. Many of the critical genes that were significantly impacted in the omics profiles will be further investigated by CRISPR-knockout or gain-of-function to understand PTGS in plant better.

Background

Posttranscriptional gene silencing (PTGS) includes the regulation of microRNA (miRNA) and short-interfering RNA (siRNA) in plant development. The DICER-LIKE 2 (DCL2)/DICER-LIKE 4 (DCL4)-mediated siRNA pathway is a major defense system to inhibit virus infection. However, different species of viruses have developed various suppressors to counteract DCL2/4-mediated siRNA defense system, becoming capable of surviving and multiplying in the infected plant, called as PTGS suppression. Viral suppressors not only suppress the siRNA defense but also inhibit miRNA regulation, resulting in the symptom development. Symptoms represent the misregulation of the miRNA phenomena, whereas a mutant virus has a defective suppressor causes mild symptoms and has a limited inhibitory effect on miRNA-regulation (Kung et al. 2014; Wu et al. 2010).

P1/HC-Pro is the first identified viral suppressor (Anandalakshmi et al. 1998; Kasschau and Carrington 1998). HC-Pro is a highly conserved protein in potyvirus that plays a major role in PTGS suppression (Kasschau and Carrington 1998; Kasschau et al. 2003; Kung et al. 2014; Valli et al. 2006). In contrast, P1 is a highly divergent protein that has variable sequences in each potyvirus. The P1 of tobacco etch virus (TEV) can enhance the HC-Pro-mediated PTGS suppression; however, little is known about the mechanism (Kasschau and Carrington 1998; Martínez and Daròs 2014; Valli et al. 2006). Martínez and Daròs (2014) demonstrated that the P1 of TEV interacts with the 60S ribosomal subunit and enhances in vitro translation.

Previous studies demonstrated that P1/HC-Pro genes of zucchini yellow mosaic virus (ZYMV) and turnip mosaic virus (TuMV) suppressed miRNA-regulation (Kung et al. 2014; Wu et al. 2010). Transgenic Arabidopsis expressing P1/HC-Pro of ZYMV (P1/HC<sup>Zy</sup> plant) or P1/HC-Pro of TuMV (P1/HC<sup>Tu</sup> plant) showed severe serrated and curling leaf phenotypes that are related to miRNA-misregulation and viral symptom development (Kung et al. 2014; Wu et al. 2010). Moreover, the FRNK motif (highly conserved amino acid sequence) of HC-Pro in TuMV and ZYMV is necessary and sufficient for PTGS suppression (Kung et al. 2014; Wu et al. 2010).

High-throughput omics approaches provide a powerful strategy to investigate whole biological systems. Label-free proteomics by mass spectrometry provide comparative protein quantitation (Wong and Cagney 2010). Moreover, Pearson gene correlation network provides a different perspective of transcriptome data to efficiently identify critical genes in the interesting pathway (Cheng et al. 2018; Liu et al. 2014).
Through heterologous P1 and HC-Pro recombination, we showed that three different potyviral P1s have a conserved function of enhancing HC-Pro-mediated PTGS suppression. The immunoprecipitation (IP) profiles of the 3 viral P1s identified VIP3/SKI8, which is involved in the degradation of RNA-induced silencing complex (RISC)-cleaved 5′-fragment RNAs. In addition, the label-free proteomics profile and western blot analysis indicated that AGO1 undergoes posttranslational degradation in P1/HC\textsubscript{Tu} plants. Transcriptome network profiles from \textit{Arabidopsis thaliana} ecotype Columbia (Col-0) vs. P1/HC\textsubscript{Tu} plants provide an overall perspective to understand the relationship between genes in mechanism of PTGS suppression. These critical genes offer new directions for further investigation of the PTGS and P1/HC-Pro-mediated suppression.

### Materials And Methods

#### Plant material and transgenic plants

\textit{Arabidopsis thaliana} ecotype Col-0 and transgenic plants, P1/HC\textsubscript{Tu} plant, and P1/HC\textsubscript{Zy} plant (Wu et al. 2010) were used in this study. Arabidopsis seeds were surface sterilized and chilled at 4ºC for two days then sown on Murashige and Skoog (MS) medium with/without suitable antibiotics. The seedlings were transferred into soil after one week of germination at one-week-old. All plants were grown at 24ºC in a growth room with 16 h of light / 8 h of dark.

#### Transgenic plant construction

For P1/HC\textsubscript{Te} plant construction, the P1/HC-Pro gene of TEV was amplified from the pTEV-At17 plasmid (Agudelo-Romero et al. 2008) by polymerase chain reaction (PCR) with primer set: PteP1 (5′-CACCATGGCCTGACATCTT-3′) and MTEHC (5′-TCCAACATTGTAAGTTTT-3′). The PCR fragment was cloned into the pENTR/D-TOPO vector (Invitrogen) to generate pENTR-P1/HC\textsubscript{Te}. The pENTR vector was transferred into the pBCo-DC vector (Kung et al. 2014) using Gateway LR Clonase II Enzyme Mix (Thermo Fisher) to generate pBCo-P1/HC\textsubscript{Te}.

For P1\textsubscript{Tu} plant construction, the TuMV infectious clone was used as a template to amplify the P1\textsubscript{Tu} gene with primer set: Ptup1/MTuP1 (5′-TCAAAAGTGACCATCACATCTT-3′), and it was then cloned into the pENTR and pBCo-DC vectors following the above procedures to generate pBCo-P1. For the HC\textsubscript{Tu} plant resistant in basta, the TuMV infectious clone was used as template to amplify the HC\textsubscript{Tu} gene with primer set: PTuHC (5′-CACCATGAGTGCAGCAGGAGCC-3′)/MTuHC, and it was then cloned into the pENTR and pBCo-DC vectors following the above procedures to generate the pBCo-HC\textsubscript{Tu} fragment. A NheI site was introduced into the fusion form of the P1HC-Pro gene (P1HC\textsubscript{Tu}–FA) to generate a F\textsubscript{362}A substitution. Furthermore, the P1 and HC-Pro genes were amplified from the TuMV infectious clone (Niu et al. 2006) and constructed under 35S promoter to create the P1\textsubscript{Tu} and HC\textsubscript{Tu} plants, respectively. The pBCo-P1/HC\textsubscript{Te}, pBCo-P1\textsubscript{Tu}, pBCo-HC\textsubscript{Tu}, and pBCo-P1HC\textsubscript{Tu}–FA binary vectors were transferred into Col-0 by the floral-dipping method with the \textit{Agrobacterium tumefaciens} AB1 strain to generate the P1/HC\textsubscript{Te}, P1\textsubscript{Tu}, and HC\textsubscript{Tu} plants, respectively.

#### Construction of recombinated P1/HC-Pro genes

The infectious clones of TuMV, ZYMV, and TEV were used as templates to generate the recombinant P1/HC-Pro constructs. The P1 cleavage site in the recombinated gene had to be preserved in the recombinated constructs and the constructs were cloned into the pBCo binary vector (Kung et al. 2014) for \textit{Agrobacterium} mediated flower-dipping transformation.

#### Antibody production

For the TuMV P1 antibody, the N-terminus of the P1 (1-190 aa) of DNA fragment was amplified with primer sets: Ptup1-Nhel (5′-TATGGCTAGCATGGCGATGTTACATTTGCAGC-3′)/MTuP1570-Xhol (5′-GGTGCTGAGGCTGCAGAGATCTCCTCCTC-3′). For the ZYMV P1 antibody, the N-terminus of the P1 (1-142 aa) DNA fragment was amplified with primer sets PZyP1- Nhel (5′-TATGGCTAGCATGGCGCTCAGTTATTG-
For the TuMV HC-Pro antibody, the internal region of the HC-Pro (1-103 aa) DNA fragment was amplified with primer sets: PTuHC-Nhel (5'-TATGGCTAGCATGGGACCTCTCGAGGAGAAGACAC-3')/MTuHC309-Xhol (5'-GGTGCTCGAGATTAGTTATCATAGGCCGAC-3'). For the ZYMV HC-Pro antibody, the internal region of the HC-Pro (1-87 aa) DNA fragment was amplified with primer sets: PZyHC-Nhel (5'-TATGGCTAGCACAGGGGAGAATTCTCAACA-3')/MZyHC261-Xhol (5'-GGTGCTCGAGAGGTTATCATAGCCGAC-3'). For the TEV HC-Pro antibody, the internal region of the HC-Pro (1-99 aa) DNA fragment was amplified with primer sets: PTeHC-Nhel (5'-TATGGCTAGCACAGGGGAGGTTATCATAGCCGAC-3')/MTe399-Xhol (5'-GGTGCTCGAGATCAACCTCTCTCATCGGTGT-3').

All of the PCR fragments were digested with Nhel and Xhol and then ligated with the same restriction enzymes-digested pET-28a vector to generate pET-P1Tu, pET-P1Zy, pET-P1Te, pET-HC11u, pET-HCZy, and pET-HCTe. All of the pET28 plasmids were transformed into the E. coli BL21 strain for recombinant protein expression. All recombinant proteins were purified by fast protein liquid chromatography (FPLC) (AKTApurifier, GE Healthcare). One mg of recombinant protein with a 1 x volume of complete Freund’s adjuvant was injected into New Zealand white rabbits for the first injection. Following three injections consisted of 1 mg of protein mixed with a 1 x volume of incomplete Freund’s adjuvant. The IgG purification was followed with protocol of Chiu et al. (2013). The IgG was collected after 4 injections for western blot detection.

**Immunoprecipitation and in-solution protein digestion**

To identify the P1-interacting proteins, the 250 mg of 10-day-old seedlings (n = 6) were homogenized with 1 mL IP buffer (25 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 5% glycerol, and a protease inhibitor (Roche)), followed by centrifugation for 10 min at 4°C. IgG of α-P1Tu, α-P1Zy, and α-P1Te was used for the in vivo IP. The IP was performed by mixing 30 µl of washed Protein A Mag Sepharose™ Xtra ferrite beads (GE), IgG (30 µl per IP reaction) and lysate. The IP reaction was carried out at 4°C with gentle mixing for 3 h. The tube was then centrifuged at 300 g to pull-down the beads, and they were washed three times with 0.3 mL wash buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 5% glycerol, 0.1% Triton-X-100, and a protease inhibitor) to remove nonspecific binding. Finally, the beads were resuspended in 50 µL elution buffer (0.1 M glycine, pH 2.0) and reaction was mixed on a rotary at 4°C for 10 min. A total of 10 µL neutralization buffer (Tris-HCl, pH 8.0) was added to neutralize the reaction.

The proteins were dissolved in 6 M urea. A total of 15 mg of proteins from each time point were used for in-solution digestion. Proteins were reduced by incubation with 10 mM dithiothreitol (DTT) for 1 h at 29 °C and alkylated by 55 mM iodoacetamide (IAA) at room temperature for 1 h. This step was quenched by 55 mM DTT at 29 °C for 45 min. The concentration of urea was diluted to 1 M before the sample was subjected to proteolysis. Protein digestion was performed overnight at 29 °C using mass spectrometry grade modified trypsin (Promega) at a 1:50 trypsin/protein ratio. After overnight incubation, 0.1% TFA was added to stop the digestion. Finally, all remaining reagents from the in-solution digestion procedure were removed using a C18 stage tip.

**LC-MS/MS Analysis**

The high-performance liquid chromatography with tandem mass spectrometric (LC-MS/MS) was performed on an Orbitrap Fusion Lumos Tribrid quadrupole-ion trap mass spectrometer (Thermo Fisher Scientific) in Instrumentation center of National Taiwan University. Peptides were separated on an Ultimate System 3000 NanoLC System (Thermo Fisher Scientific). Peptide mixtures were loaded onto a 75 µm inner diameter (ID), 25 cm length C18 Acclaim PepMap NanoLC column (Thermo Scientific) packed with 2 µm particles with a pore of 100 Å. Mobile phase A was 0.1% formic acid in water, and mobile phase B was composed of 100% acetonitrile with 0.1% formic acid. A segmented gradient was set over 90 min from 2-35% solvent B at a flow rate of 300 nL/min. Mass spectrometry analysis was performed in a data-dependent mode with Full-MS (externally calibrated to a mass accuracy of < 5 ppm, and a resolution of 120,000 at m/z = 200), followed by high-energy collision activated dissociation (HCD)-MS/MS of the most intense ions in 3 s. HCD-MS/MS (resolution of 15,000) was used to fragment multiply charged ions within a 1.4 Da isolation window at a normalized collision energy of
32. An automatic gain control (AGC) target at 5e5 and 5e4 was set for MS and MS/MS analysis, respectively, with previously selected ions dynamically excluded for 180 s. The max injection time was 50 ms.

**Identification and quantitation of proteome by label-free labeling methods**

Quantitative proteomics was performed by label-free quantitative proteomic analysis. The raw MS/MS data were searched against the UniProt Knowledgebase/Swiss-Prot *Arabidopsis thaliana* protein database (Mar 2019 version) by using the Mascot 2.3 search algorithm via the Proteome Discoverer (PD) package (version 2.2, Thermo Scientific). The search parameters were set as follows: peptide mass tolerance, 10 ppm; MS/MS ion mass tolerance, 0.02 Da; enzyme set as trypsin and allowance of up to two missed cleavages; variable modifications included oxidation on methionine, deamidation on asparagine and glutamine residues, carbamidomethylation of cysteine residues. Peptides were filtered based on a 1% FDR. Protein quantification was computed by the abundance of ions extracted from the MS spectra of the corresponding peptides. Normalization method was set to total peptide amount.

**Whole-transcriptome analysis**

Total RNAs that were isolated from 10-day-old seedlings of Col-0, *P1*<sup>Tu</sup>, *HC*<sup>Tu</sup>, and *P1/HC*<sup>Tu</sup> plants (*n* = 3) were used for whole transcriptome deep sequencing by High Throughput Sequencing Core of Academia Sinica. The sequencing was accomplished by paired-end (2 × 125) strand-specific HiSeq sequencing (Illumina). The transcriptome was analyzed by ContigView system (www.contigviews.bioagri.ntu.edu.tw) of NGS core of National Taiwan University. For the network analysis in ContigViews, the 2-fold differentially expressed genes (DEG) between Col-0 and *P1/HC*<sup>Tu</sup> plants (*n* = 3) with an 80% passing rate were selected for the assay. Reads with 2-times log<sub>10</sub> FPKM values of genes under 1.14 were trimmed. At least 10 samples from Col-0, *P1*<sup>Tu</sup>, *HC*<sup>Tu</sup>, and *P1/HC*<sup>Tu</sup> profiles (*n* = 3) were selected to calculate the Pearson correlation with a 0.95 threshold for positive relation and a 0.9 threshold for negative relation. The network data mining was performed by ContigViews.

**Ethylene detection**

Three-week-old Col-0 and *P1/HC*<sup>Tu</sup> plants (*n* = 3) were individually sealed in the 1.5 L chambers at 24ºC with 16 h light / 8 h dark. Ethylene gas samples in (1 mL) were withdrawn and collected at 4, 24, 48, and 72 h and were analyzed by GC-8A gas chromatography (Shimadzu) equipped with a flame ionization detector (FID).

**Results**

**P1 enhances the severity of the HC<sup>Tu</sup>-mediated serrated leaf phenotype and PTGS suppression**

To further investigate the functions of P1 and HC-Pro in PTGS suppression, the *P1/HC-Pro* gene of TuMV was used in this study (Fig. 1). The *P1/HC*<sup>Tu</sup> plants showed a severe serrated and curled leaf phenotype (Fig. 1A and B). The translated P1/HC-Pro protein contains an F<sub>362</sub>/S<sub>363</sub> cleavage site (Fig. 1A), which can generate separated P1 and HC-Pro proteins through P1 cleavage (Fig. 1C). We generated two individual *P1*<sup>Tu</sup> and *HC*<sup>Tu</sup> plants that expressed the P1 and the HC<sup>−Pro</sup> genes of TuMV individually (Fig. 1A and B). The *P1*<sup>Tu</sup> plant showed normal development similar to the Col-0 plants, whereas the *HC*<sup>Tu</sup> plant showed mildly serrated leaves (Fig. 1B). In addition to the difference in the severity of the leaf phenotype, the size of *HC*<sup>Tu</sup> plant was larger than that of the *P1/HC*<sup>Tu</sup> plant (Fig. 1A, and B), suggesting that the released P1 enhances the HC-Pro-mediated serrated leaf phenotype.

In addition, an F<sub>362</sub>A substitution at the F<sub>362</sub>/S<sub>363</sub>-P1 cleavage site produced a P1HC-Pro fusion protein (P1HC<sup>Tu</sup>−<sup>FA</sup>) (Fig. 1A, and C). This transgenic *P1HC*<sup>Tu</sup>−<sup>FA</sup> plant showed a normal phenotype (Fig. 1B), suggesting that the separation of P1 from HC-Pro is necessary and sufficient to develop the serrated leaf phenotype. Furthermore, a kanamycin-resistant *HC*<sup>Tu</sup> plant [HC<sup>Tu</sup> (kan) plant] was generated for crossing with the *P1*<sup>Tu</sup> plant (Basta resistant).
(Fig. 1A and B). Like the HC<sup>Tu</sup> plant, the HC<sup>Tu</sup>(kan) plant showed mildly serrated leaves (Fig. 1B). Interestingly, the P1<sup>Tu</sup><span class="math"></span> HC<sup>Tu</sup>(kan) offspring showed severely serrated and curled leaves, but the size of P1<sup>Tu</sup> × HC<sup>Tu</sup>(kan) plant was larger than that of the P1/HC<sup>Tu</sup> plant (Fig. 1B). In addition, only the P1/HC<sup>Tu</sup> plant showed high levels of the P1 and HC-Pro proteins; while the other lines, even the P1<sup>Tu</sup> × HC<sup>Tu</sup>(kan) plant, showed low levels of P1 and HC-Pro (Fig. 1C), suggesting that P1HC-Pro fusion and the separation of P1 from HC-Pro must both occur in the transgenic cells to enhance the serrated leaf phenotype.

We compared 57 potyvirus amino acid sequences of P1/HC-Pro (Fig. 2A). The alignment results showed that the sequence and length of the P1 protein in different potyviruses are highly diverse (Fig. 2A). Only the C-terminal protease activity site (black boxes) is conserved (Fig. 2A). In contrast, several conserved domains of HC-Pro were found in different species (Fig. 2A). We evaluated the phenotype of the P1/HC<sup>Tu</sup> plant and the P1/HC<sup>Te</sup> plant (Fig. 1A, and B). Both plants showed a severe serrated and curled leaf phenotype with high levels of P1 and HC-Pro (Fig. 1D). The results indicated that the P1/HC-Pro gene from various species can trigger a serrated leaf phenotype.

The next question was whether the function of the HC-Pro from each virus requires the P1 from the same species. We generated 6 recombinant P1/HC-Pro plants in which HC-Pro was fused with a heterologous P1, namely, P1<sup>Tu</sup>/HC<sup>Tu</sup>, P1<sup>Tu</sup>/HC<sup>Tu</sup>, P1<sup>Tu</sup>/HC<sup>Tu</sup>, P1<sup>Tu</sup>/HC<sup>Tu</sup>, P1<sup>Tu</sup>/HC<sup>Tu</sup>, and P1<sup>Tu</sup>/HC<sup>Tu</sup> (Fig. 2B, and C). The P1<sup>Zy</sup>/HC<sup>Tu</sup> and P1<sup>Te</sup>/HC<sup>Tu</sup> plants, similar to the other recombinant transgenic plants, showed a severe serrated leaf phenotype (Fig. 2C). In addition, the P1<sup>Zy</sup>/HC<sup>Tu</sup> and P1<sup>Te</sup>/HC<sup>Tu</sup> plants showed detectable P1 and HC-Pro expression (Fig. 1D). These results suggest that multiple P1 genes have conserved functions in enhancing the HC-Pro-mediated serrated leaf phenotype.

**HC-Pro-mediated PTGS suppression**

Previous studies demonstrated that an abnormal accumulation of miRNA and miRNA* occurs in several transgenic viral suppressor plants because suppressors interfere miRNA biogenesis (Kasschau et al. 2003; Kung et al. 2014; Wu et al. 2010). In addition to miRNA/miRNA* accumulation, miRNA targets were also upregulated in the transgenic plants because of miRNA misregulation (Kasschau et al. 2003; Kung et al. 2014; Wu et al. 2010). Therefore, phenomena of abnormal miRNA/miRNA* and target RNA accumulation are the molecular phenotypes of PTGS suppression. Except for the P1HC<sup>Tu</sup> - FA plant, all transgenic lines that contained HC<sup>Tu</sup> showed abnormal miRNA and miRNA* accumulations (Fig. 1E), confirming that HC<sup>Tu</sup> is the dominant player in PTGS suppression. Surprisingly, the P1<sup>Tu</sup> plant also showed miRNA and miRNA* accumulation through an unknown mechanism (Fig. 1E). The P1/HC<sup>Tu</sup>, P1/HC<sup>Te</sup>, and 6 recombinant P1/HC plants also showed identical miRNA/miRNA* accumulations (Fig. 2D). Transcriptome profiles also indicated that miRNA targets were upregulated in HC<sup>Tu</sup>, HC<sup>Tu</sup>(kan), P1<sup>Tu</sup> × HC<sup>Tu</sup>, and P1/HC<sup>Tu</sup> plants (Fig. 1F), suggesting that miRNA regulation was blocked by HC-Pro. However, DICER-LIKE 1 (DCL1; miR162 target) and two translation inhibition genes, APETALA 2 (AP2; miR172target) and SHORT VEGETATIVE PHASE (SVP; miR396 target), showed no change in their transcript levels (Fig. 1F). Except for the DCL1, AP2, and SVP genes, the P1/HC<sup>Tu</sup> plant suppressed most of the miRNA-target regulations (Fig. 1F). We conclude that the P1/HC<sup>Tu</sup> plant has a stronger suppressive effect than the HC<sup>Tu</sup> plants. In addition, the heterologous P1s have conserved function(s) in enhancing the HC-Pro-mediated PTGS suppression.

**Identification of host P1-interacting proteins**

We hypothesize that various P1 proteins have (a) conserved interacting protein(s) in Arabidopsis that enhance HC-Pro-mediated PTGS suppression. To identify the host P1-interacting proteins, the P1/HC<sup>Tu</sup>, P1/HC<sup>Tu</sup>, and P1/HC<sup>Te</sup> plants were used for IP with a-P1<sup>Tu</sup>, a-P1<sup>Zy</sup>, and a-P1<sup>Te</sup> antibodies, respectively. These IP eluates were analyzed by LC-MS/MS. We identified 101 cytoplasmic P1 of TuMV (P1<sup>Tu</sup>)-interacting proteins (Supplementary Data). Furthermore, we identified 56 cytoplasmic P1 of ZYMV (P1<sup>Zy</sup>)-interacting proteins and 20 cytoplasmic P1 of TEV (P1<sup>Te</sup>)-interacting proteins (Supplementary Data). Importantly, only one consensus cytoplasmic protein, VERNALIZATION INDEPENDENCE 3/ SUPERKILLER8 (VIP3/SK8; AT4G29830), was found in the IP profiles of 3 viral
P1s (Table 1). VIP3/SKI8 is a subunit of the RNA exosome complex that is required for degradation of the RISC 5′-cleavage fragment (Branscheid et al. 2015; Orban and Izaurralde 2005). In contrast, 12 consensus cytoplasmic proteins were identified in the P1Tu and P1Zy IP profiles, whereas 10 consensus proteins were identified in the P1Tu and P1Te IP profiles (Table 1). Moreover, 5 consensus cytoplasmic proteins were found in the P1Zy and P1Te IP profiles (Table 1).

### Table 1
The P1 interacting proteins

| AGI    | Protein Name | Description                                      | Interacting with |
|--------|--------------|--------------------------------------------------|------------------|
|        |              |                                                  | P1Tu  | P1Zy | P1Te |
| AT4G29830 | VIP3/SKI8  | WD repeat-containing protein                      | +a    | +    | +    |
| AT5G61780 | TSN2        | Ribonuclease TUDOR 2                              | +     |      |      |
| AT5G07350 | TSN1        | Ribonuclease TUDOR 1                              | +     |      |      |
| AT3G13300 | VSC         | VARICOSE                                         | +     |      |      |
| AT2G15430 | DdRp        | DNA-directed RNA polymerases subunit 3            | +     |      |      |
| AT3G18165 | MOS4        | Modifier of SNC1,4                               | +     |      |      |
| AT1G79280 | NUA         | Nuclear-pore anchor (NUA)                         | +     |      |      |
| AT5G53480 | Importin    | Importin subunit beta-1                          | +     |      |      |
| AT4G16143 | Importin    | Importin subunit alpha-2                         | +     |      |      |
| AT3G43300 | BIG5        | Brefeldin A-inhibited guanine nucleotide-exchange protein 5 | +     |      |      |
| AT3G47810 | VSP29       | Vacuolar protein sorting-associated protein 29   | +     |      |      |
| AT5G24780 | VSP1        | Vegetative storage protein 1                     | +     | +    |      |

Beta-D-
| Gene ID      | Gene Name                                      | Protein Name                                      |   |   |
|-------------|-----------------------------------------------|--------------------------------------------------|---|---|
| AT1G52400   | glucopyranosyl abscisate beta-glucosidase     | +                                                 | + |   |
| AT1G53310   | Phosphoenolpyruvate carboxylase 1             | +                                                 | + |   |
| AT1G16460   | Thiosulfate/3-mercaptopropane sulfurtransferase 2 | +                                                 | + |   |
| AT3G27300   | Glucose-6-phosphate 1-dehydrogenase, cytoplasmic isoform 1 | +                                                 | + |   |
| AT2G23930   | Probable small nuclear ribonucleoprotein G    | +                                                 | + |   |
| AT2G31390   | Probable fructokinase-1                       | +                                                 | + |   |
| AT3G62830   | UDP-glucuronic acid decarboxylase 2           | +                                                 | + |   |
| AT5G03630   | Monodehydroascorbate reductase 2             | +                                                 | + |   |
| AT3G52560   | Ubiquitin-conjugating enzyme E2 variant 1D    | +                                                 | + |   |
| AT1G64520   | 26S proteasome non-ATPase regulatory subunit 8 homolog A | +                                                 | + |   |
| AT1G08830   | SOD1                                          | Superoxide dismutase [Cu-Zn] 1                   | + |   |
| AT3G55620   | Eukaryotic translation initiation factor 6 – 2 | +                                                 | + |   |
| AT5G41220   | GST                                          | Glutathione S-transferase T3                     | + |   |
| AT4G24190   |                                               | Endoplasmin homolog                              | + |   |
| AT5G42980   |                                               | Thioredoxin H3                                   | + |   |
| Gene ID       | Protein Name                          | Enzyme/Function                                      | P1/Tu | HC/Tu |
|--------------|--------------------------------------|------------------------------------------------------|-------|-------|
| AT1G72730    | Eukaryotic initiation factor 4A-3    | +                                                    |       |       |
| AT1G77760    | Nitrate reductase [NADH] 1            | +                                                    |       |       |
| AT1G78370    | GST                                  | +                                                    |       |       |
| AT3G61220    | (+)-neomenthol dehydrogenase         | +                                                    |       |       |
| AT3G06650    | ATP-citrate synthase beta chain protein 1 | +                                              |       |       |
| AT5G49460    | ATP-citrate synthase beta chain protein 2 | +                                              |       |       |
| AT5G44316    | Putative UPF0051 protein ABC19        | +                                                    |       |       |
| AT3G44300    | Nitrilase 2                          | +                                                    |       |       |

The protein was identified in the relevant P1 transgenic plants and marked as “+”.

Next, we focused on P1-Tu-interacting proteins because the P1/HC<Tu> plant was the model used in this study. In the P1-Tu IP profile, two TUDOR-SN ribonucleases [(TSN1 (AT5G07350) and TSN2 (AT5G61780)] were uniquely identified 5 to 6 times in a total of 6 IP experiments with P1/HC<Tu> plants (Table 1, and Supplementary Table 1). TSN1 and TSN2 have been suggested to be involved in the regulation of uncapping mRNA and localize to processing bodies (P-bodies) and stress granules (Yan et al. 2014). These data suggested that P1-Tu might alter the function of TSN1 and TSN2. Moreover, VARICOSE (VSC; AT3G13300) and MODIFIER OF SNC1,4 (MOS4; AT3G18165), which are involved in RNA regulation, were identified in the P1-Tu IP profile (Table 1, and Supplementary Table 1). We also identified the NUCLEAR-PORE ANCHOR (NUA; AT1G79280), two IMPORTIN subunits (AT5G53480 and AT4G16143), and BREFELDIN A-INHIBITED GUANINE NUCLEOTIDE-EXCHANGE PROTEIN 5 (BIG5; AT3G43300), which are involved in nuclear and cytosolic transport (Table 1, and Supplementary Table 1) (Xue et al. 2019). Moreover, VACUOLAR PROTEIN SORTING-ASSOCIATED PROTEIN 29 (VSP29; AT3G47810) was identified, which participates in vacuolar protein trafficking and vacuolar sorting receptor recycling (Table 1, and Supplementary Table 1) (Kang et al. 2012).

Differentially expressed host proteins in transgenic plants

We performed label-free proteomics to identify the differentially expressed host proteins between Col-0 and other transgenic plants. We identified 2,757 Arabidopsis proteins in Col-0, P1<Tu>, HC<Tu>, and P1/HC<Tu> plants (Supplementary Data). We found that ADP-GLUCOSE PYROPHOSPHORYLASE (APL3; AT4G39210), 6-PHOSPHOGLUCONOLACTONASE (PGL5; AT5G24420), and TONSEKU (TSK)-ASSOCIATING PROTEIN 1 (TSA1; AT1G52410) were decreased in P1<Tu> and P1/HC<Tu> plants but were not decreased in HC<Tu> plants compared to Col-0 plants (Fig. 3A-C, panel i). However, the transcript of APL3 showed no significant difference among the various transgenic plants, whereas PGL5 and TSA1 were upregulated in HC<Tu> and P1/HC<Tu> plants compared with Col-0 plants (Fig. 3A-C, panel ii). APL3 is a starch biosynthesis enzyme, whereas PGL5 is a catalyzed enzyme in the oxidative pentose-phosphate pathway (OPPP) (Lansing et al. 2020; Liu et al. 2019). TSA1 was induced by methyl
jasmonate (MeJA) and triggers endoplasmic reticulum (ER) body formation (Geem et al. 2019; Suzuki et al. 2005). These data indicated that P1<sup>Tu</sup> might trigger APL3, PGL5, and TSA1 protein degradation through an unknown type of posttranslational regulation.

Next, we identified differentially expressed proteins between the HC<sup>Tu</sup> and P1/HC<sup>Tu</sup> plants. Nine proteins, including 2 superoxide dismutase [SOD1 (AT1G08830), and SOD2 (AT2G28190)], COPPER CHAPERONE FOR SOD1 (CCS1; AT1G12520), and ENHANCED MIRNA ACTIVITY 1/SUPER SENSITIVE TO ABA AND DROUGHT 2 (EMA1/SAD2; AT2G31660), were increased in P1/HC<sup>Tu</sup> plants compared with HC<sup>Tu</sup> plants (Fig. 3D-G, panel i). The EMA1/SAD2 contains an importin-beta domain and negatively regulates in miRNA activity and also involved in abscisic acid (ABA) signaling (Cui et al. 2016; Panda et al. 2020; Wang et al. 2011).

In contrast, 8 photosystem proteins (ATCG00340, AT1G55670, AT1G31330, AT4G12800, AT1G52230, AT1G44575, ATCG00350, and AT2G20260) were decreased in the P1<sup>Tu</sup>, HC<sup>Tu</sup>, and P1/HC<sup>Tu</sup> plants compared with Col-0 (Fig. 3H-O, panel i). However, their transcript levels were not significantly different (Fig. 3H-O, panel ii). We also found that that PATHOGENESIS-RELATED GENE 5 (PR5; AT1G75040) was decreased in P1/HC<sup>Tu</sup> plants (Fig. 3P, panel i), whereas JASMONATE RESISTANT 1 (JAR1; AT2G46370) was decreased in HC<sup>Tu</sup> and P1/HC<sup>Tu</sup> plants compared with Col-0 (Fig. 3Q, panel i). Similarity, the transcript levels of PR5 and JAR1 were not significantly different between the plants (Fig. 3P and Q, panel ii). In summary, many instances of posttranslational regulations were occurred in the P1<sup>Tu</sup>, HC<sup>Tu</sup>, and P1/HC<sup>Tu</sup> plants.

**The posttranscriptional and posttranslational regulation of miRNA targets in P1/HC<sup>Tu</sup> plants**

CCS1 is involved in copper delivery, and SOD1 and SOD2 participate in Cu/Zn superoxide dismutase activities. The transcripts of these three genes are regulated by miR398 (Bouché 2010; Sunkar et al. 2006). However, there were a high level of CCS1, SOD1, and SOD2 accumulation in the HC<sup>Tu</sup> and P1/HC<sup>Tu</sup> plants, which corresponded to their transcript levels, indicating the P1/HC-Pro-mediated PTGS suppression (Fig. 3D-F, panel ii). Indeed, the transcript level of miR168-regulated AGO1 (AT1G48410) was high in HC<sup>Tu</sup> and P1/HC<sup>Tu</sup> plants compared with Col-0 (Fig. 3R, panel ii). Surprisingly, the level of AGO1 protein was decreased via an unknown mechanism in HC<sup>Tu</sup> and P1/HC<sup>Tu</sup> plants (Fig. 3R, panel i). The western blot data also indicated that the level of AGO1 was low in P1/HC<sup>Tu</sup> plants, but was normal expression similar to Col-0 in P1/HC<sup>Zy</sup> and P1/HC<sup>Te</sup> plants (Fig. 1G). These data suggested that the P1/HC-Pro of TuMV has a specific ability to trigger the posttranslational degradation of AGO1.

**Comparative gene-to-gene network and transcriptome analysis**

In the transcriptome analysis, we constructed a gene-to-gene correlation network to study PTGS suppression from a different perspective. First, we constructed a network for Col-0 vs. P1/HC<sup>Tu</sup> plants in the ContigViews system. A list of 2-fold DGEs between Col-0 and P1/HC<sup>Tu</sup> plants was used to generate a Pearson correlation network (Fig. 4). A group of positive correlations (red lines) and a group of negative correlations (green lines) are highlighted in the network (Fig. 4). Importantly, AGO1, AGO2 (AT1G31280), and AGO3 (AT1G31290) were present in the group of negative correlations (Fig. 4). AGO2 and AGO3 were positively correlated (red line) but had an indirect correlation with AGO1 through XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 7 (XTH7; AT4G37800) (Fig. 4). Notably, the transcripts of AGO1, AGO2, and AGO3 were upregulated, but the XTH7 transcripts were downregulated in the HC<sup>Tu</sup> and P1/HC<sup>Tu</sup> plants, suggesting that the AGOs and XTH7 might have opposite functions in PTGS (Fig. 3R, panel ii; Fig. 6A-C).

Next, we made two comparative networks, which were generated by a list of 2-fold DEGs between Col-0 and HC<sup>Tu</sup> plants or between Col-0 and P1<sup>Tu</sup> plants (Fig. 5A and B). The gene positions in the comparative networks were followed with the Col-0 vs. P1/HC<sup>Tu</sup> network for comparison (Fig. 4 and Fig. 5). The gene numbers in the Col-0 vs. HC<sup>Tu</sup> network were much less than in the Col-0 vs. P1/HC<sup>Tu</sup> network (Fig. 4 and Fig. 5A). However, the main genes involved in PTGS, such as AGO1, AGO2, AGO3, and XTH7 etc., were remained in the Col-0 vs. HC<sup>Tu</sup> network (Fig. 5A). This suggested the presence of a basic network backbone in the HC<sup>Tu</sup>-mediated PTGS suppression that occurs without the effects of P1<sup>Tu</sup>. In contrast, the Col-0 vs. P1<sup>Tu</sup> network only had 7 genes in 2
small groups that also presented in parts of the Col-0 vs. \(HC^\text{Tu}\) or Col-0 vs. \(P1/HC^\text{Tu}\) networks (Fig. 4 and Fig. 5). Moreover, \(XTH7\) had less than 49 connected genes in the Col-0 vs. \(HC^\text{Tu}\) network, whereas \(XTH7\) had 61 connections in the Col-0 vs. \(P1/HC^\text{Tu}\) network (Fig. 4 and Fig. 5A). These data indicated that the \(XTH7\) connection is variable in different networks, and it might play a significant role in PTGS suppression. Overall, the comparative network analysis, it highlights the effects of \(P1^\text{Tu}\) on \(HC^\text{Tu}\)-mediated PTGS suppression. It also explains why the \(P1/HC^\text{Tu}\) plant has a severe phenotype because of how many pathways were interfered with.

**Critical genes in the Col-0 vs. \(P1/HC^\text{Tu}\) network**

The importance of \(XTH7\) is not only in number of gene connections it has or that it is connected with \(AGO1\) and \(AGO2\); \(XTH7\) also had a negative-correlation with several miRNA targets in the Col-0 vs. \(P1/HC^\text{Tu}\) network, such as 2 auxin response transcription factor genes \([ARF3\ (AT2G33860), \text{and } ARF8\ (AT5G37020)]\), \(PHOSPHATE\ 2\ (PHO2;\ AT2G33770), \text{GROWTH-REGULATING FACTOR}\ 1\ (GRF1;\ AT2G22840),\ CCS1, SOD1,\ \text{and } SOD2\ (Fig. 5). However, \(ARF3, ARF8, PHO2, GRF1, CCS1, SOD1,\ \text{and } SOD2\ formed a positive correlation in the network (Fig. 4). These miRNA target transcripts were upregulated in \(HC^\text{Tu}\) and \(P1/HC^\text{Tu}\) plants because of PTGS suppression (Fig. 3E-F; panel ii; Fig. 6D-G). Moreover, \(SEP3\ (AT1G24260)\ showed negative correlations with \(XTH7, ARF3, ARF8,\ \text{and } SOD1\ (Fig. 5). In addition, \(SEP3\ transcript levels were lower in \(P1^\text{Tu}, HC^\text{Tu},\ \text{and } P1/HC^\text{Tu}\ plants (Fig. 6H). Notably, SOD1 was shown to have a physical interaction with \(P1^\text{Tu}\) and \(P1^\text{Te}\) (Table 1) and was also highlighted in the network, suggesting the importance of SOD1 in PTGS suppression.

Four miRNA targets, including \(TARGET\ \text{OF EARLY ACTIVATION}\ \text{TAGGED}\ 2\ (TOE2;\ AT5G60120),\ \text{and } 2\ \text{squamosa}\ \text{promoter-binding protein-like genes} [SPL13A\ (AT5G50570), \text{and } SPL13B\ (AT5G50670)],\ \text{were also found in group of negative correlation area, whereas ARABIDOPSIS THALIANA HOMEBOX PROTEIN 15 (ATHB-15; AT1G52150) and PHABULOSA (PHB; AT2G34710) were found in the boundary between the positive and negative correlations groups (Fig. 4). Notably, miR172b-regulated TOE2 modules in regulating plant innate immunity (Zou et al. 2018). In Col-0 vs. \(P1/HC^\text{Tu}\) network, \(CYCLING DOF FACTOR\ 2\ (CDF2; AT5G39660),\ \text{and } 2\ \text{carbon catabolite repressor 4 (CCR4)-associated factor genes [CAF1A (AT3G44260),}\ \text{and } CAF1B (AT5G22250)]\ \text{that are involved in RNA regulation were identified in the Col-0 vs. \(P1/HC^\text{Tu}\) network (Fig. 4). CAF1A and CAF1B catalyze mRNA deadenylation, whereas CDF2 interacts with DICER-LIKE 1 (DCL1) for miRNA biogenesis (Liang et al. 2009; Sun et al. 2015; Walley et al. 2010). These genes were also upregulated in \(HC^\text{Tu}\) and \(P1/HC^\text{Tu}\) plants (Fig. 6Q, R, and Y).

Eight calcium signaling genes were identified in the group of positive correlation in the network and were significantly upregulated in \(P1/HC^\text{Tu}\) plants (Fig. 4, and Fig. 6I-P). In the network, \(CALMODULIN-LIKE\ 24\ (CML24; AT5G37770),\ \text{and } CAM-BINDING PROTWIN 60-LIKE G (CBP60G; AT5G26920) have significantly functions in the regulation of autophagy and innate immunity, respectively (Qin et al. 2018; Tsai et al. 2013). In addition, jasmonic acid (JA) signaling and defense genes were highlighted in the positive correlation and their transcripts were upregulated in \(HC^\text{Tu}\) and \(P1/HC^\text{Tu}\) plants (Fig. 4, and Fig. 6Q-V). In addition, the network indicated that \(FUMARASE\ 2\ (FUM2; AT5G50950)\ \text{and } 2\ \text{BARELY ANY MERISTEM} 2\ (BAM2; AT3G49670)\ \text{were present in the boundary region between groups of positive and negative correlation (Fig. 4). BAM2 is a CLAVATA1-related receptor kinase and promotes the differentiation of stem cells on the meristem flank (DeYoung et al. 2006). FUM2 transcripts were upregulated in \(HC^\text{Tu}\) and \(P1/HC^\text{Tu}\) plants, whereas BAM2 transcripts were decreased (Fig. 6W and X). We also showed that \(CDF2\), which is involved in miRNA biogenesis (Sun et al. 2015), is in the negative correlation group and is indirectly connected (negative correlation) with \(AGO1\) through \(HB2\ (AT3G10520)\ \text{and } XTH7\ (Fig. 4). The \(CDF2\) transcripts were upregulated in \(HC^\text{Tu}\) and \(P1/HC^\text{Tu}\) plants (Fig. 6Y). The functions of critical genes in the Col-0 vs. \(P1/HC^\text{Tu}\) network are listed in Supplementary Table 2.

**The auxin, ethylene, and ABA signaling pathway in PTGS suppression**

The auxin response can induced ethylene and concomitantly trigger ABA biosynthesis (Hansen and Grossmann 2000). Importantly, the auxin, ethylene, and ABA signaling genes could be found in the Col-0 vs. \(P1/HC^\text{Tu}\) network (Fig. 4). MiRNA-regulated \(ARF3\) and \(ARF8\) targets are also auxin response genes, which were highly expressed in \(HC^\text{Tu}\) and \(P1/HC^\text{Tu}\) plants (Fig. 6D, and E). In contrast, BAM2 expressed is antagonistic with auxin
transcriptors (Cecchetti et al. 2015) and its transcripts were downregulated in HCTu and P1/HCTu plants (Fig. 6X). Ethylene signaling genes, 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6 (ACS6; AT4G11280), SCARECROW-LIKE 13 (SCL13; AT4G17230), and 8 ethylene responsive element binding factors [ERF1 (AT4G17500), ERF4 (AT3G15210), ERF5 (AT5G47230), ERF6 (AT4G17490), ERF105 (AT5G51190), ERF104 (AT5G61600), ERF12 (AT1G28360), ERF8 (AT1G53170)] were present in group of positive correlation and their transcripts were upregulated in HCTu and P1/HCTu plants (Fig. 4; Fig. 6Z-Ai; and Supplementary Table 2).

Moreover, results from endogenous ethylene emission experiments showed higher levels over a time courses in P1/HCTu plants compared with Col-0 (Fig. 1H). ABA signaling genes, SULFATE TRANSPORTER 3;1 (SULTR3;1; AT3G51895), and 2 DIVARICATA genes [DIV1 (AT5G58900), and DIV2 (AT5G04760)] were also highlighted in the Col-0 vs. P1/HCTu network (Fig. 4) (Chen et al. 2019; Fang et al. 2018). These data suggested that P1/HC-Pro-mediated PTGS suppression has even interfere with plant hormone signaling pathways.

**Discussion**

**P1 enhances HC-Pro-mediated PTGS suppression**

In this study, we demonstrated that 3 viral P1s have a conserved function in enhancing HC-Pro-mediated PTGS suppression. From the perspective of P1-host protein interaction, VIP3/SKI8 turns over the 5'-fragment of RISC-cleaved target RNA, whereas TSN1, TSN2, and VSC are involved in mRNA decapping in stress granules and P-bodies (Branscheid et al. 2015; Deyholos et al. 2003; Gutierrez-Beltran et al. 2015; Sorenson et al. 2018; Xu and Chua 2009). Moreover, a MOS4 modifier, 2 IMPORTINs, and BIG5, which are respectively that involved in RNA splicing and RNA transportation, also interact with P1Tu (Helizon et al. 2018; Kitakura et al. 2017; Luo et al. 2013; Xu et al. 2012). In addition, EMA1/SAD2 contains an importin-beta domain has negative regulation in miRNA activity (Wang et al. 2011). EMA1/SAD2 protein levels was upregulated in P1Tu, HCTu, and P1/HCTu plants, but their transcript levels have no difference with Col-0, suggesting P1 stables or increased EMA1/SAD2 levels to help in inhibition of miRNA regulation (Fig. 3G). Moreover, transcriptome data mining also showed that the CAF1A/B deadenylases and the CDF2 DOF zinc finger protein had a strong correlation to PTGS suppression. To summarize these findings, posttranscriptional RNA regulation occurs in stress-granules and P-bodies, and many RNA regulatory components were identified among the proteins that interacted with P1 or were highlighted in the PTGS suppression network, which suggests that P1 is extremely vital for HC-Pro to enhance the suppression.

**P1/HC-Pro of TuMV specifically primes posttranslational AGO1 degradation**

AGO1 degradation has been reported to be controlled by selective autophagy (Kobayashi et al. 2019; Li et al. 2019; Michaeli et al. 2019). The P0 viral suppressor of Polerovirus is thought to trigger autophagic AGO1 degradation (Michaeli et al. 2019). In our study, P1/HC-Pro of TuMV specifically triggered AGO1 posttranslational degradation, but the same effect was not observed in P1/HCTu and P1/HCTu plants, suggesting that P1/HC-Pro triggering AGO1 degradation is a viral species dependent manner. In the other words, AGO1 degradation might not essential for the entire mechanism of P1/HC-Pro-mediated PTGS suppression, because the result is not identical in all potyviral suppressors.

Autophagy works with vacuoles to allow for the degradation of large protein complexes. VSP29 is involved in the trafficking of vacuolar proteins and in the recycling of vacuolar sorting receptors and specifically interacts with P1Tu (Table 1) (Kang et al. 2012). Moreover, CML24 interacts with AUTOPHAGY GENE 4b (ATG4b), which is primes AUTOPHAGY GENE 8 (ATG8) by removing the C-terminus and exposing a glycine residue during autophagy (Tsai et al. 2013). CML24 found to be present in the group of positive correlation of PTGS network. Therefore, we implied that P1/HC-Pro of TuMV might also trigger AGO1 posttranslational degradation through autophagy.

**Network of HC-Pro-mediated PTGS suppression**

The comparative gene correlation network provides a 4-dimensional perspective, which includes gene expression, gene correlation, position, and time course. This information is helpful to interpret and identify critical genes in pathways of interest. Through the Co-0 vs. HCTu network, we identified a basic backbone
network in HC^{Tu}-mediated PTGS suppression. However, the effects of P1-enhanced HC-Pro suppression were highlighted in Col-0 vs. P1/HC^{Tu} network upon comparing the two networks. The Col-0 vs. P1/HC^{Tu} network specifically highlighted the relationship between the AGOs in PTGS and in relationship to viral resistance. Previous studies demonstrated that AGO2 and AGO3 were upregulated to enhance the viral resistance (Alazem et al. 2017; Harvey et al. 2011; Zheng et al. 2019). AGO2 is a target of miR403, which is negatively regulated by AGO1 (Harvey et al. 2011), suggesting the upregulation of AGO2 in response to AGO1 degradation in P1/HC^{Tu} plants. However, although we have no explanation for AGO3 upregulation, we assume that the AGO2/AGO3 antiviral system was activated and complemented AGO1 degradation. Indeed, AGO2 and AGO3 are directly positively correlated in the network.

Surprisingly, several miRNA targets, such as CCS1, SOD1, SOD2, PHO2, ARF3, and GFR1, showed a positive correlation in the network. These genes were indirectly negatively correlated with AGO1 through XTH7. In addition, the other miRNA targets, such as TOE2, SPL13A/B, ATHB-15, and PHB were also presented in the network. SPL13A and SPL13B had direct positive correlation. ATHB-15 and PHB, which belong to homeodomain-leucine zipper (HD-ZIP) transcription factor (TF) family, were also having positive correlation. To summarize, the gene correlation network had significant accuracy in data mining.

Calcium signaling has been demonstrated to be involved in the suppression of gene silencing (Anandalakshmi et al. 2000; Nakahara et al. 2012). Anandalakshmi et al. (2000) demonstrated that the calmodulin-related protein (rgs-CaM) in tobacco interacts with HC-Pro, and that it suppresses gene silencing similar to HC-Pro. Nakahara et al. (2012) demonstrated that tobacco rgs-CaM counterattacked various viral suppressors by binding to RNA-binding domains. In addition, rgs-CaM triggers autophagic viral suppressor degradation (Nakahara et al. 2012). Indeed, CML24 has physical interaction with ATG4b, suggesting that there is crosstalk between calcium signaling and autophagy (Tsai et al. 2013). CML24 was present in the group of positive correlation, which was opposite to the AGOs that were present in the group of negative correlation, suggesting that calcium signaling might counteract with gene silencing.

We noted that several genes had a significant number of connected genes (> 50 connected genes), such as XTH7, FUM2, and BAM2 (Fig. 4). XTH7 has been defined as a xyloglucan endotransglycosylase/hydrolase, however, little is known about its function in PTGS suppression. In addition, the cytosolic fumarase FUM2 is essential for Arabidopsis acclimation to low temperatures (Dyson et al. 2016). BAM2 is a CLAVATA1-related receptor kinase, and only a little is known about its involvement in anther and meristem development (DeYoung et al. 2006; Hord et al. 2006). Although the functions of these genes were not explicitly linked with PTGS or defense, they were present in critical positions within the network with a large number of connected genes, which provides information for future research directions to investigate PTGS.

**Auxin and ethylene signaling in the serrated leaf phenotype**

Current studies indicated that treatment with a high-dose of auxin elicits endogenous ethylene production. In P1/HC^{Tu} plants, 3 auxin signaling genes (ARF3, ARF8, and SUTR3;1) were upregulated; therefore, we assume that is the reason why ethylene was highly accumulated with increased expression of ethylene signaling genes. In addition, Hay et al. (2006) demonstrated that auxin can initiate marginal serrations in leaves, suggesting that the serrated leaf phenotype of P1/HC^{Tu} plants might be related to endogenous auxin accumulation.

**Conclusion**

In this study, we used a transgenic plant approach to investigate the functions of P1 and HC-Pro. By mining high-throughput data from proteomic and transcriptomics profiles, P1-interacting proteins and critical genes in PTGS suppression were identified. Instead of traditional DEG identification, the comparative gene correlation network provides a four-dimensional perspective to identify critical genes and provides new ideas and directions for further investigation and finding. Recently, CRISPR technology has been applied to achieve gene knock outs. The combination of high-throughput data-mining and CRISPR technology starts a new era to further investigate P1/HC-Pro-mediated PTGS suppression.
Abbreviations

ABA: Abscisic acid; ACC: 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID; ACS6: 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 6; AGC: Automatic gain control; AP2: APETALA 2; APL3: ADP-GLUCOSE PYROPHOSPHORYLASE; ARF3: Auxin response transcription factor gene 3; ARF8: Auxin response transcription factor gene 8; ATG4b: AUTOPHAGY GENE 4b; ATG8: AUTOPHAGY GENE 8; ATHB-15: ARABIDOPSIS THALIANA HOMEОBOX PROTEIN 15; BAM2: BARELY ANY MERISTEM 2; BIG5: BREFELDIN A-INHIBITED GUANINE NUCLEOTIDE-EXCHANGE PROTEIN 5; CAF1A: Carbon catabolite represor 4 (CCR4)-associated factor gene 1А; CAF1B: Carbon catabolite represor 4 (CCR4)-associated factor gene 1B; CBP60G: CAM-BINDING PROТWIN 60-LIKE G; CCS1: COPPER CHAPERONE FOR SOD1; CDF2: CYCLING DOF FACTOR 2; CML24: CALMODULIN-LIKE 24; Col-0: Columbia; DCL1: DICER-LIKE 1; DCL2: DICER-LIKE 2; DCL4: DICER-LIKE 4; DEG: Differentially expressed genes; DIV1: DIVARICATA gene 1; DIV2: DIVARICATA gene 2; DTT: Dithiothreitol; EMA1/SAD2: ENHANCED МИRNA ACTIVITY 1/SUPER SENSITIVE TO ABA AND DROUGHT 2; ER: Triggers endoplasmic reticulum; ERF: Ethylene responsive element binding factors; FID: flame ionization detector; FPKM: The fragments per kilobase of transcript per million; FPLC: Fast protein liquid chromatography; FUM2: FUMARASE 2; GRF1: GROWTH-REGULATING FACTOR 1; HD-ZIP: Homeodomain-leucine zipper; IAA: Iodoacetamide; ID: Inner diameter; IP: Immunoprecipitation; JA: Jasmonic acid; JAR1: JASMONATE RESISTANT 1; MejA: Methyl jasmonate; miRNA: microRNA; MOS4: MODIFIER OF SNC1:4; MS: Murashige and Skoog; NUA: NUCLEAR-PORE ANCHOR; OPP: Oxidative pentose-phosphate pathway; P-bodies: Processing bodies; PCR: Polymerase chain reaction; PD: Proteome Discoverer; PGL5: 6-PHOSPHOGLUCONOLACTONASE; PHB: PHABULOSA; PHO2: PHOSPHATE 2; PR5: PATHOGENESIS-RELATED GENE 5; PTGS: Posttranscriptional gene silencing; rgs-CaM: Calmodulin-related protein; RISC: RNA-induced silencing complex; SCL13: SCARECROW-LIKE 13; siRNA: Short-interfering RNA; SOD1: Superoxide dismutase 1; SOD2: Superoxide dismutase 2; SPL13A: Squamosa promoter-binding protein-like gene 13A; SPL13B: Squamosa promoter-binding protein-like gene 13B; SULTR3: 1: SULFATE TRANSPORTER 3:1; SVP: SHORT VEGETATIVE PHASE; TEV: Tobacco etch virus; TF: Transcription factor; TOE2: TARGET OF EARLY ACTIVATION TAGGED 2; TSA1: TONSOKU (TSK)-ASSOCIATING PROTEIN 1; TSN1: TUDOR-SN ribonucleases 1; TSN2: TUDOR-SN ribonucleases 2; TuMV: Turnip mosaic virus; VIP3/SKI8: VERNALIZATION INDEPENDENCE 3/ SUPERKILLER 8; VIP3/SKI8: VERNALIZATION INDEPENDENCE 3/ SUPERKILLER B; VSC: VARICOSE; VSP29: VACUOLAR PROTEIN SORTING-ASSOCIATED PROTEIN 29; XTH7: XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 7; ZYMV: Zucchini yellow mosaic virus

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysis in this study are in this published article.

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Competing interests
The authors declare that they have no competing interests.

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**Authors’ contributions**

SFH performed the experiments, WLW, RYF, HPW, and HFL contributed the transcriptome database and network analysis, SFH produce AGO1 antibodies, HYW performed proteomics analysis, PCL observed AGO1 degradation by western blot, MS, and CTW performed ethylene detection, SFH, NS, and SSL contributed to experiment design and wrote the paper.

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P1 enhances the HCTu-mediated phenotype and HCTu suppression in miRNA-mediated regulation. (A) Schematic binary plasmids containing the various constructs that were used in this study. (B) Phenotypes of the different transgenic plants. The photographs were taken of 3-week-old seedlings. Bar, 1 cm. (C) Detection of P1 and HC-Pro of TuMV in various transgenic plants by western blotting. The asterisk (tubulin) is an internal control. (D) Detection of P1 and HC-Pro in P1ZY/HCZy, P1Te/HCTe, P1Zy/HCTu, and P1Te/HCTu plants. The @ symbol indicates CDC2 as an internal control. Two-asterisk (**) indicates cross-reaction of the -HCZy antibodies. (E) Heatmaps of miRNA and miRNA* and (F) miRNA target genes expression in various transgenic plants. Significant upregulation (Student's t test; P value <0.05) is labeled in red. Gray indicates differential expression that is not significant. (G)AGO1 protein detection in different viral P1/HC-Pro transgenic Arabidopsis plants. One asterisk (*) indicates the AGO1 isoform. Two-asterisk (**) indicates cross-reaction of the -HCZy or -HCTe antibodies. Three-asterisk (***) indicates common bands. The @ symbol indicates RUBISCO as an internal control. (H) Time courses of endogenous ethylene detection in Col-0 and P1/HCTu plants. The bars represent standard deviations (n=3).
Figure 2

Variation in potyvirus P1/HC-Pro and recombined P1/HC-Pro plants. (A) Schematic diagram of P1 and HC-Pro amino acid sequence similarity among fifty-seven Potyviruses. The black boxes indicate conserved sequence regions. (B) Schematic of the binary plasmids containing the various recombined P1/HC-Pro constructs used in this study. (C) Phenotypes of various recombinant P1/HC-Pro transgenic plants. The photographs were taken of 3-week-old seedlings. Bar, 1 cm. (D) Heatmap of abnormal miRNA and miRNA* accumulation in various recombinant P1/HC-Pro plants.
The protein and transcript levels of critical genes in various transgenic plants. (A–R) Genes that had a significant difference in protein levels (panel ii) between Col-0 and P1/HCTu plants were identified and their transcript levels were observed (panel i) in Col-0, P1Tu, HCTu, and P1/HCTu plants. The fragments per kilobase of transcript per million (FPKM) was used to represent the normalized transcript expression. The bars represent standard deviations (n=3). Normalized abundances were used for representing the protein amounts.
The gene-to-gene network of Col-0 vs. P1/HCTu plants. The gene profiles of 2-fold DEGs between Col-0 and P1/HCTu plants were used to generate the Pearson correlation network. The different circle sizes indicate the numbers of correlated genes. A positive correlation (>0.95) between the two genes is indicated by a red line, whereas a green line indicates a negative correlation (< -0.9). The red circles indicate the genes involved in calcium signaling and are grouped with a red background. The blue circles indicate that the genes involved in the defense response and are grouped with a blue background. The green circles indicate the genes involved in the PTGS pathway and are grouped with a green background. The yellow circles indicate the genes that are the miRNA targets and are grouped with a yellow background. The gray circles indicate the genes involved in JA, ABA, and ethylene biosynthesis pathways and are grouped with a gray background.
The comparative networks for Col-0 vs. P1Tu and Col-0 vs. HCTu plants. (A) The gene profiles of 2-fold DEGs between Col-0 and HCTu plants were used to generate the Pearson correlation networks. (B) The gene profiles of 2-fold DEGs between Col-0 and P1Tu plants were used to generate the network. The different circle sizes indicate the number of correlated genes. A positive correlation (> 0.95) between the two genes is indicated by a red line, whereas a green line indicates a negative correlation (< -0.9). The unpresented genes and correlation lines are indicated in gray.
Figure 6

Transcript expression comparisons of critical genes in the networks (A-AI). Genes that showed a significant connection, function, or position in the network were selected to demonstrate their transcript expression. The fragments per kilobase of transcript per million (FPKM) was used to represent the normalized transcript expression. The bars represent standard deviations (n=3).

Supplementary Files

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