Validating Plant Genes Involved in Pepper Yellow Leaf Curl Indonesia Virus Infection Using VIGS in Model Plant Nicotiana benthamiana

(Validasi Gen-gen Tanaman yang Terlibat dalam Infeksi Pepper Yellow Leaf Curl Indonesia Virus Menggunakan VIGS pada Tanaman Model Nicotiana benthamiana)

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ABSTRACT

Pepper yellow leaf curl disease caused by Pepper yellow leaf curl Indonesia virus (PepYLCIV) has become a challenge to chili pepper cultivation. Development of resistant variety by utilizing recessive resistance gene is expected to control the disease in the field. This study aimed to validate three plant genes, namely deltaCOP, hsc70, and BAM1, in PepYLCIV infection by applying Virus-induced Gene Silencing (VIGS) in a model plant, wild type Nicotiana benthamiana. PepYLCIV and construct of Tobacco rattle virus (TRV) which inducting silencing of each gene were co-inoculated into N. benthamiana plants through agroinfiltration. Gene expression and the relative amount of viral DNA were determined by quantitative reverse transcription PCR (qRT-PCR) and quantitative PCR (qPCR), respectively, at 15 days post inoculation. The results showed a decreased level of deltaCOP, hsc70, and BAM1 expressions to 66.4%, 53.0%, and 47.0%, respectively, compared to that in the control (100%). Silencing of the three genes decreased the accumulation of PepYLCIV to 0.1%, 18.4%, and 63.0%, respectively, compared to that in the control. deltaCOP and hsc70 genes were indicated to be involved in the viral infection and could be good candidate genes for obtaining chili pepper varieties resistant to PepYLCIV. This result affirmed that the reverse genetics technique could be an alternative approach for identifying plant genes involved in viral infection, including PepYLCIV. The use of an infectious clone in this study allows the virus inoculations could be carried out without rearing and maintaining its natural vector, hence reduces the risk of virus transmission to healthy plants.

Keywords: PepYLCIV, VIGS, Nicotiana benthamiana, reverse genetics, quantitative PCR.
INTRODUCTION

Chili pepper (Capsicum annuum L.) is an important vegetable commodity in Indonesia. Its fresh and dried fruits have been widely used in culinary, for cosmetic and medicinal purposes, as well as chemical irritants. Ornamental chili pepper plants are bred and grown for their attractive color. Moreover, chili pepper has been cultivated in West Java, Central Java, North Sumatra, West Sumatra, and other provinces with total production of 1,206,750 tons in 2018 (Ministry of Agriculture 2016; Central Agency on Statistics 2019). According to Indonesian Central Agency on Statistics and Directorate General of Horticulture, the planting area of chili pepper was 142,547 hectares which increased by 15.51% from 2016 to 2017, while the productivity was 8.46 t/ha, slightly decreased by 0.13% (Ministry of Agriculture 2019).

Since 2000, the occurrence of pepper yellow leaf curl disease has become one of the main challenges in chili pepper cultivation. The disease has occurred in several cultivation areas in West Java, Central Java, Special Region of Yogyakarta (DIY), Lampung, Northern Sumatra, and West Sumatra (Sulandari et al. 2006; Trisno et al. 2009; Koeda et al. 2016). The disease symptoms are very unique, including vein thickening, bright yellowing, and curl upwards leaves (Sulandari et al. 2006; Tsai et al. 2006). The disease spreads easily and causes serious yield losses, especially because the causal agent, Pepper yellow leaf curl Indonesia virus (PepYLCIV), can be transmitted by whitely Bemisia tabaci (Gennadius). This insect vector is polyphagous, distributes worldwide, and has complex genetic diversity (De Barro et al. 2008; CABI 2019). The use of synthetic chemical insecticides against the pest may cause serious health problems, harm other organisms, and contaminate the environment. Thus, cultivating resistant variety is expected to control the disease in the field and minimize the negative effects of insecticides. To date, chili pepper varieties resistant to the disease are still being underway and have not been released yet.

Virus-resistant varieties can be obtained through utilization of recessive resistance gene in crop species. It has been reported that about half of known virus resistance genes are recessively inherited (Kang et al. 2005; Truniger and Aranda 2009). The genes have been identified in cultivars and several mutagenized experimental host plants (Díaz-Pendon et al. 2004; Hashimoto et al. 2016). Suppression on the expression of these genes affected low virus infection and multiplication efficiency at the single-cell level and also in the cell-to-cell movement (Yamanaka et al. 2002; Truniger and Aranda 2009). Although the host range of virus associated with pepper yellow leaf curl disease had been studied (Sulandari et al. 2006), genes and other host factors involved in PepYLCIV infection have not been reported.

Identification of host genes can be done by forward and reverse genetic approaches using model plants, screening for interactors with viral proteins and components of protein complexes containing viral factors, genome-wide screening using the heterologous yeast system, and identification from naturally occurring resistant cultivars (Hashimoto et al. 2016). Virus-induced Gene Silencing (VIGS) is one of the reverse genetics techniques that has been successfully used in analyzing the function of biotic and abiotic stress-related genes in monocots and dicots (Scofield and Nelson 2009; Unver and Budak 2009; Gilchrist and Haughn 2010; Ramegowda et al. 2014). Lozano-Durán et al. (2011) engineered a transgenic 2IRGFP N. benthamiana plants and combined with VIGS using Tobacco rattle virus (TRV) vectors to identify the genes involved in a geminivirus infection. They identified seven genes that had potential antiviral effects, whereas the other eleven genes were required for a full infection of Tomato yellow leaf curl Sardinia virus (TYLCSV).

Three genes were analyzed in this study. These include Coatomer subunit delta (deltaCOP), heat shock cognate 70 (hsc70), and BARELY ANY MERISTEM 1 (BAM1). deltaCOP is required for protein transport, while hsc70 might play a role in rapid protein maturation, turnover during a short virus multiplication cycle, and virus cell-to-cell movement (Lozano-Durán et al. 2011). Meanwhile, Rosas-Díaz et al. (2018) reported that BAM1 protein interacts with TYLCSV C4 protein and is required for cell-to-cell movement of silencing. The expression of the three genes was required for a full infection of TYLCSV (Lozano-Durán et al. 2011). This study aimed to validate three genes (deltaCOP, hsc70, and BAM1) in PepYLCIV infection by applying VIGS in a model plant, wild type N. benthamiana.

MATERIALS AND METHODS

Preparation of Model Plant

N. benthamiana seeds were germinated on peat pellets. Two weeks after germination, the seedlings were transferred to soil media Tsuchitara® (Sumirin Agro-Products, Japan). Seedlings and plants were maintained in growth room at 25°C and illumination...
of 16 h per day (Kaido et al. 2011, 2014). Three-to-four-week-old plants were used for agroinfiltration.

**Bacterial Uses**

*Escherichia coli* strain DH5α was used for plasmid constructions, *A. tumefaciens* strain GV3101:pSoup was used for TRV vector delivery, and the infectious clone of PepYLCIV (pGreenII-p35S-PepYLCIV-DNA A+B) (Koeda et al. 2018) was used for virus inoculation. Other culture materials used were glycerol stocks containing TRV RNA 1 and RNA 2 constructs pBINTRA6 and pTV00, respectively, and TRV vector harboring the sequence of *Phytoene desaturase* gene (pTV:PDS) (Ratcliff et al. 2001).

**Construction of TRV-derived VIGS Vectors**

A cDNA pool prepared from the mature leaves of *N. benthamiana* (Kaido et al. 2014) was used as template for PCR amplification of the selected gene fragments. The primers and their sequences are listed in Table 1. The reaction mixture for PCR was prepared using KOD Plus-Ver. 2 DNA Polymerase (Toyobo, Japan). The reaction mixtures were pre-denatured at 94°C for 2 min, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 68°C for 40 s. Final extension was terminated at 72°C for 5 min. Electrophoresis was done to verify the PCR products using 1.0% (w/v) agarose gel in 1× TAE buffer.

The PCR products were then digested with *SmaI* and *BamHI* (37°C, 2 h). The fragment was inserted into pTV00 (Ratcliff et al. 2001), previously digested with the same restriction enzymes in a separate reaction (30°C, 2 h). Prior to ligation using DNA Ligation Kit Ver. 2.1 (TaKaRa Bio, Japan), pTV00 vector was treated with bacterial alkaline phosphatase (BAP) (TaKaRa Bio, Japan) at 65°C for 1 h. The Wizard® SV Gel and PCR Clean-Up System (Promega, USA) was used to purify the enzyme-digested and BAP-treated PCR products.

The ligation mixtures were transformed into *E. coli* strain DH5α by the heat shock method. *E. coli* cells carrying pTV00 derivatives (containing gene fragment) were then plated out directly on luminescence medium (LM) agar plate containing kanamycin (100 µg/ml) and incubated overnight at 37°C. Single colonies were confirmed using EmeraldAmp® MAX PCR Master Mix (TaKaRa Bio, Japan) with primer pairs TRV-RNA-110FW and reverse primer of each selected gene (Table 1). Electrophoresis was done to verify the PCR products using 1.0% (w/v) agarose gel in 1× TAE buffer.

The verified bacterial cultures of each selected gene were inoculated in 5 ml liquid Luria-Bertani (LB) media containing kanamycin (100 µg/ml) and incubated overnight at 37°C with a 250 rpm rotation rate. The overnight cultures were collected and purified using Wizard® Plus SV Miniprep DNA Purification System (Promega, USA). The OD values for each culture were measured and recorded by NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Sequencing was then carried out using BigDye™ Terminator v3.1 Cycle Sequencing Kit in an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA) according to the manufacturer's user guide.

![Table 1. List of primers and their sequences used for PCR of the three targeted genes together with other related sequences used in this study.](image-url)
Co-inoculation of Agrobacterium Carrying TRV-derived VIGS Vector and Viral Infectious Clone

pTV00 derivatives were mobilized into A. tumefaciens strain GV3101::pSoup using Gene Pulser® II Electroporation System (Bio-Rad, USA), then incubated in super optimal broth with catabolite repression (SOC) medium at 28°C for 3 h with a 200 rpm rotation rate. A portion of the suspension was plated out directly on LB agar containing kanamycin and rifampicin (100 µg/ml each) and incubated two overnights at 28°C. Bacterial colonies were picked, directly streaked on LM agar containing kanamycin and rifampicin (100 µg/ml each), and incubated at 28°C for 48 h. The Agrobacterium cultures were then inoculated in liquid LB containing acetoxytrygine (10 µl of 100 mM stock solution) and antibiotics kanamycin and rifampicin (100 µg/ml each), and incubated at 28°C overnight at 28°C with a 200 rpm rotation rate.

The overnight bacterial cultures were collected and suspended in 1 ml infiltration buffer (10 mM MES, pH 5.7; 10 mM MgSO_4). The OD_600 values for each cultures were measured and recorded by BioSpectrometer® Basic (Eppendorf AG, Germany). Agrobacterium cultures were resuspended in infiltration buffer adjusting optical density to OD_600 = 1.0, then incubated at 22°C in the dark at least for 2 h. The following combinations of Agrobacterium cultures were mixed at a volume ratio of 1:1:1, including (1) pBINTRA6 + pTV00 + infiltration buffer (mock), (2) pBINTRA6 + pTV00 + PepYLCIV, (3) pBINTRA6 + pTV::PDS + PepYLCIV, (4) pBINTRA6 + pTV::deltaCOP + PepYLCIV, (5) pBINTRA6 + pTV::hsc70 + PepYLCIV, and (6) pBINTRA6 + pTV::BAM1 + PepYLCIV.

The abaxial surfaces of the most apical full-expanded leaf of each N. benthamiana plant were infiltrated with one of the combinations of the Agrobacterium mixtures using a 1 ml syringe without a needle. The mixture was injected until the entire leaf was infiltrated. After infiltration, the plants were kept overnight at 22°C in a growth chamber, then transferred to a growth room. Co-inoculation of Agrobacterium carrying TRV-derived VIGS vector and viral infectious clone was performed in three independent experiments, three plants each, so there were three biological and technical replications.

Reverse Transcription Reaction and Quantitative PCR

Relative gene expressions and amount of viral DNA accumulations were then determined by quantitative reverse transcription PCR (qRT-PCR) and quantitative PCR (qPCR), respectively, using RNA and DNA extracted from agroinfiltrated leaves. qPCR was carried out as described (Livak and Schmittgen 2001; Lozano-Durán et al. 2011) with slight modifications. Three most apical leaves were taken from each TRV-induced silencing or control N. benthamiana plant at 15 days post infiltration (dpi). Fresh leaves were ground in liquid nitrogen to a powder using mortar and pestle at room temperature. Total DNA was isolated using DNeasy® Plant Mini Kit (QIAGEN, Germany) according to the manufacturer’s instruction. The reaction mixture for qPCR were prepared in a 0.2 ml PCR tube using TB Green™ Premix Ex Taq™ (Tli RNaseH Plus; TaKaRa, Japan) following standard protocols. A relative qPCR method using the 2^−ΔΔCT method (Livak and Schmittgen 2001) was used to compare the DNA amount of PepYLCIV coat protein gene (Table 1) among different experiments. The 25S ribosomal DNA interspacer (ITS) (Table 1) was used as the internal control.

Meanwhile, to determine the expression level of the selected genes, total RNA was isolated using PureLink® Plant RNA Reagent (Life Technologies, Thermo Fisher Scientific, USA) according to the manufacturer’s instruction. The total RNA was treated by RQ1® RNase-free DNase (Promega, USA): RNA sample (2 µg/µl) 1 µl, 10× reaction buffer 2 µl, RQ1 RNase-Free DNase 2 µl, and autoclaved ddH2O to a final volume of 20 µl, incubated at 37°C for 1 h, done twice to a final volume of 40 µl. A phenol:chloroform extraction was performed to the treated RNA samples. The RNA samples (250 ng) were then subjected to 10 µl of reverse transcription (RT) reaction using PrimeScript™ RT Master Mix (Perfect Real Time; TaKaRa Bio, Japan). After performing RT reaction, 2.0 µl of 10-fold diluted RT reaction solution was introduced as template for 25 µl qRT-PCR using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus; TaKaRa Bio, Japan). A relative qRT-PCR method using the 2^−ΔΔCT method (Livak and Schmittgen 2001) was used to compare the mRNA amount of endogenous gene (Table 1) among different experiments. The N. benthamiana EF1α gene (Table 1) was used as the internal control.
RESULTS AND DISCUSSION

TRV-derived VIGS Vector Construct Containing Target Fragment of the Three Selected Genes

Three genes were selected as candidate genes to be validated, namely deltaCOP, hsc70, and BAM1 (Figure 1). While Lozano-Durán et al. (2011) used cDNA clones obtained from the Arabidopsis Information Resource (TAIR), we used a cDNA pool of *N. benthamiana* (Kaido et al. 2014) as the template for amplifying and cloning fragments of the selected genes. Based on the sequence of primer pairs reported in their work (Lozano-Durán et al. 1997), those were NbV6.1trP70667 (1,729 bp), NbV6.1trP53333 (2,346 bp), and NbV6.1trP44051 (3,504 bp) for deltaCOP, hsc70, and BAM1 genes, respectively. The amplified fragments

**DeltaCOP**

| Gene | Fragment Size (bp) |
|------|-------------------|
| deltaCOP 532 | 586 |
| 587 | 641 |
| 642 | 696 |
| 697 | 751 |
| 752 | 806 |
| 807 | 861 |
| 862 | 916 |
| 917 | 96 |
| 972 | 1026 |
| 1027 | 1081 |
| 1082 | 1136 |
| 1137 | 1191 |
| 1192 | 1246 |
| 1247 | 1301 |
| 1302 | 1356 |

**hsc70**

| Gene | Fragment Size (bp) |
|------|-------------------|
| hsc70 597 | 641 |
| 642 | 696 |
| 697 | 751 |
| 752 | 806 |
| 807 | 861 |
| 862 | 916 |
| 917 | 96 |
| 972 | 1026 |
| 1027 | 1081 |
| 1082 | 1136 |
| 1137 | 1191 |
| 1192 | 1246 |

**BAM1**

| Gene | Fragment Size (bp) |
|------|-------------------|
| BAM1 124 | 178 |
| 179 | 233 |
| 234 | 288 |
| 289 | 343 |
| 344 | 398 |
| 399 | 453 |
| 2324 | 2378 |
| 2379 | 2433 |
| 2434 | 2543 |
| 2948 | 2598 |
| 2959 | 2653 |
| 2654 | 2708 |
| 2709 | 2763 |
| 2764 | 2818 |
| 2819 | 2873 |

![Figure 1](image-url) Fragment of *N. benthamiana* genes used in this study. Blue color: fragments used for constructs of TRV-derived vectors to induce gene silencing of *deltaCOP, hsc70,* and *BAM1.* Green color: fragments used for qRT-PCR analysis of gene expression.
were 250 bp (nt 629–878), 188 bp (nt 958–1,145), and 378 bp (nt 2,404–2,781) in length (Figure 1, blue color), and inserted into multiple cloning site (MCS) of TRV RNA 2 construct (pTV00) (Figure 2A). Lozano-Durán et al. (2011) generated and cloned 300–500 bp fragments to silence the selected genes. Although DNA fragments of 200–350 bp in length is preferred for silencing (Ramegowda et al. 2014), Hsieh et al. (2013) previously showed that the fragment size used can be as small as 78–85 bp and reduced the expression of the silenced genes by 61.5–95.8%.

In this study, we used wild type *N. benthamiana* plants combined with VIGS, a transient assay system. Gene silencing was induced for each selected gene in plants using TRV constructs (Figure 2A). TRV-based vector overcomes the problem features of *Potato virus X* (PVX), *Tobacco mosaic virus* (TMV), and *Tomato golden mosaic virus* (TGMV) which have been previously developed as VIGS vectors (Ratcliff et al. 2001). The TRV-based VIGS vector induces very mild symptoms, infects large areas of adjacent cells, and silences gene expression in growing points (Ratcliff et al. 2001). Co-inoculation with TRV does not dramatically affect the geminivirus infection in *N. benthamiana* (Lozano-Durán et al. 2011; Rosas-Díaz 2014). Agrobacterium-mediated transient expression using leaf infiltration was expected to knock down the expression of the selected genes and affect the accumulation of PepYLCIV.

**Figure 2.** Schematic of genomic organization of TRV-derived vector containing target fragment of the three selected genes, and PepYLCIV clone used in this study. (A) T-DNA organization of pBINTRA6 and pTV00 (Ratcliff et al. 2001, by modification). Lb = left border, Rb = right border, 35S = CaMV 35S promoter, T = transcriptional terminator, RdRp = RNA-dependent RNA polymerase, MP = movement protein, 16K = 16k protein, CP = coat protein, Int = intron introduced during the cloning of RNA 1, MCS = multiple cloning site where the amplified fragment of deltaCOP, hsc70, or BAM1 fragment was inserted. (B) Bipartite begomovirus components and constructed infectious clone of pGreenII-p35S-PepYLCIV-DNA-A+B (Koeda et al. 2018). AV1 = coat protein, AV2 = pre-coat protein, AC1 = replication associated protein, AC2 = transcriptional activator protein, AC3 = replication enhancer protein, AC4 = small protein involved in symptom determination and virus movement, BV1 = nuclear shuttle protein, BC1 = movement protein.
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Gene-silenced N. benthamiana Plants Co-inoculated with PepYLCIV

We used an infectious clone of PepYLCIV containing full-length sequences of DNA A and B extracted from leaf samples collected at Banda Aceh, Indonesia (Koeda et al. 2016, 2018; Figure 2B). The viral clone was named pGreenII-p35S-PepYLCIV-DNA A+B and efficiently introduced into N. benthamiana, tomato, and pepper plants by agroinoculation (Koeda et al. 2018). According to a preliminary work, PepYLCIV infection was reached and could be detected in the most apical leaves of N. benthamiana at 9 dpi (Mise 2018, unpublished data). We also used TRV vector harboring the sequence of PDS gene (TRV:PDS) as positive control for VIGS. Infecting the leaves of N. benthamiana with this vector resulted in a bleached phenotype that could be easily observed at 15 dpi, especially in the apical leaves (Figure 3). This indicated that the local VIGS assay could efficiently induce gene silencing, resulted in a systemic gene-silenced phenotype (Ratcliff et al. 2001; Zhang et al. 2014). Meanwhile, plants infiltrated with the empty vector only (no gene) showed no symptom (Figure 3, mock).

VIGS phenotypes could be seen in the apical leaves of gene-silenced N. benthamiana plants co-inoculated with PepYLCIV (Figure 3). TRV:00 plants had crinkled leaves, malformation, and reduced apical growth compared to mock-infiltrated plants (Figure 3). TRV:deltaCOP plants showed necrosis, cell death on top leaves. TRV:hs70 and TRV:BAM1 plants produced similar phenotype to that of TRV:00 plants. In addition, TRV:hs70 plants also showed slight necrosis. Senthil-Kumar et al. (2018) reported that the VIGS phenotypes could be varied and visible, such as leaf chlorosis, spotted cell death, stunted growth, leaf curling, leaf crinkling, and leaf mottling. They have developed a VIGS phenomics and functional genomics database. The database contains DNA sequence information derived from over 4,000 N. benthamiana VIGS clones and the associated silencing phenotype for approximately 1,300 genes (Senthil-Kumar et al. 2018). Such a database could be used as a reference and overcomes the disadvantage of VIGS assay since it very often does not produce a uniform silencing throughout the plant.

Gene Expression and Viral DNA Accumulation

For each of the sampled plants, three most apical leaves were harvested at 15 dpi (Figure 3). Firstly, qRT-PCR was conducted to determine the expression level of the silenced genes. The primer pairs were designed to amplify outside of the DNA fragments targeted for silencing (Figure 1, green color). The fragments of deltaCOP, hsc70, and BAM1 genes were 203 bp (nt 1,087−1,289), 184 bp (nt 644−827), and 165 bp (nt 190−354) in length, respectively. The qRT-PCR results showed a decreased expression level of deltaCOP, hsc70, and BAM1 genes to 66.4%, 53.0%, and 47.0%, respectively, compared to that in the control TRV:00 (100%) (Figure 4).

qPCR was then conducted to determine the accumulation level of viral DNA (PepYLCIV coat protein gene). Silencing of the three genes decreased the accumulation of PepYLCIV to 0.1%, 18.4%, and 0.09%, respectively.
63.0%, respectively, compared to that in the control TRV:00 (100%) (Figure 5). deltaCOP and hsc70 genes were indicated to be involved in the viral infection and could be good candidate genes for obtaining resistant chili pepper varieties. Silencing of these two genes also greatly reduced TYLCSV accumulation (Lozano-Durán et al. 2011). Wang et al. (2018) reported that knockdown of Hsc70-2 leads to drastic reduction of a tombusvirus Beet black scorch virus (BBSV) accumulation in N. benthamiana. Mutation of these two genes in chili pepper is expected to result in resistance to PepYLCIV. Our results affirmed that the reverse genetics approach could be an alternative strategy for identifying or screening plant genes involved in viral infection. VIGS assay is rapid, inexpensive, target-specific, allows to silence multiple genes, and does not involve plant transformation (Burch-Smith et al. 2004; Velásquez et al. 2009; Gilchrist and Haughn 2010; Hayward et al. 2011). The infectious clone of PepYLCIV could be used in combination with TRV-based VIGS vector. Using an infectious clone, the virus inoculations could be carried out without rearing and maintaining its natural vector. This could

Figure 4. Relative gene expression in gene-silenced N. benthamiana plants co-inoculated with PepYLCIV at 15 dpi. Expression values of the genes were estimated by qRT-PCR from the samples of Figure 3. Values are the mean of three technical replications. Bars represent standard error. Sample of TRV empty vector + PepYLCIV inoculated plants (TRV:00) was used as the calibrator, with EF1α gene was used as the internal control. The expression level of the endogenous gene was set to 100.

Figure 5. Relative amount of PepYLCIV DNA in gene-silenced N. benthamiana plants co-inoculated with PepYLCIV at 15 dpi. Expression values of the viral DNA were estimated by qPCR from the samples of Figure 3. Values are the mean of three biological and technical replications. Bars represent standard error. Sample of TRV empty vector + PepYLCIV inoculated plants (TRV:00) was used as the calibrator, with 25S ribosomal DNA interspacer (ITS) was used as the internal control. The expression level of the PepYLCIV coat protein gene was set to 100.
reduce the risk of virus transmission to healthy plants. The clone might potentially be used further in the screening of Indonesian accessions/gene pool to select chili peppers resistant to PepYLCIV.

CONCLUSIONS

Three plant genes, namely deltaCOP, hsc70, and BAM1, were validated on their involvement in PepYLCIV infection using TRV-derived VIGS vector in a model plant N. benthamiana. The expression levels of each selected gene decreased to 66.4%, 53.0%, and 47.0%, respectively, compared to that in the control (100%). Meanwhile, silencing of the three genes decreased the accumulation of PepYLCIV to 0.1%, 18.4%, and 63.0%, respectively. deltaCOP and hsc70 genes were indicated to be involved in the viral infection and could be good candidate genes for obtaining chili pepper varieties resistant to PepYLCIV. Our study also affirmed that the reverse genetics approach could be an alternative strategy for obtaining plant genes involved in viral infection, including PepYLCIV.

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AUTHOR CONTRIBUTIONS

KK: main contributor, planned and performed the experiment, and wrote the manuscript. MK: member contributor, revised the manuscript, and helped supervise the work. KM: member contributor, performed the experiment, revised the manuscript, and supervised the work.

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