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Chrysanthemum stunt viroid as a protective viroid isolate against *Columnea latent viroid* and *Pepper chat fruit viroid* in tomato plants

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Abstract The effect of prior inoculation of CSVd isolate on disease progression induced by CLVd or PCFVd isolates were observed in tomato plants. Tomato plants infected with CSVd alone showed no apparent symptoms on the leaves. In contrast, tomato plants infected with CLVd showed common symptoms at 4 weeks after inoculation, such as plant stunting, leaves epinasty, distortion and discoloration. Necrosis of the veins and substantial stunting were seen in PCFVd-infected tomato plants. Those inoculated with CLVd at the same time as the challenge inoculation had less severe symptoms than those infected with CLVd inoculation. Fewer symptoms were observed in tomato plants that had been first inoculated with CSVd and challenged with CLVd. In tomato plants inoculated with PCFVd, severe necrosis on leaf veins was observed with fewer signs of necrosis on the tomato plants that were infected with PCFVd at the same time as the challenge inoculation. However, in tomato plants infected with CSVd and challenged with PCFVd, mild discoloration on the leaf with no necrosis was detected. The results showed that CSVd could replicate when confirmed using reverse-transcription polymerase chain reaction (RT-PCR). Cross-protected tomato plants inoculated with CSVd and then challenged with PCFVd could produce fruit. On the other hand, PCFVd-infected tomato plants produced no fruit. The two cases of cross-protection bioassay, namely CSVd challenged with CLVd and CSVd challenged with PCFVd, can be helpful in the development of plants resistant to severe viroids.

Keywords: *Chrysanthemum stunt viroid* (CSVd), *Columnea latent viroid* (CLVd), Cross protection, *Pepper chat fruit viroid* (PCFVd), Tomato

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Introduction

Thailand produces around 130,000 tons of tomatoes on 6,238 hectares throughout 36 provinces (Office of Agricultural Economics, 2020). Although there have been no reports of tomato output losses associated with viroids, observations in local vegetable fields have revealed that farmers are unaware of viroid infection that could be unintentionally spreaded to other areas. Furthermore, the destruction of viroid-infected tomato seeds impacts international seed trade (Chambers *et al*., 2013). Tomato plants are susceptible to most plant diseases, including bacteria, fungi, viruses, and viroid diseases. Stunting, flower and fruit deformation, and leaf curling with necrosis on the leaf vein and petiole are the most common symptoms of tomato-infected viroids (Singh *et al*., 2003). Their primary mode of transmission is mechanical. Therefore, plant quarantine and strict hygienic practices are required to avoid the unintentional spread of viroids to different areas.

Chrysanthemum stunt viroid (CSVd) (Netwong *et al*., 2020), *Columnea latent viroid* (CLVd) (Marach, 2008), and *Pepper chat fruit viroid* (PCFVd) (Reanwarakorn *et al*., 2011) are viroid species that have been reported in Thailand. Netwong *et al*. reported the CSVd isolates collected from chrysanthemum plants exhibiting growth stunting, leaf chlorosis and yellowing, and abnormal flower production. However, when CSVd was inoculated onto tomato cv. Rutgers plants, no symptoms were observed (Netwong *et al*., 2020). In contrast to CSVd, CLVd and PCFVd caused plant distortion, severe vein necrosis, reduction in fruit size, and immature seed production in tomato plants (Reanwarakorn *et al.*, 2011; Tangkanchanapas, 2005; Tangkanchanapas *et al.*, 2013). CLVd and PCFVd are among the most common viroids threatening commercial tomato cultivar production in Thailand (Marach, 2008; Tangkanchanapas, 2005).

Viroids are known to spread primarily by both vertical (seed and pollen) and horizontal (vegetative propagation, mechanical, agricultural practices) *transmission* (Flores *et al*., 2005; Hadidi, 2003). Viroid-contaminated tools can be disinfected using different chemical substances, such as sodium hypochlorite, hydrogen peroxide, and sodium hydroxide with formaldehyde (Garnsey and Whidden, 1972; Li *et al*., 2015; Matsuura *et al.*, 2010; Olivier *et al.*, 2015; Singh *et al.*, 1989). Because of their systemic dissemination throughout the plant, heat or cold treatments have also been used for viroid elimination (Hollings and Stone, 1970; Howell *et al*., 1998; Savitri *et al*., 2013). However, if the viroid is already presented in the field, these approaches are ineffective in reducing viroid spread. Numerous transgenic strategies for viroid control have been developed which based on viroid replication

mechanism using siRNA (Kasai *et al.*, 2011), antisense RNA (Matousek *et al*., 1994), hairpin RNA (Wang *et al*., 2004), and hammerhead ribozyme (Yang *et al.*, 1997). However, these procedures are prohibited in Thailand. Due to the limitation of effective strategies to manage viroids, it is essential to develop an alternative approach to control this destructive disease once it is apparent. As a result, a non-transgenic method using a cross-protection approach to control viroid disease has possible emerged.

Cross-protection, also known as pre-immunization, is based on the use of a mild virus or viroid strain to protect a plant against a more severe virus or viroid strain (Foley *et al*., 2011; Hammond and Kovalskaya, 2017). The practical use of cross-protection is mainly applied to protect plants from viral infection (Ziebell and Carr, 2010). Successful studies have achieved to control severe infection of *Potato spindle tuber viroid* (PSTVd) and *Citrus exocortis viroid* (CEVd) in tomato plants after initially inoculation with mild PSTVd (Niblett *et al*., 1978), *Hop stunt viroid* (HSVd) in the presence of PSTVd in tomato plants (Branch *et al*., 1988), and control of severe exocortis symptoms on citron by using a mild CEVd against a severe strain (Duran-Vila and Semancik, 1990). Partial cross-protection was also demonstrated for *Tomato planta macho viroid* (TPMVd) when PSTVd was used (Galindo *et al.*, 1982).

The current study aimed to assess the possibilities of using CSVd as a protective viroid isolate against CLVd or PCFVd in tomato plants, which could contribute to establishing a cross-protection strategy to develop viroid-resistant plants.

Materials and methods

Viroid sources and plant materials

Chrysanthemum stunt viroid (CSVd) isolate RCS accession no. MF803029, *Columnea latent viroid* (CLVd) isolate NK-KUKPS1 accession no. KY235369, and *Pepper chat fruit viroid* (PCFVd) isolate LPng20-11c1 accession no. JF446893 were previously collected and stored as dried samples at the Molecular Plant Pathology Laboratory at Kasetsart University, Kamphaeng Saen campus, Thailand. Each viroid isolate was mechanically inoculated onto tomato leaves (cv. Rutgers). Viroid-infected dried leaves were ground at 1g:10 mL in 0.1M phosphate buffer (pH 9.0). Then, the viroid inoculum mixture was rubbed onto tomato leaves that had been dusted with carborundum powder. After incubating the leaves for 5 minutes, they were rinsed with distilled water. In order to be using as an inoculum, the inoculated plants were kept in an insect-proof greenhouse.

Tomato seeds (*Solanum lycopersicum* cv. Rutgers) were germinated and grown as indicator plants in 10 cm pots filled with sterilized soil in an insect-proof greenhouse. Plant sap from the infected leaves of each viroid isolate was prepared with phosphate buffer as previously described and mechanically inoculated into tomato seedlings aged 4 weeks by rubbing into the carborundum powder. The viroid-inoculated tomato plants were maintained in an insect-proof-greenhouse for observation of symptoms.

Effect of prior inoculation of a CSVd isolate on disease progression induced by CLVd or PCFVd isolates

The tomato plants were grown in 15 cm new planting bags filled with sterilized soil. Three plants were used per treatment. The different treatments was displayed in Figure 1. The uninoculated plants were used as a negative control. The prior inoculation was performed on tomato plants aged 4 weeks, whereas challenged inoculation was performed on tomato plants aged 8 weeks. The control treatments were inoculated CSVd, CLVd, and PCFVd. In the cross-protection treatments, two pairs of viroids were used as a prior inoculation of CSVd and challenge with CLVd or prior inoculation of CSVd and challenge with PCFVd. Because there was an interval between challenge inoculation, additional controls were also set up in tomato plants aged 8 weeks infected with CLVd or PCFVd. Symptom development on the plants was used to assess the efficacy of cross-protection.

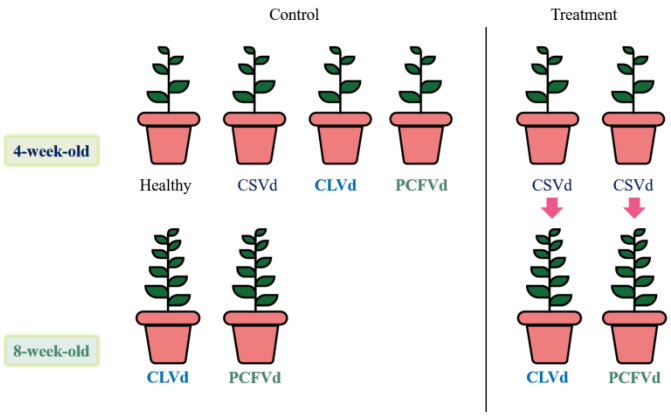

Figure 1. Cross-protection experiments: control includes uninoculated plants (healthy) as a negative control, CSVd, CLVd, and PCFVd inoculated at age 4 weeks; additional controls were CLVd, and PCFVd inoculated at age 8 weeks; treatments include tomato plants inoculated with CSVd at age 4 weeks and challenged with CLVd or PCFVd at age 8 weeks

RNA extraction

Total RNA was extracted from 100 mg young tomato leaves using a CTAB method with slight modification (Li *et al*., 2008; Reanwarakorn *et al.*, 2011). Briefly, the young tomato leaves were ground into a fine powder with liquid nitrogen and then CTAB extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1.0% Na_2SO_3, and 2.0% PVP-40) was added. The buffer mixture was incubated at 65°C for 30 minutes, then centrifuged at 13,000 rpm for 5 minutes. The supernatant was collected and mixed with phenol-chloroform: isoamyl alcohol (24:1). After centrifuging the sample at 15,000 rpm for 15 minutes, the supernatant was collected and mixed with chloroform: isoamyl alcohol (24:1) and centrifuged again at 15,000 rpm for 10 minutes. The supernatant was separated and mixed with cooled 5M NaCl and isopropanol. The samples were then chilled at -20°C overnight until further use. The RNA pellet was washed with 70% ethanol twice, dissolved in 50 µL of RNase-free dH_2O, and stored at -20°C until use.

Detection of CSVd, CLVd and PCFVd by reverse transcription-polymerase chain reaction (RT-PCR)

Thermo Scientific RevertAid Reverse Transcriptase (RT) (Thermo Scientific^{TM}) was used to synthesize cDNA using a specific primer pair (Table 1). The RT reaction mixture contained 5 µL of 2 pmol primer with 1.75 µL of RNA. The RT reaction was incubated in a thermal cycler at 96 ℃ for 5 minutes. After adding the reverse transcription buffer (2 µL), 10 mM dNTPs (1 µL) and 200 U/µL reverse transcriptase (0.25 µL), the reactions were incubated for 1 hour at 45 ℃ for the CSVd and CLVd primers and at 50 ℃ for the PCFVd primer, followed by 10 minutes at 70 ℃ for enzyme inactivation. PCR reactions were carried out using 1 µL of the cDNA, 0.25 µL of each specific primer pair (2 pmol), 3 µL of nuclease-free water, and 5 µL of GoTaq® Green Master Mix (Promega) in a total volume of 10 µL. PCR reactions were carried out under the following conditions: initial denaturation at 96 ℃ for 5 minutes, followed by 35 cycles of 96 ℃ for 40 seconds, annealing temperature (Table 1) for 40 seconds, extension step at 72 ℃ for 40 seconds, and a final extension at 72 ℃ for 7 minutes. All steps were performed according to the manufacturer's protocols in a Biometra T1 model thermocycler (Biometra GmbH). Gel electrophoresis was used to analyze the RT-PCR amplification products on 2% agarose gel. The staining dye was RedSafeTM Nucleic Acid Staining Solution (iNtRON Biotechnology). A gel documentation UV-transilluminator was used to visualize the results under UV light (SYNGENE Genesis3).

Statistical analysis

We analyzed the data using the JASP statistical software (version 0.14.1). One-way ANOVA was used to compare the means among treatments. Box plots and Tukey's honestly significant difference (HSD) were also carried out.

Results

Symptoms caused by CSVd, CLVd, and PCFVd on tomato plants

Viroid isolates (CSVd, CLVd and PCFVd) were inoculated onto tomato plants (cv. Rutgers) as inocula. CSVd was detected in the CSVd-infected tomato plants at 1 month post-inoculation. However, no symptoms were observed (Figure 2A). In the CLVd-infected tomato plants, shortened internodes, leaf distortion, leaf epinasty, and plant stunting were observed at 3 weeks post-inoculation (Figure 2B). The PCFVd-infected tomato plants displayed vein necrosis on the leaves as well as growth stunting at 2 weeks post-inoculation (Figure 2C). Infecting tomatoes with CLVd led to flower abortion, resulting in a lower yield and smaller fruit size (Figure 6A). Flowering was observed in the PCFVd-infected tomatoes, but all the flowers dropped before producing any fruit. Fruit was obtained from the CSVd-infected tomato plants, with the fruits being a similar weight and size to those obtained from healthy tomato plants.

Figure 2. Tomato leaf symptoms infected with viroids at 4 weeks post-inoculation: (A) CSVd-infected tomato plants remaining symptomless; (B) CLVd-infected tomato plants expressing leaf distortion and epinasty; (C) PCFVd-infected tomato plants expressing necrosis on leaf and leaf vein

Effect of prior inoculation of CSVd isolate on disease progression induced by CLVd or PCFVd isolates

Tomato plants infected with CSVd alone showed no apparent symptoms on the leaves (Figure 2A). In contrast, tomato plants infected with CLVd showed common symptoms at 4 weeks after inoculation, such as plant stunting,

leaves epinasty, distortion and discoloration (Figure 2B). Necrosis of the veins and substantial stunting were seen in PCFVd-infected tomato plants (Figure 2C). Those inoculated with CLVd at the same time as the challenge inoculation had less severe symptoms (average plant height of 92.97 cm) than those infected with CLVd inoculation (average plant height of 42.27 cm), as shown in Figure 3A and Figure 3B, respectively. In contrast, fewer symptoms were observed in tomato plants that had been first inoculated with CSVd and challenged with CLVd, with an average plant height of 112.67 cm (Figure 3C). In tomato plants inoculated with PCFVd, severe necrosis on leaf veins was observed, with an average plant height of 33.67 cm (Figure 4A). There were fewer signs of necrosis on the tomato plants that were infected with PCFVd at the same time as the challenge inoculation, with an average plant height of 96.1 cm (Figure 4B). However, in tomato plants infected with CSVd and challenged with PCFVd, mild discoloration on the leaf with no necrosis was detected (Figure 4C), with an average plant height of 114.23 cm (Table 2). When plant heights were compared, uninoculated (123.6 cm) and CSVd-infected plants (128.4 cm) plants were not significantly different in both the CSVd plants challenged with CLVd and the CSVd plants challenged with PCFVd. However, both the CSVd and CLVd or the PCFVd viroid isolates were replicated with a lower intensity of PCR products (Figure 5). Tomato fruits were produced by tomato plants that had been first inoculated with CSVd and challenged with CLVd (average fruit weight of 6.45 g), with no significant difference from CSVd-infected plants (average fruit weight of 7.16 g), as shown in Table 2 and Figure 6A. Initially, the PCFVd infection resulted in flowering abortion and no fruit production. However, fruits were produced on tomato plants that had been first inoculated with CSVd and challenged with PCFVd (average fruit weight of 2.37 g), as shown in Table 2 and Figure 6B.

Analysis of variance

ANOVA revealed statistically significant differences between treatments. A Tukey HSD post hoc study found significant ($p<0.001$) differences in plant height, fruit weight, and fruit width among treatments. The highest plant height was for uninoculated plants (128.4 ± 1.80). There was no significant difference between those inoculated with CSVd (123.6 ± 1.31) and those first inoculated with CSVd and challenged with CLVd (112.67 ± 2.45) or PCFVd (114.23 ± 1.50). These plants were significantly greater than plants inoculated with CLVd (42.27 ± 2.16) or PCFVd (33.67 ± 1.51) alone. The highest fruit weight was for uninoculated plants (10.56 ± 2.58), which showed no significant difference from those infected with CSVd alone (7.16 ± 5.74). Fruits obtained

from tomato plants infected with CSVd and challenged with CLVd (6.60±1.19) showed no significant difference from uninoculated plants or tomato plants challenged with PCFVd (2.37±0.40). In contrast, the size of fruits produced by tomato plants that were infected at the same time as the challenge inoculation (0.63±0.29) was significantly different from uninoculated plants and CSVd-infected tomato plants. The highest fruit width was observed in uninoculated plants (2.76±0.31), with a significant difference from other treatments except those infected with CLVd at the same time as the challenge inoculation (1.38±0.15), as shown in Table 2 and Figure 7.

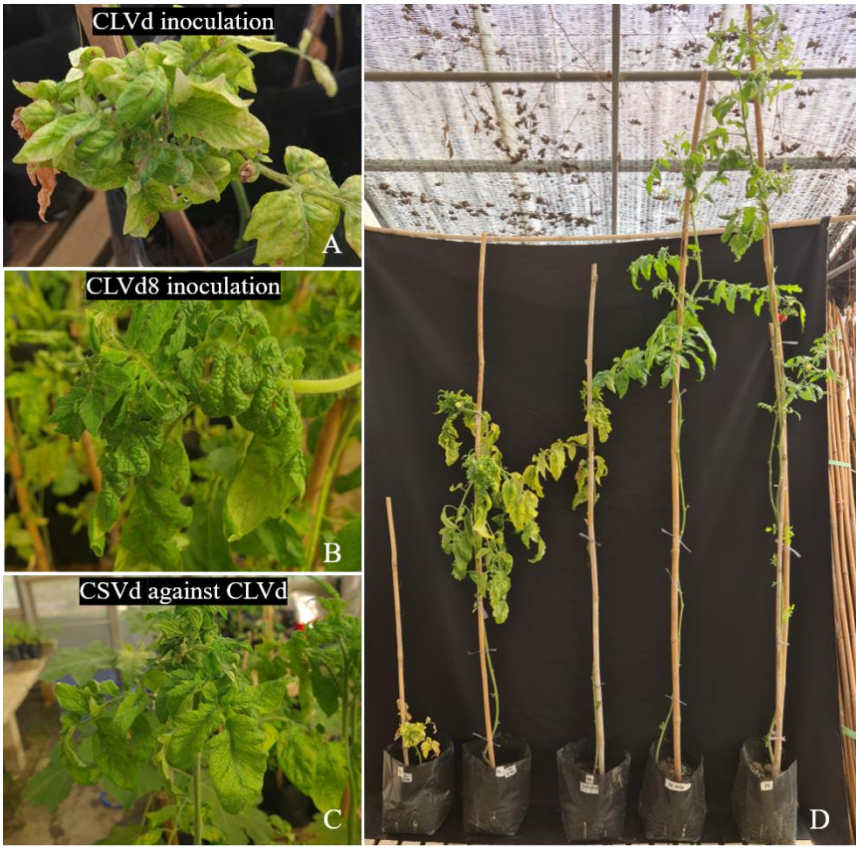

Figure 3. Cross-protection tests using a CSVd isolate and challenged with CLVd at 4 months post-inoculation**:** (A) CLVd inoculation (B) CLVd8 = CLVd inoculation at the same time as challenge inoculation; (C) CSVd inoculation and challenged with CLVd; (D) the comparison of tomato heights from left to right; CLVd inoculation, CLVd inoculation at the same time as challenge inoculation, CSVd inoculation and challenged with CLVd, CSVd inoculation, and uninoculated tomato

Figure 4. Cross-protection tests using a CSVd isolate and challenged with PCFVd at 4 months post-inoculation: (A) PCFVd inoculation; (B) PCFVd8 = PCFVd inoculation at same time as challenge inoculation; (C) CSVd inoculation and challenged with PCFVd; (D) comparison of tomato heights from left to right; PCFVd inoculation, PCFVd inoculation at the same time as challenge inoculation, CSVd inoculation and challenged with PCFVd, CSVd inoculation, and uninoculated tomato

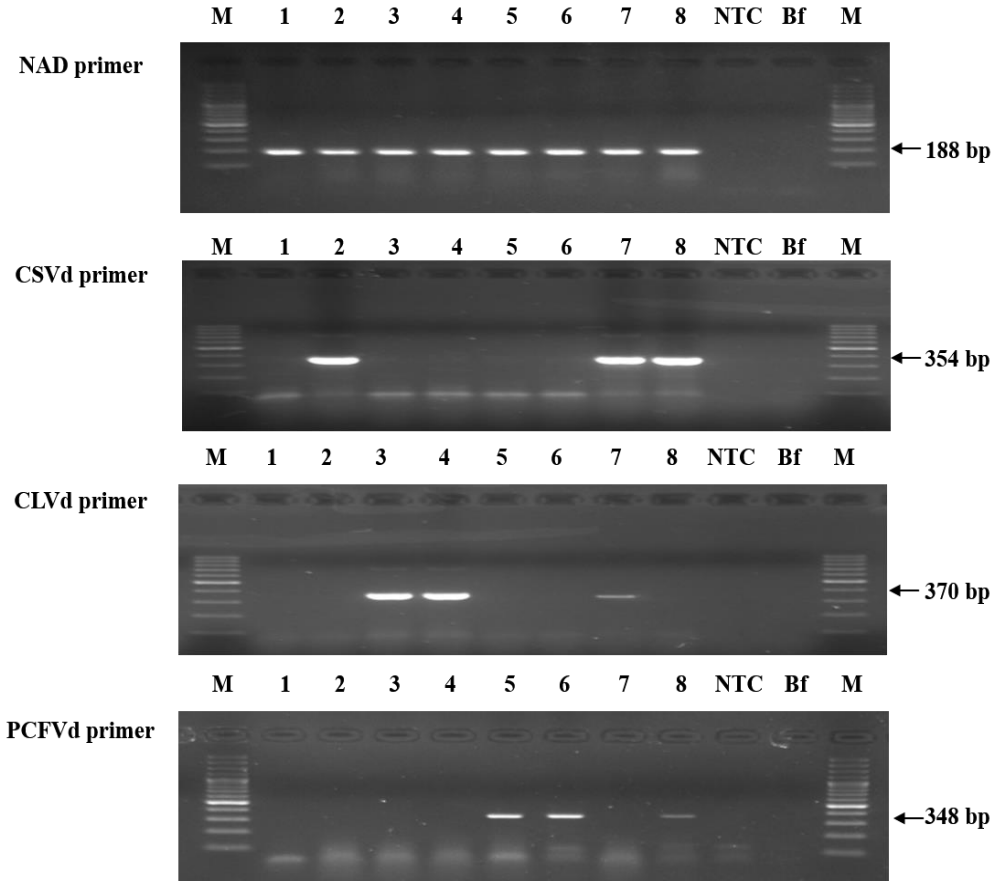

Figure 5. Electrophoresis of RT-PCR products using specific primers for NAD, CSVd, CLVd, and PCFVd: M = 100bp DNA markers; 1 = uninoculated tomato; 2 = CSVd inoculation; 3 = CLVd inoculation; 4 = CLVd inoculation at the same time as challenge inoculation; 5 = PCFVd inoculation; 6 = PCFVd inoculation at same time as challenge inoculation; 7 = CSVd inoculation and challenged with CLVd; 8 = CSVd inoculation and challenged with PCFVd; NTC = no template control during RT step; Bf = buffer control during PCR step

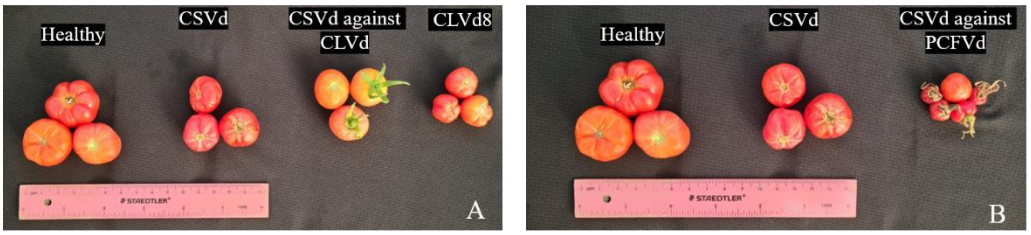

Figure 6. Reduction in tomato fruit size in each treatment: (A) tomato first inoculated with CSVd and challenged with CLVd; (B) cross-protected tomato plants challenged with PCFVd. Healthy = uninoculated tomato, CSVd = tomato inoculated with CSVd, CSVd against CLVd = tomato inoculated with CSVd and challenged with CLVd, CLVd8 = tomato inoculated with CLVd at the same time as challenged inoculation, CSVd against PCFVd = tomato inoculated with CSVd and challenged with PCFVd

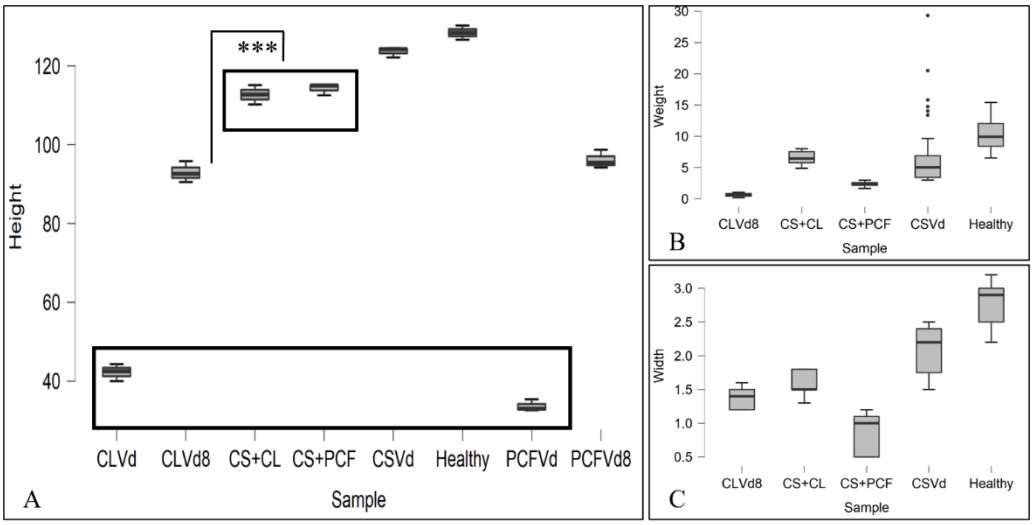

Figure 7. Box plots of one-way ANOVA and Tukey HSD comparing significant differences between treatments for plant height, fruit weight, and fruit width at $p < 0.001$ (***): (A) plant height; (B) fruit weight; (C) fruit width. CLVd = CLVd inoculation, CLVd8 = CLVd inoculation at same time as challenge inoculation, CS+CL = CSVd inoculation and challenged with CLVd, CS+PCF = CSVd inoculation and challenged with PCFVd, CSVd = CSVd inoculation, Healthy = uninoculated healthy tomato plants, PCFVd = PCFVd inoculation, PCFVd8 = PCFVd inoculation at same time as challenge inoculation

Table 1. List of oligonucleotide primers used in this study

Primer	Sequences (5'-3')	Target gene/ viroids	Annealing temp. (℃)	Amplicon (bp)	References
Nad 2-1a	GGACTCCTGACGTATACGA AGGATC	ndhB[1/]	56	188	Thompson *et al.,* 2003
Nad 2-2b	AGCAATGAGATTCCCCAAT ATCAT				
cCS1	TTAGGATTACTCCTGTCT CGCAGG	CSVd[2/]	56	354	Tangkanchanapas, 2005
hCS1	ACAGGGTTTTCACCC TTC CTT TAG				
cCL-P2	CTGCAGCCATGCAAAGA	CLVd[3/]	56	370	Marach, 2008
hCL-P2	GGTCAGGTGTGAACCAC				
PCF-Seq-F	CCGTCTTCTGACAGGAGTA ATCCC	PCFVd[4/]	60	348	Yanagisawa and Matsushita, 2017
PCF-Seq-R	ACCCGCACGGCGCTTCTC				

1/: NADH dehydrogenase ND2 subunit (internal control), 2/: = *Chrysanthemum stunt viroid*, 3/: = *Columnea latent viroid*, 4/: = *Pepper chat fruit viroid*

Table 2. Effect of prior inoculation of a CSVd isolate on disease progression induced by CLVd or PCFVd isolates (mean ± S.D.)

Treatment	Symptoms	Average plant height (cm)	Average fruit weight (g)	Average fruit width (cm)
Uninoculated plants	None	128.4 ± 1.8	10.56±2.58	2.76±0.31
CSVd inoculation	None	123.6 ± 1.31	**7**.16±5.74	2.09±0.38
CLVd inoculation	Stunted, leaf epinasty, leaf distortion, discoloration	42.27 ± 2.16	-	-
CLVd inoculation at same time as challenge inoculation	Mild leaf epinasty, mild leaf distortion	92.97 ± 2.67	0.63±0.29	1.38±0.15
CSVd against CLVd	Mild leaf distortion	112.67 ± 2.45	6.60±1.19	1.59±0.18
PCFVd inoculation	Stunted, necrosis on leaf and leaf vein	33.67±1.51	-	-
PCFVd inoculation at same time as challenge inoculation	Mild discoloration	96.1±2.33	-	-
CSVd against PCFVd	Mild stunting	114.23±1.50	2.37±0.40	0.88±0.23

Discussion

CSVd, CLVd, and PCFVd are serious viroid pathogens affecting tomato production, for which there are no genetic resistance resources available (Kovalskaya and Hammond, 2014). Breeding resistance genes into tomatoes is challenging and time-consuming. Despite several studies confirming the efficiency of a transgenic approach based on the production of the yeast dsRNA-specific ribonuclease PAC-1 to regulate the resistance to PSTVd in potato (Sano *et al*., 1997) and CSVd in chrysanthemum (Ishida *et al.*, 2002), this approach is still prohibited in Thailand. Consequently, cross-protection has emerged as a viable option in a viroid management strategy. The current study reported the interference of CSVd as a cross-protective isolate to the CLVd and PCFVd isolates in tomato plants.

Bioassays of the CLVd and PCFVd viroid isolates revealed typical symptoms on sensitive tomato plants. On the other hand, CSVd induced no symptoms following inoculation on tomato plants, although these plants reacted positively to PCR detection. Identification of a CSVd isolate provided the prospect of using this viroid to control other viroids. The use of CSVd as a protective viroid isolate against CLVd or PCFVd challenge infection results in delayed and less severe symptoms. Molecular identification tests determined that the viroids infecting tomato plants were CSVd, CLVd, and PCFVd. The protection against infection was measured by reduced signs of necrosis in the veins, leaves or petioles and by plant height. The results suggested that previous infection with a CSVd isolate could lower symptom severity in CLVd-infected tomato plants and PCFVd-infected tomato plants. Mild leaf distortion was observed in cross-protected tomato plants challenged with CLVd. Mild stunt was observed in cross-protected tomato plants challenged with PCFVd, but there was no sign of necrosis on the leaf vein, stem, or petiole. The results of this cross-protection between different viroids were consistent with those obtained by Niblett *et al*. in 1978, who discovered that a mild strain of *Potato spindle tuber viroid* (PSTVd) could prevent the expression of symptoms by severe PSTVd and CSVd (Niblett *et al*., 1978).

The current study found that both CSVd and the challenge viroid isolate (CLVd or PCFVd) were replicated with lower PCR product sensitivity (Figure 5). In contrast to the study by Singh *et al.*, pre-infection with a mild strain of PSTVd on the susceptible potato cultivar Russet Burbank, after being challenged with a severe strain of PSTVd, the latter was not detected. Furthermore, cross-protection proved insufficient in the case of the tolerant BelRus potato cultivar, in which a severe strain of PSTVd was detected (Singh *et al.*, 1990). Initially, PCFVd-infected tomato plants resulted in flowering abortion and no fruit production; however, fruits were obtained in cross-

protected tomato plants challenged with PCFVd. Consequently, cross-protection may be beneficial in increasing the tomato output in the field. Because no seeds were obtained in this study, we could not determine whether the cross-protection ability was heritable. The incomplete cross-protection observed in the study could have been due to differences in sequences between viroid isolates of different species, which agrees with Singh *et al.* in 1999, who revealed a lack of cross-protection due to less than 90% sequence similarity of PSTVd against *Tomato chlorotic dwarf viroid* (TCDVd) (Singh *et al*., 1999).

Viroids do not code for any proteins; therefore, they must rely mainly on their RNA sequence for their replication and movement throughout the plant (Flores *et al*., 2012). The mechanisms by which one viroid isolate can interfere with the replication of another viroid isolate are not completely clear. To explain this occurrence, an RNA-mediated defense mechanism was proposed in which sequence-specific targets in the form of double-strand RNA were processed by the DICER enzyme family, which produces 21–25 nucleotide double-stranded molecules (Flores *et al*., 2005; Ratcliff *et al*., 1999; Xie *et al.,* 2004). This function is equivalent to posttranscriptional gene silencing (PTGS), and the nucleotide sequences of the protecting and challenging isolates must be similar (Lin *et al*., 2007).

Based on the viral infection experiments, the following possible outcomes of cross-protection were proposed: the protecting strain failed to replicate in the plants, the protecting strain competed with a more severe variant, or the severe strain overcame the protecting strain, resulting in severe symptoms in tested plants (Zhang and Qu, 2016). The data from the present study suggested that CSVd might compete for host cells and resources with CLVd or PCFVd, as both viroids were detected (Ding, 2009; Ziebell and Carr, 2010). The CSVd ("protecting") isolate could serve as a primer for PTGS initiation, resulting in a reduction in severity and an increase in fruit production ability in cross-protected tomato plants. The current study's findings could be further used to investigate applications of a non-transgenic approach, mainly when natural resistant plants against viroids are not available.

The actions to increase the successful cross-protection are recommended as preferable, closely related viroid strains should be used to increase the probability of interaction between the protective viroid isolate and severe viroid isolates, and a more extended period of time for protective viroid isolate accumulation. So that an abundance of viroid inoculum travels throughout the entire plant with sufficient copy numbers was found as previously reported by Duran-Vila and Semancik in 1990, suggesting that the level of cross-protection was dependent upon the interval time of inoculation between mild and severe strains after CEVd had been tested (Duran-Vila and Semancik, 1990).

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