Kasetsart J. (Nat. Sci.) 37 : 453 - 459 (2003)

Evidence of Citrus Exocortis Viroid in Thailand

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ABSTRACT

A new citrus viroid variant was isolated from symptomless lime, *Citrus aurantifolia,* grown in Chainat Province. Sequencial analysis of viroid molecule was determined to be 371 nucleotides with 98.3% homology to those previously reported of citrus exocortis viroid (CEVd) . Biological assay on *Gynura aurantiaca,* an indicator host for CEVd, revealed a mild epinasty symptom on leave. Nucleotide sequence of viroid from infected *Gynura* consisted of 371 nucleotides with 99.1% similarity to citrus viroid primarily isolated from lime with three bases changed. From this study, it is concluded that this viroid isolate is a variant of CEVd and the first citrus viroid being known to Thailand.

Key words: citrus, RT-PCR, CEVd, viroids, lime

INTRODUCTION

Citrus diseases are very important problem in citrus growing areas in the world such as foot and root rot, canker, tristeza, and viroid diseases. Viroid known as the smallest pathogenic agent of the plants, is distinguished from viruses by the absence of a protein coat and a non-translated, single-stranded circular RNA genome with a relatively low molecular weight (1.3×10^6 Da) (Diener, 1987). Replication is accepted as host dependent by a rolling-circle-type mechanism (Branch and Robertson, 1984) using the transcriptional system of the host plants (Schindler and Muhlbach,1992).

Citrus viroids (CVd) have been classified on the basis of physical and biological properties into five groups (Semancik and Duran-Vila, 1991). (1) Citrus exocortis viroid (CEVd) is in a size range of 371-375 nucleotides (Gross *et al*., 1982; Visvader *et al*., 1982; Visvader and Symons, 1985) causing bark scaling on *Poncirus trifoliata* rootstock as well as dwarfing, leaf epinasty and rugosity, petiole wrinkle and necrosis (Semancik, 1988). (2) CV-I group is in a size range of 317-327 bases, CVd-Ia and CVd-Ib, known as citrus bend leaf viroid (CBLVd) (Onelge, 1996). (3) CV-II group with at least three members established in this group, CVd-IIa, CVd-IIb (cachexia disease), and CVd-IIc. They are related to the hop stunt viroid (HSVd) (Levy and Hadidi,1993, Reanwarakorn and Semancik, 1999). (4) CV-III group with four members have been reported in a size range of 280- 292 nucleotides (Semancik *et al*., 1997). (5) CV-IV group with only one member described concisting of 284 nucleotides (Puchta *et al*., 1991).

Citrus exocortis viroid is the serious citrus pathogen causing exocortis disease in the world citrus growing areas. However, none of this viroid was reported in Thailand. The objective of this work is to determine the existence of citrus exocortis

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Received date : 18/09/03 Accepted date : 20/11/03

viroid in citrus species, particularly lime, so that an effective disease control program can be established.

MATERIALS AND METHODS

Nucleic acid extraction and purification

Young shoots of lime (5 g) from Chainat Province were collected, powderized with liquid nitrogen and extracted with 15 ml water-saturated neutralized phenol and 6 ml extraction buffer. An aqueous phase was removed to a new tube after centrifugation at 7,000 rpm for 35 minutes. Nucleic acid precipitation was done by adding 3 vol. of ethanol and 10 % V/ V of 3 M sodium acetate as well as kept at –20°C for 1 hour. A pellet was resuspended in 1 ml of TKM-buffer and dialyzed overnight against TKM-buffer at 4 °C. To recover nucleic acid, 1 vol. of 4 M LiCl was added to the pellet. After standing 4 hours at 4°C, the solution was centrifuged at 7,500 rpm for 35 minutes. The supernatant was precipitated in absolute ethanol. Then, the pellet was resuspended in 1 ml TKM buffer and loaded onto CF-11 cellulose column. After ethanol precipitation, the partially-purified viroid was resuspended in 300 µl TKM-buffer for further use (Reanwarakorn and Semancik, 1998).

Viroid detection

By sequential polyacrylamide gel electrophoresis (sPAGE), a 20 µl aliquot of the partially-purified viroid samples and 8 ml of 60% glycerol were mixed and loaded into 5% polyacrylamide gel. After 3 hours electrophoresis, the gel was stained with ethidium bromide for 10 minutes as well as the areas below and above 7S RNA were removed. The piece of gel was transferred onto the top of a 5% denaturing polyacrylamide gel. Viroid-RNA bands were determined by silver staining (Reanwarakorn and Semancik, 1998).

Viroid identification

The purified samples showing viroid-RNA bands on sPAGE were subjected to reverse transcription-polymerase chain reaction (RT-PCR) using specific primers for CEVd, 5' CCGGGGATCCCTGAAGAAC 3' and 5' GGAAACCTGGAGGAAGTCG 3'. The RT-PCR products were determined by 2% agarose gel electrophoresis.

Samples representing the size of 300 – 400 nucleotides were removed from the gel, ligated to pGEM-T Easy vector (Promega, 1999) and cloned into *E. coli* (Glover and Hames,1995). After that the positive clones were sequenced to determine nucleotides for comparison with the published-CEVd sequences.

Biological assay

Gynura aurantiaca, a highly selective host for CEVd (Semancik, 1988) was used for infectivity assay of isolated viroid. Ten microlitres of viroid nucleic acid extracted from lime was inoculated to *Gynura aurantiaca* by mechanical inoculation. After 10 weeks, 5g of leaf tissues were purified and performed RT-PCR with CEVd specific primers as previously described. The RT-PCR was ligated to pGEM-T Easy vector as well as cloned into *E. coli* for nucleotide sequence determination and comparison to the nucleotide sequence of viroid isolated from lime.

RESULTS AND DISCUSSION

Viroid detection and identification

Citrus viroid isolated from lime (*Citrus aurantifolia*) displayed the viroid bands of circular and linear forms on the denaturing polyacrylamide gel after silver staining (Figure 1). From sequence analysis, the molecular size was 371 nucleotides with 98.3% sequence homology to CEVd as previously reported by Gross *et al*. (1982) (Figure 3, Table 1). Moreover, sequence analysis showed that it was 96.2% as well as 97.8% similarity to

CEV-S and CEV-A as determined by Semancik *et al*. (1993) and Visvader *et al.* (1985), respectively (Table 1). However, CEVd caused severe symptoms on tomato (Visvader and Symons, 1985) and Citron (*Citrus medica*) (Semancik *et al*., 1993) while infected lime grown in Thailand was symptomless without bark scaling. It has to be determined whether this lime is a symptomless carrier variety. In addition, there should be more than one CEVd variant detected in infecting lime since the cloned viroids were varied in sizes when subjected to *Eco*RI digestion (data not shown). Citrus exocortis viroid has been previously reported to persist in many variants in a single infected plant (Visvader and Symons, 1985) or naturally occur as a mixture of several sequence variants (Fagoaga and Duran-Vila, 1996). Since citrus exocortis viroids can cause symptomless in very broad host range, care must be taken to determine the spread of viroids in *Citrus* species as well as other crops in Thailand.

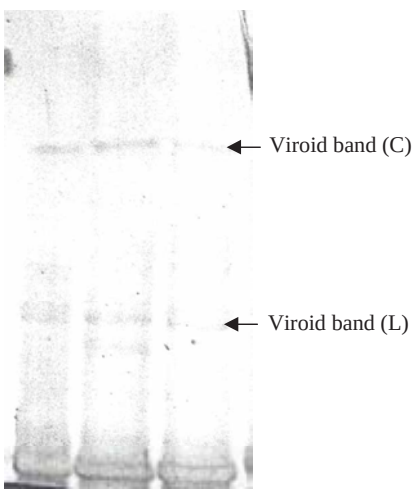

Figure 1 Viroid detection by denaturing polyacrylamild gel with silver staining. Arrows indicate the position of viroid circular (C) and linear (L) forms, respectively.

Biological assay

Infectivity of viroid isolated from lime was confirmed by Gynura infection. Leave symptoms induced by lime viroid consisted of mild epinasty after 3 months post inoculation in a greenhouse condition (Figure 5). The entity of citrus viroid on Gynura was determined by the RT-PCR with CEVd specific primers (Figure 2). The molecular size of CEVd recovered from Gynura was 371 nucleotides with 99.1% homology to the original citrus viroid of lime (Figure 3, Table 1). Viroid isolated from Gynura shared very high similarity to lime viroid only three nucleotides exchanged, $T_{99}\rightarrow A$, $G_{138}\rightarrow T$, and $C_{139}\rightarrow G$ (Table 2). The change of nucleotide at residue number 99 from

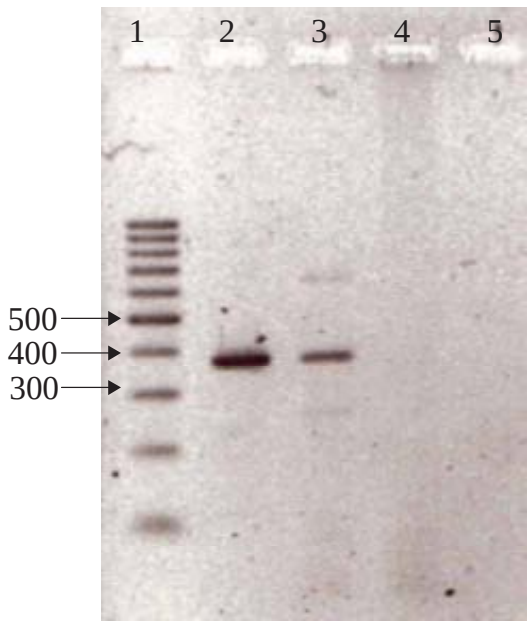

Figure 2 Viroid identification by RT-PCR using citrus exocortis viroid specific primers, molecular weight markers (lane 1), the extracts from lime (lane 2), the extracts from inoculated *Gynura* (lane 3), healthy lime (lane 4), and healthy *Gynura aurantiaca* (lane 5). Arrows indicate the molecular size ranging from 300-500 nucleotides.

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CEV-G    1 CGGGATCTTT  CTTGAGGTTC CTGTGGTGCT  CACCTGACCC TGCAGGCAGG
CEV-S      CGGGATCTTT  CTTGAGGTTC CTGTGGTGCT  CGCCTGACCC TGCAGGCAGG
CEV-A      CGGGATCTTT  CTTGAGGTTC CTGTGGTGCT  CACCTGACCC TGCAGGCAGG
CEV-l      CGGGAACTTT  CTTGAGGTTC CTGTGGTGCT  CACCTGACCC TGCAGGCAGG
CEV-g      CGGGAACTTT  CTTGAGGTTC CTGTGGTGCT  CACCTGACCC TGCAGGCAGG

CEV-G 51   AAAAGAAAAA AGAGGCGGCG GGGGAAGAAG TCCTTCAGGG ATCCCCGG -G
CEV-S      AAAAGAAAAA AGAGGCGGCG GGG -AAGAAG TCCTTCAGGG  ATCCCCGG -G
CEV-A      AAAAGAAAAA GATGGCGGCG GGGGAAGAAG TCCTTCAGGG ATCCCCGG -G
CEV-l      AAAAGAAAAA AGAGGCGGCG GGGGAAGAAG TTCTTCAGGG ATCCCCGGTG
CEV-g      AAAAGAAAAA AGAGGCGGCG GGGGAAGAAG TTCTTCAGGG ATCCCCGGAG

CEV-G 101  GAAACCTGGA GGAAGTCGAG GTCGGGGGGG A - CAGCTGCT TCGGTCGCCG
CEV-S      GAAACCTGGA GGAAGTCGAG GTCGGGGGGG ATCAGCTGCT  TCGGTCGCCG
CEV-A      GAAACCTGGA GGAAGTCGAG GTCGGGGGGG A - CAGCTGCT TCGGTCGCCG
CEV-l      GAAACCTGGA GGAAGTCGAG GTCGGGGGG - A - CAGCTGCT TCGGTCGCCG
CEV-g      GAAACCTGGA GGAAGTCGAG GTCGGGGGG - A - CAGCTTGT TCGGTCGCCG

CEV-G 151  CGGATCACTG GCGTCCAGCG GAGAAACAGG AGCTCGTCTC CTTCCTTTCG
CEV-S      CGGATCACTG GCGTCCAGCG GAGAAACAGG AGCTCGACTC CTTCCTTTCG
CEV-A      CGGATCACTG GCGTCCAGCG GAGAAACAGG AGCTCGTCTC CTTCCTTTCG
CEV-l      CGGATCACTG GCGTCCAGCG GAGAAACAGG AGCTCGTCTC CTTCCTTTCG
CEV-g      CGGATCACTG GCGTCCAGCG GAGAAACAGG AGCTCGTCTC CTTCCTTTCG

CEV-G 201  CTGCTGGCTC CACATCCGAT CGTCGCTGAA GCGCCTCGCC CCCTCGCCCG
CEV-S      CTGCTGGCTC CACATCCGAT CGTCGCTGAA GCGCCACGCC  CCCTCGCCCG
CEV-A      CTGCTGGCTC CACATCCGAT CGTCGCTGAA GCGCCTCGCC CCCTCGCCCG
CEV-l      CTGCTGGCTC CACATCCGAT CGTCGCTGAA GCGCCTCGCC CCCTCGCCCG
CEV-g      CTGCTGGCTC CACATCCGAT CGTCGCTGAA GCGCCTCGCC CCCTCGCCCG

CEV-G 251  GAGCTTCTCT CTGGATACTA CCCGGTGGAA ACAACTGAAG CTTCAACCCC
CEV-S      GAGCTTCTCT CTGGCTACTA CCCGGTGGAA ACAACTGAAG CTTCAACCCC
CEV-A      GAGCTTCTCT CTGGATACTA CCCGGTGGAA ACAACTGAAG CTTCAACCCC
CEV-l      GAGCTTCTCT CTGGAGACTA CCCGGTGGAA ACAACTGAAG CTTCAACCCC
CEV-g      GAGCTTCTCT CTGGAGACTA CCCGGTGGAA ACAACTGAAG CTTCAACCCC

CEV-G 301  AAACCGCTTT TCTTATATCT TCACTGCTCT CCGGGCGAGG GTGAAAGCCC
CEV-S      AAACCGCTTT TCTTATATCT TCACTGCTCT CCGGGCGAGG GTGAAAGCCC
CEV-A      AAACCGCTTT TCTTATATCT TCACTGCTCT CCGGGCGAGG GTGAAAGCCC
CEV-l      AAACCGCTTT TCTTGTATCT TCACTGCTCT CCGGGCGAGG GTGAAAGCCC
CEV-g      AAACCGCTTT TCTTGTATCT TCACTGCTCT CCGGGCGAGG GTGAAAGCCC

CEV-G 351  TCGGAACCCT AGATTGGGTC CCT
CEV-S      TCGGAACCCT AGATTGGGTC CCT
CEV-A      TCGGAACCCT AGATTGGGTC CCT
CEV-l      TTGGAACCCT AGATTGGGTC CCT
CEV-g      TTGGAACCCT AGATTGGGTC CCT
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CEV-G = Citrus exocortis viroid from Gross *et al*. (1982).
CEV-S = Citrus exocortis viroid from Semancik *et al.* (1993).
CEV-A = Citrus exocortis viroid from Visvader and Symons (1985).
CEV-l = Citrus exocortis viroid from lime in Thailand.
CEV-g = Citrus exocortis viroid from *Gynura aurantiaca*.

Figure 3 Sequence aligment of citrus exocortis viroid isolates.

Table 1 Sequence homologous comparison of citrus viroid isolates.

CEV isolates	% Sequence homology of CEVd isolates				
	CEV-G	CEV-S	CEV-A	CEV-l	CEV-g
CEV-l	98.3	96.2	97.8	100	99.1
CEV-g	97.5	95.6	97.3	99.1	100

CEV-G = CEVd from Gross *et al*.(1982), CEV-S = CEVd from Semancik *et al*.(1993), CEV-A = CEVd from Visvader and Symons (1985), CEV-l = CEVd isolated from lime in Thailand, and CEV-g = CEVd isolated from *Gynura aurantiaca*.

Table 2 Sequence heterogeneity of CEVd isolates.

Domain	Residue number*	CEV isolates	
		CEV-l	CEV-g
C	99	T	A
V	138	G	T
	139	C	G

* Residue number at the sites of change in either CEV-l, or CEV-g. CEV-l = CEVd isolated from lime and CEV-g = CEVd isolated from *Gynura aurantiaca*.

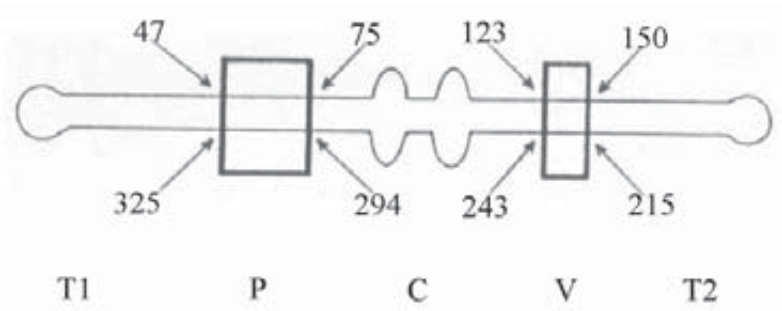

Figure 4 Diagram showing the location of domains on citrus exocortis viroid. Left-hand terminal domain (T1), Pathogenic domain (P), Conserved central domain (C), Variable domain (V), and Right- hand terminal domain (T2) (Keese and Symons, 1985).

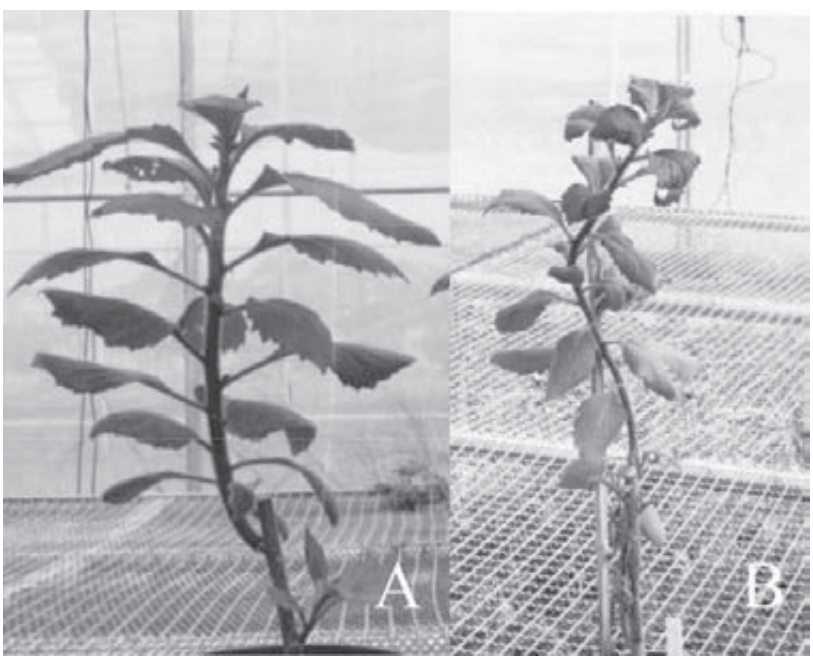

Figure 5 Leaf symptom caused by lime viroid on *Gynura aurantiaca.* Heathy (A)and inoculated (B).

T→A (Table 2) may have direct effect on the secondary structure within the C domain (Figure 4) which involves in the processing of viroid replication (Tabler and Sanger, 1984 ; Visvader *et al*., 1985). As a result, the titre of CEVd on *Gynura* was low regardless the fact that *Gynura* was reported to be a good host for CEVd (Semancik, 1988).

Variable domain is the most variable region of viroid structure (Diener, 1987). Other two exchanges in the V domain, G_{138}→T and C_{139}→ G (Table 2), may result from host interaction and cause leaf epinasty in *Gynura* (Figure 5). It will be interested to see whether or not these changes are reversible. This can be done by inoculating the viroid isolated from *Gynura* back to lime and re-isolate newly inoculated lime viroid for comparison with the wild type.

CONCLUSION

We reported here the first citrus viroid in Thailand. This viroid infected lime (*Citrus aurantifolia*) and *Gynura aurantiaca*. Lime viroid has 371 nucleotides with very high sequence homology to the severe citrus viroid variants which cause the severe symptoms on tomato and citron (*Citrus medica*). However, infected lime observed in this study was symptomless. The impacts of CEVd in lime and *Citrus* species as well as other crops remain to be further investigated.

ACKNOWLEDGEMENTS

We would like to thank the Thailand Research Fund for the Post-Doctoral Research Fellowship to Kanungnit Reanwarakorn and the Graduate School for supporting Somboon Pomma for her graduate study. *Gynura aurantiaca* is kindly provided by Prof. Dr. Semancik, UCR.

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