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cDNA Probe for Grapevine Yellow Speckle Viroid Detection

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ABSTRACT

Isolates of grapevine yellow speckle viroid (GYSVd-1) were found in Saraburi province, having 366 and 367 bp in size, and showed 96% sequence homology with Australian GYSVd-1. GYSVd cDNA probe was proven to be sensitive and highly specific to GYSVd-1. This cDNA probe could detect very low amount of the viroid even in the symptomless samples. Thus, GYSVd-1 cDNA probe would be very useful for plant quarantine or viroid free certification schemes, since the large number of samples could be tested for viroid infection with high accuracy.

Key words: grapevine, RT-PCR, GYSVd-1, viroids, cDNA probe

INTRODUCTION

Grapevines are susceptible to a number of diseases. One of them is viroid disease. Five viroids have been reported to infect grapevines, namely Grapevine yellow speckle viroid 1 (GYSVd-1), Grapevine yellow speckle viroid 2 (GYSVd-2), Hop stunt viroid (HSVd), Citrus exocortis viroid (CEVd) and Australian grapevine viroid (AGVd) (Szychowski *et al*.,1998). Only GYSVd-1 and GYSVd-2 have been known to induce yellow speckle and vein banding on the grapevine leaves (Jawanda and Chadha, 1977). Some viroid infection may appear symptomless. Therefore, viroid in symptomless carriers could be transmitted widely to other healthy crops. The detection method should be simple and sensitive enough to detect viroids in symptomless plants and in very low amount of the pathogen. Hybridization method can also be applicable for routine testing of a large number of samples (Hadidi, 1988). The control of viroid disease has to rely on an efficient detection method (Hanold, 1993). The aims of this study were 1. to study of Grauevine Yellow Speckle Viroid (GYSVd) in Thailand. 2. to produce spacific cNDA probe for GYSVd detection.

MATERIALS AND METHODS

1. Source of plant materials

Infected grapevine leaves were obtained from field-grown grapevine, growing in different areas in Thailand.

2. RNA extraction for viroids

RNA extraction was carried out using a modified method of Wan Chow Wah and Symons (1997). Grapevine leaf tissue (1.0 g) was ground in a cold mortar in 10 ml of extraction buffer (95 mM K_2HPO_4, 95 mM KH_2PO_4, 10% (w/v) sucrose, 0.15% (w/v) bovine serum albumin (BSA) fraction V, 2% (w/v) polyvinylpyrrolidone (PVP-10), 0.53% (w/v) ascorbic acid, adjusted to pH 7.6 before extraction). The leaf suspension was centrifuged at low speed (75-100 x g) for 2 min. The supernatant was pelleted and resuspended in 2

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ml TE_1 buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA) to which 0.1% (v/v) 2-mercaptoethanol and 0.65% (w/v) sodium sulphite were added to prevent browning. SDS (250 µl of 10% SDS solution) was put into the resuspension which was aliquotted into the microfuge tubes and incubated for 10 minutes at 60°C. The tubes were cooled on ice for 1 min and spin at 14,000 rpm at 4°C for 5 min. The supernatants were transferred to two fresh microfuge tubes and incubated with 400 µl of 5 M potassium acetate at -20°C for 10 min. The supernatant was precipitated and dissolved in 500 µl TE_2 buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) before precipitation with 2 M NaCl and 1 volume isopropanol. The pellet was washed in 70% ethanol, dissolved in 200 µl TE_2 as well as stored at -80°C for using further.

3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Full-length cDNA viroid was synthesized by RT-PCR technique with GYSVd specific primers. The first strand primer (V-c primer) 5′ ggacgcgaacgtgaatagg 3′ and the second strand (V-h primer) 5′ ttgaggcctggcgtaacgc 3′ are specific to the variable domain of GYSVd-1 (Szychowski *et al*., 1998).

For the reverse transcription reaction, 2 µl of the extracted RNAs were mixed with 0.7 µg cDNA primer and incubated at 80°C, for 5 min, and 37°C, for 15 min. And then it was added with the followings: 4 µl of 5X RT buffer, 2 µl of 10 mM $dNTP_S$, 0.5 µl of 100 units RT (M-MLV) enzyme and RNase free water up to 20 µl. The mixture was incubated at 42°C, for 1 hr. For the amplification step, 2 µl of RT product was combined with 2 µl of 10X PCR buffer, 0.5 µl of 10 mM $dNTP_S$, 0.6 µl of 50 mM $MgCl_2$, 100 ng of cDNA primer, 100 ng of hDNA primer, 1 unit of Taq DNA polymerase enzyme and RNase free water up to 20 µl. cDNA was amplified after an initial denaturation for 30 seconds at 92°C, in 35 cycles of 30 seconds at 92° C, 1 min at 57°C and 2 min at 72°C in PTC-100 PCR cycler. The final extension was for 15 min (Szychowski *et al*., 1998).

4. cDNA cloning and sequence analysis

Full-length double stranded cDNA (367 bp) was separated on a 2% agarose gel and purified by using Gel Extraction (Qiagen®, 2002). The purified DNA fragments were ligated into the pGEM-T easy vector to transform into *Escherichia coli* DH5α. cDNA clones were sequenced using Applied Biosystems (ABI) model 377 and subjected to ClustalW program, multiple sequence alignment with previously reported sequences.

5. cDNA probe synthesis and northern blot hybridization

Digoxigenin (Dig)-labelled cDNA probe was prepared from full-length cDNA of grapevine viroid. DIG High Prime DNA Labeling and Detection Starter Kit I^{Roche} was used in cDNA labeling for hybridization (Roche®, 1999).

Northern hybridization was modified from Sambrook and Russell (2001). The extracted RNA samples were separated on a 2% formaldehyde agarose (FA) gel in 1X FA running buffer (100 ml of 10XFA gel buffer, 20 ml of 37% formaldehyde and 880 ml RNase free water). The FA gel was rinsed with distilled water and soaked in 0.05 N NaOH for 20 min and in 20XSSC 40 min; RNA band from FA gel was transferred on to a nitrocellulose membrane and soaked in 20XSSC transfer buffer for 10 min by capillary adhesion for overnight (16-24 hrs) following the hybridization test with GYSVd-1 probe.

RESULTS

1. Plant materials and RT-PCR

Surveys of many grapevine orchards indicated that most of infected geapevines showed yellow speckle on the leaves more often than vein banding. Samples from Saraburi province showed a DNA band about 367 bp in size when detected by

RT-PCR method (data not shown).

2. cDNA cloning and sequence analysis

Two cDNA clones, each of 367 (YS-SB1) and 366 (YS-SB6) bp in length (Fig.1) and 99% sequence homology, were obtained base addition and a changed nucleotide on such sequences, were also observed as following T added at position 220 on YS-SB1 and G changed to A at position 311 on YS-SB6 (Figure 1). Both sequences were analyzed by alignment with other GYSVd-1 sequences which were reported previously on Genbank such as accession number NC_001920 (Szychowski *et al*., 1998), AF462167 (Elleuch *et al*., 2001), AB028465 (Sano *et al*., 1999), X87917 (Polivka *et al*., 1996), Z17225 (Rigden and Rezaian, 1993) and X06904 (Koltunow and Rezaian, 1988). The result showed that X06904, YS-SB1 and YS-SB6 had sequence homology score to 96% (Table1). Sequence comparison with those of known viroids including Hop stunt viroid (HSVd), Australian grapevine viroid (AGVd), Citrus exocortis viroid (CEVd) and Grapevine yellow speckle viroid 2 (GYSVd-2) were analyzed and displayed in Table 2. The comparison with other viroids showed 4, 14, 57 and 77% sequence homology with HSVd, CEVd, AGVd and GYSVd-2, respectively.

3. Sensitivity and specificity of cDNA probe

The cDNA probe (YS-SB6) could detect GYSVd-1 by using Northen hybridization (data not shown). Symptom and symptomless grapevine samples were tested by hybridization assay to confirm probe specificity. The result showed that GYSVd (YS-SB6)cDNA probe could detect 9 positive samples out of 17 samples by Northern hybridization (Figure 2). As indicated in Figure 2a, total extracted RNAs of grapevine samples generated several bands when loaded onto formaldehyde agarose but only one band hybridized with GYSVd (YS-SB6) probe (Figure 2b). This result confirmed that cDNA probe had a high specificity for GYSVd. It could be used effectively for the detection of GYSVd in symptomless samples.

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TCTCCGGATCTTCTTGCTTGTGGTTCCTGTGGTTTCACCTCGGAAGGCCGCCGCGGACCT 60
TCTCCGGATCTTCTTGCTTGTGGTTCCTGTGGTTTCACCTCGGAAGGCCGCCGCGGACCT 60

GCA-GAGAAGAAGATAGGGGCAGAGGGGGTTCGAGCCTCGTCGTCGACGAAGGGGTGCGA 119
GCA-GAGAAGAAGATAGGGGCAGAGGGGGTTCGAGCCTCGTCGTCGACGAAGGGGTGCGA 119

ACCGAGTGCCTGAGCTGGTCGACGTCCAGCTC-CCTCGGGACCACGCTGCTCTGGGCGGA 178
ACCGAGTGCCTGAGCTGGTCGACGTCCAGCTC-CCTCGGGACCACGCTGCTCTGGGCGGA 178

AGAGTCTTCTGACACTT-CTAGCCTATTCACGTTCGCGTCCTtGAGGCCTGGCGTAACGC 237
AGAGTCTTCTGACACTT-CTAGCCTATTCACGTTCGCGTCCT-GAGGCCTGGCGTAACGC 236

GGCTCTTGCCACCAGGTCGCCTCCGCTAGTCGAGCGGACTTGGTCTCTTCCGCCCAAAGC 297
GGCTCTTGCCACCAGGTCGCCTCCGCTAGTCGAGCGGACTTGGTCTCTTCCGCCCAAAGC 296

CCTTTTTCTTTCTgATCAGCTTGTTCCAACGCGCCCCGCGAGTGCAATCCCCGGAACCCC 357
CCTTTTTCTTTCTaATCAGCTTGTTCCAACGCGCCCCGCGAGTGCAATCCCCGGAACCCC 356

CGCTAAGAGG---- 367 YS-SB1
CGCTAAGAGG---- 366 YS-SB6
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Figure 1 Sequence comparison of 2 Thailand isolates GYSVd.

DISCUSSION

The yellow speckle and vein banding symptoms were similar to grapevine yellow speckle disease previous reported in Australia, Europe and America. This disease is caused by GYSVd (Rezaian, 1990; Szychowski *et al*., 1998) having a size of 367 bp in length. Thailand isolates of GYSVd are similar to Australian GYSVd-1 (Koltunow and Rezaian, 1988) 96% sequence homology. It was possible that GYSVd-1 was brought in from abroad by infected grapevine materials. Moreover, vegetative propagation of grapevine may accelerate the epidemics of GYSVd-1.

Table 1 Sequence homology comparison of YS-SB1 and YS-SB6 with other GYSVd-1.

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	NC_001920	366	2	AF462167	366	89
1	NC_001920	366	3	AB028465	367	98
1	NC_001920	366	4	X87917	366	88
1	NC_001920	366	5	Z17225	368	87
1	NC_001920	366	6	X06904	367	88
1	NC_001920	366	7	YS-SB1	367	85 *
1	NC_001920	366	8	YS-SB6	366	85 *
2	AF462167	366	3	AB028465	367	89
2	AF462167	366	4	X87917	366	98
2	AF462167	366	5	Z17225	368	96
2	AF462167	366	6	X06904	367	98
2	AF462167	366	7	YS-SB1	367	95
2	AF462167	366	8	YS-SB6	366	94
3	AB028465	367	4	X87917	366	88
3	AB028465	367	5	Z17225	368	87
3	AB028465	367	6	X06904	367	89
3	AB028465	367	7	YS-SB1	367	85
3	AB028465	367	8	YS-SB6	366	86
4	X87917	366	5	Z17225	368	95
4	X87917	366	6	X06904	367	97
4	X87917	366	7	YS-SB1	367	94
4	X87917	366	8	YS-SB6	366	94
5	Z17225	368	6	X06904	367	96
5	Z17225	368	7	YS-SB1	367	95
5	Z17225	368	8	YS-SB6	366	95
6	X06904	367	7	YS-SB1	367	96 **
6	X06904	367	8	YS-SB6	366	96 **
7	YS-SB1	367	8	YS-SB6	366	99

The accession number NC_001920, AF462167, AB028465, X87917, Z17225 and X06904 were Szychowski *et al*. (1998), Elleuch *et al*.(2001), Sano *et al*. (1999), Polivka *et al*. (1996), Rigden and Rezaian (1993) and Koltunow and Rezaian (1988), respectively. Stars indicate the minimum score (*) and maximum score (**).

Table 2 Sequence homology comparison of YS-SB1 and YS-SB6 with other viroids infecting grapevine.

SeqA	Name	Len(nt)	SeqB	Name	Len(nt)	Score
1	AY517496CEVd	372	2	NC003553AGVd	369	13
1	AY517496CEVd	372	3	X87927HSVd	297	15
1	AY517496CEVd	372	4	J04348GYSVd2	363	26
1	AY517496CEVd	372	5	YS-SB1	367	14
1	AY517496CEVd	372	6	YS-SB6	366	14
2	NC003553AGVd	369	3	X87927HSVd	297	6
2	NC003553AGVd	369	4	J04348GYSVd2	363	60
2	NC003553AGVd	369	5	YS-SB1	367	57
2	NC003553AGVd	369	6	YS-SB6	366	57
3	X87927HSVd	297	4	J04348GYSVd2	363	12
3	X87927HSVd	297	5	YS-SB1	367	4
3	X87927HSVd	297	6	YS-SB6	366	4
4	J04348GYSVd2	363	5	YS-SB 1	367	77
4	J04348GYSVd2	363	6	YS-SB 6	366	77
5	YS-SB1	367	6	YS-SB 6	366	99

The accession number AY517496, NC_003553, X87928, J04348 were CEVd (Guardo and Caruso, 2004), AGVd (Rezaian, 1990), HSVd (Polivka *et al*., 1996) and GYSVd-2 (Koltunow and Rezaian, 1989), respectively.

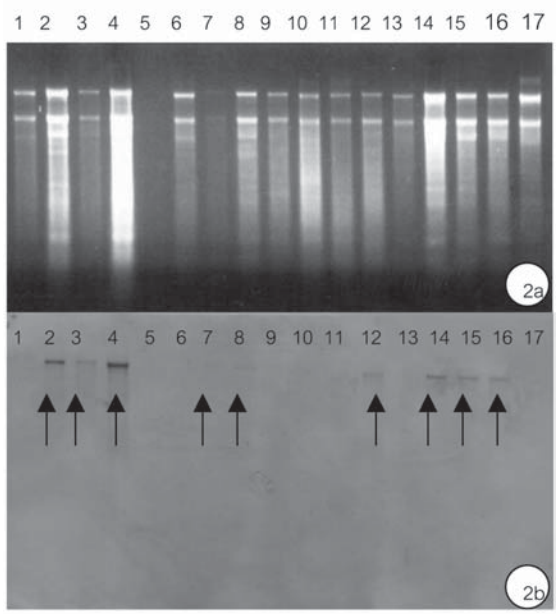

Figure 2 Northern analysis of total extracted RNAs from grapevine leaves on a 2% formaldehyde agarose gel. (2a) Lane 1, 7, 9-13 and 15-17, symtomless grapevine leaves; lane 2-6, 8 and 14, yellow speckle on leaves. (2b) Hybridization with GYSVd-1 specific probe (YS-SB6). The arrows indicate positive bands.

The YS-SB1 and YS-SB6 cDNA clones were only 1% sequence difference at the position 220 and 311 which located on the lower of variable and pathogenic domains, respectively. Positions of these changed nucleotides are very interesting because only one position can have significant impact on the virulence of disease (Qi and Ding, 2003).

The cDNA probe (YS-SB6) was specific to GYSVd because this cDNA probe could detect four symptomless samples, lane 7, 12, 15 and 16, which confirmed the presence of GYSVd-1 by RT-PCR method with GYSVd-1 specific primers (data not shown). In Figure 2 (2b), the sample in lane 5 and 6 were the ones showing yellow speckle on leaves which displayed negative results on hybridization assay. This might be the extraction method from Wan Chow Wah and Symons (1997) suggested to use for fresh leaves, but the samples in lane 5 was RNAs from frozen leaves at -20°c before extraction. For lane 6, the total extracted RNAs might be very low amount of viroids for detection with cDNA probe because they were amplified further giving a positive result by RT-PCR technique (data not shown).

Each of RNA samples subjected to formaldehyde agarose gel was extracted approximately from 40 mg infected leaves. Thus, it was demonstrated that this cDNA probe could be used for the detection of GYSVd from a small volume of tissue and a large number of samples.

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