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Detection of Citrus exocortis viroid by PCR-microplate hybridization.

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Abstrac : The polymerase chain reaction (PCR)-microplate hybridization method according to HATAYA, *et al.* (1994) was evaluated for the detection of citrus exocortis viroid (CEVd). Total nucleic acids were extracted from citrus leaves and amplified by reverse transcription (RT)-PCR with CEVd specific primer pairs. The digoxigenin (DIG)-labelled probe was prepared by PCR. The microplate hybridization were carried out according to INOUYE and HONDO (1990) and HATAYA, *et al.* (1994). By this method, CEVd could be detected in all six CEVd infected citrus samples and also from two citrus samples that were healthy by indexing with CEVd indicator plants, Etorog citron Arizona 861-S1. The practical mehtods for indexing CEVd so far known are biological assay and electrophoresis of nucleic acid extracted from citrus leaves. They require long period for detection and can not distinguish species of viroids. On the other hand, PCR-microplate hybridization provides a rapid and highly sensitive diagnostic method for CEVd.

Key words : citrus exocortis viroid, polymerase chain reaction, microplate hybridization, DIG-labelled probe

Introduction

Citrus exocortis viroid (CEVd) infects most citrus species by graft-transmission, and several citrus species, cultivars and some citrus relatives express specific symptoms. When CEVd-infected buds are grafted on a susceptible rootstock, bark scaling occurs on the rootstock and the entire tree becomes stunted. In Japan, trifoliate orange seedlings (*Poncirus trifoliata* (L.) Raf.) which is very susceptible to this viroid, have been used for rootstocks of most citrus species, so the exocortis disease has been recognized as an important disease (SEMANCIK,1980; GARNESEY and BARKLEY,1988).

CEVd indexing of imported citrus has usually been made by graft inoculation to CEVd sensitive clone of Etrog citron Arizona 861-S1 (*Citrus medica* L., ROISTACHER, *et al.*, 1977), sap inoculation to Rutgers tomato (*Lycopersicum esculentum* Mill. cv. Rutgers, NAGAO and WAKIMOTO, 1981) and nucleic acid extraction followed by return-gel electrophoresis (SCHUMACHER, *et al.*, 1986). The biological assay on indicator plants requires long period for detection and the return-gel electrophoresis can not distinguish individual species of viroids. Recently, by the advance of molecular biology, nucleotide sequences of most viroid RNAs were determined. And the hybrid-

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ization methods with non-radioactive probes and polymerase chain reaction (PCR) has been used for detection of plant viruses and viroids (VUNSH, *et al.*, 1990 and 1991; JONES, *et al.*, 1991; KOHNEN, *et al.*, 1992; HADIDI and YANG, 1990; HATAYA, *et al.*, 1992).

INOUE and HONDO (1990) developed a new hybridization method for medical use, called the microplate hybridization. HATAYA, *et al.* (1994) modified this method for detection of potato virus Y (PVY), and proved it to be about 10,000 times more sensitive than enzyme-linked immunosorbent assay (ELISA). In this paper, the PCR-microplate hybridization method according to HATAYA, *et al.*, (1994) was evaluated for detection of CEVd.

Materials and Methods

Viroid source

Six CEVd infected citrus samples and five healthy citrus samples were used (Table 1). All samples were indexed by grafting to Etrog citron Arizona 861-S1 (*Citrus medica* L.) in advance. Fresh and silica-gel dried leaves were used for total nucleic acids extraction. Purified CEVd sample (CEV-h, 0.25 μg/ml, SANO *et al*, 1986) was used as a positive control.

Total nucleic acids extraction from citrus leaves

The total nucleic acid from fresh or dried citrus leaves were extracted by following procedure. The leaves of 0.1~0.2g were crushed in 1ml of a extracting buffer containing 0.13M Tris-HC l (pH 8.9), 0.017M EDTA, 1M LiCl, 0.83% SDS (sodium dodecyl sulfate), 5% PVP and 30 μl of 2-mercaptoethanol in 1.5ml microcentrifuge tube using a plastic pestle and then centrifuged. The aqueous phase was treated with an equal volume of water saturated phenol:chloroform (1:1) mixture. After centrifugation, the nucleic acids in aqueous phase were precipitated with 0.1 volume of 3M sodium acetate (pH 5.2) and 0.6 volume of isopropyl alcohol in -30℃. Then the precipitates were extracted again with a water satulated phenol:chloroform (1:1) mixture. The total nucleic acids were precipitated again in 0.1 volume of 3M sodium acetate(pH 5.2) and 2.5 volume of ethanol, the precipitates were dissolved in 200 μl of distilled water, mixed with 50 μl of 10M LiCl, and then kept overnight on ice. Finally, insoluble RNAs in 2M LiCl were dissolved in 400 μl of distilled water.

PCR primers

Three oligodeoxynucleotide primers for PCR amplification of CEVd RNA were synthesized using an automated DNA synthesizer (Applied Biosystems Inc. Model 380B or 392). Two plus sense primers designated CEV-1P,PCEV-1P and a minus sence primer designated CEV-2M were used (Table 2 and Fig. 1).

Amplification of cDNA to CEVd RNA sequence by RT-PCR

The minus sence PCR primer, CEV-2M, was used for reverse transcription (RT) from CEVd RNA. A RT reaction mixture (20 μl), containing 4 μl of extracted RNA sample, 25pmol CEV-2M primer, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM each dNTP and 100 U M-MLV RTase(GIBCO BRL, Life Technologies), was incubated at 37℃ for 60 min. Ten μl of RT reaction mixture was added to 40 μl of PCR pre-mixture containing 7.5 mM Tris-HCl (pH 9.8), 81.25 mM KCl, 1.125mM $MgCl_2$,0.375 mg/ml bovine serum albumin (BSA), 0.075% sodium cholate, 0.075% Triton X-100, 50 pmol each of plus and minus sense

Table 1. Samples used for RT-PCR maicroplate hybridization

No.	plant	status[a]	origin
1	Etrog citron (*Citrus medica* L.)[b]	healthy	Hiroshima fruit tree research station
2	Etrog citron (*C. medica* L.)[b]	healthy	〃
3	Etrog citron (*C. medica* L.)[b]	CEVd severe strain	〃
4	Kiyomi orange (*C. sinensis* Osbeck forma kiyomi)[b]	healthy	〃
5	hassaku (*C. hassaku* hort. ex Tanaka)[b]	CEVd severe strain	〃
6	Etrog citron (*C. medica* L.)[c]	healty	Yokohama plant protection station
7	Etrog citron (*C. medica* L.)[c]	healthy	〃
8	Etrog citron (*C. medica* L.)[c]	CEVd[d]	〃
9	Etrog citron (*C. medica* L.)[c]	CEVd[d]	〃
10	Rough lemon (*C. jambhiri* Lush.)[c]	CEVd[d]	〃
11	Shikikitsu (*C. madurensis* Lour.)[c][e]	CEVd[d]	The Philippines

a) Indexed by Etrog citron Arizona 861-SI b) silicagel dried leaves c) fresh leaves d) Strains are not known e) Import permit unmber issued by Ministry of Agriculture, Forestry and Fisheries is 6Y1251

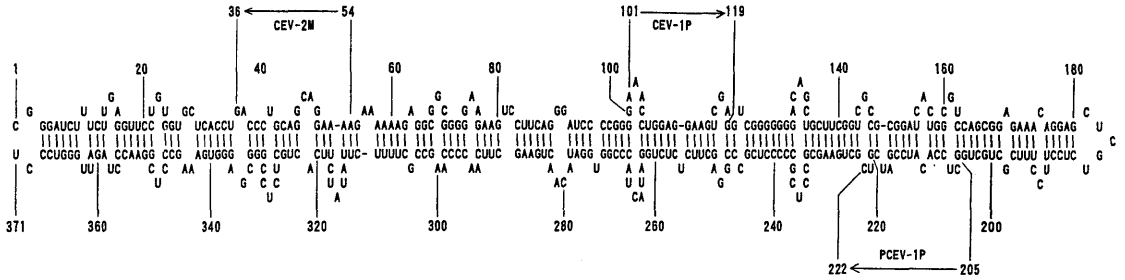

Fig. 1 Nucleotide sequence of CEVd (GROSS, et al., 1982) and annealing position of primers.

Table 2. PCR primers used

Primer	Sence	Sequence	Size	position[a]
CEV-1P	plus	5' AAACCTGGAGGAAGTCGAG 3'	19	101-119
PCEV-1P	plus	5' GCTCCACATCCGATCGTC 3'	18	205-222
CEV-2M	minus	5' TTTTCCTGCCTGCAGGGTC 3'	19	54-36

a) The numbers correspond to published nucleotide sequence numbers of CEVd (Gross et al. 1982)

primers, 187.5 μM each of dNTP and 1 U of *Tth* DNA polymerase (Toyobo Co.).

The PCR reaction mixture was overlaid with one drop of light mineral oil (Sigma Chemical Co.) to prevent evaporation. PCR amplification proceeded through 25 cycles of 30 sec denaturation at 94℃ (5 min for the first cycle), 1 min annealing at 53℃ and 2 min primer extension at 72℃ (8 min for the last cycle) using a Program Temp Control System PC-700 (Astec Co.). The reaction product was extracted with a water saturated phenol : chloroform (1:1) mixture to

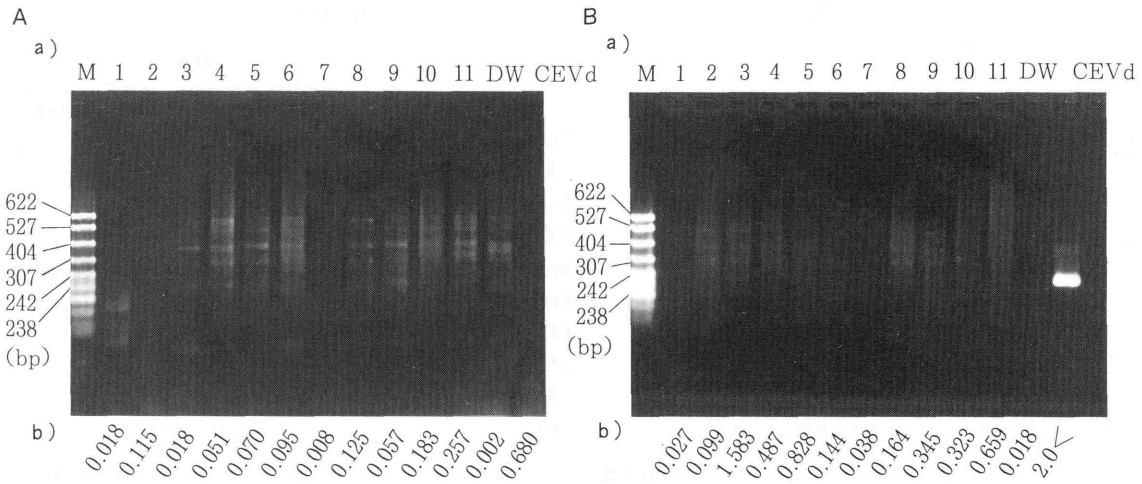

Fig. 2 Agarose gel electrophoresis (2%) of RT-PCR products and results of microplate hybridization. A : amplified with primer pair of CEV-2M and CEV-1P, B : amplified with primer pair of CEV-2M and PCEV-1P, a) : Agarose gel electrophoresis (2%) of RT-PCR products b) : absorbance values at 415 nm of microplate hybridization, lane 1~11 is each of sample No. 1~11 (see Table 1), DW: distilled water, CEVd: purified CEVd sample, M: molecular size marker (pBR322/Hpa II).

remove light mineral oil and BSA, and centrifuged. The aqueous phase was taken and mixed with 1/4 volume of 10M ammonium acetate and 2.5 volume of ethanol at -80℃ for 30 min. After centrifugation, the precipitates were dried in vacuum, and dissolved in 50 μl of TE (10 mM Tris-HCl, 1mM EDTA, pH8.0). RT-PCR products were electrophoresed in a 2% agarose gel, stained with ethidium bromide (0.5 μg/ml) and visualized using an ultraviolet transilluminator.

DIG-labelled cDNA probe for microplate hybridization

DIG-labelled cDNA probe for CEVd RNA was prepared by PCR using a pair of PCR primers, PCEV-1P and CEV-2M. cDNA amplified from purified CEVd (CEV-h, Sano, *et al.*, 1986) by RT-PCR was used as template DNA. PCR was carried out in a 50 μl reaction mixture containing 10 mM Tris-HCl (pH 8.9), 80 mM KCl, 1.5 mM $MgCl_2$, 0.5 mg/ml BSA, 0.1% sodium cholate, 0.1 % Triton X-100, 0.1mM each dGTP, dATP, dCTP, 0.065mM dTTP, 0.035mM DIG-11-dUTP (Boehringer Mannheim), 50 pmol each PCR primers, 1 U *Tth* DNA polymerase and 1 μl of RT reaction mixture. After PCR amplification, the light mineral oil was removed carefully, and DIG-labelled probes were purified by an Ultrafree C3-TTK (Millipore Ltd.) spun column to remove unreacted dNTPs, DIG-11-dUTP and primers. The probes in the upper filter cup were dissolved in 50 μl of TE. Five μl of the probe solution were electrophoresed in a 2% agarose gel. After staining with ethidium bromide (0.5 μg/ml), the DIG-labelled probe band was visualized using an ultraviolet transilluminator.

Microplate hybridization

The amplified cDNA fragments were diluted 20 fold with 10 × SSC (standard saline citrate, 1 × SSC: 0.15M NaCl, 0.015M sodium citrate, pH 7.0) containing 10 mM EDTA and denatured

at 100℃ for 5 min and then quickly chilled in ice-water. One hundred μl of the diluted DNA were pipetted into wells of a 96-well polystyrene microplate (Nunc Immunoplate II-Maxisorp). The plates were incubated at 37℃ for 2 hours. The wells were washed three times with phosphate buffered saline (PBS: 137 mM NaCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.7 mM KCl, pH 7.4) containing 0.05% Tween-20 (PBS-T). Then each well was filled with 100 μl of hybridization solution, containing the heat denatured DIG-labelled probes (2 μl/ml: 500-fold dilution), 50% formamide (E.Merck AG), 5 × SSC, 10 mM EDTA(pH 7.0), 0.1% Tween-20, 100 μg/ml of yeast tRNA. The plates were sealed with adhesive sheets and incubated overnight at 42℃. After hybridization, the wells were washed three times with PBS-T, and filled with 100 μl of alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim) which was diluted 5,000-fold with PBS-T. The plates were incubated at 37℃ for 1 hour. The wells were washed three times with PBS-T, and then 200 μl of *p*-nitrophenylphosphate (1 mg/ml in 10% diethanolamine buffer, pH 9.8) were added as substrate. The plates were incubated at room temperature for 2 hours. The absorbance value of nitrophenol obtained by hydrolysis of *p*-nitrophenylphosphate in each well were measured using a microplate reader (Corona Co.,Ltd. Model MTP-100 or Bio Rad Model 450) at 415 or 405 nm wavelenght. The average value of two wells was calculated.

Comparison of total nucleic acid extraction methods

With the aim of simplifing the extraction method, the following 5 extraction procedures were examined : (a) crude sap in distilled water, (b) samples without 2M LiCl fractination for extracting procedure as mentioned above, (c) samples of complete extracting procedure as menntioned above, (d) samples crushed in distilled water, water satulated phenol:chloroform (1:1) mixture treatment and ethanol precipitation, (e) samples crushed in extracting buffer as mentioned above, water satulated phenol:chloroform (1:1) mixture treatment and ethanol precipitation. Two CEVd infected Etrog citron and a healthy rough remon (*C. jambhiri* Lush.) were used for this experiment.

Results

RT-PCR amplification and the primer combinations

Two primer pairs, CEV-2M and CEV-1P, CEV-2M and PCEV-1P were used for RT-PCR amplification. RT-PCR products were electrophoresed in 2% agarose gel. The results of electrophoresis showed no clear bands when the primer pair of CEV-2M and CEV-1P was used (Fig. 2A-a). When the primer pair of CEV-2M and PCEV-1P was used, the clear band appeared only in the positive control, but not in other samples (Fig. 2B-a).

DIG-labelled probe by RT-PCR amplification

In order to confirm the incorporation of DIG-labels into the DNA fragments, the amplified DNA and the DIG-labelled probe were coelectrophoresed in a 2% agarose gel. As shown in Fig. 3, the DIG-labelled probe migrated slower than the corresponding DNA fragments.

Detection of CEVd by microplate hybridization

After RT-PCR amplification, RT-PCR products were detected by microplate hybridization with DIG-labelled probe. The results are shown in Fig. 2 A-b and B-b. The all CEVd infected

Table 3. Comparison of nucleic acid extraction methods

Total nucleic acid extraction method [a]	citrus samle status [b]	microplate hybridization [c]
a	CEVd-1[d]	0.025
	CEVd-2[d]	0.087
	healthy[e]	0.015
b	CEVd-1	1.729
	CEVd-2	2.0<
	healthy	0.048
c	CEVd-1	2.0<
	CEVd-2	2.0<
	haelthy	0.045
d	CEVd-1	0.048
	CEVd-2	0.026
	healthy	0.013
e	CEVd-1	1.154
	CEVd-2	0.819
	healthy	0.221

a) a : crude sap extracted in distilled water.
b : crushed in extracting buffer, water satulated phenol : chloroform(1:1) mixture treatment, isopropyl alcohol precipitation and ethanol precipitation
c : b + 2M LiCl fractionation.
d : crushed in distilled water, water satulated phenol : chloroform(1:1) mixture treatment and ethanol precipitation.
e : crushed in extracting buffer, water satulated phenol : chlorofrom(1:1) treatment and ethanol precipitation
b) Indexed by Etrog citron Arizona 861-S1.
c) Absorbance values of 405nm.
d) Etrog citron. Strains of CEVd are not known.
e) Rough remon.

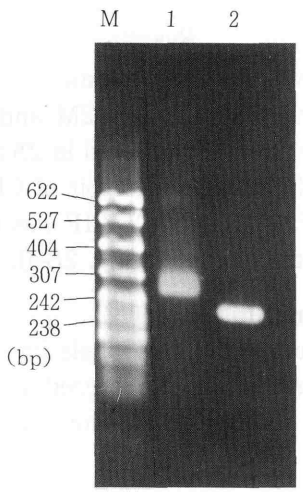

Fig. 3 Agarose gel electrophoresis (2%) of DIG labelled probe. 1 : DIG labelled probe, 2 : corresponding DNA fragments, M : molecular size marker (pBR322/Hpa II).

samples and the positive control amplified by the primer pair of CEV-2M and PCEV-1P showed positive absorbance values by microplate hybridization (Fig 2B-b). The samples without clear bands by agarose gel electrophoresis (the sample No. 3, 4, 5, 6, 8, 9, 10, 11) showed positive result. Furthermore, the sample No. 4 and No. 6 which are thought to be healthy by biological indexing also reacted positively. However, in three CEVd-infected samples (No. 8, 10, 11) and positive control amplyfied by the primer pair of CEV-2M and CEV-1P reacted positively (Fig. 2A-b). But their absorbance values were lower than those of samples amplified by the primer pair of CEV-2M and PCE1V-1P (Fig. 2B-b).

Comparison of total nucleic acid extraction methods

The results are shown in Table 3. Both CEVd-infected and healthy samples extracted by (a) and (d) methods reacted negatively. The all samples extracted by (e) method reacted positively. The samples extracted by (b) and (c) methods were positive in the infected amples, but negative in the healthy sample. The absorbance values of the infected samples by (c) method were higher than those by (b) mehtod.

Discussion

The PCR-microplate hybridization method according to HATAYA, *et al.* (1994) was evaluated for the detection of CEVd. Two pairs of primers were used for RT-PCR amplification. Clear band did not appear except for positive control when RT-PCR products were analyzed by agarose gel electrophoresis. However, the result of microplate hybridization following RT-PCR showed that the primer pair of CEV-2M and PCEV-1P is appropriate for amplification of CEVd RT-PCR. YANG, *et al.* (1992) developed RT-PCR assay for the detection of CEVd and other citrus viroid. They detected CEVd in agarose gel electrophoresis of RT-PCR products. In our tests, the bands in agarose gel electrophoresis were not always clear or multiple bands sometimes appeared. On the other hand, it was possible to detect CEVd in microplate hybridization by measuring absorbance values.

The sample No.4 and No.6 thought to be healthy by biological indexing with Etrog Citron Arizona 861-S1 reacted positively by RT-PCR microplate hybridization. This suggested that the sensitivity of microplate hybridization was higher than that of biological assay. But, further investigations will be required for defferences in CEVd strains and possibility of contamination during RT-PCR.

The most advantage of PCR-microplate hybridization is to require only few days for viroid detection that is much time-consuming as compared with biological method (ROISTACHER, *et al.*, 1977).

In general, concentration of viroid in fruits trees are relatively low. So large quantity (100~200g) of citrus leaves is needed for electrophoresis of viroid RNAs. While, microplate hybridization method requires only 0.1~0.2 g of samples.

For post-entry quarantine, it is frequently required to index a lot of samples in a short time. Therefore, total nucleic acid extraction method must be much simple and appropriate. As shown in Table 3, if crude saps or partially purified samples were used for RT-PCR amplification, the reactions were prevented or non-specific reactions were appeared. For this reason, three

procedures including crushing in extracting buffer, water satulated phenol:chloroform (1:1) mixture treatment and isopropylalchol precipitation for CEVd preparation are required.

We have tried nitrocellulose or nylon membrane for dot blot hybridization followed by RT-PCR, and found that microplate handling was easier and less time for indexing of multiple samples than nitrocellulose or nylon menbrane.

As mentioned above, PCR microplate hybridization method is a rapid and highly sensitive detection method for CEVd, and can be applied for practical indexing for the viroids.

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和 文 摘 要

PCR-マイクロプレート ハイブリダイゼーションによる カンキツエキソコーティスウイロイドの検出

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RT-PCR法で増幅したcDNAをポリスチレンマイクロプレート上で特異的プローブとハイブリダイゼーションを行うPCR-マイクロプレートハイブリダイゼーション法によりカンキツエキソコーティスウイロイド（CEVd）の検出を試みた。カンキツ葉から全核酸を抽出し，既報のCEVd塩基配列に基づいて設計したプライマーを用いてRT-PCR法による増幅を行った。RT-PCR産物を直接マイクロプレートに吸着させ，ジゴキシゲニン（DIG）標識プローブとハイブリダイゼーションを行った。プローブの作成及び標識の取り込みはPCR法によった。次にアルカリフォスファターゼ標識抗DIG抗体反応を行い，次いでアルカリフォスファターゼの基質を加え発色反応を行って吸光値を測定した。その結果，供試した11試料中，CEVd保毒6試料全てが陽性となった他，エトログシトロンアリゾナ861-S1への接ぎ木検定により健全と思われていた2試料も陽性を示した。また，RT-PCR産物の電気泳動では明瞭なバンドが認められなかった試料でもプレートハイブリダイゼーションによりCEVdを検出することができた。