UTILIZATION OF SDT-RT-PCR FOR PLANT VIRUS DETECTION

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Abstrak

Metode *simple-direct-tube* (SDT) dikembangkan untuk preparasi RNA virus atau viroid, yang selanjutnya dipakai untuk sintesis cDNA. SDT tidak memerlukan penyaringan atau sentrifugasi seperi pada ekstraksi RNA total menggunakan KIT. Metode ini juga tidak menggunakan antiserum dalam preparasi RNA virus. Di samping itu metode SDT dapat dikerjakan di laboratorium dengan temperatur ruang dan selesai dikerjakan lebih kurang dalam 25 menit. Penerapan metode ini dilanjutkan dengan reverse transcription-polymerase chain reaction (RT-PCR), dapat mendeteksi keberadaan *tomato chlorosis virus* (ToCV), *tomato infectious chlorosis virus* (TICV), *chrysanthemum virus B* (CVB), *Potyvirus* and *chrysanthemum stunt viroid* (CSVd). Hal ini menunjukkan bahwa metode SDT dapat dipakai untuk mendeteksi virus atau viroid.

Key world: Tomato chlorosis virus (ToCV), *Tomato infectious chlorosis virus* (TICV), *Chrysanthemum virus B* (CVB), *Potyvirus*, *Chrysanthemum stunt viroid* (CSVd), metode *simple direct tube*, deteksi virus, RT-PCR

1. Introduction

The reverse transcription-polymerase chain reaction (RT-PCR) and polymerase chain reaction (PCR) are powerful tools for highly sensitive detection of plant viruses with RNA and DNA genomes (Fenby *et al*., 1995; Singh, 1998). The necessary nucleotide sequences for the design of PCR primers are readily available for many plant viruses but nucleic acid extraction from plant tissues is a laborious and time-consuming step in RT-PCR procedures.

There have been many reports of simple and rapid techniques to detect plant viruses using RT PCR. Among them, direct binding (DB)-RT-PCR (Rowhani *et al*.,1995), tube capture (TC)-RT-PCR (James, 1999) and simple-direct-tube (SDT)-RT-PCR (Suehiro *et al*. 2005) are easy and useful protocols to detect plant viruses without the use of phenol-chloroform extraction and antibodies, and are based on binding virus particles to the test tubes without any initial treatment. Because of DB- and TC-RT-PCR methods require several hours to obtain virus RNAs, attempts were made to improve

these techniques so as to simplify and shorten the time required for RNA extraction. Suehiro *et al.* (2005) has been developed a protocol (named the simple-direct-tube (SDT) method) that minimizes incubation times and omits centrifuging steps to obtain viral RNA from crude sap.

In order to apply and utilize this method for the detection of various plant viruses and viroid, representative important viruses, tomato chlorosis virus (ToCV; genus *Crinivirus*), tomato infectious chlorosis virus (TICV; genus *Crinivirus*), chrysanthemum virus B (CVB; genus *Carlavirus*), *Potyvirus* and chrysanthemum stunt viroid (CSVd) were examined as well.

2.   Materials and Methods

Four plant viruses and one plant viroid, ToCV, TICV, CVB, *Potyvirus* and CSVd, collected in West Java Province, Indonesia were used in this study. ToCV and TICV were maintained in *Lycopersicon esculentum L. Potyvirus* was isolated from *Pogostemon cablin.* CVB and CSVd maintained in *Dendranthema grandiflorum* Kitam.

Virus-infected leaves were ground 1:1 (w/v) in the phosphate-buffered saline (PBS) solution containing 0.05% Tween-20 (PBST) (8 g sodium chloride, 1.15 g sodium phosphate *dibasic*, 0.2 g potassium phosphate *monobasic*, 0.2 g potassium chloride and 0.5 g Tween-20 dissolve in distilled water to 1000 ml, adjust pH to 7.4). The resulting crude sap (50 µl) was carefully placed into a PCR tube (0.5 ml, polypropylene) using a truncated tip to avoid trapping any air bubbles. After incubation at room temperature for 15 min, the sap was removed with a truncated tip. The tube was then washed twice with 50 µl of PBST to remove residual tissue. Thirty microlitres of diethylpyrocarbonate-treated water (DEPC-water) containing 15 units (U) of RNase inhibitor (Takara) was added to the tube, which was then immediately incubated at 95 °C for 1 min (denaturation), and cooled on ice for 1 min. The resulting solution was then used for RT-PCR. Crude sap from ToCV, Potyvirus and CSVd-infected leaves was used to evaluate the effect of different conditions on the standard procedure. The conditions examined was dilution of crude sap 10^1-, 10^2-, 10^3-, 10^4 - or 10^5-fold in PBST.

cDNA was generated from virus/viroid RNA by reverse transcription (RT)

reaction. The reaction mixture consisting of a total of 10 µl (1 µl RT buffer 10x, 0.35 µl DTT 50mM; 2 µl dNTP 10 mM; 0.75 µl oligo dT 10 µM; 0.35 µl MMuLV Reverse transcriptase; 0.35 µl RNAse Inhibitor ; 2 µl virus/viroid RNA) was incubated at 42 °C for 1 h. One microlitre of the synthesized cDNA was added to 24 µl of the reaction mixture containing 2.5 µl of 10 x PCR buffer, 2.5 µl of 10 x sucrose cresol, 0.5 µl dNTP 10 mM, each of the 1 µl forward and reverse primers 10 mM, 1 µl of Taq DNA polymerase (1 U/µl) and 15.5 µl of sterile distilled water. Sequences of the primers and PCR cycling conditions used in this study are listed in Table l .

Table 1. Oligonucleotide primers for used in this study

Target	Primer Sequence (5'-3')	Product size (bp)	PCR cycling conditions
ToCV	ToCV F: GTGTCAGGCCATTGTAAACCAAG ToCV R: ACAAAGCGTTTCTTTTCATAAGCAGG	405	94°C, 4 min 94°C, 1 min 62°C, 1 min 30 cycles 72°C, 1 min 72°C, 10 min
TICV	TiCV F: AATCGGTAGTGACACGAGTAGCATC TiCV R: CTTCAAACATCCTCCATCTGCC	332	94°C, 4 min 94°C, 1 min 62°C, 1 min 30 cycles 72°C, 1 min 72°C, 10 min
Potyvirus	PotyF: ATG GTH TGG TGY ATH GAR AAY GG PotyR: TGC TGC KGC YTT CAT YTG	327	94°C, 2 min 94°C, 30 s 50°C, 1 min 35 cycles 72°C, 1 min 72°C, 10 min
CVB	CVB5: CAAAGAGGTGATCATCCGTCTAG CVB3: CTCGGTTACTTTATCGCACCTAG	739	94°C, 4 min 94 °C, 30 s 45 °C, 1 min 35 cycles 72 °C, 1 min 72°C, 10 min
CSVd	CSVd-F: CAACTGAAGCTTCAACGCCTT CSVd-R: AGGATTACTCCTGTCTCGCA	250	94°C, 4 min 96 °C, 30 s 61 °C, 45 s 35 cycles 72 °C, 1 min 72°C, 5 min

3. Result and Discussion

The SDT RT-PCR method is a very convenient technique to obtain template viral RNA for RT-PCR. ToCV, TICV, CVB, *Potyvirus* and CSVd were detected by the SDT-RT-PCR method, suggesting that particles of many different viruses or viroid can be directly immobilized onto polypropylene tubes within a few minutes (Fig. 1,2). The time required is less than 25 min before cDNA synthesis can be performed even when the number of samples is increased. This incubation period can be used to prepare the reverse transcription mixture.
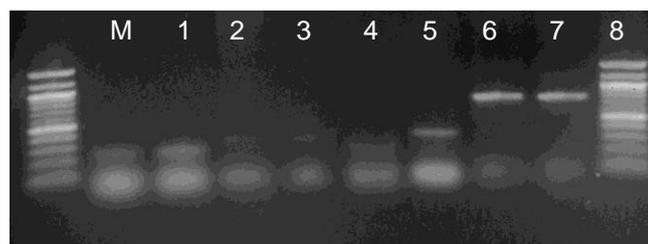

Fig. 1. Detection of CSVd (lanes 1-2), Potyvirus (lanes 3-4), TICV (lane 5), ToCV (lane 6) and CVB (lanes 7-8) by SDT-RT-PCR. Lane M: 100 bp DNA ladder (Fermentas)
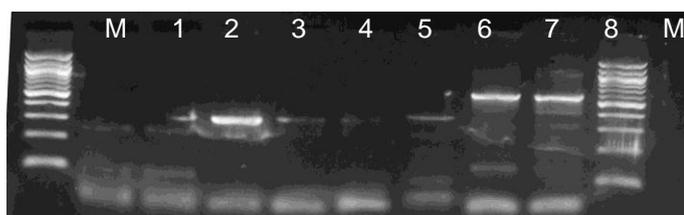

Fig. 2. Detection of CSVd (lanes 1-2), Potyvirus (lanes 3-4), ToCV (lanes 5-6) and CVB (lanes 7-8) by SDT-RT-PCR. Lane M: 100 bp DNA ladder (Fermentas)

The sensitivity of the SDT-RT-PCR method were stidied using serial dilutions of crude sap containing virus paticles. Agarose gel electrophoresis of the PCR product indicated that by the SDT-RT-PCR method were amplified when sap was diluted up to 10^3-, 10^4- and 10^5-fold for CSVd, Potyvirus and ToCV, respectively (Pig. 3,4). The DB- and TC-RT-PCR methods using direct immobilization described previously by Rowhani *et al*. (1995) and James (1999) have higher sensitivities than the SDT-RT-PCR method (Suehiro *et al*. 2005). However, much more time is required to obtain results with these methods. The objective of this study was to facilitate virus detection by obtaining template viral RNAs for RT-PCR by the shortest and simplest procedure. The SDT

method proved to be one of the fastest and most reliable protocols for detection of plant RNA viruses by RT-PCR.
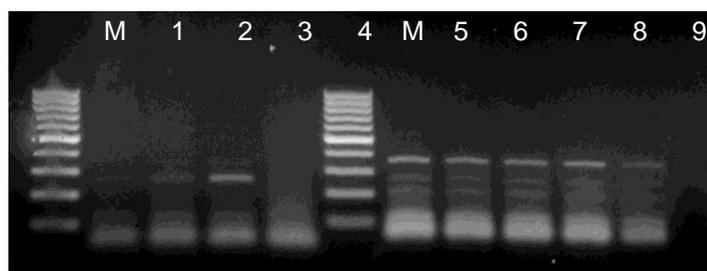

Fig. 3. Evaluation of dilution of crude sap prepared from infected leaves. Lanes 1-4; the crude sap prepared from CSVd infected leaves diluted 10^1-, 10^2-, 10^3- or 10^4-fold, respectively. Lanes 5-9; the crude sap prepared from ToCV infected leaves diluted 10^1-, 10^2-, 10^3-, 10^4- or 10^5-fold, respectively. Lane M: 100 bp DNA ladder (Fermentas)
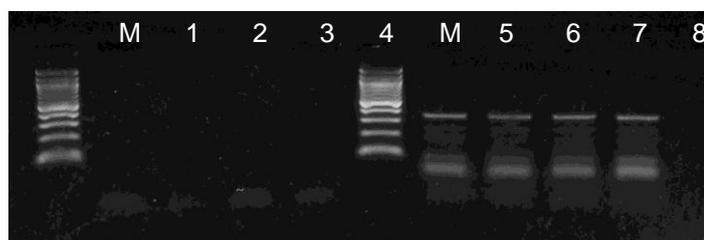

Fig. 4. Evaluation of dilution of crude sap prepared from Potyvirus infected leaves. Crude sap diluted 10^1-, 10^2-, 10^3- or 10^4-fold (lanes 5-8). Lane M: 100 bp DNA ladder (Fermentas)

The critical points for accurate detection by SDT-RT-PCR are washing the tube after crude sap incubation, to eliminate residual plant material completely; and timing the addition of DEPC-treated water and the denaturing period of the tube containing the immobilized virus particles (Suehiro *et al.* 2005). Plant species contain polyphenolic compounds or polysaccharides that affect the extraction of intact nucleic acids. It was possible to eliminate this problem by picking up visible material in the tube with a truncated tip (Thomson and Dietzgen, 1995; Suehiro *et al.* 2005). Depending on the circumstances, two or more washes may be required until the debris disappears. After washing, PBST should be completely removed using normal tips. It is important to avoid degradation of the viral RNA in the tube before cDNA synthesis, so DEPC-water should be added after preparing the RT-reaction mixture and adjusting the temperature of heat block.

Adsorption of virus particles to the tubes is thought to be caused by non-

specific attachment, causing SDT-RT-PCR is very useful tool for the detection of viruses especially when PCR primers for the target virus RNA are available but antisera against the virus particles are not. For example, the method might be available to detect viruses with universal primers for cucumoviruses (Choi *et al*., 1999) and potyviruses (Chen *et al*., 2001). It was demonstrated by Suehiro *et al.* (2005) that a large fragment at the 5' region of the TuMV genome was amplified by SDT-RT-PCR, suggesting that it may be possible to synthesize the full-length cDNA of TuMV. This research suggest that the rapidity and simplicity of the SDT method could make it useful in the detection of virus pathogens.

Acknowledgement

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