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Research Article

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Detection of *Strawberry mottle* virus by molecular hybridization

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ABSTRACT

Strawberry mottle virus (SMoV) is one of the viral pathogens affecting strawberries (Fragaria spp.) production severely worldwide. Currently, reverse transcription-polymerase chain reaction (RT-PCR) is frequently used to detect SMoV, which is sometimes not successful because of unsatisfied nucleic acid quality and the contamination of secondary metabolites resulting in poor PCR amplification. In the study, we developed a digoxigenin-labelled cDNA probe for SMoV detection by dot-blot hybridization. Total nucleic acid was extracted with modified CTAB method from strawberry plants infected by SMoV, the SMoV specific fragment was amplified with RT-PCR, which was cloned and sequenced. The cloned fragment was then labeled with digoxigenin by PCR, which is used as probe in dot-blot hybridization to test the samples infected by SMoV. The result showed that dot-blot hybridization had the same result of RT-PCR in testing SMoV. Our research provided an important alternative to RT-PCR technique for detecting SMoV. To our knowledge, this is the first report for detection of SMoV by cDNA probe. In addition, it is a potential method to test other strawberry viruses.

Key words: cDNA probe, *Strawberry mottle virus*, detection, RT-PCR, digoxigenin

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INTRODUCTION

Strawberry mottle virus (SMoV) is one of the most economically important viral pathogens infecting strawberries (*Fragaria* spp.). Losses of fruit yield and runner production in commercial strawberries due to infection by SMoV alone can be as high as 30%. In mixed infections with, for example, *Strawberry crinkle virus* (SCrV), *Strawberry vein banding virus* (SVBV) and *Strawberry mild yellow edge virus* (SMYEV), the losses can be even higher [1]. Historically, SMoV was considered to be a strain of SCrV, and it was accepted as a distinct virus in 1950s. The virus particles of SMoV are isometric, approximately 28 nm in diameter. SMoV occurs naturally in the genus *Fragaria* wherever strawberries are grown, and many strains have been reported. Recently, the complete nucleotide sequence of SMoV was elucidated, and the genome of SMoV was found to consist of RNA1 and RNA2 of 7036 and 5619 nt, respectively [1]. Based on the characterization of SMoV genome, recently, a new genus, *Sadwavirus*, was created, and SMoV, *Satsuma dwarf virus* (SDV) and *Strawberry latent ringspot virus* (SLRSV) were assigned as species in the genus [2].

The development of rapid, sensitive and specific diagnostic methods for the strawberry virus is imperative. The earliest detection method is by indexing via leaflet grafts on sensitive indicator hosts, but it is time-consuming. The serological method is a very rapid method for detection of virus. However, SMoV can't be routinely diagnosed by serological method because of lack of antiserum. Along with the nucleotide sequences of SMoV are elucidated, the reverse transcription-polymerase chain reaction (RT-PCR) method for the detection of SMoV was developed [3].

Currently, the chief limiting factor in the application of the PCR technique in routine diagnosis lies in the preparation of good quality nucleic acid, free of PCR inhibitors from strawberries. Strawberry is particularly recalcitrant to extraction of virions and nucleic acids, probably due to the large amount of secondary metabolites, such as tannins, polyphenols and polysaccharides [3].

Hybridization with cDNA or cRNA probes is an easy and powerful method for detecting plant viruses. In this paper, we develop a digoxigenin-labelled cDNA probe for detection of SMoV by dot-blot hybridization, and it will provide an important alternative to RT-PCR technique for detecting SMoV.

EXPERIMENTAL SECTION

Plant material

The strawberry cultivars 'Hokowase' was maintained in a temperature-controlled greenhouse at 20℃.

Chenopodium quinoa was mechanically inoculated with the leaf tissue of cultivars 'Hokowase', each plant was inoculated twice. The wt/vol ratio was 1:20 in phosphate buffered saline (PBS), pH 7.4, with the addition for 2% nicotine. Carborundum (600 mesh) was added on the leaf surface to facilitate delivery of the viruses into the indicator plants.

RNA extraction and RT-PCR

Extraction of total RNA from strawberry leaves by modified CTAB method was carried out as previously described [4].

The cDNA synthesis was carried out using M-MLV (Invitrogen, USA) according to the manufacturer's standard instructions. One microlitre total RNA, 1 μl random primer (9 mer) (10 μM), 1 μl dNTPs (each 2.5 mM) and 9 μl sterile distilled water were added to 0.2 ml Eppendorf tube, respectively, and incubated at 65℃ for 5 min, in succession, added 0.3 μl RNasin, 2 μl DTT (0.1 M), 4 μl buffer (5×) and 0.3 μl M-MLV (200 U/μl) to Eppendorf tube, lastly, incubated at 37℃ for 150 min.

The PCR reaction mixture contained 1 μl of first strand cDNA, 2 μl 10×Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM primers (Table 1), 0.5 U *Taq* enzyme (Promega, USA) in a total volume of 20 μl. The cycling conditions of PCR consisted of an initial denaturation step at 94℃ for 2 min, followed by 35 cycles at 94℃ for 0.5 min, 55℃ for 1 min and 72℃ for 1 min, and then a final elongation step at 72℃ for 5 min. PCR products were separated by electrophoresis in 1.5% agarose gels in 1×TBE buffer and visualized under UV light after staining with ethidium bromide.

Table 1. The primers for detection of SMoV

Primer	Sequence (5′→3′)	Target fragment (bp)
D1/D2	TAAGCGACCACGACTGTGACAAAG (Sense primer) ATTCGGTTCACGTCCTAGTCTCAC(Antisense primer)	461
D1/D3	TAAGCGACCACGACTGTGACAAAG (Sense primer) TCTTGGGCTTGGATCGTCACCTG (Antisense primer)	219

Cloning and sequencing

PCR products were excised from the gels and purified by the PCR product purification kit, then ligated into the pMD 18-T vector (TaKaRa, Japan) according to the manufacturer's instructions. The products were transformed into *Escherichia coli* DH5α and plated on LB agar. White colonies were detected by PCR, then positive colonies were sequenced.

Preparation of cDNA probe and dot-blot hybridization

Digoxigenin-labeled reaction was performed according to the manufacturer's standard instructions. The plasmid containing the detected DNA fragment was used as a template to synthesize the digoxigenin-labeled cDNA probe by PCR. Thermo cycling condition was the same as previously described. Purified the amplified fragments by Gel Extraction Kit (Sangon, China) according to the manufacturer's instructions, at last, the probe was dissolved with 40 μl TE solution (pH 8.0). The dot-blot hybridization was performed according to the manufacturer's standard instructions.

RESULTS

Detection of SMoV by RT-PCR and cloning

The 3′ non-coding region (NCR) of SMoV genome was amplified with primers D1/D3 and D1/D2 by RT-PCR. Because the primers were located in the high conserved region of RNA1 and RNA2 of genome of SMoV, both RNA1 and RNA2 could be effectively amplified. The specific 219 bp fragment was amplified with primers D1/D3 in cultivars 'Hokowase' (Fig. 1). The PCR products were purified, and ligated into the pMD 18-T vector. The ligated products were transformed into *Escherichia coli* DH5α, positive colonies were identified by PCR.

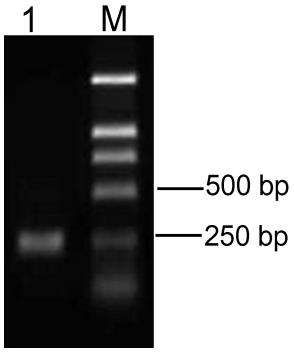

Fig. 1 Detection of SMoV by RT-PCR
M: DL2000 Marker; 1: strawberry cultivars 'Hokowase'.

DNA labeling and hybridization detection for SMoV

Total nucleic acids extracted from the positive colonies were used as the template, digoxigenin-labeled cDNA probes were generated with the primers D1/D3 according to PCR labeling technique [5]. The products of labeled probe were larger than those unlabeled through agarose gels electrophoresis, suggesting that the probe was labeled successfully.

Detection of SMoV was carried out through routine RT-PCR and hybridization to membrane blotted nucleic acids according to standard methods [1, 5], at last, the hybridized probes were immunodetected with anti-digoxigenin-AP, Fab fragments and visualized with the colorimetric substrates NBT/BCIP.

SMoV could be effectively detected by digoxigenin-labeled cDNA probes (Fig.2), and the limit of detection was dilution at 1/10, and the negative control had not obvious signal of hybridization. The hybridization detection had the same result of RT-PCR in testing SMoV.

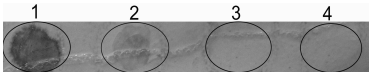

Fig. 2 SMoV was detected by digoxigenin-labeled cDNA probes

1-3: the total nucleic acid from strawberry was serially diluted to 1:1, 1:10, and 1:100, respectively; 4: negative control.

DISCUSSION

Detection of SMoV by RT-PCR has some disadvantages compared with molecular hybridization:(1) RT-PCR is prone to give false positives due to cross-contamination, and negative reactions sometimes are due to inhibition of reverse transcriptase and/or *Taq* DNA polymerase by the presence of RT-PCR inhibitors in nucleic acid; (2) primer annealing for RT-PCR is more sensitive to nucleotide variations than hybridization with large sized probes, therefore, more false negative results are easy to be obtained in RT-PCR [6]. On the whole, the hybridization detection was steadier than RT-PCR in testing SMoV.

Generally, SMoV generate only low titres in infected plants, and due to the lack of special and sensitive antisera, diagnosis of these pathogens in plants has to recur to either biological indexing or RT-PCR methods. The high reliability of the molecular hybridization assay described introduce an important alternative to serological and RT-PCR methods for detecting SMoV. This is especially important in strawberry viruses like SMoV, which lacks appropriate antisera.

In conclusion, we developed a digoxigenin-labelled cDNA probe for SMoV detection by dot-blot hybridization. The method had the same result of RT-PCR in testing SMoV. Our research provided an important alternative to RT-PCR technique for detecting SMoV. In addition, it is a potential method to test other strawberry virus.

CONCLUSION

We developed a digoxigenin-labelled cDNA probe for SMoV detection by dot-blot hybridization. The method had the same result of RT-PCR in testing SMoV. Our research provided an important alternative to RT-PCR technique for detecting SMoV. In addition, it is a potential method to test other strawberry virus.

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