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Identification of Luteovirus Nucleotide Sequences in Mild Yellow-Edge Diseased Strawberry Plants

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The availability of nucleotide sequences of the coat protein gene of *Potato leafroll virus* (PLRV) permitted the construction of DNA primers that were utilized for cDNA synthesis. Polymerase chain reaction (PCR) products of a 487 bp. and approximately 500 bp DNA fragments were amplified from nucleic acid extracts of PLRV-infected tissue and strawberry mild yellow-edge (SMYE) diseased strawberry tissue, respectively. The amplified DNA fragments were further differentiated by hybridization analysis with a cDNA probe for the coat protein gene of PLRV and restriction fragment length polymorphism (RFLP) analysis. These results suggest that a luteovirus is associated with the SMYE disease.

Keywords : Amplification, Luteovirus, PLRV, RFLP, SMYEV, strawberry.

Strawberry mild yellow-edge (SMYE) is an aphid-borne disease that causes severe loss of plant vigor, yield, and fruit quality. When its causal agent occurs in a complex with aphid-borne strawberry viruses, symptoms such as mottle, yellow leaf edge, vein banding, and crinkle occur (Fig. 1). SMYE is distributed worldwide in cultivated strawberries and is among the 50 most frequently cited plant diseases in the quarantine regulations of 124 countries (Kahn, 1989).

SMYE disease is caused by a complex of two viruses, SMYE-associated potexivirus and a putative luteovirus (SMYEV). SMYE is caused by SMYEV based on symptomatology, lack of mechanical transmission and persistent transmission of the virus in a circulative manner by the strawberry aphid *Chaetosiphon fragaefolii* Cock (Mellor and Frazer, 1970), localization of a 22-25 nm isometric virus particle in phloem cells of infected strawberry plants (Yoshikawa et al., 1984), as well as its distant serological relationship with beet western yellow luteovirus (Spiegel et al., 1986). Recently, the association of SMYEV with the disease has been reported (Jelkmann et al., 1990). This study was based on nucleotide sequence analysis of selected cDNA clones prepared from dsRNA of graft-inoculated *Rubus rosifolius* Smith as well as immunological analysis of filamentous 470-580 nm potyvirus particles in infected tissues. Potyvirus, unlike luteovirus, causes mosaic or ringspot symptoms in infected plants, transmitted by contact and are not localized in phloem cells (Francki et al., 1985).

The biology of luteovirus complicates their identification in infected tissue because these viruses are confined to phloem tissues, occur in extremely low concentrations, and are not transmitted mechanically. Recently, the luteovirus sequence data have been accumulated rapidly; several complete genome sequences as well as coat protein (CP) sequences of distinct luteoviruses have been reported (Miller et al., 1988, Mayo et al., 1989; Moon et al., 2001; van der Wilk et al., 1989; Keese et al., 1990; Vincent et al., 1990). To understand the etiology of SMYE disease, this study utilized the polymerase chain reaction (PCR) technology to determine whether the putative SMYEV is involved in the disease. Recently, PCR or RT-PCR has been valuable in the identification and detection of DNA nucleotide sequences of geminiviruses, potyviruses, luteoviruses, and viroids in infected host plant tissues (Hadidi et al., 1993; Korshineck et al., 1990; Rybicki and Hughes, 1990; Hadidi et al., 1991; Langeveld et al., 1991; Levy and Hadidi 1991; Levy et al., 1991; Kanematsu et al., 1991; Robertson et al., 1991; Wetzel et al., 1991; Montasser et al., 1992; Yang et al., 1992). To develop a similar identification method for the detection of a luteovirus involved in the etiology of SMYE disease, the CP gene of the genome of *Potato leafroll virus* (PLRV), a luteovirus, was selected to provide nucleotide sequences for the construction of primers for application in RT-PCR. In this study, the positive identification of luteovirus in nucleic acid extracts of SMYE diseased strawberry plants was reported.

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Fig. 1. Symptoms of strawberry mild yellow-edge virus (SMYEV) on strawberry (*Frgagaria ananassa* Duchesme cv "Tudla") plants (**A**), note some aborted flower buds that cause fruit yield loss and low quality strawberry fruits, compared with healthy control plants (**B**). Plants were grown vertically in stacks of cornered styrofoam pots (**C**) fertilized and watered by dripping irrigation system in a greenhouse in Omaria, Kuwait.

Materials and Methods

Source of diseased strawberry tissue. Leaf samples were collected from cultivated strawberry *Frgagaria ananassa* Duchesme cv "Tudla" naturally infected with strawberry mild yellow-edge (SMYE) disease. Plants were grown vertically in styrofoam pots stacked on top of each other, as shown in Fig. 1C, in a large greenhouse in Omaria, Kuwait.

Sources of potato leaf tissue infected with PLRV. Potato leaves infected with PLRV were obtained from naturally infected potatoes grown in commercial fields in Abdally, Kuwait and used for molecular analyses.

Nucleic acid extraction and purification from plant tissue. Total nucleic acids were isolated from strawberry or potato leaf tissue of infected and uninfected plants as described by Yang et al. (1992). Total nucleic acids from strawberry tissue were further purified on RNase-free ELUTIP-r-minicolumns containing RNA binding matrix (Schleicher and Schuell, Keene, NH, USA) as suggested by the manufacturer. Eluted nucleic acids were pre-incubated, dried *in vacuo*, dissolved in deionized water, and concentration determined (Hadidi and Yang, 1990; Yang et al., 1992).

Viral cDNA synthesis and amplification. Viral DNA was synthesized and amplified from purified viral RNA and from infected host nucleic acids as described (Hadidi and Yang, 1990; Yang et al., 1992) using a 22-mer primer [5'-GCACTGATCCT-CAGAAGAATCG-3', complimentary to PLRV RNA nucleotides 4103-4124 in the cp region (van der Wilk et al., 1989)]; and a 22-mer primer [5'-AAGAAGGCGAAGAAGGCAATCC-3' homologous to PLRV RNA nucleotides 3638-3659 in the cp region (van der Wilk et al., 1989)], resulting in 487 bp amplified PLRV cDNA fragment.

Restriction enzyme digestion of amplified cDNA fragments. Aliquots (5 µl) of the RT-PCR amplified cDNA fragments were directly digested with 2 units of *Sau*3AI or *Sin*I in total volume of 20 µl at 37°C for 2 h as recommended by the manufacturer (Gibco, BRL Life Technologies, Inc., USA).

Molecular probe. Recombinant plasmid pLM26, which contains 422 bp of PLRV CP gene, representing 68% of the gene, was supplied by L. J. Skrzeskowski, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. It was labeled with [^{32}P] dCTP (300 Ci/m mole) by nick translation (Rigby et al., 1977), and used as a molecular probe.

Analysis of RT-PCR amplified products. Aliquots (5-10 µl) of the RT-PCR products were analyzed by electrophoresis as described (Hadidi & Yang, 1990; Yang et al., 1992). Separated nucleic acids were also denatured with 0.4 M NaOH/0.6 M NaCl for 15 minutes, then transferred electrophoretically to Nytran Membranes (Schlaicher and Schuell, Inc. Keene, NH, USA), at 0.6 mA for 16 h at 4°C. Transferred nucleic acids were cross-linked to membranes by irradiation with 1200 µJ for 45 seconds in an UV crosslinker (Stratagene, LaJolla, CA, USA). Membranes were prehybridized, then hybridized with nick translated [^{32}P]-labeled PLRV cDNA probe of PLRV cp gene, washed and exposed to X-ray film with intensifying screen at -70°C as described by Hadidi et al. (1990) except that RNase A was not used during the washing process.

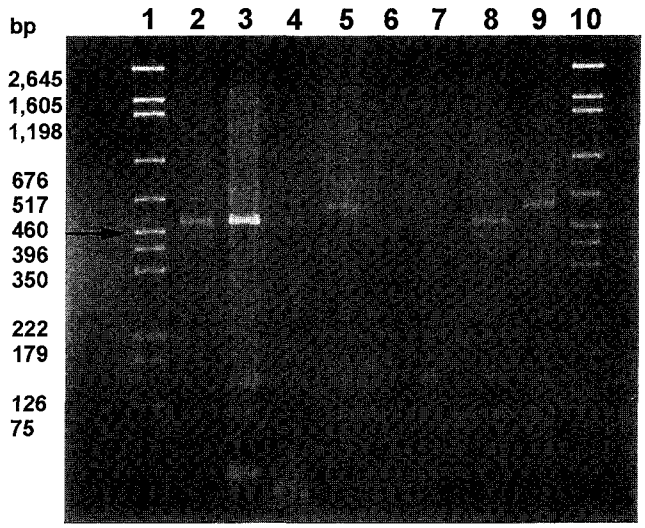

Fig. 2. Polyacrylamide gel (5%) electrophoretic analysis of RT-PCR amplified cDNA products of PLRV and SMYEV. pGEM DNA markers, arrow indicates 460 bp (lanes 1 and 10); purified PLRV RNA (lanes 2 and 8); total nucleic acids of PLRV-infected potato leaf tissue (lane 3); minicolumn-purified total nucleic acids of uninfected strawberry leaf tissue (lanes 4); minicolumn-purified total nucleic acids of SMYEV-infected strawberry leaf tissue (lanes 5 and 9); and total nucleic acids of uninfected and SMYEV-infected leaf tissue before minicolumn purification (lanes 6 and 7, respectively).

Results

The positive identification of luteovirus in nucleic acid extracts of SMYE diseased strawberry plants was performed in this study. Fig. 2 shows a polyacrylamide gel electrophoretic analysis of amplified cDNA fragments of PLRV and SMYEV. The size of PLRV cDNA fragment was, as expected, 487 bp whether the cDNA was amplified from purified PLRV RNA (lanes 2 and 8) or from total nucleic acids of PLRV-infected potato leaf tissue (lane 3). The size of SMYEV cDNA fragment, however, was slightly larger than 487 bp (lanes 5 and 9) because the electrophoretic mobility of SMYEV cDNA was slightly but distinctly slower than that of PLRV cDNA. SMYEV cDNA fragment was detected only in ELUTIP-r minicoloumn-purified total nucleic acids of infected strawberry tissue (lanes 5 and 9), but not in uninfected tissue (lane 4) or in total nucleic acids of infected and uninfected tissue before minicoloumn purification (lanes 6 and 7).

Fig. 3 shows southern blot hybridization analysis of SMYEV RT-PCR amplified cDNA fragments on minicoloumn-purified total nucleic acids from SMYEV infected strawberry leaf tissue (lane 3) as well as PLRV cDNA fragments of total nucleic acids from PLRV-infected potato

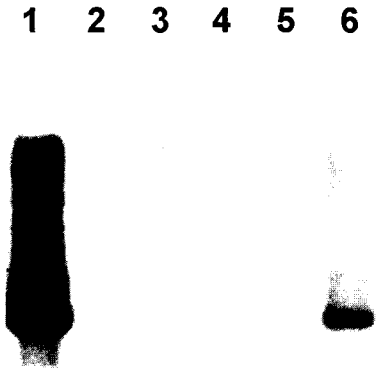

Fig. 3. Autoradiograph of Southern blot hybridization of [^{32}P]-labeled PLRV cDNA to RT-PCR amplified cDNA products of PLRV and SMYEV using total nucleic acids of PLRV-infected potato leaf tissue (lane 1) with a dimmer band on the top of the lane; minicolumn-purified total nucleic acids of uninfected (lane 2) and SMYEV-infected strawberry leaf tissue (lane 3) with a dimmer band on the top of the lane; total nucleic acids of uninfected and SMYEV-infected strawberry leaf tissue before minicolumn purification (lanes 4 and 5, respectively); and purified PLRV RNA (lane 6).

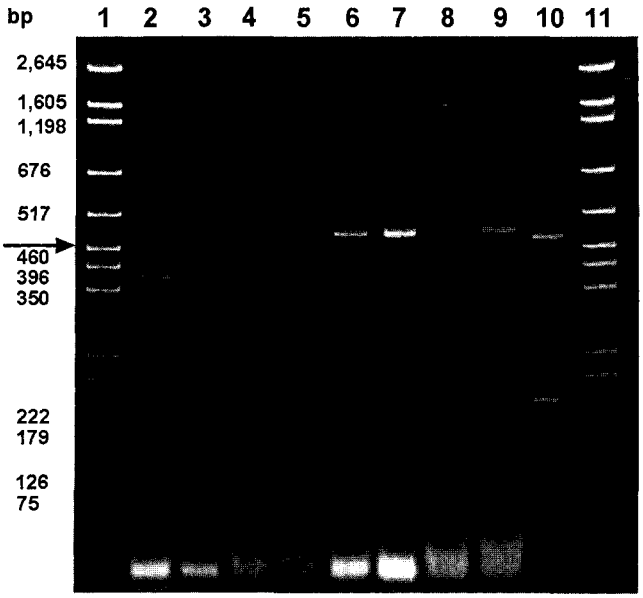

Fig. 4. RFLP analysis of amplified cDNA fragments of PLRV and SMYEV. Fragments were digested with the restriction endonuclease *Sau*3AI or *Sin*I. pGEM DNA markers, arrow indicates 460 bp (lanes 1 and11); *Sau*3AI-digested PLRV cDNA and SMYEV cDNA (lanes 4 and 8, respectively); *Sin*I-digested PLRV cDNA (lanes 2 and 5); PLRV cDNA control (lanes 3 and 10); *Sin*I-digested SMYEV cDNA (lanes 6 and 9); and SMYE cDNA control (lane 7).

leaf tissue (lane 1) in purified PLRV RNA (lane 6) using [^{32}P]-labeled PLRV cDNA probe of PLRV cp gene. The intensity of hybridization signals of SMYEV cDNA, however, was less than that of PLRV cDNA. Hybridization was not observed between the probe and the minicoloumn-purified total nucleic acids from uninfected strawberry leaf tissue (lane 2) or non-ELUTIP-r-purified total nucleic acid from SMYEV uninfected or infected tissue (Fig. 3 lanes 4 and 5, respectively).

The amplified cDNA fragments of PLRV and SMYEV were digested with the restriction endonuclase *Sau*3AI or *Sin*I and analyzed by electrophoresis on 5% polyacrylamide gel. Fig. 4 shows restriction fragment length polymorphism (RFLP) analysis of the amplified cDNAs. The restriction profile of the fragments showed the presence of identical *Sau*3AI recognition sequences in both PLRV cDNA and SMYEV cDNA (lanes 4 and 8, respectively). The restriction profile of *Sin*I-digested fragments of PLRV (lanes 2 and 5) and SMYEV (lanes 6 and 9), however, was different indicating that the SMYEV fragment does not contain the same *Sin*I recognition sequences as the PLRV fragment.

Discussion

The availability of the nucleotide sequence of the PLRV CP gene enabled the utilization of RT-PCR for the successful amplification of a virus containing similar sequences associated with SMYE disease in naturally infected strawberry plants. The specificity of the SMYEV cDNA fragment was established as follows: (1) the size of the major cDNA fragment from SMYEV-infected strawberry tissue was slightly larger than the 487 bp of PLRV; (2) specific cross hybridization of SMYEV cDNA with [^{32}P]-labeled cDNA probe of the CP gene of PLRV, as well as heterologous hybridization signals were less intense than that of homologous hybridization; (3) absence of hybridized cDNA fragments from uninfected strawberry tissue; (4) RFLP analysis revealed its similarity with that of PLRV cDNA when digested with *Sau*3AI but different when digested with *Sin*I. These results suggest that luteovirus is associated with SMYE disease and is distinct from PLRV. This finding was consistent with that of previous studies, which suggest that luteovirus is a possible etiological agent of SMYE disease in the Pacific Northwest USA (Mellor and Fazer 1970; Speigal et al., 1986) and in Japan (Yoshikawa et al., 1984).

At present, a luteovirus and a potexivirus have been implicated in the etiology of SMYE disease. It is possible that these two viruses form a complex, which results in the disease, and/or that the two viruses could be causing two

very similar and indistinguishable diseases. In each case, the geographical distribution of each virus may depend on the prevalence of each virus in strawberry germplasm and distribution of aphid vector in the area. The elucidation of the vector association and disease-causing interactions of this unusual virus complex will require further studies.

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