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Specific Primer for Detection of Jujube Witches' Broom Phytoplasma Group (16SrV) in Korea

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In order to diagnose and differentiate jujube witches' broom (JWB) phytoplasma rapidly, oligonucleotide primer pair, 16Sr(V) F/R, for polymerase chain reactions (PCRs) was designed on the basis of 16S rRNA sequences of JWB phytoplasma. The PCR employing phytoplasma universal primer pair P1/P7 consistently amplified DNA in all tested phytoplasma isolates. But no phytoplasma DNA was detected from healthy jujube seedlings. The nested PCR, the primer pair 16S(V) F/R, about 460 bp fragment, amplified DNA in all tested JWB and related phytoplasmas including ligustrum witches' broom phytoplasma of the 16S rRNA group V, but no DNA amplification was detected from other phytoplasma strains such as groups 16SrI (Aster yellows) and 16SrXII (Stolbur group) in which mulberry dwarf phytoplasma and chrysanthemum witches' broom phytoplasma belong to, respectively. The same results were obtained from both Korean and Chinese isolates of JWB phytoplasma. Nested-PCR using phytoplasma universal primer pair P1/P7 and 16SrV group-specific primer pair 16S(V) F/R could detect group V phytoplasmas rapidly and easily, in particular JWB phytoplasma.

Keywords : jujube witches' broom, oligonucleotide primer, PCR, phytoplasma

Phytoplasmas are associated with several hundreds plant species worldwide (McCoy et al., 1989). In Korea, the phytoplasmas caused serious diseases to several economically important, medical, and fruit plants. The PCR assays provide very sensitive means of detection for a wide range of phytoplasmas by using universal and specific primer pairs designed on the basis of 16S rDNA sequence (Ahrens and Seemüller, 1992; Davis and Lee, 1993; Deng and Hiruki, 1990). In addition, restriction fragment length polymorphism (RFLP) analysis of the amplified PCR product is being used to study genetic relatedness in combination with biological information, for differentiating phytoplasmas (Ahrens et al., 1992; Lee et al., 1993, 1998). On the base of analyses of 16S rDNA, phytoplasmas have been classified at least into 15 groups and over 38 subgroups (Lee et al., 1995; Montano et al., 2001). Although several phytoplasmas established phylogenetic and taxonomic relationships tree on the basis of analyses of 16S rRNA gene, 16/23S spacer region and 16S ribosomal protein genes by RFLP and sequence analysis. In some cases, however, the 16S rRNA gene sequences of related phytoplasmas are very similar, thus it is difficult to design specific PCR primers that could separate closely related phytoplasma groups. The design of phytoplasma specific group primer pairs was possible to detect and identify phytoplasma groups by PCR (Lee et al., 1994).

Jujube witches' broom (JWB) is a very serious disease in jujube tree cultivation in Asian countries such as Korea, Japan and China. JWB belongs to 16S rV-B group (Elm yellows group) based on RFLP and sequence analyses of 16S rRNA gene (Han and Cha., 2002; Lee et al., 2000). The genetic relationships based on 16S rDNA sequences between Korean and Chinese JWB phytoplasma were very similar.

The objectives of this study were to design JWB group-specific phytoplasma primers for easy differentiation of Korean and Chinese JWB phytoplasmas and the related group 16S rV phytoplasmas.

Materials and Methods

Plant materials. Jujube trees naturally infected with JWB phytoplasma was collected at Chonbuk in Korea and at Fuping in China. LiWB (ligustrum witches' broom), MD (mulberry dwarf), and ChYD (Chrysanthemum yellow dwarf) phytoplasma samples isolated from naturally infected trees. Healthy jujube trees were grown from seeds in the netted greenhouse.

DNA extraction. The DNA was extracted following Kollar et al. (1990) with minor modifications. One gram of midribs was pulverized in liquid nitrogen with a mortar and pestle. The powder was homogenized in 3 ml of CTAB extraction buffer (2.5 M NaCl, 0.5% (W/V) PVP-10 (polyvinylpyrrolidone-10) (Sigma, U.S.A), 1% (W/V) Cetavlon (hexadecyltrimethylammonium bromide), 0.5 M

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Tris-HCl (pH 8.0), 0.25 M EDTA (pH 8.0), 0.2% 2-mercaptoethanol). The suspension was incubated for 40 min. at 65°C and centrifuged for 5 min. at 1,200 g. The supernatant was extracted with an equal volume of chlorform/isoamyl alcohol (24:1) by centrifugation at 1,200 g for 5 min. The aqueous phase was mixed with 0.7 vol. of isopropanol, and left standing for 5 min. at room temperature. The resulting pellet was washed with 70% ethanol and centrifuged at 1,200 g for 5 min, and then dried under vacuum for 10 min. and resuspended by 150 μl of distilled water. The concentration of DNA in a sample was calculated with a spectrophotometer at 260 nm.

Primers and PCR. Phytoplasma universal primer pairs, P1 (Deng et al., 1991) and P7 (Schneider et al., 1995), were used to amplify a region approximately 1,800 bp in length, consist of the 16S rRNA gene, the 16S-23S rRNA intergenic spacer region and a portion of the 23S rRNA gene. The R16F2/R2 (Lee et al., 1994) was used as a nested PCR. The specific primer of group V phytoplasma, 16Sr(V)-F; 5'-CGGAGCCCCTCAAAGGTC-3', 16Sr(V)-R; 5'-ATTGAATAACGTCAAGATAG-3 was designed on the base of 16S ribosomal RNA sequence from JWB sequence. The direct PCR assays were performed using P1/P7 primer pair. The amplification products were diluted 1:30 with sterile deionized water and reamplified in nested PCR with primer 16Sr(V)F/R. Each reaction was performed in a total volume of 50 μl containing 20-30 ng/μl DNA, 150 μM of each dNTP, 2.5 mM $MgCl_2$, 0.5 μl M of each primer, 1 X PCR buffer, and 1 U of AmpliTaq Gold DNA polymerase (Perkin-Elimer).

PCR condition. The PCR was carry out thirty-six cycles following parameters step; 1 min. (5 min. for the first cycle) denaturation at 94C, annealing for 2 min. at 55C, and extension for 3 min. (10 min. in final cycle) at 72C. The nest PCR was carry out thirty cycles following parameters step; 1 min. (5 min. for the first cycle) denaturation at 94C annealing for 2 min. at 52C, and extension for 3 min. (10 min. in final cycle) at 72C after diluted 10 times of first PCR products.

PCR products were analyzed by electrophoresis in a 1% agarose gel, staining with ethidium bromide, and DNA bands were visualized using a UV transilluminator.

Results and Discussion

The phytoplasma universal primer pair P1/P7 was amplified the target DNA of expected size approximately 1.8 kb from all diseased JWB samples which collected from Korea and China. But no amplification product was obtained from asymptomatic healthy plants (Fig. 1). Nested -PCR assays with the primer pair P1/P7 followed by specific JWB group primer pair 16Sr(V)- F/16Sr(V)-R

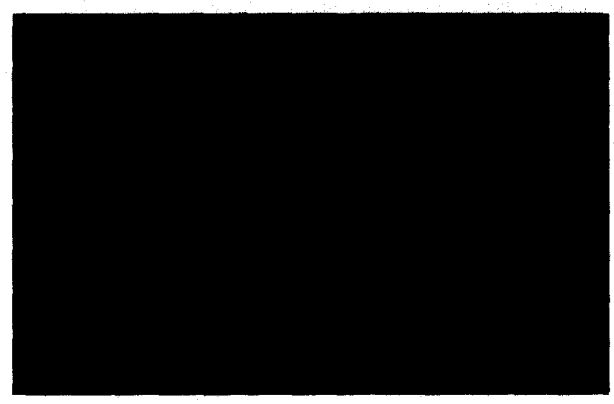

Fig. 1. Agarose gel electrophoresis of PCR products amplified from healthy and phytoplasma infected plant tissues using P1/P7 and 16S r(V)F/R primer pair. M: Molecular weight marker (100bp DNA ladder), Lanes 1~2: Jujube witches' broom (Korea), 3~4: Jujube witches' broom (China), 5: Ligustrum witches' broom, 6: Chrysanthemum yellow dwarf, 7: Mulberry dwarf, 8: Healthy jujube tree.

obtained an approximately 310bp DNA fragment from all JWB samples, but no amplification products from healthy jujube tree (Figs. 1-3). On the other hand, when amplified JWB phytoplasma samples, LiWB phytoplasma belong to 16SrV group phytoplasma, ChYD, and MD phytoplasmas belong to 16SrI group successfully amplified PCR products using P1/P7 phytoplasma universal prime pair except healthy Jujube tree. Those amplified PCR products were performed nest-PCR using JWB group specific primer pair. The expected size of the PCR products was obtained from all the tested JWB and LiWB samples but No PCR product was obtained with ChYD, JWB, and healthy jujube tree (Figs. 1 and 3).

The development of PCR assay using phytoplasma universal primer pairs based on phytoplasma DNA sequences

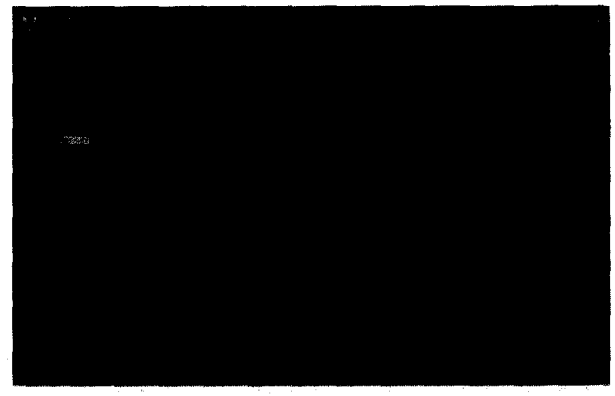

Fig. 2. Agarose gel electrophoresis of PCR products amplified from health and phytoplasma infected plant tissues using P1/P7 primer pair. M: Molecular weight marker (100bp DNA ladder), Lanes 1~6: Jujube witches' broom (Korea), 7~12: Jujube witches' broom (China), 13: Healthy jujube tree.

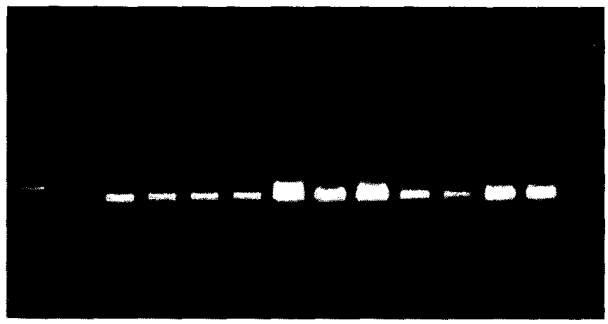

Fig. 3. Agarose gel electrophoresis of PCR products amplified from health and phytoplasma infected plant tissues using 16S r(V)F/R primer pair. M: Molecular weight marker (100bp DNA ladder), Lanes 1~6: Jujube witches' broom (Korea), 7~12: Jujube witches' broom (China), 13: Healthy jujube.

makes the detection of a broad array of phytoplasmas more sensitive. Moreover, the PCR-RFLP and PCR-nucleotide sequencing enable identification and classification of many uncharacterized phytoplsamas (Daniel et al., 1998; Davis et al., 1997; Gundersen et al., 1994; Lee et al., 2000) However, phytoplasma universal primer pairs are not applicable for epidemiological studies where more than one type of phytoplasmas are associated with given disease. In the present study, based on already known 16S rDNA sequences of phytoplasma, the 16Sr(V)F/R primer pair was developed for specific detection of phytoplasma 16S rRNA group V, especially JWB phytopalsmas. The 16Sr(V)F/R primer pair amplified both of Korean- and Chinese JWB and LiWB phytoplasmas belong to group I, but did not amplify MD and ChYD phytoplasmas belong to aster yellows (16SrI). These results clearly suggested that the 16Sr(V)F/R primer pair can be employed to distinguish jujube withces' broom with related phytoplasma isolates which belong to 16SrV group with other phytoplasmas. The nested-PCR assays using the universal primer pair P1/P7 and JWB group specific primer pair 16Sr(V)F/R, increased the sensitivity of phytoplasma detection. It is evident that the direct PCR or nested-PCR using a combination of phytoplasma universal and JWB group-specific primer pairs particularly facilitate screening processes where absolutely pathogen-free plants materials should be essential.

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