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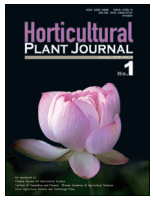

Identification and Molecular Characterization of a Phytoplasma Associated with Pomegranate Fasciation Disease

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A B S T R A C T

To confirm phytoplasma infection, samples of pomegranate (*Punica granatum* L.) plants showing symptoms of fasciation were collected from an orchard located in Tai'an, Shandong Province, China. A fragment of approximately 1.2 kb was amplified with universal primers targeting the phytoplasma 16S rRNA gene from symptomatic pomegranate plants, while no fragment was obtained from healthy plants. The phytoplasma associated with the disease was designated as pomegranate fasciation (PoF). Two representative phytoplasma 16S rDNA gene sequences (PoF-Ch01 and PoF-Ch02) had 100% nucleotide sequence identity. The 16S rDNA sequence of PoF-Ch01 and PoF-Ch02 showed the highest similarity (99.6%) to that of '*P. granatum*' phytoplasma isolate AY-PG, which belong to 16SrI-B. Further phylogenetic analysis showed that PoF-Ch01 and PoF-Ch02 belonged to a cluster of 16SrI subgroup members. *In silico* RFLP analysis indicated that PoF-Ch01 shared the highest similarity coefficient of 0.97 with reference strains of 16SrI-B, M and N. Actual RFLP analysis of both enzymes *BstUI* and *BfaI* confirmed that of the virtual RFLP analysis. Combining these results, we concluded that PoF was a member of the '*Candidatus* Phytoplasma asteris' group (16SrI), and has very close relationship with 16SrI-B subgroup.

Keywords: *Punica granatum*; fasciation disease; RFLP analysis; 16SrI group

1. Introduction

Pomegranate (*Punica granatum* L.) is an important commercial fruit crop that is extensively cultivated in parts of Asia, Mediterranean Basin, America, and Europe. Several bacterial and fungal diseases including bacterial blight (Benagi et al., 2012), canker (Liu et al., 2007) and anthracnose (Nargund et al., 2012) have been reported to threaten the pomegranate production. A few virus and virus-like diseases of pomegranate were also reported (Gomez and Pallas, 2001). Recently, phytoplasma associated with pomegranate diseases were reported in Iran and Turkey (Karimi et al., 2015; Gazel et al., 2016). The phytoplasma associated with pomegranate yellows symptoms in Iran and Turkey was related with '*Candidatus* Phytoplasma pruni' and 16SrI-B or 16SrXII-A phytoplasmas, respectively (Karimi et al., 2015; Gazel et al., 2016). So far, no phytoplasmal disease of pomegranate has been reported in China and the phytoplasma associated with pomegranate fasciation remains largely unknown.

Phytoplasmas, a class of mollicutes characterized by the absence of cell walls, is located in the sieve elements of phloem tissue (Lee et al., 2000). Owing to the difficulty in culturing phytoplasma in cell-free media, and the advances in molecular biological techniques, molecular biology-based methods including phylogenetic analysis and computer-simulated or actual

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Fig. 1 Shoots of pomegranate plants showing fasciation (A and B) and healthy-looking pomegranate plants (C)

restriction fragment length polymorphism (RFLP) analysis of highly conserved 16S rRNA gene sequences have become widely accepted for elucidating phytoplasma taxonomy (Lee et al., 1998; Zhao et al., 2009). During 2013 and 2014, ten pomegranate shoots with fasciation symptoms, which resembled typically phytoplasmal disease symptoms, were observed in China. In this study, we investigated the association of phytoplasma with symptomatic pomegranate plants.

2. Materials and methods

2.1. Diseased pomegranate tissues

Newly germinating branches of 4 years-old pomegranate tree with or without smaller foliage, stunting, and shortened internodes symptoms (Fig. 1) were sampled from suburban areas of Tai'an, Shandong Province, China, during 2013 and 2014 growing seasons. It is worth noting that some diseased shoots showed sickle apex (Fig. 1, B). For DNA extract, leaves and stems from newly germinating branches of 10 symptomatic and 4 healthy pomegranate plants were collected in 20 May 2013, respectively, as well as in 20 May 2014 for replicate test. The equivalent tissues of 4 healthy pomegranate plants were pooled together as template in each DNA extract. Double distilled water was used as template in PCR test to serve as the blank control. All ten symptomatic trees were examined for phytoplasma presence by PCR, but the data from two trees were used in the sequence analysis.

2.2. DNA extraction and nested-PCR amplification

Total DNAs were extracted with hexadecyltrimethylammonium bromide methods as described previously (Gao et al., 2011a). The existence of phytoplasma was detected by nested polymerase chain reaction (PCR) using universal primers P1/P7 (Schneider et al., 1995) and R16F2n/R16R2 (Gundersen-Rindal and Lee, 1996). Nested PCR components and amplification protocols have been described previously (Gao et al., 2011a). DNAs from asymptomatic plants were taken as negative control, while the DNAs from paulownia plants showing witches'-broom symptoms were used as positive control (Wang et al., 2010). The PCR products were analyzed by 1% agarose gel electrophoresis and were visualized under a UV transilluminator (Bio-Rad, Richmond, CA) after staining with ethidium bromide.

2.3. Cloning and sequencing of PCR products

The purified R16F2n/R16R2 PCR amplification products were cloned into a pMD18-T vector (TaKaRa, Dalian, China) following the manufacturer's instructions and then transferred into *Escherichia coli* DH5α competent cells. Six recombinant clones of two isolates were sequenced in both directions using the dideoxy chain-termination method at Shanghai Biosune Biotechnology Co. (Shanghai, China). Sequence assembly and analysis were performed using DNAStar software (Madison, WI, USA). To assess similarity percentages, multiple related sequences were aligned using ClustalW in MegAlign. A phylogenetic tree that included sequences of 18 related phytoplasma strains was constructed using the Neighbor-Joining method in MEGA 5.2. *Acholeplasma laidlawii* (GenBank accession No. M23932) was used as outgroup. Bootstrapping was performed with 1 000 replicates to evaluate the significance of internal branches in the tree.

2.4. RFLP analysis

Virtual RFLP pattern comparison of the 1.2-kb 16S rRNA gene sequences was carried out using the online software tool iPhyClassifier (http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) (Zhao et al., 2009). Recognition sites for the following 17 restriction enzymes were used in the simulated digestions: *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I. The pattern was then confirmed by an actual enzymatic RFLP analysis using distinguishing restriction enzymes (*Bst*UI and *Bfa*I) according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA), with 16S rRNA gene fragments of paulownia witches'-broom (PaWB) phytoplasma used as a control. The digested products were separated by electrophoresis on a 3% polyacrylamide gel and visualized under a UV transilluminator after ethidium bromide staining.