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Research Article

Identification and GroEl gene characterization of green petal phytoplasma infecting strawberry in Italy

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Abstract

The presence of phytoplasmas in strawberry showing malformation of the fruits together with the typical green petals symptoms was detected in some North Western Italy cultivations. Nucleic acids extracted from these plants were used in nested-PCR assays with primers amplifying 16S rDNA and GroEL sequences specific for phytoplasmas. Bands of respectively 1.2 and 1.4 kb were obtained after nested-PCR assays and RFLP analyses allowed to classify the detected phytoplasmas in the aster yellows subgroup 16SrI-C, the GroELI grouping confirms that all the strains from strawberry were identical to each other and are affiliated to GroELI-VII group. This is the first GroEL gene characterization of strawberry green petals phytoplasmas.

Keywords : Identification, polymerase chain reaction, restriction fragment length polymorphism, strawberry green petals

Introduction

Strawberry green petals (SGP) (Blattny and Blattny, 1959) is a strawberry disease transmitted by leafhoppers; the pathogen is related to the phytoplasma associated with clover phyllody (Frazier and Posnette, 1957). SGP is characterized by small and red-leaves, the infected plants bear abnormal fruits and frequently show diagnostic symptoms of virescence on flowers (Converse *et al*., 1988). The agent of this disease was molecularly identified as belonging to ribosomal subgroup 16SrI-C in Canada (Gundersen *et al*., 1996) and in the Czech Republic (Franova Honetslegrova *et al*., 1996). In Italy the presence of phytoplasmas in strawberry has been reported as associated with diverse symptomatology and 16SrI-B, aster yellows phytoplasmas were identified in Southern Italy in some cases together with 16SrI-C, clover phyllody phytoplasmas (Bertaccini *et al*., 1997; Pastore *et al*., 2002; 2006). More recently 16SrXII-A, stolbur phytoplasmas were identified in Northern Italy (Terlizzi *et al*., 2006).

A few plants showing small flowers with green petals, stunted shape and dark green leaves were observed in a private garden located in north-western Italy. Flowers were markedly virescent with green petals and also green and malformed structures in the internal part. Ripening of the fruit was very irregular and malformed fruits were produced (Fig. 1).

Material and Methods

Strawberry plants showing the above described symptoms clearly referable to phytoplasma presence, were collected in a private garden in north Italy in September 2012. Samples from freshly cut leaf midribs and pedicels of four symptomatic and one asymptomatic plants were prepared and nucleic acid was extracted following the procedure described by Lee *et al*. (1991). Polymerase chain reaction (PCR) assays and restriction fragment length polymorphism (RFLP) analyses to identify phytoplasmas were then carried out. Reference phytoplasma strains used were from naturally infected corn from Colombia (MBSCol:

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Figure 1. Symptoms related to phytoplasmas presence in flowers and fruits of strawberry. Typical green petals are present together with fruit malformations and virescences.

16SrI-B) (Duduk *et al.*, 2008) and from the collection maintained in periwinkle (Bertaccini, 2010): Plantago virescence form Germany (PVW: 16SrI-B), clover phyllody from UK (KVE: 16SrI-C) and Leontodon yellows from Italy (LEO: 16SrI-C).

Nucleic acid samples diluted in TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] to give a final concentration of 20 ng/µl were employed in direct PCR reactions as previously described (Schaff *et al.*, 1992). Reaction mixtures contain 20 ng of nucleic acid, 200 µM each dNTP and 0.4 µM primer in a total of 25 µl volume. Thirty-five PCR cycles were conducted in an automated thermal cycler. Tubes with the reaction mixture devoid of DNA templates or containing DNA from healthy periwinkle and asymptomatic strawberry were included in each experiment as negative controls. The universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) was used in direct PCR while the R16F2/R2 pair (Lee *et al.*, 1995) was employed in the nested assays to amplify the 16S rDNA. In these nested-PCR assays the products obtained with the universal primers were diluted 1:30 with sterile deionized water and used as template. The 16S rDNA sequences (about 200 ng of PCR products) amplified with primers R16F2/R2 were analyzed with the restriction endonucleases *Alu*I, *Hha*I, *Tru1*I and *Kpn*I and the collective RFLP patterns obtained were compared with those control phytoplasma strains after electrophoresis through a 7% polyacrylamide gel.

The same nucleic acid samples were also employed for amplification in nested PCR of GroEL gene with primers AYgroesF/AYampR (Mitrovic *et al.*, 2011b) that amplify a region of about 2,100 bp external to the about 1,400 bp region amplified by AYgroelF/AYgroelR (Mitrović *et al.*, 2011a). Nested PCR was carried out on amplicons diluted 1: 30. Thirty-five PCR cycles were performed for both primer pairs as previously

described (Mitrović *et al.*, 2011a). The groELI (groEL gene RFLP group I) RFLP subgrouping was performed with *Alu*I and *Tru1*I restriction enzymes (Fermentas, Vilnius, Lithuania) with analyses by electrophoresis as described above.

Results and Discussion

When the primer pair P1/P7 was used in direct PCR assays, no visible amplification products were obtained with any of the strawberry templates (data not shown), but a DNA fragment of 1.2 kb was produced from the four symptomatic strawberry samples with the primer pair R16F2/R2 in nested-PCR assays (data not shown). No amplification products were observed in the negative control devoid of template DNA or in samples containing DNA from asymptomatic strawberry and healthy periwinkle with any of the primers used. Restriction fragment length polymorphism (RFLP) analysis with the different enzymes employed showed that strawberry phytoplasma isolates had patterns identical to each others and to the reference strains LEO and KVE both belonging to subgroup 16SrI-C (Fig. 2A).

Expected length amplicons (about 1.4 kb) of partial *groEL* gene were obtained from symptomatic strawberry samples as well as from all the reference strains (data not shown). RFLP analyses with *Tru*I (Fig. 2B) and *Alu*I (data not shown) restriction enzymes yielded different profiles for reference strains belonging to 16SrI-B and undistinguishable profiles for the strains from strawberry that are identical to the reference strain belonging to GroELI group VI (Mitrovic *et al.*, 2011a).

The results obtained with all the symptomatic strawberry samples indicate that phytoplasmas associated with Italian SGP disease belong to 16SrI-C group as reported in other geographic areas. RFLP analysis of the DNA fragments amplified with both primers confirms this result, this is however the first characterization of SGP phytoplasmas on GroEl gene allowed to specifically assign the strawberry phytoplasmas to the GroELI-VII group. Very little is know in literature about GroELI characterization for the 16SrI-C phytoplasmas since all characterized strains but one were from the micropropagated collection in periwinkle (Bertaccini, 2010) and the only one field collected was from periwinkle in Serbia (Mitrović *et al.*, 2011b). However the latter belong to GroELI-VI group indicating that both strains are still present in naturally infected plants.

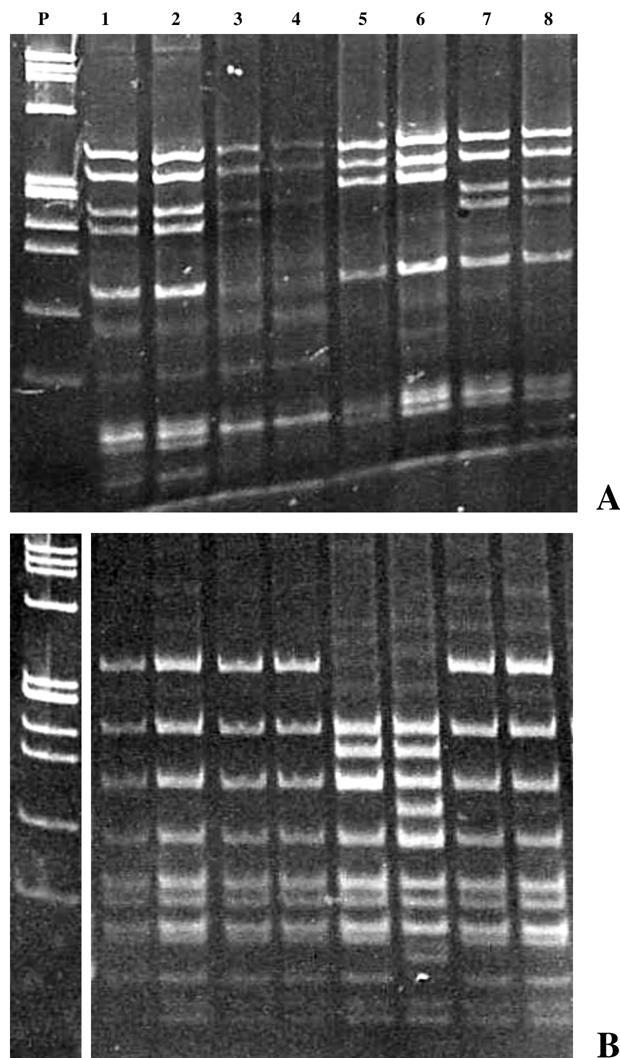

Figure 2. (A) Results in polyacrylamide gel of restriction fragment length polymorphisms (RFLP) analyses of phytoplasma 16S rDNA sequences amplified in nested PCR with primers R16F2n/R2 from strawberry samples 1, 2, 3 and 4 and reference phytoplasmas strains 5, PVW; 6, MBSCol; 7, KVE; 8, LEO; P, marker ΦX174 RF I DNA *Hae*III digest. **(B)** RFLP analysis results of phytoplasma sequences amplified in nested PCR with GroEL primers from same strawberry sample as in (A), reference phytoplasmas strains and marker are as in (A). Enzymes used in both cases is *Tru*1I.

The finding of strawberry with SGP phytoplasmas is rare in Italian crops and probably incidentally, however since these phytoplasmas are in the EU quarantine list just for this species they should monitored and all infected material must be uprooted, as it was done in this case, to avoid dangerous spreading of this disease in case of leafhopper vector presence.

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