Characterization of a 16SrII subgroup D phytoplasma strain associated with Calendula officinalis phyllody in Iran

Characterization of a 16SrII subgroup D phytoplasma strain associated with *Calendula officinalis* phyllody in Iran

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Abstract

Calendula officinalis plants with phyllody symptoms (CaoP) were observed in Yazd and Ashkezar (Yazd province, Iran) from 2013 to 2016. Twenty one symptomatic and 4 asymptomatic plants were potted individually and transferred to greenhouse for the biological and molecular characterization of associated phytoplasma. The dodder transmission to periwinkle and pot marigold plants of the CaoP agent induced in these plants virescence, phyllody and witches' broom symptoms. Total DNAs from symptomless plants and CaoP *C. officinalis* plants and from dodder inoculated periwinkles were tested by nested PCR assay with primer pairs amplifying phytoplasma ribosomal DNA. Only CaoP plants and dodder inoculated periwinkles provided positive results. RFLP analysis of the amplicons obtained in direct PCR with primers P1/P7 using *Rsa*I, *Alu*I, *Mse*I, *Hin*fI and *Hae*III restriction enzymes showed profiles identical to each other and referable to phytoplasmas in all the 21 positive samples. Six R16mF2/R16mR2

primed amplicons were selected and directly sequenced; the resulting consensus sequences had 100% of identity among each other. R16F2n/R16R2 trimmed sequences (1,250 bp) of representative samples from Yazd and Ashkezar were deposited in GenBank under accession numbers KU297202 and MH065715 respectively. BLAST search and phylogenetic analysis showed that the CaoP phytoplasma had 99% homology and clusters with phytoplasmas in group 16SrII. Computer-simulated analysis using *i*PhyClassifier suggests that the CaoP RFLP 16S rRNA gene pattern was identical to the one of 16SrII-D phytoplasmas. Since CaoP phytoplasma was shown to be molecularly identical to alfalfa witches' broom phytoplasma strains (16SrII-D) previously reported in the same geographic areas, it is possible that alfalfa plays a role in the epidemiology of CaoP disease or vice-versa.

Keywords: Alfalfa witches' broom, dodder transmission, pot marigold phyllody, RFLP

Introduction

Phytoplasmas are cell wall-less prokaryotes associated to plant diseases worldwide and transmitted mainly by leafhoppers and psyllids and very often causing quite devastating economic losses (Bertaccini et al. 2014). Characteristic disease symptoms include yellowing, discoloration, witches' broom, stunting, virescence and flower phyllody (Lee et al. 2000, Bertaccini and Duduk 2009). Ornamental plants have been reported as hosts of different phytoplasmas worldwide (Chaturvedi et al. 2010, Gera et al. 2006, Marcone et al. 1997, Omar and Alsohim, 2016, Rani et al. 2014, Wang and Hiruki 2001) and in particular in Iran *Limonium sinuatum* with phyllody and mild stunting symptoms (16SrI-C), *Gomphocarpus physocarpus* with witches' broom, dwarfing, yellowing and purpling, leaf rolling virescence, decline and seed sterility (16SrI-B), *Tanatacetum parthenium* with stunting and phyllody symptoms (16SrI-B),

Tagetes patula with witches' broom, virescence, early decline and purpling of leaves (16SrI-B), *Coreopsis lanceolata* with witches' broom, dwarfing and phyllody symptoms (16SrI-B) (Babaie et al. 2007), *Catharanthus roseus* with yellowing, dwarf, witches' broom and phyllody (16SrI-A and 16SrVI-A) (Babaie et al. 2007, Fattahi et al. 2016), *Rudbeckia hirta* with dwarfing, phyllody and virescence (16SrI-A) (Babaie et al. 2007) and *Erysimum cheiri* with phyllody and witches' broom symptoms (16SrII) have been described (Asghari Tazehkand et al. 2010).

Calendula officinalis L. (family Asteraceae), commonly known as marigold or pot marigold, is cultivated for ornamental purposes and also for many medicinal, culinary and cosmetic uses. In 2006, a phyllody disease named CaoP was observed in a collection of ornamental plants located at the Yazd Agricultural and Natural Resources Research and Education Center in Iran (Esmailzadeh Hosseini et al. 2008) adjacent to alfalfa fields showing epidemic incidence of alfalfa witches' broom (AWB) disease associated with phytoplasma presence (Esmailzadeh Hosseini et al. 2011, 2016d). Molecular and biological characterization of the phytoplasma strain associated with CaoP disease in pot marigold was therefore carried out.

Materials and methods

Plant sampling

C. officinalis plants with phytoplasma symptoms were collected from 2013 to 2016 at Yazd (31° 54' 58" N, 54°16' 41" E) and Ashkezar (32° 03' 42" N, 54° 10' 33" E) Iran, and transferred to an insect-proof greenhouse. In each area, five 250 m^2 pot marigold fields were selected randomly and sampled at five points within one square meter on a diagonal transect across each of the

fields. To calculate the disease incidence the number of symptomatic plants and the total number of plants in each sampled square meter was determined. The infection percentage was calculated multiplying the mid average of the infected plant number to the total plant number in each quadrate further multiplied by 100. Collectively 21 symptomatic and 4 asymptomatic plants were used for biological and molecular studies.

Propagation and maintenance of CaoP agent

For propagation and maintenance of the CaoP agent, transmission trials via dodder (*Cuscuta campestris* Yank.) inoculation were carried out to 21 periwinkle [*Catharanthus roseus* (L.) G. Don] and 21 pot marigold plants maintained in an insect-proof greenhouse. Dodder seeds were germinated on moist filter paper and seedlings were transferred to healthy seed-grown sugar beet (*Beta vulgaris* subsp. *vulgaris*) plants. After the colonization of the sugar beet plants, a dodder strand was connected to one symptomatic *C. officinalis* plant for three weeks colonization. This dodder was then connected with 3-4 leaves of seed grown periwinkle and healthy pot marigold plants. After four weeks the periwinkle and pot marigold plants were freed from dodder and observed for symptoms expression. Phytoplasma presence in the dodder-inoculated plants was verified by nested PCR assay using the primers described below. For long-term maintenance, the CaoP agent was transmitted by side grafting from symptomatic dodder inoculated plants to healthy young seed-grown periwinkle seedlings.

DNA extraction and polymerase chain reaction (PCR)

The total DNA extracted from 0.2 g of midrib tissue of fresh leaves from 21 symptomatic and 4 symptomless *C. officinalis* and from the tissues of dodder inoculated plants using Zhang et al.

(1998) procedure was subjected to direct and nested PCR using respectively P1/P7 (Deng and Hiruki 1991, Schneider et al. 1995) and R16mF2/R16mR2 and R16F2n/R16R2 (Gundersen and Lee 1996) primer pairs as reported by Esmailzadeh Hosseini et al. (2011). PCR products were separated in 1.2% agarose gels in 1X TBE buffer [108 g Tris-HCl, 55 g boric acid, 40 ml EDTA (0.5M), pH 8.0] and DNA bands, stained with ethidium bromide, were visualized with a UV transilluminator.

RFLP analysis

A preliminary identification of the phytoplasma associated with CaoP was obtained by restriction fragment length polymorphism (RFLP) analysis of P1/P7 PCR products (1.8 kbp fragment). The amplicons were digested separately with each of the *Rsa*I, *Alu*I, *Mse*I, *Hin*fI and *Hae*III restriction enzymes according to the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The restriction products were then separated by electrophoresis through a 2.5% agarose gel, stained by ethidium bromide and visualized with a UV transilluminator.

Virtual RFLP analysis using *i*Phyclassifier (Zhao et al. 2009) allowed then to determine the CaoP phytoplasma ribosomal subgroup affiliation. The RFLP profile of the 1,248 bp fragment (R16F2n/R16R2 region of 16S rRNA gene) of the CaoP phytoplasma strain was compared to those of phytoplasmas strains classified in 16SrII subgroups -A to –M with 17 restriction enzymes: *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, *Mbo*I (*Sau3A*I), *Mse*I, *Rsa*I, *Ssp*I and *Taq*I (Lee et al. 1998).

Sequencing and phylogenetic analysis

Selected R16mF2/R16mR2 primed nested PCR products (1,400 bp) of CaoP phytoplasma were directly sequenced using the same primers used for amplification and internal primers designed by the sequencing company. The assembling and trimming of these sequences allows to obtain fragments corresponding to the R16F2n/R2 amplicons. The homologous sequences search performed by Blast analyses at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) was used to identify the phytoplasmas closest to the strain under study. The R16F2n/R2 sequences of 16S rRNA gene of 29 phytoplasmas including CaoP were aligned and phylogenetic trees and sequence homologies were generated using MEGA7 software (Kumar et al. 2016). *Acholeplasma laidlawii* was used as an out group to root the tree after a bootstrapping analysis performed 1,000 times to estimate the stability and support for the branches.

Results

Disease incidence and symptomatology

The characteristic symptoms of CaoP disease were leaf size reduction, yellowing, flower phyllody, virescence, proliferation and sterility, proliferation of axillary buds, witches' broom and stunting (Figs. 1a, b). During the survey the average disease incidence was of 6.8%, 6.4%, 8% and 7.8% in Ashkezar fields and 10.2%, 11%, 10.4% and 12% in Yazd areas, respectively.

Dodder transmission

The success of dodder transmission was different between periwinkle and pot marigold plants: 17 and 14 out of the 21 periwinkle and pot marigold plants developed virescence, phyllody and witches' broom symptoms. Differences were also observed in the duration of the latent period in CaoP agent dodder-inoculated periwinkle and pot marigold plants since symptoms were observed respectively from 6-12 and 8-12 weeks after transmission. The CaoP phytoplasma was graft-propagated in periwinkle plants with 100% of efficiency and s virescence and phyllody symptoms appearance after 4 to 7 weeks from the grafting.

Polymerase chain reaction

After polymerase chain reaction using P1/P7 primers followed in nested reactions by R16mF2/R16mR2 and R16F2n/R16R2 primer pairs, DNA fragments of ~1.8, ~1.4 and ~1.25 kbp respectively were obtained from symptomatic *C. officinalis*, symptomatic dodder and graft inoculated periwinkles and pot marigold, but not from symptomless pot marigold or periwinkle plants (data not shown).

RFLP Analysis

Restriction fragment length polymorphism analysis of P1/P7 amplicons using *Rsa*I, *Alu*I, *Mse*I, *Hin*fI and *Hae*III restriction enzymes showed identical patterns referable to those of phytoplasmas in all the positive samples (Fig. 2). The comparison of these patterns with those previously published for P1/P7 phytoplasma amplicons (Khan et al. 2002) clearly indicate identity with the restriction profiles of phytoplasmas enclosed in group 16SrII.

Nucleotide sequence and virtual RFLP analysis

The consensus sequences of the six R16mF2/R16mR2 amplicons obtained from the selected samples collected in Ashkezar and Yazd locations provided 1.2 kbp R16F2n/R2 amplicons and resulted 100% identical to each other's independently from the collecting location or the sampling period. Two sequences of phytoplasma strains from Yazd and Ashkezar were deposited in GenBank under accession numbers KU297202 and MH065715, respectively. BLAST search of these sequences showed a 99% identity with phytoplasmas enclosed in subgroup 16SrII and the phylogenetic analysis confirmed their clustering with strains enclosed in group 16SrII in the '*Candidatus* Phytoplasma australasia'-related strains clade (NCBI GenBank accession numbers Y10097 and JQ868448) (Fig. 3). Virtual RFLP patterns derived from *in silico* digestions of CaoP phytoplasma sequences exhibited profiles referable to those of members of the 16SrII phytoplasma group and resulted identical to the pattern of phytoplasmas enclosed in the 16SrII-D subgroup (Fig. 4).

Discussion

The phytoplasma associated with *C. officinalis* phyllody in Iran was identified as a member of the 16SrII-D subgroup. The phytoplasma presence was reported in India in the same species showing virescence symptoms (Rani et al. 2014); moreover phytoplasmas belonging to peanut witches' broom group (16SrII) were detected in Saudi Arabia (Omar and Alsohim 2016) and phytoplasmas belonging to aster yellows (16SrI) group were identified in Italy and Canada, respectively (Marcone et al. 1997, Wang and Hiruki 2001).

A wide range of plant species is reported as infected by peanut witches' broom phytoplasma group (16SrII) in Iran. This phytoplasma group, according to 16S rRNA gene RFLP analyses encloses a large number of subgroups: 16SrII-A, 16SrII-B ('*Ca*. P. aurantifolia'), 16SrII-C, 16SrII-D ('*Ca*. P. australasia'), 16SrII-E, 16SrII-F, 16SrII-G, 16SrII-H, 16SrII-I, 16SrII-J, 16SrII-K, 16SrII-L (Bertaccini et al. 2014; Lee et al. 1998) and 16SrII-M (Salehi et al. 2015b). Among these groups the 16SrII-D is associated in Iran with destructive diseases such as alfalfa witches' broom (Esmailzadeh Hosseini et al. 2015a, b, c, 2016a, b, c, Salehi et al. 2011), tomato witches' broom (Salehi et al. 2014), parsley phyllody (Salehi et al. 2016d), squash phyllody (Salehi et al. 2015b), garden beet witches' broom (Mirzaie et al. 2007), sesame phyllody (Salehi et al. 2016c), sunflower phyllody (Salehi et al. 2015a), carrot witches' broom (Salehi et al. 2016b) and pomegranate little leaf (Salehi et al. 2016a). The CaoP phytoplasma strains showed patterns identical to each other in all the positive samples, that were identical to those detected in the alfalfa witches' broom phytoplasma strain (16SrII-D) (Esmailzadeh Hosseini et al. 2016a) identified as the major disease agent in alfalfa growing areas adjacent to *C. officinalis*. It is therefore possible to hypotize that alfalfa play a role in the epidemiology of *C. officinalis* phyllody disease or vice-versa.

Compliance with ethical standards

Conflict of interest. The authors declare that they have no conflict of interest.

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Fig. 1 Leaf size reduction, yellowing, phyllody, virescence, witches' broom and stunting exhibited by *Calendula officinalis* plants affected by the phyllody disease (a) compared to the healthy plants (b).

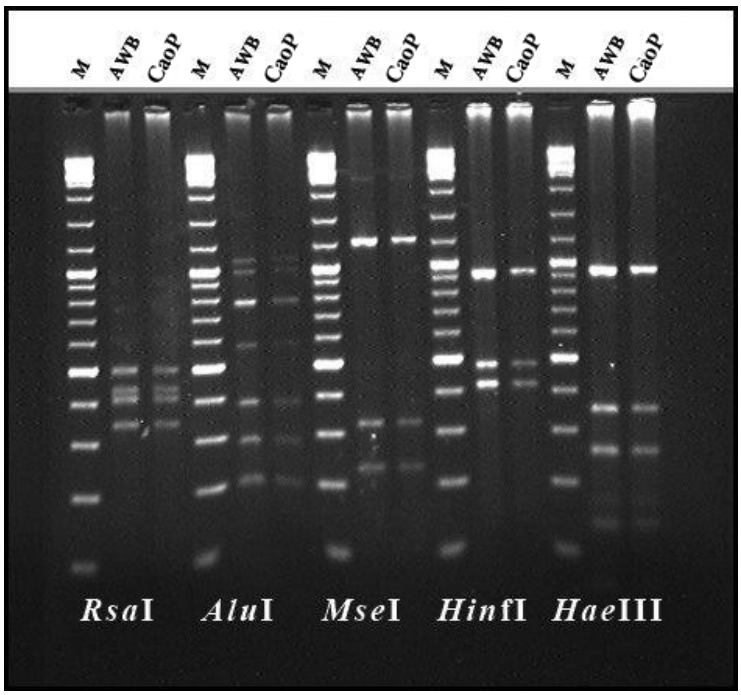

Fig. 2 Restriction fragment length polymorphism (RFLP) analysis of PCR products amplified with primers P1/P7 (1,800 bp) of *C. officinalis* phyllody (CaoP) phytoplasma and alfalfa witches' broom phytoplasma (AWB) (16SrII-D) using *Rsa*I, *Alu*I, *Mse*I, *Hin*fI and *Hae*III restriction enzymes. M: 100 bp DNA ladder.

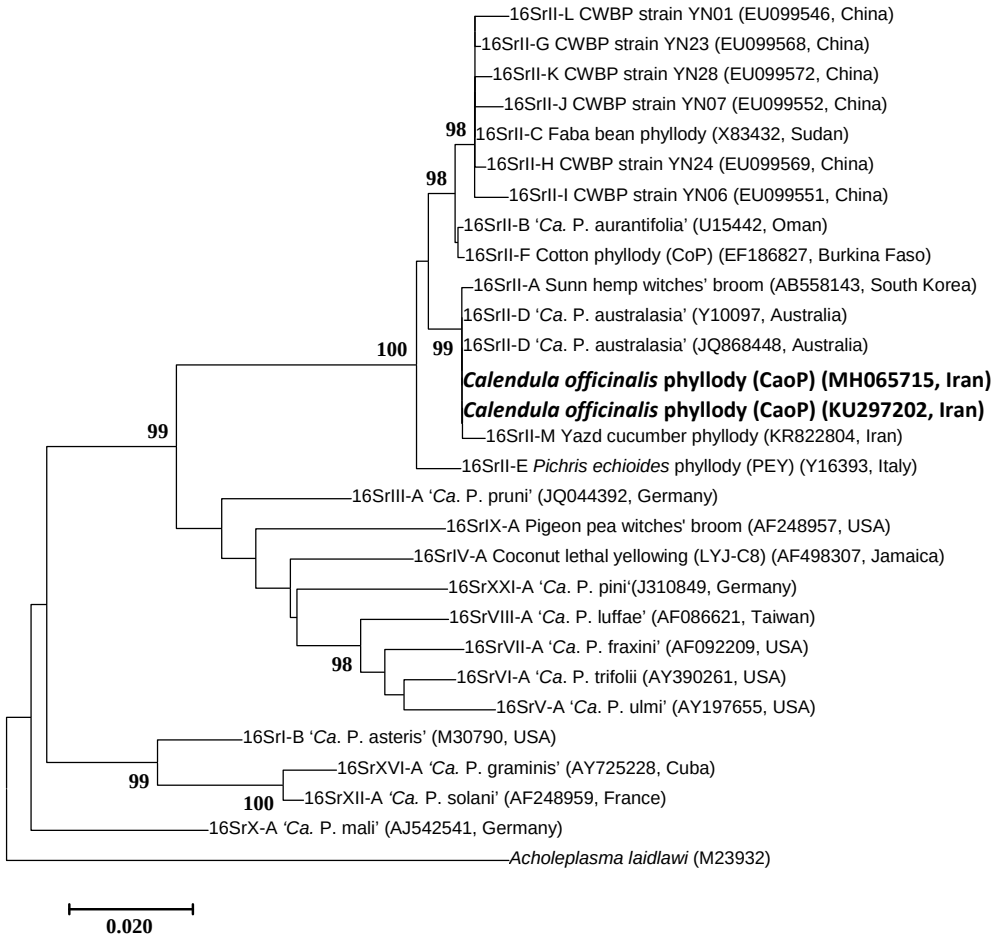

Fig. 3 Phylogenetic trees constructed using MEGA7 software (Kumar et al. 2016) by the Neighbor-Joining method (Saitu and Nei, 1987). The R16F2n/R2 sequences of 16S rRNA gene from selected phytoplasmas and *A. laidlaw*ii as the out group were used. CWBP: Cactus witches' broom phytoplasma. Bootstrapping was performed 1,000 times to estimate the stability and support for the branches and its values >95% are shown at the branch points. NCBI GenBank accession numbers for sequences are given after the phytoplasma name together with Country of detection in parenthesis. The phytoplasma ribosomal subgroups are listed before the phytoplasma names. The strains under study are in bold. The scale bar represents 2 nucleotide exchange per 100 nucleotides.

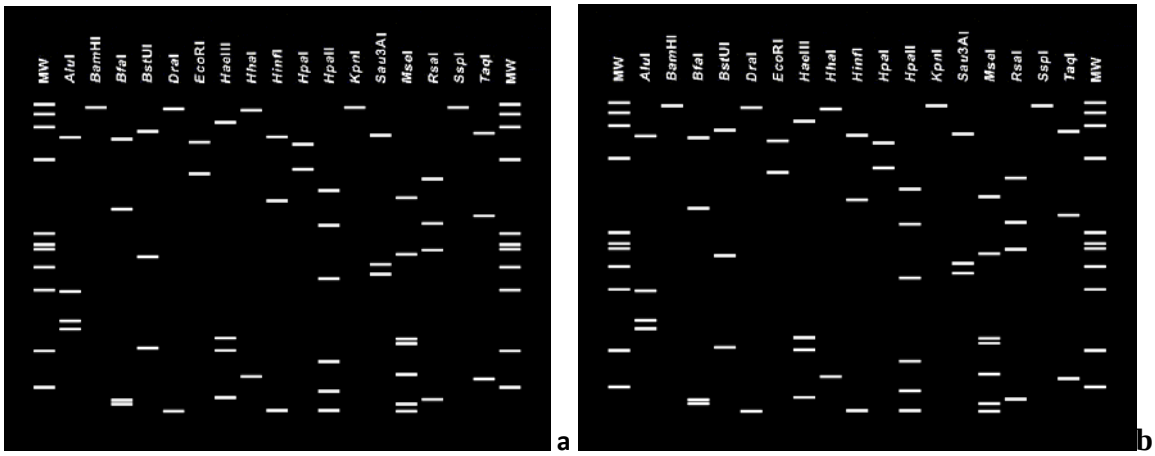

Fig. 4 Comparison of computer-simulated virtual RFLP patterns derived from *in silico* digestions of phytoplasma 16S rRNA gene (1.2 kb fragment) between *C. officinalis* phyllody phytoplasma (a) and reference strain of 16SrII subgroup D (b) '*Candidatus* Phytoplasma australasia' (GenBank accession number Y10097) using online *i*PhyClassifier program. MW, molecular weight marker phiX174 *Hind*III digested. Fragment sizes in base pair from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.