Crop Protection xxx (2017) 1–8

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Identification and characterization of a phytoplasma associated with black locust yellow disease in two provinces of Iran

Marziye Karimzade [a], Majid Siampour [b], Mohammad Zakiaghl [a, *], Mohsen Mehrvar [a]

[a] Department of Plant Pathology, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran
[b] Department of Plant Protection, College of Agriculture, Shahrekord University, Iran

ARTICLE INFO

Article history:
Received 18 December 2016
Received in revised form
10 April 2017
Accepted 7 May 2017
Available online xxx

Keywords:
Phytoplasma
Phytoplasma subgroups
Shade-tree diseases
Black locust
Iran

ABSTRACT

A phytoplasma disease was associated with black locust trees showing yellows, a devastating disease found in different geographical areas of Iran. Infected trees showed yellowing, reduced leaf size, short internodes and proliferation of buds, dieback and decline. The disease was transmitted to healthy black locust and periwinkle plants in greenhouse. DNA extractions were made from midrib of leaf tissues. Nested PCR assays with R16mF2/R16mR1 universal phytoplasma primers detected a 1.4 kb product and an 850 bp product was obtained using Fu5/Ru3 primers in double nested PCR. Phytoplasmas were detected from all symptomatic black locust trees as well as inoculated black locust and periwinkle plants. Analysis of 16S rRNA gene sequences showed that phytoplasmas associated with black locust yellows (BLYp) were related to '*Candidatus* Phytoplasma phoenicium' (group 16SrIX). Moreover, four 16S rDNA genetic isolates (BLYp-a to -d), distinguished by 1–10 single nucleotide polymorphisms (SNPs), were identified in BLYp population. Virtual RFLP analysis determined that BLYp-a represents a new tentative subgroup within the 16SrIX group; BLYp-b, BLYp-c and BLYp-d were assigned to subgroup 16SrIX-C. Actual RFLP profiles derived from *EcoR*I, *Rsa*I, *Hinf*I and *Taq*I enzymes confirmed virtual RFLP subgroup characterization. Phylogenetic analysis showed that BLYp isolates shared a recent common ancestor with some 16SrIX-C phytoplasma strains. Moreover, comparisons of 16S rRNA and *Sec*Y gene nucleotide sequence raised the question as to whether BLYp and other 16SrIX-C phytoplasmas identified in Iran are identical or closely related strains. To the best of our knowledge, this is the first report of 16SrIX phytoplasmas infecting black locust.

1. Introduction

Phytoplasmas are obligate, host dependent parasites adapted to a dual life cycle, alternating between plant and arthropod hosts (Zhao et al., 2015). They are specified by small genome size and are known as the causal agents for diseases in numerous plants. In plants, they are restricted to the phloem where they are propagated and deliver their secreted effector proteins into the plant cells. Phytoplasmas are specifically vectored by piercing-sucking homopteran insects. Phylogenetic studies showed that phytoplasmas are diverse; however, they constitute a distinct monophyletic clade within the class *Mollicutes* (Bertaccini, 2007; Hogenhout et al., 2008; Marcone, 2014).

As phytoplasma cultivation in cell-free medium has been reported only recently (Contaldo et al., 2012, 2013). Thus their classification and taxonomy are primarily based on the 16S rRNA gene sequence analysis. According to the rules established for taxonomy of uncultured organisms (Murray and Stacke-brandt, 1995), all phytoplasmas are currently accommodated in the provisional genus '*Candidatus* Phytoplasma' (IRPCM, 2004). According to the guidelines proposed by IRPCM (2004), phytoplasma strains that share less than 97.5% 16S rRNA gene sequence identity with previously defined '*Ca*. Phytoplasma' species could represent a new species. To date more than 35 '*Ca*. Phytoplasma' species have been identified (Zhao et al., 2015; Davis et al., 2016).

Actual and virtual restriction fragment length polymorphism (RFLP) analyses of 16S rRNA gene are also commonly used for grouping and classification of phytoplasmas. To date, 31 phytoplasma groups, each including subgroups have been distinguished based on the comparative RFLP patterns of 16Sr RNA gene sequences (Wei et al., 2007, 2008; Zhao et al., 2009). Moreover, sequence analysis of more variable genes, such as *rplV-rpsC*

* Corresponding author.
E-mail address: zakiaghl@ferdowsi.um.ac.ir (M. Zakiaghl).

http://dx.doi.org/10.1016/j.cropro.2017.05.010
0261-2194/

(ribosomal protein) and *SecY* genes, were needed for finer differentiation of closely related phytoplasmas (Martini et al., 2007; Lee et al., 2010). Detailed analysis of phytoplasma genomes has shown that they have high genetic potential to drive the emergence of new strains and to adapt into the wide range of plant species all around the world (Oshima et al., 2013).

Black locust (*Robinia pseudoacacia* L., Fabaceae) is a perennial deciduous plant species possessing many features that make it an important shade tree for economic and ecological purposes. Black locust is widely distributed throughout the U.S.A., Asia and Europe in forested lands, or used for afforestation, industrial wood production, bee food, soil and water conservation and nitrogen fixing improvement (Straker et al., 2015). In Iran, black locust is widely planted throughout the country as a popular tree in urban and rural green spaces (Moshki and Lamersdorf, 2011).

A disease with symptoms of yellowing and little leaf has been observed on the black locust trees in Iran over several years. The disease, termed black locust yellows (BLY), is devastating and progressive disease with high incidence in Iran. Yellowing of black locust has sometime been attributed to some micronutrient deficiencies that is a usual problem in calcareous soils of Iran (Moshki and Lamersdorf, 2011). However, the micronutrient deficiencies may not be an adequate explanation for the occurrence of the disease in Iran. A similar disease of black locust in China and U.S.A. has been found to be associated with phytoplasmas (Chapman et al., 2008; Ren et al., 2014). The symptoms of the BLY in Iran also resembled those of phytoplasma diseases. In this study, the association of phytoplasmas with black locust yellow (BLY) disease in Iran was investigated.

The main objective of this work was to focus on the role of phytoplasmas in the etiology and vast distribution of the BLY disease in the rural and urban regions of Iran. To this purpose, molecular and phylogenetic analyses for detecting and genotyping the phytoplasmas associated with black locust yellows in Iran was performed.

2. Materials and methods

2.1. Plant materials and DNA extraction

During the summer of 2014 and 2015, leaf samples were collected from thirty symptomatic and ten non-symptomatic black locust (*R. pseudoacacia*) plants in urban and rural areas of Khorasan-Razavi (northeast of Iran) and Fars (south of Iran) provinces. Nineteen plants in 2014 and 21 plants in 2015 were used in DNA extractions (Table 1). Leaf samples from the experimentally inoculated plants (by graft or dodder) were also used in DNA extractions. Total DNA was extracted from the excised veins of each sample according to Namba et al. (1993). One-hundred mg of leaf midrib was ground with a mortar containing grinding buffer (2% CTAB, 100 mM Tris pH 8, 1.4 M NaCl, 20 mM EDTA, 2% PVP-40). Isolated DNA was dissolved in TE buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA).

2.2. PCR, cloning and sequencing

Detection of phytoplasma was performed by amplification of their respective rDNA sequences in nested PCR analysis. Universal phytoplasma primers P1/P7 (Deng and Hiruki, 1991) were used for the first round of PCR. PCR was carried out in 25 μl reaction mixture using PCR master mix (Ampliqon, Denmark) and 1 μl of DNA template.

PCR conditions were initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 60s and a final extension step for 7 min at 72 °C. Amplified product was diluted at a 1:20 rate in water and 1 μl was used as template in nested PCR reactions with R16mF2/R16mR1 primer pair (Gundersen and Lee, 1996).

Third round of PCR amplification was performed using Fu5/Ru3 primer pair (Lorenz et al., 1995). The amplification conditions were programmed as above. A negative control devoid of DNA template was also used in all PCR analyses.

PCR products were run in 1.2% agarose gel and stained with DNA green viewer (Parstous, Iran).

An amplified nested PCR product of approximately 1.4 kb in size was cloned in pTG19 T/A cloning vector (Vivantis, Malaysia). Recombinant plasmid purification was performed using Plasmid DNA Isolation Kit (Denazist, Iran) and sequenced on both strands (Macrogen, South Korea).

Primer pair SecYF2(IX)/SecYR2(IX) was used for PCR amplification of *SecY* gene according to Lee et al. (2012).

2.3. 16S rRNA group/subgroup classification

The taxonomic position and 16Sr group/subgroup assignment of the detected phytoplasmas were determined by nucleotide sequence identity comparisons and *in silico* RFLP analysis of the 16S rRNA gene (Zhao et al., 2009; Zhao and Davis, 2016).

Sequences were trimmed for the F2nR2 region of the 16Sr RNA gene (1250 bp) and Basic Local Alignment Search Tool (BLAST) searched for phytoplasma group identification. Subgroup identification and virtual Restriction Fragment Length Polymorphism (RFLP) analysis was determined using standard criteria provided in the *i*Phyclassifier program (Zhao et al., 2009).

2.4. Virtual restriction fragment length polymorphism (RFLP) analysis

Virtual RFLP profiles of BLYp phytoplasmas were generated by digestion of a 1.2 kb nested PCR products with 17 restriction enzymes (*Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau3A*I, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I) in the *i*Phyclassifier program (Zhao et al., 2009).

2.5. Phylogenetic analysis

Multiple sequence alignments of *16Sr RNA* and *SecY* genes were generated using T-coffee program (Notredame et al., 2000). The generated sequence alignments were used to infer the phylogenetic trees using maximum likelihood method by PhyMl 3.0. program (Guindon et al., 2010). The best substitution model was selected using smart model selection option implemented in PhyMl program (http://www.atgc-montpellier.fr/phyml/). The validation of the branches in the tree was assessed by bootstrap analysis with 1000 replications. *Acholeplasma laidlawii* was used as an outgroup member. GenBank accession numbers of phytoplasmas are listed in Table 2.

2.6. Graft and dodder transmission

Five healthy black locust seedlings (grown from seed) were graft-inoculated with symptomatic black locust scions from naturally infected plants. The development of the symptoms on the graft-inoculated seedlings was monitored for 12 months. Dodder transmission of the disease agent to eight periwinkle seedlings was also conducted under the greenhouse conditions. The dodder (*Cuscuta campestris* Yunck.; grown from seed) was established on one of the experimentally infected (by graft inoculation) black locust seedling. The dodder established on the infected black locust was wrapped on the eight healthy periwinkle seedlings; grown

Please cite this article in press as: Karimzade, M., et al., Identification and characterization of a phytoplasma associated with black locust yellow disease in two provinces of Iran, Crop Protection (2017), http://dx.doi.org/10.1016/j.cropro.2017.05.010