Crop Protection 106 (2018) 13–22

Contents lists available at ScienceDirect

Crop Protection

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

Multilocus genotyping identifies a highly homogeneous phytoplasma lineage associated with sweet cherry virescence disease in China and its carriage by an erythroneurine leafhopper

Jiawei Wang[a,b,1], Qingzhong Liu[a,1], Wei Wei[b], Robert E. Davis[b], Yue Tan[a], Ing-Ming Lee[b], Dongzi Zhu[a], Hairong Wei[a], Yan Zhao[b,*]

[a] Shandong Institute of Pomology, Taian 271000, PR China
[b] Molecular Plant Pathology Laboratory, USDA-Agricultural Research Service, Beltsville, MD 20705, USA

ARTICLE INFO

Keywords:
Sweet cherry
Floral virescence
Phytoplasma infection
Subgroup 16SrV-B
Pathogen genotyping
Leafhopper

ABSTRACT

Phytoplasmas are a diverse group of insect-transmitted, cell wall-less bacteria that colonize plant phloem sieve elements and cause numerous diseases in economically important crops. Living a trans-kingdom parasitic life, phytoplasmas possess dynamic genomes and evolve rapidly toward formation of distinct ecological lineages in their adaptation to specific ecological niches. In an effort aimed at identification of the etiological agent responsible for a repeatedly-occurring sweet cherry virescence (SCV) disease in China, we found that the SCV disease was consistently associated with infection by a phytoplasma belonging to subgroup B of the elm yellows phytoplasma group (16SrV-B). Further analysis of genetic loci that encode important phytoplasma cellular components, including an array of ribosomal proteins and preprotein translocase subunit SecY, revealed that the SCV phytoplasma was essentially indistinguishable from the subgroup 16SrV-B phytoplasma strains responsible for jujube witches'-broom (JWB) disease and diseases of many other plants. Evidence gathered in the present study indicated that SCV-JWB phytoplasma strains formed a highly homogenous ecological lineage. The study also revealed that a polyphagous leafhopper, Táoyīdiǎnyèchán (桃一点叶蝉 in Chinese and often being cited as *Erythroneura sudra*), was able to carry the SCV phytoplasma, emphasizing a need to investigate whether this erythroneurine leafhopper plays a role in spreading the SCV phytoplasma among sweet cherry plants.

1. Introduction

Sweet cherry (*Prunus avium* L.) is a deciduous fruit tree with high nutritional and economic values. The fruit of sweet cherry tree is low in calories and rich in phyto-nutrients such as vitamins and antioxidants (USDA-ARS Nutrient Data Laboratory, 2014), offering remarkable health benefits to consumers. The timber of sweet cherry tree is both durable and pliable, making it a favorite hardwood for flooring, cabinetry, and furniture. Originating from the Black Sea/Caspian Sea region that connects western Asia to eastern Europe, sweet cherry has been widely cultivated and has become naturalized in most of the temperate and subtropical areas around the world (Janick et al., 2011). While the consumption demand is high, the production of sweet cherry is often hindered by pests and diseases attributed to fungal, viral, viroid, and bacterial infections (Ogawa et al., 1995; Xu et al., 2017). Phytoplasmas, a unique group of phloem-inhabiting, insect-transmitted cell wall-less bacteria (Weintraub and Beanland, 2006; Maejima et al., 2014), are among the pathogenic microbes that seriously impact the health and production of cherries. Phytoplasmal infection in cherry trees can trigger diseases exhibiting yellowing (Jomantiene et al., 2011), decline (Valiunas et al., 2009), virescence (Zheng et al., 2012; Wang et al., 2014a), and X-disease symptoms (Granett and Gilmer, 1971), and some of the diseases are lethal. Diverse phytoplasmas related to six distinct '*Candidatus* Phytoplasma' species including '*Ca.* Phytoplasma asteris', '*Ca.* Phytoplasma pruni', '*Ca.* Phytoplasma ziziphi', '*Ca.* Phytoplasma prunorum', '*Ca.* Phytoplasma pyri', and '*Ca.* Phytoplasma solani' have been reportedly responsible for phytoplasmal diseases in cherries (Valiunas et al., 2009; Gao et al., 2011; Bertaccini et al., 2014). Based on restriction fragment length polymorphism (RFLP) profiles of their 16S rRNA gene (16S rDNA) sequences, previously known cherry-infecting phytoplasmas belong to five different ribosomal groups (16SrI, 16SrIII, 16SrV, 16SrX, and 16SrXII). Insect vectors of most of these cherry-infecting phytoplasmas remain unknown. Early studies indicated that several leafhopper species

* Corresponding author.
E-mail address: yan.zhao@ars.usda.gov (Y. Zhao).
[1] These authors have contributed equally to the study and therefore share first authorship.

https://doi.org/10.1016/j.cropro.2017.12.005
Received 8 September 2017; Received in revised form 24 November 2017; Accepted 1 December 2017
0261-2194/ Published by Elsevier Ltd.

Table 1
Primers used in polymerase chain reactions (PCR) of this study.

Primer pair	Sequence	Target locus	Size of expected amplicon (bp)	Reference
P1/P7	F: 5′-aagagtttgatcctggctcaggatt-3′ R: 5′-cgtccttcatcggctctt-3′	partial *rrn*	1800–1850	Deng and Hiruki, 1991; Smart et al., 1996
P1A/16S-SR	F: 5′-aacgctggcggcgcgcctaatac-3′ R: 5′-ggtctgtcaaaactgaagatg-3′	partial *rrn*	1500–1550	Lee et al., 2004
R16mF2/R16mR1	F: 5′-catgcaagtcgaacgga-3′ R: 5′-cttaaccccaatcatcgac-3′	partial *rrn*	1430–1440	Gundersen and Lee, 1996
rpF1/rpR1	F: 5′-ggacataagttaggtgaattt-3′ R: 5′-acgatatttagttctttttgg-3′	*rpsS-rplV-rpsC*	1245–1389	Lim and Sears, 1992
rp(V)F1A/rp(V)R1A	F: 5′-aggcgataaaaaagtttcaaaa-3′ R: 5′-ggcattaacataatatattatg-3′	*rpsS-rplV-rpsC*	1200	Martini et al., 2007
L15F1/MapR1	F: 5′-cctggtagtggyamtggwaaaac-3′ R: 5′-attarraatatarggytcttcrtg-3′	*rplO-secY-map*	2200–2800	Lee et al., 2010
L15F1A-b/MapR1A-b	F: 5′-ggwaaaacytshggymrvgghcataaagg-3′ R: 5′-ccwatmccrtgwccdgwaaaa-3′	*rplO-secY-map*	2197–2242	Lee et al., 2010
secY(V)F1/secY(V)R4	F: 5′-agctcgtactggtggcggt-3′ R: 5′-agtgccgattttaccaatagcac-3′	*rplO-secY-map*	2111	This study[a]
secY(V)F2/secY(V)R3	F: 5′-ttcgagggtggtcaaacacct-3′ R: 5′-ccaggtttcacttgttctattcc-3′	*rplO-secY-map*	2034	This study[a]

[a] These primer pairs were designed in the present study based on an alignment of publicly available nucleotide sequences of the *rplO-secY-map* locus of group 16SrV phytoplasmas.

(*Scaphytoplus* spp.) and psyllid species (*Cacopsylla* spp.) may be involved in transmitting group 16SrIII and 16SrX phytoplasmas, respectively, among stone fruit trees including cherries (Tsai, 1979; Carraro et al., 1998; and references therein).

Prior to the present study, two sweet cherry phytoplasmal disease incidents had been reported in China (Gao et al., 2011; Wang et al., 2014a). While both incidents occurred in Shandong Province and the affected cherry trees exhibited similar floral symptoms (virescence), the etiological agents of the two disease incidents were mutually distinct. The disease in Yantai was associated with infection by a '*Ca*. Phytoplasma asteris'-related lineage (Gao et al., 2011); on the other hand, the disease in suburban Taian was linked to infection by a '*Ca*. Phytoplasma ziziphi'-related lineage (Wang et al., 2014a). In suburban Taian, after the first occurrence of sweet cherry virescence (SCV) disease in 2013 (Wang et al., 2014a), sweet cherry trees exhibiting symptoms resembling those of SCV disease were repeatedly observed in the ensuing years, indicating re-occurrence of the disease in this geographic region and signaling a need for effective measures to control the disease. Notably, the natural ecosystems of suburban Taian are rich in diversity of plant and insect species, and therefore seem highly favorable for phytoplasma niche adaptation and transmission. Numerous phytoplasmal diseases in a wide range of plant hosts have been previously reported, and diverse phytoplasma lineages affiliated with at least four distinct '*Ca*. Phytoplasma' species and four 16Sr groups (16SrI, 16SrIII, 16SrV, and 16SrXII) have been detected in the region (Liu et al., 2004, 2005; Zhao et al., 2007; Gao et al., 2012, 2014; Yu et al., 2016). Since infection sources (reservoirs) of multiple phytoplasma lineages and potential disease-transmitting insect vectors may be present in the geographic region, and since different phytoplasmas may induce visually indistinguishable symptoms in a given plant host (Davis and Sinclair, 1998; Wu et al., 2012), accurate disease diagnosis and precise identification of the infecting phytoplasma(s) is crucial to understanding the epidemiology of the SCV disease in the region and to devising sensible measures for disease management. In this communication, we report multilocus genotyping of the phytoplasma lineage that was responsible for the repeated occurrences of the SCV disease in suburban Taian. We also report that the sweet cherry-infecting phytoplasma was carried by a polyphagous erythroneurine leafhopper prevalent in the region, Táoyīdiǎnyèchán (桃一点叶蝉). Further investigations and transmission studies are warranted to determine whether this leafhopper played a role in transmitting the SCV disease.

2. Material and methods

2.1. Sample collection and DNA extraction

Leaf samples were collected from sweet cherry trees exhibiting virescence and/or witches'-broom growth symptoms in four orchards located in suburban Taian and one orchard located in Junan County, Shandong Province, China during the springs of 2013, 2014, and 2015. Two to six symptomatic branches per tree were sampled. Total DNA was extracted from 200 mg leaf midribs using a modified CTAB extraction protocol (Green et al., 1999) followed by a DNA purification step employing a commercial kit (DNeasy Plant Mini, Qiagen Inc., Valencia, CA). Leafhoppers were collected using a sweep net and the dominant species, "Táoyīdiǎnyèchán" (scientific name under debate, most frequently cited as *Erythroneura sudra* Distant), was identified based on previously established morphological criteria (Cao and Zhang, 2013). Every fifty individuals were pooled for total DNA extraction using the same protocol as for plant DNA extraction described above.

2.2. Polymerase chain reaction (PCR) and amplicon sequencing

Direct and nested PCRs were performed to target amplifications of three phytoplasma genomic loci, namely, a partial ribosomal rRNA operon (*rrn*), a ribosomal protein gene cluster *rpsS-rplV-rpsC*, and an extended *secY* locus that encodes ribosomal protein L15, preprotein translocation subunit secY, and methionine aminopeptidase (*rplO-secY-map*). The PCR primers used in this study are detailed in Table 1. Each amplification reaction was carried out in a total volume of 50 μL mixture containing 2 μL of the above prepared DNA extract, 25 pmol each of a forward and a reverse primer, 200 μM each of dATP, dCTP, dGTP and dTTP, 5 μL of 10× LA PCR Buffer II, and 2.5 units of high-fidelity TaKaRa LA Taq DNA polymerase (TaKaRa Biomedical, Otsu, Shiga, Japan). All polymerase chain reactions were carried out for 35 cycles under the conditions as previously described (Wei et al., 2011): denaturation at 95 °C for 60 s, annealing at 56 °C for 60 s, and extension at 72 °C for 75 s. The resulting PCR amplicons were cloned into pCR®II-TOPO® TA vector (Invitrogen, Carlsbad, CA, USA), and their nucleotide sequences determined using Sanger dideoxy method to achieve a minimal of 4 × coverage per base position (Macrogen, Rockville, MD, USA).