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ScienceDirect
Scientia Horticulturae 116 (2008) 130–137
SCIENTIA HORTICULTURAE
www.elsevier.com/locate/scihorti

DNA sequencing reveals false positives during the detection of aster yellows phytoplasmas in leafhoppers

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Received 7 May 2007; received in revised form 31 October 2007; accepted 5 December 2007

Abstract

During the summer of 2001 and 2002, 850 and 865 carrot plants and 926 and 2584 leafhoppers associated with aster yellow (AY)-type disease were collected from five fields in Manitoba, Canada. DNA was extracted from 999 individual leafhoppers and 381 leaf tissues from both apparently healthy and AY-like infected carrot plants. All DNA samples were examined by PCR for the presence of phytoplasmas using three universal primer pairs P1/P6, P1/P7 and R16F2n/R2 derived from phytoplasma rDNA sequences. DNA amplification with these three primer pairs generated the expected amplification products of 1.7, 1.5 and 1.2 kb, respectively. Diluted PCR products obtained using universal primer pair P1/P6 were nested with R16F2n/R16R2n. The latter set of primers amplified DNA samples from 92 carrot plants and 83 leafhopper samples. In order to assess the diversity among insect and plant phytoplasmas, nested PCR products from all 92 carrot and 83 leafhopper samples were subjected to RFLP analysis using restriction endonucleases *Kpn*I, *Mse*I, and *Hha*I. This RFLP analysis showed similar patterns among carrot and leafhopper samples. Phytoplasmas detected in most samples belonged to the subgroup 16Sr-IA. To understand why the R16F2n/R16R2n of primers did not amplify the PCR product obtained from the first PCR in the remaining samples, four PCR products of P1/P6 from two plants (representing 16Sr-IA and 16Sr-IB) and two leafhopper samples that did not amplify with nested PCR, were used for DNA sequencing.[1] The BLAST analysis of the obtained sequences showed that the PCR amplicons from the two carrot samples precisely matched with the GenBank sequences of known phytoplasmas. Alignments of these two sequences have shown very slight variations (transition/transversion ratio mean of 0.539) that would correspond to the minor differences at the 16S level between the 16Sr-IA and 16Sr-IB phytoplasma subgroups. The sequences of PCR products obtained from the two insect samples had similarity (>98%) with the sequences of phytoplasma in carrot except that their length differed from the carrot samples by 6 bp. They actually matched bacterial sequences from the GenBank, indicating that a single PCR using P1/P6 was not enough to detect phytoplasma in leafhoppers.

Keywords: Mycoplasma-like organisms; *Daucus carota* L.; DNA sequencing; Nested PCR; RFLP; *Macrosteles quadrilineatus*

1. Introduction

Yellows-type diseases caused by phytoplasmas are associated with various economical crops worldwide (Chiykowski and Sinha, 1982; Khadhair et al., 1997; Wang and Hiruki, 2001; Lee et al., 2002). These diseases have become a serious epidemiological problem for a number of major crops worldwide due to the lack of appropriate control strategies, the high mobility of their insect vectors and their large host range. They affect approximately 300 plant species in 48 families. In western Canada, several diseases caused by phytoplasmas have been reported, but aster yellows (AY) are the most commonly found and are associated with various important horticultural crops, i.e. carrots (Khadhair et al., 1997, 1998, 2001; Khadhair and Evans, 2000; Wang and Hiruki, 2001). Once the phytoplasma get into the plant, it is too late to attempt any control method. However, the management of the vector, the aster leafhopper (primarily *Macrosteles quadrilineatus*), is crucial early in and during the growing season. In Manitoba, AYs have been a great concern, in particular to the

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[1] The GenBank accession numbers for the sequences reported in this paper are: D2 (DQ679962), E4 (DQ679963), A4 (DQ679964), and B2 (DQ679965).

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doi:10.1016/j.scienta.2007.12.007

carrot growers, due to the impact of this disease on carrot yield and quality. Besides the changes in the foliage color, dwarfed and twisted petioles and a dense growth of shoots, infected carrots become slender, elongated, and covered with a dense hairy growth of secondary roots. Their taste also becomes bitter, most likely due to an increase in the content of certain phytoalexins.

Over the years, an Integrated Pest Management (IPM) strategy has compiled the use of several control methods to reduce the impact of yellows-type disease in important crops. Chemical control remains an effective method to manage aster leafhoppers. However, the effectiveness of spray rely on the timing of chemical applications, which should be based on the monitoring of the peak of the vector populations and threshold calculations. Spray upon first appearance of the leafhoppers on a weekly basis has been a common practice in the past, which has led to an excessive use of insecticides. Cultural control options available to commercial carrot growers include the planting of resistant varieties and weed control to reduce the presence of natural inoculum reservoirs. Weed removal (i.e. quackgrass, plantain chickory) helps reduce the densities of aster leafhoppers, and consequently the disease incidence.

Although pytoplasmas have been well documented in many host plants, little is known with regard to the insect–plant interactions. Interactions among phytoplasma, the vector, and the host plant play an important role in the epidemic of the disease. The infected plants serve as reservoirs for phytoplasma inoculum, and leafhopper insects transfer the pathogen from one plant to another, from field to field and between growing zones under favorable conditions. Although traditional methods, such as visual disease assessment, are commonly used in the initial diagnosis of AY, non-conventional methods such as molecular techniques are more reliable in characterizing and classifying phytoplasmas. DNA-based techniques (Deng and Hiruki, 1991; Gundersen and Lee, 1996; Firrao et al., 1994; Lee et al., 1994, 1998) are very useful for this purpose, based on both highly conserved and variable regions of the 16S rDNA. In former studies, most isolates of AY phytoplasma were found to belong to the group 16SrI (Lee et al., 1998; Marcone et al., 2001). In addition to the conventional PCR methods, recent advance in real-time PCR has allowed several authors to develop assays with high sensitivity and specificity in detecting and quantifying AY phytoplasma in different crops (Galetto et al., 2005; Torres et al., 2005; Baric et al., 2006).

We have recently described the distribution of AY phytoplasmas in both plants and insects from selected carrot fields in Manitoba, Canada, using conventional PCR assays (Wally et al., 2004). However, from an epidemiological point of view, it was critical to have an assessment of the variability of the studied populations and a sense of the pathogen movements through interactions with both their host and vector. Therefore, here we investigated the genetic relatedness among AY phytoplasma isolates in both plants and leafhoppers, using PCR products from our former study. We have also checked the specificity of the amplified PCR products from either material to avoid detection of false positives.

2. Materials and methods

2.1. Samples collection

Based on a weekly visit, carrot leaf samples with typical AY phytoplasma symptoms (leaf chlorosis, proliferation and internodes shortening, root shortening and widening, and eventually pale orange-colored and long hair-like-covered roots) were collected from five commercial carrot fields in southern Manitoba, Canada during 2001 and 2002. At each visit, a random sample of 15 asymptomatic and 15 symptomatic plants were collected from each carrot field. Leafhopper insects (i.e. *M. quadrilineatus*) were also collected from the same fields using insect sweeping net in each field visit (Wally et al., 2004). All samples including plant tissues and leafhoppers were kept in individual tubes at −80 °C until subsequent DNA analysis.

2.2. Molecular detection

2.2.1. DNA extraction from leaf tissues

Leaf tissues from both healthy-looking and symptomatic plants were frozen in liquid nitrogen and ground using pre-chilled mortar and pestle. The ground tissues from each sample were mixed with the extraction buffer (2%, w/v CTAB, 1.4 M NaCl, 20 mM ethylenediaminetetra-acetic acid (EDTA), 100 mM Tris–HCl pH 8.0, 5% (w/v) polyvinylpyrrolidone (PVPP), and 5 mM dithiothreitol), vortexed, incubated in a water bath at 65 °C and extracted twice with phenol:chloroform:isoamylalcohol (25:24:1, v/v/v), then with chloroform:isoamylalcohol (24:1, v/v). The DNA was precipitated with ice-cold isopropanol followed by centrifugation. The DNA pellet was washed with 70% (v/v) ethanol, air-dried and re-suspended in 20 μl TE buffer pH 8.0 (Tris–EDTA 10:1, v/v) (Porebski et al., 1997).

2.2.2. DNA extraction from leafhoppers

The protocol used for DNA extraction from leafhoppers was modified from Marzachi et al. (1998). Each leafhopper was placed in a 1.5 ml microcentrifuge tube and macerated with a micropestle in 200 μl of hot CTAB buffer (60 °C). After 10 min incubation, the suspension was extracted using an equal amount of chloroform:isoamylalcohol (24:1, v/v). The DNA was precipitated by 100% ethanol and centrifugation at 5000 × *g*. Each pellet was air-dried and re-suspended in 20 μl TE buffer.

2.2.3. PCR assays

Three universal primer pairs P1/P6, P1/P7, and R16F2n/R16R2n were used with each extracted DNA sample. These primer pairs were previously designed on the basis of the 16S rDNA sequence of phytoplasma (Deng and Hiruki, 1991; Gundersen and Lee, 1996). Positive (from Dr. Khadhair's collection) and negative (healthy host DNA and no-template-DNA) controls were routinely used in the PCR assays. Samples were tested using either direct PCR with the universal primer pair P1/P6 (Deng and Hiruki, 1991) or by nested PCR using either of the universal primer pairs P1/P6 or P1/P7 followed by