Detection of a Plant Pathogen in a Nonvector Insect Species by the Polymerase Chain Reaction

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ABSTRACT

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A nonculturable mycoplasmalike plant pathogen was detected in a leafhopper species capable of transmitting the pathogen as well as in a leafhopper species that does not vector the pathogen. Amplification of a pathogen-specific DNA sequence by polymerase chain reaction (PCR) revealed the presence of pathogen DNA in total nucleic acid extracts of the vector insect *Macrosteles fascifrons* and of the nonvector insect *Dalbulus maidis*, both of which had fed on plants infected by the aster yellows mycoplasmalike organism.

Mycoplasmalike organisms (MLOs) are cell wall-less prokaryotes associated with many economically important yellows diseases of plants worldwide (10). The aster yellows (AY) MLO is an important pathogen of this type, known to attack plants in over 40 different families. It is transmitted from diseased to healthy plants by grafting, dodder (*Cuscuta* spp.), or leafhoppers (1).

An important phenomenon, termed "conditioning" (9), results in the survival on novel host plants of *Dalbulus maidis* (DeLong & Wolcott) (Homoptera: Cicadellidae) after feeding on plants infected by AY MLO. Although *D. maidis* serves as a vector for different pathogens (13), it does not serve as a vector for the AY MLO. Data reporting the presence of the AY MLO in *D. maidis* that have fed on AY-infected plants have been inconclusive. Maramorosch (8) recovered the pathogen from the nonvector after the insect fed on AY-infected aster plants, whereas Purcell (16) found no evidence of pathogen acquisition. Maramorosch's (8) method for determining pathogen acquisition consisted of macerating whole insects that had fed on infected plants, followed by injecting the macerated solution into the vector species. The vector was allowed to feed on healthy aster plants, and determinations were made as to whether the plant became infected. Plant infection implied pathogen acquisition by the nonvector. Rather than using extracts from whole-insect macerations, Purcell (16) extracted the hemolymph from the nonvector after it had fed on AY-infected plants and injected the hemolymph into the vector. Several other techniques have been used for detecting MLOs infecting plants and/or insects (4).

Recently, the polymerase chain reaction (PCR) has been used as a highly sensitive method for detecting DNA (11,12). PCR has been used for pathogen detection in plant hosts (2,3,6, 14,15,17–20,22–24) and vector insects (14). Use of PCR for pathogen detection in the AY MLO-*D. maidis* system could obviate the need for time-consuming and possibly inaccurate biological assays to assess the presence of a pathogen in a nonvector. In

this study, we report detection (using PCR) of AY MLO in *D. maidis* as well as in a vector insect species *Macrosteles fascifrons* (Stål) (Homoptera: Cicadellidae).

MATERIALS AND METHODS

Vector and nonvector insects were allowed to feed on China aster (*Callistephus chinensis* (L.) Nees) plants infected with the severe strain of the aster yellows MLO (SAY MLO) for a specific time period (acquisition access period [AAP]). The AAP was followed by an incubation period, during which insects were allowed to feed only on healthy asters in the case of *M. fascifrons* or on healthy corn (*Zea mays* L. 'Aristogold Bantam Evergreen') in the case of *D. maidis*. When *D. maidis* was allowed to feed on healthy aster plants, the insect died within 4 days (9). To reduce mortality of the nonvector insect (a corn specialist) after the AAP on SAY-infected asters, we allowed *D. maidis* to feed on healthy corn during the pathogen-incubation period. Negative controls consisted of insects that fed on healthy aster (*M. fascifrons*) or on healthy corn (*D. maidis*).

Separate groups of insects were subjected to different acquisition and incubation periods. Individuals of *M. fascifrons* were given AAPs of 7 days, followed by incubation periods of 21 days (i.e., 7-21) or were given an AAP of 14 days, followed by an incubation period of 14 days (i.e., 14-14). Individuals of *D. maidis* were given AAPs of 7 or 14 days, followed by an incubation period of 14 or 7 days, respectively (i.e., 7-14 or 14-7).

Nucleic acid samples were extracted from single vector insects (*M. fascifrons*) or from batches of five insects (*M. fascifrons* or *D. maidis*). Insects were placed separately in 12-ml glass tissue homogenizers (Bellco Biotechnology, Vineland, NJ) and macerated in 400 μl of extraction buffer (100 mM Tris, 50 mM EDTA, 500 mM NaCl, pH 8.0) plus 2 μl of β-mercaptoethanol and 20 μl of 20% SDS (sodium dodecyl sulfate). The solution was transferred to a 1.5-ml Eppendorf tube and remacerated with a minipestle, followed by centrifugation at 2,000 rpm for 10 min. The supernatant was collected, and the sample was further centrifuged at 8,000 rpm for 10 min. The supernatants were combined, heated at 65 C for 5 min, and centrifuged at 14,000 rpm for 10 min. Nucleic acids were extracted from the supernatant with chloroform-isoamyl alcohol and TE-saturated phenol. To precipitate nucleic acids, 2.5 volumes of cold (−20 C) absolute ethanol was added to samples, followed by 30 min at −86 C, and 20 min of centrifugation at 14,000 rpm. The DNA pellet was dried with nitrogen gas and resuspended in 100 μl of 6× SSC (0.9 M sodium chloride, 0.09 M sodium citrate, pH 7.0).

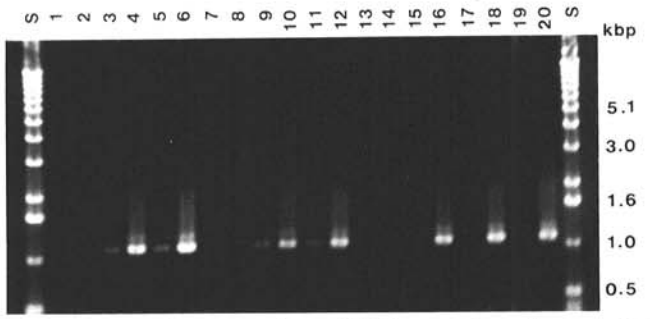

Fig. 1. Severe strain aster yellows (SAY) mycoplasmalike organism detection by polymerase chain reaction in batches of five vector insects (*Macrosteles fascifrons*). Insects were given an acquisition access period (AAP) of 7 days on SAY-infected asters, followed by an incubation period of 21 days on healthy asters (lanes 1–6); an AAP of 14 days, followed by 14 days incubation (lanes 7–12); or were maintained on healthy plants (lanes 13–18). Lanes 1, 7, and 13, undiluted nucleic acid sample. Lanes 2, 8, and 14, undiluted nucleic acid sample plus plasmid control. Lanes 3, 9, and 15, 1:10 nucleic acid dilution. Lanes 4, 10, and 16, 1:10 nucleic acid dilution plus plasmid control. Lanes 5, 11, and 17, 1:100 nucleic acid dilution. Lanes 6, 12, and 18, 1:100 nucleic acid dilution plus plasmid control. Lane 19, water control (no nucleic acid added). Lane 20, plasmid control. Lane S, standard kbp ladder.

Total nucleic acid samples extracted from vector or nonvector insects were used as template in PCR mixtures. The 50 μl of reaction mixture consisted of nucleic acid sample (undiluted or diluted 1:10 or 1:100 in double distilled water), 0.4 pmol of each primer of an AY MLO primer pair (DS19pm; 17,18), PCR buffer (1× = 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.001% gelatin), 1.25 U of Amplitaq Recombinant *Taq* DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT), and 200 μmol of each dNTP. The reaction mixture was overlaid with 45 μl of mineral oil and heated at 94 C for 2 min. The tubes were run through 35 cycles, each consisting of 1 min at 94 C (denaturation), 2 min at 50 C (annealing), and 3 min at 72 C (primer extension). After the last cycle, the reaction mixture was held at 4 C. PCR products were analyzed by electrophoresis in 1% agarose gels, followed by staining in ethidium bromide and visualization of DNA bands with a UV transilluminator. A recombinant plasmid (pAY19), containing a fragment of approximately 1 kbp of cloned AY MLO DNA, was used as a positive control template either by itself or by adding it to nucleic acid samples. A standard 1-kbp DNA ladder (Gibco BRL, Gaithersburg, MD) was used as a reference in the gels. To authenticate the amplified DNA, Southern hybridization analysis was conducted. PCR products from the agarose were alkali denatured and transferred to a nitrocellulose membrane by the method of Southern (7). The membrane was baked, prehybridized, and hybridized with a biotin-labeled probe (pAY19), as described by Davis et al (5).

RESULTS

Electrophoresis of PCR products (Figs. 1, 2, and 3) yielded a single DNA band representing a fragment of approximately 1 kbp of MLO (pathogen) DNA when the reaction mixture contained nucleic acids extracted from vector or nonvector insects that had fed on SAY MLO-infected plants. In all cases, pathogen DNA was not amplified when nucleic acid samples used in the reaction mixture were undiluted (after extraction from insects), even when the pAY19 plasmid DNA was added to the PCR

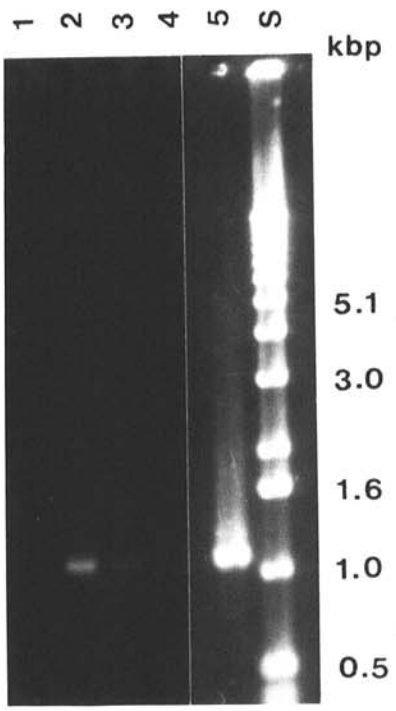

Fig. 2. Severe strain aster yellows (SAY) mycoplasmalike organism detection by polymerase chain reaction on single vector insects (*Macrosteles fascifrons*) given an acquisition access period of 7 days on SAY-infected asters, followed by 21 days incubation on healthy asters. Lane 1, undiluted nucleic acid sample. Lane 2, 1:10 nucleic acid dilution. Lane 3, 1:100 nucleic acid dilution. Lane 4, 1:1,000 nucleic acid dilution. Lane 5, plasmid control. Lane S, standard kbp ladder.

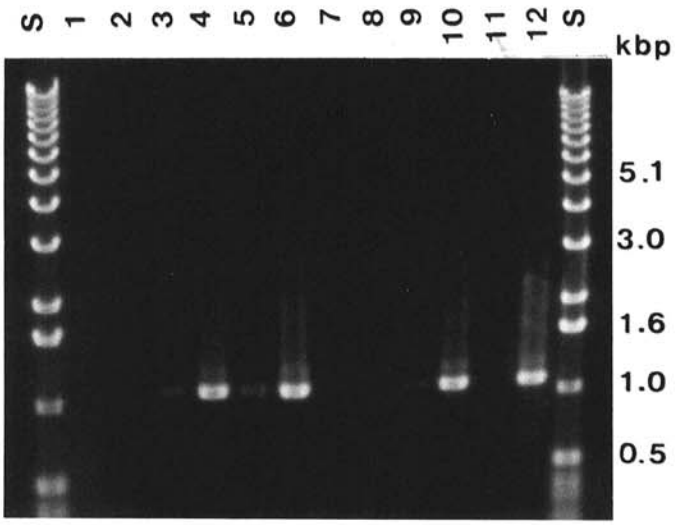

Fig. 3. Severe strain aster yellows (SAY) mycoplasmalike organism detection by polymerase chain reaction in batches of five nonvector insects (*Dalbulus maidis*). Insects were given an acquisition access period (AAP) of 7 days on SAY-infected aster plants, followed by an incubation period of 14 days on healthy corn (lanes 1–6), or an AAP of 14 days, followed by 7 days incubation (lanes 7–12). Lanes 1 and 7, undiluted nucleic acid sample. Lanes 2 and 8, undiluted nucleic acid sample plus plasmid control. Lanes 3 and 9, 1:10 nucleic acid dilution. Lanes 4 and 10, 1:10 nucleic acid dilution plus plasmid control. Lanes 5 and 11, 1:100 nucleic acid dilution. Lanes 6 and 12, 1:100 nucleic acid dilution plus plasmid control. Lane S, standard kbp ladder.

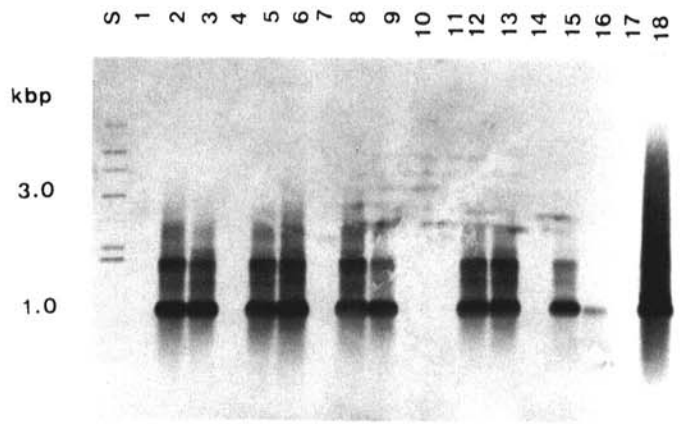

Fig. 4. Southern hybridization results. Severe strain aster yellows (SAY) mycoplasmalike organism detection in batches of five vector insects (*Macrosteles fascifrons*) (lanes 1–6) or in batches of single vector insects (lanes 7–9). Vector insects were given an acquisition access period (AAP) of 7 days on SAY-infected asters, followed by an incubation period of 21 days on healthy asters (lanes 1–3, 7–9); an AAP of 14 days, followed by 14 days incubation (lanes 4–6); or were maintained on healthy plants (lane 10). The nonvector (*Dalbulus maidis*) was extracted in batches of five insects (lanes 11–17). Nonvector insects were given an AAP of 7 days on SAY-infected asters, followed by an incubation period of 14 days on healthy corn (lanes 11–13); an AAP of 14 days, followed by 7 days incubation (lanes 14–16); or were maintained on healthy plants (lane 17). Lanes 1, 4, 7, 11, and 14, undiluted nucleic acid sample. Lanes 2, 5, 8, 10, 12, and 15, 1:10 nucleic acid dilution. Lanes 3, 6, 9, 13, and 16, 1:100 nucleic acid dilution. Lane 18, plasmid control. Lane S, standard kbp ladder.

mixture, suggesting the presence of an inhibitory substance in the nucleic acid preparations. No amplified DNA was observed in reaction mixtures containing nucleic acids from vector and nonvector insects allowed to feed only on healthy plants.

The pathogen DNA fragment was amplified in PCR mixtures containing nucleic acid extracted from vector insects that had been given an AAP of 7 or 14 days, followed by incubation periods of 21 or 14 days, respectively (Fig. 1). Pathogen detection was also positive in the case of nucleic acids extracted from single vector insects given a 7-day AAP, followed by a 21-day incubation (Fig. 2). Pathogen DNA was detected in the nonvector *D. maidis* when nucleic acids were extracted from batches of five insects given a 7-14 or 14-7 acquisition-incubation period (Fig. 3).

Results from Southern hybridization analysis with probe pAY19 (Fig. 4) show a band corresponding to the PCR product obtained from extracts of insects that fed on SAY-infected asters. Hybridization also was positive with the PCR product from the pAY19 plasmid control but not with undiluted extracts or with *M. fascifrons* that had fed only on healthy aster plants or *D. maidis* that had fed only on healthy corn plants (Fig. 4).

DISCUSSION

The survival of SAY MLO in *D. maidis* for extended periods of time is indicated by positive pathogen detection for up to 14 days after the insects were removed from the infected plants. Using biotinylated DNA probes, Vega (21) showed positive SAY MLO detection in insects that had fed on infected plants for 7 days and an increase in pathogen titer as incubation increased from 0, to 7, and to 14 days. This suggests that the pathogen multiplies in the nonvector insect. As Purcell (16) was not able to detect the pathogen in the insect's hemolymph, it seems reasonable to speculate that the pathogen does not enter the hemocoel and that multiplication occurs in the midgut. PCR may enable researchers to determine if this is the case by analyzing the midgut separately from the hemolymph. Pathogen multiplication in the midgut might be involved in the conditioning phenomenon. Maramorosch (9) suggested that pathogen multiplication might induce the production of enzymes that allow the insect to survive on previously unacceptable plants.

It is likely that PCR will become a major tool in the search for unknown insect vectors of plant pathogens, as well as in investigations of vectors of pathogens in other systems. In the search for vectors of plant viruses and MLOs, for example, PCR could be used to sample a vast array of insect species to identify those that might serve as vectors. As our results show, a nonvector insect species can carry and sustain the pathogen of interest. Thus, detection of pathogen DNA in an insect species does not necessarily indicate identification of a vector. Nevertheless, pathogen detection by PCR provides a major advantage in the search for vectors, because the technique reduces the number of potential candidate species that must be subjected to biological assay to determine transmission of the pathogen of interest.

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