JARQ 31, 281-285 (1997)

A New System for the Detection of Phytopathogenic Phytoplasmas Using PCR (Polymerase Chain Reaction) and Laser Stylectomy

Mamoru SATO, Wataru MITSUHASHI, Kenji WATANABE and Hiromu KAWAKITA

Department of Insect Physiology and Behavior, National Institute of Sericultural and Entomological Science (Tsukuba, Ibaraki, 305 Japan)

Abstract

A new system for the detection of phytoplasmas in both rice and mulberry plants using PCR assay and laser stylectomy was investigated. Pure phloem sap was collected in a capillary tube from plants with phytoplasma diseases using laser stylectomy (insect laser technique). Direct detection of phytoplasmas in the phloem sap was attempted using the polymerase chain reaction (PCR). The phloem sap was heated at 95°C for 5 min or not heated. and used directly as the DNA template for PCR amplification. Two sets of oligonucleotide primer pairs were used to amplify the target DNA fragment (length, 1.37 kb and 0.75 kb) from 16S rRNA genes of phytoplasma. The predicted band was observed for almost all the heated or unheated samples collected from diseased plants, whereas no band was detected in the samples collected from healthy plants. Each of the amplified PCR products was found to be the target DNA from 16S rRNA genes of phytoplasmas based on the analysic of their restriction enzyme digestion and determination of the DNA sequence. Thus, the new detection system using both laser stylectomy and PCR assay was found to be sensitive and specific.

Discipline: Plant disease/Biotechnology
Additional key words: rice yellow dwarf disease, mulberry dwarf disease, diagnosis, mycoplasmalike organisms

Introduction

Phytoplasmas are nonculturable *Mollicutes* associated with diseases of several hundred plant species including rice and mulberry, and are present in the sap of phloem sieve tubes. The phloem sap of rice plants can be obtained by a modified aphid technique called "laser stylectomy" in which the insect stylets of the brown planthopper are severed by YAG (yttrium-aluminum garnet) laser[5]. Furthermore, mulberry phloem sap can also be collected by laser stylectomy using *Ricania japonica*[6].

On the other hand, the use of PCR as a diagnostic tool for phytoplasma diseases has been developed. Specific oligonucleotide primer pairs designed on the basis of well-characterized cloned DNA sequences or from 16S rRNA sequences of animal mycoplasmas have been employed in the PCR detection of phytoplasmas[1-3,7,8,12]. However, there has not been any report of direct PCR detection of phytoplasmas isolated *in vitro* (outside of plant organs).

Recently, our research group has reported that phytoplasmas could be detected in the phloem sap of rice plants collected by laser stylectomy using a fluorescence technique with DNA fluorochrome 4'-6'-diamidino-2-phenylindole (DAPI)[4]. It is suggested that the new detection system of phytoplasmas could be developed by combining the laser stylectomy and PCR method.

Here, we introduce the newly developed detection system of phytopathogenic phytoplasmas in rice and mulberry plants[10,11].

Materials and methods

1) Collection of phloem sap by laser stylectomy

Rice plants with brown planthoppers (*Nilaparvata lugens*) or mulberry shoots with *Ricania japonica* were placed in front of the condensing lens of a

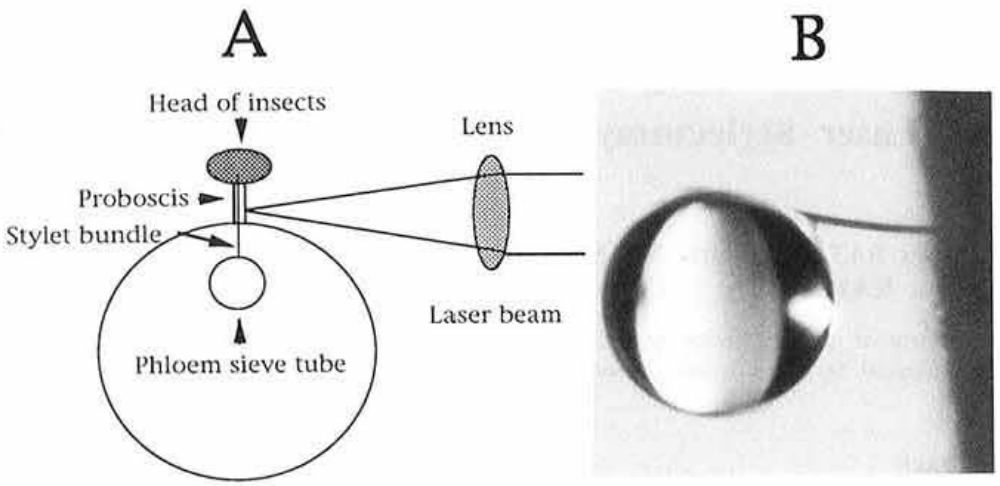

Fig. 1. Collection of phloem sap by laser stylectomy
A: Laser stylectomy system (method of collecting phloem sap by cutting insect-stylets with YAG laser beam).
B: Mulberry phloem sap exuding through the stylet bundle of *Ricania japonica* cut with YAG-laser.

YAG laser system. The horizontal beam was focused at right angle to the proboscis of the insect on the leaf sheath or shoots. The stylet bundle was cut by a pulse of the beam focused on the proboscis (Fig. 1, A). The phloem sap exuding through the severed stylet bundle inserted into the sieve element of the plant was collected in a micro-capillary tube (Fig. 1, B). A total of 0.5 to 1.0 μl of phloem sap was collected from diseased plants, while 5 to 10 μl was obtained from healthy plants. The collected phloem sap was stored at -20°C until the PCR assay was performed.

2) PCR amplification

One μl of phloem sap collected from diseased or healthy plants was diluted in 20 μl of distilled water and heated at 95°C for 5 min. A total of 2.5 μl of the heated sample was used as DNA template for the PCR assay. In another method, about 1 μl of unheated phloem sap was directly put into the PCR reaction mixture.

The amplification was performed in a 50 μl PCR reaction mixture containing 250 μM each of dATP, dGTP, dCTP and dTTP, 1 μM of each upstream and downstream primer, 5 μl of 10 × PCR reaction buffer (Promega Corp., Madison WI) and 0.5 U of taq DNA polymerase (Promega Corp). The universal primer set (SN910601, SN910502) and phytoplasma-specific primer set (SN910601, SN920204) were used in this experiment (Fig. 2). They had been designed for the detection of *Mollicutes* or phytoplasmas, respectively by Namba et al.[8]. Using the universal primer set, PCR was carried out for 50 cycles under the following conditions: first cycle: denaturation, 90 sec at 94°C; ramping, 40 sec to 60°C; annealing, 2 min at 60°C; ramping, 30 sec to 72°C; extension, 3 min at 72°C; continuing cycles: denaturation, 2 min at 94°C; final cycle; extension, 7 min at 72°C. Denaturation and annealing conditions were the same as for the first cycle. As for the phytoplasma-specific primer, thermocycling was performed for 20 cycles. The annealing temperature was 40°C and the other conditions were described above.

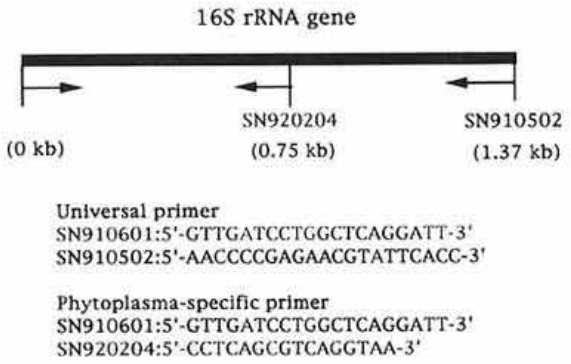

Fig. 2. Two sets of oligonucleotide primer pairs for PCR amplification of 16S rRNA fragment of phytoplasmas

3) Gel electrophoresis and restriction enzyme analysis

To analyze the PCR products by restriction

enzyme digestion, 10 μl of reaction mixture including amplified DNA was purified using Takara Suprec-02 tubes and digested by restriction enzymes such as *Eco*RV, *Hinc*II, *Hha*I, *Hae*III, and *Sma*I. The molecular weight was determined from fragments produced by the digestion of lambda phage DNA with *Hind*III (Toyobo Corp.).

4) DNA sequence analysis of amplified DNA

A part of the PCR-amplified sample DNA was purified by agarose gel electrophoresis as described previously[9]. The sequence of each DNA was determined by direct sequencing of both strands by using the 17 oligonucleotide primers with Sequenase Kit Version 2.0 (U.S. Biochemical Corp., Cleveland, OH).

Results

1) PCR detection from phloem sap of rice plants

Samples of phloem sap collected from diseased and healthy rice plants were heated and tested for PCR detection using the universal primer set (SN910601, SN910502). It was predicted that the 1.37 kb DNA fragment of the 16S rRNA gene would be amplified by PCR if the samples contained phytoplasma particles. A clear single DNA band (1.37 kb) was observed in 9 out of 10 samples collected from diseased plants. In some of the samples, phytoplasmas were detected even when the samples were diluted 100-fold or 10-fold with distilled water (data not shown). However, no band was observed for all the 10 samples collected from healthy rice plants (Table 1 and Plate 1). Similar results were obtained by the PCR assay using another phytoplasma-specific primer set (SN910601, SN920204). All the tested samples collected from diseased plants produced 0.75 kb target DNA amplified by the primers, whereas no bands were produced by the samples collected from healthy plants (Table 1, Plate 1). Similar results were obtained when unheated samples were used as DNA templates for the PCR assay. Samples collected from diseased rice plants produced a target DNA band of 1.37 kb or 0.75 kb, whereas no target DNA band was detected in samples from healthy plants (Table 1).

2) PCR detection from phloem sap of mulberry

Samples of phloem sap collected from diseased and healthy mulberry plants were tested for PCR detection using the universal primer set and phytoplasma-specific primer set. A clear single DNA

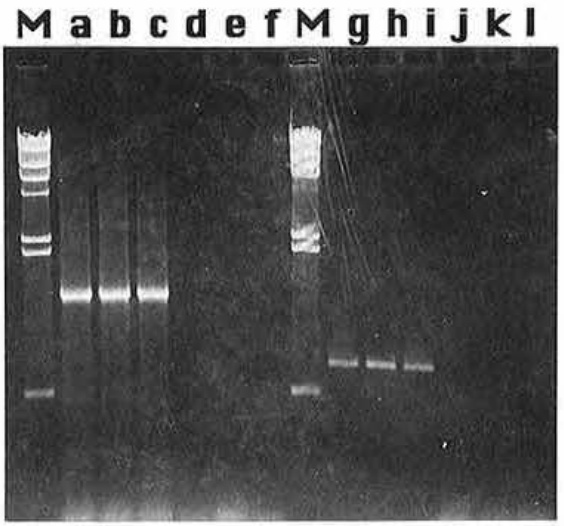

Plate 1. PCR detection of phytoplasmas in heat-treated phloem sap collected from rice plants using universal or phytoplasma-specific primer sets
M: Lambda phage DNA digested with *Hind*-III (molecular weight marker).
a,b,c: Samples collected from rice plants with yellow dwarf disease using the universal primer (1.37 kb band).
d,e,f: Samples collected from healthy rice plants using the universal primer.
g,h,i: Samples collected from rice plants with yellow dwarf disease using the specific primer (0.75 kb band).
j,k,l: Samples collected from healthy rice plants using the specific primer.

Table 1. Detection of phytoplasmas in phloem sap collected from rice plants with yellow dwarf disease or healthy rice plants by PCR assay using universal or phytoplasma-specific primer sets

Treatment of phloem sap	Universal primer		Specific primer	
	Diseased	Healthy	Diseased	Healthy
Heating[a)]	9/10[b)]	0/10	4/4	0/10
No treatment	10/11	0/10	3/3	0/10

a): The phloem sap was heated at 95°C for 5 min.
b): Figures indicate PCR-positive sample numbers to tested sample numbers.

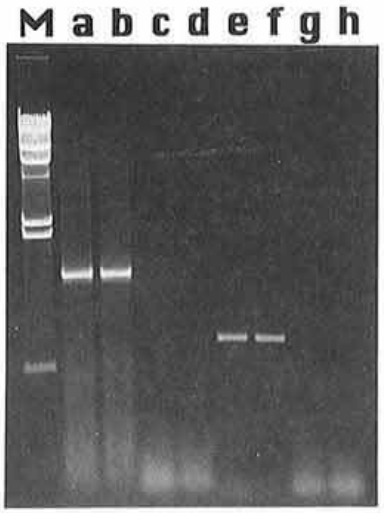

Plate 2. PCR detection of phytoplasmas in phloem sap collected for mulberry using universal or phytoplasma-specific primer sets
M: Lambda phage DNA digested with *Hind*III (molecular weight marker).
a,b: Samples collected from mulberry plants with dwarf disease using the universal primer (1.37 kb band).
c,d: Samples collected from healthy mulberry plants using the universal primer.
e,f: Samples collected from mulberry plants with dwarf disease using the phytoplasma-specific primer (0.75 kb band).
g,h: Samples collected from healthy mulberry plants using the phytoplasma-specific primer.

band (1.37 kb or 0.75 kb) was observed in 2 out of 5 samples collected from diseased plants. The presence of PCR-negative samples may be caused by a smaller population of phytoplasmas in the mulberry phloem sap than in the rice phloem sap. No band was, however, observed for all the 5 samples collected from healthy plants (Plate 2).

3) RFLP and sequences analyses of PCR products

To confirm that the DNA fragments in the phloem sap amplified by PCR had originated from 16S rRNA genes of phytoplasmas, both PCR products from "phloem sap DNA" and phytoplasma DNA extracted from rice plants with yellow dwarf disease (RYD) used as a positive control were analyzed by restriction digestion and direct sequencing.

PCR products from the phloem sap were digested with restriction enzymes. The 1.37 kb DNA from PCR using the universal primer set was clearly digested with *Hinc*II, *Hha*I and *Hae*III, but not with *Sma*I and *Eco*RV. Two new fragments digested with

Plate 3. RFLP patterns of PCR products from DNA in rice phloem sap (A) and phytoplasma DNA extracted from diseased plants (B) amplified using the universal primer set
M: Molecular weight markers (λ *Hind*III).
a: Non-digested. b: *Eco*RV-digested.
c: *Hinc*II-digested. d: *Hha*I-digested.
e: *Hae*III-digested. f: *Sma*I-digested.
Arrows: Faint DNA bands.

*Hinc*II and *Hha*I, and 3 with *Hae*III were observed although the smallest DNA fragment (77 bases) was missing from the gel. No differences were found in the RFLP patterns between "phloem sap DNA" and RYD-phytoplasma using the universal primer set and the specific primer set (Plate 3).

The 1,370-nucleotide sequence of the PCR product from the "phloem sap DNA" was determined by direct sequencing. This nucleotide sequence was compared with the 16S rRNA signature oligonucleotides of RYD-phytoplasma reported by Namba et al.[8]. No difference was detected between the nucleotide sequences (data not shown). Similar RFLP analysis was performed for the PCR products from the mulberry phloem sap. The RFLP patterns of these DNA samples were similar to that of phytoplasma DNA isolated from mulberry plants with dwarf disease (MD), indicating that they originated from MD-phytoplasma DNA present in the phloem sap.

Discussion

Our results revealed that phytoplasmas in the phloem sap collected from diseased rice and

mulberry plants by laser stylectomy could be detected by the PCR assay. This is the first report of PCR detection of phytoplasma particles suspended in phloem sap isolated outside of plants. Although the detection method of phytoplasmas using the DAPI stain described previously[4] was very useful, the new detection method was superior in terms of reliability of phytoplasma identification for the following reason: several species of bacteria were detected sometimes in the phloem sap collected by laser stylectomy (our unpublished data), and were easily stained by DAPI, while the PCR using 2 sets of primers could distinguish phytoplasmas from these bacteria (data not shown).

Furthermore, our newly developed method is unique in terms of not requiring any special phytoplasma-DNA extraction procedures to prepare template DNA. It required, but not essentially, heating (95°C, 5 min) of diluted phloem sap including phytoplasma-DNA, whereas, conventional PCR detection methods of phytoplasmas require complicated DNA-extraction procedures to prepare template DNA. Our results also showed that the plant components of the phloem sap, at least from rice and mulberry, did not inhibit PCR amplification. Therefore, this system may be applicable to many kinds of plants if the phloem sap can be collected using laser stylectomy or other methods.

This new phytoplasma detection system may be useful for studies of *in vitro* cultivation of phytoplasmas, which is a very important subject and has not yet been possible. In laser stylectomy, phytoplasmas as an inoculum of primary culture are prepared in an ideal state, since phytoplasmas are suspended in pure phloem sap of "the natural medium" of phytoplasma without any inhibitors from other plant organs except for sieve cells. Then, the growth of phytoplasmas can be monitored sensitively and specifically by the PCR assay using 2 primer sets without any DNA extraction procedures.

References

1) Ahrens, U. & Seemuller, E. (1992): Detection of DNA of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology,* **82,** 828–832.
2) Deng, S. & Hiruki, C. (1991): Amplification of 16S rRNA genes from culturable and nonculturable *Mollicutes. J. Microbiol. Methods,* **14,** 53–61.
3) Firrao, G. et al. (1993): Use of polymerase chain reaction to produce oligonucleotide probes for mycoplasmalike organisms. *Phytopathology,* **83,** 602–606.
4) Kawabe, S. et al. (1991): Detection of mycoplasmalike organisms in phloem sap collected from rice plants with yellow dwarf disease. *Ann. Phytopathol. Soc. Jpn.,* **57,** 274–277.
5) Kawabe, S. et al. (1980): Collection of rice phloem sap from stylets of homopterous insects severed by YAG laser. *Plant Cell Physiol.,* **21,** 1319–1327.
6) Kawakita, H. & Katsuhara, M. (1993): Detection of mycoplasmalike organisms in the phloem sap collected from mulberry trees with dwarf disease. *J. Seric. Sci. Jpn.,* **62,** 125–131 [In Japanese].
7) Lee, I. M. et al. (1993): Universal amplification and analysis of pathogen 16S rRNA for classification and identification of mycoplasmalike organisms. *Phytopathology,* **83,** 834–842.
8) Namba, S. et al. (1993): Detection and differentiation of plant-pathogenic mycoplasmalike organisms using polymerase chain reaction. *Phytopathology,* **83,** 786–791.
9) Sambrook, J. et al. (1982): Molecular cloning: a laboratory manual (2nd ed.). Cold Spring Harbor Laboratory Press, New York.
10) Sato, M. et al. (1996): PCR (polymerase chain reaction) detection of phytoplasma in phloem sap collected by laser stylectomy. *Ann. Phytopathol. Soc. Jpn.,* **62,** 177–180.
11) Sato, M. et al. (1996): PCR detection of mulberry dwarf disease-phytoplasmas in mulberry tissues, phloem sap collected by laser stylectomy and insect vector *Hishimonus sellatus. J. Seric. Sci. Jpn.,* **65,** 352–358.
12) Schaff, D. et al. (1992): Sensitive detection and identification of mycoplasmalike organisms in plants by polymerase chain reactions. *Biochem. Biophys. Res. Commun.,* **186,** 1503–1509.

(Received for publication, January 8, 1997)