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Detection of the Paulownia Witches' Broom Mycoplasmalike Organism by Polymerase Chain Reaction

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ABSTRACT

A polymerase chain reaction (PCR) method with high sensitivity and specificity has been developed with a pair of oligonucleotide primers designed and synthesized according to the DNA sequence specific to paulownia witches' broom mycoplasmalike organism (PWB-MLO). Under optimum conditions, i. e. 2.5mM of Mg^{2+}, 3.0 units of Taq DNA polymerase in a 100μl PCR reaction mixture and annealing at 55℃, a unique 377-bp DNA fragment was amplified after 30 cycles from all field-grown, MLO-infected paulownia tissues with or without PWB symptom. No amplified DNA was detected under the same conditions in the samples from healthy paulowina, healthy and MLO-infected jujube and rhododendron plants. With the established PCR system, the PWB-MLO DNA from approximately 2pg of crude DNA from diseased paulownia petiole tissues could be detected. This method is suitable for practical diagnosis and customs quarantine.

Key words Paulownia witches' broom, Mycoplasmalike organism, polymerase chain reaction, Pathogen diagnosis.

Mycoplasma-like organisms (MLOs) have been demonstrated to be responsible for several hundred plant diseases worldwide (McCoy R E, Caudwell A, Chang C J, et al., 1989). However, MLOs have not been cultured in vitro, and the classification of these organisms has not been determined yet. In recent years, some progress has been made in the detection and identification using serological nad nucleic acid hybridization methods (Lin C P, Chen T A, 1985; Lin C P, Chen T A, 1986; Sinha R C, Benhamou N, 1983; Sinha R C, Chiykowski L N, 1986; Kirkpatrick B C, Stenger D C, Morris T J, et al., 1987). Recent studies suggest the utility of PCR as a diagnostic tool for MLO diseases (Deng S J, Hiruki C, 1990; Ahrens U, Seemüller E, 1992; Davis R E, Lee I-M, 1993; Schaff D A, Lee I-M, Davis R E, 1990), and it has been proved to be a sensitive and specific method.

In China, paulownia is a fast-growing tree that can provid good timber and keep better ecological environment. Unfortunately, witches' broom (WB), a disease associated with MLO, has become a limiting factor in all important paulownia planting areas of China. It was estimated that PWB only had caused ¥43.02 million losses nationwide according to the statistics in 1989 (Jiang Jianping, *1990*). *In* woody plants, the distribution of MLO is highly irregular, rapid, sensitive, and practical PWB-MLO

diagnosis methods are necessary to detect and control diseased plants. Here, we developed a DNA in vitro amplification method, PCR, to sensitively detect the PWB-MLO. Futhermore, the relationship between PWB-MLO and other two MLOs was also discussed.

MATERIALS AND METHODS

1 Plant materials

Field-grown paulownia tissues with or without typical witches' broom and phyllody were collected at Xiaohongshan, Wuhan, during May, 1993. 2-year old paulownia seedlings without WB-symptom were from our laboratory greenhouse. Paulownia seedlings of tissue culture were kindly provided by Chen Weilun, Institute of Botany, Academia Sinica. MLO infected jujube leaves and rhododendron plants were collected at Jinan, Shandong, and Lushan, Jiangxi, respectively. Healthy rhododendron and jujube were from Moshan, Wuhan and Suizhou, Hubei, respectively and used as controls.

2 DNA extractions

According to Lin Mulan, et al. (1994), about 1.0g of plant tissue (leaves, petioles or stems) was ground using a mortar and pestle in 2.0ml of STE buffer (100mM sodium chloride, 100mM Tris-Cl, 10mM EDTA, pH8.0, 2.0% SDS, and 0.5% 2-mercaptoethanol). When the tissue was grpund to fine particles, the sample was extracted once with an equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1). After centrifugation, nucleic acids were precipitated by adding two-thrids volume of isopropanol to the aqueous phase. The pellet was washed with 70% ethanol, air dried at 37℃, and dissolved in 300μl of distilled water. DNA concentration was determined at OD_{260} after digesting with 40μg/ml of RNaseA at 37℃ for 30min and extracting with chloroform/isoamyl alcohol, ethanol precipitating, and washing the pellet with ethanol. The obtained results were verified by comparing the DNA preparation with a diluted series of standard λ DNA by electrophoresis. DNA extracted from healthy plants by the same extraction procedure was used as the experimental control.

5′—AATTCTTATC AAATTAAAAT CTTTGTATAA GTGGTTAATT AATTGAGTGT

PrimerA→

CGTGATTAGT TAAAACCCAT TTAACTCCTC TTAAATGAGC TGCTTTTAAA
GATTCAAACA AATCTTTTTG GTTTGTTTGA TTAAAAGGGG ATGCATTATA
GCTGTTAAAA GTTTGAGTAT CTGAATCATA AGGAGGATCA CAAAACAATA
AATCATTAAT TTGTGTTTGA GCAACTATTG TTGCGTAATC TTGGTTTTGG
ATTTTAATTT CATTCTTTTT TAAGAATAAT GTGATATTTT TAAGATTAGA
TTCGTTAATG ATAGTTGATA AACAAACATC ATTTTTTCCA TTAAACGGAG
AGTTAAATTG ATTTTTAGAA TTAACACGAT AAAGACCATC AAAACAAGTT

←PrimerB

3′-CCTTATAATA GGTTGCTTCT-5′

TTATTAAGAA AAAAAAAGCG AGCAGCTTTT GGAATATTAT CCAACGAAGA
GGTTTTTGGG CCGCGCAATT GATAAT—3′

Fig. 1 Partial sequence of PWB-MLO DNA fragment in the clone pA4. Primer positions for PCR are indicated in boxed. (A+T)% in primerA and primerB are 55% and 65%, respectively.

3 DNA in vitro amplification

A pair of PCR primer was designed and synthesized according to the sequence specific to PWB-MLO DNA (Fig. 1) (Zhang Chunli, et al., 1993). The DNA extracts prepared as above were used as PCR templates. The amplification was carried out in a 50μl of PCR reaction mixture containing 5μl of test DNA extracrts, 1.0μM each of primer, 200μM of four dNTPs, Taq DNA polymerase buffer and 1.5mM of Mg^{2+}. Taq DNA polymerase, 4×dNTPs, Taq DNA polymerase buffer and $MgCl_2$ were all from S_{ABC}, China.

4 Gel electrophoresis

When the amplification was finished, 10μl of the product were electrophoresised in 2.0% horizontal agarose gels and visualized by staining with ethidium bromide and U. V. illumination.

RESULTS

1 Optimization of PCR

To determine the optimum conditions for the PCR, the annealling temperature (Tem.) and the concentrations of Mg^{2+} and Taq DNA polymerase were selected as the controlling factors.

The 50μl reaction mixture was first denatured at 94℃ for 5min, and then added 2.0 units of Taq DNA polymerase. The mixture was overlaid with 50μl of mineral oil and subjected to 30 cycles under the following incubations: 45s of denaturation at 94℃, 55s of annealing at different temperatures (55℃, 50℃, 40℃) respectively and 60s of elongation at 72℃. In the last cycle, the extension step was 5min at 72℃. The results were illustrated as in Fig. 2A, a unique 377-bp fragment was amplified from crude DNA of PWB-MLO infected paulownia at all the annealing temperatures tested, but no DNA was amplified from healthy samples under the same conditions. Although the PCR can be undertaken at the anneling of 40℃～55℃, 55℃ was chosen as the optimum annealing temperature, because higher annealling temperature can be used to further improve the specificity of the PCR primer and shorten the time for PCR cycles.

PCRs were then undertaken at the concentrations of Mg^{2+} from 0.0mM～3.0mM and with other conditions above unchanged. The results were shown on Fig. 2B. The optimum concentration of Mg^{2+} was 2.5mM.

Under the optimum concentration of Mg^{2+} and annealing temperature, different quantity of Taq DNA polymerase was added to a 100μl reaction mixture and subjected to amplification. Fig. 2C showed that the maximum amount of amplified DNA was obtained with the concentration of Taq DNA polymerase at 3.0 units/100μl reaction mixture.

2 Detection sensitivity by PCR

The crude DNA preparation from MLO infected paulownia petioles was diluted and subjected to 35 PCR cycles. 20μl of product was electrophoresised. An amplified MLO DNA band can be clearly observed under U. V. light until a dilution of 10^{-6} (Fig. 3). Because the approximate DNA concentration of the undiluted extracts used for amplification was 0.4μg/μl, the DNA content in the final dilution was 2pg.

3 Detection of MLO in plants by PCR

Using the established PCR system, many samples were detected (Fig. 4 and Fig. 5). Specific DNA fragments were also amplified in some of the crude DNA samples extracted from paulownia without PWB-symptom, which indicated that these paulownia trees were infected by MLO, but the symptoms had not been developed yet. The weak band in the agarose gel suggested that the concentration of MLO in these plants was very low.

In our previous studies, we have found that the PWB-MLO DNA has high homology with that of jujube witches' broom (JWB) MLO and rhododendron witches' broom (RWB) MLO, which was determined by nucleic acid hybridization using pA4 as DNA probe (unpublished results). However, data from Fig. 4 showed that the PCR system using the synthesized primer pair specifically amplified PWB-MLO DNA in vitro, but no amplified DNA fragment was obtained when using JWB-and RWB-MLO DNA as templates.

DISCUSSION

It has been proved that lower MLO concentration is found in woody plants than in herbaceous plants, and is unevenly distributed (Hiruki, 1988), which needs very sensitive method to be detected. Without enrichment of MLO, the PCR system we established can easily detect PWB-MLO in field-growing trees in the dilution of 10^{-6}, that is 2pg of DNA as templates. The sensitivity is lower than that Chen, et al., (1993) reported in the detection of GY-MLO in infected periwinkle, but higher than that Ahrens et al., (1992) reproted, which needed a enrichment procedure in diagnosis of ACLR-MLO in diseased perwinkle by PCR. The reason can be attributed to the higher MLO titers in herbaceous periwinkle and the different concentrations of different MLO in the same host plant. Another reason is that the methods for DNA extraction also have great effects on the detection sensitivity by PCR. Our results showed that the sensitivity using PCR was about 500 times as sensitive as that using DNA dot hybridization in the detection of PWB-MLO in paulownia (Lin, et al., 1994).

It was reported where the paulownia was severely affected by witches' broom, the jujube tree was also usually affected by witches' broom diseases (Jin, 1980). The results obtained by DNA dot hybridization with JWB-and RWB-MLO using pA4 (a clone from PWB-NLO) as probes also show that PWB-MLO has close relationship with JWB- and RWB-MLO (unpublished data). Are they the same MLO that infected different plants? By PCR system using DNA regions from pA4 as primer pair, however, no DNA fragment was amplified from JWB- and RWB-MLO DNA, which implies that the three kinds of NLOs are closely related, but not identical. This result provides useful data for the classification of MLOs.

From the beginning of DNA preparation to the obtaining of detection results, about 6 hours are needed for 24 samples by using PCR. Much less time is consumed using this method than that using dot hybridization which needs about 2 days or more. The PCR detection system is especially practical and necessary in the quality control in the production of MLO-free paulownia cultures.

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Explanation of plates (Cover Page 3)

Upper (Fig. 2) Optimization of PCR in the detection of PWB-MLO DNA. Annealing Tem. (Fig. 2A), and the concentrations of Mg^{2+} (Fig. 2B) and Taq polymerase (Fig. 2C) were selected as the control factors. Nucleic acids extracted from healthy (H) and MLO-diseased (D) paulownia plants were used as templates.

Middle left (Fig. 3) 2.0% agarose gel electrophoresis of PCR products obtained after 35 cycles from 10-fold dilution series of crude DNA extracted from PWB-diseased paulownia petioles.

Middle right (Fig. 4) Specificity of primers for detecting PWB-MLO by PCR. Crude DNA extracted from healthy (H) and MLO-infected (D) following plants were used as templates: paulownia (P), jujube (J) and rhododendron (R). M. pGEM-3Zf(+)/Hae Ⅲ marker.

Lower (Fig. 5) Partial results of detection of PWB-MLO in paulownia plants by PCR. Crude DNA extracted from field-grown paulownia plants with or without WB symptom (1～10, 14), paulownia seedlings in green house (11～13) and paulownia tissue cultures (15～21) were used as PCR templates. M. pGEM-3Zf(+)/Hae Ⅲ marker.