Evaluation of Primer Pairs for the Reliable Diagnosis of Paulownia Witches'-Broom Disease Using a Polymerase Chain Reaction

H. Nakamura, N. Yoshikawa, and **T. Takahashi,** Faculty of Agriculture, Iwate University, Ueda 3-chome, Morioka 020, Japan; **N. Sahashi, T. Kubono,** and **T. Shoji,** Tohoku Research Center, Forestry and Forest Products Research Institute, Shimokuriyagawa, Morioka 020-01, Japan

ABSTRACT

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To establish a reliable method for detection of paulownia witches'-broom (PWB) phytoplasma by a polymerase chain reaction (PCR), several oligonucleotide primers that amplify ribosomal protein (rp) or 16S rRNA (rD) genes of PWB-phytoplasma were compared for their specificity and sensitivity in the amplification of PWB-specific fragments by PCR. Use of rp primer pairs for PCR resulted in amplified DNA fragments of expected sizes in samples from diseased leaves. A fragment was amplified from healthy samples using one primer pair (rp3/rp4), but the fragment size was different from disease-specific fragments. In contrast, all four rD primer pairs designed for the 16S rRNA gene amplified both PWB-specific DNA fragments from infected leaf samples and nonspecific fragments of the same size as PWB-specific fragments from healthy samples. Using the rp3/rp4 primer pair, we amplified a PWB-specific DNA fragment from 150 pg of nucleic acid samples. This method allowed detection at the 95% confidence level of PWB-phytoplasma from paulownia leaves from the ten asymptomatic infected trees used in this study when at least 3 leaves randomly collected in September were used.

Paulownia witches'-broom (PWB) is one of the first plant diseases reported to be caused by mycoplasma-like organisms (3) (now referred to as phytoplasmas) belonging to the class *Mollicutes* (16). PWB is a lethal disease and a serious problem in the production of paulownia (*Paulownia tomentosa* (Thunb.) Steud.) trees in Japan, Korea, China, and Taiwan (15). PWB phytoplasma is reportedly transmitted by the stink bugs *Halyomorpha mista* Uhler and *H. holys* Stal. (12,15). Infected paulownia trees are characterized by the proliferation of branches with very small yellowish leaves, i.e., witches'-brooms, followed by the dieback of branches (15). These symptoms appear first in one part of the tree, then throughout the canopy, and finally the tree dies. However, in the Tohoku District of northern Japan, which is a main production area of paulownia trees, infected paulownia do not show the typical witches'-broom. The symptoms consist of malformed flower buds with abnormally elongated calyxes and dieback of branches (15). Leaves on infected trees also do not usually show obvious symptoms. The absence of typical witches'-broom symptoms delayed the identification of the causal agent of the decline of paulownia trees in the northern part of Japan for a long time and still makes it difficult to diagnose the disease by symptom observation. For the study of the epidemiology and control of PWB, the availability of a rapid and reliable detection method is a prerequisite. Recently, we reported the amplification of ribosomal protein and 16S rRNA genes from PWB using a polymerase chain reaction (PCR) and the possibility of its use for rapid, sensitive detection of PWB (17). We sometimes encountered the amplification of nonspecific DNA fragments from healthy plants with the same size as PWB-specific fragments using a primer pair for 16S rRNA even under controlled PCR conditions, although the amount of the product was very small and the restriction fragment patterns clearly distinguished the nonspecific products from the PWB-specific ones. The amplification of nonspecific products could give misleading diagnostic results even though the amount of the amplified products is very small. In this paper, we used several primer pairs to amplify fragments of ribosomal protein or 16S rRNA genes of PWB and evaluated their specificity and sensitivity in the amplification of PWB-specific fragments by PCR. We also applied the method to detect PWB in field samples collected at different seasons.

Corresponding author: N. Yoshikawa
E-mail: yoshikawa@msv.cc.iwate-u.ac.jp

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MATERIALS AND METHODS

Plants. PWB-infected paulownia trees (10 to 20 years old) maintained at the Faculty of Agriculture, Iwate University, were used in this study. One-year paulownia seedlings were also used as PWB-free materials. Leaf and flower bud samples were stored at –30°C until DNA was extracted.

DNA extraction. Total DNA was extracted from midribs (0.1 g) of leaves from diseased and healthy paulownia trees as described previously (17). DNA was also extracted from calyxes (0.1 g) of flowers from diseased trees. The nucleic acids were suspended in 100 µl of distilled water.

Primers and PCR amplification. Eight PCR primers were synthesized on the basis of the nucleotide sequences of ribosomal protein and 16S rRNA genes of PWB and several other phytoplasmas, four oligonucleotide primers for amplification of ribosomal protein genes (rp1, rp2, rp3, and rp4) and four primers for the 16S rRNA gene (rD1, rD2, rD3, and rD4) (8,10,11, 17). The locations and sequences of primers used in this study are shown in Figure 1 and Table 1. PCR amplifications were performed as follows. The 40-µl reaction mixtures contained 2 µl of nucleic acid samples (50 to 150 ng), 4 µl of primer mixture (5 µM each), 4 µl of dNTPs (1 mM each), 0.2 µl of *Taq* DNA polymerase (5 units/µl, Amplitaq DNA polymerase, Perkin-Elmer Cetus, Norwalk, CT), 4 µl of 10× *Taq* polymerase buffer (100 mM Tris, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin) and 25.8 µl of distilled water. The mixtures were covered with 30 µl of mineral oil and 30 reaction cycles were performed with the program (95°C, 30 s; 50 or 45°C, 1 min; 72°C, 30 s for rp primers and 95°C, 30 s; 65 or 60°C, 1 min; 72°C, 30 s for rD primers). Aliquots (8 µl) of the reaction mixtures were analyzed by electrophoresis on 1% agarose gels in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4).

Sampling of materials for the detection of PWB. Ten diseased paulownia trees (10 to 20 years old) maintained in Iwate University were used in this study. A single symptomless leaf was randomly collected from each of six different branches from each tree in June and September, 1994.

RESULTS

Specificities and sensitivities of primer pairs for the detection of PWB by PCR. Results on the specificity and sensi-

tivity in the amplification of PWB-specific fragments using several primer pairs are summarized in Table 2. Use of three rp primer pairs (rp1/rp2, rp1/rp4, and rp3/rp2) amplified DNA products with expected sizes in samples from infected leaves, but not from healthy leaves using both low (45°C) and high (50°C) annealing temperatures (Table 2 and Fig. 2A). Primer pair rp3/rp4 was expected to amplify a 0.75-kbp fragment, and a disease-specific DNA fragment (0.75 kbp) was amplified in samples from infected leaves. Interestingly, in healthy control samples, a 0.6-kbp DNA fragment that was smaller than that found in infected sample was also amplified under both low and high annealing conditions (Table 2 and Fig. 2A). Disease-specific DNA (0.75 kbp) and nonspecific DNA (0.6 kbp) fragments were sometimes amplified simultaneously in samples from infected leaves (Fig. 3, lane 4). In contrast, using four combinations of rD primers (rD1/rD4, rD1/rD2, rD3/rD4, and rD3/rD2), DNA products of expected sizes were amplified in samples from both infected and healthy control leaves under low annealing stringency conditions at 60°C (Table 2 and Fig. 2B). Under high annealing stringency conditions at 65°C, two primer pairs (rD1/rD2 and rD3/rD4) did not amplify nonspecific DNA from healthy control leaves. The amount of DNA product amplified using the rD1/rD2 primer pair was larger than that using the rD3/rD4 primer pair (Table 2 and Fig. 2B). To select the most effective primer pair to amplify a PWB-specific DNA fragment, a nucleic acid preparation from infected leaves was serially diluted and subjected to PCR using the three primer pairs (rp3/rp2, rp3/rp4, and rD1/rD2) that had been found to amplify larger amounts of products than the other primer pairs. As shown in Table 2, PWB-specific products were amplified from 150 pg of nucleic acid samples for rp3/rp4 or rD1/rD2 primer pairs and from 750 pg of DNA for the rp3/rp2 primer pair. Consequently, we selected the rp3/rp4 primer pair to amplify PWB-specific DNA fragments in later experiments because nonspecific fragments (0.6 kbp) could be easily differentiated from PWB-specific DNA (0.75 kbp) on the basis of size in amplified test samples. In the case of the rD1/rD2 primer pair, it is possible that nonspecific fragments with the same size as PWB-specific fragments were amplified.

Detection of PWB-phytoplasma by PCR from leaves collected at different seasons. To establish the conditions for practical, reliable diagnosis of PWB, we determined the optimal number of leaf samples needed for testing and the best season for collecting. For leaf samples randomly collected in June, Table 3 shows that it was necessary to test at least 13 leaves from an infected tree in order to detect PWB at the 95% confidence level.

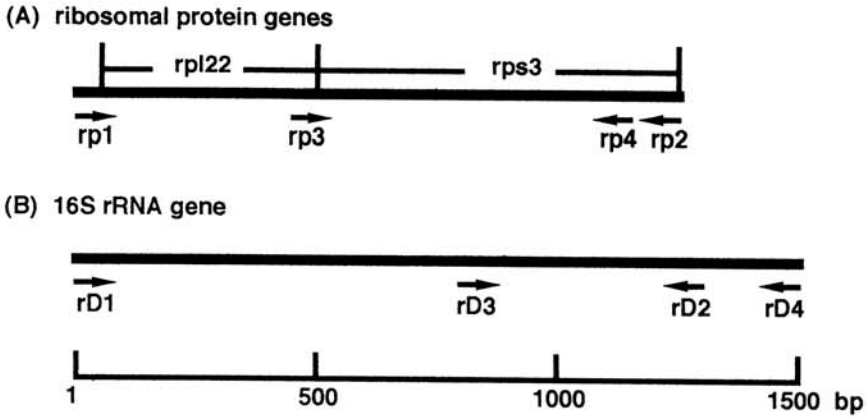

Fig. 1. Locations of primers used in this study for the polymerase chain reaction amplification of **(A)** ribosomal protein and **(B)** 16S rRNA genes of paulownia witches'-broom phytoplasma.

Table 1. Primers used for polymerase chain reaction amplification of ribosomal protein (rp) and 16S rRNA (rD) genes of paulownia witches'-broom phytoplasma

Primer[a]	Sequence (5′ to 3′)	Length (mer)	T_m (C)[b]
rp primers			
rp1	GGACATAAGTTAGGTGAATT	20	54
rp2	CGATATTTAGTTCTTTTTGG	20	52
rp3	AACTTCTAGCACAAACTTGC	20	56
rp4	GTCTGTTAGGAGTGTTAGAA	20	56
rD primers			
rD1	AGAGTTTGATCCTGGCTCAG	20	60
rD2	GAAGTCGAGTTGCAGACTTC	20	60
rD3	ACGAAAGCGTGGGGAGCAAA	20	62
rD4	GGTAGGGATACCTTGTTACG	20	60

[a] The base locations of four rp primers in the ribosomal protein genes of PWB-phytoplasma (17) are as follows: rp1 (base 1 to 20); rp2 (complementary to base 1220 to 1239); rp3 (base 431 to 450); and rp4 (complementary to base 1158 to 1177). The base locations of four rD primers in the 16S ribosomal RNA genes of the O-MLO (10) are as follows: rD1 (base 7 to 26); rD2 (complementary to base 1297 to 1316); rD3 (base 759 to 778); and rD4 (complementary to base 1489 to 1508).

[b] Each T_m was estimated by multiplying the number of A + T in the sequence of each primer residues by 2°C and the number of G + C residues by 4°C and adding the two numbers.

Table 2. Summary of the results from polymerase chain reaction (PCR) amplifications of ribosomal protein (rp) and 16S rRNA (rD) genes of paulownia witches'-broom phytoplasma using several primer pairs

Primer pairs	Amplified DNA size (kbp)	Annealing temperature (C)	Disease-specific products	Nonspecific products	Dilution end point
rp primer pairs					
rp1/rp2	1.2	50	+[a]	–	NT[b]
		45	+	–	NT
rp1/rp4	1.15	50	±	–	NT
		45	+	–	NT
rp3/rp2	0.8	50	+	–	5^{-4}
		45	+	-	NT
rp3/rp4	0.75	50	++	±[c]	5^{-5}
		45	++	+[c]	NT
rD primer pairs					
rD1/rD4	1.5	65	+	±	NT
		60	++	++	NT
rD1/rD2	1.3	65	++	–	5^{-5}
		60	++	±	NT
rD3/rD4	0.75	65	±	–	NT
		60	+	+	NT
rD3/rD2	0.55	65	++	±	NT
		60	++	+	NT

[a] ++, a large amount of PCR product was amplified; +, a small amount of product was amplified; ±, a very small amount of product was occasionally amplified; –, a product was not amplified.

[b] Not tested.

[c] The size of product was approximately 0.6 kbp.

This may be due to the uneven distribution and low concentration of PWB phytoplasma in trees. The frequency of detection increased in leaf samples collected in September (Table 3) allowed PWB detection from a sample containing 3 leaves per tree at the 95% confidence level. The amounts of DNA amplified from samples collected in September were larger than those from samples collected in June (Fig. 3). The rp3/ rp4 primer pair may be used to detect PWB from insect vectors. No nonspecific fragments with the same size as PWB-specific fragments were amplified from a stink bug (*Halyomorpha mista* Uhler) or leafhopper (*Macrosteles orientalis* Virbaste). Nonspecific products were amplified from these insects using rD primer pairs.

Malformed flower buds that develop abnormally elongated calyxes are thought to be one of the symptoms of PWB. Malformed and round normal flower buds were collected from infected trees and tested by PCR. The results revealed that a PWB-specific DNA fragment was amplified in all samples from calyxes of malformed flower buds (30 samples), but never from normally shaped flower buds (61 samples).

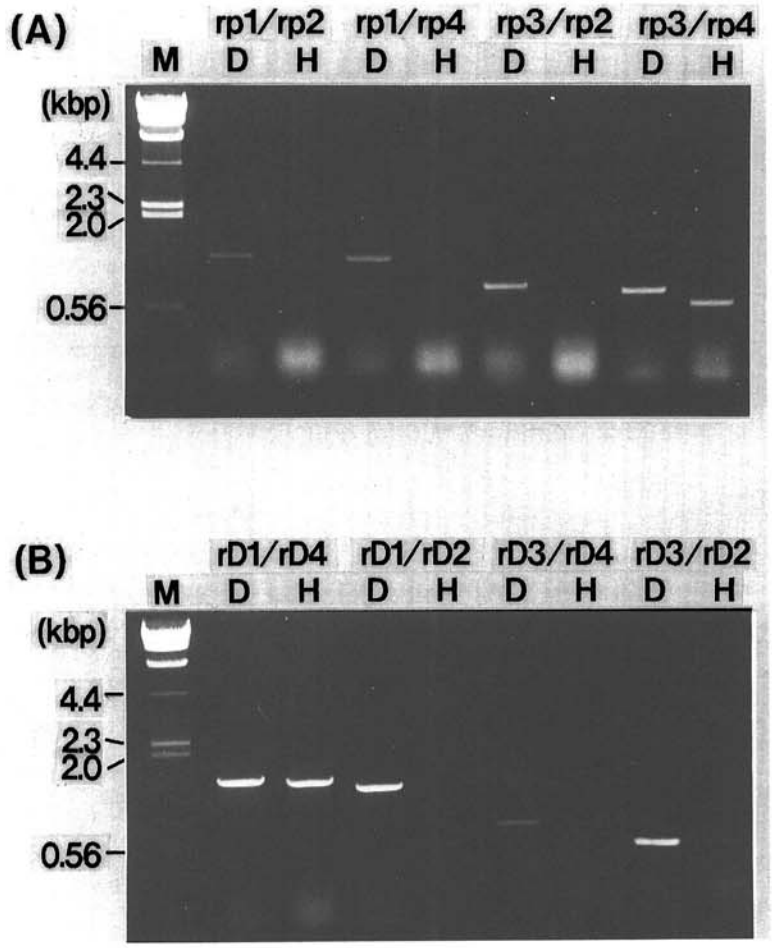

Fig. 2. Agarose gel electrophoresis of DNAs amplified by a polymerase chain reaction (PCR) from diseased (D) and healthy (H) leaf samples using several primer pairs for **(A)** ribosomal protein and **(B)** 16S rRNA genes of paulownia witches'-broom phytoplasma. PCR was conducted at annealing temperatures of **(A)** 50°C or **(B)** 60°C. Lane M, lamda DNA digested with *Hin*dIII as a size marker.

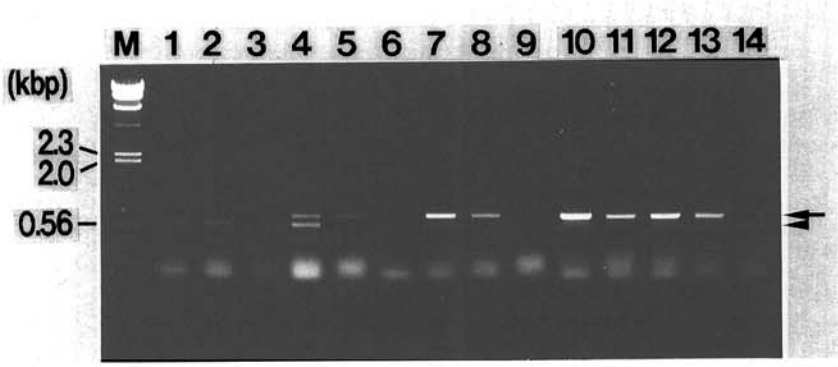

Fig. 3. Detection of paulownia witches'-broom (PWB) phytoplasma from diseased leaf samples collected in June (lanes 1 to 6) and September (lanes 7 to 12) by a polymerase chain reaction using rp3/rp4 primer pairs. Lane M, lamda DNA digested with *Hin*dIII; lane 13 and 14, infected and healthy control samples, respectively. An arrow and an arrowhead indicate PWB-specific and nonspecific DNA fragments, respectively.

DISCUSSION

PCR amplification of the 16S rRNA gene using oligonucleotide primers has been used to detect several plant-pathogenic phytoplasmas (1,2,5,9,12). However, the amplification of nonspecific fragments from healthy plants while testing for PWB and other phytoplasmas by PCR using primers for the 16S rRNA gene (1,2) has been a confounding problem. This may be due to the amplification by the 16S rRNA gene of chloroplast DNA in host plants or the presence of contaminating mollicutes from plant surfaces, as discussed elsewhere (1,2). In practical diagnosis, the absence of such nonspecific amplification is critical to obtaining reliable results. Our results indicate that the primer pairs we used for the amplification of the 16S rRNA gene generally allowed the amplification of nonspecific fragments from healthy control samples under both low and high annealing temperatures. Primers for ribosomal protein genes did not cause the nonspecific amplification. The sequences of rRNA may have base-pairing constraints that slow the diversity of rRNA, compared with the primary sequences of ribosomal protein genes (6,11). One primer pair (rp3/rp4) out of the four tested for ribosomal protein genes also allowed the amplification of nonspecific fragments, but the size of the product (0.6 kbp) was different from the PWB-specific

Table 3. Detection of paulownia witches'-broom phytoplasma from leaves of diseased paulownia trees[a]

	Month of collection (1994)	
Tree no.	June	September
1	2/6[b]	5/6
2	1/6	3/6
3	1/6	6/6
4	1/6	3/6
5	2/6	6/6
6	1/6	4/6
7	1/6	3/6
8	2/6	3/6
9	1/6	5/6
10	1/6	5/6

[a] Polymerase chain reaction was conducted using rp3/rp4 primer pairs under an annealing condition at 50°C.

[b] Number of leaf samples from which phytoplasma was detected / number of leaf samples tested.

fragment (0.75 kbp). Sensitivity using this primer pair is higher than that using other primer pairs for ribosomal protein genes, and PWB-specific fragments were detected in infected leaf samples from 150 pg of total DNA. Accordingly, we selected the rp3/rp4 primer pair for the detection of PWB from field samples.

Phytoplasmas are reported to be distributed unevenly in host plants, especially in woody plants, and their concentration changes seasonally (4,7,13,14). Frequency of PWB detection from leaf samples collected at random was higher in September than in June, indicating that PWB-phytoplasma was distributed more widely in the tree and/or the titer increased by September. From the 10 infected trees used in this study, we conclude that in June at least 13 leaves and in September 3 leaves should be collected from an infected tree to get positive results with a confidence level of 95%. These results indicate that the method is useful for the detection of PWB phytoplasma from asymptomatic paulownia leaves. The method was successfully applied to the detection of PWB phytoplasma from paulownia leaf samples collected from several fields located in Iwate, Akita, and Fukushima prefectures (data not shown). The malformation of flower buds was indirectly confirmed as a symptom of PWB. In the northern part of Japan, this floral symptom will be useful for diagnosis of PWB by symptom observation at the appropriate season. PCR using the rp3/rp4 primer pair described here is a rapid and reliable diagnostic method for detecting PWB from infected leaves and an insect vector, and may be of value in studying the epidemiology of the disease.

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