Two Groups of Phytoplasmas from Japan Distinguished on the Basis of Amplification and Restriction Analysis of 16S rDNA

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ABSTRACT

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Phytoplasmas (mycoplasma-like organisms, MLOs) associated with mitsuba (Japanese honewort) witches'-broom (JHW), garland chrysanthemum witches'-broom (GCW), eggplant dwarf (ED), tomato yellows (TY), marguerite yellows (MY), gentian witches'-broom (GW), and tsuwabuki witches'-broom (TW) in Japan were investigated by polymerase chain reaction (PCR) amplification of DNA and restriction enzyme analysis of PCR products. The phytoplasmas could be separated into two groups, one containing strains JHW, GCW, ED, TY, and MY, and the other containing strains GW and TW, corresponding to two groups previously recognized on the basis of transmission by *Macrostes striifrons* and *Scleroracrus flavovictus*, respectively. The strains transmitted by *M. striifrons* were classified in 16S rRNA gene group 16SrI, which contains aster yellows and related phytoplasma strains. Strains GW and TW were classified in group 16SrIII, which contains phytoplasmas associated with peach X-disease, clover yellow edge, and related phytoplasmas. Digestion of amplified 16S rDNA with *HpaII* indicated that strains GW and TW were affiliated with subgroup 16SrIII-B, which contains clover yellow edge phytoplasma. All seven strains were distinguished from other phytoplasmas, including those associated with clover proliferation, ash yellows, elm yellows, and beet leafhopper-transmitted virescence in North America, and Malaysian periwinkle yellows and sweet potato witches'-broom in Asia.

Numerous vegetable, ornamental, and medicinal plants in Japan are affected by diseases attributed to phytoplasmas (formerly mycoplasma-like organisms, MLOs) (8-12,16-21,23-25). The phytoplasmas in a number of these diseases are known to be transmitted either by *Macrostes striifrons* Anufriev (= *M. orientalis* Virvaste) or *Scleroracrus flavovictus* Ishihara. Two groups of phytoplasma diseases have been recognized on the basis of these transmission characteristics (24,25). The plant host ranges for both groups are very wide, and some plant species serve as hosts for phytoplasma strains in both groups, necessitating experimental insect transmission trials to determine the group affiliation of the phytoplasmas (8,10,12,18-21,23-25).

Phytoplasmas transmitted by *M. striifrons*, but not those transmitted by *S. fla-*

vovictus, were considered to be strains of aster yellows phytoplasma. This interpretation was supported by the work of Shiomi and Sugiura (24,25), who found that the North American aster yellows vector, *M. fascifrons* Stal, was capable of transmitting several Japanese phytoplasmas, including the *M. striifrons*-transmitted mitsuba (Japanese honewort) witches'-broom phytoplasma, and that *M. striifrons* transmitted American aster yellows phytoplasma. The purpose of the present study was to compare the Japanese phytoplasmas with one another and with other Asian phytoplasmas and American strains, including aster yellows phytoplasma, by polymerase chain reaction (PCR) amplification of DNA and restriction analysis of PCR products.

MATERIALS AND METHODS

Phytoplasma strains and sources.

Seven phytoplasma strains were experimentally transmitted by leafhopper vectors from naturally infected plants growing in Japan to plants of *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle) maintained in a greenhouse in Tsukuba, Japan. The strains and their original field-infected host plants and associated diseases are given in Table 1. Other phytoplasmas, used

as reference strains in DNA amplification and restriction analyses in this study, were maintained by grafting in a greenhouse-grown white-flowered clonal line of periwinkle at Beltsville, Maryland. As controls, we included in the study reference phytoplasmas representing genomic strain clusters and groups previously described (3,6,13,14). These phytoplasmas included Maryland aster yellows (AY) phytoplasma field-collected from a naturally inoculated plant of periwinkle. The remaining phytoplasmas were in singly infected plants of periwinkle unless otherwise indicated: tomato big bud (BB), clover phyllody (CPh), Canada X-disease (CX), clover yellow edge (CYE) (in Ladino clover), clover proliferation (CP), beet leafhopper-transmitted virescence (VR), ash yellows (AshY1), elm yellows (EY1), goldenrod yellows (GR1), milkweed yellows (MW1), spirea stunt (SP1), northern Italy grapevine phytoplasma (FDU), Malaysian periwinkle yellows (MPY), and sweet potato witches'-broom (SPWB).

Polymerase chain reactions. Samples (2 g) of leaves were harvested separately from healthy plants or from plants singly infected by phytoplasmas. Total nucleic acid was extracted from each sample by a previously described modification (14) of the procedure of Ausubel et al. (1). The extracted nucleic acid was quantified by spectrophotometry and by agarose gel electrophoresis. Each PCR was carried out as previously described (2,14) using one of the following pairs of oligodeoxyribonucleotide primers in a reaction mixture. Sequences for primer pairs R16F2/R16R2 (F2/R2) (14), r16F4/r16R1 (F4/R1) (2), and 16R758F/16R1232R (758F/1232R) (5) were derived from a published (15) sequence of the 16S rRNA gene of an *Oenothera*-infecting phytoplasma. Primer pair G35pm was derived from the nucleotide sequence of a cloned fragment of DNA from the Italian periwinkle virescence phytoplasma (4). Each PCR mixture consisted of a total of 50 µl that contained 25 ng of total nucleic acid extracted from plants plus 20 pmol of each primer of a pair, 1.25 units of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA), and a final concentration of 0.2 mM each of dATP, dCTP, dGTP, dTTP, 10 mM Tris-

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HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, and 0.1 mg of gelatin per ml. The reaction mix was overlaid with 25 µl of mineral oil. After an initial 2-min incubation at 94°C, the following conditions were carried out

for thirty-five cycles: 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. Following the thirty-five cycles, a final extension step of 7 min at 72°C was carried out. Five µl of reaction mixture was electrophoresed in

a 1% agarose gel in 1× TAE (0.04 M Tris acetate, pH 8.0, and 0.002 M EDTA) to analyze the PCR products.

Restriction analysis of PCR products.

Restriction analysis of PCR-amplified 16S rDNA, used previously as a rapid means to classify phytoplasma strains into groups (14,16,22), was used. Five µl of each reaction mix following PCR with primer pair 758F/1232R or primer pair F2/R2 was digested singly with 5 units of the following enzymes: *AluI* (Boehringer Mannheim, Indianapolis, IN), *MseI*, *KpnI*, *HhaI*, and *HpaII* (Sigma Chemical Co., St. Louis, MO) using the manufacturers' recommended reaction conditions with the addition of spermidine (Sigma) to a final concentration of 4 mM in a total reaction mixture of 20 µl. Digests were analyzed by electrophoresis of the 20-µl reaction mixture in a 5% polyacrylamide gel in 0.5× TBE (1× = 0.089 M Tris borate, 0.089 M boric acid, and 0.002 M EDTA). After electrophoresis, gels were stained with ethidium bromide and examined under UV transillumination. Phytoplasma group affiliations were assigned to 16S rDNA restriction fragment length polymorphism (RFLP) groups previously described (14).

RESULTS AND DISCUSSION

Results from PCR amplification of DNA sequences using primer pair F4/R1, primer pair G35pm, primer pair 758F/1232R, and primer pair F2/R2 are summarized in Table 2. Repeated experiments gave the same results. No DNA amplification was observed in reaction mixtures containing any primer pair when template consisted of DNA from healthy plants. Amplification of a DNA sequence of about 1.25 kbp was observed in reaction mixtures containing primer pair G35pm when template consisted of DNA from plants infected by AY phytoplasma, but no DNA amplification was observed when template consisted of DNA extracted from healthy plants or from plants infected by BB, CPh, VR, EY1, AshY1, CX, CYE, SPWB, GR1, MW1, SP1, FDU, or CP phytoplasmas, or by any of the seven phytoplasma strains from Japan. With primer pair F4/R1, amplification of a DNA sequence of about 700 bp was observed when template consisted of DNA extracted from plants infected by phytoplasma strains JHW, GCW, ED, TY, and MY from Japan, or by control strains AY, BB, and CPh; but no amplification was observed when template consisted of DNA from plants infected by GW, TW, EY1, VR, CP, AshY1, CYE, SPWB, GR1, MW1, SP1, FDU, MPY, or CX phytoplasmas. When the PCR was carried out using primer pair 758F/1232R or primer pair F2/R2, a DNA sequence of approximately 490 bp or 1.2 kbp, respectively, was amplified when template consisted of DNA extracted from plants infected by any of the Japanese phytoplasmas or by any of the reference phytoplasmas.

Table 1. Source host plants, associated diseases, and geographical origins of phytoplasmas investigated in this study^a

Phytoplasma designation	Host plant of origin ^b	Associated disease	Geographic origin ^c	Reference
Strains transmitted by <i>Macrosteles striifrons</i> (<i>orientalis</i>)				
JHW	<i>Cryptotaenia japonica</i> Hassk. (<i>Umbelliferae</i>) [Japanese honewort]	Mitsuba witches'-broom	Yawahara, Ibaraki	19,21
GCW	<i>Chrysanthemum coronarium</i> L. (<i>Compositae</i>)	Garland chrysanthemum witches'-broom	Furukawa, Miyagi	20, Kato et al. (unpubl.)
ED	<i>Solanum melongena</i> L. (<i>Solanaceae</i>)	Eggplant dwarf	Toyohashi, Aichi	8
TY	<i>Lycopersicon esculentum</i> L. (<i>Solanaceae</i>)	Tomato yellows	Geihoku, Hiroshima	10
MY	<i>Chrysanthemum frutescens</i> L.	Marguerite yellows	Tateyama, Chiba	11
Strains transmitted by <i>Scleroracis flavopictus</i>				
GW	<i>Gentiana</i> sp. (<i>Gentianaceae</i>)	Gentian witches'-broom	Myokokogen, Niigata	12,20,23
TW	<i>Farfugium japonicum</i> Kitamura (<i>Compositae</i>)	Tsuwabuki witches'-broom	Nichinan, Miyazaki	9

^a The phytoplasmas investigated were transmitted by *Macrosteles striifrons* (*orientalis*) or *Scleroracis flavopictus* in Japan.

^b Naturally infected field-collected host plant. Common name of plant in brackets. Names in parentheses are families of the host plants.

^c Geographical locations where phytoplasmas used in the present study were originally collected in the naturally infected host plants.

Table 2. Polymerase chain reaction amplification of DNA sequences from plants of *Catharanthus roseus* (periwinkle) infected by phytoplasmas

Phytoplasma ^a	DNA amplification with primer pair indicated ^b			
	F4/R1	G35p/m	758F/1232R	F2/R2
Japanese phytoplasmas				
JHW	+	—	+	+
GCW	+	—	+	+
ED	+	—	+	+
TY	+	—	+	+
MY	+	—	+	+
GW	+	—	+	+
TW	+	—	+	+
Reference phytoplasmas				
AY	+	+	+	+
BB	+	—	+	+
CPh	+	+	+	+
CP	—	—	+	+
AshY1	—	—	+	+
CX	—	—	+	+
CYE	—	—	+	+
VR	—	—	+	+
EY1	—	—	+	+
SPWB	—	—	+	+
MPY	ND	ND	+	+
FDU	—	—	+	+
GR1	—	—	+	+
MW1	—	—	+	+
SP1	—	—	+	+

^a Japanese phytoplasma designations are the same as those in Table 1. AY, Maryland aster yellows. BB, tomato big bud. CPh, clover phyllody. CP, clover proliferation. AshY1, ash yellows. CX, Canada X-disease. CYE, clover yellow edge. VR, beet leafhopper-transmitted virescence. EY1, elm yellows. SPWB, sweet potato witches'-broom. MPY, Malaysian periwinkle yellows. FDU, northern Italy grapevine phytoplasma. GR1, goldenrod yellows. MW1, milkweed yellows. SP1, spirea stunt.

^b +, amplification of phytoplasma-specific DNA of approximately 700 bp, 1.25 kbp, 490 bp, and 1.2 kbp in reaction mixtures containing primer pairs F4/R1, G35pm, 758F/1232R, and F2/R2, respectively. —, phytoplasma-specific DNA amplification not observed. ND, not determined.

Figure 1 shows results from restriction enzyme analysis of the 1.2-kbp 16S rDNA sequence amplified by PCR using primer pair F2/R2. The Japanese phytoplasma strains could be differentiated into two

groups on the basis of RFLPs when PCR products were digested with *AluI*, *MseI*, or *KpnI*. One pattern was exhibited by DNA from phytoplasma strains JHW, GCW, ED, TY, and MY, as well as reference strains

AY and BB; and the second pattern was exhibited by strains GW and TW, which was identical to that obtained with DNA from reference strain CX. Restriction analysis of the products from PCR primed by oligonucleotide pair 758F/1232R distinguished the same two groups (data not shown).

Since *AluI* and *MseI* restriction profiles of 16S rDNA from strains GW and TW were identical to phytoplasmas known to be affiliated with 16S rDNA group 16SrIII and the peach X-disease phytoplasma cluster (14), an additional experiment was carried out to determine possible subgroup affiliation of these Japanese phytoplasmas. Figure 2 shows results from *MseI* and *HpaII* restriction analysis of 16S rDNA amplified in PCR using primer pair F2/R2. Other phytoplasmas recently classified in group 16SrIII (6) were included for comparisons of RFLP patterns. Collective RFLP patterns of the 16S rDNA from GW and TW phytoplasmas were identical, or nearly so, to one another and to those of DNA from phytoplasma strains CYE, MW1, and FDU; but they were distinct from those of 16S rDNA from CX, GR1, and SP1 phytoplasmas (Fig. 2).

The Japanese phytoplasmas investigated in this study are associated with diseases that have been extensively researched (8–12,18–21,23–25). Symptoms observed in field-collected diseased plants and in experimentally infected periwinkle plants were typical of infections by phytoplasmas, and electron microscopy revealed the presence of phytoplasmas in phloem. Data obtained using molecular techniques in the present work are also consistent with the hypothesis that these diseases are caused by phytoplasmas. Amplification of 16S rDNA sequences in PCR is consistent with identification of the probable pathogens as phytoplasmas, since primer pairs F4/R1, 758F/1232R, and F2/R2 were derived from the published sequence (15) of 16S rDNA from a phytoplasma infecting *Oenothera* sp., and detection of phytoplasma DNA by use of these primers in PCR has been demonstrated in other work (2,5,14). The nature of the Japanese plant pathogens as phytoplasmas was further indicated by their similarity with other Asian or North American phytoplasmas in restriction patterns of amplified 16S rDNA.

Both cluster-specific DNA amplification and restriction analysis of PCR-amplified 16S rDNA indicated that two distinct groups could be distinguished among the seven Japanese phytoplasmas that we have examined. The present work is the first to document RFLP fingerprints of amplified 16S rDNA from these strains. The two strain groups that were distinguished coincided with phytoplasma groups previously designated on the basis of insect transmission (23–25). The complete correspondence between the groups based on molecular data or specificity of insect

Fig. 1. Restriction fragment length polymorphism analyses of 16S rDNA amplified in polymerase chain reactions (PCR) primed by oligonucleotide pair F2/R2. Polyacrylamide gel (5%) electrophoresis of PCR products digested with (A) *AluI*, (B) *MseI*, and (C) *KpnI*. JHW, mitsuba witches'-broom. GCW, garland chrysanthemum witches'-broom. ED, eggplant dwarf. TY, tomato yellows. MY, marguerite yellows. GW, gentian witches'-broom. TW, tsuwabuki witches'-broom. AY, Maryland aster yellows (=MDAY, AY1). BB, tomato big bud. CPh, clover phyllody. AshY1, ash yellows. CX, Canada X-disease. VR, beet leafhopper-transmitted virescence. EY, elm yellows (=EY1). CP, clover proliferation. SPWB, sweet potato witches'-broom. MPY, Malaysian periwinkle yellows. H, healthy periwinkle. S, size standard, phiX174 *HaeIII* fragments; sizes in bp.

transmission appears to favor the hypothesis that, in contrast to symptoms in diseased plants (13), characteristics of specific transmission by insects can be useful in phytoplasma classifications.

Previous studies indicated that some phytoplasmas occurring in Japan are strains of aster yellows phytoplasma (8,10,18,20,24,25). For example, the transmission of several Japanese phytoplasmas by *Macrosteles striifrons* (*orientalis*) (24,25) parallels the well-known transmission of aster yellows phytoplasma by *Macrosteles fascifrons*, vector of North American aster yellows phytoplasma. Shiomi and Sugiura (24,25) demonstrated that several Japanese phytoplasmas could also be transmitted by *M. fascifrons*, and that an American aster yellows phytoplasma strain could be transmitted by *M. striifrons*. Those findings led to the concept that aster yellows phytoplasma in North America and some phytoplasmas occurring in Japan are related strains (24,25). This concept is consistent with findings in our study. Since 16S rDNA from strains JHW, GCW, ED, TY, and MY yielded RFLP patterns that were identical to each other and typical of group 16SrI phytoplasmas, and since aster yellows strain cluster-specific oligonucleotide pair F4/R1 primed amplification of DNA from these strains, we consider the five Japanese phytoplasmas to be new members of 16SrRNA group 16SrI and the aster

yellows phytoplasma strain cluster. In a separate RFLP analysis using *HhaI* (data not shown), these strains were further identified as members of subgroup 16SrI-B. This conclusion is consistent with analysis of 16S rRNA gene nucleotide sequence data indicating strain TY to be a member of a phytoplasma group also containing paulownia witches'-broom phytoplasma (17), which belongs to group 16SrI-D (14).

GW and TW phytoplasmas are transmitted by *S. flavopictus* but not by *M. striifrons* (12,23). Thus, although these strains infect a range of plant species that is similar to the host range of the other five Japanese phytoplasma strains studied here, GW and TW do not appear to be strains of aster yellows phytoplasma on the basis of their insect transmission characteristics (9). Our results are consistent with the view that GW and TW are not strains of aster yellows phytoplasma. Instead, the data indicate that GW and TW phytoplasmas belong to the peach X-disease phytoplasma cluster and group 16SrIII, subgroup III-B.

Although this study distinguished molecular genetic differences between two groups of Japanese phytoplasmas previously separated on the basis of transmission by specific insect vectors, our work did not distinguish between subgroups of phytoplasmas as described by Shiomi and Sugiura (24), who distinguished subgroups among *M. striifrons*-transmitted phyto-

plasma strains on the basis of differential plant host ranges. While it is possible that analysis of highly conserved phytoplasma genes such as 16S rDNA provides clues to the identities of unknown insect vectors that may play a role in disease spread, study of polymorphisms in sequences less conserved than 16S rDNA will probably be necessary to reveal markers correlated with biological properties such as plant host range and type of symptom(s) induced in susceptible plant species.

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Fig. 2. Restriction fragment length polymorphism analyses of 16S rDNA amplified in polymerase chain reactions (PCR). Reaction mixtures contained primer pair F2/R2. Polyacrylamide gel (5%) electrophoresis of PCR products digested with *MseI* (left) and *HpaII* (right). CYE, clover yellow edge. FDU, northern Italy grapevine phytoplasma. SP1, spirea stunt. GR1, goldenrod yellows. MW1, milkweed yellows. Other abbreviations are the same as for Figure 1.

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