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Transovarial transmission of sugarcane white leaf phytoplasma in the insect vector *Matsumuratettix hiroglyphicus* (Matsumura)

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Abstract

White leaf is a serious disease of sugarcane caused by phytoplasma. The disease is transmitted to the plant by the leafhopper *Matsumuratettix hiroglyphicus* (Matsumura). The reservoir of phytoplasma was suspected to be weeds that grow in sugarcane farming areas because they can be infected with phytoplasma and show symptoms similar to sugarcane white leaf. However in previous work we have demonstrated by RFLP and sequencing that this is not the case. Here we have reared *M. hiroglyphicus* through two generations by feeding them phytoplasma free sugarcane grown from tissue culture. By nested-PCR followed by sequencing, we demonstrated the presence of the phytoplasma in eggs, nymphs and adults of the first and second generations thereby showing transovarial transmission. We have also shown by *in situ* PCR that phytoplasmas were widely distributed throughout the body of the insect. RFLP and sequencing showed that the same phytoplasma was present in the vector and in the plant. Together, these data point to the leafhopper *M. hiroglyphicus* as the reservoir of phytoplasma that cause sugarcane white leaf disease.

Keywords: *Matsumuratettix hiroglyphicus* (Matsumura), phytoplasma, sugarcane white leaf disease, transovarial transmission.

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Introduction

White leaf disease caused by phytoplasmas is among the most serious diseases of sugarcane, causing severe losses to the farmers of this important crop. The typical symptoms of sugarcane white leaf (SWL) disease are total chlorosis of the leaf and proliferation of tillers. It is known that phytoplasmas that cause plant diseases are transmitted and spread by insects, mainly leafhoppers and psyllids (Ploaie, 1981). It is also believed that the SWL phytoplasmas are transmitted by the leafhopper *Matsumuratettix hiroglyphicus* (Matsumura) (Matsumoto *et al*., 1968; Chen, 1974; Maramorosch *et al*., 1975). The evidence that *M. hiroglyphicus* is the disease vector derives from both laboratory experiments, which showed transmission of phytoplasma from insect to healthy plants, and epidemiological data. The identification of the reservoir of phytoplasma is also important for the management of the disease. It was believed that SWL disease is transmitted to the sugarcane by the insect from weeds that grow in sugarcane growing regions. Several kinds of gramineous weeds in the cane-growing areas such as bermuda grass (*Cynodon dactylon*), crowfoot grass (*Dactyloctenium aegyptium*) and brachiaria grass (*Brachiaria distachya*) can be infected with phytoplasmas and show the symptoms of white leaf disease (Chen *et al*., 1972; Rishi & Chen, 1989; Nakashima *et al*., 1994). However, we have shown by DNA sequencing that none of the phytoplasmas that infect the gramineous weeds was identical to that which causes SWL disease (Wongkaew *et al*., 1997) thereby excluding the gramineous weeds as the reservoir for SWL disease. Thus the reservoir of phytoplasma is either another as yet unidentified weed or the insect vector itself. The insect could be the reservoir if there is insect to insect transmission, for example through the egg. To test this hypothesis we have used *in situ* PCR and nested-PCR assays to detect and monitor the presence of SWL phytoplasma in the different life stages of insect vector *M. hiroglyphicus* through two generations.

Results

To determine whether there is transovorial transmission of phytoplasma in *M. hiroglyphicus*, we reared phytoplasma

infected insect vectors in the laboratory through two generations by feeding them healthy sugarcane. We used nested-PCR to amplify phytoplasma-specific DNA from first and second-generation adults, nymphs and eggs. Nested-PCR assays using primers MLO-X/MLO-Y followed by P1/P2 amplified a 210 bp fragment that includes part of the 16 S rRNA and part of the intergenic spacer region from twelve out of twenty founder adult *M. hiroglyphicus* templates (Fig. 1A). We were also able to detect the presence of the phytoplasma specific 210 bp DNA band in eggs, nymphal stages 1–5 (Fig. 1B) and adults of the second generation of the insect vector *M. hiroglyphicus*. Nymphs and adults were all reared on tissue culture grown disease free sugarcane plants that were shown to be phytoplasma free before insect rearing (Fig. 1C). No band was obtained from negative control DNA of the brown planthopper and from the water control. DNA template from the SWL plant used as a positive control produced the expected 210 bp specific band.

To further demonstrate the presence of phytoplasma in the insect and to describe its distribution, we carried out *in situ* PCR on *M. hiroglyphicus* sections using phytoplasma specific primers. *In situ* PCR showed a dark blue precipitate in sections from the insect vector *M. hiroglyphicus* (Fig. 2A) whereas no dark blue colour was obtained from *M. hiroglyphicus* when the PCR primers were omitted (Fig. 2B) or when sections from the brown planthoppers were used as negative controls (data not shown). Phytoplasmas were found in the salivary glands, the alimentary tract and the haemolymph throughout the whole body of both male and female insect vectors. The females had a more intense colour than the males in all these organs. No evidence of phytoplasma was found in the insect thoracic muscles.

To determine whether the phytoplasma amplified from insect and sugarcane were the same, we carried out RFLP analysis of a 700 bp DNA fragment amplified by PCR using primer pairs U1/MLO-7 and MLO-X/MLO-Y. Restriction enzymes *Taq*I, *Rsa*I and *Alu*I were not informative giving no RFLP banding pattern differences between the insect vector *M. hiroglyphicus*, SWL, sugarcane with grassy shoot disease, bermuda grass white leaf, brachiaria grass white leaf and crowfoot grass white leaf (data not shown). *Hin*fI digestion produced four different banding pattern groups (Fig. 3). The insect vector *M. hiroglyphicus* and SWL had the same DNA banding pattern, whereas those obtained with weeds were different. We sequenced twelve nested-PCR products from the spacer region of adults and eggs, and compared these sequences to those of SWL and bermuda grass white leaf, which were obtained from GenBank. The two F_1 adults, one F_1 egg, and two F_2 eggs had sequences that very closely matched that of SWL (only one nucleotide mismatch). A second and a third F_1 eggs had one and four additional mismatches, respectively. The remaining two F_2 eggs and the two F_2 adults had sequences that closely matched that of bermuda grass white leaf phytoplasma, with two and one mismatch, respectively (Fig. 4).

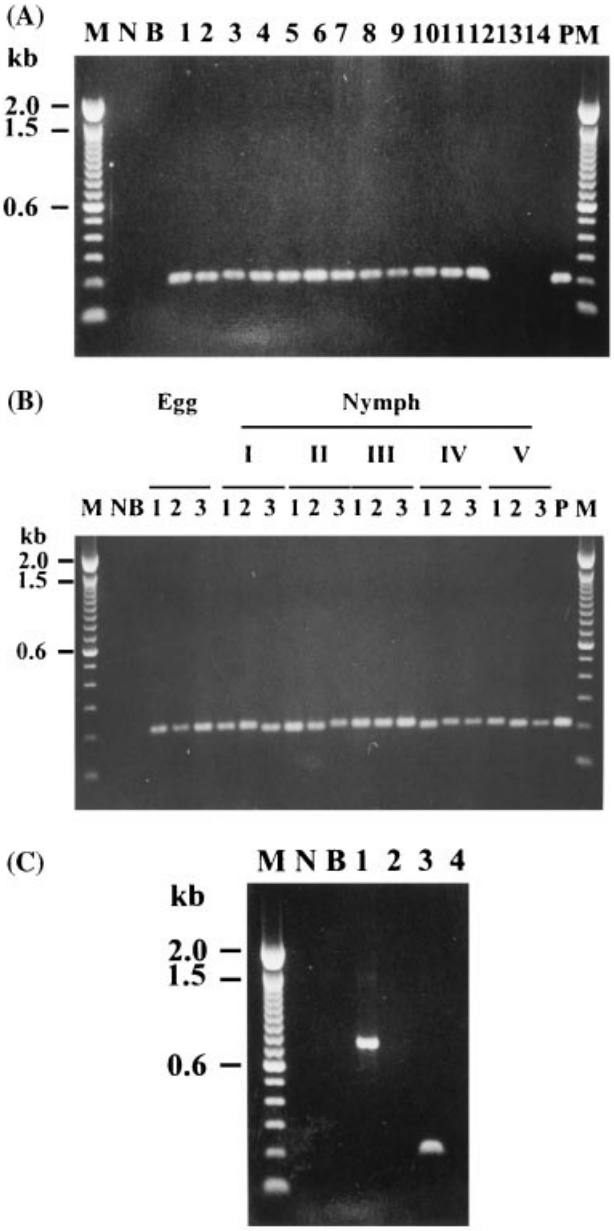

Figure 1. Agarose gel electrophoresis of insect DNA amplified by nested-PCR with primers MLO-X/MLO-Y followed by primers P1 and P2. (A) Amplification of DNA from fourteen adults. The numbers above the lanes represent different individuals; (B) amplification of DNA from three eggs and nymphal instars 1–5, three each; (C) SWL (lanes 1, 3), tissue culture sugarcane (2, 4). Samples in lanes 1, 2 were amplified with primers MLO-X/MLO-Y only. N is no insect sample (negative control), B is brown planthopper, also negative control, P is sugarcane white leaf DNA (postive control), M is the 100 bp ladder. A 210 bp band indicative of phytoplasma infection is seen with all nymphs, eggs and twelve of the F_1 adults, but is not detected in the negative controls.

Plants on which transovarially infected first and second generation insects had been reared were tested for SWL

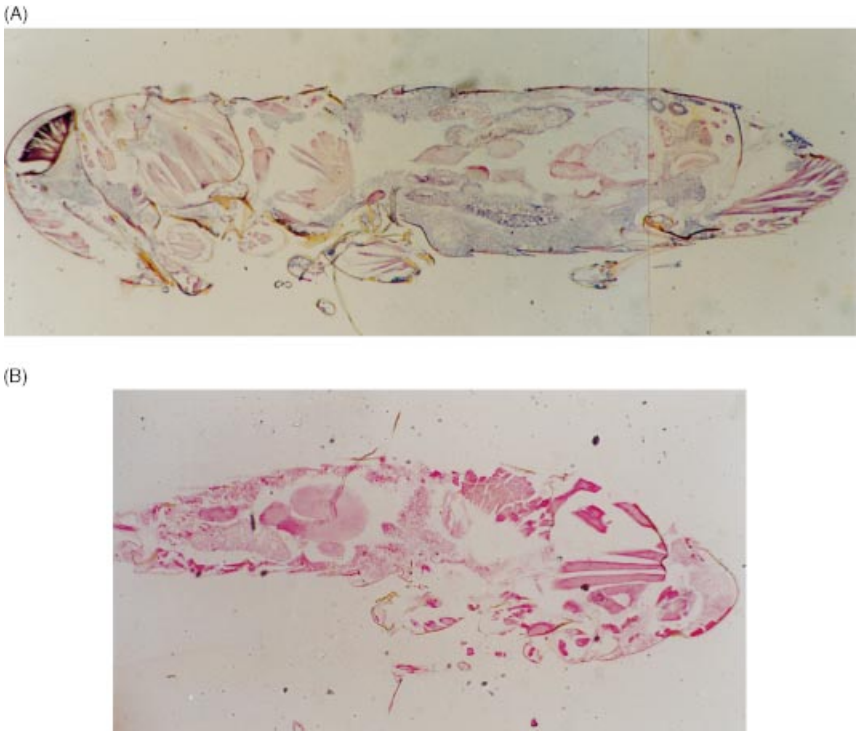

Figure 2. (A) *In situ* PCR of *M. hiroglyphicus* section. The blue colour, indicative of the presence of phytoplasma, is visible in the alimentary tracts, haemolymph, salivary glands and female reproductive organs. (B) Adjacent section treated as in (A) except that the PCR primers were omitted. No blue colour is visible in this section.

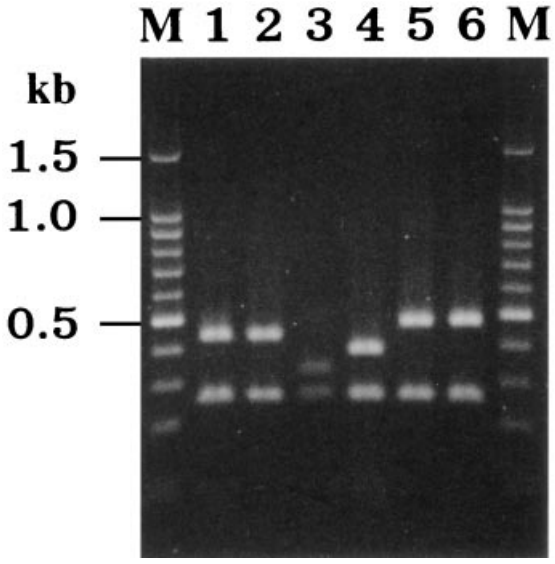

Figure 3. Agarose gel electrophoresis of insect DNA amplified by nested-PCR with primers U-1/MLO-7 then primers MLO-X/MLO-Y and digested with *Hin*fI. 1, phytoplasma from insect vector; 2, sugarcane white leaf; 3, sugarcane grassy shoot; 4, bermuda grass white leaf; 5, brachiaria grass white leaf; 6, crowfoot grass white leaf; M, 100 bp ladder.

disease by PCR with primer MLO-X and MLO-Y before and after insect infestation. The 700 bp band specific for phytoplasma infection was obtained from the four plants tested (two plants fed to each generation of insects) after but not before rearing (data not shown).

Discussion

Matsumoto *et al*. (1968) demonstrated the transmission of phytoplasma from insect to sugarcane and identified the leafhopper *M. hiroglyphicus* as the insect vector of SWL disease. The reservoir of phytoplasma is not the sugarcane plant itself as this is a seasonal crop, but was suspected to be the weeds that grow in sugarcane growing areas (Sarindu & Clark, 1993; Nakashima *et al*., 1996). However, our previous results disproved this hypothesis (Wongkaew *et al*., 1997). In this study we have set out to determine whether the insect vector itself was the reservoir. It has been stated that there is no evidence of transovarial passage of SWL phytoplasma agent in this vector (Rishi & Chen, 1989; Lee & Davis, 1992) but no data were shown to back this statement. Our study, using a sensitive nested-PCR assay with SWL phytoplasma specific primer pairs revealed the presence of the phytoplasma in adults, nymphs and eggs of *M. hiroglyphicus.* This is the first demonstration of the presence of SWL phytoplasma agent throughout the life cycle of *M. hiroglyphicus* and of transovarial transmission. Other plant pathogenic phytoplasma have been detected in nymphs and adults of other leafhopper vectors (Chiykowski, 1991). In addition, Alma *et al*. (1997) suggested transovarial transmission of the phytoplasma that causes aster yellow in leafhoppers. Kawakita *et al*. (2000) showed transovarial transmission of mulberry dwarf phytoplasma in another leafhopper vector.

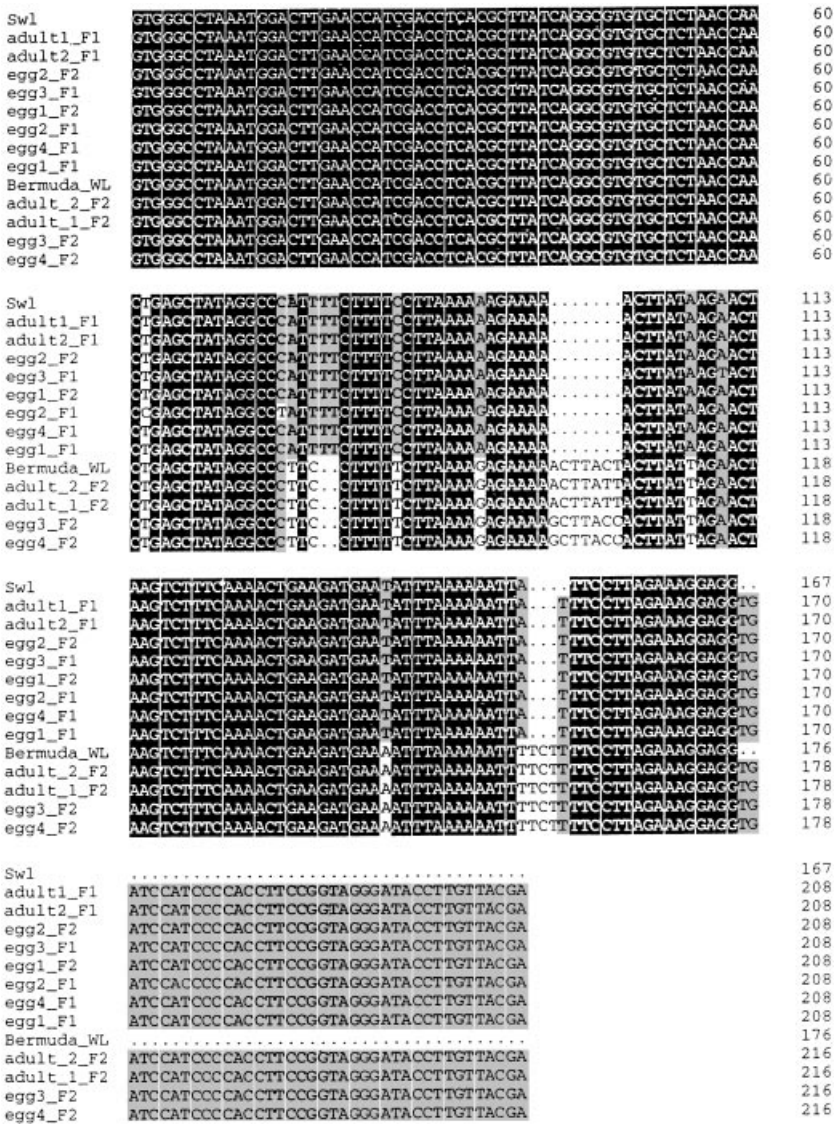

Figure 4. Alignment of DNA sequences from the spacer region of phytoplasma DNA amplified from four adults and eight eggs of *M. hiroglyphicus*, and compared to the sequence of sugarcane white leaf and bermuda white leaf.

Previously, no alternative host plant for SWL phytoplasma has been found. Gramineous weeds in the sugarcane fields that show white leaf symptoms from phytoplasma pathogenic agent are not the SWL phytoplasma plant reservoirs (Wongkaew *et al*., 1997). RFLP shows that the phytoplasmas in the insect vector are different from those of the infected weeds but identical to those of the SWL. Our results indicate that *M. hiroglyphicus* can act as the reservoir for SWL phytoplasma. There is no evidence that the phytoplasma pathogen affects either the longevity or fecundity of the leafhopper vector (Chiykowski, 1991).

The DNA sequence amplified from the hypervariable spacer region of phytoplasma from the insect vector and sugarcane were very similar, but not identical. These minor differences in sequence may reflect population to population variations. The insect had phytoplasma sequence similar to that which cause SWL and also those that infect bermuda grass. This is not surprising because the insects were wild caught, and could have been infected with either weed or SWL phytoplasma. Multiple infection of the same insect with two strains of phytoplasma is also a possibility. Mixed phytoplasma infections in plants and insects with no obvious interference in the hosts and vectors have been reported (Lee *et al*., 1995; Palmano & Firrao, 2000). The bermuda grass white leaf phytoplasma was amplified only from F_2 eggs and adults whereas SWL phytoplasma was amplified from F_1 and F_2 eggs but only F_1 adults. This reduction in the frequency of SWL phytoplasma relative to bermuda grass phytoplasma from first to second generation suggests that transovarial transmission may be more difficult for the SWL agent. However the small number of samples precludes definite conclusions.

The localization of phytoplasma in the insect vector *M. hiroglyphicus* body was done by using non-radioactive

in situ PCR. The phytoplasma in *M. hiroglyphicus* is in the alimentary tracts, haemolymph, salivary glands and female reproductive organs. A study of phytoplasma in *M. hiroglyphicus* by transmission electron microscopy showed that the phytoplasmas of various shapes and sizes between 500 and 1500 nm were found in the mouth, alimentary tract and haemolymph throughout the insect vector body (Hanboonsong *et al.*, 2000). While the phytoplasma detected by *in situ* PCR could be either of the sugarcane or bermuda grass white leaf agent, or both, these results do show propagation of the phytoplasma in the insect body.

The insect males showed weaker signs of infection by phytoplasma than the females. Beanland *et al.* (1999) also reported that the female leafhoppers were more efficient in the transmission of aster yellow phytoplasma than males. It is possible that the female leafhopper *M. hiroglyphicus* is also able to transmit the SWL phytoplasma more efficiently than the male leafhopper. However, more studies are required to prove or disprove this hypothesis.

No studies have been conducted on the mechanism of multiplication of phytoplasma in the leafhopper *M. hiroglyphicus*. However, previous work has indicated that the transmission of phytoplasmas by other leafhopper species is propagative, in that the phytoplasmas are ingested and pass into the body organs where they circulate and multiply (Lherminier *et al*., 1989; Lefol *et al*., 1993; Lefol *et al*., 1994; Fletcher *et al*., 1998). Our *in situ* PCR studies suggest that phytoplasmas in *M. hiroglyphicus* have a similar cycle in the insect body as described in other leafhoppers.

If the insect vector is the reservoir of phytoplasma, it is important to determine whether insects that acquired the disease through transovarial transmission can infect sugarcane. Preliminary data indicate that this is the case. PCR analysis of four sugarcane plants on which transovarially infected insects were reared produced the diagnostic band for the phytoplama agent. These results need to be confirmed with a larger number of plants and with field experiments.

Adult *M. hiroglyphicus* live for 35–40 days. The eggs hatch 12–14 days after laying and the nymphs reach the adult stage after 12–14 days (Phisitkul *et al*., 1989). In the sugarcane fields of north-east Thailand, *M. hiroglyphicus* occurs abundantly from June to July and the disease is more severe during that time (Phisitkul *et al*., 1989). In this region, sugarcane is usually planted in April and harvested 8–12 months later, depending on the variety. The seedling period is usually 2–4 months after planting. Thus there is a 2–4 month period in which the insects cannot be infected by feeding on sugarcane. During that period, the phytoplasma could be transmitted transovarially through two generations. Infected second generation insects can then infect the newly grown sugarcane and the plant to insect transmission of the disease would resume.

The evidence that *M. hiroglyphicus* is not only the vector but also the reservoir of the SWL phytoplasma has important implications for the control of the disease, because it appears that the control of the vector is the most effective if not the only way to stop the spread of SWL disease**.**

Experimental procedures

Rearing of insect vector Matsumuratettix hiroglyphicus *(Matsumura)*

M. hiroglyphicus was collected by light traps from sugarcane fields in Udonthani province in north-east Thailand. A colony of adult insects was reared on phytoplasma free sugarcane tissue cultured plants. The plants and insects were caged and maintained at 25 °C. The newly laid eggs were collected from the soil near the plants and transferred to new healthy plants until they became adults. The plants were tested by PCR to confirm their disease-free status before being used for insect rearing. Eggs, first to fifth instar nymphs and F_1 adults were singly collected in Eppendorf tubes and kept at –80 °C until DNA extraction. F_1 adults were maintained to an F_2 on the phytoplasma free sugarcane tissue cultured plants. To do this, a few eggs were moved to new healthy plants and allowed to hatch. The F_2 eggs, nymphs and adults were singly collected and DNA extracted. Because it was not possible to determine in advance which *M. hiroglyphicus* were infected with phytoplasma, we chose to use brown planthopper *Nilaparvata lugens* (Stal.), which is non-vector of phytoplasmas, as a negative control.

DNA extraction

Eggs, newly hatched nymphs from first to fifth instar and adults were ground singly in Eppendorf tubes with DNA extraction buffer (200 mM Tris pH 8.0, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml Proteinase K). The samples were left overnight at 37 °C. One volume of phenol-chloroform-isoamyl alcohol (25 : 24 : 1) was added, followed by 5 min of centrifugation at 13 000 ***g***. The supernatants were collected, one volume of chloroform-isoamyl (24 : 1) was added and the tubes were centrifuged at 13 000 ***g*** for 5 min. The supernatants were transferred to a 1.5-ml Eppendorf tube and one-tenth volume of 3 M sodium acetate pH 5.2 was added, then the samples were kept at –20 °C for 30 min. One volume of isopropanol was added and left at 4 °C for 2 h. After incubation, the tubes were centrifuged at 13 000 ***g*** for 20 min at 4 °C, and the supernatant was discarded. The pellet was washed with 70% ethanol, air dried, resuspended in TE buffer (10 mM Tris, 1 mM EDTA), and stored at –20 °C until use. In parallel, and following the same DNA extraction procedure, negative controls without insect samples were obtained.

PCR amplification

The PCR assays were carried out by using two sets of oligonucleotide primers whose sequences matched the 16S rRNA and 23S rRNA of phytoplasmas (Namba *et al*., 1993; Wongkaew *et al*., 1997). The first set consisted of primers MLO-X, 5′-GTTAGGTTAAGTCCTAAAACGAGC-3′, and MLO-Y, 5′-GTGCCAAGGCATCCACTGTATGCC-3′ and amplified a 700 bp DNA fragment. The nested-PCR used a second set of primers near the spacer region, the sequences of which were P1, 5′-GTCGTAACAAGGTATCCCTACCGG-3′, and P2, 5′-GGTGGGCCTAAATGGACTTGAACC-3′. PCR amplification was performed in a 25 µl reaction mixture containing 20–25 ng of insect genomic DNA, 0.2 mM each

dNTP, 0.25 μM of each primer, 1 U *Taq* DNA polymerase (Promega Corporation, Madison, USA) in 1× PCR reaction buffer (supplied by the manufacturer) containing 1.5 mM $MgCl_2$. Thirty-five PCR cycles were conducted in an Autogene (Grant Instruments, Cambridge, UK) thermocycler. Each cycle consisted of 1 min (5 min for first cycle) denaturation at 92 °C, 30 s annealing at 60 °C and 1.30 min (10 min for the last cycle) extension at 72 °C. The nested-PCR assays were carried out using 1 μl of the first PCR product (diluted 1 : 50 in sterile deionized water) as template. A total of forty cycles were conducted for nested-PCR under the same condition of denaturation and extension as first PCR, but used 30 s annealing at 68 °C. Samples of no insect DNA and genomic DNA from brown planthopper, which is non-vector of phytoplasmas, were used as negative controls. The positive control was DNA from SWL. PCR products were analysed by electrophoresis through a 1.5% agarose gel in TBE buffer (0.089 M Tris, 0.089 M boric acid and 0.002 M EDTA) containing 0.5 μg/ml ethidium bromide.

In situ *PCR*

In situ PCR was conducted to confirm the presence and determine distribution of the phytoplasma in the insect vector body. Adult *M. hiroglyphicus* were fed on SWL plant whereas the adult *N. lugens*, used as a negative control, were fed on phytoplasma free rice plants. The insects were anaesthetized with CO_2. Whole insects bodies were fixed with 4% freshly prepared buffered paraformaldehyde (prepared in PBS) for 1 h, washed with 3× PBS, then rinsed with sterile water. The insect bodies were embedded in paraffin and cut in 4 μm thin longitudinal sections showing the body of the insect from the mouth to the anus. These sections were adhered to aminoalkylsilane-coated slides. The paraffin was removed with fresh xylene, followed by rehydration in 100% ethanol. Tissue sections were then permeabilized with 10 μg/ml proteinase K for 1 h at 37 °C then heated at 95 °C for 3 min to inactivate the proteinase K. Amplification of phytoplasma DNA was carried out on the slide, using primer pairs P1 and P2 (see above) and performed on a Perkin-Elmer thermocycler adapted for *in situ* PCR. Twenty-five microliters of PCR reaction mixture was used for each sample. The mixture contained 1× PCR buffer (10 mM Tris-HCl at pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin), 1 μM primers, 200 μM dNTP mix, 100 μM digoxigenin-11–2′-deoxyuridine-5′ triphosphate (Boehringer, Mannheim, Germany), 2% bovin serum albumin, and 2.5 U Taq DNA polymerase. Coverslips were applied and sealed with nail polish. The slides were placed on a heat block and covered with mineral oil. Amplification conditions were 93 °C for 1 min, 68 °C for 30 s and 72 °C for 1 min for a total of thirty cycles. After amplification, the slides were washed with 2× SSC. PCR products were detected by adding an alkaline phosphatase conjugated antiDIG polyclonal antibody (dilution 1 : 200) and incubation at room temperature for 30 min. After a thorough rinse, the alkaline phosphatase substrates (nitroblue terrazolium and 5-bromo-chloro-3-indolylphosphate) were added and in the presence of alkaline phosphatase a dark blue precipitate was formed. Color deposition was checked and photographed under a light microscope. *In situ* PCR of *M. hiroglyphicus* sections without primers was used as an additional negative control.

RFLP analysis of PCR products

DNA from each life cycle stage of *M. hiroglyphicus* and SWL was amplified with two primer sets. The first set was U-1, 5′-GTTTGATCCTGGCTCAGGATT-3′, MLO-7, 5′-CGTCCTTC ATCGGCTCTT-3′, and the second set was MLO-X, 5′-GTTAGGTTAAGTCCTAAAACGAGC-3′, MLO-Y, 5′-GTGCCAAGGCATCCACTGTATGCC-3′ from the 16S rRNA and 23S rRNA regions of phytoplasma. PCR products of 700 bp in size were digested separately with the four restriction endonucleases *Alu*I, *Hin*fI, *Rsa*I and *Taq*I (Boehringer, Mannheim, Germany), following manufacturers instructions. Digested DNA fragments were separated in a 2% agarose gel in TBE containing 0.5 μg/ml of ethidium bromide and visualized under an UV transilluminator.

Sequencing

The 210 bp products of the nested-PCR amplification of *M. hiroglyphicus* from various stages of the life cycle were cloned into a pGEMT vector (Promega, Madison, USA), and sequenced using an ABI Prism automated sequencer. The sequencing data from both *M. hiroglyphicus* and SWL were aligned and analysed by using DNAMAN version 4.03 (Lynnon Corporation, Quebec, Canada).

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