Theor Appl Genet (1997) 95: 660–663

P. Wongkaew · Y. Hanboonsong · P. Sirithorn
C. Choosai · S. Boonkrong · T. Tinnangwattana
R. Kitchareonpanya · S. Damak

Differentiation of phytoplasmas associated with sugarcane and gramineous weed white leaf disease and sugarcane grassy shoot disease by RFLP and sequencing

Received: 15 April 1997/Accepted: 18 April 1997

Abstract Restriction fragment length polymorphism (RFLP) and sequencing were used to elucidate the genetic relationship between phytoplasmas that cause white lead disease and grassy shoot disease in sugarcane and white leaf disease in gramineous weeds found in the cane-growing areas (Crowfoot grass, Bermuda grass and Brachiaria grass). A 1.35-kb DNA fragment encoding for the 16*s* rRNA was amplified by PCR using universal primers and analysed by digestion with nine restriction endonucleases. A DNA fragment containing the 3′ end of the 16*s* rRNA and the spacer region between the 16*s* rRNA and the tRNA(Ile) was amplified by PCR and sequenced. Analysis of the RFLP patterns and of the sequence showed that grassy shoot and white leaf diseases in sugarcane are caused by two different phytoplasmas. Sequence analysis of phytoplasma DNA obtained from three species of weeds showing symptoms of white leaf disease failed to detect any organism that is identical to those infecting the sugarcane. Moreover the phytoplasma species that infect the three types of gramineous weeds, although closely related, are nevertheless different

Key words Phytoplasma · Sugarcane · White leaf disease · Grassy shoot disease · Ribosomal RNA

Communicated by G. Wenzel

P. Wongkaew · Y. Hanboonsong (✉) · P. Sirithorn
C. Choosai · S. Boonkrong
Faculty of Agriculture, Khon Kaen University,
Khon Kaen 40002, Thailand

T. Tinnangwattana · R. Kitchareonpanya
Northeastern Sugarcane Agricultural Centre, Office of the Cane and Sugar Board, Ministry of Industry, Thailand

S. Damak
Centre for Molecular Biology, Lincoln University, Canterbury, New Zealand

Introduction

Phytoplasmas, commonly known as mycoplasma-like organisms (MLOs), are believed to be associated with numerous plant diseases affecting more than 300 plant species worldwide (McCoy et al. 1989; Kollar et al. 1990). In Thailand phytoplasmas cause serious diseases to several crops and result in considerable losses to the economy. Sugarcane white leaf (SWL) and grassy shoot (SGS) diseases are severe diseases of sugarcane that are caused by MLOs. SWL disease is characterised by leaf chlorosis and proliferation of tillers. Plants with SGS disease are dwarfed and develop witches' broom-type symptoms by producing a large number of tillers (Chona et al. 1960; Sarosh 1986; Rishi and Chen 1989). Each year phytoplasma diseases, especially SWL, cause losses of over 100 million Baths to the Thai sugarcane industry.

In the cane-growing areas, several kinds of gramineous plants, such as Bermuda grass (*Cynodon dactylon*), Crowfoot grass (*Dactyloctenium aegyptium*) and Brachiaria grass (*Brachiaria distachya*), also show the symptoms of white leaf disease associated with phytoplasma infection (Nakashima et al. 1994). Weeds have been suspected to be the reservoir of phytoplasma from which sugarcane plants could be re-infected (Sarindu and Clark 1993; Nakashima et al. 1996) but this has never been proven. While SWL phytoplasmas are transmitted by the leafhopper *Matsumuratettix hiroglyphucus* (Matsumoto et al. 1968; Chen 1974; Maramorosch et al. 1975), insect vectors that transmit phytoplasmas from and to the other gramineous plants are still unknown. The transmission of phytoplasma by leafhopper from SWL to other weeds and from affected weeds to healthy sugarcane was attempted but failed (Nakashima et al. 1994). This suggested that different phytoplasmas specifically infect the different plants.

The genetic relationships between various phytoplasmas that affect sugarcane and gramineous weeds have not been totally elucidated. Antisera raised

against phytoplasma causing SWL cross react with tissues from plants with SGS but not with gramineous weeds with symptoms of white leaf disease (Viswanathan 1997). Antibodies against a Bermuda grass phytoplasma isolate did not react with isolates from the two other gramineous weeds, not with SWL (Sarindu and Clark 1993). Restriction fragment length polymorphism (RFLP) studies of the phytoplasma 16*s* rDNA region have shown the same restriction pattern with DNA amplified from three different gramineous weeds with white leaf symptoms but a different pattern with DNA from sugarcane with SWL (Nakashima et al. 1996).

Here we describe the use of RLFP and sequencing to elucidate the precise genetic relationships among phytoplasmas infecting sugarcane and gramineous weeds in the Northeast of Thailand.

Materials and methods

Sources of phytoplasmas

Samples of sugarcane showing white leaf disease symptoms, as well as healthy plants, were collected from fields in the Khon Kaen and Udonthani provinces in the Northeast of Thailand. A variety of gramineous weeds with white leaf symptoms and sugarcane plants with grassy shoots were also collected from the same locations.

DNA extraction

Total DNA of healthy and infected plants was extracted from the midribs of the leaves according to the CTAB method (Kollar et al. 1990; Nakashima et al. 1991).

PCR amplification

Two oligonucleotide primer sets were used. The first set consisted of universal primers based on the sequence of the 16*s* rRNA of phytoplasmas (Deng and Hiruki 1991; Namba et al. 1993). The sequence of the primers was 5′ GTTTGATCCTGGCTCAGGATT 3′ (position 1–21 in the rRNA) and 5′ AACCCCGAGAACGTATTCACC 3′ (position 1368–1348). The second set consisted of one primer complementary to the 16*s* rRNA (primer MLO1, 5′ CAGGTGGTGCATGGTTGTCGTC 3′, position 1020–1041) and one primer complementary to the tRNA sequence in the spacer region (MLO2, 5′ GAACCACCGACCTCACGCTTATC 3′).

PCR amplification was performed in a 25-µl reaction mixture containing 0.2 mM each of dATP, dGTP, dCTP and dTTP, 0.25 µm of each primer, 1 U of *Taq* DNA polymerase (Promega Corporation, Madison, USA) in 1 × PCR reaction buffer (supplied by the manufacturer) containing 1.5 mM of $MgCl_2$. Thirty PCR cycles were conducted in an Autogene (Grant Instruments, Cambridge, UK) thermocycler. Each cycle consisted of 1 min denaturation at 94°C, 30 s annealing at 55°C (60°C for MLO1 and MLO2) and 1 min extension at 72°C.

RFLP analysis of PCR products

Each PCR product was digested separately with nine restriction endonucleases (Boehringer, Mannhein, Germany), *Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII, *Hpa*I, *Hpa*II, *Msp*I, *Rsa*I and *Taq*I, and electrophoresed through a 1.5% agarose gel in TBE (0.089 M Tris, 0.089 M boric acid and 0.002 M EDTA) containing 0.5 µg/ml of ethidium bromide. Following electrophoresis, the gel was examined under UV light at 300 nm using a transilluminator.

Sequencing

The product of PCR amplification of DNA with primers MLO1 and MLO2 was purified with a Wizard PCR product purification kit (Promega Corp, Madison, USA) and then sequenced using primer MLO2 and the Silver Sequence DNA kit (Promega Corp, Madison, USA) according to the manufacturer's instructions. Twenty five nanograms of DNA were used for each sequencing reaction. The cycling parameters were 10 s at 94°C, 30 s at 60°C and 1 min at 70°C for 60 cycles. The product of the sequencing reaction was run in a 6% polyacrylamide denaturing gel and the bands were detected by silver staining following instructions provided with the sequencing kit.

Sequence analysis

DNA sequences were aligned using the Clustal method (Higgins and Sharp 1988) and a DNAMAN programme (Lynnon Biosoft, Quebec, Canada). Aligned sequences and a dendrogram were generated.

Results

PCR amplification of DNA from SGS, SWL, Bermuda grass, Crowfoot grass and Brachiaria grass with white leaf disease, using the universal primers, resulted in a 1.35-kb fragment (data not shown).

Restriction digestion of the PCR product with *Hpa*II showed a different banding pattern in SGS, compared with the other plants (Fig. 1). As shown in Fig. 1 a, *Hpa*II digest of PCR-amplified DNA from all plants except SGS resulted in three bands of about 850 bp, 470 bp and 70 bp, respectively; whereas digestion of SGS DNA resulted in three bands of about 850 bp, 420 bp, and 70 bp suggesting that there is one additional *Hpa*II site in the DNA fragment amplified from SGS. In SGS the 70-bp band was probably a doublet. A faint band of about 470 bp was also present suggesting the possibility of a mixed population of phytoplasma in the plants showing the grassy shoots symptoms. The same banding pattern was obtained after digestion of the PCR products with *Msp*I (data not shown).

PCR products of SWL, SGS, Bermuda grass, Crowfoot grass and Brachiaria grass digested with *Taq*I revealed different fragment profiles between the phytoplasmas associated with sugarcane diseases and three species of gramineous plants with white leaf symptoms (Fig. 1 b). The other restriction digestions did not show any difference between the various plants (the product of *Rsa*I digestion is shown in Fig. 1 c).

PCR amplification of DNA from affected plants using the MLO1 and MLO2 primers resulted in a 0.65-kb fragment (data not shown). No PCR amplification was obtained with both sets of primers when healthy sugarcane plants were used (data not shown).

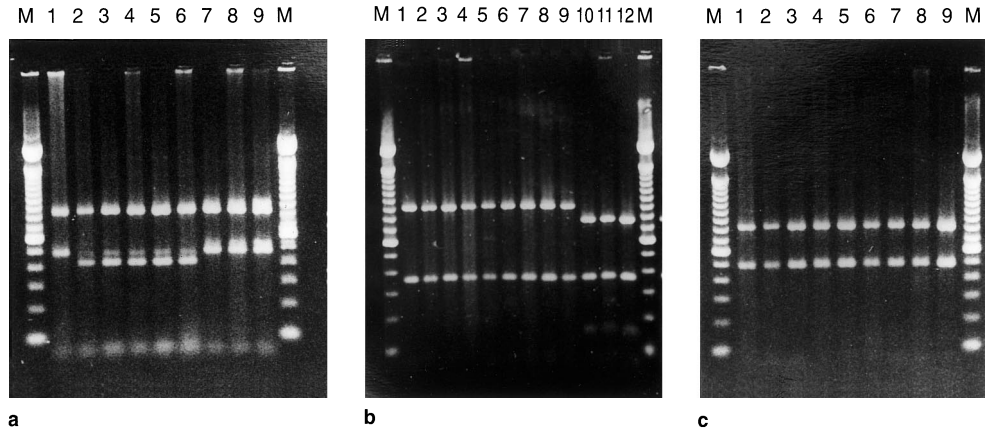

Fig. 1 Agarose-gel electrophoresis of PCR-amplified DNA from various phytoplasma-infected plants digested with *Hpa*II (**a**), *Taq*I (**b**) and *Rsa*I (**c**). **a, c** *M* 100-bp size marker; *1* SWL; *2–6* SGS; *7* Bermuda grass/white leaf; *8* Crowfoot grass/white leaf; *9* Brachiaria grass/white leaf. *b M* 100-bp size marker; *1–4* SWL; *5–9* SGS; *10* Bermuda grass/white leaf; *11* Crowfoot grass/white leaf; *12* Brachiaria grass/white leaf. Run in 1.5% agarose gels in TBE buffer

```
ORIGIN
BERMUD    AAAAACTTACTACTTATTAGAACTAAGTCTTTC.AAAACTGAAGAT.GAAAATTTAAAAA   58
CROWFO    ---------t----------------------.------------.-------------   58
BRACHI    ---------t----------------------.------------.-------------   58
SGS       ----.......------a---------------.------------.-------------   51
SWL       ----.......------a---------------a------------a---t---------   53

BERMUD    TTTCTTTTCCTTAGAAAGGAGGTGATCCATCCCCACCTTCCGGTAGGGATACCTTGTTAC  118
CROWFO    -------------------------g----------------------------------  118
BRACHI    ----------------------------------------------------------  118
SGS       -a...-------------------------------------------------------  108
SWL       -a...-------------------------------------------------------  110

BERMUD    GACTTAACCCCAATCATCGATCCTACCTTAGACAGTTCCCTCTTCCGAAGAAGTTAGGCT  178
CROWFO    ---------------------------------g--------------------------  178
BRACHI    ---------------------------------g--------------------------  178
SGS       ---------------------------------ga-------------------------  168
SWL       ---------------------------------g--------------------------  170

BERMUD    ACCGTTTTTGAGTATTACCAACTT
CROWFO    --------c---------------
BRACH     --------c---------------
SGS       --------c---------------
SWL       ------------------------
```

Fig. 2 Alignment of DNA sequence from the 3′ end of the 16*s* rRNA and the spacer region of phytoplasmas infecting sugarcane and gramineous weeds. *Bermud* Bermuda grass; *Crowfo* Crowfoot grass; *Brachi* Brachiara grass; *SGS* sugarcane with grassy shoot; *SWL* sugarcane with white leaf

To further investigate the variation between phytoplasmas from various plants showing white leaf symptoms, DNA amplified with primers MLO1 and MLO2 was sequenced using primer MLO2. Approximately 195 bp of readable sequence, including about 130 base pairs of the variable regions at the 3′ end of the 16*s* rRNA gene and 62 base pairs of the spacer region between the tRNA(Ile) and the 16*s* rRNA, was obtained. Sequence alignment showed that all phytoplasmas infecting the various plants were different (Fig. 2). Also phytoplasmas from SGS were different from SWL. DNA was extracted from three sugarcane plants from different varieties (Uthon 1 and Ratoon) with grassy shoot disease. The sequence obtained was identical with all three plants (data not shown). The 16*s* rRNA sequence obtained from DNA amplified from SWL was identical to that deposited in GenBank. A dendrogram generated from the sequence showed that phytoplasmas from SWL and SGS clustered in one group and those from the gramineous weeds in another (Fig. 3).

Discussion

RFLP analysis of PCR-amplified DNA using universal primers showed a similar banding pattern in SWL and three different weeds with white leaf disease, but a different banding pattern in SGS with *Hpa*II. The faint 470-bp band (Fig. 1a) also suggests a mixed population of phytoplasma in grassy shoot disease. While the banding pattern generated by restriction digestion of SGS DNA with *Hpa*II could have been the result of a partial digestion, this is unlikely to be the case as other bands that could have resulted from a partial digestion are absent and the banding pattern is identical in the five sugarcane plants with SGS.

The faint 470-bp band seen in the *Hpa*II digests could also have been the result of contamination from other PCR products; for example from PCR-amplified SWL DNA. Again, this is unlikely since the negative controls of the PCR (no DNA and healthy plant) did not show any band (data not shown). Thus the most

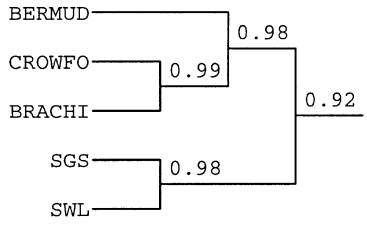

Fig. 3 Dendrogram of phytoplasma infecting sugarcane and gramineous weeds generated from sequence data. Abbreviations are as in Fig. 2

likely explanation for the banding pattern of PCR-amplified DNA from SGS is multiple infection by different phytoplasma groups. One group had a banding pattern similar to that of SWL whereas the other group had a different pattern. The group with the different banding pattern is predominant, as judged by the respective intensity of the bands. Multiple phytoplasma infection based on an analysis of the banding pattern of restriction enzyme-digested PCR products has been reported in sesame plants (Nakashima et al. 1996) and grapevines (Bianco et al. 1993).

Analysis of the sequence of the 3′ end of the 16*s* rRNA and of the spacer region confirmed that grassy shoot and white leaf diseases in sugarcane are caused by two different phytoplasmas. We have observed that several sugarcane plants first develop grassy shoot symptoms then white leaf symptoms (unpublished) suggesting that SGS is an early developmental stage of SWL and is caused by the same phytoplasma. The sequence data obtained here shows that this is not the case.

The sequence analysis also showed that each weed was infected by a different phytoplasma which was also different from those of sugarcane with white leaf or grassy shoot diseases. It has been suggested that weeds growing in the same area as sugarcane may be infected with the SWL phytoplasma and could transmit the disease from the weeds to sugarcane. However, transmission of phytoplasma by leafhopper from SWL to other weeds and from affected weeds to healthy sugarcane plants was attempted but failed (Nakashima et al. 1994). Our data are in line with Nakashima's observations in showing that white leaf disease is caused by different phytoplasmas in sugarcane and in the three weeds examined. Thus more plant species need to be screened to identity the reservoir of phytoplasma from which the insect vectors transmit the disease to sugarcane plants.

In summary, these data show that grassy shoot and white leaf diseases in sugarcane are caused by two different phytoplasmas. Sequencing of phytoplasma DNA obtained from three species of weeds showing symptoms of white leaf disease failed to detect any phytoplasma that is identical to those infecting the sugarcane. Moreover, we have shown that the phytoplasma species that affect three types of gramineous weeds, although closely related, are nevertheless different.

Acknowledgements This work was part of the "Management of Sugarcane White Leaf Disease" project funded by the Thailand Research Fund.

References

Bianco PA, Davis RE, Prince JP, Lee I-M, Gundersen DE, Fortusini A, Belli G (1993) Double and single infections by ester yellows and elm yellow MLOs in grapevines with symptoms characteristic of flavescence doree. Riv Patol Veg 3:69–82

Chen CT (1974) Sugarcane white leaf disease in Thailand and Taiwan. Sugarcane Pathologists' Newslet 11/12:23

Chona BL, Capoo SP, Varma PM, Seth ML (1960) Grassy shoot disease of sugarcane. Indian Phytopathol 13:37–47

Deng S, Hiruki C (1991) Amplification of 16s rRNA genes from culturable and nonculturable mollicutes. J Microbiol Methods 14:53–61

Higgins DG, Sharp PM (1988) CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 73:237–244

Kollar A, Seemuller E, Bonnet F, Saillard, C, Bove JM (1990) Isolation of the DNA of various plant pathogenic mycoplasa-like phytoplasmas from infected plants. Phytopathology 80:233–237

Maramorosch K, Kimura M, Chareonridhi S (1975) Mycoplasma-like organisms associated with white leaf disease in Thailand. FAO Plant Pro Bull 23:137–139

Matsumoto T, Lee CS, Teng WS (1968) Studies on sugarcane white leaf disease of Taiwan with special reference to transmission by a leafhopper, *Epitettix hiroglyphicus* Mats. Proc Soc Sugarcane Technol 13:1090–1099

McCoy RE, Caudwell A, Chang CJ, Chen A, Chiyknowski LN, Cousin MT, Dale JL, de Leeuw GTN, Golino DA, Hackett KJ, Kirkpatrick BC, Marwitz R, Petzold H, Sinha RC, Sugiura M, Whitcomb RF, Yang IL, Zhu BM, Seemuler E (1989) Plant diseases associated with mucoplasma-like organisms. In: Whitcomb RF, Tully JG (eds) The mycoplasmas, vol. 5. Spiroplasma, acholeplasma and mycoplasmas of plants and arthropods. Academic Press, London New York, pp 545–640

Nakashima K, Challeprom W, Wongkaew P, Sirithorn P (1994) Detection of a mucoplasma-like organism associated with white leaf disease of sugarcane in Thailand using DNA probes. JIRCAS Jour 1:57–67

Nakashima K, Hayashi T, Chaleeprom W, Wongkaew P, Sirithorn P (1996) Complex phytoplasma flora in Northest Thailand as revealed by 16*s* rDNA analysis. Ann Phytopathol Soc Jpn 62:57–60

Nakashima K, Kato S, Iwanami S, Murata N (1991) Cloning and detection of chromosomal and extrachromosomal DNA from mycoplasma-like organisms that cause yellow dwarf disease of rice. Appl Environ Microbiol 57:3570–3575

Namba S, Kato S, Iwanami S, Oyaizu H, Shiozawa H, Tsuchizaki T (1993) Detection and differentiation of plant-pathogenic mycoplasmlike organisms using polymerase chain reaction. Phytopathology 83:786–791

Rishi N, Chen CT (1989) Grassy shoot and white leaf disease. In: Ricaus BC, Egan BT (eds) Diseases of sugarcane. Elsevier Science Publisher, Amsterdam, pp 289–300

Sarindu N, Clark MF (1993) Antibody production and identiy of MLOs associated with sugarcane whiteleaf disease and bermuda-grass whiteleaf disease from Thailand. Plant Pathol 42:396–402

Sarosh, Misra SR, Kishan-Singh, Singh K (1986) Anatomical studies on sugarcane affected with grassy shoot disease. Indian Phytopath 39:499–502

Viswanathan R (1997) Detection of phytoplasmas associated with grassy shoot disease of sugarcane by ELISA techniques. J Plant Dis Protect 104:9–16