Molecular Detection and Characterization of

Phytoplasmas That Cause Sugarcane White Leaf Disease

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Abstract

Sugarcane white leaf disease (SCWL) is one of the most destructive sugarcane diseases in Thailand. The disease is caused by wall-less microorganisms resembling mycoplasmas, called phytoplasmas. As it has not been possible to culture phytoplasmas in vitro, there has been a lack of information about the phytoplasmas, and it has been difficult to detect the pathogens. DNA of SCWL phytoplasmas was isolated from diseased sugarcane plants and the fragments were cloned in Escherichia coli. By using the cloned DNA fragments as probes, we could detect SCWL phytoplasmas from host plants and insect vectors by a hybridization assay. Rapid detection using PCR also enabled us to detect the ribosomal DNA (rDNA) fragments of the phytoplasmas. Hybridization assay and sequence analysis of rDNA of the phytoplasmas revealed that the genome of the SCWL phytoplasmas and that of the rice yellow dwarf (RYD) phytoplasmas are close to each other. The rDNA analysis showed that the SCWL phytoplasmas are also related to the sugarcane grassy shoot phytoplasmas and other phytoplasmas associated with white leaf diseases in gramineous weeds in Northeast Thailand phylogenetically, but that they are not identical. Sequence analysis of the cloned fragments of the SCWL phytoplasmas revealed that the phytoplasmas have circular 2.6-kb extrachromosomal DNAs. Hybridization assay indicated that the extrachromosomal DNAs of SCWL phytoplasmas and those of the RYD phytoplasmas

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and phytoplasmas associated with white leaf symptoms in gramineous weeds shared a considerable nucleotide sequence homology, whereas little homology with those of sesame phyllody phytoplasmas and aster yellows-type phytoplasmas. RFLP analysis revealed that the extrachromosomal DNAs showed a polymorphism among isolates collected within an individual field. It is assumed that the DNA-based detection methods and molecular studies on the phytoplasmas may contribute to the development of methods of control of the SCWL disease.

Additional key words: mycoplasma-like organism (MLO), detection, hybridization, PCR, ribosomal DNA, extrachromosomal DNA

Introduction

Sugarcane white leaf disease (SCWL) was first recorded in Thailand in 1954[27] and in Taiwan in 1958[26], while in Japan it was reported in 1986[1]. In Thailand, SCWL is widely spread in major cane-growing areas and is one of the most destructive sugarcane diseases[49]. However, extensive control undertaken by growers proved effective in Taiwan, and the disease now occurs only sporadically. In Japan, this disease occurred in Tanegashima island, but it has now disappeared. The typical symptoms of this disease consist of the appearance of leaves with total chlorosis, pronounced stunting and profuse tillering (Fig. 1A-C)[38, 43]. Discoloration first appears on young leaves (Table 1), or budding leaves, and profused tillers. These symptoms are very similar to those of yellow dwarf disease of rice[38]. The disease is caused by a wall-less microorganisms resembling mycoplasmas (Fig. 1D), called phytoplasmas, commonly known as mycoplasma-like organisms (MLOs). The phytoplasma cells are located in sieve elements with a size ranging from 0.1 to 1.0 μm, and are transmitted by the leafhopper *Matsumuratettix hiroglyphicus*. Like many other plant diseases caused by phytoplasmas, the development of SCWL symptoms requires a long incubation period (more than one month). The insect vectors also require an incubation period of about one month to transmit the pathogen and to allow the detection of phytoplasmas[38, 43]. So far it has not been possible to culture phytoplasmas in vitro. Therefore, there

is a lack of information about the phytoplasmas, including the genetic and phylogenetic relationships among the SCWL phytoplasmas, other phytoplasmas and culturable mycoplasmas (mollicutes)[42].

DNA technology has enabled to amplify or clone fragments of phytoplasma chromosomal or extrachromosomal DNA (plasmids) from phytoplasma-infected plants and insects[22]. Dot or Southern hybridization and polymerase chain reaction (PCR) methods have been used for the detection and genetic/phylogenetic studies of some phytoplasmas[4, 7-9, 12-25, 30-40, 45-47, 49, 50]. In an attempt to detect the pathogen from host plants and insect vectors, we cloned random fragments of DNA of the SCWL phytoplasmas collected from Khon Kaen, Northeast Thailand[30]. To reveal the phylogenetic and epidemiological characteristics of the phytoplasmas, we analyzed the PCR-amplified 16S ribosomal RNA gene (rDNA) of the phytoplasmas[34, 35]. The extrachromosomal DNA of the SCWL phytoplasmas has also been analyzed[31, 33].

Isolation and cloning of SCWL phytoplasma DNA.

To isolate phytoplasmas from the phloem of the infected plants, we extracted DNA from crude vascular bundles of sugarcane plants with SCWL-disease[30]. DNA was purified using bisbenzimide-CsCl equilibrium density gradient centrifugation[16]. There were two bands, a main band and an upper faint band. Main bands represent the DNA of

healthy plants. Therefore, we assumed that the faint band originated from the phytoplasmas. The DNA of the SCWL phytoplasmas was concentrated by repeated centrifugation. The isolated DNA was digested with the restriction enzyme *Hin*dIII, and was cloned into a plasmid (Bluescript) and *Escherichia coli* (NM522) system. By differential hybridization, most of the recombinant plasmids reacted with the labeled DNA of the SCWL phytoplasmas but not with the labeled DNA of healthy sugarcane plants. Therefore, the fragments of these recombinant plasmids were considered to be derived from the DNA of the SCWL phytoplasmas.
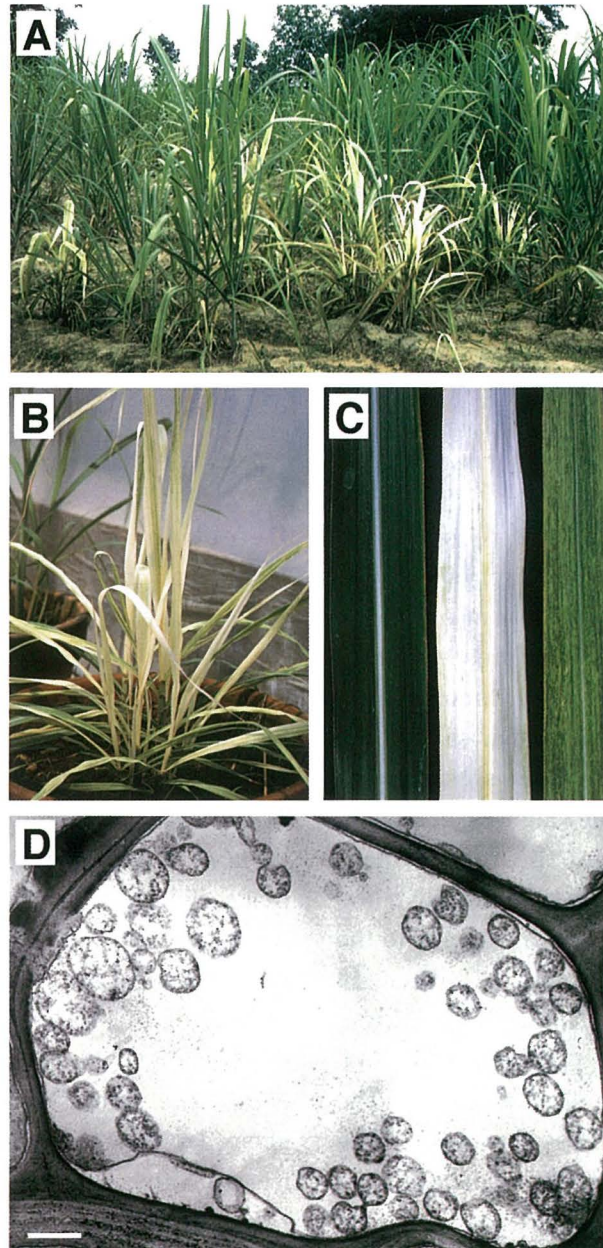

Fig. 1. Symptoms and pathogens of sugarcane white leaf disease (SCWL). (A) A sugarcane field infected with the SCWL phytoplasmas in Khon Kaen, Northeast, Thailand. (B) White leaves occurring from the phytoplasma-infected cutting of the sugarcane collected in Khon Kaen. (C) Comparison of color of a leaf of healthy sugarcane (left), a newly emerging leaf of the phytoplasma-infected sugarcane plants (center) and the older leaf of the infected plants (right). (D) Causal agent, phytoplasma of SCWL. Bar represents 0.5 μm.

Table 1. Distribution of chtromosomal DNA and extrachromosomal DNA of SCWL phytoplasmas in sugarcane plans[a]

Position[a]	Healthy plants			Infected plants		
	Chl[b]	C[c]	EC[c]	Chl[b]	C[c]	EC[c]
2nd leaf from top	28.6±2.3	0	0 (8%)[d]	2.2±0.9	0.43±0.04	0.92±0.16
4th leaf from top	33.4±2.3	0	0	7.7±4.9 (23%)[d]	0.44±0.11	1.59±0.08
6th leaf from top	32.8±4.3	0	0	16.9±5.0 (52%)[d]	0.51±0.01	1.83±0.04
Stem	–	0	0	–	0.73±0.04	2.01±0.23
Root	–	0	0	–	0.10±0.01	1.00±0.14
Cutting	–	0	0	–	0.01±0.01	0.04±0.02

a Leaves were counted the top developing leaf to the bottom old leaf.

b Chlorophyll content (Chl) was measured with a chlorophyll meter (Minolta SPAD-502). Unit: SPAD. The value was expressed as chlorophyll content ± standard error.

c Each value indicates the relativa absorbance (A_{633}) of the hybridization spots on the X-ray film measured with a laser densitometer. C, relative absorbance of hybridization signal between the chromosomal DNA probe S28 of SCWL phytoplasmas and DNA extracted from 0.1 mg of the tissur. EC, relative absorbance of hybridizarion signal between the extrachromosomal DNA probe S1 of SCWL phytoplasmas and DNA extracted from 0.1 mg of the tissue. We can compare these absorbance values as they were measured simultaneously.

d Percentage of chlorophyll content of the infected leaves against that of the healthy leaves is shown.

Approximately 60% of the recombinant plasmids showed hybridization signals with the labeled DNA of the SCWL phytoplasmas, and the signals were more than 10 times stronger compared with those of other recombinant plasmids[30]. The estimated molecular sizes of the cloned inserts showing stronger signals were about 2.5 kb, and of those showing weaker signals, 1.6-3.5 kb. The inserts of the recombinant plasmids with strong signals hybridized with a low-molecular-weight DNA (ca. 2.5 kb) specific to the disease, whereas the inserts of other 14 recombinant plasmids hybridized with a high-molecular-weight DNA (> 23 kb) of SCWL phytoplasmas specific to the disease (Fig. 2). The genome size of the mollicutes is considered to be 500-1,500 kb[10, 42]. Therefore, we assumed that the inserts of the recombinant plasmids showing strong signals with the DNA of the diseased plants were derived from the extrachromosomal DNA of the SCWL phytoplasmas, and those of the other 14 recombinant plasmids were derived from the chromosomal DNA of the SCWL phytoplasmas.

Detection of SCWL phytoplasmas in sugarcane plants and insect vectors

Detection of the phytoplasmas has been based on the electron-microscopic examination of ultra-thin sections and transmission by insect vectors. However, such a method is not practical. Transmission tests are of limited value due to the latency and long incubation period in insects. Ultra-thin sectioning is a tedious procedure and requires expensive equipment. Progress has been made in the serological assay of phytoplasmas. Polyclonal antisera have been produced with partially purified SCWL phytoplasma antigens from infected sugarcane plants[44]. However, these antisera showed substantial cross-reactions with antigens from healthy plants. Therefore, an efficient and reliable method of detecting the pathogen is urgently needed for the selection of healthy cutting shoots, disease forecasting, quarantine procedures, screening of resistant cultivars, development of methods to control the disease and basic research on the behavior of the pathogens.
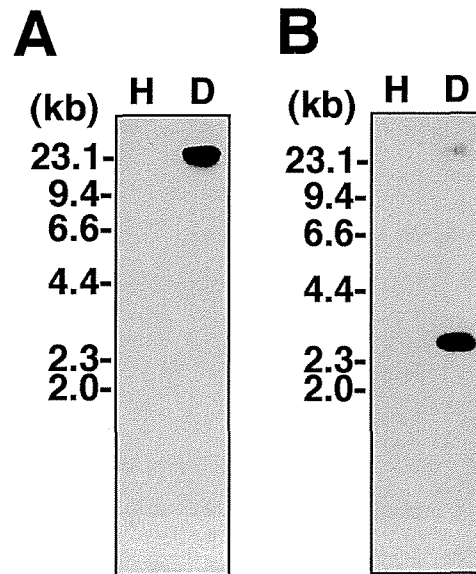
Fig. 2. Southern hybridization of undigested DNA from healthy sugarcane plants and sugarcane plants with white leaf disease
30). DNA from healthy sugarcane plants (H) and DNA from sugarcane plants with white leaf disease in Khon Kaen,
Thailand (D) were electrophoresed and blotted onto a nylon membrane. The membrane was hybridized with DNA
probe S28(A), and then hybridized with DNA probe S1 (B).

Chromosomal and extrachromosomal DNAs of the phytoplasmas were detected in SCWL-infected plant tissues by dot hybridization using a peroxidase-labeled probe S28 and probe S1[30] (Fig. 3). The extrachromosomal DNA probe gave higher hybridization signals than the chromosomal DNA probe. These results indicate that the copy number of the extrachromosomal DNA was considerably larger than that of the chromosomal DNA of SCWL within a cell of phytoplasmas, which is in agreement with previous data of RYD phytoplasmas[36]. DNA extracted from sugarcane plants with white leaves collected from Khon Kaen and Lampang (more than 200 leaves) was hybridized with the extrachromosomal DNA probe S1 and chromosomal DNA probe S28. These probes did not give out signals with healthy DNA specimens. Moreover, no signals were obtained using DNA from sugarcane leaves with white-line symptoms caused by Xanthomonas albilineans and mosaic leaves caused by sugarcane mosaic virus with these DNA probes (Fig. 3).

Chromosomal and extrachromosomal DNAs of the SCWL phytoplasmas were detected from all parts of the diseased plants maintained in a greenhouse using the DNA probes S28 and S1 (Table 1). In these specimens, young leaves showed severe chlorosis, and the older leaves recovered and showed green-yellow or white stripe symptoms. However, the DNA concentration of the phytoplasmas in younger leaves with severe chlorosis was not always high. As reported earlier, there was no correlation between the amount of rice yellow dwarf (RYD) phytoplasmas and the severity of chlorosis in RYD-infected tissues[32]. In such cases, the difference in the symptoms may be due to the growth stage of the leaves, rather than to the concentration of phytoplasmas. Otherwise it is possible that symptom-inducing metabolites synthesized by RYD or SCWL phytoplasmas had translocated through the phloem elements to the meristems.

Phytoplasma DNA was detected in insect vectors *M. hiroglyphicus* collected from the sugarcane fields infected with SCWL in Khon Kaen by using the extrachromosomal DNA probe S1[30]. Hybridization signals were distinct when the extrachromosomal DNA probe S1 was used, whereas the signals were faint when the chromosomal DNA probe S28 was used.
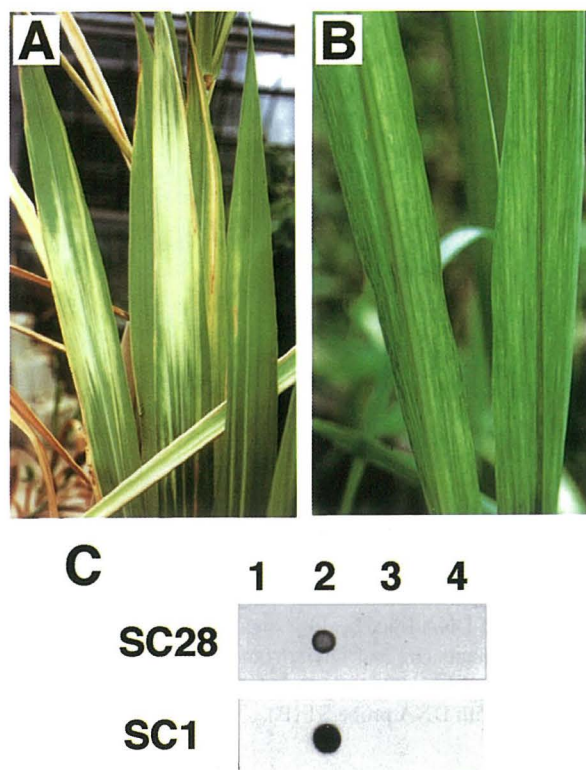
Fig. 3. Dot hybridization of the peroxidase-labeled, cloned DNA probes with the DNA from healthy and diseased sugarcane plants. (A) Symptoms of sugarcane plants infected with Xanthomonas albilineans. (B) Symptoms of sugarcane plants infected with sugarcane mosaic virus. (C) Detection of DNA of the SCWL phytoplasmas from sugarcane plants by dot hybridization using the chromosomal DNA probe S28 and the extrachromosomal DNA probe S1. DNA were extracted from healthy plants (1), plants infected with SCWL phytoplasmas (2), plants infected with *Xanthomonas albilineans* (3) and plants infected with sugarcane mosaic virus (4). DNA specimen extracted from 1 mg of tissues was spotted on nylon membranes and hybridized with the probes. Hybridization signals were detected by the ECL method (Amersham).

Our results indicated that the non-radioactive peroxidase-labeled extrachromosomal as well as chromosomal DNA probes are effective in assaying SCWL phytoplasmas. Extrachromosomal DNA probes gave much stronger signals than chromosomal DNA probes. It is considered that the extrachromosomal DNA probes may be a useful tool to detect the SCWL phytoplasmas, because they were isolated from all of the SCWL specimens and insect vectors of SCWL phytoplasma.

Recently, regions of the rRNA operon, including the complete 16S rDNA, the 16S/23S rDNA spacer region, a portion of 23S rDNA, and other genes of some phytoplasmas have been sequenced[12, 13, 18, 25, 40, 47]. These sequences have been used to develop PCR-based detection assays, which are specific for the phytoplasmas[7, 12, 21, 23, 24, 39, 45, 47, 49, 50]. Namba *et al.* (1993) developed a universal primer set to amplify the 16S rDNA of phytoplasmas[39]. We were able to detect the 16S rDNA of the SCWL phytoplasma from SCWL-infected sugarcane plants by the PCR method using the primer set[34, 35]. It is concluded that the PCR and the hybridization assays are effective tools to detect the SCWL phytoplasmas.

Phylogenetic relationship of SCWL phytoplasmas with other phytoplasmas and culturable mollicutes

The taxonomy of the phytoplasmas is problematic due to lack of molecular characterization of the phytoplasmas. They have been classified on the basis of the characteristics of the host-parasite interaction such as symptoms,

plant host range, and insect vectors. Although the symptoms of SCWL are similar to those of RYD, the pathogens are different based on the difference in pathogenicity and insect vectors[38, 43]. Until recently no direct method was available to utilize the variation of DNA in phytoplasmas. Several research groups have attempted to classify phytoplasmas in the light of DNA homology analyzed by random DNA probes[14, 16, 22, 37]. We examined the homology of 14 chromosomal DNA probes derived from a Khon Kaen (Northeast Thailand) isolate of SCWL phytoplasmas with the DNA of a Lampang (North Thailand) isolate of SCWL phytoplasmas, RYD phytoplasma isolates from Japan and Thailand, other types of phytoplasmas and some culturable mollicutes[30]. The data revealed that the SCWL phytoplasmas of Thailand shared a greater nucleotide sequence homology with the RYD phytoplasmas than with other phytoplasmas. The results also indicate that

SCWL phytoplasmas are not related to some culturable mycoplasmas and spiroplasmas.

Sequence analysis of the PCR-amplified rDNA fragments has been found to be useful for the phylogenetic analysis of phytoplasmas. The sequence data of the 16S rDNA of phytoplasmas suggested that phytoplasmas are more closely related to each other than they are to the major groups of culturable mollicutes[25, 40, 47]. Seemüller *et al.* (1994) reported a phylogenetic tree based on the sequence analysis of 16S rDNAs which showed five clusters of major phytoplasmas including SCWL designed as: (i) the aster yellows strain cluster, (ii) the apple proliferation strain cluster, (iii) the western-X disease strain cluster, (iv) the SCWL strain cluster and (v) the elm yellows strain cluster[47]. The SCWL cluster consists of the SCWL phytoplasmas, leafhopper strain BVK, and the RYD phytoplasmas (strain MLO-III) (Fig. 4).
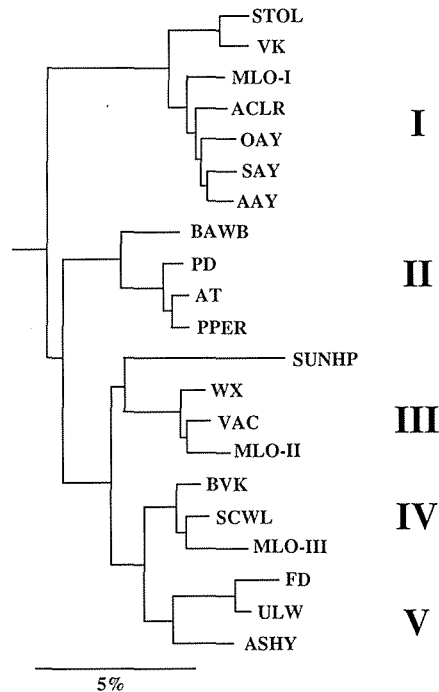

Fig. 4. Phylogenetic tree for the major phytoplasmas 47). Bar = evolutionary distance of 5%. The following strains were included: stolbur strain STOL; grapevine yellows (Vergilbungskrankheit) strain VK; onion yellows strain MLO-I; apricot chlorotic leafroll strain ACLR; oenothera aster yellows strain OAY; severe American aster yellows strain SAY; American aster yellows AAY; black alder witches' broom strain BAWB; pear decline strain PD; apple proliferation strain AT; European stone fruit yellows strain OOER; sunhemp witches' broom strain SUNHP; western X-disease strain WX; vaccinium witches' broom strain VAC; tsuwabuki witches' broom strain MLO-II; strain BVK from a leafhopper; sugarcane white leaf strain SCWL; rice yellow dwarf strain MLO-III; flavescence dorée strain FD; elm yellows strain ULW; and ash yellows strain ASHY.

Phylogenetic relationship among SCWL and other phytoplasmas in sugarcane fields

Many gramineous weeds showing white leaf symptoms were present in the vicinity of sugarcane fields infected with SCWL in Thailand and Taiwan[5, 38, 43]. Phytoplasmas were detected in these weeds by electron microscopy in Taiwan[5]. The insect vector of SCWL phytoplasmas did not transmit the SCWL phytoplasmas from the diseased sugarcane plants to the weeds or from the weeds to healthy

sugarcanes plants[43]. Several kinds of phytoplasma-infected plants were reported from fields with upland crops such as sugarcane and sesame in Northeast Thailand[34]. At least three other kinds of gramineous plants with white leaf symptoms were reported as: i) *Brachiaria* sp. (BraWL) (Fig. 5A), ii) Crowfoot grass (*Dactyloctenium aegypcium*) (DacWL) (Fig. 5B), and iii) Bermuda grass (*Cynodon dactylon*) (BGWL) (Fig. 5C). Moreover, several species with witches' broom symptoms were reported in *Indigofera juncea* (IWB),
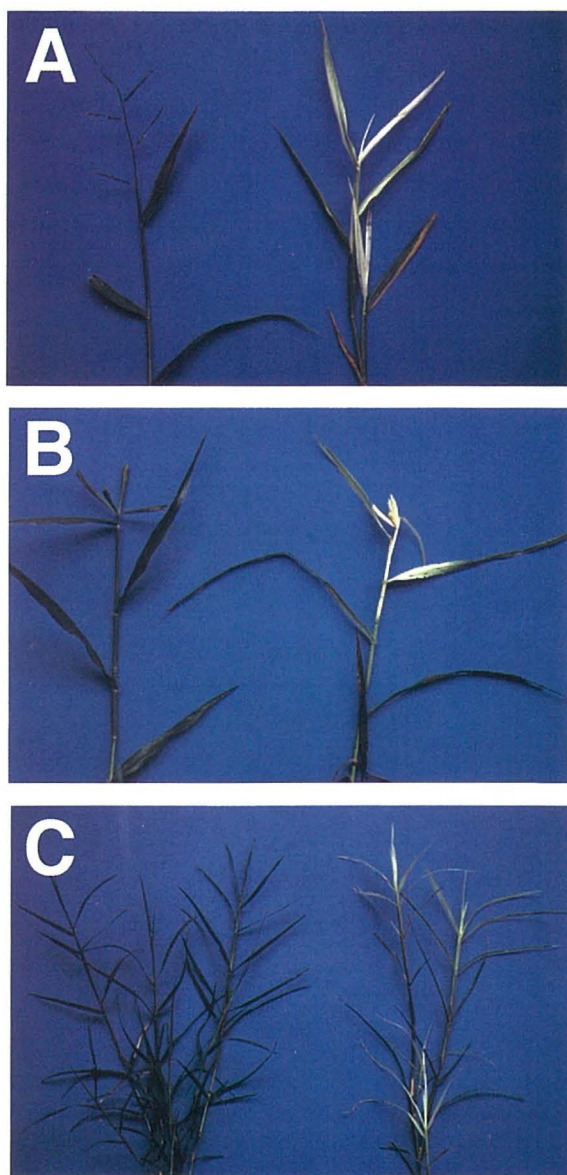

Fig. 5. White leaf symptoms of *Brachiaria* sp. (BraWL) (A), Crowfoot grass (*Dactyloctenium aegypcium*) (DacWL) (B), and Bermuda grass (*Cynodon dactylon*) (BGWL) (C) in Khon Kaen, Thailand.

Crotalaria incana (CWB), and *Trianthema portulacastrum* (TWB), besides sesame phyllody (SP) and *Richardia* sp. with phyllody (RP). Sarindu and Clark (1993) reported that polyclonal antibodies against a SCWL phytoplasma isolate did not react with other white leaf-associated isolates like BraWL, DacWL, and BGWL phytoplasma isolates, and antibodies against the BGWL phytoplasma isolate did not react with the SCWL, BraWL, DacWL, and BGWL phytoplasma isolates in Thailand[44]. However, the genetic relationship among the SCWL phytoplasmas and other phytoplasmas in the same fields has not been determined.

We performed a hybridization assay using DNA probes for the SCWL phytoplasmas with spots of the phytoplasma-infected plants in Khon Kaen[34]. Chromosomal DNA probe S28 derived from the SCWL phytoplasma isolate hybridized with the BraWL, BraWL, DacWL, DacWL, BGWL, and BGWL isolates, but not with the SP, RP, IWB, CWB, and TWB isolates.

To analyze the phylogenetic relationship of the pathogens, we compared the restriction profiles of the 16S rDNA sequence of phytoplasmas of the phytoplasma-affected plants[34]. Comparing the restriction enzymes, *Rsa* I or *Alu* I, fragment profiles of PCR-amplified 16S rDNA segments of the phytoplasmas revealed that the phytoplasmas associated with white leaf symptoms (SCWL, BraWL, DacWL, and BGWL) were related to a RYD phytoplasma, while the phytoplasmas associated with phyllody and witches' broom (SP, RP, IWB, CWB, and TWB) were closely related to a gentian witches' broom (GWB) phytoplasma[38]. These results indicated that SCWL, BraWL, DacWL, and BGWL phytoplasmas are probably members of the MLO-III cluster (Fig. 4)[39, 40], and SP, RP, IWB, CWB, and TWB phytoplasmas are members of the MLO-II cluster (Fig. 4)[39, 40]. Zreik *et al.* (1995) reported that the SP phytoplasmas showed genomic similarities with sunhemp phyllody phytoplasmas (SUNHP)[50]. SP phytoplasmas may belong to the MLO-II and SUNHP phylogenetic subgroup (Fig. 4).

Restriction enzyme *Acc* I fragment profiles of the PCR-amplified 16S rDNA of the SCWL phytoplasmas were different from those of BraWL, DacWL, BGWL, or RYD[35]. Moreover, fragment profiles of phytoplasmas associated with phyllody and witches' broom diseases in Northeast Thailand were different from those associated with white leaf diseases. Fragment profiles of the SP phytoplasmas were the same as those of the RP phytoplasmas, and the electrophoretic mobility of the majority, though not all, of DNA bands was the same as that of IWB, CWB, and TWB. The findings suggest that the SCWL phytoplasmas are different from the other phytoplasmas associated with other gramineous plants with white leaf symptoms, which is in agreement with the results of serological studies[44]. It is not possible to differentiate RYD, BraWL, DacWL, and BGWL phytoplasmas by restriction analysis. Since these phytoplasmas are considered to be related phylogenetically, the analysis of the 16S rDNA of phytoplasmas may enough to resolve the complexity of the phytoplasmas in fields though several kinds of the phytoplasmas associated with the same kind of symptoms occurred in a small patch of field.

Sugarcane grassy shoot disease (SGS), caused by a phytoplasma, is fairly widespread in India and Thailand[43]. The disease induces premature profuse tillering followed by yellowing of the leaves in sugarcane plants. Wongkaew *et al.* (1997) reported the differentiation of phytoplasmas associated with white leaf diseases of sugarcane and gramineous weeds and grassy shoot of sugarcane by the application of various molecular techniques like RFLP and sequencing of rRNA gene (Fig. 6)[49]. They concluded that SCWL and SGS are caused by different phytoplasmas. Sequence analysis of DNA obtained from BraWL, DacWL, and BGWL phytoplasmas did not enable to identify organisms similar to those infecting sugarcane and it is assumed that SGS may be caused by multiple phytoplasma groups[49]. Multiple phytoplasma infections have been reported in several other cases[4, 21, 35].
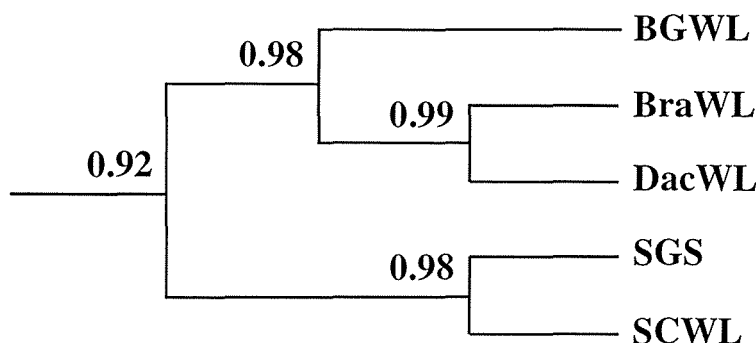
Fig. 6. Dendrogram of phytoplasmas infecting sugarcane and gramineous weeds generated from sequence data of the 3' end of the 16S rRNA and the spacer region of the phytoplasmas 49). The following strains were included: Bermuda grass white leaf strain BGWL, Dactyloctenium grass (Crowfoot grass) white leaf strain DacWL, Brachiaria grass white leaf strain BraWL; sugarcane grassy shoot strain SGS; and sugarcane white leaf strain SCWL.

Analysis of extrachromosomal DNA of SCWL phytoplasmas

The existence of plasmids or extrachromosomal DNA has been reported in some phytoplasmas and mollicutes[8, 9, 12, 15, 17, 19, 22, 30-38, 46]. Although partial nucleotide sequences of plasmids associated with the aster yellow group of phytoplasmas have been reported[12], information on the characteristics of extrachromosomal DNAs of the phytoplasmas is insufficient for determining its role in biological functions. To address this problem, we determined the sequence of the cloned extrachromosomal DNA of a Thai isolate of SCWL phytoplasmas[33].

Sequence analysis revealed that one of the extrachromosomal DNA clones, S1, was 2651 bp long, having *Hin*d III sites at both ends. By applying the inverse PCR method, we obtained inverse DNA fragments from one end of the S1 sequence to the other end of the sequence from the infected plant DNA. DNA was not amplified using healthy plant DNA as template. The sequence analysis of the amplified DNA revealed that the *Hin*d III site of one end of the S1 sequence is the same as that of *Hin*d III site of the other end. These data suggest that the extrachromosomal DNA is a circular DNA and that its length is 2645 bp. Our data are in agreement with those of Klinkong and Seemüller (1993) who reported that the extrachromosomal DNA of SCWL

phytoplasmas shows a closed circular form based on two-dimensional electrophoresis[15].

GC content (G + C ratio) of the sequence of the extrachromosomal DNA of SCWL phytoplasmas was only 22.2 % (A : 41.4 %, T : 36.4 %, C : 9.6 %, G : 12.6 %). The physical map of the extrachromosomal DNA is shown in Fig. 7. There were at least five putative open reading frames (ORFs) (Table 2). ORF 1 (positions 204 to 1250) and ORF 2 (positions 1750 to 2148) were coded in one direction of the sequence (Fig. 7A and Table 2). ORF 3 (positions 2012 to 1731), ORF 4 (positions 1369 to 1124) and ORF 5 (positions 466 to 233) were coded in the complementary sequence (Fig. 7B and Table 2). The ORF 4 stops at a TGA codon in the case of the unique codon-usage system of *Mycoplasma* spp. for the SCWL phytoplasmas[29], whereas the ORF 4 reads through the TGA codon for tryptophan and stops at the next stop codon TAA (ORF 4': positions 1369 to 983, Table 2) in the case of the universal codon-usage system of prokaryotes such as *Acholeplasma* spp.[29]. In the codon usage pattern of the other kinds of mollicutes[29], the GC content of the third base of the codons of each ORF exceeded 70 % (Table 2). The length of the other ORFs was shorter than 200 bp. The ORF 1 extended over 1047 bp, and encoded a polypeptide with 349 amino acids (Mr 41,494; hereafter referred to as the 41.5 K protein).

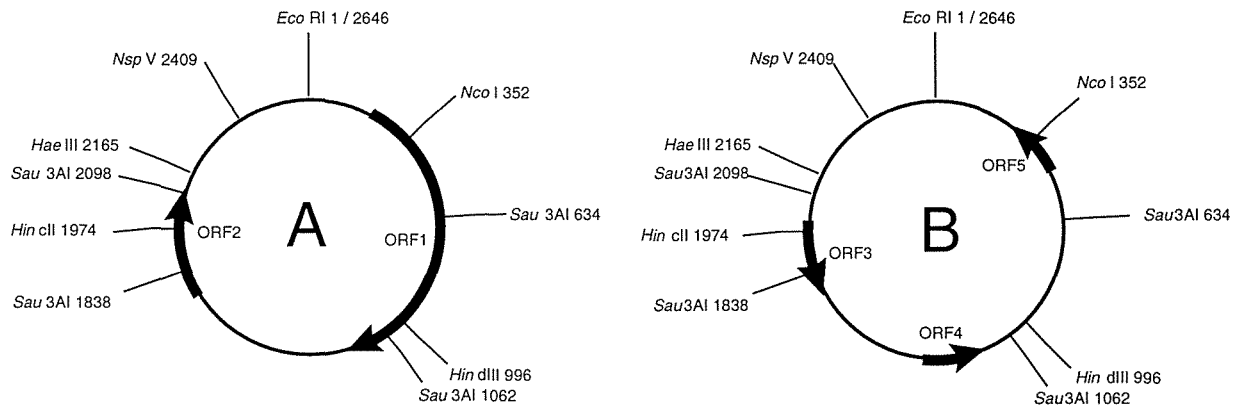The amino acid sequence was compared with

Fig. 7. Physical map of the extrachromosomal DNA of the SCWL phytoplasma 33). Circles A and B show the cutting sites of typical restriction enzymes and putative ORFs. Circle B shows the ORFs coded in the complementary sequence opposite to the direction in circle A.

Table 2. Summary of the ORFs of the extrachromosomal DNA of the SCWL phytoplasma

	Nucleotide Size	Termination Vodon	3rd Codon (A+T)	Peptide Size	Mr
ORF1	1047 bp	TAA	81%	349 a.a.	41,494
ORF2	399 bp	TAA	87%	133 a.a.	15,875
ORF3	282 bp	TAA	84%	94 a.a.	11,050
ORF4	246 bp	TGA	79%	82 a.a.	9,839
(ORF4'	234 bp	TAA	81%	129 a.a.	15,183)
ORF5	234 bp	TAA	73%	78 a.a.	8,860

a sequence database. The region between amino acids 204 and 269 of the 41.5 K protein and AL1 proteins of geminiviruses such as potato yellow mosaic virus (PYMV)[6] or AL1-like proteins of tobacco nuclear genome[3], was homologous in the sequence. The highest homology was 38.7 % between the region from amino acids 214 to 269 of the 41.5 K protein and the region from amino acids 140 to 201 of the AL1 protein of PYMV. The AL1 proteins are reported to be necessary for viruses to synthesize their genomic DNA segments. These results suggest that the 41.5 K protein of the ORF 1 may play a role in the replication of the extrachromosomal DNA. On the other hand, the predicted amino acid sequences of the other ORFs were compared with the database, but no similar proteins were found.

All the SCWL phytoplasma isolates collected from Thailand showed homology in their extrachromosomal DNAs. These molecules may play an important role in pathogenesis as in other cases of plant and animal pathogenic bacteria[41]. Denes and Sinha (1992) suggested that the extrachromosomal DNA of clover phyllody phytoplasmas played a certain role in the transmission by insect vectors[9]. Further studies are needed to clarify the function of the extrachromosomal DNAs of SCWL and other phytoplasmas.

Results from preferential hybridization of extrachromosomal DNA probes of SCWL and other phytoplasmas (RYD, SP, and AY phytoplasmas) with DNA of several phytoplasma-infected plants are summarized in Table 3[31]. Hybridization data indicated that the extrachromosomal DNAs of RYD, SCWL, and other phytoplasmas associated with white leaf diseases of gramineous plants were related to each other, and different from DNAs of phytoplasmas associated with other types of symptoms.

Table 3. Preferential dot hybridization of extrachromosomal DNA probes of four phytoplasmas with DNA preparations from plants infected with 12 phytoplasmas[a]

Source of phyoplasma DNA	DNA probe			
	Sugarcane white leaf S1	Rice yellow dwarf R32	Seame phyllody SP49 and 51	Aster yellows PAY45
Sugarcane whith leaf (Khon Kaen, Thailand)	++	w/-_b	-	-
Brachiaria white leaf (Khon Kaen, Thailand)	+	w/-_b	-	-
Dactyloctenium white leaf (Khon Kaen, Thailand)	+	w/-_b	-	-
Bermudagrass white leaf (Khon Kaen, Thailand)	++	++	-	-
Rice yellow dwarf (Tochigi, Japan)	++	++	-	-
Rice yellow dwarf (Chachoengsao, Thailand)	++	++	-	-
Sesame phyllody (Khon kaen, Thailand)	-	-	++	-
Rice orange leaf (Los Banos, Philippines)	-	-	-	++
Onion yellows (Saitama, Japan)	-	-	-	++
Paulownia witches' broom (Ibaraki, Japan)	-	-	-	++
Aater yellows (California, USA)	-	-	-	+
Gentian witches' broom (Fukushima, Japan)	-	-	-	-

a DNAs from several phytolasma-infected plants were blotted onto nylon membranes, and probed with extrachromosomal DNA probes of rice yellow dwarf, sugarcane white leaf, sesame phyllody, and aster yellows phytoplasmas. ++, strong hybridization signal; +, moderate hybridization signal; w, weak hybridization signal; -, no signal.

b Some specimens hybridized weakly with probe R32.

Extrachromosomal DNAs of rice orange leaf (ROL), onion yellows (OY), and paulownia witches' broom (PWB) phytoplasmas from Asia were related to those of AY phytoplasmas in USA. Extrachromosomal DNA of the SP phytoplasmas is different from that of all of them. The extrachromosomal DNA of phytoplasmas may thus be involved in the gene flow among phytoplasmas of gramineous plants and in the interactions between phytoplasmas and their hosts. Extrachromosomal DNA may even be related to the symptom expression because all the phytoplasmas that hybridized with the DNA probes of SCWL phytoplasmas were associated with chlorosis symptoms of leaves.

Extrachromosomal DNAs of SCWL phytoplasmas were polymorphic among the isolates collected from Khon Kaen, Thailand (Fig. 8)[31]. We reported that the extrachromosomal DNA of RYD phytoplasmas was also polymorphic among isolates collected from a small field in Japan[31]. However, we could not observe a polymorphism in the chromosomal DNA of SCWL

or RYD phytoplasmas. It is known that viral genomes such as the smallest fragment of rice dwarf phytoreovirus genome and satellite RNA of cucumber mosaic cucumovirus collected in a small field are polymorphic[2, 28]. Small molecules associated with virus and microorganisms including phytoplasmas may multiply rapidly and accumulate various sequences as far as they remain functional.

Conclusion and Prospects

The DNA-based detection methods of SCWL phytoplasmas and information about the genetic/phylogenetic characteristics of the SCWL and other phytoplasmas may contribute to the promotion of research on the epidemiology and plant-microbe interaction of the agent, and eventually to the development of methods of control of the SCWL disease.

In recent years there has been a growing interest in the use of molecular techniques as a tool for solving genetic and phylogenetic problems of
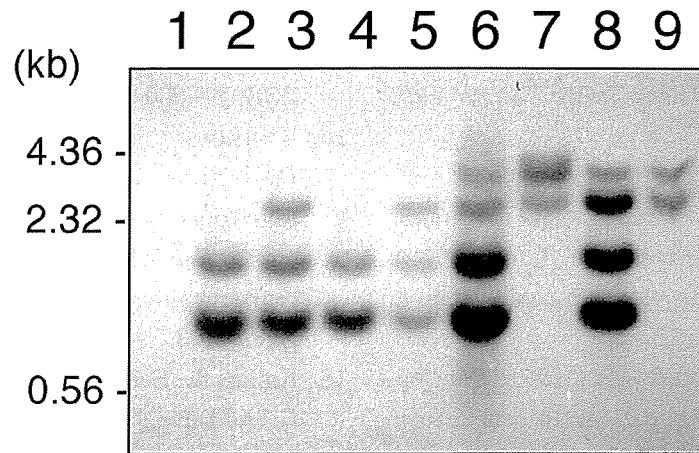
Fig. 8. Variation in extrachromosomal DNA from SCWL phytoplasma isolates collected in a field in Khon Kaen, Thailand 31). DNA from eight SCWL isolates (lanes 2-9) collected in the field and healthy sugarcane plants (lane 1) was digested with Hind III and EcoR I, electrophoresed with 0.7% agarose gel, blotted onto a nylon membrane, and hybridized with the extrachromosomal DNA probe S1.

phytoplasmas. Firrao et al. (1996) reported a physical map of the chromosome (genome) of the western X-disease phytoplasma[10]. The results suggested that the phytoplasma chromosome is circular and consists of approximately 670 kb. Thus it is possible to clone all the chromosomal DNA of the phytoplasma and determine the sequence of whole phytoplasma chromosomes. Analysis of the phytoplasma chromosomes may provide a framework for characterizing the genes important in pathogenicity and/or in vitro culture.

Crop varieties resistant to phytoplasmas are urgently needed. Garnier (1997)[11] has proposed a unique approach to develop phytoplasma-resistant varieties. She suggested that since the growth and metabolism of mollicutes are inhibited by antibodies, future control of these agents in plants could be considered. Indeed, it has been shown recently that transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack[48]. Transgenic tobacco plants expressing an antibody against the stolbur phytoplasmas have been developed[11]. They will now face the challenge to resist phytoplasmas. Biotechnology will facilitate the development of control methods as well as basic studies of phytoplasmas including SCWL phytoplasmas.

References

1) Arai, K. and Ujihara, K. (1989). Studies on sugarcane white leaf disease which occurred in Tanegashima-island. Bull. Fac. Agricul. Kagoshima Univ. 39: 9-16 [in Japanese].

2) Aranda, M. A., Edaile, A. and Fernando-Garcia-Arenal. (1993). Genetic variability and evolution of the satellite RNA of cucumber mosaic virus during natural epidemics. J. Virol. 67: 5896-5901.

3) Bejarano, E. R., Khashoggi, A., Witty, M. and Lichtenstein, C. (1996). Integration of multiple repeats of geminivirus DNA into the nuclear genome of tobacco during evolution. Proc. Natl. Acad. Sci. USA 93: 759-764.

4) Bianco, P. A., Davis, R. E., Prince, J. P., Lee, I.-M., Gundersen, D. E., Fortusini, A. and Belli, G. (1993). Double and single infections by aster yellows and elm yellows MLOs in grapevines with symptoms characteristic of flavescence doree. Riv. Patol. Veg. 3: 69-82.

5) Chen, C. T., Lee, C. S. and Chen, M. L. (1972).

Mycoplasma-like organisms in *Cynodon dactylon* and *Brachiaria distachya* affected by white leaf diseases. *Rep. Taiwan Sugar Exp. Stn.* **56**: 49-55.

6) Coutts, R. H. A., Coffin, R. S., Roberts, E. J. F. and Hamilton, W. D. O. (1991). The nucleotide sequence of the infectious cloned DNA components of potato yellow mosaic virus. *J. Gen. Virol.* **72**: 1515-1520.

7) Davis, R. E. and Lee, I.-M. (1993). Cluster-specific polymerase chain reaction amplification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. *Phytopathology* **83**: 1008-1011.

8) Denes, A. S. and Sinha, R. C. (1991). Extrachromosomal DNA elements of plant pathogenic mycoplasmalike organisms. *Can. J. Plant Pathol.* **13**:26-32.

9) Denes, A. S. and Sinha, R. C. (1992). Alteration of clover phyllody mycoplasma DNA after *in vitro* culturing of phyllody-diseased clover. *Can. J. Plant Pathol.* **14**: 189-196.

10) Firrao, G., Smart, C. D. and Kirkpatrick, B. C. (1996). Physical map of the Western X-disease phytoplasma chromosome. *J. Bacteriol.* **178**: 3985-3988.

11) Garnier, M. (1997). Non-cultivable phytopathogenic mycoplasmas: characterization, detection and perspectives for control. *Wien. Klin. Wochenschr.* **109**: 613-617.

12) Goodwin, P. H., Xue, B. G., Kuske, C. R. and Sears, M. K. (1994). Amplification of plasmid DNA to detect plant pathogenic mycoplasmalike organisms. *Ann. Appl. Biol.* **124**: 27-36.

13) Gundersen, D. E., Lee, I.-M., Schaff, D. A., Harrison, N. A., Chang, C. J., Davis, R. E. and Klingsbury, D. T. (1996). Genetic diversity and differentiation among phytoplasma strain in 16S rRNA groups I (aster yellows and related phytoplasmas) and III (X-disease and related phytoplasmas). *Int. J. Syst. Bacteriol.* **46**: 64-75.

14) Kirkpatrick, B. C., Stenger, D. C., Morris, T. J. and Purcell, A. H. (1987). Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. *Science* **238**: 197-200.

15) Klinkong, S. and Seemüller, E. (1993). Detection and differentiation of the mycoplasmalike organism associated with sugarcane white leaf disease using cloned extrachromosomal DNA probe. *Kasetsart J.* **27**: 98-103.

16) Kollar, A., Seemüller, E., Bonnet, F., Saillard, C. and Bove, J. M. (1990). Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. *Phytopathology* **80**: 233-237.

17) Kuske, C. R. and Kirkpatrick, B. C. (1990). Identification and characterization of plasmids from the western aster yellows mycoplasmalike organism. *J. Bacteriol.* **172**: 1628-1633.

18) Kuske, C. R. and Kirkpatrick, B. C. (1992). Phylogenetic relationships between the western aster yellows mycoplasmalike organism and other prokaryotes established by 16S rRNA gene sequence. *Inter. J. Syst. Bacteriol.* **42**: 226-233.

19) Kuske, C. R., Kirkpatrick, B. C., Davis, M. J. and Seemüller, E. (1991). DNA hybridization between western aster yellows mycoplasmalike organism plasmid and extrachromosomal DNA from other plant pathogenic mycoplasmalike organisms. *Mol. Plant-Microbe Interact.* **4**: 75-80.

20) Kuske, C. R., Kirkpatrick, B. C., Davis, M. J. and Seemüller, E. (1991). Differentiation of virescence phytoplasmas using western aster yellows mycoplasma-like organism chromosomal DNA probes and restriction fragment length polymorphism analysis. *J. Gen. Microbiol.* **137**: 153-159.

21) Lee, I.-M., Bertaccini, A., Vibio, M. and Gundersen, D. E. (1995). Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. *Phytopathology* **85**: 728-735.

22) Lee, I.-M. and Davis, R. E. (1992).

Mycoplasmas which infect plants and insects. *In* Mycoplasmas-Molecular biology and pathogenesis. ed. Maniloff, J., McElhancy, R. N., Finch, L. R., and Baseman, J. B., American Society for Microbiology, Washington, D. C., 379-390.

23) Lee, I.-M., Gunderson, D. E., Hammond, R. W. and Davis, R. E. (1994). Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology* **84**: 559-566.

24) Lee, I.-M., Hammond, R. W., Davis, R. E. and Gundersen, D. E. (1993). Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology* **83**: 834-842.

25) Lim, P.-O. and Sears, B. B. (1989). 16S rRNA sequence indicates that plant-pathogenic mycoplasmalike organisms are evolutionary distinct from animal mycoplasmas. *J. Bacteriol.* **171**: 5901-5906.

26) Ling, K. C. (1962). White leaf disease of sugarcane. *Taiwan Sugar,* **9**: 1-5.

27) Mangelsdorf, A. J. (1962). A research program for the Thailand sugar industry. Department of Agriculture, Bangkok, 1-16.

28) Murao, K., Suda, N., Uyeda, I., Isogai, M., Suga, H., Yamada, N., Kimura, I. and Shikata, E. (1994). Genetic heterogeneity of rice dwarf phytoreovirus field isolates and nucleotide sequences of variants of genome segment 12. *J. Gen. Virol.* **75**: 1843-1848.

29) Muto, A., Andachi, Y., Yamao, F., Tanaka, R. and Osawa, S. (1992). Transcription and translation. *In* Mycoplasmas - Molecular biology and pathogenesis. ed. Maniloff, J., McElhancy, R. N., Finch, L. R., and Baseman, J. B., American Society for Microbiology, Washington, D. C., 331-347.

30) Nakashima, K., Chaleeprom, W., Wongkaew, P. and Sirithorn, P. (1994). Detection of mycoplasma-like organism associated with white leaf disease of sugarcane in Thailand

using DNA probes. *JIRCAS J.* **1**: 57-67.

31) Nakashima, K. and Hayashi, T. (1995). Extrachromosomal DNAs of rice yellow dwarf and sugarcane white leaf phytoplasmas. *Ann. Phytopathol. Soc. Jpn* **61**: 456-462.

32) Nakashima, K. and Hayashi, T. (1997). Multiplication and distribution of rice yellow dwarf phytoplasma in infected tissues of rice and green rice leafhopper *Nephotettix cincticeps. Ann. Phytopathol. Soc. Jpn* **61**: 451-455.

33) Nakashima, K. and Hayashi, T. (1997). Sequence analysis of extrachromosomal DNA of sugarcane white leaf phytoplasma. *Ann. Phytopathol. Soc. Jpn* **63**: 21-25.

34) Nakashima, K., Hayashi, T., Chaleeprom, W., Wongkaew, P. and Sirithorn, P. (1995). Detection of DNA of phytoplasmas associated with phyllody disease of sesame in Thailand. *Ann. Phytopathol. Soc. Jpn* **61**: 519-528.

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

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

37) Nakashima, K., Kato, S., Iwanami, S. and Murata, N. (1993). DNA probes reveal relatedness of rice yellow dwarf mycoplasmalike organisms (MLOs) and distinguish them from other MLOs. *Appl. Environ. Microbiol.* **59**: 1206-1212.

38) Nakashima, K. and Murata, N. (1993). Destructive plant diseases caused by mycoplasma-like organisms in Asia. *Outlook on Agriculture* **22**: 53-58.

39) Namba, S., Kato, S., Iwanami, S., Oyaizu, H., Shiozawa, H. and Tsuchizaki, T. (1993). Detection and differentiation of plant-pathogenic mycoplasmalike organisms using

polymerase chain reaction. *Phytopathology* **83**: 786-791.

40) Namba, S., Oyaizu, H., Kato, S., Iwanami, S. and Tsuchizaki, T. (1993). Phylogenetic diversity of phytopathogenic mycoplasma-like organisms. *Int. J. Syst. Bacteriol.* **43**: 461-467.

41) Panopolous, N. and Peet, R. C. (1985). The molecular genetics of plant pathogenic bacteria and their plasmids. *Ann. Rev. Phytopathol.* **23**: 381-419.

42) Razin, S. (1992). Mycoplasma Taxonomy and Ecology. *In* Mycoplasmas - Molecular biology and pathogenesis. ed. Maniloff, J., McElhancy, R. N., Finch L. R., and Baseman, J. B., American Society for Microbiology, Washington, D. C., 3-22.

43) Rishi, N. and Chen, C. T. (1989). Grassy shoot and white leaf diseases. pp. 289-300, *In* Diseases of sugarcane - Major Diseases. ed. Ricaud, B. C., Egan, B. T., Gillaspie, A. G. Jr and Hughes, C. G., Elsvier, Amsterdam, pp. 399.

44) Sarindu, N. and Clark, M. F. (1993). Antibody production and identity of MLOs associated with sugar-cane whiteleaf disease and bermuda-grass whiteleaf disease from Thailand. *Plant Pathol.* **42**: 396-402.

45) Schneider, B., Ahrens, U., Kirkpatrick, B. C. and Seemüller, E. (1993). Classification of plant-pathogenic mycoplasma-like organisms using restriction-site analysis of PCR-amplified 16S rDNA. *J. Gen. Microbiol.* **139**: 519-527.

46) Schneider, B., Maurer, R., Saillard, C., Kirkpatrick, B. C. and Seemüller, E. (1992). Occurrence and relatedness of extrachromosomal DNAs in plant pathogenic mycoplasmalike organisms. *Mol. Plant-Microbe Interact.* **5**: 489-495.

47) Seemüller, E., Schneider, B., Maurer, R., Ahrens, U., Dire, X., Kison, H., Lorenz, K.-H., Firrao, G., Avinent, L., Sears, B. B. and Stackebrandt (1994). Phylogenetic classification of phytopathogenic mollicutes by sequence analysis of 16S ribosomal DNA. *Int. J. Syst. Bacteriol.* **44**: 440-446.

48) Tavladoraki, P., Benvenuto, E., Trinca, S., De Martinis, D., Cattaneo, A. and Galeffi, P. (1993) Transgenic plants expressing a functional single-chain Fv antibody are specifically protected from virus attack. *Nature* **366**: 469-472.

49) Wongkaew, P., Hanboonsong, Y., Sirithorn, P., Choosai, C., Boonkrong, S., Tinnangwattana, T., Kitchareonpanya, R. and Damak, S. (1997). Differentiation of phytoplasmas associated with sugarcane and gramineous weed white leaf disease and sugarcane grassy shoot disease by RFLP and sequencing. *Theor. Appl. Genet.* **95**: 660-663.

50) Zreik, L., Carle, P., Bove, J. M. and Garnier, M. (1995). Characterization of the mycoplasmalike organism associated with witches' broom of lime and proposition of a *Candidatus* taxon for the organism, "*Candidatus* phytoplasma aurantifolia". *Int. J. Syst. Bacteriol.* **45**: 449-453.

分子生物学的手法を用いたサトウキビ白葉病
ファイトプラズマの検出と性状解析

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摘　要

　サトウキビ白葉病（SCWL）は，タイのサトウキビにおいて最も重要な病害である。この病害は，細胞壁を欠いた微生物であるマイコプラズマーファイトプラズマーより引き起こされている。ファイトプラズマを人工培養することができないために，病原体の性状の詳細は不明で，病原体を検出することも困難であった。我々は，SCWLファイトプラズマのDNAをサトウキビから分離し，そのDNA断片を大腸菌でクローニングした。これらの断片をDNAプローブとして用いたハイブリダイゼーションアッセイにより，植物および媒介昆虫から病原体を簡便に検出することが可能になった。さらにPCR法の適用により，SCWLファイトプラズマのリボソームRNA遺伝子（rDNA）を迅速に検出することも可能になった。ハイブリダイゼーションアッセイとrDNAの解析から，SCWLファイトプラズマのゲノムはイネ黄萎病（RYD）ファイトプラズマのゲノムと似ていることが示された。またrDNAの解析から，SCWLファイトプラズマは，東北タイで発生しているサトウキビグラッシーシュートファイトプラズマや，白葉症状の雑草で見出されたファイトプラズマに近縁であるが，それらは同一ではないことも示された。一方SCWLファイトプラズマのDNA断片の解析から，このファイトプラズマは長さ約2.6 kbの環状の染色体外DNA（プラスミド）を持つことが明らかになった。この染色体外DNAは，RYDファイトプラズマの染色体外DNAや，白葉症状の雑草で見出されたファイトプラズマが持つ染色体外DNAと相同性が高いが，ゴマ葉化病ファイトプラズマが持つ染色体外DNAや，アスターイエローズ型のファイトプラズマが持つ染色体外DNAとの相同性は低いことが示された。RFLP解析により，この染色体外DNAはサトウキビ畑において構造が多様であることが明らかになった。開発されたファイトプラズマDNAの検出法やファイトプラズマのDNAに関する研究は，サトウキビ白葉病を防除するために役立つことが期待される。

キーワード：サトウキビ白葉病，ファイトプラズマ，DNA，迅速診断，遺伝学的性状

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