Detection of Mycoplasma-like Organisms Associated with White Leaf Disease of Sugarcane in Thailand using DNA Probes*

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Abstract

Sugarcane white leaf disease (SCWL) is one of the most destructive diseases of sugarcane in Thailand. An efficient and reliable method to detect the pathogen is urgently needed for the development of methods of control of the disease. We isolated the DNA of the causal agent, a mycoplasma-like organism (MLO), cloned random DNA fragments, and used them as DNA probes to detect the pathogen rapidly and easily. DNA of the SCWL MLO was isolated from the host DNA by repeated bisbenzimide-CsCl equilibrium density gradient centrifugation. The DNA fragments cut by restriction enzyme *Hin*dIII were cloned in a plasmid-*Escherichia coli* system. Southern hybridization analysis revealed that the inserts of some recombinant plasmids consisted of fragments of chromosomal DNA of the MLO, whereas the inserts of the other recombinant plasmids consisted of fragments of extrachromosomal DNA of the MLO. Cloned DNA probes were successfully applied in dot hybridization to detect the MLO in sugarcane plants and in the insect vector *Matsumuratettix hiroglyphicus*. Dot hybridization using the DNA probes indicated that SCWL MLO and MLOs of gramineous plants including rice yellow dwarf MLO shared a greater nucleotide sequence homology with one another than with other MLOs. The method developed was found to be useful to detect the pathogen infecting sugarcane plants in the fields of the Khon Kaen area in Thailand.

Additional key words : MLO, dot hybridization

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Introduction

In Thailand, sugarcane white leaf disease (SCWL) widely occurs in major cane-growing areas and is one of the most destructive sugarcane diseases[12,13,20]. The typical symptoms of this disease consist of total chlorosis in the spindle portion and profuse tillering. The disease is caused by a mycoplasma-like organism (MLO) transmitted by leafhoppers *Matsumuratettix hiroglyphicus*[12,20]. Because so far it has not been possible to culture MLOs *in vitro*, there is a lack of information about the MLOs, including the genetic and phylogenetic relationship among the SCWL MLO, other MLOs and culturable mycoplasmas (mollicutes). Like many other plant diseases caused by MLOs, 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 MLOs[20].

Detection of the MLO 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 the 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 MLOs. Polyclonal antisera have been produced with partially purified SCWL MLO antigens from infected sugarcane plants[22]. 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 of control of the disease and basic research on the behavior of the pathogens. The major purpose of our study is to develop methods of detection of SCWL MLO and eventually to develop methods of control of the SCWL disease.

A hybridization method using DNA probes and a polymerase chain reaction (PCR) method have been developed and used in the assay and genetic/phylogenetic studies of some MLOs[2,4-6,8-11,17,18,22,23]. In the present study, we cloned random fragments of DNA of the SCWL MLO collected in Khon Kaen, Northeast Thailand, and attempted to detect the pathogen from host plants and insect vectors. We examined the homology of 14 chromosomal and three extrachromosomal DNA probes of SCWL MLO with two isolates of SCWL MLO, three isolates of MLOs of gramineous weeds with white leaf symptoms collected in Northeast Thailand, two isolates of rice yellow dwarf (RYD) MLO, nine of other MLOs and four of different species of culturable mollicutes. It was shown that SCWL MLO and MLOs of gramineous plants including RYD MLO formed a genetic cluster distinct from any other MLOs of other kinds of plants. The method developed was found to be effective in detecting the pathogen in small samples from various portions of sugarcane plants grown in the Khon Kaen area.

Materials and methods

Materials. MLO isolates used in the experiments are shown in Table 1. A Khon Kaen isolate and a Lampang isolate of sugarcane (*Saccharum officinarum*) white leaf (SCWL-K and SCWL-L) were collected from diseased plants in Khon Kaen, Northeast Thailand, and Lampang, North Thailand, respectively. Gramineous weeds, *Brachiaria* species, *Dactyloctenium aegyptium* and Bermuda grass (*Cynodon dactylon*), with white leaf symptoms (BraWL, DacWL and BGWL) were collected in sugarcane fields infected with SCWL in Khon Kaen, Thailand. A Tochigi isolate of rice (*Oryza sativa*) yellow dwarf (RYD-To)[14-16] was collected from Ohtawara in Tochigi Prefecture, Japan and a Chachoengsao isolate (RYD-Th) in Chachoengsao, Thailand[15,16] was provided by the Department of Agriculture, Thailand. These isolates were maintained in rice plants sequentially renewed and inoculated with MLO-infected *Nephotettix cincticeps*. Plant materials infected with an onion

Table 1 MLO isolates and culturable mollicutes used in the experiments

Code	MLO isolates/Culturable mollicutes	Origin
	MLO isolates	
SCWL-K	Sugarcane white leaf MLO	Khon Kaen, Thailand
SCWL-L	Sugarcane white leaf MLO	Lampang, Thailand
BraWL	*Brachiaria* white leaf MLO	Khon Kaen, Thailand
DacWL	*Dactyoctenium* white leaf MLO	Khon Kaen, Thailand
BGWL	Bermuda grass white leaf MLO	Khon Kaen, Thailand
RYD-To	Rice yellow dwarf MLO	Tochigi, Japan
RYD-Th	Rice yellow dwarf MLO	Chachoengsao, Thailand
OY	Onion yellows MLO	Saitama, Japan
CWB	Cineraria witches' broom MLO	Saitama, Japan
JHWB	Japanese hornwort witches' broom MLO	Ibaraki, Japan
WDY	Water dropwort yellows MLO	Ibaraki, Japan
GWB	Gentian witches' broom MLO	Fukushima, Japan
UD	Udo dwarf MLO	Niigata, Japan
TWB	Tsuwabuki witches' broom MLO	Miyazaki, Japan
PWX	Peach western X MLO	California, USA
PD	Pear decline tree MLO	California, USA
	Culturable mollicutes	
SC	*Spiroplasma citri* Morocco-R8-A2	
SK	*Spiroplasma kunkelii* E275	
MH	*Mycoplasma hominis* PG21	
MO	*Mycoplasma orale* CH19299	

(*Allium cepa*) yellows (OY) MLO, a cineraria (*Senecio cruentus*) witches' broom (CWB) MLO, a Japanese hornwort (*Cryptotaenia japonica*) witches' broom (JHWB) MLO, a water dropwort (*Oenanthe javanica*) yellows (WDY) MLO, a gentian (*Gentiana triflora*) witches' broom (GWB) MLO, an udo (*Aralia cordata*) dwarf (UD) MLO, and a tsuwabuki (*Forfugium japonicum*) witches' broom (TWB) MLO were provided by the National Agriculture Research Center, Tsukuba, Japan[15,16]

Total DNA was extracted from the plants showing typical symptoms. Presence of MLOs in the plant tissues was confirmed by electron microscopy and/or transmissibility of vector insects.

Nucleic acids extracted from periwinkle (*Catharanthus roseus*) with peach (*Prunus persica*) western X (PWX) MLOs, and pear (*Pyrus* species) decline (PD) tree MLOs were provided by the University of California, Davis, USA.

Spiroplasma citri Morocco-R8-A2, S. *kunkelii* E275, *Mycoplasma hominis* PG21 and *M. orale* CH19299 were cultured as described elsewhere[15].

Sugarcane leaves with white streak (leaf scald) caused by *Xanthomonas albilineans* and leaves with mosaic symptoms caused by sugarcane mosaic virus were collected from sugarcane fields in Khon Kaen, Thailand and Tanegashima island, Japan.

Electron Microscopy. Sugarcane leaves with white leaf symptom collected in Khon Kaen were cut into approximately 1 x 2 mm pieces and prefixed with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.6) for two hours at room temperature. The specimens were rinsed with the buffer containing 0.5% sucrose and then postfixed with 2% buffered osmium tetroxide for 2 hrs at 4℃. Afterwards, the specimens were dehydrated in a graded series of

ethanol and embedded in Spurr-resin. Ultra-thin sections were cut with a diamond knife, stained with uranyl acetate and lead nitrate, and observed under a Hitachi H-7000 transmission electron microscope.

DNA extraction. Sugarcane leaves showing typical symptoms were surface-sterilized with 1% sodium hypochlorite for 5 min and rinsed twice in sterile distilled water. Total DNA was extracted from crude vascular bundles separated from the midribs of the leaves by the CTAB method[6,14]. The DNA of SCWL MLO was purified by repeated bisbenzimide-CsCl equilibrium density gradient centrifugation[6,14].

Molecular cloning of DNA fragments of MLO. Cloning was performed according to standard procedures[21]. MLO DNA digested with restriction nuclease *Hin*dIII. Resulting fragments were electrophoresed in 0.7% agarose with a low-melting-temperature. The DNA fragments larger than 1.0 kb extracted from the gel and ligated with *Hin*dIII-digested plasmid Bluescript KS II+ (Stratagene). Then the recombinant plasmids were electroporated into cells of E. *coli* NM522 by using a BioRad Gene Pulsar according to the instructions of the manufacturers. Transformants were screened for MLO insert DNA by a differential dot hybridization assay as described elsewhere[14].

Preparation of DNA probes. DNA segments of recombinant plasmid were amplified from the vector plasmid by a PCR procedure[15]. The amplified DNA molecules were labeled with horseradish peroxidase using the Amersham ECL gene detection system according to the instructions of the manufacturers.

Dot hybridization. DNA extracted from approximately equal amounts of MLO-infected and healthy plant tissues (twofold dilution from DNA extracted from 10 mg of tissues) were denatured and blotted onto nylon membranes (Hybond N+ ; Amersham). DNA samples from *M. hiroglyphicus* collected in the sugarcane fields infected with SCWL in Khon Kaen, Thailand were also denatured and spotted onto the membrane with MLO-free *N. cincticeps* serving as a control. The membranes were hybridized with peroxidase-labeled DNA probes. Hybridization was performed by using Amersham ECL gene detection system. After hybridization, the membrane was washed under high stringency conditions as described elsewhere[15]. Hybridization with more than two probes was successively conducted on the same membrane without interference. The method allowed the easy comparison of the signal. The A_{633} values of the hybridization spots on the film were measured with an LKB laser densitometer 2222-020 UltroScan XL.

Southern hybridization. Undigested DNA specimens extracted from healthy and MLO-infected plant tissues (ca. 10 mg) were electrophoresed in a 0.7% agarose gel in TBE buffer[21], transferred to a nylon membrane (Hybond N ; Amersham) by a capillary method[21] and hybridized with the DNA probes. Hybridization and signal detection were performed as described above.

Results

Identification of MLOs. Sugarcane plants showing white leaf symptoms were collected in Khon Kaen, Northeast Thailand (Fig.1A). The typical MLO cell structures were observed in sieve elements from the ultra-thin sections of the diseased plants by electron microscopy (Fig.1B). They lacked cell walls and were amorphous. The approximate size was 0.1-1.0μm.

Isolation of MLO DNA. Bisbenzimide-CsCl equilibrium density gradient centrifugation of the DNA from crude vascular bundles of sugarcane plants with SCWL-disease revealed a main band and an upper faint band, whereas that of the DNA of

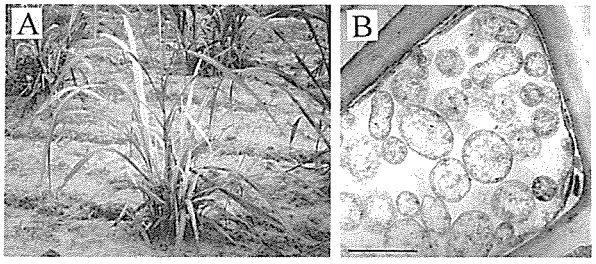

Fig.1. Symptoms and pathogens of sugarcane white leaf disease (SCWL). (A) Symptoms of SCWL in Khon Kaen, Northeast Thailand. (B) Causal agent, mycoplasma-like organism (MLO) of SCWL. Bar represents 0.5 μm

healthy plants showed only the main band (data not shown). As the upper band is specific to diseased plants, the DNA of the band is assumed to correspond to the DNA of SCWL MLO. The DNA of SCWL MLO was concentrated by repeated centrifugation. Less than 1.0 μg of MLO DNA was recovered from 50-100 μg of the total DNA per gram tissue of the diseased plants (fresh weight).

Cloning of MLO DNA. More than 200 transformants were recovered by cloning of the DNA fragments of SCWL MLO. By differential hybridization, 43 of 50 recombinant plasmids reacted with the peroxidase-labeled DNA of SCWL MLO but not with the labeled DNA of healthy sugarcane plants. Twenty-six recombinant plasmids showed hybridization signals with the labeled DNA of SCWL MLO more than 10 times stronger compared with the other 17 recombinant plasmids. The estimated molecular sizes of the cloned inserts showing stronger signals were all 2.5 kb, and of those showing weaker signals 1.6-3.5 kb. Three recombinant plasmids showing relatively strong positive signals and 14 recombinant plasmids showing relatively weak signals with the labeled DNA of SCWL-MLOs were selected from 43 positive clones (Table 2). The inserts of three recombinant

Table 2 Summary of results from preferential dot hybridization of DNA probes of SCWL MLO to DNA preparations from several MLO-infected plants and culturable mollicutesa

Sammple		Chromosomal DNA probe								Extrachromosomal DNA probe
		S28 S38	S47	S6 S31	S19	S21	S10	S46	S50	S 3 S23	S43 S44	S1 S37	S49
MLO isolates	SCWL-K	+	+	+	+	+	+	+	+	+	
	SCWL-L	+	+	+	+	+	+	+	+	+	
	BraWL	+	+	+	+	+	+	+	+	+	
	DacWL	+	+	+	+	+	+	+	+	+	
	BGWL	+	+	+	+	+	+	+	+	+	
	RYD-To	+	+	+	+	+	+	+	+	+	
	RYD-Th	+	+	+	+	+	+	+	+	+	
	OY	−	+	−	+	+	−	+	+	−	
	CWB	−	+	−	+	+	+	+	+	−	
	JHWB	−	+	−	−	+	+	+	+	−	
	WDY	−	+	+	+	+	+	+	+	−	
	GWB	−	−	+	+	+	+	+	+	−	
	UD	−	−	+	+	+	+	+	+	−	
	TWB	−	−	+	+	+	+	+	+	−	
	PWX	−	−	−	−	−	+	−	+	−	
	PD	−	−	−	−	−	+	+	+	−	
Mollicutes	SC	−	−	−	−	−	−	−	−	−	
	SK	−	−	−	−	−	−	−	−	−	
	MH	−	−	−	−	−	−	−	−	−	
	MO	−	−	−	−	−	−	−	−	−	

a +, positive hybridization signal;-, negative hybridization signal.

plasmids with the 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 MLO specific to the disease. The typical hybridization patterns are shown in Fig.2. The genome size of the mollicutes is considered to be 500-1,500 kb[20]. Therefore, we assume that the inserts of the recombinant plasmids showing strong signals with the DNA of the diseased plants were derived from the extrachromosomal DNA of SCWL MLO, and those of the other 14 recombinant plasmids were derived from the chromosomal DNA of SCWL MLO.

Detection of MLO in plants. Chromosomal DNA of MLOs was detected in a minimum of 0.13 mg of sugarcane tissue by using a S28 probe (Fig.3A), whereas extrachromosomal DNA was detected in a minimum of less than 0.01 mg of plant tissue by using a Sl probe (Fig.3B). Although the minimum amount varied depending on the specimens, the extrachromosomal DNA probe gave higher hybridization signals than the chromosomal DNA probe. All of the DNA specimens extracted from white leaves collected in Khon Kaen and Lampang (more than 200 leaves) hybridized with the extrachromosomal DNA probe Sl as well as chromosomal DNA probe S28. These probes did not give out signals with healthy DNA specimens. Green leaves from sugarcane hills without white leaf disease, leaves with white-line symptoms caused by *Xanthomonas albilineans* and mosaic leaves caused by sugarcane mosaic virus did not react with these DNA probes (data not shown). Chromosomal and extrachromosomal DNA of SCWL MLO were detected from all parts of the diseased plants using the DNA probes S28 and Sl (Table 3). The amounts of DNA of SCWL MLO in leaf blades were larger than those in leaf sheaths. The youngest 1-3 leaves showed severe chlorosis (chlorophyll contents were ca. 0-10 SPAD) and the older leaves showed green-yellow (chlorophyll contents were ca. 10-20 SPAD)

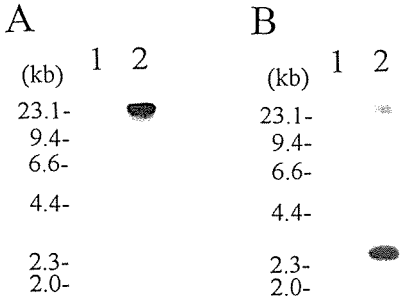

Fig.2. Southern hybridization of undigested DNA from healthy sugarcane plants and sugarcane plants with white leaf disease. DNA from healthy sugarcane plants (lane 1) and DNA from sugarcane plants with white leaf disease in Khon Kaen, Thailand (lane 2) were electrophoresed and blotted onto a nylon membrane. The membrane was hybridized with DNA probe S28 (A), and then hybridized with DNA probe Sl (B)

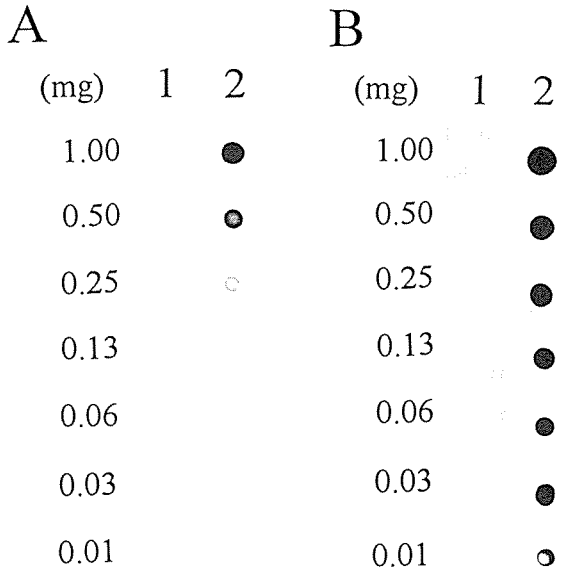

Fig.3. Dot hybridization of the peroxidase-labeled, cloned DNA probes of SCWL MLO with the DNA extracted from healthy sugarcane plants (column 1) and sugarcane plants with white leaf disease in Khon Kaen, Thailand (column 2). Twofold serial dilutions of the extracted DNA samples were blotted (top to bottom) onto nylon membranes. The membranes were hybridized with the labeled chromosomal probe S28 (A) and hybridized with the labeled extrachromosomal probe S1 (B). Numerals indicate weight of fresh leaves per dot.

Table 3 Distribution of chromosomal DNA and extrachromosomal DNA of SCWL MLO in sugarcane plants[a]

Position[b]		Healthy plant		Diseased plant No.1		Diseased plant No.2		Diseased plant No.3	
		C[c]	EC[c]	C	EC	C	EC	C	EC
2nd leaf from top	LB	0	0	0.36	0.79	0.45	0.72	0.50	1.24
	LS	0	0	0.01	0.08	0.01	0.07	0.05	0.39
4th leaf from top	LB	0	0	0.56	1.64	0.23	1.44	0.53	1.70
	LS	0	0	0.10	0.66	0.23	0.92	0.20	0.66
6th leaf from LS	LB	0	0	0.50	1.83	0.53	1.90	0.51	1.77
	LS	0	0	0.11	0.72	0.10	0.72	0.15	0.79
Stem		0	0	0.80	2.42	0.68	1.97	0.72	1.64
Root		0	0	0.09	0.72	0.11	1.11	0.10	1.18
Cutting		0	0	0.01	0.02	0.01	0.02	0.01	0.08

a Each value indicates the relative absorbance (A_{633}) of the hybridization spots on the film measured with a laser densitometer.

b Leaves were counted from the top developing leaf to the bottom old leaf. LB, leaf blade, LS, leaf sheath.

c C, relative absorbance of hybridization signal between the chromosomal DNA probe S28 of SCWL MLO and DNA extracted from 0.1 mg of the tissue. EC, relative absorbance of hybridization signal between the extrachromosomal DNA probe Sl of SCWL MLO and DNA extracted from 0.1 mg of the tissue. We can compare these absorbance values because they were measured simultaneously.

or white stripe symptoms. The chlorophyll contents exceeded 20 SPAD in healthy sugarcane leaves. There was no relation between the amounts of DNA of MLO and the severity of the chlorosis symptoms.

Detection of MLO in insects. MLO DNA was detected in insect vectors *M. hiroglyphicus* (Fig.4A) collected in the sugarcane fields infected with SCWL in Khon Kaen by using the extrachromosomal DNA probe Sl (Fig.4B). Hybridization signals were distinct when the extrachromosomal DNA probe Sl was used, whereas the signals were faint when the chromosomal DNA probe S28 was used.

Relatedness among SCWL MLO isolates, other MLOs and other culturable mollicutes. Genetic differentiation of MLO isolaes was examined by comparing their preferential hybridization with various probes. Nucleic acids extracted from plants infected with various MLOs including SCWL MLO isolates from Khon Kaen and Lampang were dot-hybridized with various probes. The results are summarized in Table 2. None of the 14 chromosomal DNA probes and of the three extrachromosomal DNA probes exhibited non-specific hybridization with healthy plant specimens. All of the DNA probes of SCWL MLO hybridized with two isolates of SCWL MLO and MLOs of the other gramineous plants including RYD MLO. None of the extrachromosomal DNA probes hybridized with the MLOs of other kinds of plants. Three chromosomal DNA probes reacted only with MLO isolates of gramineous plants, and other chromosomal DNA probes reacted with some MLOs of other kinds of plants (OY,CWB,JHWB,WDY,GWB,UD,TWB,PWX and PD MLOs). No signals were observed between culturable mollicutes (*S.kunkelii, S.citri, M.hominis,*

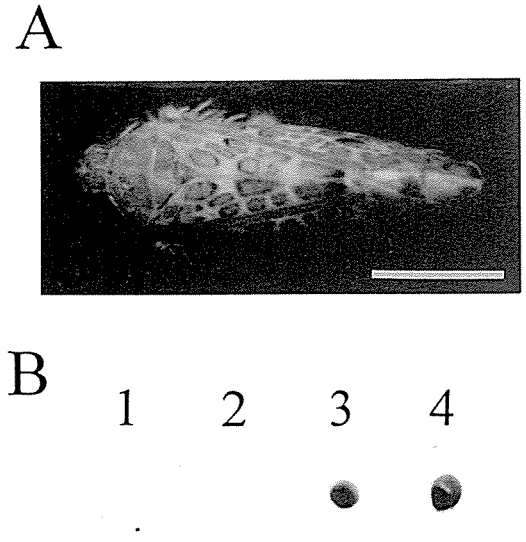

Fig.4. Detection of SCWL MLO in leafhoppers *Matsumuratettix hiroglyphycus*. (A) Leafhopper *M. hiroglyphycus* collected from sugarcane fields with SCWL in Khon Kaen, Thailand. Bar represents 1 mm. (B) DNA samples from MLO-free leafhoppers *Nephotettix cincticeps* (1 and 2), and those from *M. hiroglyphicus* (3 and 4) were spotted on a membrane. The membrane was hybridized with labeled extrachromosomal DNA probe Sl. DNA of one spot derived from two insects

and *M.orale*) and chromosomal as well as extrachromosomal DNA probes of SCWL MLO.

Discussion

The present study revealed that the non-radioactive peroxidase-labeled extrachromosomal as well as chromosomal DNA probes are effective in assaying SCWL MLO. These probes hybridized with the leaves, stems and roots of all the sugarcane plants affected with SCWL disease, but not with those of healthy and other sugarcane plants with bacterial and viral diseases. Extrachromosomal DNA probes gave much stronger signals than chromosomal DNA probes. These results which indicated that the copy number of the extrachromosomal DNA was considerably larger than that of chromosomal DNA of SCWL MLO within a cell of MLO were similar to the findings in the case of RYD MLO[14]. As the extrachromosomal DNA was detected from all of the SCWL specimens and insect vectors of SCWL MLO used in the experiment, it is considered that the extrachromosomal DNA probe may be a useful tool to detect the SCWL MLO.

Existence of plasmids or extrachromosomal DNAs has been reported in several MLOs and mollicutes[3,7,8,24]. Previous work also indicated that RYD MLO harbored extrachromosomal DNAs[14,15]. It is interesting to note that a sequence of the extrachromosomal DNA of SCWL MLO showed a homology with those of MLOs of gramineous plants including RYD MLO. The extrachromosomal DNA of MLOs may thus be involved in the genetic flow among MLOs of gramineous plants and in the interactions between MLOs and their hosts such as insect transmissibility as indicated in the extrachromosomal DNA of clover phyllody MLO[4]. Extrachromosomal DNA may even be related to the symptom expression because all the MLOs that hybridized with the DNA probes of SCWL MLO were associated with chlorosis symptoms of leaves.

Due to the technical difficulty in characterzing MLOs bacteriologically, their taxonomy has not been well established. They have been classified essentially 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 of these diseases are different based on the difference in pathogenicity and insect vectors[16]. Many gramineous weeds showing white leaf symptoms were present in the vicinity of sugarcane fields infected with SCWL in Thailand and Taiwan[1,19]. White leaf disease of *Brachiaria* sp.,*Dactyloctenium aegyptium* and Bermuda grass was observed near sugarcane fields infected with SCWL in Northeast Thailand. MLOs were detected in these weeds by electron microscopy in Taiwan[1,19]. The insect vector of SCWL MLO failed to transmit the SCWL MLO from the diseased sugarcane plants to the Bermuda grass and to transmit the MLOs from the Bermuda grass to healthy sugarcanes plants[20]. Polyclonal antibodies against SCWL MLO did not react with the MLOs of the weeds, and

antibodies against the MLO of the Bermuda grass did not react with SCWL MLO and MLOs of the other weeds[22]. These data indicate the SCWL MLO is different from the MLOs of the weeds. For introducing a genetic concept in the classification of these organisms, it would be important to gain information on DNA homology. Several research groups have discussed the classification of MLOs in the light of DNA homology analyzed by random DNA probes[8,10,15]. We examined the homology of DNA probes derived from a Khon Kaen (Northeast Thailand) isolate of SCWL MLO with the DNA of a Lampang (North Thailand) isolate of SCWL MLO, some MLOs associated with gramineous weeds with white leaf symptoms, RYD MLO isolates from Japan and Thailand, other types of MLOs and some culturable mollicutes. It was clearly shown that the SCWL MLOs of Thailand shared a greater nucleotide sequence homology with the MLOs of gramineous plants including RYD MLO than with other MLOs. There were no relations between SCWL MLO and some culturable mycoplasmas and spiroplasmas. Recenthly Schneider *et al.* have revealed that SCWL and BGWL were phylogenetically related and different from other MLOs such as aster yellows based on the analysis of the structure of 16S ribosomal RNA (rRNA) sequences[23]. Namba *et al.* reported that RYD MLOs were phylogenetically different from other MLOs in Japan by comparing the sequences of 16S rRNA genes[18]. Our hybridization results indicated that SCWL MLO, RYD MLO, BGWL MLO and other MLOs of gramineous weeds were related to each other genetically. The phylogenetic diversity of MLOs of gramineous plants awaits further study.

By using DNA probes, we were able to detect MLOs from 0.1 mg of tissue and a few insect vectors within two days. This method may thus be useful for indexing cuttings, quarantine procedures, development of methods of control of the disease and basic research. We, however, could not develop SCWL MLO-specific DNA probes as they reacted with all the MLOs associated with gramineous plants. In the sugarcane fields of Thailand, we observed a large number of gramineous weeds with white leaf symptoms. For epidemiological investigations on the SCWL MLO, the current study suggests that it is necessary to develop a method of detection specific to SCWL MLO by modifying the DNA probes or by using primers for polymerase chain reaction (PCR) amplification of the sequences of the DNA probes or 16S rRNA sequences for MLOs.

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References

1) Chen, C. T. *et al.*(1972). Mycoplasma-like organisms in *Cynodon dactylon* and *Brachiaria distachya* affected by white leaf diseases. *Rep. Taiwan Sugar Exp. Stn.* 56 : 49-55.

2) Davis, R. E.& 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.

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

4) Denes, A. S.& 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.

5) Kirkpatrick, B. C. *et al.*(1987). Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. *Science*

238 : 197-200.
6) Kollar, A. *et al.*(1990). Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. *Phytopathology* 80 : 233-237.
7) Kuske, C. R.& Kirkpatrick, B. C.(1990). Identification and characterization of plasmids from the western aster yellows mycoplasmalike organism. *J. Bacteriol.* 172 : 1628-1633.
8) Kuske, C. R. et al.(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.
9) Kuske, C. R. *et al.* (1991). Differentiation of virescence MLOs western aster yellows mycoplasma-like organism chromosomal DNA probes and restriction fragment length polymorphism analysis. *J. Gen. Microbiol.* 137 : 153-159.
10) Lee, I.-M.& Davis, R. E.(1988). Detection and investigation of genetic relatedness among aster yellows and other mycoplasmalike organisms by using cloned DNA and RNA probes. *Mol. Plant-Microbe Interact.* 1 : 303-310.
11) Lee, I.-M. et al.(1993). Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. *Phytopathology* 83 : 834-842.
12) Lin, S. C.& Lee, C. S.(1968). Studies on sugarcane white leaf disease. I. Causal organism. *Rep. Taiwan Sugar Exp. Stn.* 47 : 129-138.
13) Mangelsdorf, A. J.(1962). A research program for the Thailand sugar industry. Department of Agriculture, Bangkok. 1-16.
14) Nakashima, K. *et al.* (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.
15) Nakashima, K. *et al.*(1993). DNA probes reveal relatedness of rice yellow dwarf mycoplasmalike organisms (MLOs) and distinguish them from other MLOs. *Appl. Environ. Microbiol.* 59 : 1206-1212.
16) Nakashima, K.& Murata, N.(1993). Destructive plant diseases caused by mycoplasma-like organisms in Asia. *Outlook on Agriculture* 22 : 53-58.
17) Namba, S. *et al.*(1993). Detection and differentiation of plant-pathogenic mycoplasmalike organisms using polymerase chain reaction. *Phytopathology* 83 : 786-791.
18) Namba, S. *et al.*(1993). Phylogenetic diversity of phytopathogenic mycoplasmalike organisms. *Int. J. Syst. Bacteriol.* 43 : 461-467.
19) Razin, S.(1992). Mycoplasma Taxonomy and Ecology. In Mycoplasma. ed. J. Maniloff *et al.* American Society for Microbiology. Washington, D. C. 3-22.
20) Rishi, N.& Chen, C. T.(1989). Grassy shoot and white leaf diseases. In Diseases of sugarcane. ed. Ricaud, B. C.& Egan, B. T.,Elsevier Science Publisher, Amsterdam. 289-300.
21) Sambrook, J.,T. *et al.* (1989). Molecular Cloning : A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22) Sarindu. N.& Clark, M. F.(1993). Antibody production and identity of MLOs associated with sugar-cane white leaf disease and bermuda-grass white leaf disease from Thailand. *Plant Pathology* 42 : 396-402.
23) Schneider, B. et al.(1992). Occurrence and relatedness of extrachromosomal DNAs in plant pathogenic mycoplasmalike organisms. *Mol. Plant-Microbe Interact.* 5 : 489-495.
24) Schneider, B. *et al.*(1993). Classification of plant-pathogenic mycoplasma-like organisms using restriction site analysis of PCR-amplified 16S rDNA. *J. Gen. Microbiol.* 139 : 519-527.

DNAプローブによるタイのサトウキビ白葉病病原マイコプラズマ様微生物の検出

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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とって最も重要な病害である。本病の病原体を検出するための，有効で信頼性の高い方法が，強く求められていた。我々は，本病の病原体であるマイコプラズマ様微生物（MLO）のDNAを分離し，そのランダムなDNAをクローニングして，DNAプローブとして用いることにより，病原体を迅速かつ簡便に検出することができた。SCWL MLOのDNAは，ビスベンズイミドー塩化セシウム平衡密度勾配遠心を繰り返すことにより，宿主のDNAから分離された。制限酵素*Hin*dⅢで切断して得られたDNA断片は，プラスミドー大腸菌の系にクローニングされた。サザンハイブダイゼーションにより，いくつかの組み換えプラスミド挿入断片は，MLOの染色体断片からなり，他の組み換えプラスミド挿入断片は，MLOの染色体外DNA断片からなることが明らかになった。クローン化したDNAプローブを用いたドットハイブリダイゼーションにより，サトウキビ及び媒介昆虫*Matsumuratettix hiroglyphicus*からMLOを検出することができた。これらのDNAプローブを用いたドットハイブルダイゼーションにより，SCWL MLOは，イネ黄萎病MLOを含むイネ科植物のMLOと塩基配列の相同性は高いが，他の種類のMLOとの相同性は低いことが示唆された。本検出法をタイ国コンケン地区のSCWL MLOの検出に適用し，極少量の植物組織からの病原体検出において，極めて優れていることを確認した。

キーワード：MLO，ドットハイブリダイゼーション