タイにおけるカンキツおよびミカンキジラミからのカンキツグリーニング病病原体の検出

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Detection of Citrus Greening Organism in Citrus Plants and Psylla *Diaphorina citri* in Thailand*

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Abstract

Citrus greening disease caused by greening organisms (GOs; *Liberobacter* sp.) is one of the most destructive diseases of citrus in Thailand. We applied the PCR method to the detection of 16S rDNA fragments of GOs in leaves with seven kinds of symptoms, mottling (Type I), mild chlorosis with green veins (Type II), severe chlorosis with green veins (Type III), pale green color in young leaves (Type IV), vein yellowing (Type V), vein corking (Type VI) and unclear symptoms (Type VII) collected from GO-infected citrus trees in Thailand. The GO DNA was high in leaves with Types I, II, III, V and VI symptoms. We also detected GO DNA in an insect vector *Diaphorina citri*. Sequence analysis of the amplified 16S rDNA fragment of seven Thai isolates collected from six major citrus-producing areas revealed that the sequences of the 16S rDNA fragments of these isolates were the same and were very similar to that of the Indian isolate of *L. asiaticum*.

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Key words: citrus greening, *Liberobacter asiaticum*, 16S rDNA, PCR, detection, nucleotide sequence.

INTRODUCTION

Citrus greening disease that infests citrus-growing areas of the African and Asian tropics[3,4] is one of the most destructive diseases of citrus in Thailand. A prokaryote associated with greening disease was found in sieve elements enclosed by a 25 nm thick envelope, which was different from the unit membrane envelope characteristic of phytoplasmas (7-10 nm thickness)[13]. These organisms were called bacterium-like organisms (BLOs), or greening organisms (GOs). The organisms are transmitted by two psyllid insect vectors *Diaphorina citri* Kuw. and *Trioza erytreae* Del Guercio in Asia and Africa, respectively[8,9]. Because GOs could not be cultured *in vitro*., microbiological and genetic information about the GOs was not available until Jagoueix *et al.* (1994) determined the nucleotide sequences of the 16S rDNA from the Indian and African isolates of the GOs. They reported that the GOs are members of the α subdivision of the *Proteobacteria*[7]. Placing the GOs in a new genus, they proposed *Liberobacter asiaticum* and *Liberobacter africanum* for Asian and African types, respectively.

GOs have been detected by electron-microscopy and transmission by grafting or insect vector, all of which are tedious and time-consuming. To control such diseases and to study pathogen behavior, efficient and reliable detection methods are needed. The recently developed hybridization procedure using DNA probes specific to GOs was a first step towards this goal[5,14,15]. DNA hybridization analysis indicated that only *L. asiaticum* is present in the 11 Asian countries examined, *i.e.* India, Nepal, Sri Lanka, Vietnam, Cambodia, Malaysia, Indonesia, Thailand, Philippines, Taiwan and China[5]. The polymerase chain reaction (PCR) procedure for amplifying 16S rDNA fragments using GO-specific primers enabled the detection of GO isolates in periwinkle plants[7]. We previously applied this method for the detection of 16S rDNA fragments of Thai GO isolates, Nakorn Pathom, Rangsit and Nan isolates maintained in citrus plants[11]. A simplified DNA extraction procedure enabled us to get DNA for PCR within 20 min and to detect the GO within 4.5 hr.

In the present paper, we studied the relationship between symptoms and distribution of the GOs in man-

* The nucleotide sequence data reported here will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB008809.

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darin 'Somkeowan' plants and used our PCR procedure in the detection of GOs from several kinds of citrus plants infected with Thai isolates, from other kinds of plants such as *Murraya paniculata* (L.) Jack. and from the insect vector psylla *D. citri*. We also analyzed the nucleotide sequence of the 16S rDNA fragments of seven Thai isolates collected from six major citrus-producing areas.

MATERIALS AND METHODS

Materials One hundred fifteen mandarin *Citrus reticulata* Blanco leaves having seven kinds of symptoms (Type I-VII in Plate I-B-H; Table 1) were collected from 15 GO-infected 'Somkeowan' trees (4-5 years old) from the orchards of the Nan Horticultural Research Station and five GO-infected 'Somkeowan' trees (4-5 years old) in other orchards in Nan, Thailand on 5 August 1997. Seven leaves showing seven kinds of symptoms, types I to VII, from each tree were collected. *M. paniculata*, eggplants *Solanum melongena* L., and peppers *Capsicum annuum* L. that showed greening disease-like symptoms and the insect vector psylla *D. citri* were also collected from the orchards in Nan on the same date. Other Thai isolates were collected from mandarins, pomelos *C. grandis* (L.) Osb. and sweet oranges *C. sinensis* (L.) Osb. before 1996 as shown in Table 2. These isolates have been maintained in mandarins, sweet oranges or rough lemons *C. jumbhiri* Lush. in the screen house of the Department of Agriculture (DOA), Thailand.

DNA extraction and detection of GO DNA DNA of plants was extracted from the midrib of each leaf. The 16S rDNA fragments of GOs were amplified from the extracted DNA by the PCR method[12]. DNA of insects was extracted by either the mannitol[10] or CTAB

Table 1. Relationship between symptoms and amount of pathogen in leaves of GO-infected trees of mandarin 'Somkeowan' in Nan, Thailand

Symptoms		Score of amount of GOs					Total number	Score average
Type	Description	− or ±	+1	+2	+3	+4		
I	Mottling	0 0%	2 10%	5 25%	10 50%	3 15%	20	2.7
II	Mild chlorosis with green vein	0 0%	1 5%	6 30%	7 35%	6 30%	20	2.9
III	Severe chlorosis with green vein	0 0%	2 11%	1 5%	12 63%	4 21%	19	2.9
IV	Pale green color in young leaves	2 10%	3 15%	8 40%	6 30%	1 5%	20	2.1
V	Vein yellowing	0 0%	0 0%	3 43%	3 43%	1 14%	7	2.7
VI	Vein corking	0 0%	0 0%	2 29%	1 14%	4 57%	7	3.3
VII	Unclear symptoms	0 0%	7 35%	5 25%	4 20%	4 20%	20	2.3
	Total	2 2%	15 13%	30 27%	43 38%	23 20%	113	2.6

Table 2. GO isolates maintained at Department of Agriculture (DOA), Thailand.

Isolate	Collection description				Maintain[a]
	Cultivar[a]	Symptoms	Site[b]	Date	
Yala	MD 'Chogun'	yellowing, decline	Yalang Dis, Pattani Pro	27 Apr. 1991	SO 'Madam Vinous'
Phetchabun	MD 'Somkleng'	yellowing	Kaoko Dis, Phetchbun Pro	26 Nov. 1990	MD 'Ponkan'
Phichit	PM 'Kaothongdee'	chlorosis, dieback	Muan Dis, Phichit Pro	3 Aug. 1995	RL
Rangsit	MD 'Somkeowan'	mottling, yellowing	Nongsua Dis, Pathum Thani Pro	26 June 1993	MD 'Ponkan'
Nakorn Pathom	SO	mottling, chlorosis	Sampran Dis, Nakorn Pathom Pro	1985	SO 'Madam Vinous' or RL

a) MD: mandarin *Citrus reticulata* Blanco, PM: pomelo *C. grandis* (L.) Osb., SO: sweet orange *C. sinensis* (L.) Osb., RL: rough lemon *C. jambhiri* Lush.

b) Dis: district, Pro: province

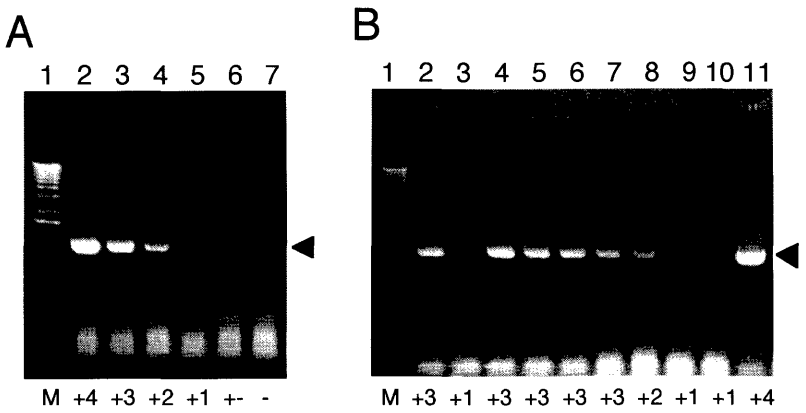

Fig. 1. Detection of 16S rDNA fragments of GOs from mandarin 'Somkeowan' plants in Nan, Thailand. (A) Quantitative estimation of the GO level in leaves using PCR. Lane 1, DNA marker; lane 2, amplified DNA from DNA of GO-infected leaves; lane 3, amplified DNA from DNA of GO-infected leaves /DNA of healthy leaves (1/10); lane 4, amplified DNA from DNA of GO-infected leaves /DNA of healthy leaves (1/100); lane 5, amplified DNA from DNA of GO-infected leaves /DNA of healthy leaves (1/1000); lane 6, amplified DNA from DNA of GO-infected leaves /DNA of healthy leaves (1/10,000); lane 7, amplified DNA from DNA of healthy leaves. (B) Example of the detection of GO DNA from an individual leaf. Lane 1; DNA marker; lanes 2-11, amplified GO DNA from DNA of an individual leaf by PCR.

method[12].

DNA sequencing The purified double-stranded PCR product of 16S rDNA fragments was used as a template for direct sequencing with an auto-sequencer (ABI 373 DNA Sequencer STRETCH). A commercial reagent kit (PRISM Dye Termination Cycle Sequencing Ready Reaction Kit) was used according to manufacturer's instructions. Four primers, OI1 : 5′-GCGCGTATGCAATACGAGCGGCA-3′; OI2c : 5′-GCCTCGCGACTTCGCAACCCAT-3′; GO16S501-520 : 5′-AGGCGGCGATTAAGTTAGAG-3′; GO16S750-731 : 5′-ACAGCTAGCACTCATCGTTT-3′ were used for sequencing.

RESULTS

Symptoms of citrus greening disease

Typical symptoms of greening disease in mandarin trees are shown in Plate I. Seven kinds of symptoms (Type I-VII) were observed in GO-infected 'Somkeowan' trees in orchards in Nan, Thailand. Plates I-B to I-H represent types I to VII as mottling, mild chlorosis with green vein, severe chlorosis with green veins, pale green color in young leaves, vein yellowing, vein corking and unclear symptoms, respectively. Leaves showing types I-IV were abundant in GO-infected 'Somkeowan' trees, but leaves with type V and VI symptoms were rare (Table 1). Type IV was found only in young and budding leaves, type III was found in relatively young leaves and types I, II, V and VI were found in older leaves. Leaves with unclear symptoms (Type VII) were older than leaves with the other types of symptoms.

Detection of the GO DNA in citrus leaves

We examined the quantitative ability of our PCR method. DNA extracted from veins of GO-infected leaves (Fig. 1A, lane 2), from veins of healthy leaves (Fig. 1A, lane 7), and a mixture from veins of infected and healthy leaves at several dilutions (Fig. 1A, lane 3-6) as templates for PCR was used. We suspect that the amount of the PCR products reflects the amount of GOs in veins of each leaf because the amplified DNA dependent on the DNA template from GO-infected leaves (Fig. 1A). We indexed the amount of the pathogen into +4, +3, +2, +1, ±, and −. We tried to index the pathogen from leaves with different types of symptoms and to estimate the amount of GOs based on the results from the dilution experiments shown in Fig. 1A. Figure 1B shows an example of such an estimation. The results are summarized in Table 1. The average score in Table 1 corresponded to the average score for the amount of GOs. The titer of GOs was relatively high in leaves having several kinds of symptoms such as mottling (Type I; Plate I-B), chlorosis with green veins (Type II and III; Plate I-C and D), vein yellowing (Type V; Plate I-F) and vein corking (Type VI; Plate I-G), but was relatively low in pale green young leaves (Type IV; Plate I-E) and in leaves with unclear symptoms (Type VII; Plate I-H).

Detection of the GO from several varieties of citrus with greening symptoms infected with other Thai isolates

16S rDNA fragments of GOs were isolated by the PCR methods from varieties of citrus plants infected with the Thai isolates (Table 2); sweet orange 'Madam Vinous' leaves with mottling (Type I) and vein corking (Type VI) infected with a Yala isolate, mandarin 'Ponkan' leaves with mottling (Type I) and yellowing (Type II and III) infected with a Phechabun isolate, rough lemon leaves with mottling (Type I) and yellowing (Type II and III) infected with a Phichit isolate, mandarin 'Ponkan' leaves with vein corking (Type VI) infected with a Rangsit isolate, and sweet orange 'Madam Vinous' leaves with mottling (Type I) infected with a Nakorn

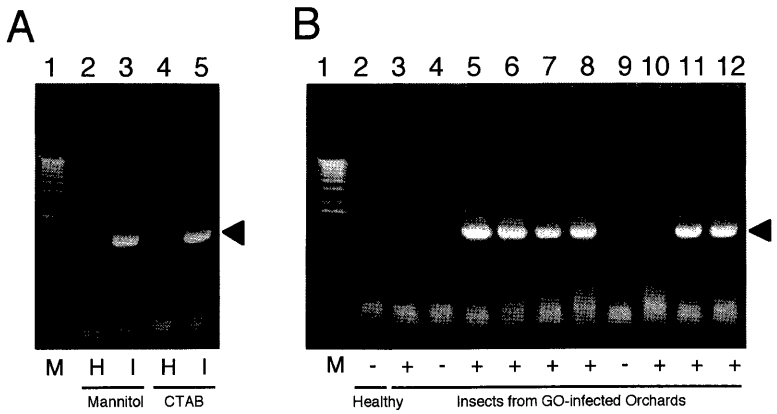

Fig. 2. Detection of 16S rDNA fragments of GOs from psylla *Diaphorina citri* in Nan, Thailand. (A) Examination of PCR detection from ten psylla. Lane 1, DNA marker ; lanes 2 and 3, mannitol method ; lanes 4 and 5, CTAB method ; lanes 2 and 4, amplified GO DNA from DNA of healthy psylla ; lanes 3 and 5, amplified GO DNA from DNA of psylla collected in GO-infected orchards in Nan, Thailand. (B) Example of the detection from one phyllid *D. citri*. Lane 1, DNA marker ; lane 2, amplified GO DNA from DNA of a healthy psylla ; lanes 3-12, amplified GO DNA from DNA of an individual psyllid collected in GO-infested orchards in Nan, Thailand.

Pathom isolate (data not shown).

Detection of the GO from other genera of plants with greening-like symptoms

As the vector insects display a preference for *Murraya* spp.[3], which tend to flush all year round, the *Murraya* spp. might be an alternative host of the GOs. We collected the leaves of *Murraya paniculata* plants, eggplants and pepper plants in the GO-infected orchard in Nan. Some leaves showed chlorosis with green veins, which was similar to greening disease of citrus (*cf.* Type III, Plate I-D). We could not detect the pathogen using PCR with extracted DNA from *Murraya* plants, eggplants or pepper plants with greening-like symptoms (data not shown). Therefore, we suspected that these symptoms were caused by nutrient deficiency or some other reason, and that the other genera, such as *Murraya paniculata*, might not be alternative host plants of GOs.

Detection of the GO from psylla

We compared two kinds of DNA extraction methods, the mannitol method and CTAB method, for psylla *D. citri*. GO DNA was amplified from DNA extracted from psylla collected from infected orchards using both methods (Fig. 2A, lanes 3 and 5). However, DNA was not amplified from healthy psylla kept in an isolated chamber (Fig. 2A, lanes 2 and 4). The CTAB method was preferred to isolate DNA from insects as well as plants, since it is easier than the mannitol method. Figure 2B shows DNA of the GOs isolated from an insect. We could isolate GO DNA from only one insect. Twenty five insects were GO-positive among 30 insects (83.3 %) collected in orchards in Nan.

Sequence analysis of the 16S rDNA of GO Thai isolates

16S rDNA fragments of two Nan isolates, one of which was collected at the Nan Horticultural Research Station and the other in an orchard in Nan, as well as a Yala isolate, a Phetchanun isolate, a Phichit isolate, a Rangsit isolate, and a Nakorn Pathom isolate were sequenced. Nucleotide sequences of these isolates were the same (Fig. 3). We observed a 98.8 % homology between the 16S rDNA of Thai isolates of GO and that of the Poona isolate (India) of *L. asiaticum*[7] and 97.5% homology between the 16S rDNA of Thai isolates of GO and that of the Nelspruit isolate (Africa) of *L. africanum*[7]. Because these results showed that Thai isolates were very close to the Indian isolate of *L. asiaticum*, we suspected that the Thai and Indian isolates were *L. asiaticum*.

DISCUSSION

Greening disease can be recognized through several kinds of symptoms (Plate I). Table 1 showed that GOs are distributed throughout leaves of GO-infected citrus plants. The symptoms of chlorosis with green veins (Type II and III ; Plate I-C and D) would seem to be opposite of symptoms of vein yellowing (Type V ; Plate I-F) and vein corking (Type VI ; Plate I-G), but DNA of GOs were isolated from all the leaves (Table 1). The differences in leaf symptoms may be affected by the growth stage of the leaves when they are infected, or by physiological and environmental conditions.

Both GOs and phytoplasmas multiply in phloem of the plants and are transmitted by insect vectors. Hence their pathological characters are very similar. Kuske *et al.* (1992) reported that the highest concentration of two strains of aster yellows (AY) phytoplasma were consistently detected in the expanding, symptomatic shoots of infected periwinkle plants[6]. Similarly, strawberry plants infected with clover phyllody phytoplasma had the highest phytoplasma titer in areas with symptoms[1]. Pathogen titers and symptom severity were also closely correlated in peach and chokecherry infected with eastern X-phytoplasma[2]. In all these cases, the highest concentrations of phytoplasmas occurred in regions of infected plants where symptoms were most severe, sug-

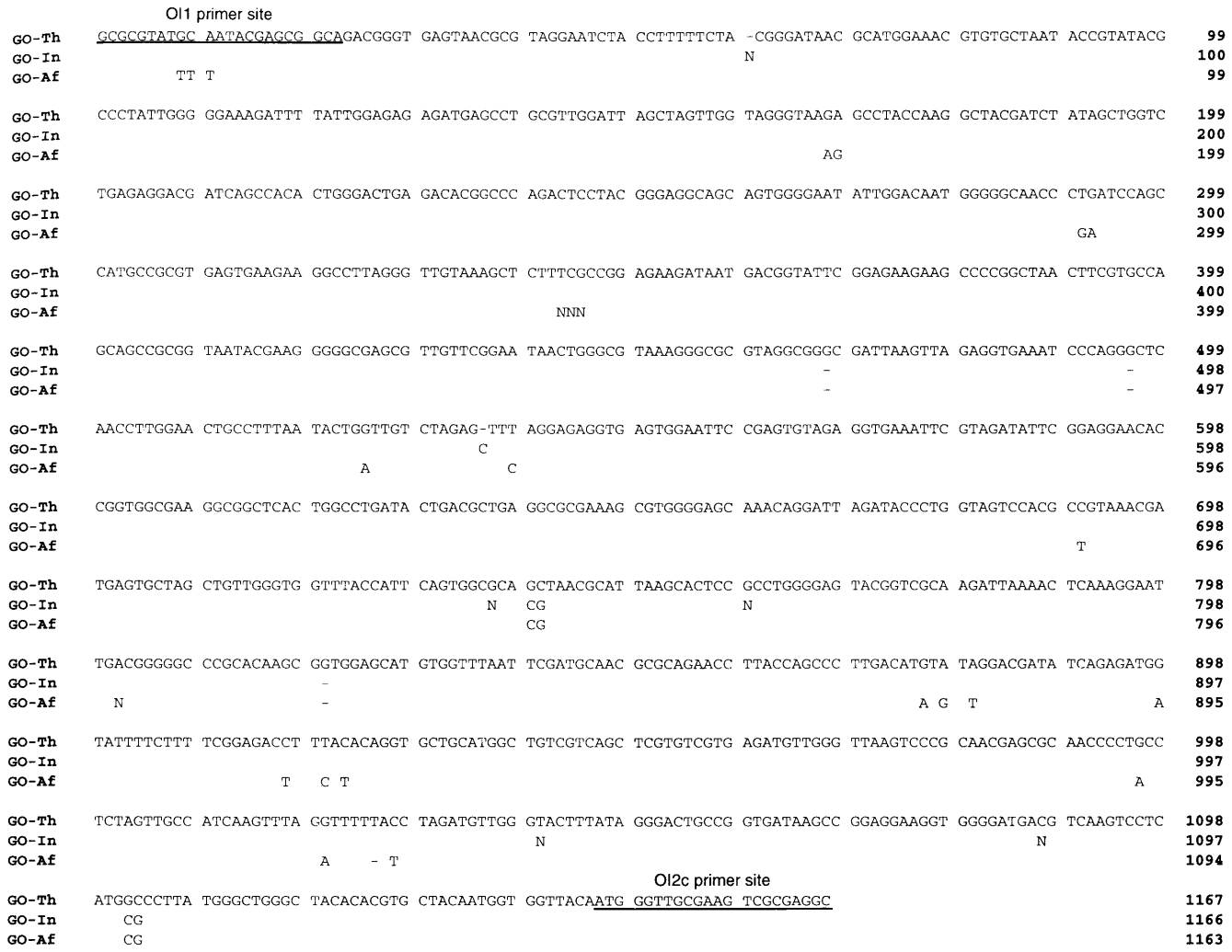

Fig. 3. Sequence alignment of 16S rDNA fragments amplified from Thai, Indian and African isolates of GO. A blank indicates that the nucleotide is the same as that in the sequence of the Thai isolate of GOs (GO-Th). Gaps, included to maximize the alignment, are indicated by dashes. The numbers refer to the positions of the nucleotides from the 5′ terminus of the sequences shown. GO-Th, Thai GO isolates (two Nan, one Yala, one Phetchabun, one Phichit, one Rangsit and one Nakorn Pathom isolates); GO-In, Indian GO isolate (Poona isolate); GO-Af, African GO isolate (Nelspruit isolate).

gesting that these phytoplasmas might colonize locally and synthesize metabolites which induce localized symptoms.

On the other hand, we reported that the amount of rice yellow dwarf (RYD) phytoplasma was not correlated with the severity of chlorosis in RYD-infected tissues[10]. Similar results were obtained in sugarcane with sugarcane white leaf (SCWL) phytoplasma[11]. Apparently, phytoplasma concentration in newly emerging leaves with severe chlorosis was not always high. In these cases, possible symptom-inducing metabolites synthesized by RYD or SCWL phytoplasmas may be translocated through the phloem elements from other parts of plants to the meristems.

Based on these reports, determination of the relationship between symptom severity and distribution of GOs is not simple. The GOs were present at relatively levels in leaves with severe symptoms such as chlorosis (Type III) and corking (Type VI). However, the GO concentration was low in some leaves with severe symptoms, but was high in some leaves with unclear symptoms (Table 1). As the leaves with unclear symptoms were relatively old, and the leaves with the more severe symptoms were relatively young, the difference in symptoms may be a result of host maturity at the time of infection, rather than of the GO concentration. Developing younger leaves infected with GOs may show more severe symptoms, whereas mature older infected leaves may have unclear symptoms even though GOs multiply in almost all the leaves.

Sequence analysis of 16S rDNA fragments of seven kinds of Thai isolates indicated that all the isolates are same, and are closer to *L. asiaticum* than *L. africanum*. The sequence of a Nakorn Pathom isolate determined by the direct sequencing method was different in four sites from the sequence of the Nakorn Pathom isolate as shown in a previous report[12]. We suspected that the sequence in that report contained artifacts since we

determined the sequence of only one clone contained the PCR-amplified fragments. Sequence data of 16S rDNA fragments of these isolates indicated that *L. asiaticum* occurs in several kinds of citrus plants such as the mandarins 'Somkeowan', 'Chogun', 'Somkleng', and the pomelo 'Kaothongdee' in almost all citrus-producing provinces in Thailand (*cf.* Table 2). Furthermore, these results revealed that *L. asiaticum* can cause chlorosis and dieback symptoms on pomelo tree, although pomelo have been suspected to be tolerant to the typical Asian strain that occurs on other varieties of citrus[3].

It was earlier report that GOs could be detected within 4.5 hr from citrus plants maintained in a greenhouse by the PCR method[12]. Our present study revealed that GOs can be detected from one leaf of citrus plants in orchards or plants maintained in a screen house under hot conditions in Thailand. As the amount of GOs of leaves with type I (mottling), II (mild chlorosis with green veins), III (severe chlorosis with green veins), V (vein yellowing) and VI (vein corking) were relatively high, leaves with these symptoms were suitable for the detection. Furthermore, GOs can also be detected from an individual insect vector by the PCR method. Although the cost of PCR and DNA hybridization methods is higher than that of an immunological assay such as ELISA, it can provide reliable data within a short time. The detection of GOs using the PCR should facilitate epidemiological studies which will lead to their control.

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Plate I

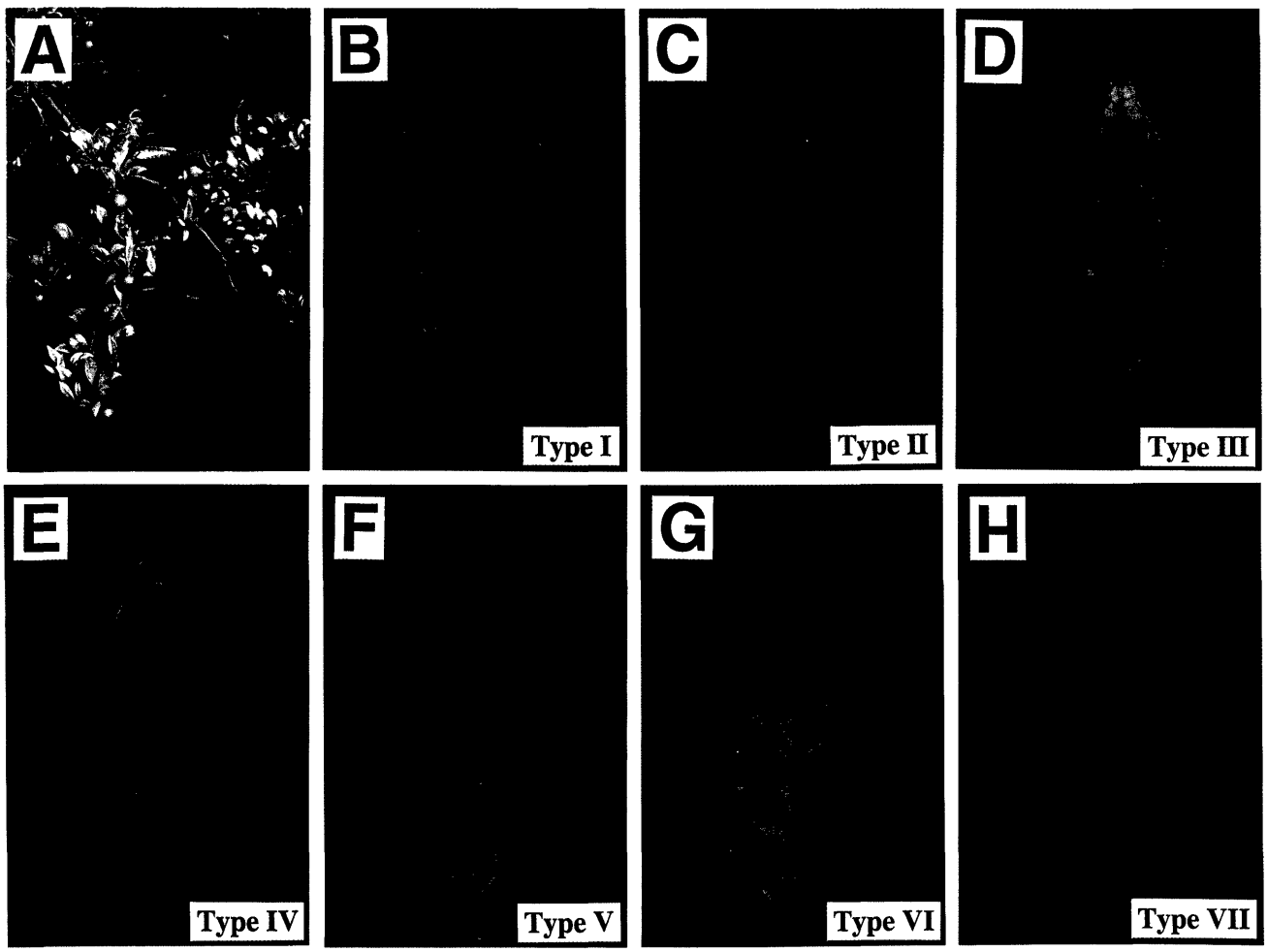

Plate I Typical symptoms of citrus greening disease of mandarin 'Somkeowan' plants in Nan, Thailand : (A) Shoots showed typical symptoms, (B) Type I symptoms, (C) Type II symptoms, (D) Type III symptoms, (E) Type IV symptoms, (F) Type V symptoms, (G) Type VI symptoms and (H) Type VII symptoms.

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和 文 摘 要

中島一雄・大津善弘・Maitree PROMMINTARA：タイにおけるカンキツおよびミカンキジラミからのカンキツグリーニング病病原体の検出

カンキツグリーニング病は，熱帯地域のカンキツ類に最も大きい被害をもたらしている。我々は，PCR 法の適用により，タイ国ナン市のグリーニング病病原体（GO ; *Liberobacter* sp.）感染カンキツ樹において発現していた 7 種の症状，すなわち黄色斑紋（タイプ I），葉脈に緑色を残した穏やかな黄化（タイプ II），葉脈に緑色を残した激しい黄化（タイプ III），新葉の黄緑化（タイプ IV），葉脈黄化（タイプ V），葉脈のコルク化（タイプ VI），および不明瞭な症状（タイプ VII）の葉から GO の 16S rDNA を検出できた。タイプ I, II, III, V, VI の症状を呈した葉からは多量の GO DNA を検出できた。また，媒介昆虫ミカンキジラミ 1 匹からも GO の DNA を検出することもできた。タイのカンキツ産地 6 カ所から採集された 7 種の GO 株の 16S rDNA 断片の塩基配列を解析した結果，これらの配列は全く同じであり，タイ株は *L. asiaticum* インド株と類似していることが明らかになった。