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Strain identification and distribution of citrus Huanglongbing bacteria in Taiwan

Chia-Hsin TSAI, Ting-Hsuan HUNG, and Hong-Ji SU*

Department of Plant Pathology and Microbiology, National Taiwan University, Taipei 106, Taiwan

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ABSTRACT. The 48 representative Huanglongbing bacteria (HLBB) isolates were selected from 457 disease samples collected from Huanglongbing (HLB)-diseased citrus trees grown in seven main citrus-producing areas, including tropical and subtropical regions of Taiwan. After indexing and eliminating the citrus viruses, the selected HLBB isolates, free from the viruses, were used for identification of HLBB strains based on a pathogenicity and virulence test with the following differential citrus cultivars: Ponkan mandarin (*Citrus reticulata* Blanco), Liucheng sweet orange (*C. sinensis* Osb.), Wentan pummelo (*C. grandis* f. *buntan* Hay.), and Eureka lemon (*C. limon* Burm.). Four strains of HLBB were identified. Strain I showed pathogenicity on mandarin and sweet orange by inducing typical HLB symptoms. Strain II showed high virulence on all differential cultivars and multiplied fast in all cultivars. Strain III caused intermediate symptoms on mandarin and sweet orange and mild symptoms on pummelo, but did not infect Eureka lemon. Mild strain IV infected mandarin and sweet orange without causing symptoms and was rarely isolated. Strain II, which attacked all citrus cultivars grown in Taiwan, was found to dominate over the other strains in the field. Strains III and I were second in quantity. One-third (32.6%) of the diseased samples showing HLB-like symptoms in the field survey were found to be infected by HLB pathogen in PCR detection. Only 1.9% of healthy-looking citrus trees surveyed, including Wentan pummelo (5%) and Eureka lemon (5.7%), were infected by HLBB. The HLBB-isolates collected from mandarin, tangor, sweet orange, and kumquat (*Fortunella margarita* [Lour.] Swingle) were commonly co-infected with *Citrus tristeza closterovirus* (CTV) and/or *Citrus tatter leaf capillovirus* (CTLV). About 32% of the HLBB-infected trees examined were infected with HLBB only. Most HLB-affected mandarin (66.7%) and tangor (69%) trees were also infected by CTV while CTV was rarely detected in HLBB-infected pummelo (5%) or lemon (0%).

Keywords: Huanglongbing; HLBB strain; Pathogenicity.

INTRODUCTION

Citrus greening was first reported in South Africa in 1947, while a similar disease known as "Huanglongbing" (HLB) was already found in China in 1943. This psyllid-borne virus-like disease also known as Likubin caused by *Candidatus* Liberibacter asiaticus has been devastating the citrus production in Taiwan since 1951 (Matsumoto et al., 1961). The Asian heat-tolerant form of Huanglongbing bacteria (HLBB) has seriously affected citrus trees in the tropical and subtropical regions of Asia, and recently in North and South America as well. The HLB caused great damage to the citrus industry by shortening tree lifespan and lowering fruit yield and quality. The HLBB was transmitted by the Asian psyllid (*Diaphorinia citri*) in a persistent manner without transovarial passage. Chinese box orange (*Severinia buxifolia*) was found to be the alternative host of the HLB pathogen and vector psyllid. No HLBB multiplication was detected in Jasmine orange (*Murraya paniculata*) or Curly leaf (*Murraya koenigii*) (Hung et al., 2000b). Most citrus cultivars, except pummelo, were susceptible to the Asian form of HLB before 1971. However, pummelo became infected by a new HLB strain in Taiwan and Southeast Asia in 1970s. The pummelo cultivars grown in Philippines, Malaysia, Southern China, Vietnam, Thailand, Sri Lanka, Bangladesh, and Cambodia have become susceptible to HLB in recent decades. It is caused by a nonculturable fastidious bacteria inhabiting the sieve tube and is referred to as *Candidatus* Liberibacter (Jagoueix et al., 1994). The HLB disease retards growth of the plant and causes serious yellowing and decline of citrus trees, atrophy, and incomplete colouring of mature fruit. The pathogen can be categorized into two forms, Asian and African, based on the influence of temperature on symptom expression. The Asian form caused by "*Ca*. Liberibacter asiaticus," the symptoms of which can occur at temperatures above 30°C, is heat-tolerant, and the African form caused by "*Ca*. Liberibacter africanus," the symptoms of which

*Corresponding author: E-mail: hjsu@ntu.edu.tw; Fax: +886-2-23625717; Tel: +886-2-33664095.

appear above 30°C, is heat-sensitive (Bové et al., 1974). In Taiwan, severe leaf yellowing was first noticed in Ponkan mandarin (PM), Tankan tangor (Tan), and Liucheng sweet orange (LSO), but not in Wentan pummelo (WP) in the field in 1951 (Matsumoto et al., 1961). The pummelo cultivar formerly resistant to HLB eventually became infected and displayed HLB symptoms about 30 years after HLB first appeared (Su and Wu, 1979; Huang and Chang, 1980). The kumquat (*Fortunella margarita* (Lour.) Swingle), which was formerly resistant to HLB, recently became infected and displayed yellow mottling symptoms in 2006 (Tsai et al., 2006). It was assumed that the change of host range may have been due to the evolution of HLB strains in pathogenicity. The present research work was mainly aimed at isolation and differentiation of HLB pathogen stains in Taiwan by pathogenicity and virulence characterization. In this study, the adequate differential cultivars were selected for identifying strains of HLBB isolates collected from tropical and subtropical regions.

MATERIALS AND METHODS

HLBB isolates and sources

Totally 457 diseased samples showing HLB-like symptoms were collected from seven main citrus producing areas over Taiwan island, i.e., 85 from the Hsinchu area growing Ponkan mandarin (PM) and Tankan tangor (Tan); 49 from the Miaoli, Tan area; 77 from Chiayi, cultivating Wentan pummelo (WP); 93 from the Hualien WP area; 42 from the Tainan Liucheng sweet orange (LSO) producing area; 53 from Pingtung, cultivating Eureka lemon (EL), and 42 from the Yilan kumquat producing area (Figure 1). The HLB isolates were selected as representative ones after indexing of HLB, CTV and CTLV with PCR, ELISA and RT-PCR, respectively. Many healthy-looking samples were also collected from all citrus producing areas to detect the latent HLBB, CTV, and CTLV infections.

Pathological characterization of HLBB isolates for strain identification

To avoid interaction between CTLV and CTV, the HLBB-infected samples without CTLV and CTV, or with only mild CTV, were selected as HLBB isolates for strain identification. The HLBB isolates with CTV infection became free from CTV by passage through filter plants like Trifoliate orange (*Poncirus trifoliate* (L.) Raf.), which is immune to CTV (Yoshida et al., 1983), or Pummelo, which is hypersensitive to most CTV strains except the pummelo stem-pitting strain (Tsai et al., 1993). The 48 Taiwanese representative isolates of HLBB were selected from 457 diseased samples. Among many citrus cultivars, Ponkan mandarin (PM) and Liucheng sweet orange (LSO) were found to be most susceptible to HLBB by showing distinct yellow mottling symptoms. Wentant pummelo (WP), formerly immune to HLBB became infected in 1971 in Taiwan. The Eureka lemon (EL) cultivar, originally quite resistant to HLBB, became susceptible and displayed classic HLB symptoms in recent years (Matsumoto et al., 1961). Therefore, PM, LSO, WP and EL were used as differential cultivars of the HLBB strain. HLBB isolates were subjected to pathogenicity tests with these four differential citrus cultivars. Healthy seedlings about 15 cm tall were inoculated by grafting with diseased buds or leaf petioles. More than two trial buds were grafted onto each differential plant. For each HLBB isolate, four replicates of each differential cultivar were used. The test citrus seedlings were grown in an insect-proof greenhouse

Figure 1. Agroecological areas of HLB epidemic in Taiwan island. PM and Tan are grown in 1 (Hsinchu) and 2 (Miaoli). WP and other pummelo are grown in areas 3 (Chiayi) and 5 (Hualien). LSO is grown in area 6 (Chiayi) EL is grown in area 7 (Pingtung) and Kq is centralized in area 4 (Yilan). Bold line crossing area 3 and 5 is Tropic of Cancer. Region above bold line is subtropical, and region below bold line is tropical. ^ denotes the Central Mountain Range.

with an air-cooling system. The symptom development on each test plant was observed and recorded each month after inoculation. Multiplication dynamics of HLB pathogen in test plants was analyzed by PCR each month until 12 months after inoculation. Disease severity was graded as follows: 0 = no symptom; 1 = mild chlorosis without dwarfing; 2 = intermediate chlorotic mottling with moderate dwarfing; 3 = severe HLB symptoms, including yellow mottle, leaf hardening, and curling with distinct dwarfing.

Genomic DNA isolation and HLBB-PCR detection

The nucleic acid extracts to be used as the templates for PCR detection were prepared by using the method described by Hung et al. (1999). Leaf midrib (500 mg) was powdered in liquid nitrogen, and each sample was suspended in 1.5 ml of DNA extraction buffer [1 M Tris-HCl (pH 8.0) 0.5 M EDTA, 5 M NaCl, 1% N-Lauroylsarcosine] and transferred to a 1.5 ml Eppendorf tube. After incubation at 55°C for 1 h, the sample was centrifuged at 4,000 *g* for 5 min. The supernatanat (800 µl) was collected, and 100 µl 5 M NaCl and 100 µl 10% CTAB (hexadecyl-trimethyl-ammonium-bromide) in 0.7 M NaCl were added. The mixture was incubated at 65°C for 10 min. The sample was subjected to one cycle of chloroform/isoamyl alcohol (24:1) extraction, and the aqueous supernatant was then reextracted by an additional cycle of phenol/chloroform/isoamyl alcohol (25:24:1). The nucleic acids were precipitated by mixing 600 µl of the supernatant with 360 µl isopropanol followed by centrifugation at 12,000 *g* for 10 min. The pellets were washed with 70% ethanol, dried, and resuspended in 150 µl TE buffer as template solution.

Two µl of the extract as template was pipeted into 25 µl tube containing PCR mixture. The amplification of HLBB-DNA in PCR cycle with the primer pairs, consisted of the forward primer 5′-CAC CGA AGA TAT GGA CAA CA-3′ and the reverse primer 5′-GAG GTT CTT GTG GTT TTT CTG-3′ (Hung et al., 1999). The amplification of HLBB specific DNA fragment (226-bp) in the PCR thermal cycle consisted of 94°C for 3 min, 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min followed by a 72°C extension for 10 min. Reactions were carried out in PCR Thermal Cycler AB 2720 followed by electrophoresis analysis. The PCR products were quantified using the densitometer supplied by AlphaEase®FC Image Analysis Software. The density of the PCR products was determined and represented by a pixel (picture element) value with a range from 0 to 255.

Genomic RNA extraction and RT-PCR for CTLV detection

RNeasy Mini Kit (Qiagen, Hilden, Germany) was used for preparing the total RNA from diseased samples. Two c-DNA primers for the RT-PCR-based detection of CTLV were designed from GenBank (accession No. AY646511) (Lin and Hung, 2004). The primer pair, consisting of the forward primer 5′-GGA AGA CTC ACA TAG ACC CG -3′and the reverse primer 5′-TAC TCT CCG AAC CTG CCT C-3′, was used for proceeding one-step RT-PCR to amplify a CTLV-specific 636-bp fragment (Hung et al., 1999a; Hung et al., 2000; Lin and Hung, 2004).

ELISA tests for CTV detection

Monoclonal antibody (3E10) recognizing a common epitope of the CTV strain was used for indexing CTV in all diseased samples by a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Tsai and Su, 1991; Tsai et al., 1993).

RESULTS

Identification of HLBB strains by pathogenicity

The HLBB strains were differentiated based on the criteria of pathological characterization including host range, pathogenicity and virulence. Multiplication of HLBB was shown with PCR test by pixel value. Taiwan HLBB isolates were categorized into four strains. They were identified according to the disease index in symptom expression on the four differential citrus cultivars and PCR detection of HLBB multiplication 12 month after inoculation (Table 1). Strain I caused severe yellow-mottling and typical HLB symptoms on susceptible Ponkan mandarin (PM) and Liucheng sweet orange (LSO) cultivars but did not infect Wentan pummelo (WP) or Eureka lemon (EL) cultivars (Figure 2). No multiplication of HLBB in the latter two cultivars was confirmed by PCR detection. Strain II showed high virulence of pathogenicity in all cultivars. It induced severe HLB symptoms, including leaf yellow-mottling and curling, vein-yellowing

Table 1. The pathogenicity characterization of HLBB isolates according to disease index and PCR detection of HLBB infection.

Strain	Differential cultivars of citrus[a]			
	PM	LSO	WP	EL
I	3 /+++[a]	3 / +++	0 / -	0 / -
II	3 / +++	3 / +++	3 / +++	3 / +++
III	2 / ++	2 / ++	1 / +	0 / -
IV	0 / +	0 / +	0 / -	0 / -

[a]Disease index / PCR index. Disease index was graded 12 months after inoculation: 0, healthy looking without symptoms; 1, mild chlorotic symptoms; 2, intermediate symptoms including chlorosis, mottling with intermediate dwarfing; 3, typical greening symptoms including leaf yellow mottle and curling, vein-yellowing with distinct dwarfing. Pixel value (density count) index of the HLBB-specific band on agarose gel electrophoresis: -, pixel value< 50; +, 50 ~ <110; ++, 110 ~ < 170; +++, 170 ~ < 230. Differential cultivars were Ponkan mandarin (PM), Liucheng sweet orange (LSO), Wentan pummelo (WP), and Eureka lemon (EL).

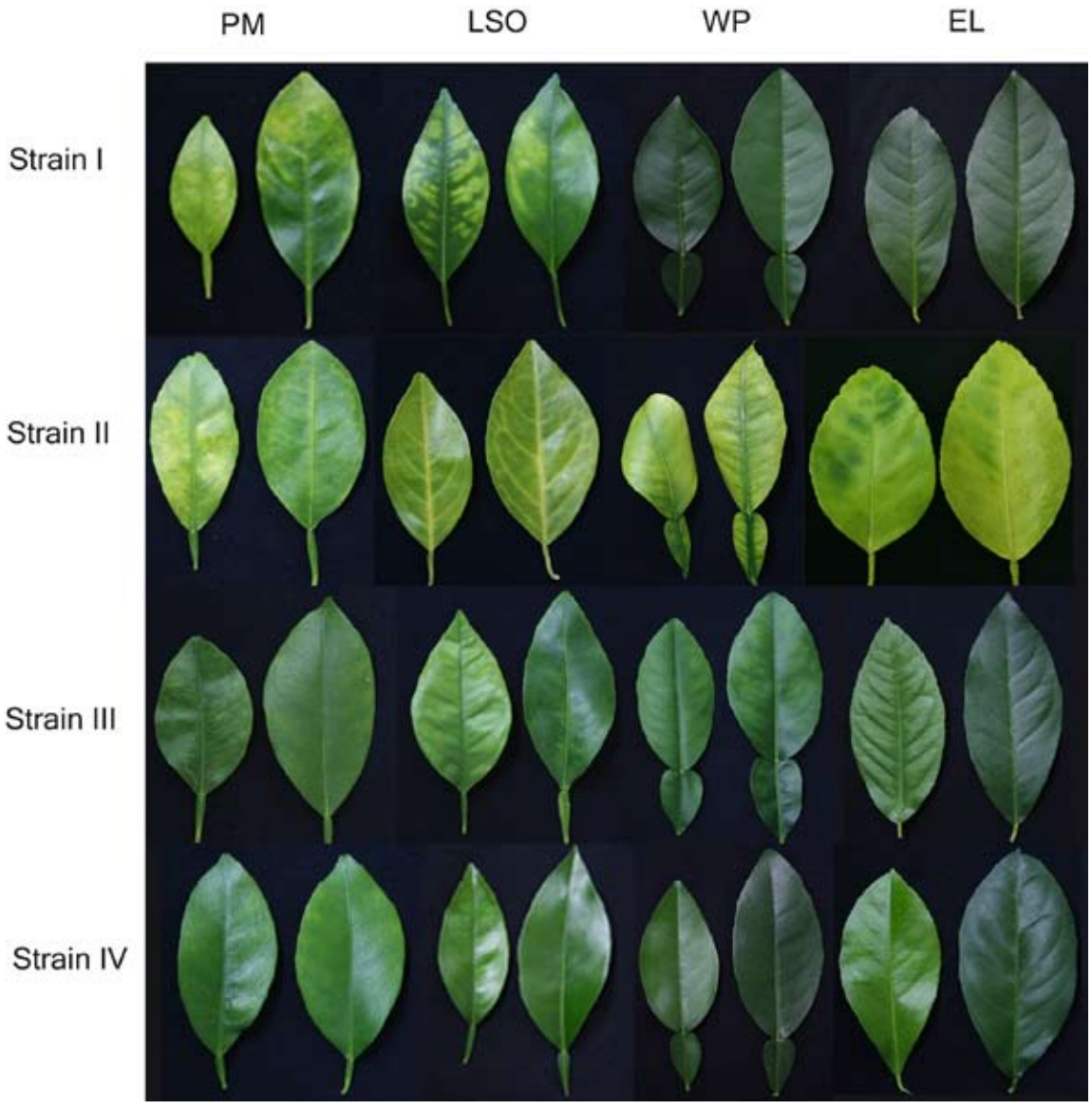

Figure 2. Different types and severities of the symptoms induced by the four HLBB strains (I-Ⅳ) in four differential citrus cultivars of Ponkan mandarin (PM), Liucheng sweet orange (LSO), Wentan pummelo (WP) and Eureka lemon (EL) under greenhouse conditions during 2005-2006.

and distinct dwarfing. Multiplication of the strain II was demonstrated by high pixel value in PCR detection of HLBB. Strain III, the intermediate virulent strain, infected PM, LSO and WP, but not EL, and caused leaf-chlorosis with mild vein enation. No yellow mottling or typical HLB symptoms were induced by strain III. Strain IV was a mild strain infecting PM and LSO without symptoms and did not infect WP or EL. This mild strain was not commonly isolated.

Detectable duration of HLB pathogen and incubation period of symptom appearance

The detectable duration, indicated by the multiplication rate, and the incubation period, indicated by the symptom expression after infection, were affected by pathogen strains and host cultivars. The multiplication rate of the pathogen in each differential cultivar was monitored by PCR. The first HLBB detection by PCR and incubation period of the first symptom appearance were shown in Table 2. Strain I propagated faster in PM than LSO as indicated by detectable durations of 2 and 3 months, respectively, determined by PCR analysis after inoculation. A 3-month inoculation period was demonstrated in both cultivars. No infection of strain I was detected in WP or EL. The multiplication rate of strain II was most rapid in all the tested cultivars. The strain II HLBB was detected within 2 months in mandarin (PM) and 3 months in the other test cultivars. It induced symptoms in 3 months on susceptible PM and LSO cultivars and in 4 months on tolerant WP and EL cultivars. Strain III was detected in PM within 3 months and in LSO and WP within 4 months. Its incubation period was 5 months in PM and LSO, and

Table 2. Detectable duration of pathogen by PCR-monitoring and incubation period of symptom appearance by HLBB strains in four differential cultivars, inspected monthly after graft-inoculation.

Strain		Months after inoculation			
		PM	LSO	WP	EL
I	PCR / Sym[a]	2 / 3	3 / 3	- / -	- / -
II	PCR / Sym.	2 / 3	3 / 3	3 / 4	3 / 4
III	PCR / Sym.	3 / 5	4 / 5	4 / 6	- / -
IV	PCR / Sym.	3 / N	5 / N	- / -	- / -

[a]First positive detection of PCR / First month of symptom appearance (incubation period) Differential citrus cultivars mentioned in Table 1.
N: symptomless.

6 months in WP. The EL plant was immune to strain III. The mild strain IV showed latent infection in mandarin (PM) and sweet orange (LSO) and multiplied slowly with a detectable duration of 3~5 months. However, it disappeared in some test plants 1~2 years after inoculation. Based on the multiplication rate and incubation period, strain II was the most virulent strain, and it dominated over the other strains in Taiwan. Strains I and III, showing moderate virulence, were detected at a lower rate. Generally speaking the incubation period of susceptible cultivars such as PM and LSO was shorter than those of tolerant cultivars such as WT and EL.

Distribution of HLBB strains on Taiwan island

A total of 457 diseased samples were collected from seven major citrus-greening areas on the island of Taiwan, and 48 representative HLBB isolates were selected for strain identification by pathogenicity assay within 12 months. The infection and replication of HLBB were confirmed by PCR (Table 3). The strain II, detected in 68.8% of the samples, was isolated most commonly from all cultivars grown in Taiwan, including mandarin (PM), tangor (Tan), sweet orange (LSO), pummelo (WP), kumquat, and lemon (EL). Strain III, with an 18.3% detection rate, was isolated mainly from WP and rarely from PM and Tan grown in northern, eastern, and central parts of Taiwan. Strain I, with an 8.3% detection rate, was isolated from PM and Tan from old citrus-growing Hsinchu and Miaoli areas in northern Taiwan. Mild strain IV, with a 4.2% detection rate, was rarely isolated from tangor grown in Hsinchu. Only strain II was detected in EL in the tropical Pingtong area while all four strains were found in the subtropical areas.

HLB incidence and complex infection of HLBB with viruses in the field

The citrus trees were commonly infected by HLBB, citrus tristeza closterovirus (CTV), and tatter leaf capillovirus (CTLV) (Su and Cheon, 1984; Tsai et al., 1991). One third (32.6%) of the suspected citrus trees with HLB-like symptoms were indexed to be infected by HLBB. About 32% of HLBB-infected trees were infected by HLBB only, and the other HLBB-infected trees (68%) were also mix-infected with CTV (24.8%), CTLV (27.5%) or CTV+CTLV (15%) (Table 4). Over half of the suspected Eureka lemon trees (52%) grown in the tropical Pingtung area were infected by HLBB while about 30% of the other suspected citrus trees were infected by HLBB on the whole island. The samples of HLBB-infected trees free from the viruses were used for the isolate sources of HLBB strains. The frequency of HLBB/viruses mix-infection was varied in citrus cultivars. All the citrus trees of mandarin (PM), tangor (Tan), sweet orange (LSO), and kumguat were infected by HLBB in combination with CTV and/or CTLV. No viruses were detected in half of the HLBB-infected pummelo (WP) (50.8%) or lemon (EL) (61.5%) trees. The HLB-affected mandarin (66.7%) and tangor (69%) trees were frequently mix-infected with CTV. However, CTV was rarely detected in pummelo (4.8%) and lemon (0%). The CTLV was found commonly infecting WP (44.4%), kumquat (30%), and EL (38.5%)

Table 3. The proportion and distribution of HLBB strains over the island of Taiwan via pathogenicity tests with 48 representative HLBB isolates obtained from various localities.

Locality (Prefecture)	Citrus cultivar	No. of HLBB isolates	Strains of HLBB			
			Strain I	Strain II	Strain III	Strain IV
Hsinchu (N)	PM	5	1	3	1	0
	Tan	7	1	4	0	2
Miaoli (N)	Tan	5	2	2	1	0
Chiayi (M)	WP	9	0	6	3	0
Yilan (E)	Kq	4	0	4	0	0
Hualien (E)	WP	7	0	3	4	0
Tainan (S)	LSO	5	0	5	0	0
Pingtung (S)	EL	6	0	6	0	0
Total/percentage		48	4 (8.3%)	33 (68.8%)	9 (18.8%)	2 (4.2%)

N, northern; M, central; S, southern; E, eastern part of Taiwan.

Table 4. Complex infection of HLBB with CTV and CTLV in the main citrus-growing areas.

Locality (Prefecture)	Citrus cultivars	HLBB-infected plants / No. of indexed plants[a] (%)	Complex infection of citrus viruses in HLBB-positive samples (%)			
			HLBB	HLBB+CTV	HLBB+CTLV	HLBB+CTV+CTLV
Hsinchu	PM	9/29 (31.0%)	0/9 (0%)	6/9 (66.7%)	0/9 (0%)	3/9 (33.3%)
	Tan	16/56(28.6%)	0/16 (0%)	11/16 (68.9%)	0/16 (0%)	5/16 (31.2%)
Miaoli	Tan	13/49 (26.5%)	0/13 (0%)	9/13 (69.2%)	0/13 (0%)	4/13 (31.8%)
Chiayi	WP	30/77 (39.0%)	14/30 (46.7%)	2/30 (6.7%)	14/30 (46.7%)	0/25 (0%)
Yilan	Kq	10/60 (16.7%)	0/10 (0%)	4/10 (40.0%)	3/10 (30.0%)	3/10 (30.0%)
Hualien	WP	33/93 (35.5%)	18/33 (54.5%)	1/33 (3.0%)	14/33 (42.4%)	0/29 (0%)
Tainan	LSO	12/42 (28.6%)	0/12 (0%)	4/12 (33.3%)	0/12 (0%)	8/12 (66.7%)
Pingtung	EL	26/50 (52.0%)	16/26 (61.5%)	0/26 (0%)	10/26(38.5%)	0/26 (0%)
Total		149/457 (32.6%)	48/149 (32.2%)	37/149 (24.8%)	41/149 (27.5%)	23/149 (15.4%)

[a]The citrus trees showing HLB-like symptoms including chlorosis, vein-yellowing, yellowing and yellow-mottling which may be induced by HLBB and/or the other causes.

while PM (33.3%), Tan (31.2%), and LSO (66.7%) were frequently infected by HLBB with a complex infection of CTV plus CTLV.

Latent infection of HLBB, CTV, and CTLV in citrus trees in the field

The infection of HLBB and citrus viruses was detected in citrus samples collected from healthy-looking citrus trees of different cultivars grown in seven counties in Taiwan (Table 5). In total, only 1.9% trees of healthy-looking 305 samples—including 4 of 80 WP-samples (5%) grown in Chiayi and Hualien and 2 of 35 EL-samples (5.7%) grown in tropical Pingtung area—were infected by HLBB without a mix-infection of CTV and CTLV. HLBB was not detected in the 30 healthy-looking PM-trees from Hsinchu, 70 Tan-trees from Miaoli, 50 kumquat trees from Yilan, or 40 LSO-trees from Tainan. About 32.7% of healthy-looking citrus trees were infected by CTV. A high percentage of PM trees (73.3%, Hsinchu) and Tankan trees (68.6%, Miaoli) were commonly infected by CTV without visible symptoms. CTV was not detected in healthy WP and EL trees. A single infection of CTLV was detected frequently in WP (52.5%, Chiayi and Hualien) and EL trees (54.3%, Pingtong) while about 20% of surveyed trees were infected singly with CTLV. A mixed-infection of CTLV plus CTV was commonly detected in LSO (70%, Tainan) and kumquat (64%, Yilan), and in a certain amount of Tan (31.4%, Miaoli) and PM (26.7%, Hsinchu).

DISCUSSION

Citrus Huanglongbing was first found in China in 1943. The virus-like disease was first noticed in Taiwan in 1951, six years after the Second World War (Matsumoto et al., 1961). The HLB disease became epidemic after introduction, possibly from China circa 1945. HLB has been devastating the citrus production and causing great damage to the citrus industry by shortening the tree

Table 5. Incidence of latent infection of HLBB, CTV, and CTLV in symptomless citrus trees.

Citrus cultivars[a]	Locality (Prefecture)	Sample no.	HLBB and citrus viruses in symptomless trees			
			HLBB	CTV	CTLV	CTV+CTLV
PM	Hsinchu	30	0/30 (0%)	22/30 (73.3%)	0/30 (0%)	8/30 (26.7%)
Tan	Miaoli	70	0/70 (0%)	48/70 (68.6%)	0/70 (0%)	22/70 (31.4%)
WP	Chiayi, Hualien	80	4/80 (5.0%)	0/80 (0%)	42/80 (52.5%)	0/80 (0%)
Kq	Yilan	50	0/50 (0%)	18/50 (36.0%)	0/50 (0%)	32/50 (64.0%)
LSO	Tainan	40	0/40 (0%)	12/40 (30.0%)	0/40 (0%)	28/40 (70.0%)
EL	Pingtung	35	2/35 (5.7%)	0/35 (0%)	19/35 (54.3%)	0/35 (0%)
Total/percentage		305	6/305 (1.9 %)	100/305 (32.7%)	61/305 (20%)	90/305 (29.5%)

[a]The test samples collected from the healthy-looking citrus trees of different cultivars including Ponkan mandarin (PM), Tankan tangor (Tan), Wentan pummelo (WP), kumquat (Kq), Liucheng sweet orange (LSO), and Eureka lemon (EL).

lifespan and lowering fruit yield and quality in Taiwan. The heat-tolerant form of HLBB has severely affected citrus trees in the tropical and subtropical regions of Asia and recently in South and North America (Coletta et al., 2004; Zhou et al., 2007). The systemic HLB disease is caused by non-culturable fastidious bacteria. It easily becomes epidemic because of common transmission through vegetative-propagated seedlings and is rapid spread by psyllid vector.

Taiwan has both tropical and subtropical regions. The Central Mountain Range separates the island into west and east regions. This special topography and geographical feature causes diversity in climates. Due to this diversity, different citrus cultivars were planted in different areas. For example, PM and Tan were concentrated in northwest region, and WP was concentrated in southwest and southeast region while EL was only planted in southern Taiwan. HLBB-infected leaves collected from PM, Tan, and LSO were all mix-infected with CTV or CTLV. This may be why these HLB-infected trees showed severe symptoms and declined quickly in the field. Among the 457 HLB-like trees surveyed, only approximately 30% were caused by HLBB. Other factors, including root rot, stem borer (*Anoplophora macularia* [Thomson]), and nutrient deficiency, also caused HLB-like symptoms including chlorosis of leaves, vein-yellowing, and tree decline.

In this report, HLBB isolates were separated into different strains for the first time. Strain I was specific to PM, Tan and LSO cultivars in our pathogenicity tests. This pathogenicity character was similar to the HLB observed from 1951 to 1970 in Taiwan (Su and Wu, 1979; Huang and Chang, 1980). Severe HLB symptoms were only found in PM, Tan, and LSO, but not in WP. Therefore, strain I was similar to the original type observed in the field in 1951. HLB was a virus-like and systemic disease. The cultivar limitation of strain was also found in CTV. CTV pummelo stem-pitting strain (Pum/SP) found in southeast Taiwan caused severe symptoms, including bunchy twigs and curly leaves and stem-pitting on twigs, trunk, and roots in WP, but it could not infect LSO and PM. Sweet orange stem-pitting strain (SO/SP), Mandarin stem-pitting strain (Mand/SP), and ordinary seedling-yellow strain (SY) could not persist in WP trees (Tsai et al., 1993). HLBB strain II, a highly virulent strain, was able to infect the formerly immune WP and the other cultivars, and it rapidly propagated in all seedlings to cause severe yellowing symptoms in pathogenicity tests. In the field, this predominant strain usually existed in trees showing severe symptoms, especially in tolerant cultivars, and could therefore be easily located. Strain II was assumed to have evolved from strain I. Strain III was an intermediate strain that could be found in trees which had been infected by HLB for several years, but still maintained their growth and capacity to produce fruit. Trees in the field infected with strain III usually showed intermediate or mild symptoms, and this observed phenomenon was in accordance with our pathogenicity tests. Strain IV was a mild strain and only found in Tan producing areas. The trees in the field infected with strain IV were usually symptomless and differential cultivar tests also showed the same degree of symptoms 12 months after inoculation. The mild strain IV was used to test its ability to cross-protect citrus plants, but it did not show a protective effect against the severe strains in our preliminary trials. In view of HLBB strain evolutions, molecular characterization of Taiwanese strains is expected to be investigated in comparison with foreign strains through international collaboration.

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台灣柑橘黃龍病之系統鑑別與分佈

蔡佳欣　洪挺軒　蘇鴻基

國立台灣大學 植物病理與微生物學系

本試驗自台灣熱帶與亞熱帶地區 7 柑橘主要產區，從疑似黃龍病株採集 457 個病材料，以聚合酶連鎖反應 (PCR) 偵測黃龍病菌 (Huanglongbing bacteria = HLBB)，並經過濾植物去除病毒後，得到 48 個無病毒之黃龍病代表分離株以進行病原性與毒性之生物檢定。將各代表分離株嫁接接種在四種不同感病性之柑橘鑑別品種包括有椪柑 (*Citrus reticulate* Blanco)、柳橙 (*C. sinensis* Osb.)、文旦 (*C. grandis* f. *buntan* Hay.) 及檸檬 (*C. limon* Burm) 實生苗，觀察其發病狀況。共鑑別出四種黃龍病菌系統，系統 I 僅對椪柑與柳橙具高病原性並產生典型黃龍病病徵。系統 II 對四種鑑別品種皆具高毒性，並可快速增殖其內。系統 III 在椪柑與柳橙引起中度病徵，在文旦僅出現輕度病徵，然而卻無法感染檸檬。系統 IV 則僅可感染椪柑及柳橙，幾無病徵表現。經調查台灣田間病菌系統分佈，系統 II 為目前分佈廣之最優勢族群，系統 III 及 I 次之。田間採集疑似黃龍病材料中，實際上約三分之一感染黃龍病 (32.6%)，而看似健康之各品種柑橘株僅 1.9% 感染黃龍病菌，並只在文旦 (5%) 與檸檬株 (5.7%) 發現。於本次收集之黃龍病材料中，椪柑、桶柑、柳橙與金柑 (*Fortunella margarita* (Lour.) Swingle) 普遍複合感染了柑橘萎縮病毒 (*Citrus tristeza closterovirus* = CTV) 與破葉病毒 (*Citrus tatter leaf capillovirus* = CTLV)。單獨感染黃龍病者佔 32%。椪柑及桶柑之黃龍病株大多複合感染 CTV，但於文旦與檸檬之黃龍病株則少見 CTV 之複合感染。

關鍵詞：柑橘黃龍病；柑橘黃龍病菌系統；病原性。