Identification of Alternative Hosts of the Fastidious Bacterium Causing Citrus Greening Disease

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With 3 figures

Received June 10, 1999; accepted December 13, 1999

Keywords: alternative hosts, citrus greening, dot hybridization, DNA probe, fastidious bacterium

Abstract
Citrus greening is a severe disease caused by a fastidious bacterium (GFB) residing in the sieve tubes of its hosts. It is an epidemic disease and is spread by insect vectors. In Asia, the Asian citrus psyllid (Diaphorina citri Kuwayama) is the vector for GFB. For the epidemiological study, an investigation of alternative hosts of GFB was made. Four suitable hosts of the Asian psyllid that are considered as possible alternative hosts of GFB were investigated on graft-inoculation tests. The multiplication of GFB in plants was monitored by dot hybridization using a GFB-specific DNA probe developed previously by us. The results demonstrate that GFB can replicate in Chinese box orange (Severinia buxifolia) and wood apple (Limonia acidissima), but not in common jasmin orange (Murraya paniculata var. paniculata) and curry leaf (Murraya euchrestifolia). Chinese box orange is a good host in which GFB replicates as well as it does in its citrus hosts. Wood apple is a transient host in which GFB exists temporarily and disappears several months later. Common jasmin orange and curry leaf are not hosts of GFB as they showed no detectable signals in dot hybridization tests throughout 1 year of experimentation.

Introduction
Citrus greening is a very severe disease in Asia. It is caused by a nonculturable fastidious bacterium that inhabits the sieve tubes of its hosts (Garnier et al., 1973). The pathogen infects citrus trees of almost all cultivars, and causes substantial economic losses to the citrus industry by shortening the lifespan of the infected trees. The insect vector, Asian citrus psyllid (Diaphorina citri Kuwayama), spreads this disease in Asia (Capoor et al., 1985; Chen et al., 1973; Aubert, 1987; Gottwald et al., 1989). It has been an important epidemic disease and has been difficult to control in Taiwan and several other Asian countries. Furthermore, citrus greening has a long incubation period, and many latently infected citrus plants occur in the field (McClean, 1970; Huang, 1979). Development of an accurate diagnostic technique becomes the first step for disease control. However, the fastidious bacterium (GFB) causing citrus greening is difficult to detect because of its low concentration and uneven distribution in its environment.
natural hosts (da Graca, 1991). Fortunately, the application of DNA probes overcomes the difficulty of GFB detection. A standard protocol using a DNA probe has been developed by the authors and successfully applied in GFB detection for several years (Hung et al., 1999a). This method has also been applied in ecological studies of citrus greening to obtain some answers to epidemiological questions that could not be determined by conventional methods.

In Taiwan, there are three major strategies for the control of citrus greening: (1) the establishment of pathogen-free nursery systems; (2) vector (psyllids) control; and (3) elimination of the inoculum sources including infected citrus trees and alternative hosts of GFB. At present, there is no detailed research about alternative hosts of GFB. To investigate alternative hosts of GFB, four suitable hosts for Asian citrus psyllids have been tested. These four plants are the common jasmin orange (Murraya paniculata var. paniculata), curry leaf (Murraya euchrestifolia), wood apple (Limonia acidissima), and Chinese box orange (Severinia buxifolia). They all belong to the Rutaceae family.

Common jasmin orange (CJO) and curry leaf (CL) are two members of Genus Murraya. CJO is a shrub that is widely distributed in tropical Asia, and commonly cultivated as a hedge plant. CL differs from CJO in many characteristics, especially in smell. CL is widely cultivated in countries such as Malaysia and India where curry is commonly consumed. Both CJO and CL are favoured hosts of psyllids (Lin et al., 1973; Chakraborty et al., 1976; Miyakawa, 1980). In Taiwan, CJO is very popular whereas CL cultivation is rare.

Wood apple (WA) has not yet been cultivated in Taiwan, but it is commonly grown as an ornamental plant in Thailand, India and Indonesia and it is also a known host for psyllids.

Chinese box orange (CBO) is a spinous shrub distributed in India, Malaysia, Vietnam, southern China, the Philippines, Taiwan and Japan. It is often seen in citrus orchards and has proved to be an important host of psyllids in Taiwan (Lin et al., 1973).

The ability of GFB to survive in these four test plants was investigated using the graft-inoculation test. The occurrence and multiplication of GFB in hosts were monitored by dot hybridization tests using a GFB-specific DNA probe. In this paper, it is shown that GFB can inhabit other hosts in addition to citrus hosts. This discovery offers important information for the control of citrus greening disease.

Materials and Methods

Plant materials

Four suitable hosts of psyllids, CJO, CL, WA, and CBO, were chosen for graft-inoculation tests. The plants were obtained from seeds; those of CJO and CBO were collected from local plants in Taiwan whereas the seeds of CL and WA were exotic, CL being obtained from Malaysia and WA from Thailand. Valencia sweet orange (Citrus sinensis L.) was used as the control citrus host in this experiment. The healthy citrus plants were obtained using a technique of shoot tip grafting (Murashige et al., 1972; Su and Chu, 1984). A GFB-infected Valencia plant was the inoculum source to supply the GFB-infected scions for the graft-inoculation tests. All experimental plants were under insect-proof control in a greenhouse.

Graft-inoculation

The budwood-grafting method referred to in other publications (Su and Chu, 1984; Yoshida, 1996) was chosen for the graft-inoculation. The GFB-infected citrus scions were grafted onto seedlings of CJO, CL, WA and CBO. After grafting, these test plants were sampled and DNA preparations were made monthly for the detection of GFB. The occurrence and multiplication of GFB were monitored by dot hybridization tests using a GFB-specific DNA probe. Observations and records of symptom development were also carried out monthly. For reverse tests, GFB-infected scions of alternative hosts were grafted onto the citrus plant (Valencia sweet orange). Grafting was judged successful when the scions survived for more than 3 months after grafting. The growth of the grafted scions was graded good, poor, or no growth by comparing its growth to that of citrus grafted on citrus.

Preparation of nucleic acid samples for dot hybridization tests

The nucleic acid samples used in dot hybridization tests were prepared using a method described by Lee and Davis (1988) and Lee et al. (1988, 1990) with minor modifications. Five grams of leaf midribs were powdered in liquid nitrogen and resuspended in 15 ml GFB extraction buffer [300 mM mannitol, 50 mM Tris-HCl (pH 7.4), 5 mM ethylenediaminetetraacetic acid (EDTA), 4 mM β-mercaptoethanol]. After low speed centrifugation (4000 × g for 10 min), the supernatant was centrifuged at 12000 × g for 15 min to pellet the GFB. This GFB pellet was resuspended in DNA extraction buffer [0.1 M Tris-HCl (pH 8.0), 0.05 M EDTA, 0.5 M NaCl, 0.1% N-lauroylsarcosine], incubated at 55 °C for 1 h and centrifuged at 4000 × g for 10 min to remove debris. The supernatant was mixed with 1% cetyltrimethylammonium bromide (CTAB) and incubated at 65 °C for 10 min. The sample was clarified by chloroform: isoamyl alcohol (25:1) and phenol: chloroform: isoamyl alcohol (25:24:1) extraction, and then mixed with 0.6 volumes of isopropanol to pellet DNAs. The DNA pellet was suspended in 6 × SSC solution [0.9 M NaCl, 0.09 M sodium citrate, (pH 7.0)] to make 3 μg/μl for GFB detection. All DNA samples from monthly collections were stored at −20 °C.

GFB-specific DNA probe

A cloned GFB-specific DNA fragment (0.24 kb), which was ligated into plasmid pBS (Stratagene Biotec., La Jolla, CA, USA), was chosen to develop a GFB DNA probe. This probe has demonstrated its specificity and sensitivity, and has been successfully used in the detection of GFB in various citrus cultivars (Hung et al., 1999a). A polymerase chain reaction (PCR)-labelling method was used in the preparation of a biotinylated GFB-specific DNA probe as described by Panaud et al. (1993) with slight modification. Amplification was performed in 50 μl
reaction mixture containing 50 ng each of the two opposing primers (T3 primer: 5'-AAT TAA CCC TCA CTA AAG GG-3'; T7 primer: 5'-GTA ATA CGA CTC ACT ATA GCC C-3'), 100 ng recombinant plasmids as templates, 0.2 mM each of three dNTPs (dATP, dCTP, dGTP), 0.18 mM dATP, 0.02 mM biotin-7-dATP (Gibco BRL, Gaithersburg, MD, USA), and 2.5 U Taq polymerase in incubation buffer [100 mM Tris (pH 8.3); 500 mM KCl; 2 mM MgCl₂, and 0.01% gelatin, w/v]. The reaction mixture was overlaid with 30 μl mineral oil. The thermal cycles were run for 30 cycles (denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min). All thermal cycles of PCR were performed on a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Foster City, CA, USA). The PCR product was purified by HiPure PCR Product Purification Kit (Boehringer Mannheim, Mannheim, Germany) and suspended in 50 μl TE buffer (pH 8.0).

**Dot hybridization**

For dot hybridization, DNA samples in 6 × SSC solution were denatured by boiling for 10 min. Three microlitres of DNA, after four-fold serial dilution in 6 × SSC (starting concentration at 3 μg DNA/μl), were dot blotted on nylon membranes. The membranes were air dried, baked at 80°C for 2 h under vacuum. Pre-hybridizations were performed at 68°C for 1 h in hybridization solution: 5 × SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, 1% (w/v) blocking reagent (Boehringer Mannheim). Hybridizations were performed at 68°C for 12–16 h in hybridization solution plus denatured DNA probes (50 ng/ml). The bound probes were detected with BluGENE detection system (Gibco BRL) according to the manufacturer's instruction.

**Results**

**Graft compatibility**

The four test plants were different in graft compatibility. The WA and CBO were compatible with citrus and so the citrus scions survived, and grew on the WA and CBO plants. The CJO and CL were less compatible with citrus and so the citrus scions could survive on CJO and CL, but they did not grow well.

**Multiplication of GFB into four suitable hosts of psyllid vector**

After graft-inoculation, four test plants were sampled and DNA was extracted every month. The multiplication of GFB in test plants was monitored by dot hybridization tests using a GFB-specific probe. CJO and CL showed negative results with no hybridization signal appearing during 1 year of experimentation. GFB cannot replicate in CJO and CL according to the results of dot hybridization.

The WA and CBO showed two different positive results (Fig. 1a, b). In the case of WA, GFB could be detected from the fifth to tenth month after graft-inoculation. The strength of the dot hybridization signal (judged by serial dilution tests) showed that the signal reached a peak at the seventh month and declined quickly. No signal appeared at the eleventh and twelfth months. GFB could be detected only between the fifth and tenth month, and its detection signals were much weaker than the positive control of GFB-infected citrus samples.

The CBO showed apparently positive results in this experiment. When compared with citrus (Fig. 1c), the pattern of dot hybridization in serial dilution is similar. The first signal appeared at the third month in CBO with the signal maintaining a high strength after reaching a peak at the eighth month. It was the same as citrus (Valencia sweet orange) control, but with citrus having slightly stronger signals than CBO.

For the convenience of data analysis, the hypothetical dynamic curves of GFB replication were drawn according to the results of dot hybridization tests in WA, CBO and citrus (Fig. 2). The curve of WA is an inverted ‘V’ shape (Fig. 2a), which increased linearly from the fourth to the seventh month and decreased linearly from the seventh to the tenth month. The WA curve returned to the zero at the tenth month. The curves of both CBO and citrus are the standard growth curves (Fig. 2b, c) which include lag, log and stationary phases. The first 2 months are the lag phase, in which the concentration of GFB in hosts is too low to be detected by dot hybridization tests. The following 6 months (second to eighth) are the log phase, in which GFB could be detected and its concentration increased linearly. The stationary phase began at the eighth month when the concentration of GFB reached a maximum.

**Symptom expression**

In addition to GFB detection using a DNA probe, a record of symptom-expression was also made in the present experiment. Similar to the results of dot hybridization, CJO and CL showed negative results. No symptom occurs on CJO or CL 12 months after graft-inoculation. WA showed mild yellowing symptoms at the sixth month, apparent yellowing symptoms at the ninth month, and recovered slightly from yellowing at the twelfth month. CBO showed mild chlorosis symptoms at the sixth month, evident chlorosis at the ninth month, and additional leaf-hardening and vein enation at the twelfth month. The Valencia sweet orange, which was used for the positive control of symptoms, showed yellowing symptoms at the sixth month, severe yellowing and leaf-hardening at the ninth month, and additional vein enation at the twelfth month.

**GFB transmission from CBO back to citrus host by graft-inoculation**

According to the results in Fig. 1, GFB can survive for over 1 year only in CBO hosts. CBO was shown to be a good recipient plant of GFB in this graft-inoculation experiment. To understand whether CBO is qualified to be a good donor of GFB or not, another experiment of graft-inoculation was made. A healthy citrus plant (Valencia sweet orange) was inoculated by grafting with GFB-infected CBO scions. GFB multiplication was also monitored by dot hybridization tests, and the result is shown in Fig. 3. GFB can be transmitted from CBO back to the citrus host. GFB infection in this citrus host was
first detected by dot hybridization tests at the third month after graft-inoculation, and the signals reached a maximal strength at the eighth month. The pattern of dot hybridization in serial dilution was close to Fig. 2c where the citrus plant was graft-inoculated with GFB-infected citrus scions. GFB-infected CBO scions approximately have the same ability of inoculation as citrus scions.

**GFB detection of CBO samples collected from the field**
In the spring of 1999, eight CBO samples were collected from the field near diseased citrus orchards in southern Taiwan to test for GFB. The dot hybridization test showed that one sample (CBO2) from the field showed positive (Fig. 4), which indicated that there are GFB-infected CBO plants actually existing in the field.

**Discussion**
Our experiment demonstrates that GFB may exist persistently in CBO just as in citrus plants. In graft-inoculation tests, CBO was a susceptible recipient plant in which GFB could survive and replicate; it was also a qualified donor plant that can transmit GFB to citrus hosts by grafting. CBO could be an alternative host of GFB according to these results.

CJO and CL are two members of the genus Murraya. They are suitable hosts for psyllids but not for GFB. Although GFB-infected scions of citrus can survive on them, GFB cannot be transferred into them. No signal appeared in CJO and CL when graft-inoculation and dot hybridization were performed.

Interestingly, GFB can slightly replicate in WA but not survive for a long time. In these experiments, GFB in WA could be detected only from the fifth to tenth month after grafting. It indicates that WA is a non-persistent host of GFB.

For further identification, the experiment of vector transmission has been continued since January 1999. In the study of vector transmission, the viruliferous psyllids
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Fig. 1 The hypothetic dynamic curves of the fluctuation of GFB replication in wood apple (a), Chinese box orange (b), and Valencia sweet orange (c). The curves are drawn according to the end point signal of serial dilution in dot hybridization tests (shown in Fig. 1a, b, c). The annotation is the same as described in Fig. 1.

are the donors of GFB instead of GFB-infected scions. This experiment is more difficult and time-consuming than graft-inoculation tests. It is likely to be finished by next spring. However, it has already been established that the preliminary results are identical to the results in this paper.

Multiplication of GFB in its alternative hosts appears to be linked to graft compatibility between citrus and the alternative hosts. According to the results described above, CJO and CL have poor graft compatibility to citrus scions and GFB seems to dislike inhabiting them.

In contrast, WA and CBO have good graft compatibility to citrus and GFB can replicate in them. However, WA is a transient host of GFB, which suggests that graft compatibility is probably just one of the qualifying conditions to become a host of GFB.

Alternative hosts always play an important role in an epidemic disease, but they are often neglected in epidemiological studies especially when they cannot be recognized. Alternative hosts are concealed havens for pathogen survival. Based on the results reported in this paper, CBO should be regarded as an alternative host of GFB. The data of GFB detection in the samples collected from the field verify that there is GFB-infected CBO already existing naturally. This discovery has not yet appeared in any other reports. On the other hand, CBO is not only an alternative host of GFB, but also a possible host of citrus tristeza closterovirus (CTV). Yoshida reported that CBO is susceptible to CTV in his experiments with graft-inoculation (Yoshida, 1996). Tsai discovered CBO is also a suitable host for the brown citrus aphid (Toxoptera citricida) that is the most efficient CTV vector (Tsai, 1998). CTV causes citrus tristeza disease which is another world-wide disease of citrus. Greening and tristeza are considered as two of the most severe diseases in Asia. It is strongly advised that CBO plants in the citrus orchard and its vicinity should be eliminated for the control of citrus greening and tristeza disease.

Among the four suitable hosts of psyllids, CJO and

Fig. 2 The hypothetic dynamic curves of the fluctuation of GFB replication in wood apple (a), Chinese box orange (b), and Valencia sweet orange (c). The curves are drawn according to the end point signal of serial dilution in dot hybridization tests (shown in Fig. 1a, b, c). The annotation is the same as described in Fig. 1.

Fig. 3 Dot hybridization test using a biotinylated GFB-specific DNA probe for the detection of GFB in the samples of Chinese box orange (CBO) collected from the fields in southern Taiwan. Lane Vd, a diseased Valencia sample for positive control of citrus; Lane Vh, a healthy Valencia sample for negative control of citrus; Lane BOd, a GFB-infected CBO sample via graft-inoculation for positive control of CBO; Lane BOh, a healthy CBO sample for negative control of CBO; Lane BO1~BO8, the test samples of CBO collected from the fields. The number 1~256 indicate reciprocals of four-fold dilution from starting concentration 3 μg/μl of nucleic acid samples.

Fig. 4 Dot hybridization test using a biotinylated GFB-specific DNA probe for the detection of GFB in the samples of Chinese box orange (CBO) collected from the fields in southern Taiwan. Lane Vd, a diseased Valencia sample for positive control of citrus; Lane Vh, a healthy Valencia sample for negative control of citrus; Lane BOd, a GFB-infected CBO sample via graft-inoculation for positive control of CBO; Lane BOh, a healthy CBO sample for negative control of CBO; Lane BO1~BO8, the test samples of CBO collected from the fields. The number 1~256 indicate reciprocals of four-fold dilution from starting concentration 3 μg/μl of nucleic acid samples.
CBO are often seen in Taiwan. CJO is a common hedge plant cultivated everywhere in Taiwan. Fortunately, the present experimental data show negative results of GFB replication in CJO. CJO was originally considered to be a possible host of GFB (da Graca, 1991; Guo and Deng, 1998; Singh and Nimbalkar, 1977), but the present data suggest that CJO should not be a host of GFB. Most of the CBO plants are wild in Taiwan. The character of CBO in the epidemiology of citrus greening will be further understood when the authors complete their next research project that includes transmission experiments by psyllid vector, recording seasonal fluctuation of psyllids population on CBO plants, and investigation of GFB-infected percentages of CBO plants in fields. The psyllid-transmission experiment will also be applied in CJO, CL and WA to reconfirm their resistance or tolerance to GFB infection.

According to local records of flora, CL is rare and WA is absent in Taiwan. However, they are prevalent and important in tropical Asia. CL is a spice, which is considered as a necessity in several south-eastern Asian countries. Several samples of CL were obtained from Malaysia, but GFB was never detected in them. The present data also show negative results in GFB detection via graft-inoculation tests. WA is normally used as an ornamental plant for landscaping in several Asian countries. The present results indicate that WA is not a good host for GFB, although GFB can temporarily replicate in it. Therefore, for the control of greening it would be advisable to avoid planting WA in the vicinity of citrus orchards.

The external symptom expression seems to be somewhat similar to the GFB-infection. CJO and CL did not show symptoms after graft-inoculation, which corresponds with the negative results from dot hybridization tests. CBO showed chlorosis, leaf-hardening and vein enation symptoms 1 year after grafting. Dot hybridization tests verify that the symptoms of CBO and GFB infection are correlative. WA was shown to be a transient host of GFB, but the yellowing symptoms on WA were still induced. Interestingly, WA recovered from yellowing symptoms when GFB was declining in WA. These data show that symptom-expression may be related to infection and replication of GFB.

All the detections of GFB in this research were conducted by DNA probes with dot hybridization tests. This method has its limit in the sensitivity for GFB detection. Recently, a faster and more sensitive assay using polymerase chain reaction to detect GFB in its hosts has been developed (Hung et al., 1999b). Therefore, another experiment is being carried out to reconfirm the data obtained from this paper using the PCR-based method. This research is not yet complete, but the preliminary results correspond with those presented in this paper.

Literature


