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REVIEW

Occurrence and Control of Citrus Greening (Huanglongbing) in Japan

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Abstract

Citrus greening (huanglongbing) is established in majority of the subtropical and tropical citrus-producing areas of the world. In Japan, the disease causes damage to citrus grown on the subtropical islands and threatens the major citrus production areas on the main islands of Kyushu, Shikoku, and Honshu. Since the initial identification of the disease on a small island near Taiwan, wide-ranging programs and collaboration among scientists, plant quarantine officials, and administrators to combat the disease have been undertaken. This paper reviews the history of the disease and development of countermeasures in Japan. The scientific insight and crucial technical tools that promote control practices are summarized. Implications for the control of the disease in other countries and future research directions are also discussed.

Discipline: Agricultural Environment
Additional key words: *Citrus depressa* Hayata, *Diaphorina citri* Kuwayama, *Murraya paniculata* (L.) Jack

Introduction

Greening (huanglongbing) is the most serious disease of citrus that limits production in the subtropical and tropical citrus-producing areas of the world (Gottwald 2010). Before its identification, the disease was known by a variety of names: yellow shoot (huanglongbing) in China; likubin (decline) in Taiwan; dieback in India; leaf mottle in the Philippines; vein phloem degeneration in Indonesia; and yellow branch, blotchy mottle, or greening in South Africa. As it became clear that all these diseases were similar, the term "greening" was widely adopted (da Graça 1991). The disease is caused by three phloem-limited bacteria: "*Candidatus* Liberibacter asiaticus" (CLas), "*C.* Liberibacter africanus" (CLaf), and "*C.* Liberibacter americanus" (CLam) (Bové 2006, Gottwald 2010). CLas and CLam are predominantly transmitted by the Asian citrus psyllid (ACP; *Diaphorina citri* Kuwayama), whereas CLaf is transmitted by the African citrus psyllid *Trioza erytreae* Del Guercio. In Asia, including Japan, the prevalent pathogen and vector are CLas and ACP, respectively (Bové 2006, da Graça 1991). This review summarizes the occurrence of citrus greening and the development of control measures in Japan.

History of citrus greening in Japan

It has long been known that greening occurs wherever the vector insects occur on citrus and citrus relatives. In Japan, ACP is widespread in the subtropical islands (Nansei Islands) that extend between Taiwan and Kyushu main island. Orange jasmine, *Murraya paniculata* (L.) Jack, is a favored host of ACP and is widely planted as a hedge in residential areas on the islands. The southern and northern islands are within Okinawa and Kagoshima Prefectures, respectively (Fig. 1).

Citrus greening was not recorded in these islands until the 1980s. The presence of ACP and the absence of citrus greening was a mystery among citrus researchers at the time, and some foreign scientists strongly recommended a comprehensive field investigation in these islands (Miyakawa, personal communication).

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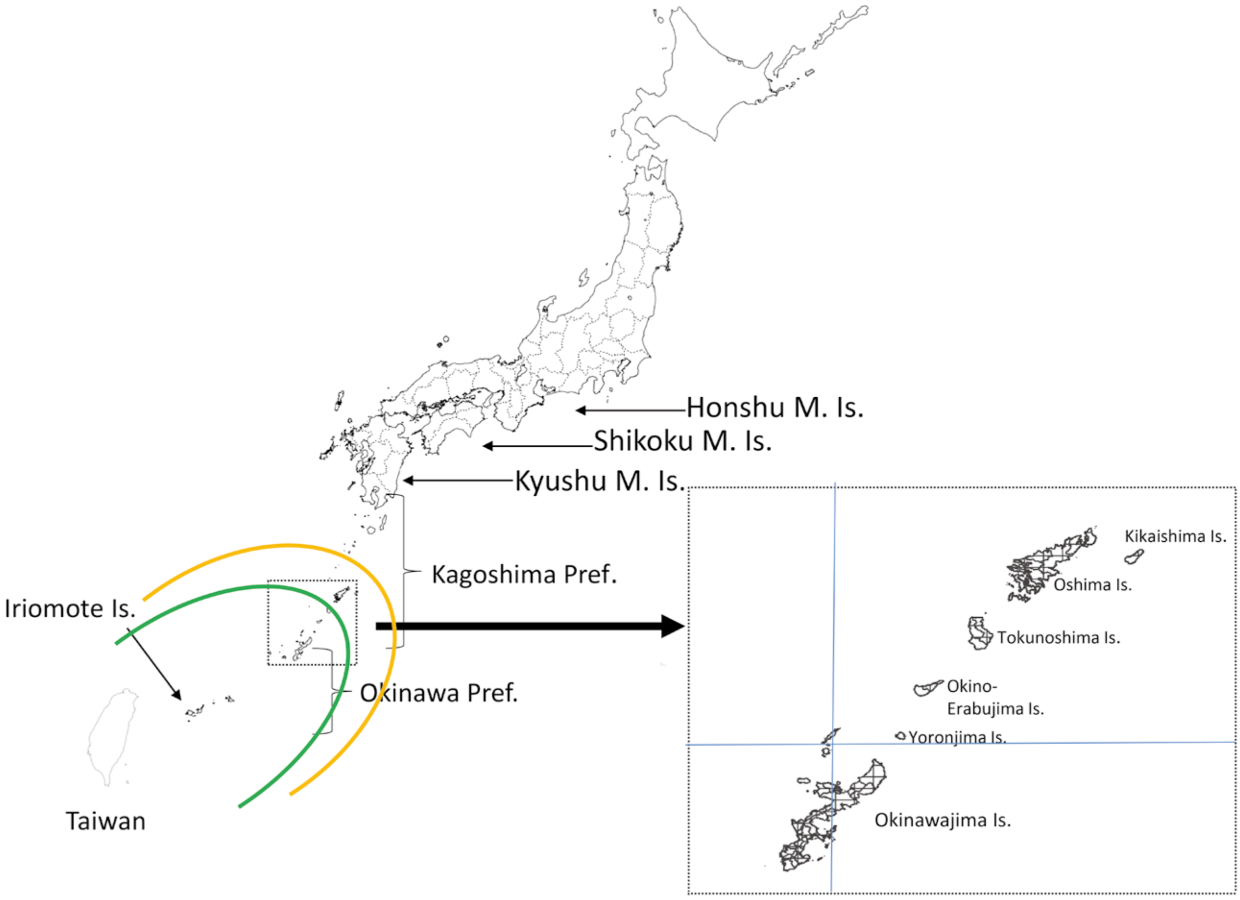

Fig. 1. Location of the subtropical islands (Nansei Islands) of Japan
The subtropical islands that extend between the Kyushu main island and Taiwan are shown together with the names of the islands mentioned in the text. The current northern distribution limits of citrus greening and its vector insect are approximately indicated with an orange line and green line, respectively. Blue horizontal and vertical lines in the inset approximately indicate latitude 27.00°N and longitude 128.00°E, respectively.

Following this advice, T. Miyakawa conducted repeated field surveys for the disease on these islands. Initial investigations in commercial citrus orchards failed to find the disease. Subsequently, in 1988, the disease was detected in two trees of flat lemon (*Citrus depressa* Hayata), a local sour citrus: one in a small-scale orchard and the other in a residential garden on Iriomotejima Island (Miyakawa & Tsuno 1989; Miyakawa, personal communication). It thereafter became clear that citrus greening was more prevalent in residential areas than in commercial orchards in Okinawa and Kagoshima Prefectures (Naito et al. 2001, Shinohara et al. 2006, Toguchi & Kawano 1997). In hindsight, if the investigation had been concentrated on residential gardens, citrus greening would have been detected much earlier (Miyakawa, personal communication). It is therefore reasonable to assume that citrus greening was endemic in some subtropical islands of Japan before 1988.

Following the initial identification of citrus greening in Japan, the plant quarantine office immediately felled the two infected trees. Field investigations were conducted to search for additional infected trees on Iriomotejima Island, the neighboring Ishigakijima Island, and Okinawajima Island (often called Okinawa Main Island), but no infected trees were detected (Toguchi & Kawano 1997). With the assistance of Prof. Hong-Ji Su of National Taiwan University, further investigation revealed additional infected trees on Iriomotejima Island in 1993 (Toguchi & Kawano 1997) and Okinawajima Island in 1994. These findings prompted a wide-scale field investigation on the *D. citri*-infested subtropical islands in Okinawa Prefecture from 1994 to 1997. The results indicated the occurrence of citrus greening on five islands (Toguchi & Kawano 1997). Further surveys revealed its occurrence on all but two inhabited islands (Minami-Daitojima and Kita-Daitojima Islands; Naito et al. 2001) in Okinawa Prefecture. In Kagoshima Prefecture, the disease was first detected on Yoronjima Island in 2003 (Shinohara et al. 2006) and later identified on the islands located to the south of Oshima Island (often called Amami-Oshima Island). Despite aggressive field surveys, the disease has not been detected to date on Oshima Island, where the vector insects are extremely common (Toguchi & Kawano 1997). Until 2012, when the disease was successfully eradicated, the foremost front of citrus greening, which was equivalent to the northern limit of the disease, was Kikaishima Island (Anonymous 2012). At present, the northern limit is Tokunoshima Island (Fig. 1).

Countermeasures: Different approaches in Kagoshima and Okinawa Prefectures

It is comparatively easy to eradicate an invading disease when it is limitedly distributed. An eradication program for a widespread disease is inefficient at best. Citrus greening spread unnoticed in many islands in Okinawa, whereas the disease was restricted to limited areas in the southern islands among the subtropical islands in Kagoshima. Against this backdrop, complete eradication was attempted in Kagoshima. Complete eradication has the advantage of possible return to the original, restriction-free cultivation practices; however, its disadvantage is its high cost and the intensity required in sampling suspicious trees. Contrarily, semi-eradication was a practical option in Okinawa. It requires less intensity in sampling suspicious trees; i.e., a few instances of overlooking infected trees do not lead to the collapse of the whole project. The disadvantage is the prolonged practice period. The countermeasures in the residential areas of Okinawa consisted of a broad field survey and felling of infected trees. At high altitudes in the northern part of Okinawajima Island, where ACP occurrence is rare, some areas have been kept almost citrus greening-free by carefully monitoring and restricting the movement of nursery trees, ensuring that the yields in flat lemon orchards remain unaffected (Hosokawa et al. 2019).

Successful eradication program on Kikaishima Island, Kagoshima

Unless specified otherwise by references, all data cited here were sourced from the "Report on the results of the urgent control of citrus greening in Kikai Town," released by the Forestry and Fisheries Promotion Division, Oshima Branch Office, of the Kagoshima Prefecture Government (Anonymous 2012).

1. Preliminary field survey (invasion monitoring program) from 2002 to 2007

Kikaishima Island in Kagoshima Prefecture is located in the northern parts of the subtropical islands of Japan. Citrus greening had not been detected for many years despite the abundance of ACP on the island (Fig. 1). On Kikaishima Island, the plant quarantine office started a preliminary field survey, designated as an "invasion monitoring program," in 2002. Under the program, four citrus greening-infected trees were detected at one location in Oasato village section in 2003. These trees were immediately felled. A further field survey using a combination of visual assessment and polymerase chain reaction (PCR) analysis was conducted over the entire island, most intensively in Oasato and the neighboring Nishime village sections, from 2003 to 2006. The movement of citrus trees and budwood was controlled by a town by-law from 2004, and the infected trees were promptly removed after detection. During the survey period, all trees in Oasato village section (~2,000 trees) were visually assessed, and suspicious trees were tested for infection using PCR. The results showed that 24 trees (including the original detected trees) were infected with CLas. All infected trees were immediately felled after a positive PCR test. Concurrently, all trees in Nishime village section (~1,000 trees) were visually assessed, and suspicious trees were tested using PCR. All the analyzed samples from Nishime village section tested negative for CLas (Table 1). The precise number of citrus trees assessed in Oasato and Nishime were never released in publicly accessible documents; the approximate numbers recorded here (2,000 trees in Oasato and 1,000 trees in Nishime) are the author's estimate based on the scattered data included in the "Report on the results of the urgent control of citrus greening in Kikai Town" (Anonymous 2012). Additionally, 36,178 trees in 3,835 locations were visually assessed outside of Oasato and Nishime, and 1,355 suspicious trees were tested for infection using PCR. All these samples tested negative for CLas (Table 1). The results of the invasion monitoring program suggested that citrus greening was restricted to Oasato village section on Kikaishima Island (Anonymous 2012).

2. Eradication program from 2007 to 2012

The limited occurrence and small number of infected trees on Kikaishima Island indicated that eradication was feasible. The formal eradication program based on the Ministerial Ordinance concerning the urgent

Table 1. Number of *Candidatus* Liberibacter asiaticus-infected trees detected on Kikaishima Island from 2002 to 2012[a]

	Oasado				Nishime				Others				Remarks
Year[b]	No. of locations visited	No. of trees observed[c]	No. of trees sampled for PCR	No. of PCR-positive trees	No. of locations visited	No. of trees observed	No. of trees sampled for PCR	No. of PCR-positive trees	No. of locations visited	No. of trees observed	No. of trees sampled for PCR	No. of PCR-positive trees	
2002	7	155	3	0	2	62	1	0	126	2,750	50	0	Invasion monitoring program
2003	95	1,471	404	5	29	655	111	0	658	8,944	510	0	Invasion monitoring program
2004	100	1,344	586	6	41	881	67	0	1,240	9,429	249	0	Invasion monitoring program
2005	30	441	177	2	0	0	0	0	1,122	8,614	328	0	Invasion monitoring program
2006	223	3,511	301	11	55	1,022	45	0	689	6,441	218	0	Invasion monitoring program
2007	341	5,683	495	4	87	1,632	69	0	1,089	9,529	431	0	Eradication program, first stage
2008	364	6,096	922	0	90	1,756	67	0	1,075	12,494	431	0	Eradication program, first stage
2009	372	6,064	958	0	90	1,696	270	0	1,252	13,428	833	0	Eradication program, first stage
2010	376	5,872	1,188	0	89	1,775	314	0	1,292	14,395	1,663	0	Eradication program, first stage
2011-1[d]	0	0	0	0	0	0	0	0	25	1,090	107	0	Eradication program, first stage
2011-2[d]	250	3,632	283	0	60	1,153	75	0	2,893	12,521	1,026	0	Eradication program, second stage

[a] All data were cited from the "Report on the results of the urgent control of citrus greening in Kikai Town" (Anonymous 2012) released by the Forestry and Fisheries Promotion.
[b] The Japanese fiscal year that begins in April 1 and ends in March 31
[c] The author estimates that ~2,000 and ~600 citrus trees were assessed in Oasato and Nishime, respectively, from 2002 to 2011. The same trees were examined twice or thrice a year; thus, the number of trees were counted twice or thrice. Some trees were not examined at all or examined only once a year in some years.
[d] There were overlapping periods between the first and second stages of the eradication program in 2011. The results of the first and second stages were indicated as 2011-1 and 2011-2, respectively.

control of citrus greening (Ministry of Agriculture, Forestry and Fisheries Ordinance No. 8, 2007) under the Plant Protection Act (No. 151, 1950), Japan, was initiated in April 2007 and terminated in March 2012. The program consisted of restriction on the movement of citrus trees, budwood, and ACPs from Kikaishima Island, as well as the urgent control of citrus greening on Kikaishima Island. The eradication program was implemented using a two-stage approach; the first stage (from April 2007 to May 2011) was to eradicate infected trees from Kikaishima Island, and that in the second stage from March 2011 to March 2012 was to confirm the absence of the disease (April and May 2011 represented a period of overlap between the first and second stages). The control measures applied in the first stage comprised a combination of field surveys, felling of infected trees, and applications of insecticides. In the second stage, field surveys were conducted in a more intensive manner to verify the eradication of citrus greening from Kikaishima Island.

The field surveys in the first stage were conducted with different degrees of intensity in accordance with the expected disease incidence. A more intensive survey was performed in areas located within 500 m from the originally detected infected trees. These areas corresponded with Oasato and Nishime village sections, and all citrus trees were visually assessed thrice a year in both village sections. Contrarily, a less intensive survey was practiced outside of these village sections; ~30% of all citrus trees were sampled in each year and observed for disease symptoms to ensure that all citrus trees were examined at least once in three years. In all cases, suspicious trees were tested for infection using PCR.

All PCR-positive and neighboring citrus trees that grew within a ~5-m radius of the PCR-positive trees were immediately felled after spraying fenitrothion to prevent the dissemination of viruliferous ACPs. All neighboring trees were purchased by the eradication program before felling. The stumps of the felled trees were bored with drills, and undiluted glyphosate was applied. Similarly, all neighboring citrus trees that grew within a ~5-m radius of PCR-positive trees detected from 2003 to 2007 were removed with compensation.

Insecticide was applied to all citrus and orange jasmine trees that grew within a 500-m radius centered on the infected trees in the Oasato and Nishime village sections thrice a year to reduce the transmission of CLas.

3. First stage of the eradication program

During the first stage of the eradication program, ~2,000 trees in ~120 locations in Oasato village section were visually assessed, and suspicious trees were tested using PCR thrice a year. As shown in Table 1, the numbers of assessed trees and locations fluctuated annually, mainly because of germination and death of seedlings in these years (the locations and trees examined in Oasato from 2007 to 2011, shown in Table 1, were counted three times because the same locations and trees were examined three times a year). During the first stage of the eradication program, infected trees were detected only in 2007 and not in the subsequent years (Table 1). The four infected trees were located within 60 m from the site of infestation confirmed in the invasion monitoring program from 2003 to 2006.

In the same period, ~600 trees in ~30 locations in Nishime village section were visually assessed, and suspicious trees were tested using PCR thrice a year. The numbers of the assessed trees and locations fluctuated annually. Additionally, the locations and trees examined in Nishime from 2007 to 2011, shown in Table 1, were counted three times. All samples tested negative for CLas (Table 1).

In the areas outside of Oasato and Nishime, all citrus trees were visually assessed at least once in these years, and suspicious trees were tested using PCR. All samples tested negative for CLas. Table 1 shows that 50,906 trees were visually assessed from 2007 to 2011, but the author assumes that some samples were counted twice and that the true number of citrus trees assessed in the areas outside of Oasato and Nishime might be ~37,000.

As mentioned, the 24 infected trees detected in the invasion monitoring program from 2002 to 2006 were felled before the eradication program started in 2007. In the eradication program, all 47 trees that grew within 5 m from the 24 infected trees were felled. Furthermore, the 4 infected trees that were detected in the eradication program and the 10 trees that grew within 5 m from the 4 infected trees were felled. The felling was completed by March 2008. The economic value of each tree was estimated and purchased by the program before felling.

4. Second stage of the eradication program

In the first stage of the eradication program, infected trees were detected in the first year, but no additional infected trees were discovered in the following three years. This result indicated that all infected trees and potentially infected trees (in the vicinity of the infected trees) were located and removed from Kikaishima Island. Following this assumption, the second stage of the eradication program was designed to verify the absence of infected trees on Kikaishima Island.

In the second stage, field surveys were conducted more precisely than that in the first stage. In Oasato and Nishime village sections, which were located within 500 m from all infected trees that were detected, all citrus trees were visually assessed twice a year, and suspicious trees were subjected to more sensitive real-time PCR (RT-PCR; Okuda et al. 2009) and less sensitive but more robust conventional PCR (Jagoueix et al. 1996). It was assumed that a citrus tree develops symptoms two years after infection with CLas (Yamamura et al. 2016). In the Oasato village section, the last infected tree with symptoms was felled in March 2008. Therefore, a tree that remained latently infected in March 2008 should have developed symptoms by March 2010. A similar theory was applied for the detection in Nishime village section, where no CLas-infected tree was found. Field surveys on all 2,502 trees in Oasato and Nishime village sections in 2010 and 2011 showed that all suspicious trees tested negative for CLas, which strongly indicated that CLas was absent in these areas.

The sampling numbers needed to detect CLas-infected trees with 95% probability outside of Oasato and Nishime were calculated based on an epidemiology model of citrus greening and a mathematical sampling theory developed by Yamamura et al. (2016). The mathematical sampling theory is common in equations difficult for non-mathematicians to understand. Therefore, the author sought to describe the theory deliberately in an intuitive manner. The readers are referred to Yamamura et al. (2016) for a precise explanation of the theory. The number of CLas-infected trees was assumed to triple every year by insect transmission, and the latent infection period was estimated to be two years. Outside of Oasato and Nishime, under the eradication program practice, no chemical was sprayed from April 2007 to March 2011, leaving potential for the insect transmission of CLas. Suppose an infected tree was present in 2007 outside of Oasato and Nishime, the number of infected trees in 2009 would be 9 ($= 1 \times 3 \times 3$). After the two-year latent infection period, these nine trees would be symptomatic in 2011; thus, the number of CLas-positive symptomatic trees after four years in 2011 would be nine. The field survey in the first stage revealed that 36,975 citrus trees were located outside of Oasato and Nishime on Kikaishima Island (Yamamura et al. 2016). Now, the probability of choosing only healthy plants by a random sampling of an arbitrary number of trees out of the total number of trees can be calculated using a combination formula or in practice using an approximation formula. For example, the author calculated that the probability of choosing only from the 36,966 ($= 36{,}975 - 9$) healthy plants and not from the 9 infected trees by random sampling of 10,500 trees out of the total 36,975 trees is ~4.9%. Returning to Yamamura et al.'s theory, it was calculated that if 29% of all citrus trees outside of Oasato and Nishime were sampled in 2011, the probability of missing potentially infected trees was less than 5%. Therefore, the number of plants that should be visually assessed was calculated as 36,975 × 0.29 = 10,723 plants. Subsequently, 12,521 (> 10,723) plants were visually examined in 2011, followed by RT-PCR testing in combination with PCR of samples from 1,384 suspicious trees, of which no tree tested positive. This result indicated that no infected tree was located outside of Oasato and Nishime with 5% probability of occurrence of an undesirable event (i.e., more than one infected tree remained) in 2009.

Based on the data that indicated that no infected trees occurred inside and outside of Oasato and Nishime (i.e., on the entire island), the eradication of citrus greening was officially declared by the Ministry of Agriculture, Forestry and Fisheries in March 2012 (Anonymous 2012). This was the first successful campaign in the world to eradicate citrus greening from an entire island.

An additional eradication program is currently operating on Tokunoshima Island, located to the south of Kikaishima Island. Further eradication programs are also planned to eradicate citrus greening from all islands in Kagoshima Prefecture (Amami Shimbun Newspaper 2018).

Control programs in Okinawa

Following the first detection of citrus greening on Okinawajima Island in 1994, field surveys were conducted in various parts of the island (Toguchi & Kawano 1997). It soon became clear that the island was heavily infested with the disease. The disease incidence was apparently much higher in residential areas than in commercial citrus orchards. The primary control measures consisted of felling the diseased trees and chemical spraying to reduce the populations of citrus psyllids (Uechi et al. 2019). The approach is similar to that adopted in Kagoshima. Remarkably, the control program has been successful in some northern areas of the island. One example is the establishment of disease-free areas (semi-eradicated areas, where the absence of the disease is carefully monitored by the local government, although the confirmation program by the plant quarantine office has not been completed) in the commercial citrus orchards of Ogimi village.

Growers make a high profit by producing flat lemon in the mountainous heights of Ogimi village. In contrast to the lower coastal residential areas, citrus greening occurs only occasionally around these areas, probably because the cliffs that separate the heights from the coastal areas obstruct the movement of viruliferous citrus psyllids. Taking advantage of the geography, the establishment of greening-free citrus-producing orchards was attempted in this area. The control measures consisted of strictly restricting the importation of citrus trees and budwood from citrus greening-infested areas, regularly inspecting suspicious trees and ACPs, and routinely chemical spraying citrus orchards to reduce the population of citrus psyllids. The Oshikawa, Oganeku, Ogimi, and Nerome village sections have been free of citrus greening and citrus psyllids since 2010 and have been designated as “caution zones” for citrus greening invasion (Hosokawa et al. 2019). Commercial citrus production has been maintained in the mountainous heights in the caution zone. In the Ogimi village office, loop-mediated isothermal amplification (LAMP) (Okuda et al. 2005) was introduced as a diagnostic test for the detection of citrus greening. This detection method is simple and sensitive and has been routinely used by nonspecialists in the village office.

In other parts of Okinawa, a find-and-slash approach has been applied under various control programs since the initial identification of the disease in 1989. The occurrence of citrus greening has been reduced with the execution of these control programs, although the disease remains extensively spread to be eradicated.

Development of new techniques, insight, theories, and their application for control

So far, citrus greening has been endemic to relatively small islands in Japan. Even in the disease-infested islands, the disease mainly occurred in residential areas, and there have been sporadic occurrences in the main citrus-producing areas. Taking advantage of the relative simplicity of the situation, Japanese researchers took a shrewd approach to first rapidly develop tools needed for the eradication and semi-eradication and then explore more sophisticated methods to prepare for more serious situations expected in the future.

1. Detection methods

As mentioned in the preceding section, a find-and-slash approach has been a primary countermeasure against citrus greening in Japan. Accordingly, a simple and reliable detection method has always been desired. Given that the symptoms of citrus greening are similar to those induced by physiological disorders, visual assessment is inaccurate. Until the 1980s, the only tools for confirming CLas infection were biological indexing using susceptible citrus cultivars and observing ultrathin sections using a transmission electron microscope. These tools played a critical role in the first detection of citrus greening in Japan (Miyakawa & Tsuno

1989) but were too time-consuming for large-scale detection. Additionally, the titer of CLas is extremely low in citrus tissues; therefore, developing a conventional immunological detection procedure, such as enzyme-linked immunosorbent assay, which is highly suitable for large-scale detection, has proved extremely difficult (Bové 2006). A breakthrough was achieved by the availability of molecular detection techniques, including DNA hybridization, PCR, RT-PCR, and LAMP. The introduction of DNA hybridization using a CLas-specific probe developed in Taiwan (Hung et al. 1999) accelerated the detection of CLas in many areas of Okinawa Prefecture in the early 1990s, supplemented by the introduction of PCR detection using the OI1/OI2c primers to amplify 16S rDNA (Jagoueix et al. 1994). The introduction of PCR detection greatly facilitated the detection of diseased trees in many areas of Kagoshima and Okinawa (Naito et al. 2001, Shinohara et al. 2006).

Although PCR detection surpassed all other classic detection methods, more sensitive, less time-consuming and less labor-intensive methods are indispensable. This is particularly true for detecting CLas at an early stage of infection when CLas is sporadically distributed in the plant. The sensitivity and robustness of detection were improved using a new primer set targeting a different fragment of 16S rDNA in Japan (Fujikawa & Iwanami 2012). Primers that target the fragments of the ribosomal protein genes of the β operon have been reported from foreign countries (Hocquellet et al. 1999, Hoy et al. 2001). Nested PCR, which is essentially more sensitive than conventional PCR, was also developed for the sensitive detection of CLas (Ding et al. 2005). Subsequently, RT-PCR detection was developed (Li et al. 2006, Okuda et al. 2009). This method is not only more sensitive than the original PCR detection but also less time consuming and labor-intensive because it dispenses with the need to visualize the PCR products by agarose gel electrophoresis and staining. The introduction of RT-PCR detection following the protocol reported by Okuda et al. (2009) aided the efficient field surveys of citrus greening in the eradication program on Kikaishima Island, Kagoshima (Anonymous 2012).

One drawback of the PCR and RT-PCR detection is the requirement for expensive laboratory equipment, such as a thermal cycler and electrophoresis apparatus. The LAMP procedure, another highly efficient DNA amplification technique, does not require a thermal cycler or electrophoresis apparatus and therefore is suitable for underequipped laboratories, including those found in extension centers and quarantine offices. Characteristically, the visualization of the amplified DNA products is performed without opening the reaction tubes in both LAMP and RT-PCR. For this reason, the risk of contamination is greatly reduced, an additional advantage of these techniques. The LAMP-based detection of CLas was developed for these reasons (Okuda et al. 2005, 2008). A commercial kit for the detection of CLas is available (Nippon Gene 2020) and has been routinely used in the Ogimi village office in Okinawa for monitoring the CLas-free status in greening-free citrus-producing orchards, as described above.

The early removal of infected trees is crucial for the success of the eradication and quarantine programs. For this reason, it is desirable to detect CLas from all trees at an early stage of infection. It has often been observed that trees that tested negative for CLas subsequently tested positive after a few months, strongly indicating that some negative results for the first test might have been false negatives. These false-negative results cannot be eliminated even with the use of a sensitive test such as RT-PCR. To overcome this problem, more sophisticated detection methods have been proposed in Japan. Some techniques have focused on the timing and portion of sampling (Iwanami et al. 2009, Kanamaru et al. 2016), whereas others have focused on choosing a more reliable primer set (Fujikawa & Iwanami 2012) and using cultivation to enhance sensitivity (Fujiwara et al. 2018). Many other detection methods have been reported abroad (Das et al. 2019, Valdés et al. 2016). The efficacy of these new techniques has not yet been confirmed in Japan. The validation of these techniques by a neutral international third party is desired, and the best techniques should be introduced for practical use in Japan and other countries.

In addition to sensitivity, simplicity is important in detecting CLas, especially in a large-scale eradication and quarantine program. The time and labor requirements in DNA amplification and detection are greatly reduced by LAMP (Okuda et al. 2005, Okuda et al. 2008). However, the preparation of template DNA from plants remains time consuming and labor demanding; thus, a simple technique has been developed (Fujikawa et al. 2013). This technique was successfully incorporated for the simple and sensitive detection of CLas by direct PCR using the primer set Las606 and LSS. Unfortunately, the DNA template prepared in this manner is not applicable for LAMP detection.

Simple and sensitive tests are indispensable in the middle and final stages of eradication and quarantine programs. Contrastingly, the most precise detection method should be applied when the occurrence of CLas is confirmed for the first time in a new area, island, or country. The most precise and practical detection of CLas may be realized using a combination of two fundamentally different methods (e.g., molecular, serological, or biological). Indeed, the first occurrence of citrus greening in Japan was confirmed by a combination of biological indexing and electron microscopic observation (Miyakawa & Tsuno 1989).

2. Other diagnostic methods

Massive starch accumulation characterizes CLas infection in leaves. An iodine starch-staining method developed in Taiwan (Su, personal communication) for the diagnosis of citrus greening was introduced in Okinawa (Taba et al. 2006). The simple test promoted the detection of many infected trees in Okinawa. Similar tests are used for a primary screening of infected trees in Taiwan, the USA, and elsewhere (Etxeberria et al. 2007, Su 2008).

The leaves of CLas-infected trees often develop symptoms resembling those induced by zinc or iron deficiency. Following the preliminary work on Réunion (Aubert 1979), a precise analysis of microelement concentrations in CLas-infected leaves of Satsuma mandarin and Tankan mandarin in Japan and Siem mandarin in Indonesia was conducted. Typically, the concentrations of particular microelements (zinc and iron) rather than the concentrations of element in general are reduced by CLas infection (Masaoka et al. 2011). This insight is applicable for the simple initial screening of CLas-infected trees, although such a practice has never been used in orchards. Furthermore, it suggests that the symptoms of citrus greening might be reduced by supplying zinc and iron through foliar sprays and the trunk or as a soil drench. A recent study supports this hypothesis (Inoue et al. 2020).

Some recently developed diagnostic tools use cutting-edge technologies, such as remote sensing, image processing, and spectroscopy (Das et al. 2019). Simple and sensitive methods have also been explored. One of the most promising simple detection methods is canine olfactory detection (Gottwald et al. 2020). Diagnosis or detection by sniffer dogs has been widely used for many diseases and pests, including various cancers and fire ants (Lin et al. 2011). The approach is worth trying for the simple diagnosis of citrus greening in many countries, including Japan.

3. Characterization of the CLas genome and its implications

The whole-genome sequencing of CLas has been a challenge for many years because of the difficulty in preparing pure pathogen DNA. Only several short pathogen-specific fragments that were occasionally obtained had been sequenced (Jagoueix et al. 1994, Okuda et al. 2005, Planet et al. 1995, Subandiyah et al. 2000). The development of next-generation sequencing technology in the early 2000s, which enabled the sequencing of the DNA of a pathogen in crude sap, remarkably overcame this difficulty. The first whole-genome sequence of CLas was reported for the US strain psy62 (Duan et al. 2009), followed by the whole-genome sequencing of several other strains (Katoh et al. 2014, Li et al. 2020, Zheng et al. 2014). The whole-genome sequence of Ishi-1, a Japanese strain from Ishigaki Island in Okinawa, revealed unique features, such as a smaller genome size and the insertion or deletion of several large fragments (Katoh et al. 2014). The greatest difference in Ishi-1 is the absence of a 33-kbp fragment that encodes 40 genes, including the bacteriophage-type DNA polymerase gene between the SC1 and SC2 genes in the prophage region. The 33-kbp fragment is retained in the psy62 and UF506 strains in Florida and the gypsy strain in China (Katoh et al. 2014). These results suggested that the 33-kbp fragment is associated with neither pathogenicity nor transmissibility because Ishi-1 induces severe symptoms on citrus and is propagated to a high titer in the vector insect. This is in sharp contrast to the UF506 strain, in which the SC1 and SC2 genes flanking the bacteriophage-type DNA polymerase gene are suspected to be important for infection and virulence expression (Zhang et al. 2011). It is likely that Ishi-1 carries different virulence factors (Katoh et al. 2014).

It is remarkable that the majority of Japanese strains lack the bacteriophage-type DNA polymerase gene, whereas most strains of CLas isolated in other countries retain the gene (Tomimura et al. 2009). It is also noteworthy that strains lacking the bacteriophage-type DNA polymerase gene are distributed in the northern areas of the subtropical islands of Japan, whereas strains harboring the bacteriophage-type DNA polymerase gene are distributed in the south-westernmost islands of Japan that are close to Taiwan (Tomimura et al. 2010). It is surmised that a typical Japanese strain, such as Ishi-1, might have evolved in Taiwan and the neighboring islands in Japan. Furthermore, the upstream region of the bacteriophage-type DNA polymerase is frequently exchanged among strains in Asia through recombination (Furuya et al. 2010, 2011). This insight might be investigated to infer the evolution history of CLas strains.

It should be noted that the whole-genome sequences of CLaf and CLam have also been reported (Lin et al. 2015, Wulff et al. 2014). Neither CLaf nor CLam is relevant to citrus greening in Japan and other Asian countries to date. However, Asian countries should not ignore the threat of these pathogens because ACPs transmit both CLaf and CLam (da Graça 1991, Lopes et al. 2013).

4. Development of differentiation markers and their application

The analysis of the whole-genome sequence of the CLas Ishi-1 strain has shown that many simple sequence repeats (SSRs; also termed variable tandem repeats) are scattered in the genome. SSRs in bacterial genomes are poorly recognized and have been reported in several bacterial species only, including *Yersinia pestis*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Bacillus anthracis*, and *Xylella fastidiosa* (Katoh et al. 2011). In the Ishi-1 genome, 27 perfect SSRs with 4-63 nucleotides per unit are present (Katoh et al. 2011). Four highly polymorphic SSR loci are useful to differentiate 84 isolates from Japan, 4 isolates from Taiwan, and 12 isolates from Indonesia (Katoh et al. 2011). The SSR markers also differentiate 13 Indian, 3 East Timorese, 1 Papuan, and 8 Floridian strains (Katoh et al. 2012). These results indicate that SSR markers might be a useful tool to monitor an invasive strain and estimate genetic diversity, which is indispensable in a quarantine program.

The estimation of the genetic diversity of CLas in Japan using SSR markers has shown that CLas in the islands near Taiwan are as divergent as in Taiwan, whereas CLas sequences show high homology at its northern limit in Kagoshima (Katoh et al. 2011). Generally, a pathogen is genetically highly homogeneous soon after invading a new location, whereas genetic diversity increases with time after the invasion event. Based on this assumption, the data indicate that CLas was first distributed in the islands near Taiwan concurrently with Taiwan itself long before 1988 and gradually dispersed northward; the invasion of Kikaishima took place shortly before citrus greening was recognized in the 2000s. Furthermore, changes in repetition in some SSRs are associated with psyllid transmission (Katoh et al. 2015). The “psyllid transmission marker” might be applied to estimate the rate of psyllid transmission among CLas populations in the field.

5. Design of eradication programs

In an eradication program, confirming the absence of the disease after implementing the eradication measures is a crucial step. To be absolutely thorough, all samples (all citrus trees in the quarantine area) should be tested. However, this is impracticable and expensive. Instead, it is reasonable to adopt an appropriately reduced sampling that accepts a consumer’s risk (the probability of the occurrence of undesirable events) of 0.05 (Yamamura et al. 2016). As mentioned, a statistical theory for calculating the required sample size was developed and supported the eradication program on Kikaishima Island, Kagoshima (Yamamura et al. 2016). This theory is applicable to other eradication programs for citrus greening in Japan and abroad.

6. Insight on vector ecology and application to predict possible invasion sites

Historical analysis revealed that ACP and citrus greening appeared almost simultaneously in Brazil and the USA (Manjunath et al. 2008). This association would be applicable to any further outbreak in the future. ACPs are visible and therefore are more readily detectable than CLas, for which detection at an early stage of infestation is technically difficult. For this reason, it makes sense to predict the likely locations for ACP establishment beforehand and intensively monitor the areas in a prevention program. ACP overwinters where the average daily minimum temperature in the coldest month is 4.5°C or higher (Ashihara 2007). These areas correspond to “frost-free” zones and are distributed along the coasts of not only small subtropical islands but also some of the main islands of Japan, including the Kyushu main island (NARO 2007). These frost-free zones on the Kyushu main island are presently under regular inspection in the quarantine program (Miyazaki Plant Protection and Fertilizer Inspection Center 2020). ACP was eliminated in 2014 by immediate and extensive chemical spraying in the emergency quarantine program as soon as an outbreak was discovered in Ibusuki on the Kyushu main island (Shimotsu et al. 2015).

7. Insight on transmission of CLas by nymphs of ACP

Since the first report of CLas transmission by ACP (McClean & Oberholzer 1965), limited information on the transmission efficiency has been produced. Some researchers have failed to transmit CLas using adult ACPs in spite of repeated attempts (M. Koizumi, personal communication). The mystery was solved by the quantitative analysis of CLas in the body parts of ACP (Inoue et al. 2009). This work clearly demonstrated that the concentration of CLas was significantly increased in psyllids that fed on infected plants as nymphs, and the emergent adults transmitted CLas efficiently. Contrarily, the concentration of CLas did not significantly increase in adults that fed on infected plants, and CLas was not transmitted at all. These results indicate that an outbreak of citrus greening is still preventable soon after a chance introduction of only adult ACPs in a new area through, for example, wind dispersal. This insight is extremely useful in planning a rational and realistic plant quarantine program.

8. Culture of CLas in vitro

CLas characterization had been greatly hampered by its fastidious nature. Early work to culture CLas failed or conceivably cultured a different organism (da Graça 1991). In the 1980s and 1990s, developing the culture medium for CLas was less difficult than validating the bacterium because no simple tool for identifying CLas was available. In the early 2000s, two culture methods for CLas were reported with validation using PCR detection in the USA (Davis et al. 2008, Parker et al. 2014). Both methods apparently rely on unknown metabolic compounds either from coculturing bacteria or from citrus juice. In contrast, a culture medium composed only of commercially available reagents was developed in Japan (Fujiwara et al. 2018). The Ishi-1 strain extracted from infected citrus leaves consistently propagated on the medium after several passages on this medium, which indicated that CLas survival on the culture medium does not depend upon compounds from citrus sap (Fujiwara et al. 2018).

The culture of a fastidious organism, such as CLas, not only is an attractive scientific goal but also is extremely important for the development of control measures. Thousands of chemicals may be screened against CLas on the medium, the pathogenicity of a CLas strain may be evaluated by the quantitative inoculation of a citrus host plant after culture on the medium, and viable CLas can be sensitively detected by a combination of culture in vitro and reverse-transcription PCR amplification. Accordingly, continuing efforts to optimize culture systems to execute a more sophisticated quarantine program in the future is extremely important.

9. Vector control

Vector suppression is critical in both disease eradication and disease mitigation. For this reason, intensive efforts were made to screen insecticides effective against ACP (Hayashikawa et al. 2006, Yasuda et al. 2006). Approximately 10 insecticides and miticides, including mineral oil emulsion, a fatty acid glyceride emulsion, fenitrothion, several neonicotinoids (clothianidin, dinotefuran, imidacloprid, and thiamethoxam), pyrimidifen, and milbemectin, are currently registered for controlling ACP on citrus, and in some cases, orange jasmine in Japan.

Developing insecticide resistance, which has been apparently induced by intense insecticide use, has become a serious concern in Florida, USA (Tiwari et al. 2011). No insecticide resistance of ACP has yet been observed in Japan. However, Japan and other countries should learn from the American experience.

10. Is orange jasmine a host?

An early study in Japan showed that orange jasmine is not a host of CLas (Miyakawa 1980). After the 2000s, some authors in China, Brazil, and the USA claimed that orange jasmine can be a host of CLas (Cifuentes-Arenas et al. 2019, Damsteegt et al. 2010, Deng et al. 2007, Zhou et al. 2007), whereas other authors concluded that it is not a host (Hung et al. 2000, Dai et al. 2005).

It is possible that orange jasmine is genetically divergent and that previous studies used different lines of orange jasmine that differ in degree of susceptibility to CLas. Keeping in mind that there is no report of an orange jasmine tree that persistently harbors CLas for several years, it is reasonable to state that orange jasmine is generally not a host of CLas, but some lines of orange jasmine might act as a transient host.

11. Treatment of infected trees

Electron microscopic observation of infected cells from diseased citrus trees in the 1970s suggested that the pathogen morphologically resembled a mycoplasma-like organism (MLO; now designated a phytoplasma). Given that an MLO is sensitive to tetracycline, it was assumed that citrus greening might be controlled using tetracycline. During the 1970s and 1980s, many trials on trunk injection of antibiotics were conducted in Taiwan, the Philippines, India, and other countries, mainly using not only tetracycline-based compounds but also other chemicals, such as penicillin-based compounds (da Graça 1991). The results suggested that tetracycline and penicillin were effective in reducing symptoms, although the phytotoxicity of some formulas and the labor of annual trunk injection remained problematic.

After the outbreak of citrus greening in the USA, the chemical control of the disease attracted interest, and not only tetracycline and penicillin but also chemicals, such as sulfonamides, tolfenamic acid, and kasugamycin, were trialed. The results attained to date are similar to those reported in the 1970s in Asian countries (Blaustein et al. 2018).

In Japan, trunk injection of antibiotics has never been attempted because the find-and-slash approach has been a primary strategy of control due to the general public concern on the overuse of antibiotics in agriculture. CLas-free scions were obtained by bud-grafting infected buds on ponkan (*Citrus reticulata* Blanco) seedlings after dipping the buds in an aqueous penicillin solution of 0.2 mg/mL or a higher concentration (Miyakawa 1979).

The technique might be useful for introducing a new citrus cultivar from a potentially CLas-infested area because it easily eliminates CLas from contaminated budwood.

It has long been known that the foliar symptoms of citrus greening are similar to those of iron, zinc, and manganese deficiency. Iron and zinc concentrations are reduced in CLas-infected leaves (Masaoka et al. 2011). Interestingly, following this study, the treatment of infected trees by spraying several formulas of iron ion aqueous solutions was trialed, but it had little effect, which suggested that citrus trees do not readily absorb iron ion aqueous solutions (the author's unpublished observation). Recently, it was demonstrated that foliar application of a high-Fe^{2+} citrate solution can restore the vigorous growth of CLas-infected trees (Inoue et al. 2020). However, the efficacy has been demonstrated only in potted plants in a greenhouse, and field trials are yet to be performed.

12. Search for CLas-tolerant cultivars

Field observations in the Philippines in the 1960s suggested that citrus cultivars showed variation in susceptibility to citrus greening; the largest number of plants without symptoms of citrus greening was observed among selections of trifoliate orange (*Poncirus trifoliata* (L.) Raf.) and its hybrids, followed by lemon (*C. limon* (L.) Osbeck) and sweet lime (*C. limettioides* Tanaka) (Gonzales et al. 1972). In India, rough lemon (*C. jambhiri* Lush.), sweet lime, and pomelo (*C. grandis* Osbeck) are tolerant, and trifoliate orange is fairly tolerant to citrus greening (da Graça 1991). The tolerance of trifoliate orange is also reported in Japan (Miyakawa 1980). Under a joint research project between the Tropical Agricultural Research Center (the former organization of Japan International Research Center for Agricultural Sciences), Japan, and the Department of Agriculture, Thailand, 43 citrus cultivars grown in the field at Nan Horticultural Research Station, Thailand, were evaluated for their resistance to citrus greening (Koizumi et al. 1993). The authors claimed that Wilking, Ellendale, Pet-yala, Beauty, Onesco, Pong-chieng-ga (Tankan), Nian-ju, and sweet orange were the most susceptible; many mandarins, including Fairchild, Murcott, Kinnow, Clementine, Fremont, Ponkan, King, and Som-keo-wan, showed moderate susceptibility; Queen mandarin, Avon Ever Bearing (calamondin), and rough lemon demonstrated mild susceptibility; and Ladu mandarin and Som-pan mandarin exhibited the strongest resistance with good growth and few disease symptoms and yielded healthy fruits.

The responses of 30 citrus genotypes to CLas were examined under controlled conditions in a greenhouse or growth room (Folimonova et al. 2009). Eureka lemon (*C. limonia* Osbeck), Persian lime (*C. aurantifolia* (Christm.) Swingle), Carrizo citrange (× *Citroncirus webberi* J. W. Ingram & H. E. Moore), and *Severinia buxifolia* (Poiret) Ten. were tolerant, whereas trifoliate orange exhibited inconsistent tolerance. In a graft transmission test in a greenhouse, pathogen transmission was lower for limes, mandarins, or Swingle citrumelo than for three cultivars of sweet oranges and Murcott tangor, suggesting that susceptibility differs among citrus species (Lopes & Frare 2008). Some citrus × trifoliate orange hybrids, including Carrizo citrange, US-897 (Cleopatra mandarin × trifoliate orange 'Flying Dragon'), and US-942 (Sunki mandarin × trifoliate orange 'Flying Dragon'), have been shown to be tolerant (Albrecht & Bowman 2012). Recently, it was shown that the US-942 expresses a 67-amino-acid-peptide (SAMP), which is predicted to have antimicrobial activity by analogy to *Arabidopsis* heat-stable protein (HS, Huang et al. 2021). Further, long-term observation in a heavily infested field in Florida indicated tolerance in the citrus relative genera *Microcitrus* and *Eremocitrus* (Ramadugu et al. 2016); subsequently, it was shown that the SAMP from *M. australasica* Swingle had the strongest effect on inhibiting bacterial growth in plants (Huang et al. 2021).

A comprehensive interpretation of the experimental tolerance to CLas is challenging because tolerance has been evaluated with different criteria using different methods. Therefore, researchers interested in using tolerant cultivars to mitigate the damage of citrus greening should reexamine the tolerance in the intended target environments.

Development of sustainable citrus culture in heavily infested orchards in the Mekong Delta

Prevention is a primary control measure in Japan because citrus greening is endemic in the subtropical islands, whereas the major citrus-producing areas in the main islands, including Kyushu, are free of the disease. A rare study on developing a disease mitigation measure was conducted in the Mekong Delta under a joint research program between Vietnam and Japan. Monthly or bimonthly application of neonicotinoids was effective for controlling ACP. Remarkably, interplanting with guava prevented the occurrence of citrus greening almost completely for about one and a half years after planting, although almost all trees were infected after two and a half years, irrespective of the presence of guava, indicating that guava interplanting was effective for one year at most (Ichinose et al. 2012). Guava interplanting might be an attractive sustainable disease mitigation measure in other Southeast Asian countries (Iwanami et al. 2013).

Future directions

Citrus greening has been successfully contained in residential areas and adjacent minor citrus-producing areas in the subtropical islands of Japan. Eradication programs are successfully underway. The present situation provides stakeholders with optimism that citrus greening will be gradually overcome in Japan. However, the cost of eradication in Kagoshima and the maintenance of disease-free areas in Okinawa will be increasingly unbearable in the near future. Furthermore, with global warming, many coastal areas of the Honshu, Kyushu, and Shikoku main islands will become more vulnerable to citrus greening. For this reason, it is expected that a future control strategy should comprise two elements: sustainable containment and early monitoring combined with immediate eradication. Future research directions should naturally target these two elements.

1. Sustainable containment

Following the eradication programs on Kikaishima and Tokunoshima Islands, additional programs are planned on at least two islands (Okino-Erabujima and Yoronjima) in Kagoshima. In Okinawa, expanding the citrus greening-free zone (caution zone) beyond Ogimi village to Nago City is planned. If the current find-and-slash approach is continued, the cost of these programs will intolerably increase year by year. Developing techniques that reduce the cost of detecting and felling infected trees is a high priority. Such techniques may include an automatic disease monitoring system that uses machine learning in combination with a drone or a satellite, a theory to reduce the control area and number of trees sampled for eradication, and formulating biological control agents effective against ACP.

In the long run, the development and introduction of disease-tolerant commercial cultivars may be an option in the heavily affected areas in Okinawa for sustainable containment. However, caution is required because a majority of the apparently tolerant cultivars may harbor CLas and act as reservoirs of the disease. Ideally, a practical tolerant cultivar would not harbor CLas at all (i.e., immune). Such immunity is difficult to identify among natural citrus cultivars and is conferred only by genetic modification. Modification using the CRISPR/Cas gene-editing systems may be a favorable option to develop new citrus cultivars immune to CLas.

2. Early warning system

At present, a frost-free zone in the coastal areas of the Kyushu main island is monitored for ACP occurrences under a plant quarantine program. Both theory and experience have shown that the detection of ACPs on orange jasmine is the first sign of a possible outbreak of citrus greening in a new area. Therefore, precise mapping of orange jasmine trees is crucial to an effective early warning. Currently, mapping of orange jasmine trees is performed through on-site confirmation that heavily depends on the knowledgeable experts of local governments. Fewer such personnel will be available in the future as government spending will inevitably reduce concomitant with the shrinking population of Japan; an automatic mapping system using machine learning of images is a suitable alternative to consider.

The simple and precise detection of ACPs on orange jasmine trees is also indispensable. One promising technique may be the detection of ACP DNA from sticky plate traps on orange jasmine trees (Fujiwara et al. 2017). The visit frequency to monitoring sites required may be drastically reduced by applying this method. Early warnings work better as ACP is detected earlier when the population is small. Therefore, further improvement in the sensitivity of ACP detection on orange jasmine trees, including the optimization of the number and position of the traps, is desirable.

3. Basic research as the foundation of a breakthrough control

Since the first detection of citrus greening in Japan in 1988, research on the disease has mainly focused on the development of urgent and practical control measures. Collaterally, certain basic research has also been conducted. Among the most important accomplishments of basic research is the establishment of a stable culture system for CLas on medium in vitro (Fujiwara et al. 2018). It is expected that the screening of a variety of chemicals that show anti-CLas activity will be greatly promoted using the culture system. Crucial to the successful development of the new culture medium in Japan is to supplement the lacking metabolic compounds of CLas deduced from the genome sequence. Thus, an additional important basic research focus is the genome sequencing of diverse strains of CLas. Thirty-five whole-genome sequences were available from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/) at the time of the preparation of this manuscript (in 2020). The majority of these genome sequences are derived from CLas strains in the USA, and only a small number is derived from strains in Japan and China. The unique genome organization of the Ishi-1 isolate in Japan strongly indicates that some Southeast Asian isolates of CLas may show a more divergent genome organization, and each isolate may require different supplementary compounds on an artificial medium. Accordingly, additional genome sequencing of Southeast Asian isolates of CLas may be needed. Specifically, at least two major genomic strains of CLas are present in Japan, and several are extant in Asia (Tomimura et al. 2009, Miyata et al. 2011). As the genomes of these different strains of CLas are revealed, the lacking metabolic compounds will be deduced, and consequently, the optimization of the culture medium for each strain may become possible.

Conclusion

Citrus greening was first identified in Japan in 1988. Subsequently, personnel from national research institutes; local governments; universities; plant quarantine offices; and the Ministry of Agriculture, Forestry and Fisheries have collaborated to develop and execute control measures. One important accomplishment is unquestionably the eradication of the disease from Kikaishima Island, which is the first successful example in the world. Additionally, the establishment of a citrus greening-free zone (caution zone) in Okinawa and the implementation of an early warning system in the frost-free zone in the coastal areas of Kyushu to protect the main citrus-producing areas are examples of effective plant quarantine practices. The vital factors in these successes are as follows: i) collaboration of scientists, plant quarantine officials, administrators, and residents; ii) immediate implementation of plant quarantine; and iii) introduction of the most advanced techniques. Finally, it should be noted that the disease was first detected by the local government and university researchers for whom citrus greening is not a primary job responsibility. Without their unselfish action and awareness, the occurrence of citrus greening in the subtropical islands would have remained unnoticed for longer and controlling the disease would have been much more difficult.

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