CITRUS GREENING DISEASE

Citrus Greening Disease and the Greening Bacterium

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ABSTRACT. Citrus greening is one of the most severe diseases of citrus. It is widespread throughout Southern and Eastern Africa and in Asia from Pakistan to China. The disease is also present in the southwestern part of the Arabian Peninsula. The disease is due to a bacterium-like organism (BLO) restricted to the phloem sieve tube elements. This bacterium has a membraneous cell wall of the gram-negative type and belongs to the α subdivision of the proteobacteria. The greening BLO has not been cultured. Symptoms of greening are not specific and are often confused with mineral deficiencies. Confirmation that a citrus tree is affected by greening has, up to now, relied on the electron microscopical detection of the greening BLO. In 1987, monoclonal antibodies (MAs) were produced against the greening BLO from Poona (India). Since then, MAs specific for China and South African greening BLOs have been obtained. However, these MAs are not useful for general diagnosis, because they are highly strain specific. Recently, DNA probes have been obtained for the detection of the BLO. One of these could detect all greening BLO strains tested, with a sensitivity at least equivalent to that of electron microscopy.

The greening BLO is transmitted by two psyllid vectors, *Trioza erytreae* in Africa and *Diaphorina citri* in Asia. The two vectors are present in the Arabian Peninsula, Mauritius and Reunion islands. However, in Reunion the biological control of the two psyllids by their respective parasites (*Tamarixia radiata* and *T. dryi*) has been successfully achieved. In certain countries of South and Central America (Brazil, Argentina, Honduras) *D. citri* is present but greening is absent.

Greening disease has been known in China for more than 100 yr and was reported for the first time from this country by Reinking in 1919 under the name of Huanglungbin (yellow shoot disease) (28, 38). Later, in 1937, a disease with similar symptoms was described in South Africa and was called greening disease (43). Since then, the disease has been reported in many countries under different names: likubin (decline) in Taiwan, citrus dieback in India, leaf mottling in the Philippines, and vein phloem degeneration in Indonesia. The name greening is the commonly used name. The disease affects all citrus varieties and is widespread.

Greening disease has been studied for almost a century, and the major results of this work will be reviewed. While a review on greening appeared in 1991 (9), more recent investigations, based on cellular and molecular techniques, have led to the characterization and detection of the greening pathogen, and these will be described. Perspectives for future work will be also outlined.

THE FIRST CENTURY: 1890-1986.

Symptomatology. Trees affected are generally stunted, have a sparce yellow foliage, and show twig dieback. Sometimes, at early stages of infection, symptoms are seen only on one part of the canopy, hence the name of the disease in China (yellow shoot). The most characteristic symptom is leaf mottle (normal-green patches on a pale green background). This symptom is conspicuous on sweet orange leaves, but less prominent on mandarin leaves. Leaf mottle is not specific of greening, as stubborn-affected leaves show similar symptoms but it is the best foliar symptom for the diagnosis of greening. Zinc-deficiency-like symptoms are commonly associated with greening, resulting in its confusion with nutritional problems.

Fruits on severely affected trees are small, lopsided and poorly colored,

remaining dull green, hence the name greening is given to the disease in South Africa. Occasionally, color development on affected fruits starts at the peduncular end rather than at the stylar end as it is the case in normal fruits. This symptom is referred to as color inversion or "red nose"!. Variable numbers of aborted seeds are present in fruits of affected sweet orange and mandarin trees.

Greening is a graft-transmissible disease. Because of the mineral deficiency and yellowing symptoms associated with greening, the disease was first considered to be caused by water logging and/or micronutrient deficiencies. In China, the disease was first shown to be graft-transmissible in 1956 by Lin (28). However, this work remained unknown outside China for many years. Independently, Mc Lean and Oberholzer (33) demonstrated, in 1965, that South African greening was graft-transmissible.

Psyllid vectors of the greening agent; biological control. When Mc Lean and Oberholzer (32) demonstrated that greening disease was graft-transmissible, they also established that the disease was naturally transmitted by the African citrus psylla, *Trioza erytreae* (33). Shortly thereafter, the Asian citrus psylla, *Diaphorina citri* was shown to transmit the disease in the Philippines (29, 40) and in India (8). These two psylla vectors occur together in only four places in the world: Reunion and Mauritius, in the Indian Ocean, and Sta Helena in the Alantic ocean and along the western border between Saudi Arabia and Yemen in the Arabian Peninsula. It was shown experimentally, that each psylla species is able to transmit both African and Asian greening BLOs (27, 31).

Biological control of the two psyllas was achieved successfully in Reunion island with hymenopterous psylla parasites: *Tamarixia (ex-tetrastichus) radiata* introduced from India against *D. citri*, and *Tamarixia dryi* introduced from South Africa against *T. erytreae* (3). Control of the psyllas together with eradication of greening-affected trees has led to the elimination of citrus greening disease in Reunion. Unfortunately, biological control of the psyllas cannot be achieved in most countries because the psylla parasites have their own parasitic insects. Care was taken not to introduce these hyperparasites into Reunion when biological control was initiated.

The greening agent: a bacterium-like organism (BLO). Graft and insect transmissions of greening disease had initially led to the conclusion that an infectious agent, probably a virus, was responsible for the disease. Laflèche and Bové (25, 26) were the first to show, by electron microscopy, that a microorganism, not a virus, was present in the phloem sieve tubes of greening-affected trees. They first thought that it was a mycoplasma-like organism (MLO) as MLOs were known since 1967 to infect phloem of numerous plants (10). However, a comparative study of the greening agent and the citrus stubborn agent, a genuine mycoplasma, showed the former to have a 25nm-thick cell envelope, more than twice the thickness of the unit membrrane envelope of the latter (39). Later, the bacterial nature of the greening organism (GO) was demonstrated (13, 14, 20, 35) and it was subsequently shown to have a membraneous peptidoglycan-containing cell wall of the gram negative type (16, 17). Indirect evidence for its bacterial nature was also deduced from the observation that penicillin treatment of greening-affected greenhouse and orchard trees resulted in symptom remission (2, 5).

Several groups have tried to culture the greening bacterium, with some claims of apparent success having appeared (12, 22, 23, 36, 41). In some cases, mycoplasmas were cultured and supposed to be the greening organism even though its bacterial nature had already been demonstrated; in other cases, gram negative bacteria were cultured but could not be proven to be the GO.

Currently, the GO remains uncultured and, by analogy with the uncul-

tured plant-pathogenic mycoplasma-like organisms (MLOs), the GO is called a bacterium-like organism (BLO).

Geographical distribution. In Asia, greening disease is largely distributed from Pakistan to China, and was found recently in a southern island of Japan (34). In Africa it is mainly restricted to southern and eastern countries even though greening is also present in Cameroon (4). Greening affects two countries of the Arabian peninsula, Yemen and Saudi Arabia, as well as Sri Lanka, Madagascar, Reunion, and Mauritius in the Indian Ocean.

In Africa, Yemen and Madagascar, greening occurs only in cool areas, at elevations higher than 1000 m, and is transmitted by *T. erytreae*. In Asia and Saudi Arabia greening is also present at low elevations under warm temperatures, and is transmitted by *D. citri*. Experiments performed under phytotron conditions showed that there are indeed two forms of greening, namely the African, heat sensitive form and the Asian heat tolerant form (6).

Rutaceous and non-rutaceous hosts. The greening BLO infects all citrus with some variations in susceptibility according to species. In addition, other rutaceous plants such as *Murraya paniculata*, *Atalantia missionis*, and *Swinglea glutinosa* are good hosts for the psyllas vectors (1, 42). However, there is no evidence for the presence of the BLO in these plants.

As the greening BLO cannot be obtained in culture, it was necessary to find an experimental host easier to handle and work with than citrus. Periwinkle is known to be a good host for phloem-restricted prokaryotes, and the successful transmission of Indian and South African BLO strains to periwinkle by dodder was achieved in 1983 (15). In periwinkle, the greening BLO reaches much higher titers than in citrus. The transmission of the greening BLO from China (24), the Philippines (37) and Thailand (Garnier, unpublished data) has also been achieved.

Dodder transmission of the greening BLO to *Nicotiana xanthi* has been obtained recently. Symptoms are severe even though the titer of BLOs in this plant is low (Garnier, unpublished data).

RECENT WORK: 1987 - 1991.

Development of monoclonal antibodies. Because the greening BLO has not been cultured, it was difficult to characterize the organism and to produce specific reagents for its detection. In 1987, we developed a general procedure to produce monoclonal antibodies (MAs) against phloem-restricted prokaryotes (30). In this procedure, purified phloem tissue from infected periwinkle plants is used as immunogen, and the hybridomas producing MAs specific for a given pathogenic prokaryote are screened by differential ELISA or Immunofluorescence (IF). When this method was used with greening-infected periwinkle plants, MAs were produced, first against an Indian (Poona) strain of the BLO (21), then against strains from China (Fujian) (18) and South Africa (Nelspruit) (Garnier, *et al.*, unpublished data). A total of ten different MAs have now been obtained in our laboratory.

Discovery of BLO serotypes. The use of MAs for the detection of the greening BLO by DAS-ELISA and/or IF showed that they were highly strain specific and recognized almost exclusively the strain used for immunization. These results indicated the existence of different serotypes. Even in a single country, different serotypes were found in close proximity. MA 10A6 (produced against the Poona strain of the GO) was the only MA able to recognize strains from several countries. This MA was used to purify, by immunoaffinity chromatography, the antigenic protein, called P42, against which it was directed. After purification, P42, was used to produce MAs by *in vitro* immunization of spleen cells. Three new MAs were obtained which recognized a larger number of strains than those previously produced by *in vitro* immunization (11). In particular, one of them, MA 1A5, could recognize all the Asian strains tested except those

from China, but it did not react with the African strain. This MA has to be evaluated on a larger scale, and may be useful in the detection of Asian strains of the BLO.

Purification of the greenig BLO. Besides its use in the purification of protein P42, MA 10A6 was used to isolate intact greening BLOs. The purified BLOs were studied by IF and electron microscopy (EM), and were shown to have a filamentous morphology with length of 1 to 4μm and a diameter of 0.15 to 0.3 μm. Round forms were also observed with a diameter of 1.0μm (45). This is in agreement with the morphology of the greening BLO as seen *in situ* in the phloem of infected citrus or periwinkle plants. Immunofluorescence and immunogold labeling of the purified BLO have shown that P42 is a surface protein, evenly distributed on both the round and filamentous forms. This also indicates that the two forms represent different morphologies of the same BLO.

Cloning and characterization of BLO-DNA fragments. Because MAs are too specific for the detection of all strains of the greening BLO, we have developed BLO-DNA probes (46). For this purpose, we have purified total phloem DNA from periwinkle plants infected with the Poona (India) strain of the BLO. This DNA was digested with restriction endonuclease *Hind* III and the resulting DNA fragments were cloned in the replicative form (RF) of the phage M13mp18 used as the cloning vector. Three recombinant phages p3, p19, and P10, containing Poona-BLO DNA inserts were selected by differential hybridization against DNA extracted from healthy and greening-infected periwinkle midribs. The inserts of p3, p19 and p10, were respectively 2.6 kbp, 1.0 kbp and 0.6 kbp in length and named In 2.6, In 1.0 and In 0.6. When the cloned DNA fragments were used as probes in Southern or dot hybridization experiments done at high stringency (60 C, 0.1 SSC), the smallest insert, In 0.6, hybridized only with DNA of periwinkle or citrus plants infected with the homologous Poona strain. In 1.0 gave positive hybridization signals with all Asian greening strains tested, except a Taiwan strain, and In 2.6 hybridized with all Asian strains tested. Under these conditions (high stringency), none of the probes hybridized with DNA extracted from healthy plants nor with DNA extracted from the African (Nelspruit) strain.

In order to characterize the three inserts, they were sequenced and the putative coding sequences were compared to those of known genes (47). This showed that In 2.6 contains the genes of the *nus*G-*rpl*KAJL-*rpo*BC operon. *nus*G codes for a protein involved in transcription antitermination, *rpl*K, A, J, and L for ribosomal proteins L11, L1, L10, L12 respectively, and *rpo*B and C for RNA polymerase subunits β and β'. In the greening BLO, these genes occur in the same order as in other eubacterial species. This confirms for the first time, on the molecular level, that the greening BLO is an eubacterium. In 1.0 contains genes for a bacteriophage-type DNA polymerase; no known genes could be identified in In 0.6.

Asian and African BLOs: different strains or different species? In 2.6 contains genes for conserved proteins (ribosomal proteins), explaining why it hybridized with all Asian BLO strains tested. However, In 2.6 did not hybridized with the African (Nelspruit) strain at high stringency. This shows that Asian and African strains of the BLO are different as suggested previously by biological (6) and serological properties (11, 18). To evaluate the relatedness between Asian and African greening BLOs, hybridizations at intermediate stringency (32 C, 0.1 SSC) were performed (48). Under these conditions, In 2.6 hybridized with the African strain. From the hybridization results we calculated that there was at least 30% mismatches between the Poona-BLO gene cluster and its counterpart in the Nelspruit strain. Whether the African and Asian BLOs tested are different strains, different bacterial species or even different genera needs further investigation.

In order to characterize the greening BLO, and to more precisely determine its phylogenetic position, the gene coding for the 16S ribosomal RNA (16S rDNA) was cloned and sequenced (19). The BLO (Poona strain) was first immunocaptured from a plant extract with MA 10A6, previously coated onto the surface of an eppendorf tube. The 16S rDNA was then amplified by PCR, using universal primers of the 16S rDNA for amplification of prokaryotic 16S rDNA. Sequence analysis of the 16S rDNA has revealed that the greening BLO belongs to the α subdivision of the proteobacteria, a phylogenetic group containing plant symbionts, and human or animal bacteria having arthropod vectors. Many bacteria of this group are not available in culture. We propose that the greening BLO represents a new genus in this group.

DNA probes: detection of the BLO in orchard trees and psyllas by dot blot hybridization. Since In 2.6 hybridizes with the DNA extracted from all Asian BLO strains tested, we therefore used it as a probe for the detection of the greening BLO in orchard trees. Dot-blot hybridizations were performed on 53 field-grown citrus trees of various regions in India (Delhi, Mysore, Orissa, Rajasthan and Uttar Pradesh) (7, 44). EM detection of the greening-BLO was also performed on the same samples. Thirty-nine trees were found positive by hybridization and 28 by EM. Only one tree was EM-positive but negative by hybridization. Symptomless trees and trees showing symptoms of mosaic virus infection gave negative results with both techniques. These results indicate that In 2.6 is at least as sensitive as EM to detect the greening BLO in field trees. Probe In 2.6 was also used to detect the greening-BLO in individual psyllas directly blotted by crushing on a nylon membrane. This showed that 30% of the psyllas collected in Malaysia in September 1991 were infected by the BLO while in May and October 1992, the percentage of infected psyllas was only 3 to 5%. These preliminary results indicate that In 2.6 can be efficiently used to study the epidemiology of greening disease.

CONCLUSIONS AND PERSPECTIVES FOR FUTURE WORK

The work carried out in recent years had two main objectives: development of detection procedures and characterization of the greening BLO.

Even though the greening BLO has not been cultured, monoclonal antibodies against surface antigens have been produced and DNA fragments of the BLO genome have been cloned and sequenced. For the first time, reagents which enable detection of all strains of the GO are available. Greening BLO-infected trees can now be distinguished from mineral deficiency-affected trees, and gene amplification by polymerase chain reaction PCR can easily be developed if a technique more sensitive than DNA hybridization is required to detect the greening BLO. In addition, as the presence of the greening BLO can be determined in individual psyllas, the percentage of infected psyllas in a population can be determined throughout the year. This opens new approaches for epidemiology.

Characterization of the greening BLO by 16S rDNA sequencing showed that the BLO belongs to the α subdivision of the proteobacteria. Even though many bacteria in this group have not been cultured, some media supporting the growth of certain of these bacteria can be evaluated for the BLO.

Finally, we have obtained BLO-infected tobacco plants. This will allow evaluation of genes that might control the BLO. Indeed, it is much easier to produce transgenic tobacco than citrus plants, and production of tobacco plants transgenic for lyzozyme is underway.

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