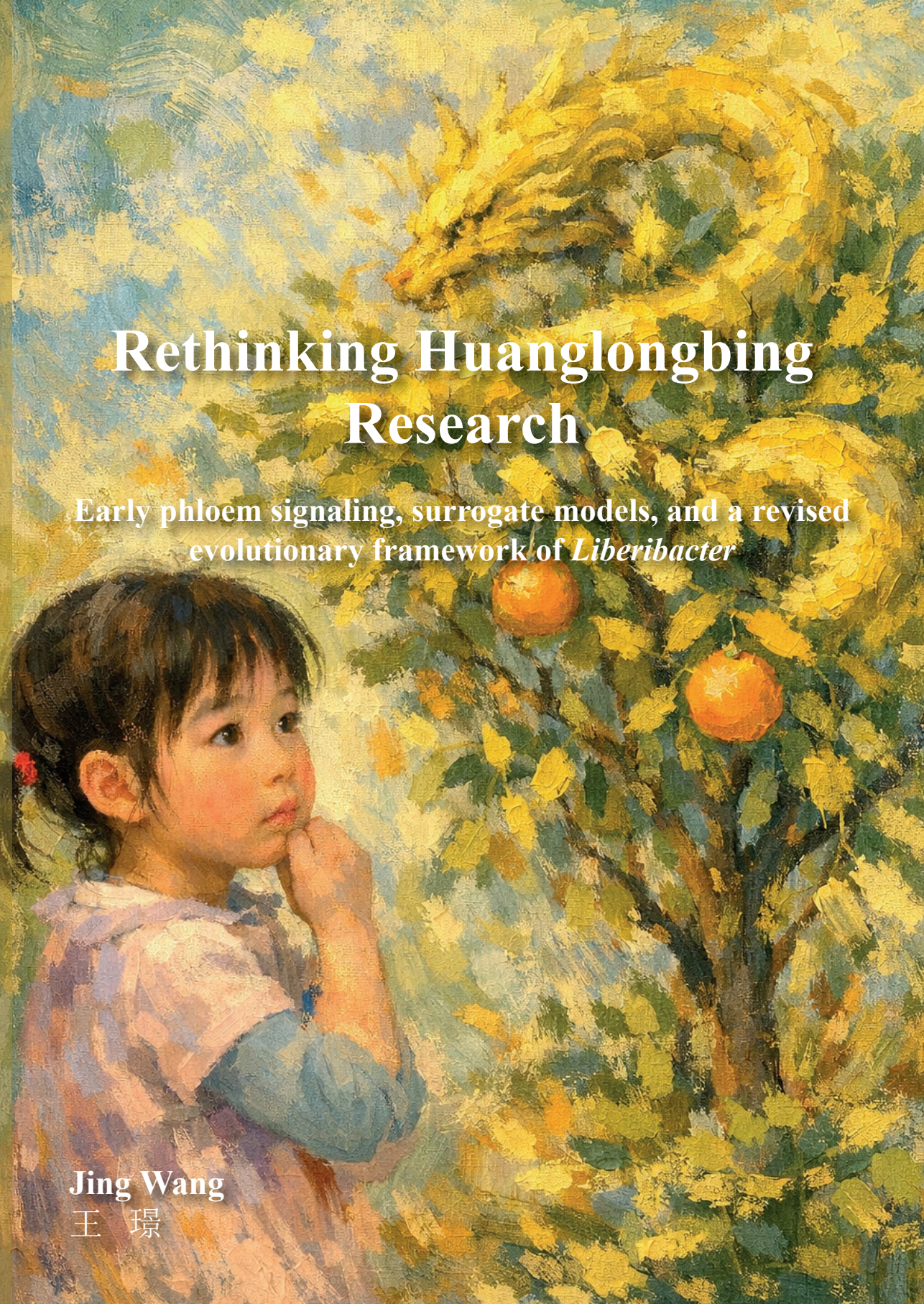


An oil painting of a young girl with dark hair and bangs, wearing a pink and blue striped shirt. She is looking up at a tree with yellow leaves and two orange fruits. The background is a mix of yellow and blue brushstrokes.

Rethinking Huanglongbing Research

Early phloem signaling, surrogate models, and a revised evolutionary framework of *Liberibacter*

Jing Wang

王 璟

Propositions

1. Candidatus *Liberibacter* is a *Rhizobium*.
(this thesis)
2. The rhizobia-legume nodule symbiosis is an effective tool to study Candidatus *Liberibacter* genes .
(this thesis)
3. Toxicology on isolated compounds misjudges safety of traditional Chinese medicine.
(Han et al., 2019, *Frontiers in Pharmacology*, 10, 648)
4. Hallucinations in AI make it essential that outcomes can be validated.
(Farquhar et al., 2024, *Nature*, 630(8017), 625-630.)
5. Praise is worthless in a system where criticism is suppressed.
6. A statement's force comes more from authority of the speaker than its content.

**Propositions belong to the thesis, entitled
Rethinking Huanglongbing Research: Early phloem signaling, surrogate models,
and a revised evolutionary framework of *Liberibacter***

Jing Wang

Wageningen, 13rd May 2025

Rethinking Huanglongbing Research

**Early phloem signaling, surrogate models,
and a revised evolutionary framework of *Liberibacter***

Jing Wang

Thesis committee

Promotor

Prof. Dr T. Bisseling
Professor of Molecular Biology
Wageningen University & Research

Co-promotor

Prof. Dr X. Cheng
Principal Investigator, Agricultural Genomics Institute
Shenzhen Chinese Academy of Agricultural Sciences, China

Other members

Prof. Dr H Spaink, Leiden University
Prof. Dr M Medema, Wageningen University & Research
Dr *ir.* M Joosten, Wageningen University & Research
Dr *ir.* V Vleeshouwers, Wageningen University & Research

This research was conducted under the auspices of the Graduate School Experimental Plant Sciences(EPS).

Rethinking Huanglongbing Research

Early phloem signaling, surrogate models,
and a revised evolutionary framework of *Liberibacter*

Jing Wang

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr C. Kroeze,
in the presence of the Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 13 May 2026
at 1.00 p.m. in the Omnia Auditorium.

Jing Wang

Rethinking Huanglongbing Research: Early phloem signaling, surrogate models,
and a revised evolutionary framework of *Liberibacter*,

131 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2026)

With references, with summary in English

This research was conducted in Laboratory of Molecular Biology, Wageningen University &
Research, with support from China Scholarship Council.

Financial support from Wageningen University for printing this thesis
is greatly appreciated.

Cover design by: Jing Wang

Printed by: ProefschriftMaken || www.proefschriftmaken.nl

CONTENTS

Chapter 1	General Introduction	09
Chapter 2	MiG-based transmission of <i>Ca. Liberibacter asiaticus</i> enables studies of host-pathogen interaction at early infection stages of HLB	29
Chapter 3	Comparative expression analysis of homologous genes from <i>Liberibacter asiaticus</i> and the symbiotic plasmid pSymA of <i>Sinorhizobium</i>	49
Chapter 4	Targeting a CLas gene in planta using a rhizobial-legume model	73
Chapter 5	Phylogenetic analysis based on most conserved AMPHORA proteins shows that <i>Liberibacter</i> is nested in the <i>Rhizobium</i> genus	91
Chapter 6	General Discussion	107
Summary		125
Acknowledgements		129

1

General Introduction

Jing Wang

Laboratory of Molecular Biology, Department of Plant Sciences, Graduate School
Experimental Plant Sciences, Wageningen University & Research, Wageningen
6708 PB, the Netherlands.

Huanglongbing (HLB), also known as citrus greening disease, is a devastating bacterial disease that affects citrus trees. It is caused by the bacterium *Candidatus Liberibacter spp.* and is spread by the Asian citrus psyllid *Diaphorina citri*. Infected trees exhibit yellowing of leaves, which are misshapen, and the fruits are bitter. Ultimately, the trees die. There is no cure for HLB, making it a significant threat to the global citrus industry, as it severely impacts fruit production. In this Introduction, I will introduce the background of HLB research from several aspects: Early history of HLB research; The severe impact of HLB; Strategies for controlling HLB; Molecular pathogenesis studies.

1. EARLY HISTORY OF HLB RESEARCH

HLB was first observed in South China by Reinking in 1919, and it was named “yellow shoot”(Reinking, 1919). By 1936, the disease had become a serious problem in the region and was locally named “Huang黄 Long龙 Bing病”, which means “yellow dragon disease”. However, due to the regional accent in Chaozhou, the local people actually referred to it as “Huang黄 Nen嫩 Bing病”, where “Nen” refers to the tender shoots, thus meaning the disease of yellow tender shoots. Phytopathologist Kung Hsiang Lin(林孔湘) from the South China Agricultural University dedicated much of his research from the 1940s to the late 1950s to HLB. Through interactions with farmers in Guangdong province’s Chaozhou district, Lin discovered that HLB had been affecting the region since the late 19th century. In 1956, Lin’s groundbreaking research demonstrated that HLB was transmissible via graft-inoculation, a significant step in understanding how the disease spreads (Lin, 1956). Due to Lin’s contributions, the International Organization of Citrus Virologists (IOCV) decided at its 13th conference in Fuzhou (Fujian Province, China) in 1995 that the official name of the disease would be “huanglongbing”. Today, huanglongbing is used worldwide to refer to this disease. This was the first time, on an international level, that a plant disease was named using Chinese pinyin.

In South Africa, the disease was observed as early as 1928/1929, where it was known as “yellow branch” in the western Transvaal and “greening” in the eastern Transvaal. Initially, it was believed to be related to mineral nutrition deficiencies (van der Merwe and Andersen, 1937). However, it was first in the 1960s that the true nature of the disease was understood, linking it to a transmissible pathogen like the one found in China (McClellan and Oberholzer, 1965).

Despite these early observations and local research, HLB remained relatively unknown in the Western scientific community until the mid-20th century. Ciccarone, an Italian phytopathologist, played a crucial role in bringing attention to Lin’s work by publishing a report in 1957 (Ciccarone, 1957), by which Western scientists also became aware of HLB.

In 1965, McClean and Oberholzer in South Africa confirmed the transmission of the greening agent by graft-inoculation and identified the African citrus psyllid as a vector. In 1967, further research in India and the Philippines demonstrated the role of the Asian citrus psyllid in transmitting HLB (Martinez and Wallace, 1967). Together, these studies have brought HLB to global attention.

2. THE SEVERE IMPACT OF HLB

The severe impact of HLB on the citrus industry is caused by its rapid and widespread transmission, as well as its serious harm to the health of citrus trees. HLB can be transmitted in two ways: short-distance transmission mediated by insects and long-distance transmission by human activities. The Asian citrus psyllid, *Diaphorina citri*, and the African citrus psyllid, *Trioza erytreae*, are the insect vectors for HLB. These insects feed on infected citrus phloem sap, acquiring the HLB pathogen, and injecting it into healthy plants during subsequent feedings.

The industrial propagation of citrus trees typically involves grafting branches from citrus varieties that produce excellent fruit onto rootstocks of citrus species that are well-adapted to the local soil and are resistant to diseases. China is a major exporter of citrus fruit seedlings, and during the times when HLB was not well recognized, many HLB-infected seedlings were exported to other countries, leading to global spreading. Additionally, some citrus psyllid nymphs most likely have been co-transported.

The pathogens causing HLB are the bacteria *Candidatus Liberibacter asiaticus* (CLas), *Candidatus Liberibacter africanus* (CLaf), and *Candidatus Liberibacter americanus* (CLam). Among these, CLas has caused the most severe HLB outbreaks, likely because both CLas and its psyllid vector are heat-tolerant (Bové et al., 1974; Lopes et al., 2009). HLB in Asia and Oceania is primarily caused by CLas.

In 2004, HLB was first reported in Brazil, caused by CLam carried by Asian psyllids (Coletta-Filho et al., 2004; Teixeira et al., 2005). Due to the higher heat tolerance of CLas and its ability to reach higher bacterial loads in citrus trees, it gained a competitive advantage, and by 2008, CLas had been massively spread in Brazil. (Lopes et al., 2009). CLas is also responsible for the devastating impact on the citrus industry in Florida, USA (Graham et al., 2020).

CLaf and its vector, the African psyllid, are heat sensitive (Bové et al., 1974) and therefore only spread in regions where temperatures are below 30°C. They are mainly found in high-altitude areas in, for example, South Africa, Nigeria, and Madagascar (Catling, 1969). CLas and the Asian psyllid, generally, are geographically separated from CLaf and its vector. However, there are certain islands where both coexist (Garnier et al., 1996). For instance, on the Indian Ocean islands of Réunion and Mauritius, there is a wide variation in temperature related to altitude.

On Réunion Island, CLas and the Asian psyllid are predominantly found at altitudes below 500 meters, whereas CLaf and the African psyllid are found at altitudes above 500 meters.

Some citrus-growing regions are currently free from HLB, even though citrus psyllids have already been detected in these areas. For example, the African psyllid has been observed in Portugal and Spain, while the Asian psyllid has been detected in Argentina, Honduras, as well as in regions of the Caribbean and the West Indies (Vaccaro, 1994; Burckhardt and Martinez, 1989; Etienne et al., 1998). The Mediterranean countries are the only major citrus-growing regions that have, so far, remained free of the Candidatus *Liberibacter* bacteria.

Candidatus *Liberibacter* can infect all citrus species, including all agricultural varieties. Initially, infected trees may show one or more “yellow shoots” that gradually expand into larger yellow or pale green branches (Fig. 1H). The bacteria eventually spread throughout the entire canopy. As the disease progresses, the affected branches may exhibit defoliation, twig dieback, and fruit drop. The leaves often display blotchy mottle, characterized by multiple shades of yellow, pale green, and dark green blending together without sharp boundaries (Fig. 1F). This pattern is distinctive and helps to differentiate HLB from other types of mottling and nutrient deficiencies.

Fruits from HLB-infected trees often show an unusual color pattern, where the orange color starts developing from the stem end (peduncle) while the opposite end (stylar end) stays green (Fig. 1I). These affected fruits are usually smaller in size, misshapen (lopsided), and often contain undeveloped (aborted) seeds. Additionally, the area where the fruit attaches to the stem (peduncle insertion point) appears sunken, which is caused by abnormal thickening of the white inner peel (albedo) – a typical sign of HLB.

Leaf symptoms of HLB include not only changes in color, but they also become thicker and obtain a leathery texture. The midribs and lateral veins may sometimes become enlarged, swollen, and corky. In some cases, the leaves may only exhibit symptoms similar to zinc deficiency, remaining small and turning uniformly yellow over time. Histologically, localized pockets of necrotic phloem are scattered through the leaves, a consistent symptom of HLB.

Due to the irreversible damage HLB causes to citrus trees, its impact on the citrus industry is devastating. In China, starting in the 1930s, citrus production in the Pearl River Delta and Hanjiang Delta of Guangdong province experienced a sharp decline due to this disease (Jiang et al., 1935). This necessitated frequent tree replacements, leading to increased management costs and reduced profit (Zhang et al., 2023). By the mid-20th century, HLB had spread to Fujian and Guangxi provinces, (Luo et al., 2023). From the late 1970s to the 1980s, the disease severely impacted citrus orchards in Sichuan, Jiangxi, and Yunnan, later reaching Hunan, Guizhou, and Zhejiang after 1980 (Fan et al., 2009). Currently, 11 out of the 19 citrus-growing provinces in China, which account for more than 80% of the country’s citrus cultivation area, have been affected by HLB (Fan et al., 2009).

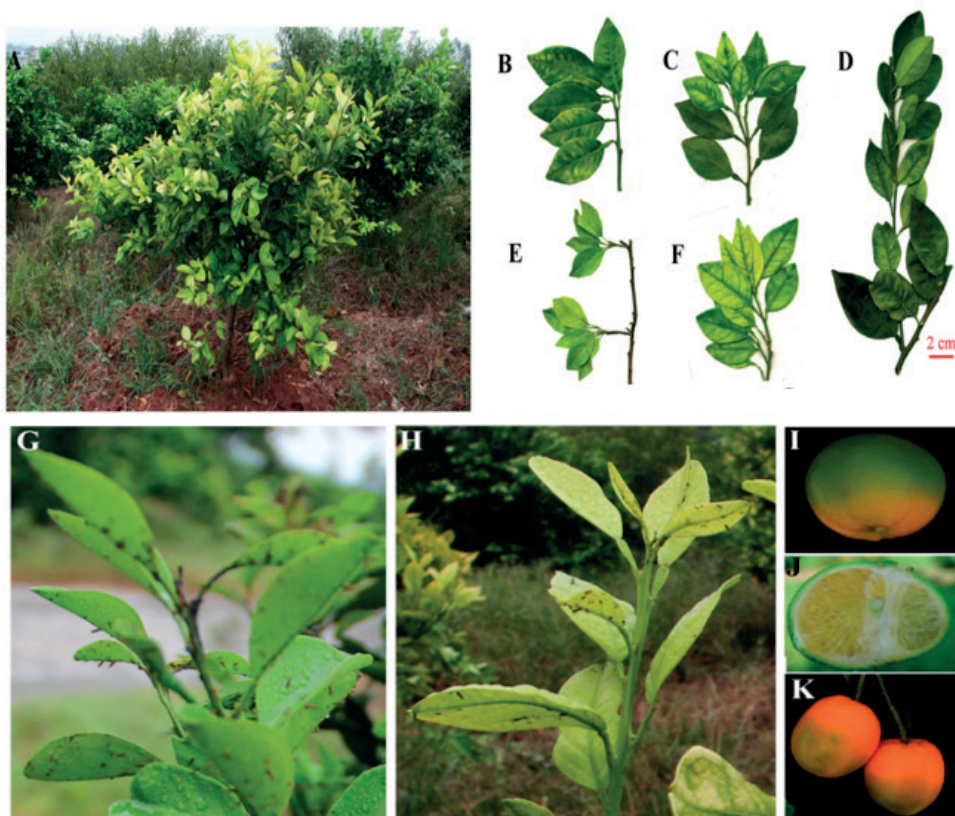


Figure 1: Sweet orange tree infected with HLB.

A: Newly emerged shoots with yellow leaves.

B-F: Branches affected by HLB.

G, H: Comparison between a healthy and a chlorotic branch.

I-K: Fruits affected by HLB.

HLB has significantly impacted the U.S. citrus industry, particularly in Florida and California. Citrus contributes 16% to the total value of U.S. fruit production (USDA–ERS, 2018). In Florida, the state with the largest orange production, citrus acreage has declined by 30%, and production has decreased by 74%. This occurred since HLB was discovered in 2005, and it resulted in economic damage of over \$1 billion per year, and about 5,000 jobs were lost annually (Court et al., 2017). As a result of Florida's production decline, California has become the largest citrus producer in the US, despite HLB being discovered there in 2012. To combat the disease, California has implemented aggressive management efforts, including pesticide applications, biological control, early detection programs, and an eradication program for diseased trees. Overall, HLB poses a great challenge to citrus growers, causing substantial economic losses and threatening the sustainability of citrus cultivation.

3. STRATEGIES FOR CONTROLLING HLB

3.1. Current control strategies

3.1.1. Psyllid control and quarantine measures

Current HLB control relies heavily on managing its vector, the Asian citrus psyllid. Insecticides form the backbone of this strategy, targeting both adults and psyllid larvae. Broad-spectrum insecticides such as Diflubenzuron, Spinetoram, Fenpropathrin, Malathion, and Dimethoate are widely used. These insecticides are applied using conventional ground air-blast applicators for high-volume sprays and truck-mounted applicators for low-volume mist sprays, which offer cost-effective and extensive coverage (Hoffmann et al., 2010). Systemic insecticides, including neonicotinoids like clothianidin, imidacloprid, and thiamethoxam, provide long-lasting protection, sometimes up to 9 weeks, with imidacloprid achieving 50 to 90% control of Asian citrus psyllid adults through foliar application (Tiwari and Stelinski, 2013; Ichinose et al., 2010). Additionally, insect growth regulators like pyriproxyfen, buprofezin, and diflubenzuron disrupt the development of Asian citrus psyllid nymphs, strengthening the overall control strategy (Inoue et al., 2009).

In China, the application of up to 13 insecticide sprays per year has effectively managed HLB by significantly suppressing Asian citrus psyllid populations (Roistacher, 1996). This intensive approach includes both broad-spectrum and systemic insecticides, tailored to local conditions and pest pressure. In Florida, insecticides that allow for the use of smaller amounts have proven effective. (Hoffmann et al., 2010; Powell et al., 2007). Insecticides such as abamectin, carbaryl, diflubenzuron, dimethoate, fenpropathrin, malathion, spirotetramat, and zeta-cypermethrin are applied in small amounts, making the treatment more cost-effective. Systemic insecticides are also applied via soil drench and drip irrigation (Boina and Bloomquist, 2015). Studies in Florida have shown significant yield increases in citrus orchards treated with insecticides, with economic benefits outweighing the costs (Stelinski et al., 2009). However, intensive use can lead to resistance in Asian citrus psyllid populations and potential secondary pest outbreaks, emphasizing the need for integrated pest management practices. Additionally, while these strategies effectively control Asian citrus psyllid, their frequent application can harm non-target organisms, including beneficial insects and other wildlife, and lead to the accumulation of chemical residues in soil and water, posing risks to broader ecosystems and human health.

3.1.2. Eradication of infected trees and replacement with disease-free plants

Systematically removing and destroying HLB-infected trees helps to decrease the overall presence of CLAs, thereby slowing HLB spreading. The success of this method depends largely on the implementation scale. Large-scale, coordinated efforts are necessary to ensure effectiveness, as small-scale or incomplete efforts are insufficient due to the high mobility of psyllids. However, this strategy is not cost-effective and requires coordination among grow-

ers. For example, Muraro estimated that eradicating infected trees increases maintenance costs about \$450 per acre/year (Muraro 2010). These factors make implementation challenging despite the effectiveness of these methods.

3.1.3. Using antibiotics

Controlling HLB in citrus trees through antibiotics involves several key strategies and considerations. Antibiotics have been used to manage bacterial diseases in fruit trees, and though only a few antibiotics are commercially available for plant disease treatment (McManus and Stockwell, 2001), they have shown significant promise. Effective antibiotics like ampicillin, carbenicillin, and penicillin have antibacterial properties, but are not yet commercially used for HLB (Zhang et al., 2010; Zhang et al., 2011; Zhang et al., 2012; Zhang et al., 2014; Le et al., 2006). The treatment criteria for HLB include the ability of antibiotics to be active within the plant, easily delivered to the phloem, non-phytotoxic, thermally stable, and with minimal potential for pathogens to develop resistance. Earlier applications, such as tetracycline trunk injections in the 1970s, were discontinued due to phytotoxicity and high labor costs (Su and Chang, 1974). In contrast, combinations like penicillin and streptomycin have demonstrated superior efficacy in suppressing CLas, especially in greenhouse conditions (Zhang et al., 2011). Despite these benefits, the use of antibiotics is restricted by concerns over phytotoxicity, residue accumulation in fruit, the development of antibiotic-resistant bacteria, potential killing of plant and soil microbiome members, and high treatment costs.

While current HLB control strategies have shown varying degrees of success, they each come with significant drawbacks. Intensive insecticide use can lead to resistance in psyllid populations, harm non-target organisms, and contribute to environmental contamination. Tree eradication, though effective in reducing pathogen spread, is costly and requires large-scale coordination, making it unsustainable for many growers. Antibiotic treatments face challenges related to phytotoxicity, regulatory concerns, and the potential for antibiotic resistance. Overall, these methods serve only as stopgap measures rather than long-term solutions. Therefore, the development of a lasting, effective, and economically viable strategy to eradicate HLB is urgently needed.

3.2. Attempts to develop new approaches

Developing genetically resistant citrus varieties is considered the most sustainable long-term solution for managing HLB. However, natural resistance in commercially viable citrus cultivars is extremely limited, and conventional breeding approaches face significant challenges due to the long juvenile phase of citrus trees, which can last from 4 to 12 years. Additionally, resistance traits found in related species such as *Poncirus trifoliata* have been difficult to introduce into elite citrus cultivars, due to genetic incompatibility and the lack of robust rootstock/scion combinations.

To overcome these limitations, molecular genetic engineering approaches have been explored. For example, the overexpression of *Arabidopsis thaliana* *NPR1* (*AtNPR1*), a key regulator of systemic acquired resistance (SAR), in citrus plants has been studied (Dutt et al., 2015). *NPR1* enhances the plant's defense system by activating a broad spectrum of pathogenesis-related genes. Transgenic sweet orange cultivars expressing *AtNPR1* under the control of a constitutive (*CaMV* 35S) or phloem-specific (*AtSUC2*) promoter exhibit significantly reduced HLB symptoms. Some transgenic lines even remained disease-free for up to 36 months in field trials under high disease pressure (Dutt et al., 2015). However, the constitutive induction of SAR, also with a phloem-specific promoter, will most likely result in a plant-wide constitutive expression of defense genes. Previously, it has been shown that constitutively expressed defense genes have a negative effect on plant growth (He et al., 2022; Mengarelli et al., 2021; Wang and Eulgem, 2024). See also below

Another strategy involves the overexpression of genes encoding a peptide with anti-microbial activity. For example, modified plant thionins, which possess direct antimicrobial properties against Cas. Transgenic citrus plants expressing a thionin gene showed reduced bacterial titers and disease severity (Hao et al., 2016). Another example is a novel class of stable antimicrobial peptides (SAMPs), derived from HLB-tolerant citrus relatives. These peptides exhibit potent antibacterial activity against CLAs, while simultaneously activating the plant's innate immune responses (Huang et al., 2021). However, in both cases, constitutive expression will affect the microbiome and negatively affect plant growth.

A disadvantage of peptides with antimicrobial activity is the lack of specificity. Therefore, a search for a more CLAs specific approach is attractive. A promising strategy for a more specific targeting of CLAs is the targeted inhibition of its transcription regulators, which control key bacterial functions necessary for virulence (Barnett et al., 2019). A more detailed description is provided later.

The use of defense-related genes, although not specifically targeting CLAs, can be markedly improved when their expression is only at the right moment and the right place. Constitutively induced SAR will result in significant energy trade-offs, diverting resources away from essential physiological processes such as growth, flowering, and fruit production. For example, overexpression of *NPR1* in *Arabidopsis* leads to stunted growth and reduced yield (Dutt et al., 2015), making it unsuitable for commercial citrus production.

Constitutive expression of genes encoding antimicrobial compounds will have, to some extent, similar energy trade-off problems, and their expression in fruits seems unacceptable. Further, they might kill members of the root microbiomes. Therefore, the use of defense-related genes requires a more refined and condition-dependent approach.

A promising alternative is the use of a suitable promoter to create citrus plants that induce localized antimicrobial responses specifically in the phloem cells upon their infection by CLAs. This approach would require the promoters of genes that are exclusively induced in the phloem cells immediately upon infection by CLAs of phloem cells. Such genes can be identified by transcriptome analyses. However, currently available transcriptome studies are based on whole leaves with obvious HLB symptoms, making it difficult to identify genes induced in the phloem during the early stages of HLB infection (Curtolo et al., 2020; Ribeiro et al., 2023). Additionally, a reproducible and controlled experimental system for testing early-stage HLB infection has not yet been established.

Future research should focus on identifying reliable phloem-specific promoters activated by CLAs infection and exploring SAMPs with higher specificity against CLAs.

3.3. Using alternative research systems

Since CLAs cannot be cultured in vitro, traditional antimicrobial screening methods are not possible. To overcome this limitation, alternative research systems have been established to study CLAs. One approach utilized *Sinorhizobium meliloti*. CLAs genes, such as *LdtR*, *RpoH*, and *VisNR*, that encode transcription regulators, were expressed in *S. meliloti* together with a construct based on a promoter of one of their target genes driving the expression of *GFP*. This system enables rapid identification of small molecules that inhibit these transcription factors that are essential for CLAs virulence. Screening over 120,000 compounds has led to the discovery of several inhibitors that selectively inhibit CLAs transcription factors. This approach offers a specific antimicrobial strategy that targets CLAs with specificity.

Another approach uses hairy root cultures, which can support the intracellular growth of CLAs and other Candidatus *Liberibacter* species. By inducing hairy root formation on infected citrus and potato explants, using *Agrobacterium rhizogenes*, a platform for testing antimicrobial treatments was established. This system allows for the evaluation of approaches such as introduction of resistance genes and application of chemical treatments. Additionally, it provides opportunities to study plant-CLAs interactions.

Additionally, *Catharanthus roseus* (periwinkle) has been widely used as a surrogate host for CLAs. CLAs can be transmitted to periwinkle by dodder. In periwinkle, it replicates and induces systemic symptoms, including leaf yellowing and stunting. Its small size, rapid growth, and high susceptibility make periwinkle a convenient model for studying CLAs colonization and host responses. Importantly, CLAs titers in periwinkle can reach high levels, facilitating downstream molecular analyses. Periwinkle has also proven useful for evaluating the efficacy of antimicrobial compounds and gene-based interventions in a whole-plant context.

While these alternative systems have significantly expanded our experimental capabilities, they each have limitations. Heterologous bacteria like *S. meliloti* lack the cellular complex-

ity of plant systems, and periwinkle is taxonomically distant from citrus and may not fully recapitulate phloem-specific interactions or defense responses. Hairy root cultures, although valuable for root-focused studies, exclude shoot tissues and differ physiologically from intact citrus organs.

Therefore, a system that enables rapid, reproducible CLas infection in citrus seedlings—while preserving the natural host and full plant architecture—would provide a more representative platform for research on mechanisms underlying the interaction and antimicrobial screening.

4. MOLECULAR PATHOGENESIS STUDIES

Despite the advancements in the aforementioned new HLB treatment approaches, a comprehensive understanding of CLas pathogenesis at the molecular level remains crucial for the development of effective control strategies. The unique biology of CLas, including its phloem-limited lifestyle and lack of an in vitro culture system, has posed significant challenges to research. Recent molecular pathogenesis studies have begun to shed light on the intricate interactions between CLas and citrus hosts, revealing key virulence factors, host immune responses, and molecular pathways that influence disease progression. These insights are instrumental in guiding the design of new ways of protection and genetic resistance strategies for long-term HLB management.

To establish a foundation for these molecular investigations, it is essential to first understand how CLas infects citrus plants in natural environments. After being transmitted by the Asian citrus psyllid during feeding on young citrus shoots, CLas initially amplifies in the phloem of leaf tissues (Raiol-Junior et al., 2021). From these leaves it is, rapidly translocated to the roots, CLas accumulates to high titers in the roots well before any foliar symptoms become apparent, suggesting that early root colonization is a critical step for subsequent systemic spread (Johnson et al., 2014). Once root infection is established, CLas moves acropetally back into the canopy, leading to widespread colonization of shoots and leaves (Raiol-Junior et al., 2021).

This implies that the defense response induced in the phloem is too low for a long time to stop the bacteria from moving through the plant. A general response to bacterial infection in plants is programmed cell death (PCD), by which the invaded bacteria are kept very locally and cannot spread (Coll et al., 2011). The systemic infection by CLas shows that such a rapidly induced programmed cell death is not induced.

One possible explanation for the lack of PCD in phloem cells during HLB progression is the ability of CLas to actively suppress or evade early host immune signaling. Studies have identified several CLas-secreted effectors, such as *SDE1*, that interfere with host defense pathways, including suppressing reactive oxygen species (ROS) bursts and salicylic acid-

mediated responses, which are critical for initiating localized cell death (Pitino et al., 2018; Clark et al., 2018). Additionally, CLas infection has been reported to induce the upregulation of citrus *WRKY* transcription factors (e.g., *WRKY40*), some of which act as negative regulators of defense, potentially dampening the activation of PCD during early stages (Hu et al., 2017; Martinelli et al., 2013).

Another factor contributing to lack of PCD could be the phloem's inherently limited capacity for mounting robust immune responses. Phloem is a low-oxygen, low-protein environment specialized for transport, with reduced numbers of nucleated cells, which may constrain the speed and intensity of defense responses compared to mesophyll tissues (Kehr, 2006; Will & Van Bel, 2006).

The death of phloem cells during advanced stages of HLB has profound consequences for the transport of photosynthates, hormones, and other signaling molecules. The collapse of sieve elements and companion cells impairs assimilate flow, causing starch accumulation in leaves (Etxeberria et al., 2009; Fan et al., 2010).

These secondary effects underscore that, beyond direct bacterial colonization, the physiological collapse of the phloem network is central to the devastating impact of HLB on citrus trees.

5. TRANSCRIPTOMIC RESPONSE DURING CITRUS HLB INFECTION

All published transcriptome studies of citrus HLB have been conducted on leaves from field or greenhouse-grown citrus plants displaying visible disease symptoms (Curtolo et al., 2020; Liu et al., 2019; Ribeiro et al., 2023; Wang et al., 2016). These studies typically compare symptomatic leaves with healthy controls to identify differentially expressed genes (DEGs) associated with HLB. However, by the time visible symptoms appear, infected leaves have already suffered extensive physiological and structural damage—especially in the phloem—making it impossible to disentangle the primary host transcriptional responses directly triggered by initial CLas infection from secondary transcriptional changes caused by advanced disease progression. Further, the use of whole-leaf samples containing multiple tissue types does not allow the detection of phloem specific early responses. As a result, these studies consistently report strong downregulation of photosynthesis-related genes (Curtolo et al., 2020; Liu et al., 2024; Ribeiro et al., 2023), changes that likely reflect general decline due to late-stage disease rather than primary defense mechanisms.

These transcriptomic datasets have revealed transcriptional reprogramming in pathways involved in starch and carbohydrate metabolism, hormone signaling, and reactive oxygen species (ROS) production (Curtolo et al., 2020; Liu et al., 2024; Ribeiro et al., 2023). However,

it remains unclear whether these alterations represent genuine immune responses to CLas or are instead secondary consequences of severe phloem dysfunction and energy depletion. Comparative studies between HLB-tolerant and susceptible citrus genotypes underscore this ambiguity: tolerant cultivars, such as ‘LB8-9’ Sugar Belle mandarin, show elevated expression of genes involved in ROS scavenging and phloem repair, while susceptible varieties accumulate damaging ROS and exhibit more pronounced phloem degeneration (Robeldoe et al., 2023; Ribeiro et al., 2023). Yet these differences mainly reflect improved tolerance to phloem collapse not a primary, CLas-specific resistance mechanism that prevents bacterial colonization.

The model plant *Catharanthus roseus* (periwinkle) has also been used in HLB studies, as it rapidly develops HLB-like symptoms when infected with CLas via the parasitic plant dodder. However, transcriptomic comparisons in periwinkle similarly rely on symptomatic versus healthy leaves, and they also reveal suppression of photosynthesis genes indicative of secondary disease effects (Liu et al., 2019). Together, these observations highlight a consistent pattern across current transcriptome studies: they predominantly capture secondary responses associated with late-stage symptoms, failing to resolve the primary molecular events induced upon CLas entry into phloem tissues.

To uncover the key regulatory genes and pathways activated during the earliest stages of infection—those that could provide effective, specific resistance targets—controlled experimental systems enabling synchronized, reproducible inoculation and precise tracking of early infection dynamics are urgently needed. By focusing on phloem-specific early transcriptional changes, such systems could identify primary host responses directly induced by CLas, paving the way for breeding or engineering citrus varieties with genuine, targeted resistance rather than merely improved tolerance to phloem blockage or reducing secondary effects.

6. GENOME OF CLAS

The genome of CLas was first determined by Duan et al. (2009) through metagenomic sequencing of DNA extracted from infected Asian citrus psyllids, revealing a small circular chromosome of approximately 1.23 Mb with a GC content of 36.5%—features typical of obligate intracellular bacteria. Subsequent comparative analyses with other *Liberibacter* species, including Candidatus *Liberibacter africanus* (CLaf) and Candidatus *Liberibacter americanus* (CLam), showed that CLas shares ~93% and ~88% of its genes with CLaf and CLam, respectively, but still harbors dozens of unique genes potentially linked to host adaptation and virulence (Hartung et al., 2011; Thapa et al., 2020).

Interestingly, *Liberibacter crescens* is the only culturable member of the genus and possesses a slightly larger genome (~1.5 Mb), but this still seems a strongly reduced genome. This explains why a very complex medium is required for its culture.

Phylogenetic phylogenomic comparisons place *Liberibacter* species within the Rhizobiales, where free-living and symbiotic relatives such as *Sinorhizobium meliloti* (6.7 Mb genome) and *Bradyrhizobium japonicum* (9.1 Mb) genomes encode complex regulatory networks, extensive metabolic repertoires (Hartung et al., 2011).

In contrast, CLas as well as *Liberibacter crescens* have a strongly reduced genome, losing genes for de novo synthesis of purines, pyrimidines, multiple amino acids (e.g., tryptophan, valine), and key DNA repair enzymes. This extreme genome reduction likely reflects a long evolutionary trajectory under relaxed selective pressure to maintain functions not essential within the protected, nutrient-rich niches of the phloem or insect vector.

The high conservation of core genes across CLas, CLaf, and CLam supports a scenario of early specialization in a common ancestor followed by divergence (Hartung et al., 2011; Leonard et al., 2012). CLas retains genes encoding ABC transporters. These are located in the membrane and are involved in the uptake of nutrients. As CLas highly depends on these it is clear that there is a high selective pressure to maintain these genes required for the uptake of such compounds. It will also be essential to maintain a mechanism to suppress the defense response of the lack of a Type III secretion system commonly used by Gram-negative pathogens to inject effectors into host cells. However, CLas has a Sec-dependent secretion system (Duan et al., 2009) by which CLas can use a suite of Sec-delivered effectors (SDEs). Many SDEs encoding genes are upregulated in citrus in comparison to psyllids as host (Thapa et al., 2020). Notably, effectors like *SDE1* have been shown to inhibit citrus papain-like cysteine proteases, promote early senescence, and enhance bacterial colonization by accelerating nutrient remobilization from senescing tissues, ultimately contributing to symptom development (Clark et al., 2020). These findings highlight that CLas has retained key mechanisms that enable it to rely on host-derived nutrients and effectively suppress host defenses, ensuring its successful colonization and systemic spread.

In conclusion, although significant progress has been made in understanding the biology, epidemiology, and control strategies of HLB, many fundamental questions remain unresolved. The complex interaction between CLas and its citrus hosts, especially the early molecular events occurring within the phloem during initial infection, is still poorly understood. Current research efforts, though valuable, are limited by reliance on whole-leaf samples and field conditions that obscure tissue-specific and early-stage responses. A deeper and more precise understanding of the molecular mechanisms underlying HLB pathogenesis, host immune responses, and tolerance traits is urgently needed. Addressing these gaps will not only enable the development of more effective and sustainable control strategies but also provide critical insights for engineering citrus varieties with durable resistance. Future studies focusing on early, phloem-specific transcriptomic profiling and functional validation of candidate genes or drugs will be crucial steps toward achieving long-term solutions to the HLB crisis.

REFERENCES

- Abd-Elgawad, M., El-Mougy, N., El-Gamal, N., Abdel-Kader, M., & Mohamed, M.** (2010). Protective treatments against soil-borne pathogens in citrus orchards. *J Plant Prot Res*, 50, 477–484.
- Alvarez, S, E Rohrig, D Solís, & M H Thomas** (2016). Citrus greening disease (huanglongbing) in florida: Economic impact, management and the potential for biological control. *Agr. Res.*, 5, 109–118.
- Barnett, M. J., Solow-Cordero, D. E., & Long, S. R.** (2019). A high-throughput system to identify inhibitors of *Candidatus Liberibacter asiaticus* transcription regulators. *Proceedings of the National Academy of Sciences*, 116(36), 18009–18014.
- Blaustein, R, A., Lorca, G, L., Meyer, J, L., Gonzalez, C, F., Teplitski, & M** (2017). Defining the core citrus leaf and root associated microbiota: Factors associated with community structure and implications for managing HLB (citrus greening) disease. *Appl. Environ. Microbiol.*, 83, e00210–17. doi:10.1128/AEM.00210–17
- Boina, D. R., & Bloomquist, J. R.** (2015). Chemical control of the Asian citrus psyllid and of huanglongbing disease in citrus. *Pest Management Science*, 71(6), 808–823.
- Bové, J, M., Calavan, E, C., Capoor, S, P., Cortez, R, E., Schwarz, & R, E.** (1974). Influence of temperature on symptoms of california stubborn, south african greening, indian citrus decline and philippines leaf mottling diseases. *Proc. 6th Conf. IOCV*, 12–15, Univ. Calif., Div. Agr. Sci., Riverside. CA.
- Burckhardt, D, Martinez, & M** (1989). Note sur la presence au honduras d'un redoutable ennemi des citrus: *Diaphorina citri* kuwayama (hom. Psylloidea Psyllidae). *Bulletin de la Société Entomologique de France*, 94, 65–66.
- Catling, H. D.** (1969). The bionomics of the South African citrus psylla, *Trioza erythrae* (Del Guercio) (Homoptera: Psyllidae) I. The influence of the flushing rhythm of citrus and factors which regulate flushing. *Journal of the entomological Society of Southern Africa*, 32(1), 191–208.
- Cazorla, F, M., & J Mercado-Blanco** (2016). Biological control of tree and woody plant diseases. An impossible task? *BioControl*, 61, 233–242.
- Chen, X, M Triana, & P A Stansly** (2017). Optimizing production of tamarixia radiata (hymenoptera: Eulophidae), a parasitoid of the citrus greening disease vector *Diaphorina citri* (hemiptera: Psylloidea). *Biol. Control*, 105, 13–18.
- Ciccarone, & A** (1957). Il “giallume” (“yellow shoot”) degli agrumi in cina. *Revista di Agrumicoltura*, 2, 45–50.
- Clark, K., Franco, J. Y., Schwizer, S., Pang, Z., Hawara, E., Liebrand, T. W., ... & Ma, W.** (2018). An effector from the Huanglongbing-associated pathogen targets citrus proteases. *Nature communications*, 9(1), 1718.
- Clark, K. J., Pang, Z., Trinh, J., Wang, N., & Ma, W.** (2020). Sec-delivered effector 1 (*SDE1*) of ‘*Candidatus Liberibacter asiaticus*’ promotes citrus huanglongbing. *Molecular Plant-Microbe Interactions*, 33(12), 1394–1404.
- Coletta-Filho, H, D., Targon, M, L. P. N., Takita, M, A., de Negri Jr, J, D., Pompeu, J, Machado, & M, A.** (2004). First report of the causal agent of huanglongbing (“*Candidatus Liberibacter asiaticus*”) in brazil. *Plant Disease*, 88, 1382.
- Coll, N. S., Eppe, P. D. J. L., & Dangel, J. L.** (2011). Programmed cell death in the plant immune system. *Cell Death & Differentiation*, 18(8), 1247–1256.
- Court, C, D., Hodges, A, W., Rahmani, M, Spreen, & T, H.** (2017). Economic contributions of the florida citrus industry in 2015–16. FE1021. UF/IFAS Extension, Gainesville, FL.
- Curtolo, M., de Souza Pacheco, I., Boava, L. P., Takita, M. A., Granato, L. M., Galdeano, D. M., ... & Machado, M. A.** (2020). Wide-ranging transcriptomic analysis of

- Poncirus trifoliata, Citrus sunki, Citrus sinensis and contrasting hybrids reveals HLB tolerance mechanisms. Scientific Reports, 10(1), 20865.
- Duan, Y, Zhou, L, Hall, D, G., Li, W, Dodapaneni, H, Lin, H, Liu, L, Vahling, C, M., Gabriel, D, W., Williams, K, P., Dickerman, A, Sun, Y, Gottwald, & T** (2009). Complete genome sequence of citrushuanglongbing bacterium, 'Candidatus *Liberibacter asiaticus*' obtained through metagenomics. Mol. Plant-Microbe Interact., 22, 1011–1020.
- Dutt, M, G Barthe, M Irely, & J Grosser** (2015). Transgenic citrus expressing an arabidopsis NPR1 gene exhibit enhanced resistance against huanglongbing (HLB; citrus greening). PLoS One, 10, e0137134.
- Ehsani, R, Dewdney, M, Johnson, & E** (2016). Controlling HLB with thermotherapy. What have we learned so far? Citrus Industry News Sept.: 26–28.
- Etienne, J, Burckhardt, D, Grapin, & C** (1998). *Diaphorina citri* (kuwayama) en guadeloupe, premier signalement pour les caraïbes. Bulletin de la Société Entomologique de France, 103, 32.
- Etzeberria, E., Gonzalez, P., Achor, D., & Albrigo, G.** (2009). Anatomical distribution of abnormally high levels of starch in HLB-affected Valencia orange trees. Physiological and Molecular Plant Pathology, 74(1), 76–83.
- FAN G, LIU B, WU R, et al.** Thirty years of research on citrus Huanglongbing in China[J]. Fujian Journal of Agricultural Sciences, 2009, 24 (2): 183–190.
- Fan, J., Chen, C., Yu, Q., Khalaf, A., Achor, D. S., Brlansky, R. H., ... & Gmitter Jr, F. G.** (2012). Comparative transcriptional and anatomical analyses of tolerant rough lemon and susceptible sweet orange in response to 'Candidatus *Liberibacter asiaticus*' infection. Molecular plant-microbe interactions, 25(11), 1396–1407.
- Fujiwara, K, Iwanami, T, Fujikawa, & T** (2018). Alterations of Candidatus *Liberibacter asiaticus*-associated microbiota decrease survival of ca. *L. asiaticus* in invitro assays. Front.Microbiol.,9,3089.doi:10.3389/fmicb.2018.03089
- Garnier, M, Jagoueix, S, Toorawa, P, Grisoni, M, Mallessard, R, Dookun, A, Saumtally, S, Autrey, J, C., Bové, & J, M.** (1996). Both huanglongbing (greening) liberobacter species are present in mauritius and reunion. In: Proc. 13th Conf. IOCV, 392–94, IOCV, Univ. Calif., Riverside, CA.
- Gmitter Jr, F., Grosser, J., Castle, W., & Moore, G.** (2007). A comprehensive citrus genetic improvement program. Citrus genetics, breeding and biotechnology. CAB International, Oxfordshire, pp 9–18.
- Gottwald, T, R., J V da Gracxa, & R B Bassanezi** (2007). Citrus huanglongbing: The pathogen and its impact. Plant Health Prog., 6, 1–18.
- Gottwald, T, R., J H Graham, M S Irely, T G McCollum, & B W Wood** (2012). Inconsequential effect of nutritional treatments on huanglongbing control, fruit quality, bacterial titer and disease progress. Crop Prot., 36, 73–82.
- Graham, J., Gottwald, T., & Setamou, M.** (2020). Status of huanglongbing (HLB) outbreaks in Florida, California and Texas. Tropical Plant Pathology, 45(3), 265–278.
- Hao, G, E Stover, & G Gupta** (2016). Overexpression of a modified plant thionin enhances disease resistance to citrus canker and huanglongbing (HLB). Front. Plant Sci., 7, 1078.
- Hartung, J. S., Paul, C., Achor, D., & Brlansky, R. H.** (2010). Colonization of dodder, *Cuscuta indecora*, by 'Candidatus *Liberibacter asiaticus*' and 'Ca. *L. americanus*'. Phytopathology, 100(8), 756–762.
- Hartung, J. S., Shao, J., & Kuykendall, L. D.** (2011). Comparison of the 'Ca. *Liberibacter asiaticus*' genome adapted for an intracellular lifestyle with other members of the Rhizobiales. PLoS one, 6(8), e23289.
- He, Z., Webster, S., & He, S. Y.** (2022). Growth-defense trade-offs in plants. Current Biology, 32(12), R634–R639.

- Hoffman, M. T., M S Doud, L Williams, M -Q Zhang, F Ding, E Stover, D Hall, S Zhang, L Jones, & M Gooch** (2013). Heat treatment eliminates 'Candidatus *Liberibacter asiaticus*' from infected citrus trees under controlled conditions. *Phytopathology*, 103, 15–22.
- Hoffmann, C., Fritz, B., Martin, D., Atwood, R., Hurner, T., Ledebuhr, M., Tandy, M., Jackson JL, & Wisler, G.** (2010). Evaluation of lowvolume sprayers used in asian citrus psyllid control applications. *Hort-Technology*, 20, 632–638.
- Hu, Y., Zhong, X., Liu, X., Lou, B., Zhou, C., & Wang, X.** (2017). Comparative transcriptome analysis unveils the tolerance mechanisms of *Citrus hystrix* in response to 'Candidatus *Liberibacter asiaticus*' infection. *PLoS One*, 12(12), e0189229.
- Huang, C. Y., Araujo, K., Sánchez, J. N., Kund, G., Trumble, J., Roper, C., ... & Jin, H.** (2021). A stable antimicrobial peptide with dual functions of treating and preventing citrus Huanglongbing. *Proceedings of the National Academy of Sciences*, 118(6), e2019628118.
- Ichinose, K., Miyazi, K., Matsuhira, K., Yasuda, K., Sadoyama, Y., Tuan DH, & van Bang, D.** (2010). Unreliable pesticide control of the vector psyllid *Diaphorina citri* (hemiptera: Psyllidae) for the reduction of microorganism disease transmission. *J Environ Sci Health B*, 45, 466–472.
- Inoue, H., Ohnishi, J., Ito, T., Tomimura, K., Miyata, S., Iwanami, T., & Ashihara, W.** (2009). Enhanced proliferation and efficient transmission of Candidatus *Liberibacter asiaticus* by adult *Diaphorina citri* after acquisition feeding in the nymphal stage. *Ann Appl Biol*, 155, 29–36.
- Jia, H., Zhang, Y., Orbović, V, Xu, J, White, F, F., Jones, J, B., Wang, & N** (2017). Genome editing of the disease susceptibility gene *csLOB 1* in citrus confers resistance to citrus canker plant biotechnol. *J.* 15 817 823.
- Jiang, Z., Li, M., & Wang, H.** (1935). Introduction of citrus cultivation in Guangdong. *Lingnan Agric. J.* 2, 105-164.
- Johnson, E. G., Wu, J., Bright, D. B., & Graham, J. H.** (2014). Association of 'Candidatus *Liberibacter asiaticus*' root infection, but not phloem plugging with root loss on huanglongbing-affected trees prior to appearance of foliar symptoms. *Plant pathology*, 63(2), 290-298.
- Kean, S., Soyong, K., & To-anun, C.** (2010). Application of biological fungicides to control citrus root rot under field condition in cambodia. *J Agric Technol*, 6, 219–230.
- Kehr, J.** (2006). Phloem sap proteins: their identities and potential roles in the interaction between plants and phloem-feeding insects. *Journal of experimental botany*, 57(4), 767-774.
- le Roux, H., van Vuuren, S., Pretorius, M., & Buitendag, C.** (2006). Management of huanglongbing in south africa. *Proc Huanglongbing-Greening Intl Workshop 2006. Ribeirão*, pp. 43– 47.
- Ledford, H.** (2017). Engineered virus in line to battle citrus disease. *Nature*, 545, 277-278.
- Leonard, M. T., Fagen, J. R., Davis-Richardson, A. G., Davis, M. J., & Triplett, E. W.** (2012). Complete genome sequence of *Liberibacter crescens* BT-1. *Standards in genomic sciences*, 7(2), 271-283.
- Lin, & Kung Hsiang** (1956). Yellow shoot of citrus (in chinese). *Acta Phytopathologica Sinica*, 2, 1–12.
- Lin, & Kung Hsiang** (1956). Etiological studies of yellow shoot of citrus (in chinese). 42. *Acta Phytopathologica Sinica*, 2, 12–42.
- Liu, C., Chang, X., Li, F., Yan, Y., Zuo, X., Huang, G., & Li, R.** (2024). Transcriptome analysis of *Citrus sinensis* reveals potential responsive events triggered by Candidatus *Liberibacter asiaticus*. *Protoplasma*, 261(3), 499-512.
- Liu, X., Zheng, Y., Wang-Pruski, G., Gan, Y., Zhang, B., Hu, Q., ... & Liu, L.** (2019). Transcriptome profiling of periwinkle infected with Huanglongbing ('Candidatus *Liberibacter asiaticus*'). *European Journal of Plant Pathology*, 153(3), 891-906.
- Lopes, S, A., Frare, G, F., Bertolini, E, Cambra, M, Fernandes, G. N., Ayres, A, J., Marin,**

- D, R., Bové, & J, M.** (2009). *Liberibacters* associated with citrus huanglongbing in brazil: Candidatus *Liberibacter asiaticus* is heat tolerant, candidatus *Liberibacter americanus* is heat sensitive. Plant Disease.
- Lopez, J, A., Durborow, & S, L.** (2015). Huanglongbing and the california citrus industry: A cost comparison of do nothing vs. do something management practices Tex. J. Agr. Nat. Resour. 27 51 68.
- Luo, L., Zhang, L., Yu, G., & Liu, G.** (2023). Impact of Huanglongbing on Citrus Orchards: A Spatiotemporal Study in Xunwu County, Jiangxi Province. Agriculture, 14(1), 55.
- Ma, W., Pang, Z., Huang, X., Xu, J., Pandey, S. S., Li, J., ... & Wang, N.** (2022). Citrus Huanglongbing is a pathogen-triggered immune disease that can be mitigated with antioxidants and gibberellin. Nature Communications, 13(1), 529.
- Martinez, A, L., Wallace, & J, M.** (1967). Citrus leaf mottle-yellows disease in the philippines and transmission of the causal virus by a psyllid, *Diaphorina citri*. Plant Disease Reporter, 51, 692–695.
- Martinelli, F., Reagan, R. L., Uratsu, S. L., Phu, M. L., Albrecht, U., Zhao, W., ... & Dandekar, A. M.** (2013). Gene regulatory networks elucidating huanglongbing disease mechanisms. PloS one, 8(9), e74256.
- Mengarelli, D. A., Roldán Tewes, L., Balazadeh, S., & Zanol, M. I.** (2021). FITNESS acts as a negative regulator of immunity and influences the plant reproductive output after *Pseudomonas syringae* infection. Frontiers in Plant Science, 12, 606791.
- McClellan, A, P. D., Oberholzer, & P. C. J.** (1965). Greening disease of sweet orange: Evidence that it is caused by a transmissible virus. South Africa Journal of Agricultural Science, 8, 253–276.
- McClellan, A, P. D., Oberholzer, & P. C. J.** (1965). Citrus psylla, a vector of the greening disease of sweet orange. South Africa Journal of Agricultural Science, 8, 297–298.
- McManus PS, & Stockwell VO** (2001). Antibiotic use for plant disease management in the united states. Peach, 2, 2, 900.
- Morgan, K, T., R E Rouse, & R C Ebel** (2016). Foliar applications of essential nutrients on growth and yield of ‘valencia’ sweet orange infected with huanglongbing. HortScience, 51, 1482–1493.
- Muraro, & R, P.** (2010). Costs of managing HLB and citrus black spot. Presented at Citrus Expo, Ft. Meyers, FL. 19 May 2010.
- Nemec, S., Datnoff, L., & Strandberg, J.** (1996). Efficacy of biocontrol agents in planting mixes to colonize plant roots and control root diseases of vegetables and citrus. Crop Prot, 15, 735–742.
- Ozores-Hampton, M, F M Roka, R E Rouse, & P Roberts** (2017). Dual treatment tested for HLB trees. Citrus Industry News. May: 28–31.
- Pagliaccia, D, Shi, J, Pang, Z, Hawara, E, Clark, K, Thapa, S, P., DeFrancesco, A, D., Liu, J, Tran, T -T, Bodaghi, S, Folimonova, S, Y., Ancona, V, Mulchandani, A, Coaker, G, Wang, N, Vidalakis, G, AndMa, & W** (2017). A pathogen secreted protein as a detection marker forcitrus huanglongbing. Front. Microbiol., 8, 2041.
- Postali Parra, J. R., Alves, G. R., Ferreira Diniz, A. J., & Vieira, J. M.** (2016). *Tamarixia radiata* (Hymenoptera: Eulophidae) x *Diaphorina citri* (Hemiptera: Liviidae): mass rearing and potential use of the parasitoid in Brazil. Journal of Integrated Pest Management, 7(1).
- Pitino, M., Allen, V., & Duan, Y.** (2018). LasΔ5315 effector induces extreme starch accumulation and chlorosis as Ca. *Liberibacter asiaticus* infection in Nicotiana benthamiana. Frontiers in Plant Science, 9, 113.
- Powell, C. A., Burton, M. S., Pelosi, R. A., Ritenour, M. A., & Bullock, R. C.** (2007). Effects of insecticides on Asian citrus psyllid (Hemiptera: Psyllidae) populations in a Florida citrus grove. Plant Health Progress, 8(1), 6.

- Qureshi, J. A., & P A Stansly** (2011). Three homopteran pests of citrus as prey for the convergent lady beetle: Suitability and preference. *Environ. Entomol.*, 40, 1503–1510.
- Raiol-Junior, L. L., Cifuentes-Arenas, J. C., de Carvalho, E. V., Girardi, E. A., & Lopes, S. A.** (2021). Evidence that ‘Candidatus *Liberibacter asiaticus*’ moves predominantly toward new tissue growth in citrus plants. *Plant Disease*, 105(1), 34–42.
- Reinking, & O, A.** (1919). Diseases of economic plants in southern china. *Philippines Agric.*, 8, 109–135.
- Ribeiro, C., Xu, J., Hendrich, C., Pandey, S. S., Yu, Q., Gmitter Jr, F. G., & Wang, N.** (2023). Seasonal transcriptome profiling of susceptible and tolerant citrus cultivars to citrus Huanglongbing. *Phytopathology*, 113(2), 286–298.
- Robledo, J., Welker, S., Shtein, I., Bernardini, C., Vincent, C., & Levy, A.** (2023). Phloem and xylem responses are both implicated in HLB tolerance of Sugar Belle. *Phytopathology*.
- Roistacher, C.** (1996). The economics of living with citrus diseases: Huanglongbing (greening) in thailand. *Proc 13th Conference of the International Organization of Citrus Virologists (IOCV) University of California, Riverside*, pp 279–285.
- Roka, F. R., Muraro, A. M., & Morris, A.** (2010, October). Economics of HLB management: Pull trees or spray nutritionals. In *International Citrus Economics Conference*, Orlando, FL.
- Rouse, R. E., M Ozores-Hampton, F M Roka, & P Roberts** (2017). Rehabilitation of huanglongbing affected citrus trees using severe pruning and enhanced foliar nutritional treatments. *Hort Science*, 52, 972–978.
- Stansly, P., & Roka, F.** (2013). Economics of HLB vector control and foliar nutrition. *Citrus Industry*. Jun, 1–2.
- Stansly, P. A., H A Arevalo, J A Qureshi, M M Jones, K Hendricks, P D Roberts, & F M Roka** (2014). Vector control and foliar nutrition to maintain economic sustainability of bearing citrus in florida groves affected by huan glongbing. *Pest Manag. Sci.*, 70, 415–426.
- Stelinski LL RM, Yates, Y., Dewdney, M., & Spann, T.** (2009). Low volume applications: An emerging technology in the citrus industry. 2009. Accessed 15–02–2017.
- Su, H., & Chang, S.** (1974). Electron microscopical study on the heat and tetracycline response, and ultra-structure of the pathogen complex causing citrus likubin disease. *Proc 8th Int Congr Electron Microscopy*, 2, 628–629.
- Tansey, J. A., P Vanaclocha, C Monzo, M Jones, & P A Stansly** (2017). Costs and benefits of insecticide and foliar nutrient applications to huanglongbing-infected citrus trees. *Pest Manag. Sci.*, 73, 904–916.
- Teixeira, D. C., Danet, J. L., Eveillard, S, Martins, E. C., de Jesus Junior, W. C., Yamamoto, P. T., Lopes, S. A., Bassanezi, R. B., Ayres, A. J., Saillard, C, Bové, & J. M.** (2005). Citrus huanglongbing in são paulo state, brazil: PCR detection of the “Candidatus” *Liberibacter* species associated with the disease. *Molecular and Cellular Probes*, 19; 173–179.
- Teixeira, D. C., Lopes, S. A., Yamamoto, P. T., Eveillard, S, Martins, E. C., de Jesus Junior, W. C., Bassanezi, R. B., Ayres, A. J., Danet, J. L., Saillard, C, Bové, & J. M.** (2005). PCR detection of the two *Liberibacter* species associated with citrus huanglongbing (HLB) in são paulo state, brazil, *proc. 16th conf. IOCV*, 432–438, IOCV, Riverside, CA.
- Teixeira, D. C., Saillard, C, Eveillard, S, Danet, J. L., Ayres, A. J., Bové, & J. M.** (2005). Candidatus *Liberibacter americanus* sp. nov., associated with citrus huanglongbing (greening disease) in São Paulo State, Brazil. *Intern. J. Syst. Evol. Microbiol.*, 55, 1857–1862.
- Teixeira, D. C., Saillard, C, Eveillard, S., Danet, L. J., Ayres, A. J., Bové, & J. M.** (2005). A new *Liberibacter* species, Candidatus *Liberibacter americanus* sp. nov., is associated with citrus huanglongbing (greening

- disease) in São Paulo state, Brazil. Proc. 16th conf. IOCV, 325–340, IOCV, Riverside, CA.
- Teixeira, D. C., Ayres, A. J., Kitajima, E. W., Tanaka, F. A. O., Danet, J. L., Jagoueix-Eveillard, S., Saillard, C., Bové, & J. M. (2005).** First report of a huanglongbing-like disease of citrus in sao paulo state, brazil, and association of a new *Liberibacter* species, “candidatus *Liberibacter americanus*”, with the disease. *Plant Disease*, 89, 107.
- Thapa, S. P., De Francesco, A., Trinh, J., Gurung, F. B., Pang, Z., Vidalakis, G., ... & Coaker, G. (2020).** Genome-wide analyses of *Liberibacter* species provides insights into evolution, phylogenetic relationships, and virulence factors. *Molecular plant pathology*, 21(5), 716–731.
- Tiwari, S., & Stelinski LL (2013).** Effects of cyantraniliprole, a novel anthranilic diamide insecticide, against asian citrus psyllid under laboratory and field conditions. *Pest Manag Sci*, 69, 1066–1072.
- U S Department Of Agriculture, Economic Research Service (USDA, & ERS (2018).**
- Vaccaro, & N, C. (1994).** Comportamiento y diffusion de la “chicharrita de los citrus” *Diaphorina citri* en la zona citrica de entre rios. *Información Citricota. CITRUS vol. 2 – INTA - E. E. A.*
- van der Merwe, A. J., Andersen, & F. G. (1937).** Chromium and manganese toxicity. Is it important in Transvaal citrus greening? *Farming South Africa*, 12, 439–440.
- Wang, J., & Eulgem, T. (2024).** Growth deficiency and enhanced basal immunity in *Arabidopsis thaliana* mutants of EDM2, EDM3 and IBM2 are genetically interlinked. *Plos one*, 19(2), e0291705.
- Wang, N, E A Pierson, J C Setubal, J Xu, J G Levy, Y Zhang, J Li, L T Rangel, J Martins, & Jr (2017).** The Candidatus *Liberibacter*–host interface: Insights into pathogenesis mechanisms and disease control. *Annu. Rev. Phytopathol.*, 55, 451–482.
- Will, T., & van Bel, A. J. (2006).** Physical and chemical interactions between aphids and plants. *Journal of experimental botany*, 57(4), 729–737.
- Zhang, M., Duan, Y., Zhou, L., Turechek WW, Stover, E., & Powell CA (2010).** Screening molecules for control of citrus huanglongbing using an optimized regeneration system for ‘candidatus *Liberibacter asiaticus*’-infected periwinkle (*Catharanthus roseus*) cuttings. *Phytopathology*, 100, 239–245.
- Zhang, M., Powell CA, Zhou, L., He, Z., Stover, E., & Duan, Y. (2011).** Chemical compounds effective against the citrus huanglongbing bacterium ‘Candidatus *Liberibacter asiaticus*’ in planta. *Phytopathology*, 101, 1097–1103.
- Zhang, M., Powell CA, Guo, Y., Doud MS, & Duan, Y. (2012).** A graftbased chemotherapy method for screening effective molecules and rescuing huanglongbing-affected citrus plants. *Phytopathology*, 102, 567–574.
- Zhang, M., Guo, Y., Powell CA, Doud MS, Yang, C., & Duan, Y. (2014).** Effective antibiotics against ‘Candidatus *Liberibacter asiaticus*’ in HLB-affected citrus plants identified via the graft-based evaluation. *PLoS One*, 9, e111032.
- Zhang, Y, J Xu, N Riera, T Jin, J Li, & N Wang (2017).** Huanglongbing impairs the rhizosphere-torhizoplane enrichment process of the citrus rootassociated microbiome. *Microbiome*, 5, 97.
- Zhang, J., Liu, Y., Gao, J., Yuan, C., Zhan, X., Cui, X., ... & Xu, M. (2023).** Current epidemic situation and control status of citrus Huanglongbing in Guangdong China: The space–time pattern analysis of specific orchards. *Life*, 13(3), 749.

MiG-based transmission of *Ca. Liberibacter asiaticus* enables studies of host-pathogen interaction at early infection stages of HLB

Feng Zhang,^{1,2,3,†} Jing Wang,^{4,†} Nan Cai,^{1,2,3,†} Lei Gong,^{1,2,3} Yunlin Cao,^{1,2,3} Zhengmin Yang,^{1,2,3} Songliang Jiang,^{1,2,3} Yi Li,^{1,2,3} Jianming Luo,^{1,2,3} Jian Han,⁵ Ziniu Deng,^{3,6} Shunyuan Xiao,⁷ Alessandra Gentile,^{3,8,*} Ton Bisseling,^{4,9,*} and Xianfeng Ma^{1,2,3,*}

¹Engineering Research Centre of Education Ministry for Germplasm Innovation and Breeding New Varieties of Horticultural Crops, College of Horticulture, Hunan Agricultural University, 410128, Changsha, China.

²Yuelushan Laboratory, Changsha 410128, China.

³National Center for Citrus Improvement, College of Horticulture, Hunan Agricultural University, Changsha 410128, China.

⁴Laboratory of Molecular Biology, Department of Plant Sciences, Graduate School Experimental Plant Sciences, Wageningen University & Research, Wageningen 6708 PB, the Netherlands.

⁵Hunan Horticultural Research Institute, Hunan Academy of Agricultural Sciences, Changsha 410125, China.

⁶NanLing Institute of Citrus Industry, Chenzhou 423000, China

⁷Institute for Bioscience and Biotechnology Research & Department of Plant Sciences and Landscape Architecture, University of Maryland College Park, Rockville MD 20850, USA.

⁸Department of Agriculture, Food and Environment, University of Catania, Via S. Sofia 100, Italy

⁹College of Grassland, Resources and Environment, Inner Mongolia Agricultural University, Hohhot 010011, China

†These authors contributed equally

*Corresponding authors: Xianfeng Ma (ma8006@hunau.edu.cn), Ton Bisseling (ton.bisseling@wur.nl), Alessandra Gentile (alessandra.gentile@unict.it)

Abstract

Huanglongbing (HLB), caused by the phloem-limited bacterium *Candidatus Liberibacter asiaticus* (CLAs), represents a formidable challenge to global citrus cultivation. Traditional inoculation methods are labor-intensive and time-consuming, limiting the study of early infection dynamics and the comprehensive exploration of pathogen-host interactions. In this study, we established a rapid modified micrografting (MiG) system using 10-day-old *Citrus maxima* seedlings grafted with buds from HLB-symptomatic mature *C. sinensis*. This method achieved a high graft survival rate and significantly accelerated the invasion of CLAs, with early detection in lateral roots at 15 days post-inoculation (dpi) and systemic infection in roots and leaves by 40 dpi. In contrast, conventional mature-grafting (MaG) showed no symptoms at 90 dpi, with only weak CLAs titer detected in roots and undetectable levels in leaves. The MiG system enabled precise sampling of infected phloem tissues at early infection stages. Laser-capture microdissection (LCM) combined with transcriptomics identified 72 previously unreported phloem-specific early response genes during CLAs infection. These genes provide new insights into early host-pathogen interactions and phloem-specific defense mechanisms. Collectively, the MiG inoculation system provides a fast, synchronized, and efficient platform for unraveling early-stage pathogenesis of HLB and other vascular-limited diseases, facilitating the development of targeted control strategies.

INTRODUCTION

Huanglongbing (HLB), commonly known as citrus greening disease, is one of the most devastating threats to global citrus production, causing leaf mottling, fruit deformation, root decline, and eventually tree death (Bové et al., 2003; Wang and Trivedi, 2013). The disease is caused by the bacterium of the genus *Candidatus Liberibacter*, among which three main species are recognized, *Candidatus Liberibacter asiaticus* (CLas), *C. Liberibacter africanus* (CLaf), and *C. Liberibacter americanus* (CLam) (Bové and Barros, 2006). CLas is a phloem-limited, intracellular bacterium that is primarily transmitted by the Asian citrus psyllid (*Diaphorina citri*) (Shengeliya, 1988; Graham et al., 2024). Virtually all commercial citrus cultivars are susceptible to HLB, and no cure or resistant variety is available (Hu et al., 2021). Therefore, it is imperative to investigate the molecular mechanisms underlying HLB pathogenesis to support the development of effective control strategies. However, progress has been hindered by several technical challenges. Firstly, CLas cannot be robustly cultured outside its hosts. Although CLas bacteria have been maintained in a dynamic growth state for over 20 months, they exhibit limited growth in subcultures (Zheng et al., 2024). This constraint severely restricts the development of controlled experimental systems (Graham et al., 2024). Secondly, most reported HLB-related studies utilized field-collected symptomatic plant materials — especially leaves, twigs, or fruits — for experimental analyses (Clark et al., 2018). However, such samples are frequently confounded by environmental variability and/or co-infection with multiple pathogens, especially viruses and viroids, thereby complicating experimental outcomes (Fu et al., 2017), and rendering them unsuitable for studying early-stage host-pathogen interactions.

In addition, commonly used inoculation methods, including psyllid-mediated transmission and conventional grafting (Zhao et al., 2025), present significant limitations. Psyllid transmission often yields inconsistent infection, with positive rates ranging from 10% and 30% (Gasparoto et al., 2022), while the grafting of CLas-infected budwood onto 1-year-old citrus plants or mature plants (hereafter referred to as mature-grafting, MaG) requires at least four months for detectable CLas presence in leaves, with severe HLB symptoms typically manifesting after one year (Huang et al., 2021; Cui et al., 2022; Gasparoto et al., 2022). Such protracted timelines significantly hinder interrogation of early colonization events and preclude the spatial-temporal resolution needed to dissect the initial molecular interactions between the host and the pathogen (Jiang et al., 2019). Therefore, establishment of a novel CLas-citrus interaction system, particularly one that allows access to early infection stages, is vital to address these questions, as current HLB investigation methods largely fail to provide this access.

Micrografting (MiG) generally refers to grafting a young small shoot tip onto an appropriate rootstock either in vitro or in vivo (Shengeliya, 1988; Sanjana et al., 2024). In vitro MiG has been widely employed to obtain virus-free mother trees of fruit cultivars for propagation

(Jonard et al., 1983; Wang et al., 2022). In this study, we developed a modified MiG approach, grafting lignified scions from mature citrus plants onto naive 10-day-old citrus seedlings. This configuration inverts the traditional source-sink relationship, with the lignified scion acting as the primary carbon source, thereby directing phloem flow toward the juvenile seedling (Tournier et al., 2006; Comtet et al., 2017). We hypothesize that this directed phloem transport may enhance the translocation of CLAs into the recipient seedling, reducing the latency period and accelerating systemic infection compared to MaG methods, in which infected budwoods were grafted onto mature citrus trees. Furthermore, MiG may enable early detection of CLAs infections, providing a tractable system to study initial host-pathogen interactions.

To test this hypothesis, we established a MiG-based inoculation system to investigate CLAs transmission dynamics. We combined laser-capture microdissection (LCM) with RNA sequencing to analyze phloem tissues from infected seedlings and uninfected controls, aiming to capture spatiotemporally precise transcriptional changes at the onset of infection. This MiG-LCM approach seeks to uncover early regulatory targets associated with HLB resistance, offering a scalable framework for studying CLAs-citrus interactions and other phloem-limited plant pathogens.

RESULTS

1. Comparison of micro- and mature-grafting efficiency

To develop an efficient inoculation system for CLAs, we compared MiG and MaG. In MiG, lignified scions (~2 cm) bear axillary buds from 1-year-old *Citrus sinensis* (L.) Osbeck was grafted onto 10-day-old *C. maxima* (Burm.) Merr. seedlings. In MaG, similar scions were grafted onto 1-year-old *C. maxima* plants (Fig. 1A). Both methods achieved successful graft unions, with callus formation observed at vascular junctions (Fig. 1, B, D, and E). Graft survival rate exceeded 85% at 90 days after grafting (DAG) for both MiG and MaG (Fig. 1C), indicating robust vascular connections essential for CLAs translocation from scion to recipient plant.

2. MiG accelerates CLAs infection compared to MaG

As a next step, we compared MiG and MaG for their efficiency/speed in the development of HLB symptoms upon inoculation with a CLAs-infected scion. 2-cm-long lignified scions with visible axillary buds and attached leaves were excised from HLB-symptomatic *C. sinensis*, and these were grafted using MiG and MaG, respectively (Fig. 2, A-C). The inclusion of leaves on the scions was designed to enhance phloem-mediated CLAs translocation, as leaves serve as active source tissues for photosynthate transport. At 90 dpi, MiG-inoculated plants displayed characteristic HLB symptoms, including leaf mottling and yellowing (Fig. 2C), whereas MaG-inoculated plants remained asymptomatic (Fig. 2D).

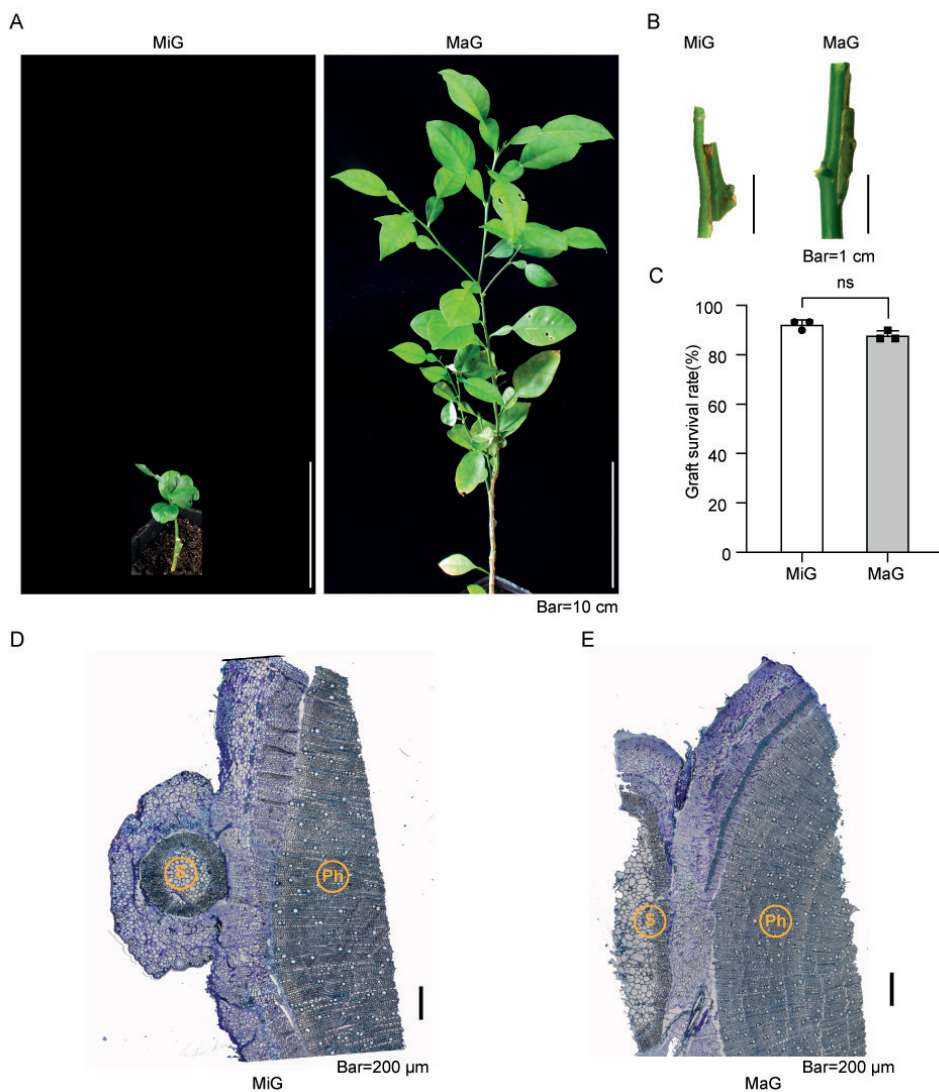


Figure 1. MiG achieves successful graft union formation and high survival rates in citrus.

A: Photographs of MiG of 10-day-old *C. maxima* seedlings and MaG of 1-year-old *C. maxima* seedlings grafted with 1-year-old *C. sinensis* scions.

B: Representative graft junctions in MiG and MaG at 10 DAG.

C: Survival rates of MiG and MaG plants at 90 DAG. Error bars denote $\pm$ SE ($n=3$); ns, not significant (Duncan's, $P<0.05$).

D: Semithin toluidine-blue-stained sections through the graft union at 10 DAG in MiG. Ph: Phloem; S: Scion.

E: Semithin toluidine-blue-stained sections through the graft union at 10 DAG in MaG.

S, scion; S: Scion. MiG, micrografting; MaG, mature-grafting.

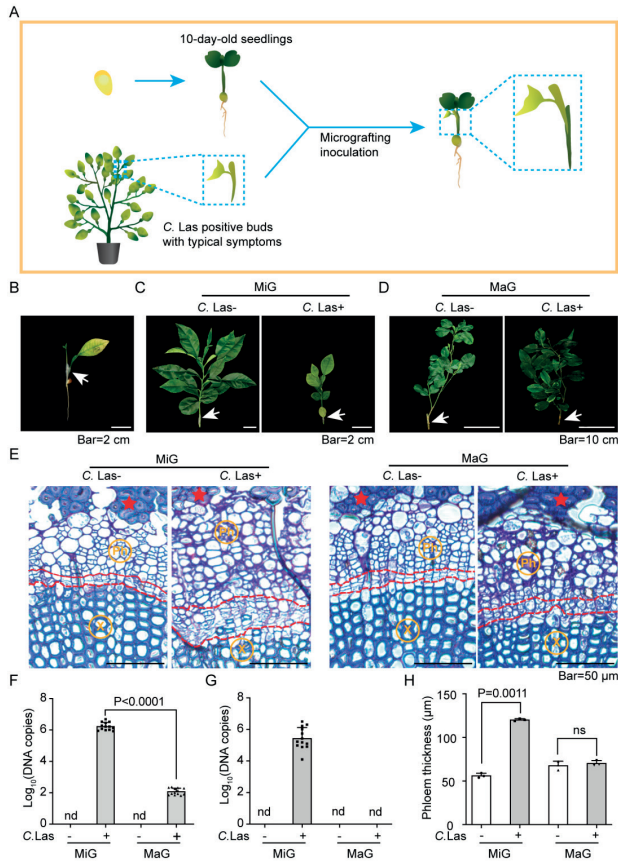


Figure 2. Comparison of *CLas* infection in *C. maxima* at 90 dpi via MiG and MaG.

A: Schematic of the MiG inoculation process. Leaf-bearing scions exhibiting typical HLB symptoms were harvested from mature *C. sinensis* branches and grafted onto 10-day-old *C. maxima* seedlings to establish *CLas* infection.

B: Representative image of *C. maxima* seedling inoculated with *CLas* via MiG. White arrow indicates the micrografting junction.

C: Typical HLB symptoms (leaf mottling and yellowing) in MiG-inoculated plants at 90 dpi.

D: Asymptomatic leaves of MaG-inoculated plants at 90 dpi. Scion leaves frequently fall off after a period of infection in MaG.

E: Light micrographs of leaf midrib transverse sections showing anatomical responses to *CLas* infection in MiG- and MaG-inoculated plants and uninfected controls (*CLas*-) at 90 dpi. Phloem (Ph) and xylem (X) are indicated, respectively; red dashed lines outline the cambium zone; asterisks (★) mark parenchyma cells.

F: *CLas* titers in roots of inoculated plants at 90 dpi, showing ~10⁵-fold higher titers in MiG compared to MaG (n = 10; Duncan's test, P < 0.05).

G: *CLas* titers in leaves of inoculated plants at 90 dpi, determined by qPCR, with detectable titers in MiG but not MaG (n = 10; Duncan's test, P < 0.05).

H: Quantification of phloem thickness in the leaf midrib across treatments. Data are presented as mean ± SE (Duncan's, P < 0.05, n = 3).

MiG, micrografting; MaG, mature-grafting; C. Las-, uninfected control; C. Las+, *CLas*-infected.

Semithin cross-sections through the midrib of leaves at 90 dpi revealed that uninoculated controls (both MiG and MaG) had a vascular cambium of 2-3 cell layers (Fig. 2E). In MaG-inoculated plants, the cambium expanded moderately to 4-5 layers. However, in MiG-inoculated plants infected with CLAs exhibited a pronounced expansion to 8-9 cell layers, with increased phloem tissue (Fig. 2E, H).

Quantitative PCR (qPCR) analysis revealed stark differences in CLAs titers between MiG and MaG. In MiG-inoculated plants, CLAs was readily detected in both leaves and roots, with titers reaching $\sim 10^6$ copies/ μg DNA in roots and 10^6 - 10^7 copies/ μg DNA in leaves, correlating with severe HLB symptoms (Fig. 2E, F, and G). In contrast, MaG-inoculated plants showed negligible CLAs titers in roots (<10 copies/ μg DNA, $\sim 10^5$ -fold lower than MiG) and undetectable levels in leaves, with no HLB symptoms observed (Fig. 2E, F, and G).

So, MiG resulted in a faster infection with CLAs than MaG, making it the more efficient method for HLB research.

3. Temporal dynamics of CLAs infection in MiG citrus plants

To investigate the progression of CLAs infection and its organ-specific colonization over time, *C. maxima* seedlings were inoculated by MiG with CLAs-infected *C. sinensis* scions. Leaf and roots samples were collected from grafted plants at 15, 20, 30, 40, 60, and 90 dpi from ten individual plants per time point. Bacterial titers were quantified via qPCR in the uppermost leaf (UL, defined as the first fully expanded leaf at 15-30 dpi or the most recently fully expanded leaf from 40 dpi onward), the oldest leaf (OL, the lowest leaf), and 1-cm apical segments of lateral roots (LR) and primary roots (PR) (Fig. 3A). Infection efficiency was defined as the percentage of replicates ($n = 10$) with detectable CLAs by qPCR (Fig. 3B).

At 15 dpi, CLAs was detected in 20% of the LR samples, with a low bacterial titer of approximately 10^2 copies/ μg DNA, while it remained undetectable in PR and all leaves. This showed that the lateral roots are the primary accumulation site of CLAs. By 20 dpi, both LR and PR showed significantly increased titers ($\sim 10^4$ - 10^5 copies/ μg DNA), with detection rates of 80% and 70%, respectively. In the aboveground organs, CLAs was detected in 20% of the OL and UL, with titers still below 10^3 copies/ μg DNA. At 30 dpi, root infection became more established, with both LR and PR maintaining high detection rates (80-70%) and titers rising to 10^5 - 10^6 copies/ μg DNA. In contrast, UL and OL showed slower increases in infection, with significantly lower titers compared to roots. At 40 dpi, CLAs was detectable in almost all replicates across all sample types. LR and PR achieved titers stabilized around 10^6 copies/ μg DNA. Despite its recent emergence (being <10 days old at this point), UL already exhibited a 90% infection efficiency with titers comparable to roots ($\sim 10^6$ copies/ μg DNA), indicating a rapid colonization. OL maintained the lowest titers among all tissues and organs during early to mid-infection stages. From 60 dpi onwards, CLAs infection saturated in all organs, reaching 100% detection and titers approaching $\sim 10^7$ copies/ μg DNA at 90 dpi (Fig. 3, B and C).

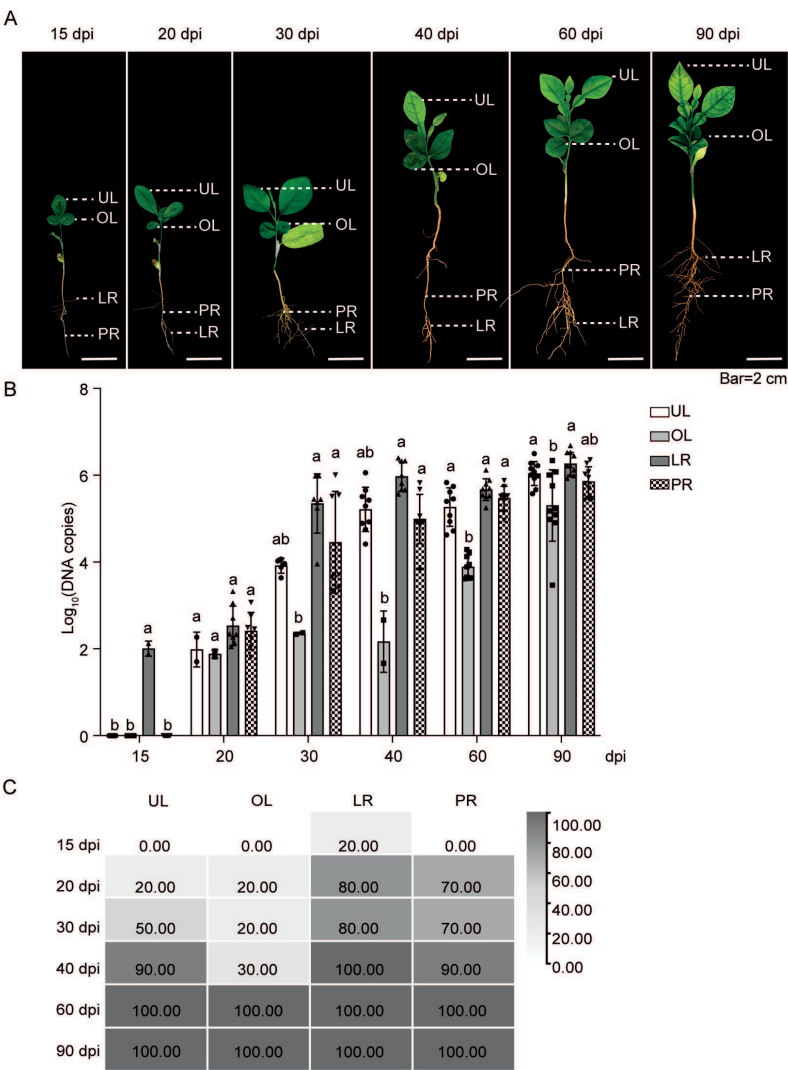


Figure 3. Temporal dynamics of *CLas* infection in MiG *C. maxima* seedlings.

A: Progression of symptoms and tissue-sampling points in 10-day-old *C. maxima* seedlings MiG with HLB-symptomatic *C. sinensis* scions. Different parts of the seedlings were labeled: uppermost leaf (UL), oldest leaf (OL), primary root (PR), and lateral roots (LR). Images represent representative seedlings at 15, 20, 30, 40, 60, and 90 dpi.

B: Bacterial titers of *CLas* measured by qPCR in leaves and roots across time points in four tissues. Titer values represent mean $\pm$ SE of the average values of the *CLas* positive samples. Values represent log₁₀-transformed copy numbers of *CLas* 16S rDNA. Different letters indicate statistically significant differences among tissues or organs at the same time point ($P < 0.05$, one-way ANOVA followed by Duncan's multiple comparison test, $n = 10$).

C: Infection rates (%) of *CLas* in different tissues across sampling time points. Values represent the proportion of *CLas*-positive samples among destructively harvested plants ($n=10$). Grayscale indicates infection frequency.

4. Identification of novel CLas-induced citrus genes

MiG-assisted CLas transmission provides, for the first time, the possibility to identify genes involved in early stages of the infection. Previous studies have identified gene expression at later stages of infection, typically after 2 months (Gao et al., 2023), 6 months (Curtolo et al., 2020), or upon the appearance of clear disease symptoms (Ribeiro et al., 2023; Liu et al., 2024). These studies primarily relied on symptom-based sampling or time points several months post-inoculation. Therefore, we assessed whether MiG could be used to identify more biologically relevant genes induced by CLas infection that have not been reported before. We analysed the leaf transcriptional changes at a stage in which disease symptoms had just appeared. These transcriptional changes were analyzed and compared with the leaf transcriptome obtained from trees with clear HLB symptoms in an orchard.

To ascertain whether the leaves from the orchard are at a later stage of infection than the micrografted material, we performed a cytological analysis of the phloem tissue at 40 dpi of MiG when HLB symptoms just began to appear in leaves of the CLas-inoculated tree. Semithin cross sections through the veins showed that the cambium of MiG-transmitted, CLas-positive leaves consists of 3-5 layers (Fig. 4, B and F), whereas in the uninoculated control consists of 2-3 cell layers (Fig. 4, A and D). In the leaves from the orchard, extensive lysis of phloem cells had occurred, resulting in the formation of lacunae (Fig. 4, C and F). This showed that in the leaves from the orchard, HLB had advanced to a markedly later stage than the 40 dpi MiG sample.

As CLas primarily infects phloem tissue, we focused on the transcriptome changes induced in this tissue. LCM was employed to isolate phloem tissue (Fig. 4G). qPCR showed that the CLas 16S rRNA levels in phloem samples were about 70 times higher than in whole leaves, and CLas enrichment in the phloem tissue was similar in leaves of the 40 dpi MiG and orchard samples (Fig. 4H). As expected, CLas was barely detectable in the combined pith and xylem tissues in both 40 dpi and orchard samples (Fig. 4H).

Differentially expressed genes (DEGs) were identified by comparing the transcriptome of the 40 dpi and orchard sample with that of uninoculated samples. We first identified 300 DEGs with the highest fold change ($P < 0.05$) from the comparison between the 40 dpi-MiG samples and the uninoculated control. Among them, 187 DEGs (with 112 DEGs downregulated and 75 DEGs upregulated) had not been reported in previous transcriptome studies (Curtolo et al., 2020; Ribeiro et al., 2023; Liu et al., 2024). Next, we wondered if the 187 DEGs were also differentially expressed in the orchard sample. The comparative analysis showed that 72 DEGs of the 187 DEGs were uniquely identified between the 40 dpi samples in comparison with the control sample (Fig. 5). These DEGs are most likely involved in the early responses of phloem cells to CLas infection.

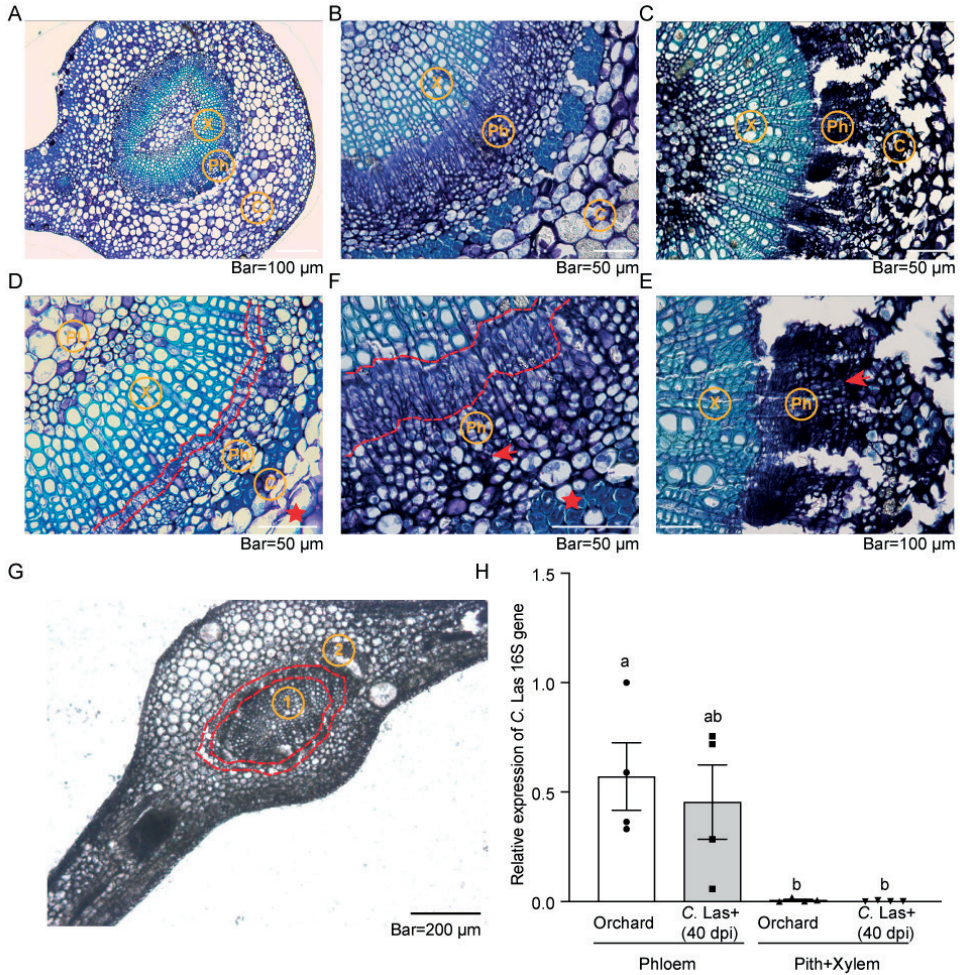


Figure 4. Cross-sections and LCM of *C. sinensis* leaf veins.

A: Healthy *C. sinensis* from the control group (not inoculated with CLAs).

B: *C. sinensis* at 40 dpi with CLAs.

C: *C. sinensis* sampled from the orchard.

D, E, F: Magnifications of regions A, B, and C, respectively.

The red triangle indicates cell wall thickening, the red pentagram marks fiber cells, and the red line denotes the boundary between the cambium and the xylem/phloem. PI: pith; X: xylem; Ph: phloem; C: cortex. G) Schematic of LCM, showed the sequential excision of the phloem ring ① and the pith and xylem ②. The red dashed lines indicate the cutting boundaries, with the phloem ring ① excised first, followed by the pith and xylem ②. The yellow numbers correspond to the tissues removed in this order for molecular analysis. H) The difference in CLAs titer between the phloem and the combined pith and xylem tissues at 40 dpi in MiG and orchard plants, as illustrated by the schematic in G. Different letters indicate statistically significant differences among tissues or organs at the same time point ($P < 0.05$, one-way ANOVA followed by Duncan's multiple comparison test, $n=4$).

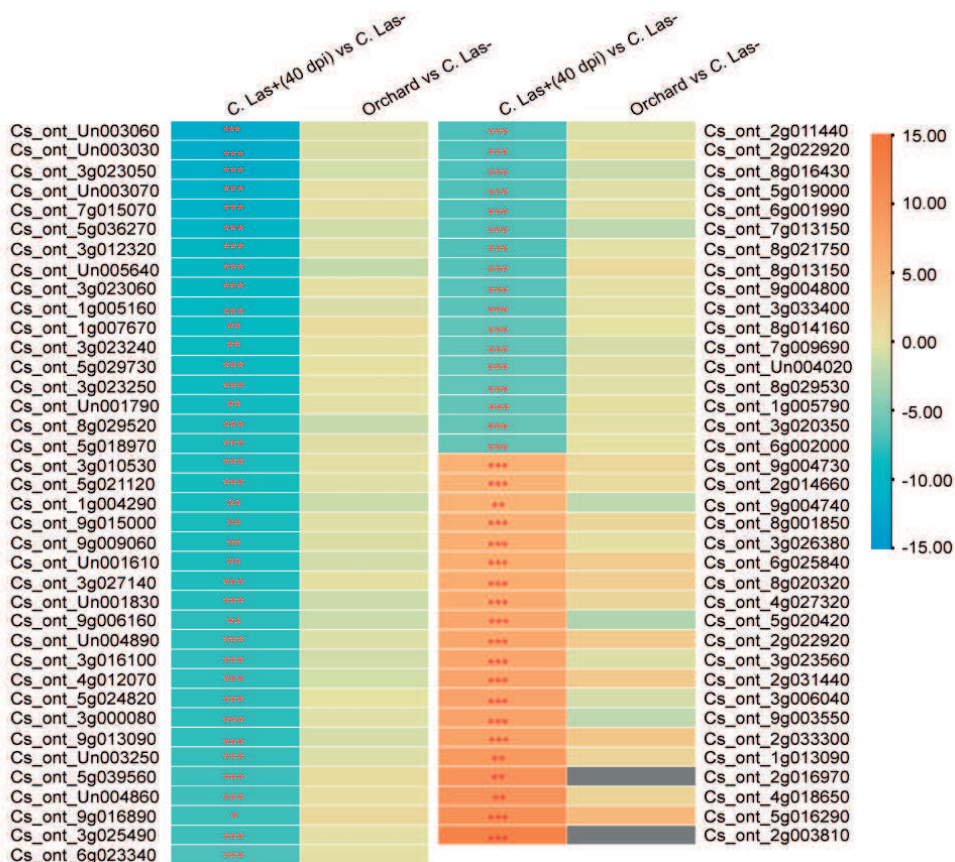


Figure 5. Comparison of the most significantly upregulated or downregulated DEGs with the highest fold changes in the phloem during the early stage of HLB with their expression profiles in phloem samples collected from orchard-grown trees. One asterisk indicates FDR-adjusted $0.01 < P \leq 0.05$; two asterisks indicate $0.001 < P \leq 0.01$; three asterisks indicate $P \leq 0.001$, based on Baggerly's test followed by Benjamini-Hochberg FDR correction implemented. MiG, micrografting; C. Las-, uninfected control; C. Las+, CLas-infected.

In conclusion, MiG-assisted transmission of CLas, in combination with LCM-assisted phloem-transcriptome, provides a novel enabling strategy to study host mechanisms involved in early responses to CLas infection.

DISCUSSION

In this study, we established a MiG method that enables efficient CLas inoculation and transmission in citrus seedlings. Coupled with the use of LCM to obtain phloem tissue, this approach facilitates the investigation of early host-pathogen interactions, allowing the identification of previously unreported, early responsive genes. Because the HLB inoculum

source in this study was a citrus donor plant confirmed to harbor without common citrus viruses such as CTV and CYVCV, we expect high specificity in CLas transmission, thereby eliminating potential confounding effects from potential co-infections of the citrus seedlings by other unknown pathogens.

The use of uniform 10-day-old citrus seedlings, each bearing three fully developed leaves, ensures developmental similarity across replicates. This allows easy tracking of both the leaves present at the time of inoculation and those formed thereafter, enabling the monitoring of infection dynamics. A similar study will be difficult, if not impossible, to achieve with traditional MaG. As in this latter case, shoot architecture is rather complex, making it not possible to select leaves of recipient plants in replicates to represent successive stages of infection.

Using the MiG approach, we obtained a total of 60 inoculated seedlings for assessing infection dynamics (Fig. 3). Approximately 50% of the UL tested positive for CLas at 30 dpi. At 40 dpi, nearly all UL were infected, exhibiting highly similar bacterial titers. Initial HLB symptoms also appeared around this time. When molecular analyses are needed, small midvein samples from UL can be collected non-destructively for qPCR confirmation, allowing precise tracking of infection progression. In contrast, none of the ten MaG-inoculated plants exhibited visible symptoms at 90 dpi, and qPCR analysis of UL revealed no detectable CLas titers at this time point. The first signs of HLB emerged around 6 months post-inoculation, when 2 out of 10 plants showed mild chlorosis on apical leaves of a single branch. By 9 months, 4 plants showed more pronounced symptoms. Severe symptoms, including widespread leaf yellowing and canopy thinning, developed progressively in all 10 plants between 12 and 24-months post-inoculation. This extended latency and variability in symptom onset are consistent with previous reports indicating that symptom development in MaG plants typically occurs over a period of 6 months to 2 years (Coletta-Filho et al., 2014; Zheng et al., 2024). These findings demonstrate that MiG greatly shortens the duration required for symptom development compared to MaG. Further, it also reduces spatial requirements, making it a more practical and efficient system for studying early-stage HLB pathogenesis.

The MiG system enables precise monitoring of infection dynamics and thereby offers a powerful platform for identifying genes involved in the early stages of CLas infection. At 40 dpi, our transcriptomic analysis revealed 187 DEGs that had not been reported in previous HLB-related studies, despite extensive omics research conducted over the past decade using leaves exhibiting pronounced symptoms (Albrecht and Bowman, 2012; Martinelli et al., 2013; Killiny et al., 2018). Among these 187 newly identified DEGs, 115 were detected exclusively in LCM phloem tissue, but not in whole-leaf samples, suggesting that their expression is most likely phloem-specific. This highlights the advantage of combining the MiG system with phloem-targeted transcriptomics for uncovering genes that have not been previously identified. Overall, our findings demonstrate the potential of MiG to uncover early host responses to CLas infection. In future studies, we aim to extend this approach by analyzing

temporal and tissue-specific transcriptomic changes to gain deeper insights into early host-pathogen interactions.

The utility of the MiG platform likely extends beyond citrus-HLB to other vascular diseases caused by phloem-limited, intracellular pathogens that are difficult or impossible to culture. Many such pathogens share the experimental challenges observed in HLB studies: long latency, inconsistent symptom expression, and a lack of efficient inoculation methods (Jiang et al., 2019). For example, *Candidatus Phytoplasma asteris*, the causal agent of aster yellows in various crops, and *Candidatus Phytoplasma ziziphi*, which causes jujube witches' broom, are both phloem-restricted and transmitted by insect vectors, making synchronized infection studies difficult (Bové et al., 2003; Weintraub and Beanland, 2006), and *Xylella fastidiosa*, which causes Pierce's disease in grapevine and olive quick decline syndrome in *Olea europaea*, is xylem- or phloem-limited and currently lacks robust systems for controlled early-stage infection studies (Sicard et al., 2018). The MiG-LCM approach developed in our study offers a potential solution to develop efficient experimental systems.

Together, the integration of MiG and LCM provides a robust framework for dissecting early-stage molecular changes during CLas infection. This approach has the potential to discover phloem-specific, early responsive genes, which will provide novel insight. Also, it will facilitate the identification of candidate promoters that could drive the expression of antimicrobial peptides at the onset of infection. Such targeted expression strategies hold promises for genetic improvement of citrus varieties with improved resistance to HLB and for advancing precision intervention at the earliest stages of pathogen colonization.

METHODS

Plant material

To eliminate microbial contaminants and promote uniform germination, seeds of *C. maxima* and *C. sinensis* were surface-sterilized. The procedure included soaking the seeds in 0.1 mol/L NaOH for 5 minutes to remove pectin, and then thorough rinsing with distilled water, followed by a 10-minute hot water treatment at 52°C. Next, the seeds were sequentially disinfected in 75% ethanol for 30 seconds and in 1% NaClO for 15 minutes, and finally rinsed 5 times with sterile water.

10-day-old and 1-year-old *C. maxima* seedlings were used as recipient plants for MiG and MaG grafting, respectively. HLB infected scion materials were obtained from 1-year-old *C. sinensis* seedlings exhibiting severe and consistent HLB symptoms, such as chlorotic mottling on mature leaves. These plants were screened via RT-PCR and qPCR to exclude co-infections with Citrus tristeza virus (CTV) (Kokane et al., 2021), Citrus tatter leaf virus (CTLV) (Cowell et al., 2017), and Citrus yellow vein clearing virus (CYVCV) (Chen et al.,

2016). Only HLB infected plants that were not positive to the above mentioned viruses were used as scion source.

Plant inoculation via MiG and MaG

For MiG inoculation, scions consisting of ~2 cm lignified stem segments with a single axillary bud were excised from infected *C. sinensis* donor plants. Ten-day-old *C. maxima* seedlings, 4-5 cm tall with two leaves, were selected as recipient hosts. A longitudinal slit (~2 cm) was made along the hypocotyl of each seedling using a sterile scalpel, and the scion was inserted with tight cambium alignment. The graft junction was wrapped with Parafilm® M and the seedlings were placed under transparent domes to maintain high humidity for graft union healing. For MaG-inoculation, the similar HLB infected scions were grafted onto 1-year-old *C. maxima* seedling using the same grafting procedure as described for MiG. All plants were grown in sterilized vermiculite under controlled greenhouse conditions (25-30°C with 60%-80% relative humidity).

Grafting survival rates and healing efficiency of MiG and MaG seedlings were statistically analyzed 90 DAG. For both grafting types (MiG and MaG), survival data were recorded from 10 individual seedlings per replicate, with three independent biological replicates.

CLas detection and quantification

To detect CLas, total DNA was extracted from the leaf veins or root tissues using the cetyltrimethylammonium bromide (CTAB) method (Bai et al., 2013), and the DNA concentration was adjusted to 100 ng/μL for all samples. Quantitative real-time PCR (qPCR) was performed using the CLas-specific primer pair HLBas/HLBr, Citrus cytochrome oxidase (COX) was used as an internal reference control to normalize DNA quality and amplification efficiency (Li et al., 2006).

For absolute quantification of CLas copy numbers, a standard curve was established based on a series of 102- to 107-fold gradient dilutions of a plasmid containing the CLas 16S rRNA gene fragment. The plasmid DNA was spiked into 100 ng of total genomic DNA extracted from healthy citrus tissues, and qPCR was performed to determine the corresponding Ct values. Three technical replicates were conducted for each sample (Ma et al., 2022). Quantification of CLas was conducted using the following equation: $Y = 0.2916X + 12.15$.

Histology of vascular tissues

For detailed cellular observations, sections of citrus leaf veins were prepared using a meticulous process involving fixation in FAA solution, a series of ethanol dehydrations, and embedding in Technovit 7100 resin (Her-aeus Kulzer, Hanau, Germany), and cut into 3-μm-thick sections (Fujinami et al., 2017). Sections were stained with a toluidine blue staining solution, and examined under a light microscope (Axio Imager M2; Zeiss, Germany) to analyze the organization of the leaf midvein.

Analysis of CLas Distribution and Inoculation Efficiency in MiG Seedlings

To assess CLas colonization patterns and inoculation efficiency, leaf and root samples were collected from MiG-inoculated *C. maxima* seedlings at 15, 20, 30, 40, 60, and 90 dpi. At each time point, four distinct tissues were sampled from each seedling, including the uppermost leaf (UL, the newest fully expanded leaf present at sampling), the oldest leaf (OL, the lowest fully expanded leaf), 1 cm apical segments of lateral roots (LR), and primary roots (PR). Total DNA was extracted from these tissues using the CTAB method, and CLas titers were quantified by qPCR. For each time point, ten independent seedlings were sampled, and CLas detection was performed separately for each of the four tissues per plant. Based on qPCR results, the infection rate (percentage of CLas-positive seedlings) was calculated for each tissue type and time point.

Laser microdissection

Leaf veins from three sample types—40 dpi CLas-infected, orchard-derived infected, and uninoculated controls—were carefully cleaned and fixed in Farmer's fixative (75% ethanol: 25% acetic acid, v/v) at 4°C for 16 hours. Fixed veins were subsequently dehydrated through an ethanol gradient consisting of 75% ethanol (30 minutes), 85% ethanol (30 minutes), and 100% ethanol (90 minutes). Steedman's wax was prepared by combining 90% polyethylene glycol 400 distearate with 10% 1-hexadecanol, melted and mixed at 65°C for 2 hours. Dehydrated vein samples were first infiltrated overnight with a 1:1 mixture of 100% ethanol and molten Steedman's wax, followed by three rounds of incubation in 100% Steedman's wax at 40°C for 2 hours each. The infiltrated tissues were then placed onto aluminum plates to allow wax solidification. Transverse sections of 14 µm thickness were cut using a Leica RJ2035 or RM2255 rotary microtome, and floated onto sterile water supplemented with 40 mmol/L DTT, mounted onto UV-treated 4 µm PEN-membrane slides (Leica Microsystems, <https://www.leica-microsystems.com/>). Wax was removed by rinsing slides three times in 100% ethanol for 3 minutes each. Slides were air-dried immediately prior to laser microdissection. Phloem rings, forming a circular pattern within the vascular bundle, were selectively excised using a Leica LMD7000 laser microdissection system. Approximately 50 phloem rings were collected per biological replicate, directly into the cap of a 0.5 mL RNase-free microcentrifuge tube containing 70 µL of RLT buffer (Qiagen RNeasy Micro Kit, <https://www.qiagen.com>) supplemented with 40 mmol/L DTT.

RNA isolation

To assess RNA quality from LMC of phloem tissues, residual tissue left on each slide after microdissection was first analyzed. Total RNA was extracted from these residual tissues using the Qiagen RNeasy Micro Kit following the manufacturer's instructions. RNA concentration was measured using the Qubit 2.0 Fluorometer (Invitrogen), and RNA integrity was evaluated via agarose gel electrophoresis. Only slides showing relatively high RNA yields and clear 28S/18S rRNA bands in residual tissues were selected for downstream RNA isolation from

the corresponding microdissection of phloem samples. RNA extraction was performed using the Qiagen RNeasy Micro Kit under RNase-free conditions according to the manufacturer's protocol. For cDNA synthesis and quality control, 2 μ L of each RNA sample was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). The resulting cDNA was pre-amplified using the SsoAdvanced™ PreAmp Supermix (Bio-Rad). Amplification efficiency and target representation were verified by qPCR, using citrus COX as a host gene control and CLas 16S rRNA as a pathogen marker. Finally, RNA samples from the four best biological replicates per sampling condition based on RNA quality (Agilent 2100 Bioanalyzer profiles) and qPCR performance-were used for RNA-seq library preparation.

Transcriptome sequencing and data analysis

Libraries were constructed using the Trio RNA-Seq™ Library Preparation Kit (Tecan; link). The constructed libraries were first quality-checked using the Illumina MiSeq platform, which confirmed high-quality read distribution across all 12 libraries. Subsequently, all libraries were subjected to paired-end sequencing (2×150 bp) on an Illumina NovaSeq platform, generating an average of 40 million reads per sample. Sequencing was performed by Bioscience, Wageningen University & Research. Raw sequencing reads were mapped to the Citrus sinensis genome v3.0 (Zhong et al., 2024) using the CLC Genomics Workbench v10.0.1 (Qiagen). All mapping parameters were set to default values. Transcript abundance was quantified as TPM (transcripts per million) following the method described by (Wagner et al., 2012). Differential gene expression analysis was also performed using the CLC Genomics Workbench with default statistical settings.

Quantification and statistical analysis

Data are presented as mean $\pm$ standard error (SE). Statistical analysis was performed using SPSS software. To compare group means, one-way analysis of variance (ANOVA) followed by Duncan's multiple range test or independent-sample t-tests were applied. Statistical significance was determined at a threshold of $P < 0.05$.

REFERENCES

- Albrecht, U., and Bowman, K.D. (2012). Transcriptional response of susceptible and tolerant citrus to infection with *Candidatus Liberibacter asiaticus*. *Plant Sci* 185-186, 118-130.
- Bai, J., Baldwin, E., Liao, H.L., Zhao, W., Kostenyuk, I., Burns, J., and Irely, M. (2013). Extraction of DNA from orange juice, and detection of bacterium *Candidatus Liberibacter asiaticus* by real-time PCR. *J Agric Food Chem* 61, 9339-9346.
- Bové, J.M., and Barros, A.P.D. (2006). Huanglongbing: A destructive, newly emerging, century-old disease of citrus. *J Plant Pathol.* 88(1).
- Bové, J.M., Renaudin, J., Saillard, C., Foissac, X., and Garnier, M. (2003). *Spiroplasma citri*, a plant pathogenic mollicute: relationships with its two hosts, the plant and the leafhopper vector. *Annu Rev Phytopathol* 41, 483-500.
- Chen, H., Zhou, Y., Wang, X., Zhou, C., Yang, X., and Li, Z. (2016). Detection of Citrus yellow vein clearing virus by Quantitative Real-time RT-PCR. *Horticultural Plant Journal* 2, 188-192.
- Clark, K., Franco, J.Y., Schwizer, S., Pang, Z., Hawara, E., Liebrand, T.W.H., Pagliaccia, D., Zeng, L., Gurung, F.B., Wang, P., Shi, J., Wang, Y., Ancona, V., van der Hoorn, R.A.L., Wang, N., Coaker, G., and Ma, W. (2018). An effector from the Huanglongbing-associated pathogen targets citrus proteases. *Nat Commun* 9, 1718.
- Coletta-Filho, H.D., Daugherty, M.P., Ferreira, C., and Lopes, J.R. (2014). Temporal progression of 'Candidatus *Liberibacter asiaticus*' infection in citrus and acquisition efficiency by *Diaphorina citri*. *Phytopathology* 104, 416-421.
- Comtet, J., Jensen, K.H., Turgeon, R., Stroock, A.D., and Hosoi, A.E. (2017). Passive phloem loading and long-distance transport in a synthetic tree-on-a-chip. *Nat Plants* 3, 17032.
- Cowell, S.J., Harper, S.J., and Dawson, W.O. (2017). A real-time RT-qPCR assay for the detection of Citrus tatter leaf virus. *J Virolog. Methods* 244, 29-31.
- Cui, X., Zhang, J., Liu, Y., Luo, X., Deng, X., Zhang, S., and Xu, M. (2022). Comparison of different grafting methods on the effect of 'Candidatus *Liberibacter asiaticus*' transmission. *Fruit Research* 2, 140-146.
- Curtolo, M., de Souza Pacheco, I., Boava, L.P., Takita, M.A., Granato, L.M., Galdeano, D.M., de Souza, A.A., Cristofani-Yaly, M., and Machado, M.A. (2020). Wide-ranging transcriptomic analysis of *Poncirus trifoliata*, *Citrus sunki*, *Citrus sinensis* and contrasting hybrids reveals HLB tolerance mechanisms. *Sci Rep* 10, 20865.
- Fu, S., Shao, J., Zhou, C., and Hartung, J.S. (2017). Co-infection of sweet orange with severe and mild strains of *Citrus tristeza* virus is overwhelmingly dominated by the severe strain on both the transcriptional and biological levels. *Front Plant Sci* 8, 1419.
- Fujinami, R., Yamada, T., Nakajima, A., Takagi, S., Idogawa, A., Kawakami, E., Tsutsumi, M., and Imaichi, R. (2017). Root apical meristem diversity in extant lycophytes and implications for root origins. *New Phytol* 215, 1210-1220.
- Gao, C., Li, C., Li, Z., Liu, Y., Li, J., Guo, J., Mao, J., Fang, F., Wang, C., Deng, X., and Zheng, Z. (2023). Comparative transcriptome profiling of susceptible and tolerant citrus species at early and late stage of infection by "Candidatus *Liberibacter asiaticus*". *Front Plant Sci* 14, 1191029.
- Gasparoto, M.C.G., Primiano, I.V., Bassanezi, R.B., Lourenço, S.A., Montesino, L.H., Wulff, N.A., Martins, E.C., Filho, A.B., and Amorim, L. (2022). Prevalent transmission of 'Candidatus *Liberibacter asiaticus*' over 'Ca. *Liberibacter americanus*' in a long-term controlled environment. *Phytopathology* 112, 180-188.

- Graham, J.H., Bassanezi, R.B., Dawson, W.O., and Dantzler, R.** (2024). Management of Huanglongbing of citrus: lessons from São Paulo and Florida. *Annu Rev Phytopathol* 62, 243-262.
- Hu, B., Rao, M.J., Deng, X., Pandey, S.S., Hendrich, C., Ding, F., Wang, N., and Xu, Q.** (2021). Molecular signatures between citrus and Candidatus *Liberibacter asiaticus*. *PLoS Pathog* 17, e1010071.
- Huang, C.Y., Araujo, K., Sánchez, J.N., Kund, G., Trumble, J., Roper, C., Godfrey, K.E., and Jin, H.** (2021). A stable antimicrobial peptide with dual functions of treating and preventing citrus Huanglongbing. *Proc Natl Acad Sci U S A* 118.
- Jiang, Y., Zhang, C.X., Chen, R., and He, S.Y.** (2019). Challenging battles of plants with phloem-feeding insects and prokaryotic pathogens. *Proc Natl Acad Sci U S A* 116, 23390-23397.
- Jonard, R., Hugard, J., Macheix, J.J., Martinez, J., Mosella-Chancel, L., Poessel, J.L., and Villemur, P.** (1983). In vitro micrografting and its applications to fruit science. *Scientia Horticulturae* 20, 147-159.
- Killiny, N., Jones, S.E., Nehela, Y., Hijaz, F., Dutt, M., Gmitter, F.G., and Grosser, J.W.** (2018). All roads lead to Rome: Towards understanding different avenues of tolerance to huanglongbing in citrus cultivars. *Plant Physiol Biochem* 129, 1-10.
- Kokane, S.B., Misra, P., Kokane, A.D., Gubyad, M.G., Warghane, A.J., Surwase, D., Reddy, M.K., and Ghosh, D.K.** (2021). Development of a real-time RT-PCR method for the detection of Citrus tristeza virus (CTV) and its implication in studying virus distribution in planta. *3 Biotech* 11, 431.
- Li, W., Hartung, J.S., and Levy, L.** (2006). Quantitative real-time PCR for detection and identification of Candidatus *Liberibacter* species associated with citrus huanglongbing. *J Microbiol Methods* 66, 104-115.
- Liu, C., Chang, X., Li, F., Yan, Y., Zuo, X., Huang, G., and Li, R.** (2024). Transcriptome analysis of Citrus sinensis reveals potential responsive events triggered by Candidatus *Liberibacter asiaticus*. *Protoplasma* 261, 499-512.
- Ma, W., Pang, Z., Huang, X., Xu, J., Pandey, S.S., Li, J., Achor, D.S., Vasconcelos, F.N.C., Hendrich, C., Huang, Y., Wang, W., Lee, D., Stanton, D., and Wang, N.** (2022). Citrus Huanglongbing is a pathogen-triggered immune disease that can be mitigated with antioxidants and gibberellin. *Nat Commun* 13, 529.
- Martinelli, F., Reagan, R.L., Uratsu, S.L., Phu, M.L., Albrecht, U., Zhao, W., Davis, C.E., Bowman, K.D., and Dandekar, A.M.** (2013). Gene regulatory networks elucidating huanglongbing disease mechanisms. *PLoS One* 8, e74256.
- Ribeiro, C., Xu, J., Hendrich, C., Pandey, S.S., Yu, Q., Gmitter, F.G., Jr., and Wang, N.** (2023). Seasonal transcriptome profiling of susceptible and tolerant citrus cultivars to citrus Huanglongbing. *Phytopathology* 113, 286-298.
- Sanjana, M., Shanmugasundaram, K.A., Muthuvel, I., Meenakshisundaram, P., and Amirtham, D.** (2024). Grafting horizons: A review on exploring micrografting for enhanced plant performance. *Annals of Phytomedicine An International Journal* 13.
- Shengeliya, I.N., and Butenko, R.G.** (1988). Micrografting as a means of producing virus-free citrus plants. *Soviet PlantPhysiology* 35, 149-153.
- Sicard, A., Zeilinger, A.R., Vanhove, M., Scharrel, T.E., Beal, D.J., Daugherty, M.P., and Almeida, R.P.P.** (2018). *Xylella fastidiosa*: insights into an emerging plant pathogen. *Annu Rev Phytopathol* 56, 181-202.
- Tournier, B., Tabler, M., and Kalantidis, K.** (2006). Phloem flow strongly influences the systemic spread of silencing in GFP *Nicotiana benthamiana* plants. *Plant J* 47, 383-394.
- Wagner, G.P., Kin, K., and Lynch, V.J.** (2012). Measurement of mRNA abundance using RNA-seq data: RPKM measure is incon-

sistent among samples. *Theory Biosci* 131, 281-285.

Wang, M.-R., Bettoni, J.C., Zhang, A.L., Lu, X., Zhang, D., and Wang, Q.-C. (2022). In vitro micrografting of horticultural plants: method development and the use for micropropagation. *Horticulturae* 8(7):576.

Wang, N., and Trivedi, P. (2013). Citrus huanglongbing: a newly relevant disease presents unprecedented challenges. *Phytopathology* 103, 652-665.

Weintraub, P.G., and Beanland, L. (2006). Insect vectors of phytoplasmas. *Annu Rev Entomol* 51, 91-111.

Zhao, P., Yang, H., Sun, Y., Zhang, J., Gao, K., Wu, J., Zhu, C., Yin, C., Chen, X., Liu, Q., Xia, Q., Li, Q., Xiao, H., Sun, H.X., Zhang, X., Yi, L., Zhou, C., Kliebenstein, D.J., Fang, R., Wang, X., and Ye, J. (2025). Targeted *MYC2* stabilization confers citrus Huanglongbing resistance. *Science* 388, 191-198.

Zheng, D., Armstrong, C.M., Yao, W., Wu, B., Luo, W., Powell, C., Hunter, W., Luo, F., Gabriel, D., and Duan, Y. (2024). Towards the completion of Koch's postulates for the citrus huanglongbing bacterium, Candidatus *Liberibacter asiaticus*. *Hortic Res* 11, uhae011.

Zhong, C., Yang, X., Niu, J., Zhou, X., Zhou, J., Pan, G., Sun, Z., Chen, J., Cao, K., and Luan, M. (2024). Transcriptome analysis of *Citrus Aurantium* L. to study synephrine biosynthesis during developmental stages. *PeerJ* 12, e17965.

3

Comparative expression analysis of homologous genes from *Liberibacter asiaticus* and the symbiotic plasmid pSymA of *Sinorhizobium*

Jing Wang¹, Rene Geurts¹, Ton Bisseling^{1,2,*}

¹Laboratory of Molecular Biology, Department of Plant Sciences, Graduate School Experimental Plant Sciences, Wageningen University & Research, Wageningen 6708 PB, the Netherlands.

²College of Grassland, Resources and Environment, Inner Mongolia Agricultural University, Hohhot 010011, China

*Corresponding authors: Ton Bisseling (ton.bisseling@wur.nl)

Abstract

Huanglongbing (HLB), caused by Candidatus Liberibacter asiaticus (CLas), is a destructive citrus disease, but functional analysis of CLas genes remains challenging due to its unculturable nature. Given the close phylogenetic relationship between CLas and Sinorhizobium meliloti (Sme), we compared the activity of promoters from CLas and the symbiotic megaplasmid pSymA within the Sme–Medicago truncatula root nodule system. We selected 79 CLas–pSymA homologous gene pairs, of which 28 dual-promoter reporter constructs were introduced into Sme and analyzed in nodules by confocal microscopy. For 12 gene pairs, promoter activity was detectable in planta. Strikingly, none of the homologous pairs displayed identical expression patterns. In several cases, CLas promoters were active in infection threads and symbiosomes, while their pSymA counterparts were weak or inactive. Conversely, other pairs showed strong pSymA promoter activity restricted to infection threads, with little or no expression from the CLas promoter. These results indicate that, although CLas and pSymA genes encode proteins with conserved homology, their regulatory control has diverged substantially. We propose that the evolutionary adaptation of CLas to the stable intracellular phloem niche reshaped its promoter activity, leading to distinct expression logic compared to the dynamic regulation of Sme symbiotic genes in nodules. This divergence highlights the importance of examining CLas genes in heterologous in planta systems, providing new insight into the regulatory evolution of this pathogen.

INTRODUCTION

Huanglongbing (HLB), also named citrus greening disease, is a highly destructive bacterial disease affecting citrus plants (Bové, J. M., 2006). The pathogen *Candidatus Liberibacter asiaticus* (CLas), which causes HLB, belongs to the Rhizobiaceae family (Hartung et al., 2011; Thapa et al., 2020). CLas cannot be cultured; therefore, the function of several CLas genes has been studied in its close relative *Sinorhizobium meliloti* (Sme, Barnett et al., 2019). They both have an intracellular lifestyle in their host. Therefore, the expression of CLas genes and their orthologues of Sme might be regulated in a similar way. However, whether this is the case has never been determined. Here, we studied the expression patterns in Sme induced root nodules of 12 pairs of homologous genes of CLas and the symbiotic mega-plasmid pSymA of Sme.

CLas has two hosts, citrus trees and psyllid insects. It lives intracellular within sieve element cells of the phloem of citrus trees. When CLas is ingested by psyllids from the phloem, they occupy the salivary glands of the psyllids, where they are hosted in an intercellular and intracellular manner. Citrus psyllids are the vector that transfers CLas from infected trees to other trees. When CLas is inside the phloem sieve element cells of citrus trees, both sieve element cells and their companion cells first remain biologically active. However, CLas triggers an immune response, and this leads to phloem blockage and death of sieve element cells, slowly killing the tree. The mechanisms by which CLas triggers the citrus immune response are still unclear.

Due to the current lack of effective strategies to treat HLB, more in-depth research targeting CLas is necessary. However, the unculturable nature of CLas severely hampers research. In chapter 4 of this thesis, we describe that CLas belongs to the genus *Rhizobium*, which is part of the Rhizobiaceae. This family encompasses several genera containing species that are able to establish a nitrogen-fixing root nodule symbiosis with legumes. Collectively, they are named rhizobia. Based on the close relationship between CLas and rhizobia, and because of the intracellular lifestyle of rhizobia in legume nodules, CLas genes have been studied by comparison with rhizobial genes. For example, in 2012, Kuykendall et al. identified 514 genes that are shared between CLas and five rhizobiales species. Within the Rhizobiaceae family, Sme is used as a model organism for which excellent molecular genetic tools are available. Previously, Sme has been used to study CLas genes. For example, Barnett et al. described a strategy using Sme as a host to identify compounds that inhibit the activity of CLas transcription activators (Barnett et al., 2019).

In Sme, most of the genes that support nodule symbiosis are located on the symbiotic mega-plasmid pSymA. Kuykendall et al. Identified 182 pairs of homologous genes between CLas and pSymA of Sme. Due to the phenomenon where a single CLas gene is homologous to multiple Sme genes or a single Sme gene is homologous to multiple CLas genes, the number of genes in CLas or Sme that are homologous to the other is less than 182 (Kuykendall et al., 2012). The high homology of the protein sequences indicates that these genes may have conserved func-

tions. However, whether the mechanism controlling their expression is not known. We will compare the expression patterns of these pSymA/ CLas gene pairs. To do this, we will utilize root nodules formed by the interaction of *Sme* and *Medicago truncatula* (*Medicago*).

The rhizobia-legume nodule symbiosis starts with the activation of the rhizobial nodulation genes (Geurts & Bisseling, 2002). This results in the biosynthesis and secretion of lipochito-oligosaccharides that are named Nod factor. Upon perception of Nod factors by the host, the nodulation process is set in motion. In *Medicago*, it starts with the curling of root hairs. In these curls, rhizobia become entrapped and induce the formation of a tube-like structure, the infection thread (IT), which is bound by a plant membrane, and cell wall. The ITs grow to the base of the root hairs and a new IT is formed in the underlying cortical cells. The rhizobia enter the plant through these ITs. In parallel, mitotic activity is induced in the inner root layers. In *Medicago*, it starts with divisions in the pericycle and this is followed by mitotic activation of endodermal and inner cortical cells. These dividing cells form a nodule primordium. The ITs grow towards these primordia. There, they enter nodule primordium cells, and rhizobia are released from the ITs into the cytoplasm of the host cell. During this process, they become surrounded by a membrane of the host, and in this way, organelle-like structures are formed that are named symbiosomes. Subsequently, symbiosomes divide and differentiate into elongated structures that fix nitrogen.

Medicago forms indeterminate nodules with a persistent meristem at their apex. This meristem adds cells to the different nodule tissues. In this way, a developmental gradient is formed, from the apex to the root attachment point. Indeterminate nodules are divided into zones. Next to the meristem is the infection zone where rhizobia are released, and symbiosomes divide and start to differentiate. The infection zone is followed by the fixation zone. At the transition from infection into fixation zone, genes encoding subunits of nitrogenase, the enzyme responsible for nitrogen fixation, are rapidly induced and starch accumulates in the infected cells.

We made use of this developmental gradient along the longitudinal axis of *Medicago* nodules. This allowed us to study the expression during nodule development of pairs of CLas and pSymA genes in a single nodule section. The high homology between the CLas and *Sme* genes strongly suggests that they have a common evolutionary origin. By introducing the CLas and pSymA genes in *Sme* and comparing their expression pattern in nodules, we could determine to what extent the mechanisms controlling their expression have been maintained.

Our results show that for all the promoter pairs, their expression patterns are distinct. When both the CLas and pSymA promoters are active within the nodules, their high-intensity expression locations differ. In other cases, either the CLas promoter or the pSymA promoter is active exclusively. This suggests that during the rapid evolution of CLas in a stable and distinct environment, significant changes occurred in its promoters, resulting in a gene expression pattern that differs markedly from its homologous genes on pSymA.

RESULTS

1. Selection of CLas genes that have close homology to pSymA genes

Our aim was to compare the expression patterns of homologous gene pairs of pSymA and CLas. pSymA genes often have a homologue on the Sme genome. We decided to focus on CLas genes, whose homology to the pSymA gene is higher than to that of the Sme chromosome. To determine which CLas genes are more homologous to the pSymA gene, we used the 182 protein sequences of CLas to identify the genes it shares with pSymA. First, we made 182 single gene evolutionary trees to select the closest homologue of the following strains: *Bartonella henselae* str. Houston-1, *Bradyrhizobium japonicum* USDA6, *Candidatus Liberibacter africanus* PTSAPSY, *Candidatus Liberibacter americanus* str. Sao Paulo, *Candidatus Liberibacter solanacearum* CLso-ZC1, *Agrobacterium tumefaciens* LBA4213, *Rhizobium etli* CFN42, and *Sinorhizobium meliloti* 2011 (chromosome). For each strain, the protein with the lowest E-value in the matching hit obtained by BLAST was selected. These eight proteins were aligned together with the proteins from CLas and pSymA using the Geneious alignment software. Thus, a Neighbor-Joining evolutionary tree, based on this alignment, was made using Geneious tree builder. The evolutionary trees obtained were rooted using *Bradyrhizobium japonicum* USDA6, and the relationship between CLas, pSymA, and *Sinorhizobium meliloti* 2011 (Chromosome) was determined. 79 CLas genes were more homologous to the pSymA gene than to that of the *Sinorhizobium* chromosome. Among them, 57 genes of the CLas cluster together with homologous genes from three other *Liberibacter* species and pSymA. An example, NP_436514 from pSymA, is shown in Figure S1. It clusters together with proteins from CLas (YP_003064948) and three other *Liberibacter* species. Additionally, 22 genes of the CLas cluster together with genes from the other three *Liberibacter* species, pSymA, and *Bartonella*. *Bartonella* is a pathogenic bacterium carried by insects such as fleas and is located within animal cells. Figure S2 shows an example, NP_436272 from pSymA.

Further, there are 103 CLas proteins that are closer to the protein on the *Sinorhizobium* chromosome. Figure S3 gives two examples showing that the pSymA gene is located adjacent to the cluster formed by *Liberibacter* spp. genes and the gene of the chromosome of *Sinorhizobium*. Table S1 gives the IDs of the 79 selected pairs of homologous proteins of CLas and pSymA and their putative function.

2. Vector assembly

For the 79 pairs of CLas and pSymA genes, vectors were assembled to examine their expression patterns. We created a vector harboring a CLas gene promoter to drive *sfGFP*, while the promoter of the homologous pSymA gene drives *mCherry*. The structure of this vector is two expression cassettes positioned in opposite directions to prevent read-through (Fig. S6). Some pSymA genes are located in the same operon. So they share the same promoter, which reduced the number of Sme promoters that needed to be tested. Also, some CLas genes occur in the same operon. Additionally, some CLas genes are homologous to multiple pSymA

genes, and vice versa. These factors have led to a reduction in the number of promoters that need to be tested. 38 promoters of CLas needed to be tested instead of 79, while for Sme, the number of promoters that had to be tested is 72. For the Sme promoter sequences, we cloned them from purified total Sme DNA. Since CLas DNA was not available, we synthesized the CLas promoter sequences.

35 promoter sequences were successfully synthesized, and 3 failed. Their corresponding 35 Sme promoter sequences were successfully cloned. Finally, 28 vectors were successfully constructed, and they are listed in Table S3.

3. Comparison of the expression patterns of gene pairs of CLas and pSymA.

To compare the expression patterns of homologous gene pairs, we introduced the expression vectors into Sme and inoculated it on *Medicago*. Root nodules were harvested 14 days post inoculation, and the gene expression patterns were studied. 100uM-thick longitudinal sections of the root nodules were obtained using a vibratome, and they were analysed by confocal microscopy. The green fluorescent signal originated from the *sfGFP* protein coding part driven by the CLas promoter, while the red fluorescent signal originated from the *mCherry* protein coding part driven by the pSymA promoter.

First, we examined nodules formed by wild-type Sme CL150 to test whether auto fluorescence was visible when using the wavelengths to visualize *sfGFP* and *mCherry* (Fig. S4). The central tissue of the nodule is composed of infected cells, containing numerous symbiosomes, and uninfected cells. Uninfected cells in the fixation zone exhibit autofluorescence at both wavelengths, but it was not present in the infected cells.

We used the LacZ promoter to drive *sfGFP* and *mCherry* as a positive control to test whether the fluorescent signals could be observed in nodules. We adjusted the intensity of the laser, as well as the scanning time and signal strength enhancement, to make the intensities of the two signals approximately the same. In this case, the overlapping red and green fluorescence signals appear yellow. As shown in Figure S5, the red and green fluorescent signals are clearly visible in the infection threads that are present in the infected cells, and in the symbiosomes within the infected cells. Autofluorescence in uninfected cells is not visible under these scanning conditions.

Out of the 28 vectors we constructed, genes of 12 vectors were expressed at a sufficiently high level to be detected. These can be categorized into three groups:

1. Both the CLas and pSymA promoters are expressed.
2. Only the CLas promoter is expressed.
3. Only the pSymA promoter is expressed.

One vector, MW005, expressed both the CLas and pSymA constructs. It carries the promoter of the CLas gene YP_003065261 driving *sfGFP* and the native promoter of the pSymA gene NP_435311 driving *mCherry*. As shown in Figure 1, genes from both CLas and pSymA were expressed in the nodules, but their expression patterns were different. The expression level of the CLas gene in ITs and symbiosomes is weak, resulting in *sfGFP* fluorescence that is only slightly stronger than that of the autofluorescence of uninfected cells. On the other hand, the pSymA construct exhibited a higher expression level in the ITs and symbiosomes within the infection zone. In the fixation zone, the expression intensity of the pSymA construct decreases within the symbiosomes, and it maintains a high expression level in the ITs.

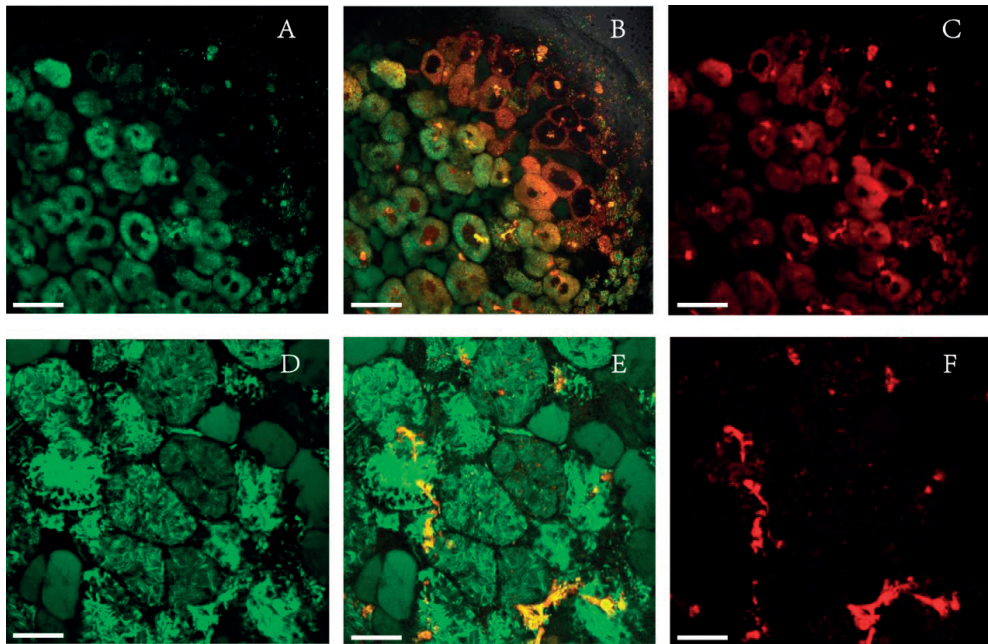


Figure 1: Comparison of expression patterns of pairs of CLas and *S. meliloti* promoters

Confocal microscopy of nodules formed by the *S. meliloti* CL150 strain carrying MW005, showing CLas-promoter-*sfGFP* and Sme-promoter-*mCherry* fluorescence. The CLas promoter shows higher activity than the Sme promoter in the fixation zone, whereas the Sme promoter is more strongly expressed in the infection zone and infection threads.

A,D: Image made using the *sfGFP* wavelength

C,F: Image using the *mCherry* wavelength

B,E: The overlap of both fluorescent images and the bright field image.

A-C are magnified views of the infection zone, **D-F** are magnified views of the fixation zone. Red arrow indicates infection thread. Bar: A-C: 50µm, D-F: 25µm.

There are two vectors, MW004 and MW007, which result in similar expression patterns. In both cases, only the CLas construct is expressed. MW004 carries the native promoter of the CLas gene YP_003065051 and the promoter of pSymA gene NP_435305. In MW007, it is YP_003065009 (CLas) and NP_435551 (pSymA). Figure 2 shows the expression of the con-

structs on MW004. In this figure, *sfGFP* driven by the CLas promoter results in a relatively high expression in Sme located within the ITs and in symbiosomes. In contrast, *mCherry* driven by the pSymA promoter is hardly expressed. Only in a few ITs is a low signal detectable.

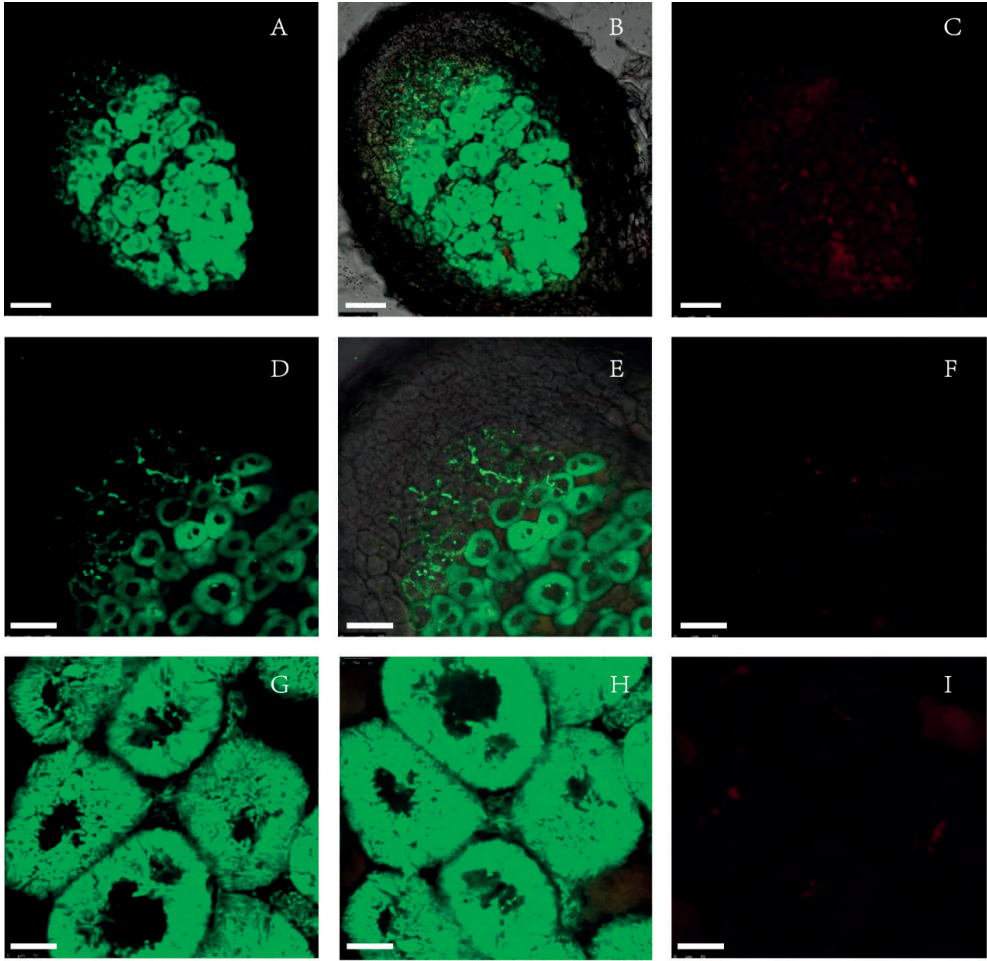


Figure 2: Confocal microscopy of nodules formed by the *S. meliloti* CL150 strain carrying MW004, showing CLas-promoter-*sfGFP* and Sme-promoter-*mCherry* fluorescence. Only the CLas promoter-driven signal is detectable, and its expression spans from the infection zone to the fixation zone.

A,D,G: Image made using the *sfGFP* wavelength

C,E,I: Image using the *mCherry* wavelength

B,E,H: The overlap of both fluorescent images and the bright field image.

A-C are views of whole nodule, **D-F** are magnified views of the infection zone, **G-I** are magnified views of the fixation zone. Red arrow indicates infection thread.

Bar: A-C: 75µm, D-F: 50µm, G-I: 25µm

There are nine vectors exhibiting a similar expression pattern, where only the construct of pSymA is expressed. They are MW003, MW006, MW010, MW018, MW022, MW023,

MW025, MW026, and MW028. Taking MW003 as an example, it carries the promoter of the CLas gene YP_003064586 and the promoter of the pSymA gene NP_436105. As shown in Figure 3, the red fluorescence emitted by Sme is in the ITs in both infection and fixation zones. In the infection zone, The pSymA construct is expressed in the symbiosomes in the infection zone, albeit at a level markedly lower than in the IT. In the fixations zone it is below the detection level. In contrast, *sfGFP* driven by the CLas promoter is not visible.

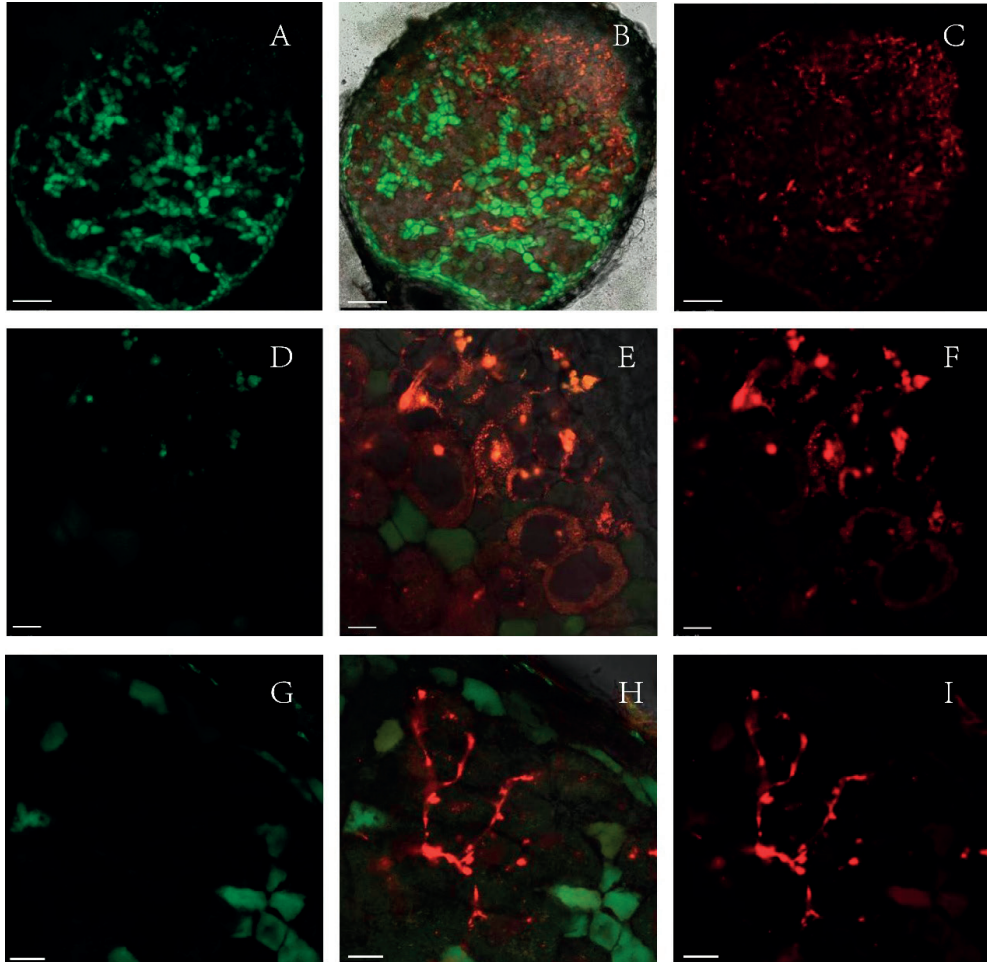


Figure 3: Confocal microscopy of nodules formed by the *S. meliloti* CL150 strain carrying MW003, showing CLas-promoter-*sfGFP* and Sme-promoter-*mCherry* fluorescence. Only the Sme promoter-driven signal is detectable, and its expression is confined to the infection zone and infection threads.

A,D,G: Image made using the *sfGFP* wavelength

C,F,I: Image using the *mCherry* wavelength

B,E,H: The overlap of both fluorescent images and the bright field image.

A-C are views of whole nodule, **D-F** are magnified views of the infection zone, **G-I** are magnified views of the fixation zone. **A-C:** 100μm, **D-F:** 10μm, **G-I:** 25μm

DISCUSSION

We studied the expression of 12 pSymA and CLas promoters in root nodules. Although the proteins encoded by the corresponding genes are similar, the mechanisms controlling their expression are very different.

The homology of the proteins encoded by the gene pairs varies between 41% and 73%, strongly suggesting that they have a similar biochemical function. However, none of the gene pairs have a similar gene expression pattern, and only in one case are both gene constructs expressed. In this case, the expression in the ITs is similar, but the expression in the symbiosomes has a different pattern along the nodule longitudinal axis. Based on the large difference in the expression patterns, we conclude that the mechanisms by which the expression of these genes is regulated are very different.

Many rhizobia are able to form root nodules on legumes and in all these cases are accommodated inside host cells. However, it is very likely that this property has been obtained by horizontal gene transfer. These rhizobia, for example *Bradyrhizobium*, are often identified as endophytes in non-legumes. In most cases, these rhizobia lack the genes required to establish a nitrogen-fixing root nodule symbiosis. Therefore, the endophytic nature seems more ancestral than the root nodule symbiosis. The ability to live in phloem cells is within the Rhizobiaceae restricted to *Liberibacter* species. Therefore, it is very likely that this intracellular lifestyle evolved independently. This might explain why the mechanisms controlling these genes are very different. *Sinorhizobium* gene expression in nodules is tightly regulated to meet symbiotic needs, focusing on nitrogen fixation and host compatibility. In the fixation zone, *Sinorhizobium* transforms into bacteroids, which are larger, polyploid, and specialized for nitrogen fixation, reducing general bacterial functions. This specialization is influenced by host plant factors like NCR peptides, which modulate bacteroid behavior to maintain symbiosis. The ten tested *Sinorhizobium* genes, involved in metabolism, regulation, and transport, are downregulated in bacteroids, indicating their minor role in nitrogen fixation and adaptation to the endosymbiotic lifestyle. This precise regulation of Sme gene expression in nodules evolved through long-term symbiosis with host plants and based on our results, it does not exist in CLas.

This study employed experimental methods to test the function of promoters from CLas and Sme to investigate gene expression. In addition, we examined previously published transcriptome expression data of CLas and Sme in the host to provide comparative evidence. As shown in Table S4, the gene expression levels of CLas living in the citrus phloem sieve element cells (Roux et al., 2014) and the gene expression levels of Sme in various parts of *Medicago* nodules (FI: Meristem, FIId&FIIP: Infection zone, IZ&FIIL: Fixation zone; ref) are provided. Based on the comparison of transcriptome data, the correlation between CLas and Sme gene expression levels is low (Pearson Correlation coefficient = 0.08, p-Value = 0.814).

This indicates that, consistent with our experimental results, the transcriptome data suggests that their gene expression patterns in their respective host cells are different. When we compared the gene expression patterns of Sme obtained from our experiments with Roux's transcriptome data, the match was not high. For example, our experimental results show that Sme genes are more strongly expressed in the bacteroides of the Infection zone compared to the Fixation zone. However, Roux's data indicate that NP_435551 is expressed at 82.46% in the Fixation zone of the entire nodule. Additionally, eight other genes have more than half of their expression levels in the Fixation zone of the entire nodule. This discrepancy may be due to Roux dividing the root nodules into parts such as meristem, infection zone, and fixation zone, while our observed expression patterns are mainly categorized as being in ITs or in the symbiosomes within the infection zone or fixation zone. These two different classification methods have resulted in the mismatch of expression patterns.

Kuykendall used protein sequence similarity methods to identify 182 genes on the pSymA symbiotic megaplasmid that are homologous to those in CLas, suggesting that these genes, key genetic components related to symbiosis and host interaction, might still be conserved and functionally significant. These genes have copies of the chromosome of Sme. It is likely that the genes important for the endoparasitic lifestyle of CLas are more conserved on CLas and the pSymA plasmid relative to the genes on the chromosome of Sme, due to selective pressure. Therefore, we constructed evolutionary trees for the 182 genes and found that 79 of these genes in CLas are more closely related to the genes on the pSymA plasmid than to those on the chromosome of Sme. This was also the starting range for our expression testing this time. It's noteworthy that 22 genes are conserved among CLas, the pSymA plasmid, and *Bartonella henselae*, which is an intracellular parasitic bacterium found in animal cells. These genes include five associated with energy production and electron transport, eleven transporter genes, three cellular signaling and response genes, and two structural and metabolic enzyme genes. Overall, these genes encode proteins that ensure the bacteria can efficiently produce energy, transport necessary molecules, respond to environmental signals, and maintain cellular integrity. All these functions are essential for their survival and ability to establish symbiotic or pathogenic relationships, illustrating a core set of capabilities crucial across different host environments and relationships.

In this study, we found that the promoter activity for homologous genes in Sme and CLas differs. Sme genes are regulated and alter their expression levels in different environments within nodules, whereas CLas genes maintain a similar level of expression regardless of environmental changes. This indicates that in the process of adapting to different survival strategies, the regulatory mechanisms of gene expression have diverged, even though protein functions may have remained similar.

METHODS

Plant material and inoculation with rhizobia

Medicago truncatula cv. Jemalong A17 was used as the wild-type genotype. Seedlings were cultivated in perlite that had been saturated with low-nitrate Fåhræus medium [0.25 mM $\text{Ca}(\text{NO}_3)_2$] as described by Catoira et al. (2000). Plants were maintained at 21°C under a 16h light/8h dark photoperiod. After 1 week of growth, seedlings were inoculated with *Sinorhizobium meliloti* strain CL150.

Vector construction

Vector design and assembly were performed using the Golden Gate-based MoClo Tool Kit. The *Liberibacter* promoter driving *sfGFP* expression was cloned into the Level 1 vector PICH47811, whereas the *Sinorhizobium* promoter driving *mCherry* expression was cloned into the Level 1 vector PICH47732. Both expression cassettes were subsequently combined in a one-step assembly reaction and inserted into the Level 2 vector POGG216 in opposite orientations.

Phylogenetic Analysis and Promoter Cloning

Genomes were downloaded from the NCBI database. Bayesian phylogenetic inference was conducted using MrBayes v3.2. Sequence alignments were generated in Geneious software, and regions with substantial ambiguity (gaps > 5 bp) were manually removed. Promoter regions were predicted using BDGP and PromPredict software, and broader overlapping prediction intervals were selected. For genes located within operons, the promoter of the entire operon was used. For each gene, the region from the predicted transcriptional start site to the upstream sequence immediately preceding the start codon was cloned as its promoter.

Microscopy

Bright-field and fluorescence confocal images of nodules were acquired using a Leica SP8 microscope. Excitation wavelengths of 488 nm and 587 nm were used for *sfGFP* and *mCherry*, respectively.

REFERENCES

- Barnett, M. J., Solow-Cordero, D. E., & Long, S. R.** (2019). A high-throughput system to identify inhibitors of Candidatus *Liberibacter asiaticus* transcription regulators. *Proceedings of the National Academy of Sciences*, 116(36), 18009–18014.
- Catoira, R., Galera, C., de Billy, F., Penmetsa, R.V., Journet, E., Maillet, F., Rosenberg, C., Cook, D., Gough, C., and Dénarié, J.** (2000). Four genes of *Medicago truncatula* controlling components of a Nod factor transduction pathway. *Plant Cell*, 12: 1647–1665.
- Geurts, R., & Bisseling, T.** (2002). Rhizobium Nod factor perception and signalling. *The Plant Cell*, 14(Suppl. 1), S239–S249.
- Kuykendall, L. D., Shao, J. Y., & Hartung, J. S.** (2012). Conservation of gene order and content in the circular chromosomes of ‘Candidatus *Liberibacter asiaticus*’ and other Rhizobiales. *PLoS ONE*, 7(4), e34673.
- Kuykendall, L. D., Shao, J. Y., & Hartung, J. S.** (2012). ‘Candidatus *Liberibacter asiaticus*’ proteins orthologous with pSymA-encoded proteins of *Sinorhizobium meliloti*: Hypothetical roles in plant host interaction. *PLoS ONE*, 7(6), e38725.
- Roux, B., Rodde, N., Jardinaud, M. F., et al.** (2014). An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. *The Plant Journal*, 77(6), 817–837.
- Weber, E., Engler, C., Gruetzner, R., Werner, S., & Marillonnet, S.** (2011). A modular cloning system for standardized assembly of multigene constructs. *PloS one*, 6(2), e16765.

SUPPLEMENTARY DATA

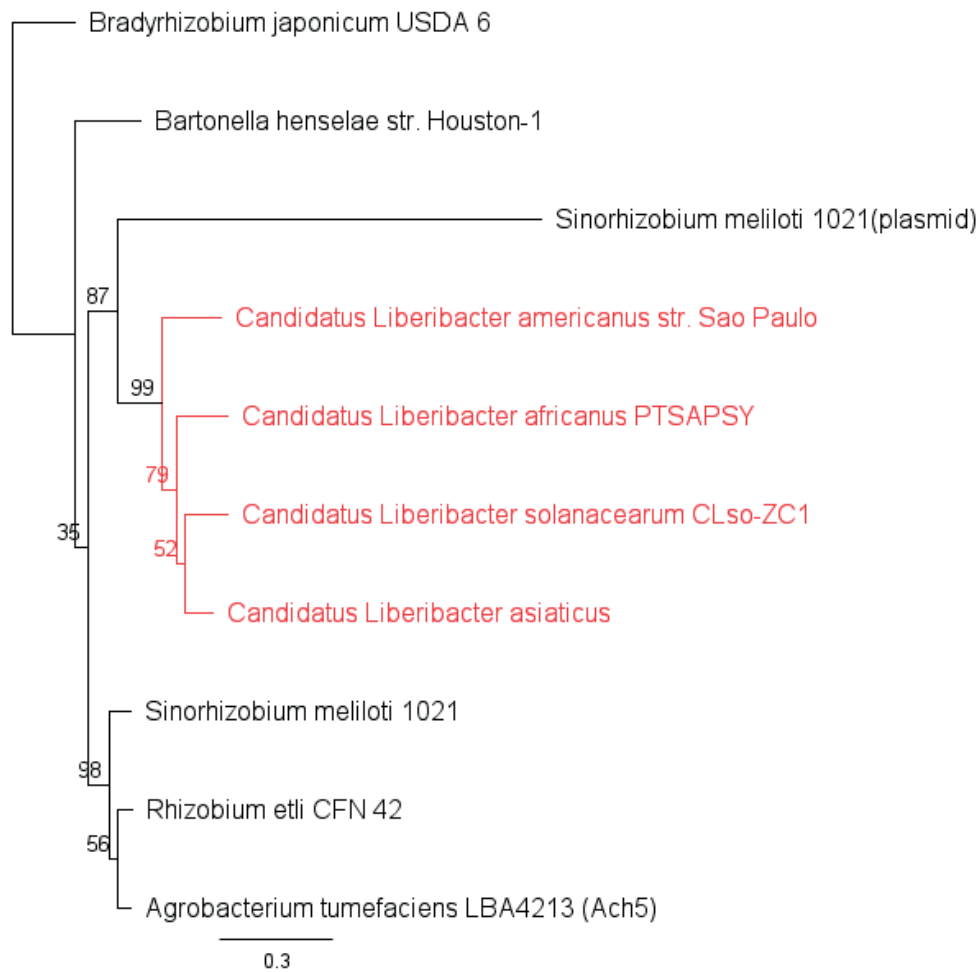


Figure S1: Neighborhood evolutionary tree obtained by Geneious tree builder. Evolutionary tree of NP_436514 from pSymA and YP_003064948 from CLas with homologous proteins from the other strains. Bootstrap values are marked on the nodes (replicate number: 100).

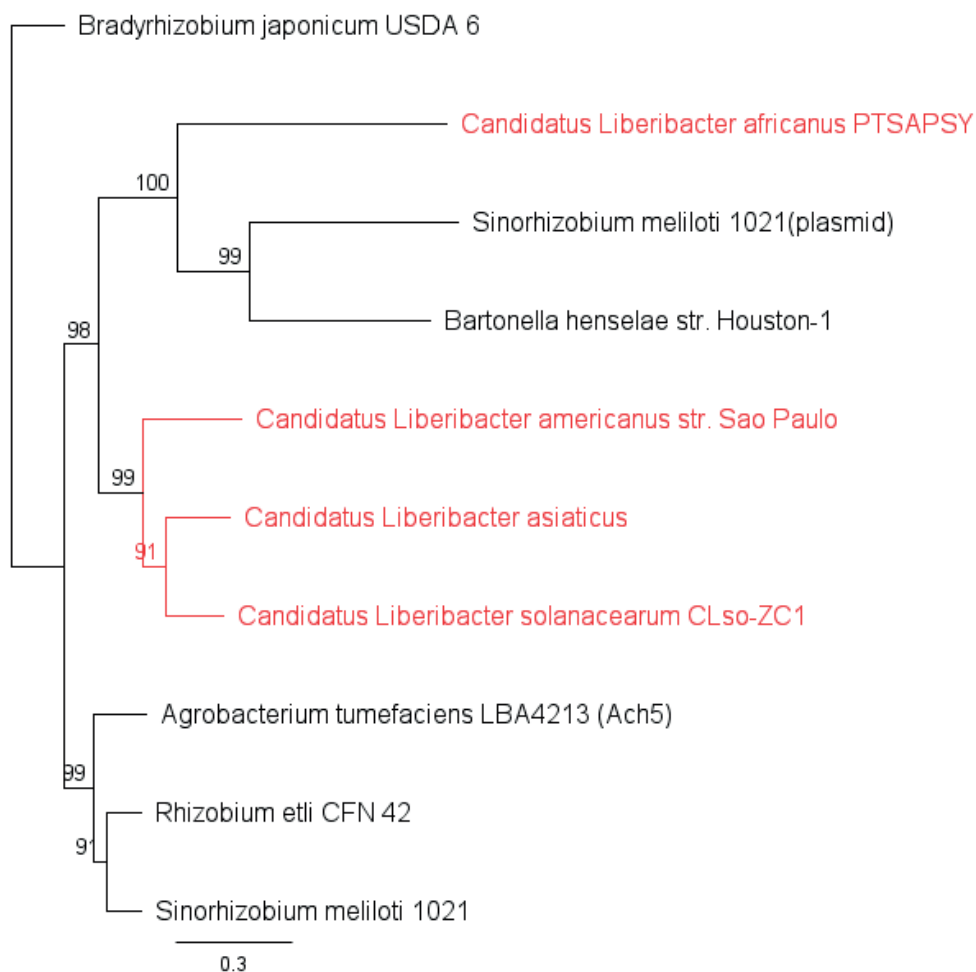


Figure S2: Neighborhood evolutionary tree obtained by Geneious tree builder. Evolutionary tree of NP_436272 from pSymA and YP_003064552 from CLas with homologous proteins from the other strains. Bootstrap values are marked on the nodes (replicate number: 100).

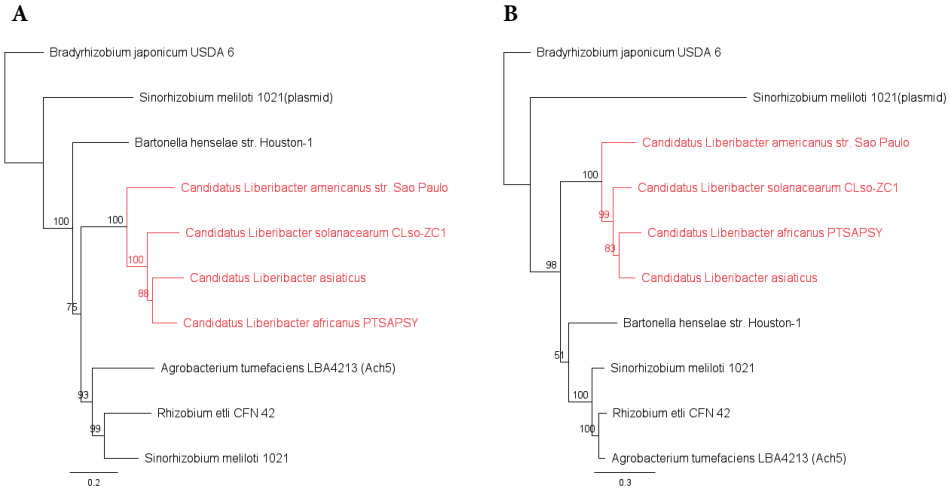


Figure S3: Neighborhood evolutionary tree obtained by Geneious Tree Builder.

A: Evolutionary tree of NP_436482.1 from pSymA and YP_003065516 from CLas with homologous proteins from other strains.

B: Evolutionary tree of NP_436477.1 of pSymA and YP_003064695 of CLas with homologous proteins from the other strains.

Bootstrap values are marked on the nodes (replicate number: 100).

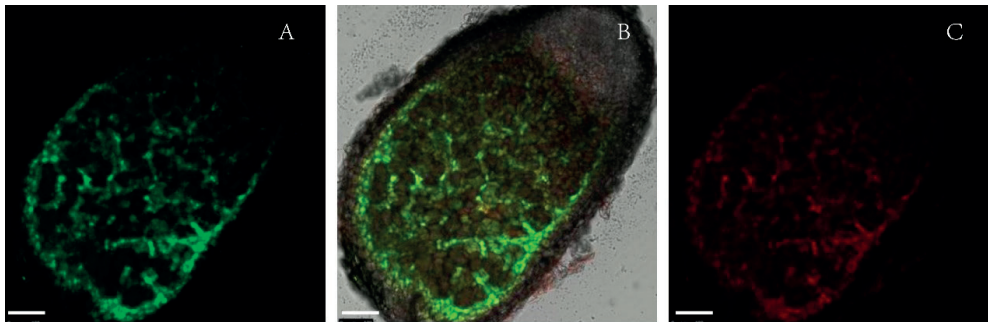


Figure S4: Confocal microscopy of nodules formed by the *S. meliloti* CL150 strain. Only autofluorescence is visible, with autofluorescence present in uninfected cells. Bar: 100µm

A: Image made using the *sfGFP* wavelength

C: Image using the *mCherry* wavelength

B: The overlap of both fluorescent images and the bright field image.

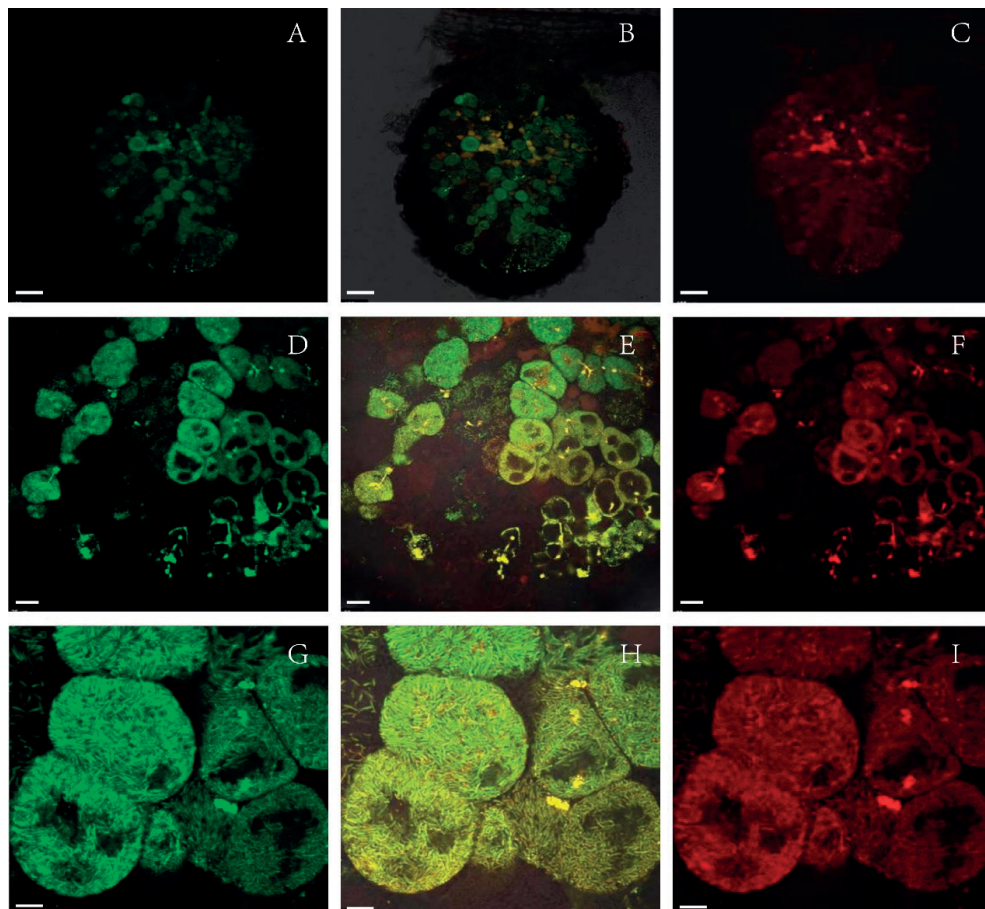


Figure S5: Confocal microscopy of nodules formed by the *S. meliloti* CL150 strain carrying MW001. Both red and green fluorescence are driven by the *lacZ* promoter, and are continuously expressed from the infection zone through the fixation zone. Bar: A-C: 100 μ m, D-F: 25 μ m, G-I: 10 μ m.

A,D,G: Image made using the *sfGFP* wavelength

C,F,I: Image using the *mCherry* wavelength

B,E,H: The overlap of both fluorescent images and the bright field image.

A-C are views of whole nodule, D-F are magnified views of the infection zone, G-I are magnified views of the fixation zone.



Figure S6: Schematic diagram of plasmid structure. *sfGFP* driven by the CLas promoter and *mCherry* driven by the Sme promoter are oriented in opposite directions on the plasmid.

Table S1: ID of 79 pairs of homologous proteins of CLas and pSymA and their putative function

Protein ID in Sinorhizobium	Protein ID in Liberibacter	Description
Genes of four Liberibacters, pSymA and Bartonella that clustered		
1 NP_435691	YP_003065437	FixC oxidoreductase
2 NP_435692	YP_003064782	FixB electron transfer flavoprotein alpha chain
3 NP_435693	YP_003064781	FixA electron transfer flavoprotein beta chain
4 NP_435771.1	YP_003065017	Putative pyridoxal-phosphate-dependent amino-transferase
5 NP_435818	YP_003065171	hypothetical protein
6 NP_435955	YP_003065137	VirB11-like ATPase
7 NP_435998.1	YP_003064552	ABC transporter,ATP-binding protein
8 NP_436023.1	YP_003065157	ABC transporter,ATP-binding protein,involved in transport of sugar phosphates (glucose-6-phosphate and glycerol-3-phosphate)
9 NP_436029.1	YP_003064586	ABC transporter,ATP-binding protein
10 NP_436067.1	YP_003065157	ABC transporter,ATP-binding protein
11 NP_436080	YP_003065265	NuoA2 NADH:quinone oxidoreductase subunit A 2
12 NP_436081	YP_003065280	NuoN2 NADH:quinone oxidoreductase subunit N 2
13 NP_436094.1	YP_003065306	Sensor histidine kinase

14	NP_436145.2	YP_003064586	ABC transporter, ATP-binding protein, involved in amino acid or peptide transport
15	NP_436146.1	YP_003064552	ABC transporter, ATP-binding protein, involved in amino acid or peptide transport
16	NP_436258.1	YP_003065195	ArgE acetylornithine deacetylase
17	NP_436272.1	YP_003064552	ABC transporter, ATP-binding protein
18	NP_436405.1	YP_003065157	ABC transporter, ATP-binding protein
19	NP_436481	YP_003065165	MrcA2 penicillin-binding protein
20	NP_436497.1	YP_003065306	Response regulator
21	NP_436525.1	YP_003065157	ABC transporter, ATP-binding protein
22	NP_436535.1	YP_003064686	ABC transporter ATP-binding protein
Genes of four <i>Liberibacters</i> and pSymA that clustered			
23	NP_435712.1	YP_003064946	Node beta ketoacyl ACP synthase
24	NP_435715	YP_003064676	NodQ1 ATP-sulfurylase large subunit-APS kinase
25	NP_435856.2	YP_003065113	DegP4 protease like protein
26	NP_436058	YP_003064695	Transcriptional regulator, LysR family
27	NP_436072	YP_003065274	NADH-quinone oxidoreductase subunit I 2
28	NP_436078	YP_003065267	NuoC2 NADH:quinone oxidoreductase subunit C2
29	NP_436079	YP_003065266	NuoB2 NADH:quinone oxidoreductase subunit B 2
30	NP_436082	YP_003065279	NuoM2 NADH:quinone oxidoreductase subunit M 2
31	NP_436087	YP_003065275	NuoJ2 NADH:quinone oxidoreductase subunit J 2
32	NP_436136	YP_003064695	Transcriptional regulator, LysR family
33	NP_436253.1	YP_003064695	Transcriptional regulator, LysR family
34	NP_436422.1	YP_003064948	short chain alcohol dehydrogenase-related dehydrogenase
35	NP_436485.1	YP_003065492	Diguanylate cyclase
36	NP_436495	YP_003065365	UvrD2 DNA helicase
37	NP_436514.1	YP_003064948	oxidoreductase
38	NP_435253.1	YP_003064676	Selenocysteine-specific elongation factor
39	NP_435281.1	YP_003064586	Putative pentose monosaccharide ABC transporter, ATP-binding protein
40	NP_435282.1	YP_003064948	short chain alcohol dehydrogenase-related dehydrogenase
41	NP_435305.2	YP_003065051	DnaJ/CbpA-type protein
42	NP_435311.1	YP_003065261	1 kDa chaperonin
43	NP_435312.1	YP_003065326	Hypothetical protein
44	NP_435342.1	YP_003065326	CspA7 cold shock protein

45	NP_435352.1	YP_003064586	ABC transporter,ATP-binding protein,likely involved in transport of galactose and,to a lesser extent,L-arabinose,glucose,D-arabinose,L-fucose and D-fucose
46	NP_435360.1	YP_003065009	ABC transporter,ATP-binding protein
47	NP_435419.1	YP_003064948	short chain alcohol dehydrogenase-related dehydrogenase
48	NP_435423.1	YP_003064750	short chain alcohol dehydrogenase-related dehydrogenase
49	NP_435501.1	YP_003064878	ThrC2 threonine synthase
50	NP_435513.1	YP_003064586	ABC transporter,ATP-binding protein
51	NP_435536	YP_003064695	Transcriptional regulator,LysR family
52	NP_435549.2	YP_003065316	adenylate/guanylate cyclase
53	NP_435551.1	YP_003065009	Nitrate transporter,ATP binding protein
54	NP_435608	YP_003064907	Putative cation efflux protein
55	NP_435624.1	YP_003065009	ABC transporter ATP-binding protein
56	NP_435637.1	YP_003065326	CspA6 cold shock protein
57	NP_435641.1	YP_003065262	groEL2 chaperonin
58	NP_435642.1	YP_003065261	groES2 chaperonin
59	NP_435689	YP_003065329	NifA transcriptional activator
60	NP_435713.1	YP_003064948	NodG 3-oxoacyl-(acyl carrier protein) reductase
61	NP_435728	YP_003065356	NodM Glucosamine--fructose-6-phosphate aminotransferase
62	NP_435764.1	YP_003064901	Aminotransferase,class III
63	NP_435891.1	YP_003065157	NosF ATPase
64	NP_436007	YP_003064782	EtfA2 electron-transport flavoprotein,alpha-subunit
65	NP_436008	YP_003064781	EtfB2 electron transport flavoprotein,beta subunit
66	NP_436085	YP_003065278	oxidoreductase
67	NP_436098	YP_003065137	CpaF2 pilus assembly protein
68	NP_436214.1	YP_003065009	ABC transporter,ATP-binding protein
69	NP_436219.1	YP_003064845	Aminotransferase
70	NP_436237	YP_003065245	Kup2 Potassium uptake protein
71	NP_436267.1	YP_003064901	Aminotransferase
72	NP_436297.2	YP_003064775	Na ⁺ /H ⁺ antiporter
73	NP_436341.1	YP_003065009	ABC transporter,ATP-binding protein
74	NP_436370	YP_003065245	Hypothetical protein
75	NP_436381.1	YP_003065009	ABC transporter,ATP-binding protein
76	NP_436382.1	YP_003064586	ABC transporter,ATP-binding protein
77	NP_436431.1	YP_003064586	ABC transporter,ATP-binding protein
78	NP_436437.1	YP_003065009	ABC transporter,ATP-binding protein
79	NP_436498.1	YP_003064826	KdpD histidine kinase

Table S2: homologous gene pairs awaiting verification of expression patterns. For each gene pair, whether its promoter is the same as a previous promoter is indicated.

	Protein ID in Sino-rhizobium	Protein ID in Liberibacter	shared promoter with other Sme Protein?	Same promoter with previous CLas Protein?
1	NP_435253.1	YP_003064676		
2	NP_435281.1	YP_003064586		
3	NP_435282.1	YP_003064948	same promoter as NP_435281.1	
4	NP_435305.2	YP_003065051		
5	NP_435311.1	YP_003065261		
6	NP_435312.1	YP_003065326		
7	NP_435342.1	YP_003065326		
8	NP_435352.1	YP_003064586		
9	NP_435360.1	YP_003065009		
10	NP_435419.1	YP_003064948		
11	NP_435423.1	YP_003064750	same promoter with NP_435419.1	
12	NP_435501.1	YP_003064878		
13	NP_435513.1	YP_003064586		
14	NP_435536	YP_003064695		
15	NP_435549.2	YP_003065316		
16	NP_435551.1	YP_003065009		
17	NP_435608	YP_003064907		
18	NP_435624.1	YP_003065009		
19	NP_435637.1	YP_003065326		
20	NP_435641.1	YP_003065262		
21	NP_435642.1	YP_003065261	same promoter with NP_435641.1	
22	NP_435689	YP_003065329		
23	NP_435691	YP_003065437		
24	NP_435692	YP_003064782	same promoter with NP_435691	
25	NP_435693	YP_003064781	same promoter with NP_435691	Same promoter with YP_003064782
26	NP_435712.1	YP_003064946		
27	NP_435713.1	YP_003064948	same promoter with NP_435712.1	
28	NP_435715	YP_003064676		
29	NP_435728	YP_003065356		
30	NP_435764.1	YP_003064901		
31	NP_435771.1	YP_003065017		
32	NP_435818	YP_003065171		
33	NP_435856.2	YP_003065113		
34	NP_435891.1	YP_003065157		

35	NP_435955	YP_003065137		
36	NP_435998.1	YP_003064552		
37	NP_436007	YP_003064782		
38	NP_436008	YP_003064781	same promoter with NP_436007	
39	NP_436023.1	YP_003065157		
40	NP_436029.1	YP_003064586		
41	NP_436058	YP_003064695		
42	NP_436067.1	YP_003065157		
43	NP_436072	YP_003065274		
44	NP_436078	YP_003065267		
45	NP_436079	YP_003065266	same promoter with NP_436078	Same promoter with YP_003065267
46	NP_436080	YP_003065265	same promoter with NP_436078	Same promoter with YP_003065267
47	NP_436081	YP_003065280		
48	NP_436082	YP_003065279	same promoter with NP_436081	Same promoter with YP_003065280
49	NP_436085	YP_003065278	same promoter with NP_436081	Same promoter with YP_003065280
50	NP_436087	YP_003065275	same promoter with NP_436081	Same promoter with YP_003065280
51	NP_436094.1	YP_003065306		
52	NP_436098	YP_003065137		
53	NP_436136	YP_003064695		
54	NP_436145.2	YP_003064586		
55	NP_436146.1	YP_003064552	same promoter with NP_436145.2	
56	NP_436214.1	YP_003065009		
57	NP_436219.1	YP_003064845		
58	NP_436237	YP_003065245		
59	NP_436253.1	YP_003064695		
60	NP_436258.1	YP_003065195		
61	NP_436267.1	YP_003064901		
62	NP_436272.1	YP_003064552	same promoter with NP_436267.1	
63	NP_436297.2	YP_003064775		
64	NP_436341.1	YP_003065009		
65	NP_436370	YP_003065245		
66	NP_436381.1	YP_003065009		
67	NP_436382.1	YP_003064586	same promoter with NP_436381.1	
68	NP_436405.1	YP_003065157		
69	NP_436422.1	YP_003064948		

70	NP_436431.1	YP_003064586	
71	NP_436437.1	YP_003065009	same promoter with NP_436431.1
72	NP_436481	YP_003065165	
73	NP_436485.1	YP_003065492	
74	NP_436495	YP_003065365	
75	NP_436497.1	YP_003065306	
76	NP_436498.1	YP_003064826	same promoter with NP_436497.1
77	NP_436514.1	YP_003064948	
78	NP_436525.1	YP_003065157	
79	NP_436535.1	YP_003064686	

Table S3: GoldenGate Level 2 vectors carrying homologous gene pairs from CLas and PsymA.

Number	Protein ID of <i>Liberibacter</i> gene	Protein ID in <i>Sinorhizobium</i>	Vector ID
1	YP_003064676	NP_435253	MW002
2	YP_003064948	NP_435713	MW003
3	YP_003065051	NP_435305	MW004
4	YP_003065261	NP_435311	MW005
5	YP_003065326	NP_435312	MW006
6	YP_003065009	NP_435551	MW007
7	YP_003064750	NP_435423	MW008
8	YP_003064878	NP_435501	MW009
9	YP_003064695	NP_436253	MW010
10	YP_003065316	NP_435549	MW011
11	YP_003064907	NP_435608	MW012
12	YP_003065329	NP_435689	MW013
13	YP_003064946	NP_435713	MW014
14	YP_003065356	NP_435728	MW015
15	YP_003064901	NP_436267	MW016
16	YP_003065017	NP_435771	MW017
17	YP_003065171	NP_435818	MW018
18	YP_003065113	NP_435856	MW019
19	YP_003065157	NP_435718	MW020
20	YP_003065137	NP_436098	MW021
21	YP_003064552	NP_435492	MW022
22	YP_003065274	NP_436072	MW023
23	YP_003065306	NP_436497	MW024
24	YP_003065245	NP_436237	MW025
25	YP_003065195	NP_436258	MW026
26	YP_003065165	NP_436481	MW027
27	YP_003065492	NP_436485	MW028
28	YP_003064686	NP_436535	MW029

4

Targeting a CLas gene in planta using a rhizobial-legume model

Jing Wang¹, Feng Zhang^{2,3,4}, Rene Geurts¹, Huchen Li^{5,*}, Ton Bisseling^{1,6,*}

¹Laboratory of Molecular Biology, Department of Plant Sciences, Graduate School Experimental Plant Sciences, Wageningen University & Research, Wageningen 6708 PB, the Netherlands.

²Engineering Research Centre of Education Ministry for Germplasm Innovation and Breeding New Varieties of Horticultural Crops, College of Horticulture, Hunan Agricultural University, 410128, Changsha, China.

³Yuelushan Laboratory, Changsha 410128, China.

⁴National Center for Citrus Improvement, College of Horticulture, Hunan Agricultural University, Changsha 410128, China.

⁵College of Grassland, Resources and Environment, Inner Mongolia Agricultural University, Hohhot 010011, China

⁶College of Plant Science and Technology, Beijing University of Agriculture, Beijing, 102206, China

*Corresponding authors: Huchen Li (), Ton Bisseling (ton.bisseling@wur.nl)

Abstract

Huanglongbing (HLB), caused by the phloem-limited bacterium Candidatus Liberibacter asiaticus (CLas), remains one of the most severe threats to global citrus production. Progress in functional validation of CLas genes and drug screening is hindered by the lack of a robust in planta system. Here, we employed the Sinorhizobium meliloti (Sme)–Medicago truncatula root nodule symbiosis as a heterologous platform to investigate the function of the stress-response transcription factor RpoH (RNA polymerase sigma-32 factor) from CLas. Deletion of the native Sme RpoH caused a strong reduction in nodule number, arrest of primordia development, premature senescence, impaired nitrogen fixation, and extensive bacterial death within infection threads. Complementation with CLas RpoH partially rescued these defects, restoring nodule formation and partially improving bacteroid differentiation. Importantly, the previously identified CLas RpoH inhibitor rosiglitazone maleate reverted the complemented strain to the mutant phenotype and significantly reduced CLas titers in citrus seedlings. Together, these findings demonstrate that CLas RpoH is essential for bacterial survival in planta and can be targeted by small molecules. Moreover, the Sme–Medicago root nodule system provides a tractable, high-throughput platform to functionally analyze CLas genes and screen candidate drugs for HLB management.

INTRODUCTION

HLB, also known as citrus greening disease, is caused by three *Liberibacter* species belonging to the α -proteobacteria: CLas, 'Ca. *L. africanus*', and 'Ca. *L. americanus*'. CLas is widely spread and currently threatens citrus production globally (Bové, J. M., 2006).

The obligate biotrophic nature of CLas hampers studies on the mechanism underlying HLB as well as the development of strategies to cure the disease. Current research focuses on blocking the immune activation of citrus or on screening for drugs that inhibit CLas. Ma et al. have shown that applying gibberellins or antioxidants reduces citrus immune response and alleviates HLB symptoms (Ma et al., 2022). However, downregulation of the immune response fails to eliminate CLas and reduces the resistance to other diseases. Further gibberellins can affect the growth of the plant. Progress in validating resistance genes or candidate treatments in citrus has been slowed by the extended time required to establish transgenic citrus lines and to observe reliable disease phenotypes, which often takes 6–12 months after CLas inoculation. For example, Zhao et al. (2025) evaluated *PUB21* and *PUB21DN* functions in citrus trees only after year-long infection assays under greenhouse and field conditions. Such timeframes hinder rapid functional validation and iterative screening of therapeutic candidates. To circumvent the lack of a CLas culture system, two studies have employed *Candidatus Liberibacter crescens* and *Sinorhizobium meliloti*—free-living, culturable Alpha-proteobacteria that are phylogenetically close to CLas within the order Rhizobiales—as surrogate models for drug screening (Barnett et al., 2019; Duan et al., 2009). They evaluated the inhibiting ability of antimicrobial peptides or chemicals on these relatives at the free-living stage. However, CLas lives inside plant phloem sieve elements, an intracellular environment, so different from ex planta culturing. Therefore, there is a need for an efficient in planta system that allows high-throughput screenings.

Sme can establish a nitrogen-fixing root nodule symbiosis with for example, *Medicago truncatula* (*Medicago*) (Geurts and Bisseling, 2002). In root nodules, Sme is intracellularly hosted, like CLas. Nodule formation involves two major processes, nodule organogenesis and rhizobial infection. Nodule organogenesis in *Medicago* starts with the mitotic activation of pericycle cells, and this is followed by cell division in the inner cortex and endodermis. In this way, a nodule primordium is formed (Xiao et al., 2014). The infection process starts with the curling of root hairs, by which the rhizobia become entrapped in the curl. There, they induce the formation of a tube-like structure. This is the infection thread, by which the bacteria can enter the root hair and subsequently the infection threads traverse outer cortical cells towards nodule primordia. When the infection thread has reached a primordium, the cells at the apex continue to divide and form an apical meristem. The other cells stop dividing, and rhizobia are released from the branched infection thread into the cytoplasm of primordium cells. During this process, they become surrounded by a membrane of the host, and in this way, symbiosomes are formed (Roth and Stacey, 1989). Subsequently, the meristem continu-

ously adds new cells to the different nodule tissues, including the central tissue containing the infected cells. The infection threads enter cells derived from the meristem, and rhizobial release occurs in 1-2 cell layers adjacent to the meristem (Moling et al., 2014). Upon release, the symbiosomes divide. Ultimately, the infected cells contain thousands of symbiosomes (Jones et al., 2007). The rhizobia in the infected cells are named bacteroids, which undergo significant morphological and physiological changes compared to their free-living form, including enlargement and a loss of reproductive capability (Mergaert et al., 2006).

Due to the indeterminate growth of *Medicago* nodules, a gradient of developmental stages is present from the apical meristem to the root attachment point. Therefore, the nodules can be divided into different zones (Mergaert et al., 2006). Adjacent to the apical meristem is the infection zone. There rhizobia are released, and symbiosomes divide and differentiate into elongated structures. The infection zone is followed by the fixation zone, visualized by starch granule accumulation at the cell periphery of the infected cells. At the transition of infection into fixation zone, the *nif* genes are expressed by which the symbiosomes differentiate into nitrogen-fixing “organelles”. 6-8 cell layers at the basis of the fixation zone are derived from the nodule primordium, whereas all other cell layers are derived from the meristem (Xiao et al., 2014). Therefore, nodule formation provides an in planta system by which the intracellular life of Sme could be studied. Based on the intracellular lifestyle of Sme and its close phylogenetic relationship with CLas nodules have the potential to be an in planta system to study CLas genes.

The orthologous transcription factors (TFs) of CLas and Sme have been identified. When heterogeneously expressed 5 CLas TFs in Sme mutants of corresponding orthologs could (partly) rescue the growth defect of free-living Sme mutants under certain stresses. More importantly, small molecules that specifically inhibit 3 of the 5 TFs (*VisNR*, *LdtR* and *RpoH*) were identified. Here, we used the root nodule symbiosis as an in planta system to examine whether CLas TFs can be targeted in planta with the previously identified molecules.

RESULTS

1. Mutation of *SmeRpoH* affects nodule formation.

To test whether mutation of *VisNR*, *LdtR*, or *RpoH* ($\Delta visNR$, $\Delta ldtR$, or $\Delta RpoH$) in Sme has a nodule phenotype, they were inoculated on *Medicago* seedlings. *RpoH* is encoded by two homologous genes (*RpoH1* and *RpoH2*) in Sme, so the $\Delta RpoH$ strain we used was a double mutant. Nodule number was counted at 14 days post-inoculation (dpi). Wild-type (WT) Sme formed on average 24.5 nodules per plant. $\Delta ldtR$, $\Delta visNR$ mutants formed a similar number of nodules as WT. Their mature nodules were, in general, elongated and pink, resembles to that of WT (Fig. 1A). In contrast, the $\Delta RpoH$ mutant formed, on average, 15.4 nodules, which was significantly lower than that of WT (Fig. 1G). $\Delta RpoH$ nodules were

rather small, spherical, and white (Fig. 1B), indicating a developmental defect. One extra orthologous TFs (*LsrB*), for which no inhibitor was identified (Melanie et al., 2019), also resulted in a reduced nodule number and developmental defect. Given that *RpoH* inhibiting drugs have been identified and has nodule phenotype, we selected $\Delta RpoH$ for further study.

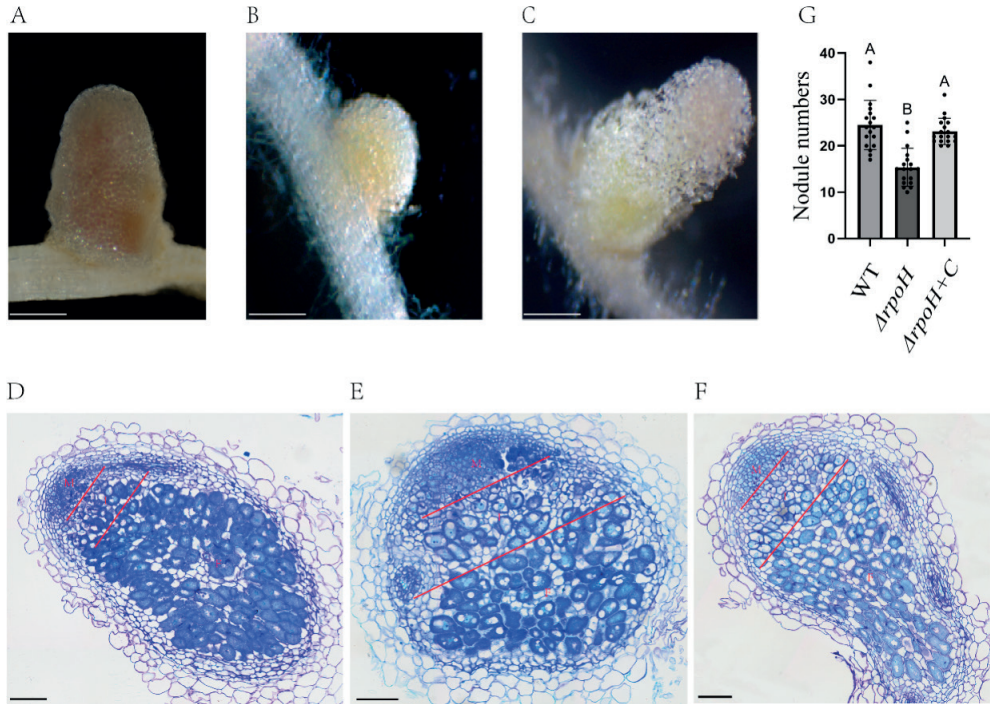


Figure 1: The $\Delta RpoH$ mutation reduces nodule number and alters nodule morphology (spherical), while *CLas* *RpoH* restores nodule formation.

A-C: Phenotype of 14 days nodules formed with WT (A), $\Delta RpoH$ Sme (B) and $\Delta RpoH RpoH_{CLas}$ (C). Bar: 2mm

D-F: Longitudinal sections of 14-day-old nodules with WT (D), $\Delta RpoH$ Sme (E) and $\Delta RpoH RpoH_{CLas}$ (F). Bar: 100 μ m. Red lines indicate the boundaries between the meristem (M), infection zone (I), and fixation zone (F).

G: The number of nodules counted 14 days

2. $\Delta RpoH$ causes an arrest at an early stage of nodule primordium development.

The reduced nodule number induced by $\Delta RpoH$ might be due to an arrest at an early stage of nodule primordium development. To test whether nodule primordium development was affected, the *Medicago ENOD11::GUS* stable line was used. The *ENOD11* gene is expressed in nodule primordia, but not in lateral root primordia, by which the two primordium types can easily be distinguished. WT Sme and $\Delta RpoH$ mutants were spot inoculated at the susceptible zone of ~40 roots. At 5 dpi, the root segment at the inoculation site was harvested and fixed af-

ter GUS staining. Nodule primordia were analyzed in longitudinal sections. The nodule primordium developmental stages were classified according to Xiao et al.. If a root segment had more than one nodule primordium, only the most developed one is counted. In roots inoculated with WT Sme, 88.4% of the nodule primordia reached at least stage III, and 62.8% of the nodule primordia reached at least stage V. In contrast, the majority of nodule primordia induced by $\Delta RpoH$, were in stage I or stage II (56%), and only a few (16%) primordia had developed to stage IV or V (Fig. 2A). In addition to this, we used EdU staining, to mark cells that were synthesizing DNA, in nodule primordia at 5dpi (Li et al., 2021). In this way, we could detect whether the development of the nodule primordia was arrested. All WT primordia had labeled cells, and about 44% of primordium cells were stained. A few (3 out of 29) $\Delta RpoH$ nodule primordia had no labeled cells, and the percentage of labeled cells was reduced to 19% in all $\Delta RpoH$ primordia (Fig. 2B). This showed that some of the nodule primordia induced by $\Delta RpoH$ were blocked at an early stage of development, or the reduce number of EdU stained cells means that their development is slow and they might become arrested at a later stage of primordium development, resulting in a reduced nodule number at 14dpi (Fig. 1G).

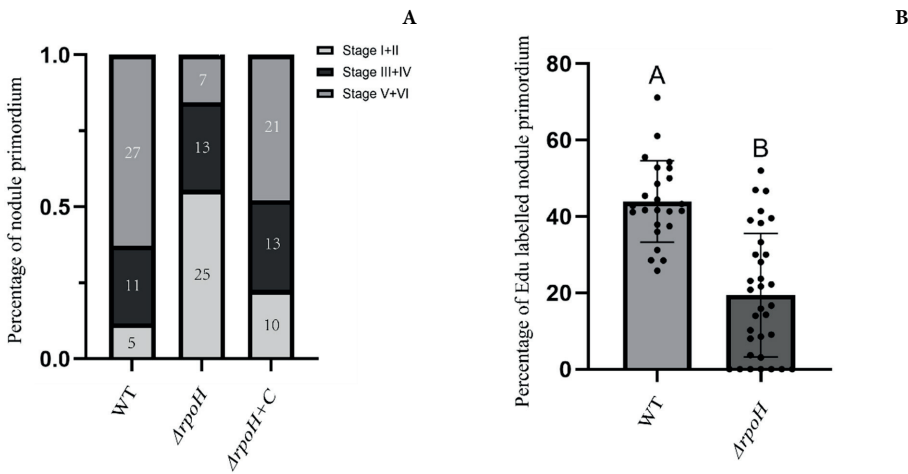


Figure 2: The $\Delta RpoH$ mutation delays nodule primordium development, whereas CLas $RpoH$ restores normal development.

A: Nodule primordium formed by WT, $\Delta RpoH$ Sme and $\Delta RpoH+RpoH_{CLas+}$. The number on each stage indicate the number of primordium that developed to that stage.

B: The percentage of cells labelled by EdU in each nodule primordium. The horizontal lines mark the mean values, and the vertical lines indicate the standard deviation.

3. $\Delta RpoH$ mutation affects the development of bacteroids.

The reduced nodule size and an arrest at the primordium stage strongly suggest that the viability of the Sme $\Delta RpoH$ strain was affected in the nodule (primordium). To examine whether this is the case, we analyzed semi-thin longitudinal sections of nodules collected at 14 dpi. $\Delta RpoH$ nodules have, like WT nodules, an apical meristem of ~6 cell layers (Figs. 1D,

E). At the transition from infection to fixation zone, starch granules rapidly accumulate in the infected cells (Redondo et al., 2009). Therefore, to identify the nitrogen-fixing zone, we used Lugol to stain starch (Trinick M J, 1973, Fig. S1). Longitudinal sections of WT nodules ($n=20$) showed that they had 17.7 ± 4.3 cell layers in the fixation zone. In $\Delta RpoH$ nodules, 60% (24/40) of them showed a premature senescence. The fixation zone of the other 40% (16/40) $\Delta RpoH$ nodules, in which premature senescence had not occurred, was composed of 7.8 ± 2.4 layers (Fig. 3C). These layers are not derived from the apical meristem, but most likely from the nodule primordium. This suggests that cells derived from the meristem of $\Delta RpoH$ nodules did not (yet) develop into fixation zone cells. In the 1–2 cell layers adjacent to the meristem within the infection zone, Sme is released from infection threads, and they become elongated in the proximal infection zone and during the switch to the fixation zone. To determine whether bacteroid development in the fixation zone was affected, we quantified the length of bacteroids, as well as the expression level of *NifH*, by real-time qPCR. The length of bacteroids in the fixation zone in WT nodules was $13.9 \pm 1.1 \mu\text{m}$ ($n=$), whereas in $\Delta RpoH$ nodules this was significantly reduced to $6.2 \pm 0.5 \mu\text{m}$ (Fig. 3D). Additionally, in $\Delta RpoH$ nodules, *NifH* expression level was about 2% of that of WT nodules (Fig. 3E).

As cells in the infection zone of $\Delta RpoH$ nodules are unable to develop into the fixation zone, we questioned whether these bacteroids are alive. To investigate this, we performed the live/dead staining of bacteria. In all WT ($n=20$) nodules, the bacteria were alive both in the infection thread and in the infected cells (Fig. 3A). In 19 out of 20 $\Delta RpoH$ nodules, a large number of rhizobia in the infection threads are dead. However, a small number of viable rhizobia were still observed within the infection threads (Fig. 3B). In the fixation zone (of all $\Delta RpoH$ nodules), prior to the onset of early senescence, the bacteroids within the infected cells are alive. However, in the infection threads within the fixation zone, many Sme cells are dead. In nodules where early senescence has occurred, no fluorescent signals of dead bacteroids are observed in the infected cells within the fixation zone, likely due to DNA degradation of the dead bacteroids.

In conclusion, our findings show that in $\Delta RpoH$ nodules, many Sme's in the infection threads are dead. In the fixation zone of $\Delta RpoH$ nodules, bacteroids remain alive until early senescence, but many Sme die within the infection threads. These data support the notion that *RpoH* TF is needed for Sme to survive in planta.

4. Gene expression of Sme $\Delta RpoH$ in nodules/*RpoH* targets at free living stage display different expression in nodules

It has been shown that 4 heat shock genes (*GroEL*, *IbpA*, *Smc03794*, and *ThiC*), 2 ROS response-related genes (*OxyR* and *Smb20964*), and 2 pH response-related genes (*DctA* and *Smc01505*) are positively regulated by *RpoH* in free-living Sme under certain stress conditions (de Lucena, 2010; Ono et al., 2001; Lehman and Long, 2018). We studied whether their regulation still depended on *RpoH* within nodules. We extracted total RNA from WT and

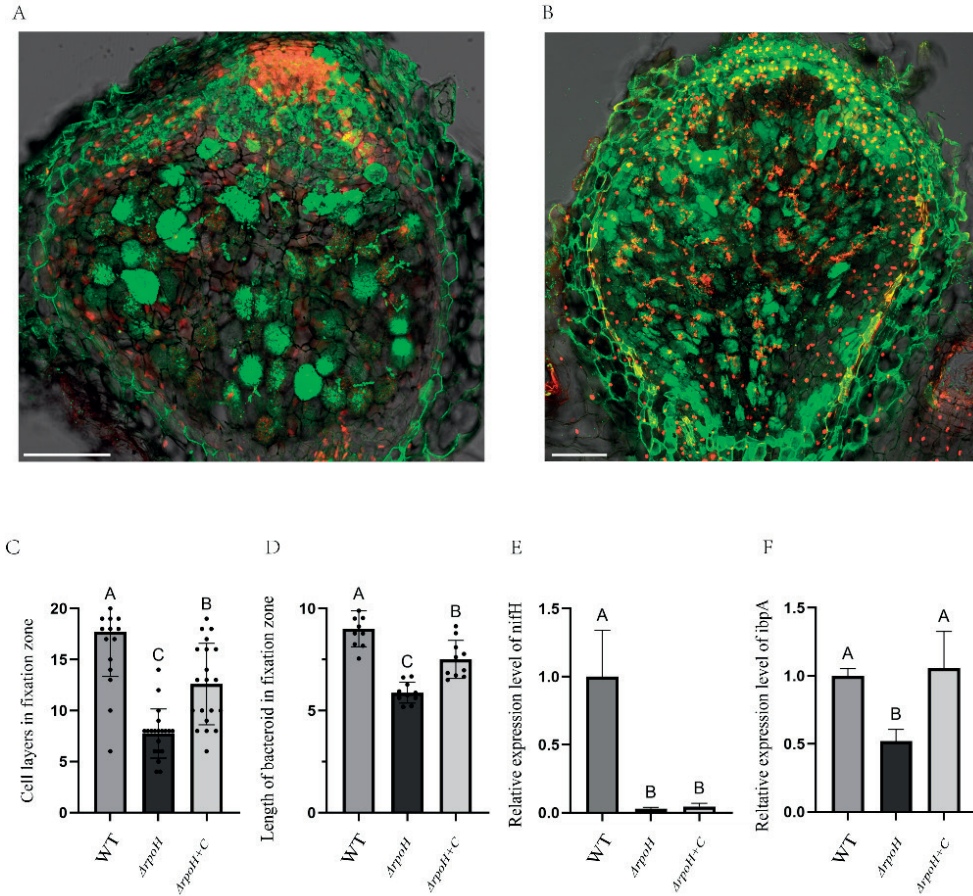


Figure 3: Complementation of $\Delta RpoH$ with CLas *RpoH*

The $\Delta RpoH$ mutation leads to incomplete bacteroid differentiation, while CLas *RpoH* partially restores bacteroid development

A-B: Longitudinal sections of 14-day-old nodules live/dead staining. In WT (A), Sme in ITs and infected cells are alive (green), whereas in $\Delta RpoH$ (B), there is massive death (red) of Sme in ITs. Bar: 100 μm.

C: Statistics of cell layers in the fixation zone of 14-day-old nodules.

D: Statistics of bacteroid length (μm) of 14-day-old nodules.

E: Relative expression level of the *nifH* gene in 14-day-old nodules.

F: Relative expression level of the *ibpA* gene in 14-day-old nodules.

$\Delta RpoH$ nodules at 14 dpi. qPCR showed that *IbpA*, *Smc03794*, *ThiC*, and *DctA* exhibited decreased expression in $\Delta RpoH$ compared to WT nodules (Fig. 3F and S2). In contrast, *Smc01505* showed a substantial increase in expression in the $\Delta RpoH$. Other genes, including *GroEL*, *OxyR*, and *Smb20964*, had a similar expression in WT and mutant (Fig. S2). Thus, several genes that are positively regulated by *RpoH* in free-living Sme exhibit distinct expression patterns in nodules, demonstrating that *RpoH* controls a different set of targets in planta.

5. CLas *RpoH* can partially complement nodulation defects caused by $\Delta RpoH$

As described above, $\Delta RpoH$ causes several nodule phenotypes: nodule number is decreased, a reduction of the number of cell layers in the fixation zone, bacteroids remain rather small in the fixation zone, markedly reduced expression of *NifH* and 4 genes that are regulated by *RpoH* have a markedly reduced expression level, traits that possibly could be complemented by CLas *RpoH* and thus can serve as indicators for drug testing. To test this, we introduced the *RpoH_{CLas}* gene of CLas driven by the Sme *RpoH* promoter, in the Sme $\Delta RpoH$ mutant. This strain, ($\Delta RpoH_{RpoHCLas}$ ($\Delta RpoH + pSme::RpoH_{CLas}$)) was inoculated on *Medicago*, and formed on average 23.1 nodules per plant (n=14) at 14 dpi. The nodule number equals that formed with the Sme WT strain (24.5 ± 5.3 , Fig. 1G). We also studied whether nodule primordium development could be rescued at 5 dpi. 77% (34/44) nodule primordia developed beyond stage II and 48% (21/44) of these developed to stage IV or stage V. This is similar to WT (Fig. 2A). 20 out of 50 nodules that were formed by $\Delta RpoH_{RpoHCLas}$ were elongated, and on average of 12.6 (26 nodules) cell layers were present in their fixation zone (Fig. 3C). This was lower than in the WT (17.7), but significantly higher than in the $\Delta RpoH$ mutant (7.8). In the fixation zone, the length of the bacteroid was $\sim 8.1 \mu\text{m}$ (Fig. 3D), lower than the WT ($\sim 13.92 \mu\text{m}$) but higher than the $\Delta RpoH$ mutant ($\sim 6.2 \mu\text{m}$). Live/death staining showed that half of the $\Delta RpoH_{RpoHCLas}$ nodules (10 out of 20) had no death of Sme in the infection thread, whereas in 19 nodules, $\Delta RpoH$ Sme in infection threads are dead. As shown in Fig. 3E, *NifH* was hardly expressed in $\Delta RpoH_{RpoHCLas}$ and of the genes regulated by *RpoH*, only the expression of *IbpA* was rescued (Fig. 3F).

In conclusion, introducing the *RpoH* gene from CLas into the Sme $\Delta RpoH$ mutant partially restored nodule traits, including nodule number, primordium development, and the number of cell layers in the fixation zone. Although bacteroid length and cell viability improved, they did not fully reach WT levels. Additionally, only *IbpA* expression was rescued, whereas the expression level of *NifH* and other *RpoH*-regulated genes remained low. This suggests that because CLas *RpoH* can partially substitute for native Sme *RpoH*, it may serve as a functional proxy for drug testing.

6. Rosiglitazone maleate inhibits CLas *RpoH* in root nodules

Previously, it was shown that bortezomib, ChemDiv D244-0326, and rosiglitazone maleate can effectively inhibit *RpoH* in freeliving bacteria. We first tested whether these drugs can inhibit Sme's *RpoH*. Ten-day-old plants were inoculated with Sme. At 3dpi, a final concentration of 20 μM of the drug was applied to the plant roots, thereby avoiding direct contact between the drug and Sme. Due to the severe plant growth inhibition caused by bortezomib, we did not use it in subsequent tests. When drugs were added to the root, 20 μM rosiglitazone maleate caused defects in nodule development, leading to a decrease in the number of nodules (Fig. 4A, 15.6 compared to 25.1 of Mock), and most of them were spherical white nodules (72.2% compared to 18.9% of Mock). In contrast, ChemDiv D244-0326 had

no effect on nodule formation (nodule numbers: 24.8, 91.3% pink nodules). To detect the inhibitory effect of rosiglitazone maleate on CLas *RpoH*, we used the same method to apply rosiglitazone maleate to the *Medicago* roots inoculated with $\Delta RpoH_{RpoHCLas}$. This showed that 20 μ M rosiglitazone maleate causes the percentage of elongated nodules of $\Delta RpoH_{RpoHCLas}$ root nodules (Fig. 4A, 25.6%) to revert to the $\Delta RpoH$ phenotype (6.1%). The fixation zone of $\Delta RpoH_{RpoHCLas}$ nodules with rosiglitazone maleate treatment has fewer cell layers (7.9 ± 2.4 , $n=20$), resembling to that in $\Delta RpoH$ nodules (Fig. 3C). Further, we quantified the expression level of *IbpA*. 3 *Medicago* plants were examined with nodules formed by $\Delta RpoH_{RpoHCLas}$ without drug treatment, and 3 plants after the addition of rosiglitazone maleate. This showed that 20 μ M rosiglitazone maleate reduced expression of *IbpA* (Fig. 4B).

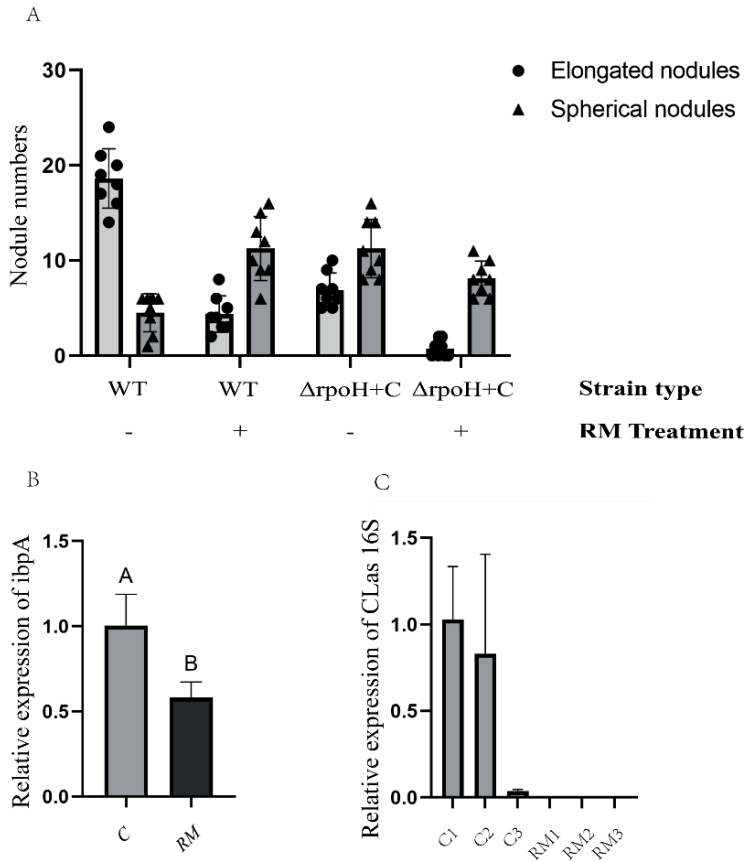


Figure 4: Rosiglitazone maleate suppresses *RpoH*

Rosiglitazone maleate suppresses *RpoH* activity in both nodules and citrus tissues.

A: Rosiglitazone maleate caused a reduction in nodule number and alterations in nodule morphology at 14 days.

B: Rosiglitazone maleate suppresses the expression of the *ibpA* gene of *S. meliloti* in root nodules.

C: Rosiglitazone maleate reduces CLas titers in infected citrus seedlings.

7. Rosiglitazone maleate inhibits CLas *RpoH* in Citrus seedlings

To evaluate whether rosiglitazone maleate exerts a similar inhibitory effect on CLas *RpoH* in citrus roots, we tested its activity in CLas-infected citrus seedlings. Utilizing the rapid citrus seedling transformation system developed in Chapter 2, CLas-infected citrus scions were grafted onto 10-day-old *C. maxima* seedlings. At 40 days post-grafting, six seedlings exhibiting similar HLB symptoms and growth status were selected for the assay. Three seedlings were cultured in liquid medium supplemented with 60 μ M rosiglitazone maleate, while the remaining three were maintained in medium without the compound. After 60 days, CLas was detected in the roots of all seedlings grown in medium without rosiglitazone maleate, whereas no CLas was detected in the roots of treated seedlings (Fig. 4C).

DISCUSSION

In this study, we demonstrated that the life of Sme in *Medicago* root nodules relied on the *RpoH* transcription factor. Most Sme *RpoH* cannot survive in the infection threads, after release, the alive ones cannot fully differentiate and have an early senescence phenotype. Complementation by CLas *RpoH* can partially rescue the developmental defects of *Medicago* $\Delta RpoH$ nodules. The application of rosiglitazone maleate to the roots of *Medicago* effectively inhibited Sme and CLas *RpoH* activity within the nodules, indicated by a reduction of *IbpA* expression as well as a similar nodule phenotype to $\Delta RpoH$. Further the application of rosiglitazone maleate significantly reduced the abundance of CLas in citrus. We conclude that the *S. meliloti*–*Medicago* root nodule system provides a practical and tractable in planta platform for evaluating drugs targeting CLas.

Targets of *RpoH* show different expression between free living stage and root nodules, only *IbpA* is consistent, indicating that an in planta method is needed.

In addition to *RpoH*, we found that CLas *LsrB* could compensate for the impaired root nodule formation observed in the Sme $\Delta lsrB$ mutant (Fig. S1), indicating that the *Medicago* nodulation system may also be suitable for testing drugs targeting CLas *LsrB*. So far, only six CLas TFs orthologs have been examined under free-living conditions. However, if future knockout mutants of the corresponding Sme TFs exhibit distinct nodule phenotypes, they could also be incorporated into the drug screening pipeline. For example, Sme *CpdR1*—an orthologue of a CLas regulator—was shown to affect nodule development, and its loss-of-function mutation leads to unelongated nodules and altered nodule morphology (Kobayashi et al., 2009).

Our study was focused on the ability of drugs to target CLas *RpoH* in root nodules. This required a more detailed analysis of the $\Delta RpoH$ nodule phenotype, and this provided some novel insight into the role of *RpoH* in nodulation. Previous studies have reported that mutations in Sme's *RpoH* lead to premature nodule senescence. This is confirmed by our study. We

also observed that several genes related to immune response and senescence are upregulated, whereas defense suppression genes are downregulated (Fig. S1).

We showed that *RpoH*, in nodules, is the highest expressed in infection threads (Fig. S2). Previously, it was shown that *RpoH* is expressed in the complete nodule (Oke et al., 2001). This study was done on intact nodules by which most likely the expression in infection threads could not be observed. The high expression level in infection threads indicates that the rhizobia are exposed to stress. This is also shown by the death of rhizobia in the infection threads. It is unclear what kind of stress they experience.

In WT nodules, the bacteroid undergoes a major developmental switch at the transition from infection to fixation zone; among others, they rapidly express *nif* genes and their length markedly increases. This developmental switch seems to be almost completely blocked in the $\Delta RpoH$ nodules. The underlying reason is not clear, but it might be the sub-optimal physiological condition of the bacteroids.

The ultimate goal for controlling HLB is to cultivate HLB-resistant citrus varieties, a task that is extremely challenging due to the lack of knowledge about the molecular interactions between CLAs and citrus phloem tissues. Utilizing the extensive knowledge of Rhizobiaceae, close relatives of CLAs, and using *Sinorhizobium meliloti* as a heterologous model for research is an important interim measure. Here, we introduce the *Sinorhizobium-Medicago* symbiotic nodulation system as a method to further study the interactions between CLAs genes and plants. This approach could inspire future tests of drugs, the effects of CLAs genes on the host, and the antagonistic effects of citrus resistance genes on CLAs genes.

METHODS

Plant material and inoculation with rhizobia

Medicago truncatula ecotype Jemalong A17 and the ENOD11::GUS stable reporter line (Journet et al., 2001) were used in this study. Seedlings were cultivated either in perlite saturated with low-nitrate Fåhræus medium [0.25 mM $\text{Ca}(\text{NO}_3)_2$] as described by Catoira et al. (2000), or on agarose-based BNM medium plates (Ehrhardt et al., 1992), and maintained at 21°C under a 16 h light/8 h dark photoperiod. *Sinorhizobium meliloti* CL150 cultures were induced with 10 mM luteolin for 24 h prior to inoculation. Mature nodules were collected at 14 dpi from plants grown in perlite, whereas nodule primordia were harvested at 5 dpi from spot-inoculated roots of plants grown on BNM plates.

Constructs

The *Sinorhizobium meliloti* wild-type strain and the deletion mutants $\Delta visNR$, $\Delta ldtR$, $\Delta RpoH$, and $\Delta lsrB$ were previously generated and described by Barnett et al. (2019). The complemen-

tation construct for $\Delta RpoH$ was assembled using the Golden Gate–based MoClo Tool Kit. The *Sinorhizobium RpoH* coding sequence together with its native promoter was amplified by PCR from boiled liquid cultures of *Sinorhizobium* CL150(wild type). The *Liberibacter RpoH* coding sequence and its promoter region were synthesized by Twist Bioscience. For both species, promoter regions were defined as the 2-kb sequence upstream of the start codon. The Level 2 vector pOGG026 was used as the final expression construct.*RpoHRpoH*

Gene expression and RNA-Seq

Total RNA from nodules was isolated using the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized from 1 μ g of total RNA using random hexamer primers and the iScript Select cDNA Synthesis Kit (Bio-Rad), following the manufacturer's instructions. Quantitative real-time PCR was performed in 10- μ L reaction volumes using SYBR Green Supermix (Bio-Rad). *rkpK* and *16S* rRNA were used as reference genes.

Three independent biological replicates were analyzed. For RNA-seq, total RNA was extracted as described above. RNA sequencing was performed by BGI Tech Solutions (Hong Kong) on a HiSeq 2000 platform. Reads were mapped to the *Medicago truncatula* reference genome using CLC Genomics Workbench (QIAGEN, Denmark), and transcript abundance was quantified as transcripts per million (TPM).

EdU staining

Medicago truncatula seedlings were grown on BNM plates and spot-inoculated with *Sinorhizobium meliloti* CL150. After 5 days, inoculated root segments (0.3 cm) were excised and submerged in liquid BNM medium supplemented with 1 g L⁻¹ D-glucose. Samples were incubated with 10 mM EdU for 2 h on a shaker. Subsequent washing and staining steps were performed according to the manufacturer's instructions for the Click-iT EdU Imaging Kit (Thermo Fisher).

Microscopy and imaging

Root fragments and nodules were fixed in 4% (w/v) paraformaldehyde and 5% (v/v) glutaraldehyde in 50 mM phosphate buffer (pH 7.4). Samples were then washed three times for 15 min each with 0.1 M phosphate buffer, washed once with water for 15 min, and dehydrated for 10 min in a graded ethanol series (10%, 30%, 50%, 70%, 90%, and 100%, v/v). Tissues were subsequently embedded in Technovit 7100 resin (Heraeus Kulzer). Sections of 5 μ m thickness were cut using a Leica RJ2035 microtome, stained with 0.05% Toluidine Blue (Sigma), mounted in Euparal (Carl Roth), and examined using a Leica AU5500B microscope equipped with a DFC425c camera.

Confocal imaging was performed using a Leica SP8 confocal laser scanning microscope (Leica, Germany). SYTOX Green (Live/Dead assay) and EdU fluorescence were detected

using an excitation wavelength of 488 nm. Propidium iodide (Live/Dead assay) was detected using an excitation wavelength of 536 nm.

Live/Dead staining

Live/dead staining of nodules was performed using the LIVE/DEAD Cell Viability Kit (Thermo Fisher) according to the manufacturer's instructions.

Drug test in citrus

To evaluate the inhibitory effect of rosiglitazone maleate on CLas *RpoH* within citrus roots, a rapid seedling-based evaluation system was employed. Fresh *Citrus maxima* seeds were prepared by manually removing the outer seed coats (testae) and placed in Petri dishes lined with moistened filter paper for germination. Upon the radicles reaching a length of approximately 2 cm, the germinated seedlings were transferred to a hydroponic system for further growth. Following a 10-day establishment period in the hydroponic culture, CLas-positive infected scions were grafted onto these *C. maxima* seedlings using the rapid citrus seedling transformation .

At 40 dpi, six seedlings exhibiting uniform HLB symptoms and consistent growth status were selected for the pharmacological assay. Three of these seedlings were cultured in liquid medium supplemented with 60 μ M rosiglitazone maleate, while the remaining three seedlings served as a control group, maintained in the same medium without the addition of the compound. After an additional 60 days of treatment, root tissues were harvested from all experimental plants. The presence and bacterial titer of CLas in the roots were subsequently quantified via qPCR to determine the in vivo efficacy of the treatment.

REFERENCES

- Barnett, M. J., Solow-Cordero, D. E., & Long, S. R. (2019). A high-throughput system to identify inhibitors of *Candidatus Liberibacter asiaticus* transcription regulators. *Proceedings of the National Academy of Sciences*, 116(36), 18009–18014.
- Bové, J. M. (2006). Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. *Journal of Plant Pathology*, 88(1), 7–37.
- de Lucena, D. K. C., Pühler, A., & Weidner, S. (2010). The role of sigma factor *RpoH1* in the pH stress response of *Sinorhizobium meliloti*. *BMC Microbiology*, 10, 265.
- Duan, Y., Zhou, L., Hall, D. G., Li, W., Dodapaneni, H., Lin, H., Liu, L., Vahling, C. M., Gabriel, D. W., Williams, K. P., Dickerman, A., Sun, Y., & Gottwald, T. (2009). Complete genome sequence of citrus huanglongbing bacterium, “*Candidatus Liberibacter asiaticus*,” obtained through metagenomics. *Molecular Plant-Microbe Interactions*, 22(8), 1011–1020.
- Geurts, R., & Bisseling, T. (2002). Rhizobium Nod factor perception and signalling. *The Plant Cell*, 14(Suppl.), S239–S249.
- Huang, C.-Y., Araujo, K., Niño Sánchez, J., Kund, G., Trumble, J., Roper, C., Godfrey, K. E., & Jin, H. (2021). A stable antimicrobial peptide with dual functions of treating and preventing citrus Huanglongbing. *Proceedings of the National Academy of Sciences*, 118(6), e2019628118.
- Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., & Walker, G. C. (2007). How rhizobial symbionts invade plants: The *Sinorhizobium-Medicago* model. *Nature Reviews Microbiology*, 5(8), 619–633.
- Kobayashi, H., De Nisco, N. J., Chien, P., Simmons, L. A., & Walker, G. C. (2009). *Sinorhizobium meliloti* CpdR1 is critical for co-ordinating cell cycle progression and the symbiotic chronic infection. *Molecular Microbiology*, 73(4), 586–600.
- Lehman, A. P., & Long, S. R. (2018). OxyR-dependent transcription response of *Sinorhizobium meliloti* to oxidative stress. *Journal of Bacteriology*, 200(7), e00622–17.
- Li, H., Schilderink, S., Cao, Q., Kulikova, O., & Bisseling, T. (2021). Plant-specific histone deacetylases are essential for early and late stages of *Medicago* nodule development. *Plant Physiology*, 186(3), 1591–1605.
- Ma, W., Pang, Z., Huang, X., Xu, J., Pandey, S. S., Li, J., Achor, D. S., Vasconcelos, F. N. C., Hendrich, C., Huang, Y., & Wang, W. (2022). Citrus Huanglongbing is a pathogen-triggered immune disease that can be mitigated with antioxidants and gibberellin. *Nature Communications*, 13, 529.
- Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Kondorosi, A., & Kondorosi, E. (2006). Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium–legume symbiosis. *Proceedings of the National Academy of Sciences*, 103(13), 5230–5235.
- Moling, S., Pietraszewska-Bogiel, A., Postma, M., Fedorova, E. E., Hink, M. A., Limpens, E. H. M., Gadella, T. W. J., & Bisseling, T. (2014). Nod factor receptors form heteromeric complexes and are essential for intracellular infection in *Medicago* nodules. *The Plant Cell*, 26(10), 4188–4199.
- Oke, V., Rushing, B. G., Fisher, E. J., Moghadam-Tabrizi, M., & Long, S. R. (2001). Identification of the heat-shock sigma factor *RpoH* and a second *RpoH*-like protein in *Sinorhizobium meliloti*. *Microbiology*, 147(9), 2399–2408.
- Ono, Y., Mitsui, H., Sato, T., & Minamisawa, K. (2001). Two *RpoH* homologs responsible for the expression of heat shock protein genes in *Sinorhizobium meliloti*. *Molecular and General Genetics*, 264(6), 902–912.
- Redondo, F. J., de la Peña, T. C., Morcillo, C. N., Lucas, M. M., & Pueyo, J. J. (2009). Overexpression of flavodoxin in bacteroids induces changes in antioxidant metabolism leading to delayed senescence and starch

accumulation in alfalfa root nodules. *Plant Physiology*, 149(2), 1166–1178.

- Roth, L. E., & Stacey, G.** (1989). Bacterium release into host cells of nitrogen-fixing soybean nodules: The symbiosome membrane comes from three sources. *European Journal of Cell Biology*, 49(1), 13–23.
- Trinick, M. J.** (1973). Symbiosis between *Rhizobium* and the non-legume, *Trema aspera*. *Nature*, 244, 459–460.
- Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., Farkas, A., Mikulass, K., Nagy, A., Tiricz, H., Satiat-Jeunemaitre, B., Alunni, B., Bourge, M., Kucho, K.-I., Abe, M., Kereszt, A., Maroti, G., Uchiumi, T., Kondorosi, E., & Mergaert, P.** (2010). Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science*, 327(5969), 1122–1126.
- Xiao, T. T., Schilderink, S., Moling, S., Deinum, E. E., Kondorosi, E., Franssen, H., Kulikova, O., Niebel, A., & Bisseling, T.** (2014). Fate map of *Medicago truncatula* root nodules. *Development*, 141(18), 3517–3528.
- Zhao, P., Yang, H., Sun, Y., Zhang, J., Gao, K., Wu, J., Zhu, C., Yin, C., Chen, X., Liu, Q., et al.** (2025). Targeted *MYC2* stabilization confers citrus Huanglongbing resistance. *Science*, 388(6743), 191–198.

SUPPLEMENTARY DATA

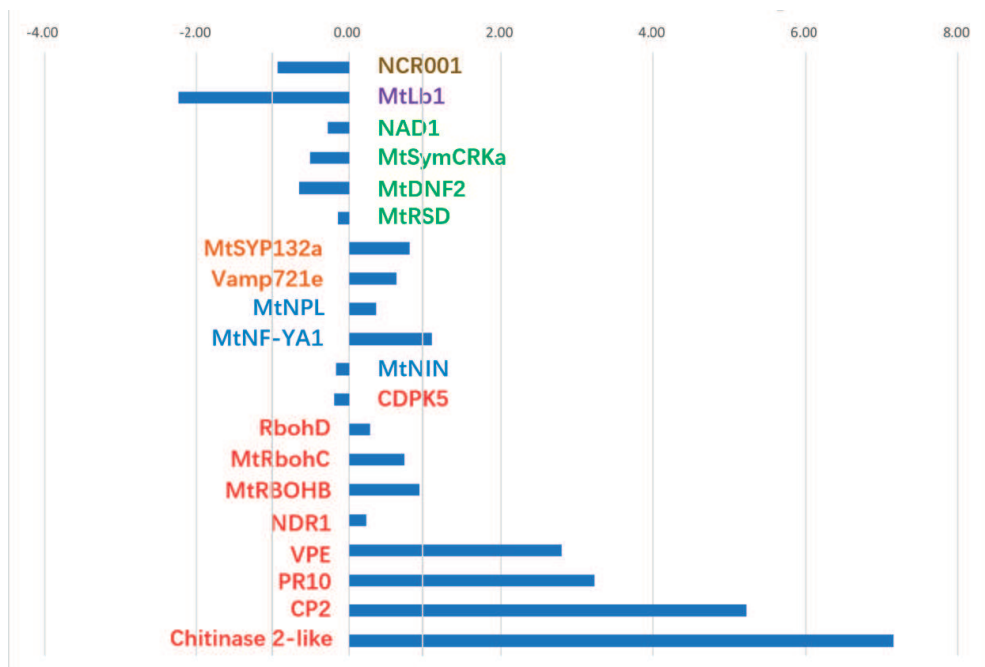


Figure S1: The transcriptome of the nodules formed with $\Delta RpoH$ mutant shows enhanced defense. Fold change is indicated in log2 scale. Green: Genes involved in suppressing the immune response; Brown: Genes related to the release of Sme from infection threads; Blue: Key transcription factors involved in nodule formation; Red: Genes related to immune response and senescence.

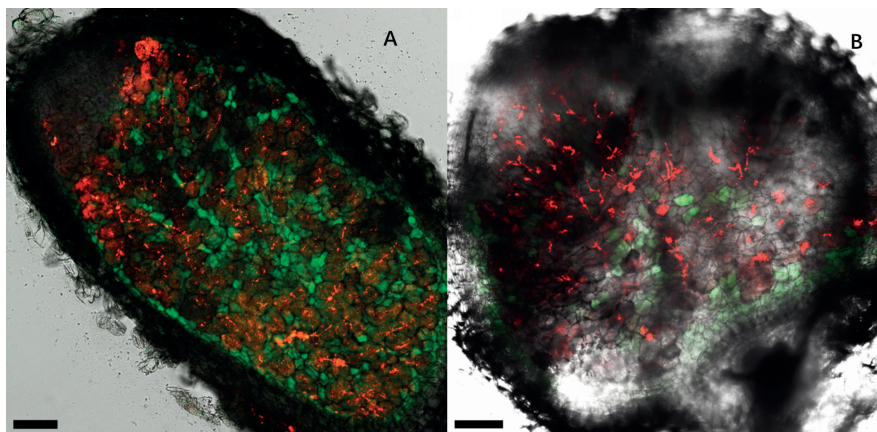


Figure S2: Expression of *RpoH-mCherry*

Expression of the *mCherry* driven by the *RpoH* promoter in 14-day-old nodules.

A: in the background of WT Sme.

B: in the background of $\Delta RpoH$ Sme.

Bar: 100 μ m.

5

Phylogenetic analysis based on most conserved AMPHORA proteins shows that *Liberibacter* is nested in the *Rhizobium* genus

Jing Wang¹, Joel Klein¹, Lucas Schmitz¹, Ton Bisseling^{1,2,*}, Rene Geurts^{1,*}

¹Laboratory of Molecular Biology, Department of Plant Sciences, Graduate School Experimental Plant Sciences, Wageningen University & Research, Wageningen 6708 PB, the Netherlands.

²College of Grassland, Resources and Environment, Inner Mongolia Agricultural University, Hohhot 010011, China

*Corresponding authors: Rene Geurts (rene.geurts@wur.nl), Ton Bisseling (ton.bisseling@wur.nl)

Abstract

Huanglongbing (HLB), caused by Candidatus Liberibacter spp., poses a severe threat to citrus production worldwide. Despite their importance, the evolutionary placement of Liberibacter within the Rhizobiaceae remains uncertain due to their highly reduced genomes, defective DNA repair, and accelerated evolutionary rates, which generate long-branch attraction (LBA) in phylogenetic analyses. Here, we reconstructed phylogenies using ~1,600 α -proteobacterial genomes and both AMPHORA and BUSCO gene sets. Trees based on the most conserved AMPHORA proteins consistently nested Liberibacter within the genus Rhizobium, whereas trees using less conserved genes or BUSCO markers placed Liberibacter as a deep-branching lineage, likely reflecting LBA artifacts. Comparative Ka/Ks analyses confirmed that divergence levels of selected marker sets correlated with the phylogenetic position of Liberibacter, with the most conserved genes resolving their placement within Rhizobium. These findings demonstrate that Liberibacter species are not an independent genus but represent a specialized lineage of Rhizobium that diverged early and adapted to an intracellular lifestyle in phloem and insect hosts. This refined phylogenetic framework provides a more accurate evolutionary context for studying Liberibacter biology and may guide the use of rhizobial surrogates in HLB research.

INTRODUCTION

Huanglongbing (HLB), also called citrus greening disease, is a highly detrimental disease affecting citrus trees. It causes (among others) yellowing of leaves and fruits of poor quality, and ultimately it will kill the plants (Lin, 1956). In the last decade in the US, HLB led to a 21% drop in fresh citrus fruit and a 72% decline in juice production (Dala-Paula et al., 2019).

HLB is associated with three species of Candidatus *Liberibacter*: Ca. *L. asiaticus* (CLas), Ca. *L. africanus* (CLaf), and Ca. *L. americanus* (CLam). Other members of the genus include Ca. *L. solanacearum* (CLso), which causes zebra chip disease of potato, and *L. crescens* (Lcr), the only species cultured to date and not known to be pathogenic to citrus. Transmission occurs via citrus psyllids: during feeding, psyllids acquire the bacterium from infected plants and can subsequently transmit it when they feed again. The bacteria are injected directly into phloem sieve elements through the psyllid stylet; once introduced, they colonize the phloem, disrupt assimilate transport, and induce the characteristic systemic symptoms of HLB.

These pathogenic bacteria cannot be cultured, which hampers research on them. Therefore, it is attractive to do some research on closely related model species. This would require evolutionary trees in which they are accurately positioned. However, due to their defective DNA repair system, the genomes of *Liberibacter* species evolve very fast, by making it not trivial to determine the evolutionary history of this genus.

The 16S rRNA sequences of the three HLB pathogens and Clcr, the only culturable bacterium among *Liberibacter*, were used to show that they belong to the Rhizobiaceae family (Dodapaneni et al. 2008; Williams et al. 2007). So in the family that also contains genera, like *Rhizobium* and *Sinorhizobium*, that can form a nitrogen fixing nodule symbiosis with legume plants.

In 2009, Duan et al. successfully assembled the genome of CLas through metagenomic sequencing of psyllids hosting these bacteria. The genomes of CLam and CLaf were obtained using a comparable metagenomic sequencing approach (Wulff et al., 2014; Lin et al., 2015). Genomes of Candidatus *Liberibacter* spp are small. For instance, the genome size of CLas is only 1.23 Mb, whereas the genome of *Sinorhizobium meliloti* is about 3.6 Mb. Due to the lifestyle of *Liberibacter* in a nutrient-rich environment, the plant phloem and insect salivary gland, they could lose a major part of their genome as there was no pressure to maintain several pathways. CLas lacks a complete restriction-modification system, which results in CLas being susceptible to prophage invasion and leads to phage-mediated recombination events in its genome. In addition, CLas is also impaired in DNA proofreading and repair (Duan et al., 2009). As a result, the CLas genome rapidly evolves, and this phenomenon holds true for other *Liberibacter* species as well.

After obtaining the CLas genome, Duan et al. used a Bayesian phylogenetic analysis to produce an evolutionary tree of α -Proteobacteria, including 25 Rhizobiales strains, and it was based on a common set of 94 proteins. These 94 proteins were selected from Sobral's work in which a tree for 72 α -Proteobacteria strains was made (Williams et al. 2007). These proteins were selected because they are single-copy in these strains, and there are no signs of horizontal gene transfer. In this tree, CLas was an early branch of the Rhizobiaceae, which makes *Liberibacter* a separate genus. In 2020, Thapa et al. made an evolutionary tree including 38 *Liberibacter* strains and 8 Rhizobiales strains with the aim to explore how *Liberibacter* evolved pathogenicity (Thapa et al., 2020). This phylogenetic tree is constructed based on 345 orthologous genes using the maximum likelihood method. It showed that the clade formed by *Liberibacter* spp. is separated from the clade composed of *Sinorhizobium*, *Agrobacterium*, *Rhizobium*, *Bartonella*, and *Brucella*. In Duan's tree, CLas exhibits an extraordinarily long branch length, which is most likely caused by its fast rate of evolution. This excessive branch length may confound the genetic history information contained in CLas's DNA sequence. In the tree of Thapa et al., *Liberibacter* branch lengths were also long. They introduced more *Liberibacter* strains, which all clustered together instead of being located within the Rhizobiaceae, as they are in the tree of Duan et al. They are juxtaposed with the cluster consisting of Rhizobiaceae, *Brucella*, and *Bartonella*. This suggested that the *Liberibacter* strains cluster together due to the Long-Branch Attraction(LBA) problem. The LBA problem arises when data are analysed from different species, of which regions of the genome evolved in some species with a different rate than in others. This can lead to the erroneous clustering of distantly related species into a single group (Johannes Bergsten, 2005).

To obtain a more accurate phylogenetic tree, we initially included a larger number of genomes (~1600 α -Proteobacteria) to achieve more precise node sequences and minimize the issue of LBA. Based on this, we employed two methods for tree construction: one using 31 highly conserved AMPHORA proteins and the other using 54 BUSCO proteins. The results of these two methods differed, with the phylogenetic tree based on BUSCO proteins resembling previously published structures. We found that the structure of the phylogenetic tree is related to the conservation level of the proteins. Using the 16 most conserved AMPHORA proteins for tree construction, the results indicated that *Liberibacter* belongs to *Rhizobium* rather than being a separate genus.

RESULTS

1. Phylogenetic tree based on the AMPHORA gene set

First, we constructed an evolutionary tree using the AMPHORA gene set from a large number of genomes (Wu et al., 2012). The AMPHORA gene set includes 31 conserved house-keeping genes, typically single-copy, such as *rplA*, *rplB*, *DnaG*, and *rpoB*, which are critical for essential biological processes and commonly used in bacterial evolutionary studies.

Previous studies placed *Liberibacter* spp. in the Rhizobiales order within α -Proteobacteria. Unlike earlier studies using up to 25 Rhizobiales species, we included ~1600 α -Proteobacteria genomes (including ~540 Rhizobiales species) from the NCBI database, plus 9 β -Proteobacteria species as an outgroup. Twelve genomes were excluded due to incomplete AMPHORA genes. The AMPHORA2 software extracted protein-encoding sequences, which were then aligned using Clustal Omega. Poorly aligned or incomplete regions were manually removed. The concatenated DNA sequences of the 31 genes were used to construct a phylogenetic tree with the IQ-TREE software using the maximum likelihood method and bootstrap analysis with 100 replications to examine the Rhizobiales branches.

The evolutionary tree (Fig. S1a) showed that strains belonging to certain orders and families, in general, clustered well. Some strains were classified in an order, different from the published classification. This may be due to the use of the AMPHORA gene set. Previous classifications have often been based only on the 16S rRNA gene sequence. So it is also possible that previous classifications of these species are not correct. Figure S1b is the part of the tree containing the Rhizobiales species. Three clusters can be seen in this part of the tree. Cluster1 contains the *Sinorhizobium/Ensifer* group and some *Rhizobium* species intermingled with *Pararhizobium*, *Shinella*, and a *Mycoplana* strains. Cluster2 is composed of an *Agrobacterium* group, some *Rhizobium* groups, and a few other Rhizobiales species. Cluster3 contains a group with five *Liberibacter* strains that are nested in a large *Rhizobium* group. Notably, nodes that separate these 3 clusters exhibited a bootstrap support value of 100.

This is the first analysis that indicates that *Liberibacter* spp. does not form a separate genus but might be part of the *Rhizobium* genus. Next, we constructed a tree using a method that considers as many proteins as possible to test if we would obtain the same results.

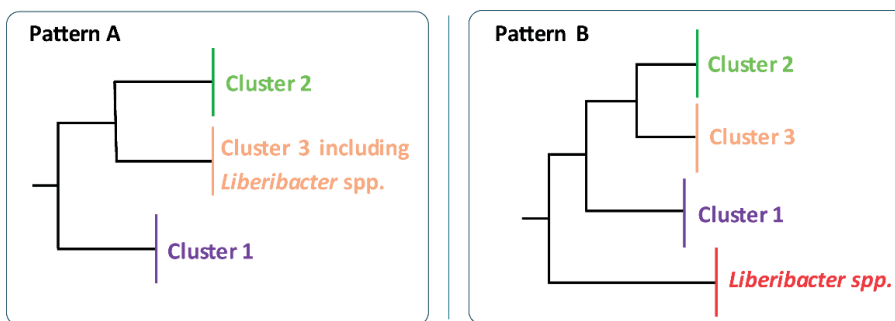


Figure 1: A schematic diagram illustrating two patterns of the relationship of three clusters and *Liberibacter* spp. in a single gene tree.

2. Phylogenetic tree based on a BUSCO gene set

To test the effect of using a larger gene set on the evolutionary tree, we increased the number of orthologous genes while using the same reference genomes. We built the tree based on

single-copy genes present in all selected genomes, identified using BUSCO (Benchmarking Universal Single-Copy Orthologs) software.

We evaluated the genomes with the BUSCO proteobacteria_obd10 lineage gene set, containing 219 marker genes. Genomes containing more than 80% of these marker genes were retained. Although *Liberibacter* spp. Genomes contain about 70% of these markers due to gene loss; they were included. After screening, 1370 genomes from the AMPHORA tree were retained, and 54 single-copy orthologous genes were identified. These gene sequences were aligned, trimmed, concatenated, and used to build an evolutionary tree with IQ-TREE software using the maximum likelihood method and 1000 bootstrap replicates.

The distribution of orders in this tree is similar to that in the AMPHORA tree (Fig. S2A), but the distribution of genera within Rhizobiales is different (Fig. S2B). The distribution of the 3 clusters is the same as in the AMPHORA tree. However, *Liberibacter* spp. are not located within cluster 3, but at the root outside of the node consisting of the 3 clusters. This difference is most likely due to the different gene set that was used. The BUSCO gene set included AMPHORA genes and other genes. It is possible that the differences in the evolutionary trees are due to different levels of conservation of their protein-encoding DNA sequences. To test this hypothesis, we made several evolutionary trees and tested whether the AMPHORA and BUSCO patterns correlated with a level of conservation.

3. The structure of the phylogenetic tree is related to the conservation level of the proteins used

The position of *Liberibacter* in the two previously obtained phylogenetic trees can be summarized into two patterns (Fig. 1). The phylogenetic tree based on AMPHORA proteins corresponds to pattern A, where *Liberibacter* is located within cluster 3, along with the *Rhizobium* genus. In contrast, the tree based on BUSCO proteins corresponds to pattern B, where *Liberibacter* is located outside the three clusters. When constructing RAXML trees using individual AMPHORA proteins, the position of *Liberibacter* in the resulting single-gene phylogenetic trees showed that 16 proteins followed pattern A, while 15 proteins followed pattern B (Table S1). To test the relationship between the conservation level of these genes and the structure of the phylogenetic tree, we calculated their nonsynonymous to synonymous substitutions (Ka/Ks, ω) ratios. Due to the generally low evolutionary pressure on *Liberibacter* proteins, the Ka/Ks ratio for *Liberibacter* will always be rather high. Therefore, we determined the difference in conservation of a *Liberibacter* gene with that of *Rhizobium*. This was done by comparing the Ka/Ks ratio of *Liberibacter* with that of this gene in *Rhizobium*. To calculate this value ($\omega_{Liberibacter}/\omega_{Rhizobium}$), we used a simple evolutionary tree, as shown in Figure 2A, containing *Liberibacter asiaticus* and its closest relative, *Liberibacter africanus*, and two *Rhizobium* species, *Rhizobium indicum* and *Rhizobium indigoferae*. Both *Rhizobium* species are located in Cluster 3. The Ka/Ks value of *Liberibacter asiaticus* related to node C was named ω_1 , and the mean Ka/Ks value of *Rhizobium indicum* and *Rhizobium indigoferae*

related to node A was named ω_2 . We calculated the ratio of ω_1 to ω_2 for each gene; the larger the ratio, the lower the pressure in *Liberibacter* relative to *Rhizobium* to maintain the sequence. The group of AMPHORA genes that have pattern A, has a mean ω_1/ω_2 ratio of 2.3324 (Fig. 2B, two of them with ω_2 of 0 are excluded). In contrast, the group of AMPHORA genes which have pattern B, have a mean ratio of 5.95 (seven of them with ω_2 of 0 and one outlier are excluded). Subsequently, we calculated the value for 12 random BUSCO genes, which were not AMPHORA genes, resulting in a mean ratio of 16.5315.

We verified our theory by constructing species trees from these gene sets. The tree from 16 AMPHORA Pattern A genes resembled the AMPHORA tree, with *Liberibacter* spp. in the same position (Fig. 3). The trees from 15 AMPHORA Pattern B genes and 12 BUSCO genes resembled the BUSCO tree, with *Liberibacter* spp. outside cluster 3. Thus, genes with similar evolutionary rates between *Liberibacter* and *Rhizobium* resulted in *Liberibacter* spp. being nested within the *Rhizobium* cluster.

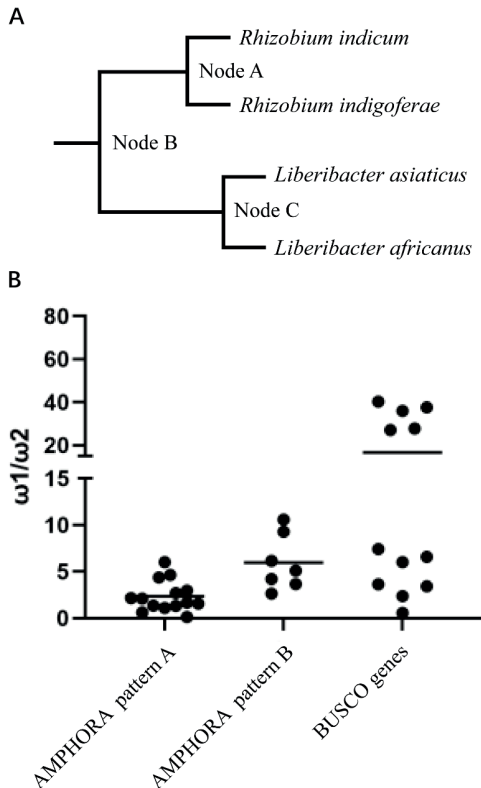


Figure2: Single-gene Ka/Ks analyses

Single-gene Ka/Ks analyses show substantial differences between AMPHORA- and BUSCO-selected genes.

A: Schematic diagram of the evolutionary tree topology used in the calculation of Ka/Ks values.

B: The ratio of Ka/Ks value of *Liberibacter* (ω_1) and Ka/Ks value of *Rhizobium* (ω_2) for each gene from the three groups.

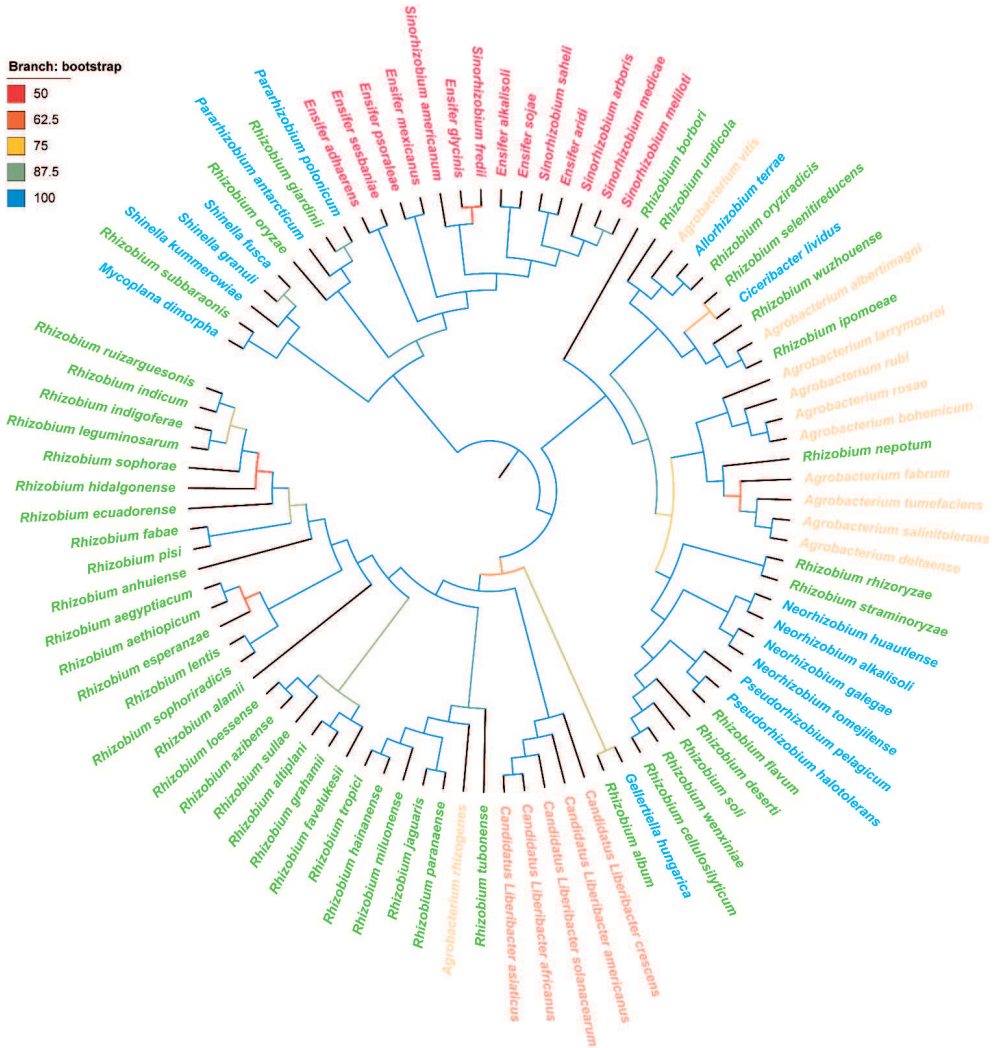


Figure3: Species tree made by the most conserved AMPHORA pattern A genes.

DISCUSSION

In this study, we have shown that *Liberibacter* species do not constitute an independent genus located at the root of the Rhizobiaceae family, but are nested within the genus *Rhizobium*. The phylogenetic tree in which *Liberibacter* formed a monophyletic group with *Rhizobium* required the use of genes that are among the least diverged *Liberibacter* genes.

In previous phylogenetic studies, *Liberibacter* species were in a cluster positioned outside the one formed by other Rhizobiaceae genera. We hypothesized that this was due to long-branch attraction and tried to overcome this problem by increasing the molecular datasets,

such as the number of species as well as the number of orthologous genes. This was also done in other studies aiming to avoid long-branch attraction. For example, Philippe et al., in their study on the evolutionary history of Acoelomorph flatworms, used more reference genomes. Additionally, in the phylogenetic study of Cupressoideae by Qu et al., coalescent phylogenetic reconstruction methods were applied and an increased number of orthologous genes. In both cases, the long-branch attraction problem was solved. However, in our study, using more than 1600 α -Proteobacteria species in combination with 54 BUSCO genes was not sufficient to avoid long branch attraction. In this tree, the *Liberibacter* species clustered with *Marteella* species, bacteria that(only) have in common with *Liberibacter* that they undergo rapid evolution. This indicated that long-branch attraction had occurred. Using the more conserved AMPHORA genes, *Liberibacter* species formed a monophyletic group with the genus *Rhizobium*. The *Liberibacter* species formed a single cluster and became separated from *Marteella*. The role of the conservation of the genes in avoiding long branch attraction was underlined by using 2 groups of AMPHORA genes. The tree obtained with the set with the most conserved genes had no sign of long branch attraction, and *Liberibacter* was nested within the *Rhizobium* genus. In contrast, in the one obtained with the least conserved genes, the *Liberibacter* cluster was located at the root of the Rhizobiaceae family. The branch length of *Liberibacter asiaticus* was 0.078 using the conserved set and 0.103 for the less conserved ones, indicating the role of branch length in wrong positioning. Also, in another study, long-branch attraction could be avoided by using more conserved genes. In a study on the phylogeny of gastropod mollusks, it was shown that removing fast-evolving sites helped to alleviate the issue of long-branch attraction (Uribe et al., 2019).

Using either the complete AMPHORA gene set or a more conserved subset of these resulted in an identical position and adjacent branches. This suggests that this is the most probable position and long-branch attraction is sufficiently avoided. In this optimized phylogenetic tree, *Liberibacter* spp. form a single cluster located within the *Rhizobium* genus. This indicates that all *Liberibacter* spp. evolved from a common ancestor shared with *Rhizobium* spp. The position in the tree indicates that they diverged early from other *Rhizobium* spp. This evolutionary history also makes it not possible to identify *Rhizobium* species with the closest phylogenetic relationship to *Liberibacter* spp. We hypothesise that an ancient *Rhizobium* species with an endophytic lifestyle is the ancestor. However, we cannot exclude the possibility that *Liberibacter* spp. evolved from different *Rhizobium* ancestors, but due to their still rather long branches, they cluster.

We show here for the first time that *Liberibacter* is not a separate genus but is nested within the genus *Rhizobium*, which means that *Liberibacter* is more closely related to *Rhizobium* than *Sinorhizobium* and *Agrobacterium*. This helps in identifying potential therapeutic targets and developing prevention and control strategies. The adjustments made to the evolutionary history of *Liberibacter* spp. in this study contribute to greater accuracy in subsequent research.

REFERENCES

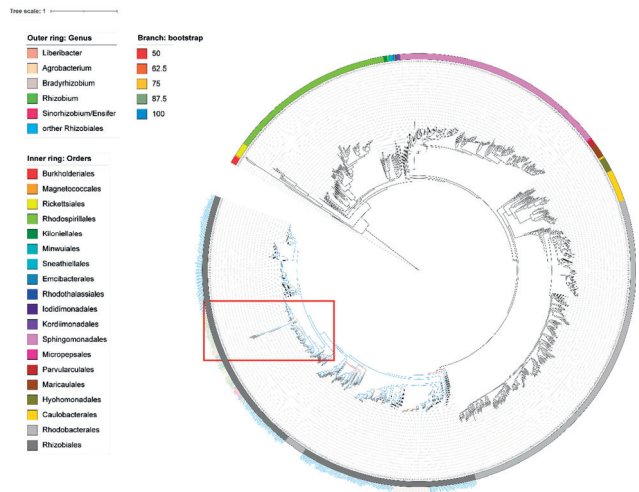
- Batarseh, T.N., Batarseh, S.N., Morales-Cruz, A., et al.** (2023). Comparative genomics of the *Liberibacter* genus reveals widespread diversity in genomic content and positive selection history. *Frontiers in Microbiology* 14.
- Bergsten, J.** (2005). A review of long-branch attraction. *Cladistics* 21, 163–193.
- Bocchetta, M., Gribaldo, S., Sanangelantoni, A., et al.** (2000). Phylogenetic depth of the bacterial genera *Aquifex* and *Thermotoga* inferred from analysis of ribosomal protein, elongation factor, and RNA polymerase subunit sequences. *Journal of Molecular Evolution* 50, 366–380.
- Bruno, E.M., Campbell, B., and Etxeberria, E.** (2019). Impacts of citrus greening on citrus production and juice processing in Florida. USDA Economic Research Service Report 197, 1–32.
- Dala-Paula, B.M., Plotto, A., Bai, J., et al.** (2019). Effect of huanglongbing or greening disease on orange juice quality, a review. *Frontiers in Plant Science* 9, 1976.
- Doddapaneni, H., Liao, H., Lin, H., Bai, X., Zhao, X., Civerolo, E.L., Irey, M., Coletta-Filho, H., and Pietersen, G.** (2008). Comparative phylogenomics and multi-gene cluster analyses of the citrus huanglongbing (HLB)-associated bacterium Candidatus *Liberibacter*. *BMC Research Notes* 1, 72.
- Duan, Y., Zhou, L., Hall, D.G., Li, W., Doddapaneni, H., Lin, H., Liu, L., Vahling, C.M., Gabriel, D.W., Williams, K.P., Dickerman, A., Sun, Y., and Gottwald, T.R.** (2009). Complete genome sequence of Candidatus *Liberibacter asiaticus*, the bacterium associated with citrus Huanglongbing. *Molecular Plant-Microbe Interactions* 22, 1011–1020.
- Lafliche, D., and Bové, J.M.** (1970). Structures de type mycoplasme dans les feuilles d'orangers atteints de la maladie du greening. *Comptes Rendus de l'Académie des Sciences Série D* 270, 1915–1917.
- Lin, K.H.** (1956). Yellow shoot of citrus. Symptomatology. Investigations in the cause of huanglongbing. *Natural Journal of Plant Pathology* 88, 7–37.
- Lin, H., Pietersen, G., Han, C., Read, D.A., Lou, B.H., Gupta, G., et al.** (2015). Complete genome sequence of Candidatus *Liberibacter africanus*, a bacterium associated with citrus huanglongbing. *Microbiology Resource Announcements* 3, e00733–15.
- Philippe, H., Brinkmann, H., Copley, R.R., et al.** (2011). Acoelomorph flatworms are deuterostomes related to *Xenoturbella*. *Nature* 470, 255–258.
- Portik, D.M., and Wiens, J.J.** (2021). Do alignment and trimming methods matter for phylogenomic (UCE) analyses? *Systematic Biology* 70, 440–462.
- Qu, X.J., Jin, J.J., Chaw, S.M., et al.** (2017). Multiple measures could alleviate long-branch attraction in phylogenomic reconstruction of Cupressoideae (Cupressaceae). *Scientific Reports* 7, 41005.
- Thapa, S.P., De Francesco, A., Trinh, J., Gurung, F.B., Pang, Z., Vidalakis, G., Wang, N., Ancona, V., Ma, W., and Coaker, G.** (2020). Genome-wide analyses of *Liberibacter* species provide insights into evolution, phylogenetic relationships, and virulence factors. *Molecular Plant Pathology* 21, 716–731.
- Uribe, J.E., Irisarri, I., Templado, J., et al.** (2019). New patellogastropod mitogenomes help counteracting long-branch attraction in the deep phylogeny of gastropod mollusks. *Molecular Phylogenetics and Evolution* 133, 12–23.
- Williams, K.P., Sobral, B.W., and Dickerman, A.W.** (2007). A robust species tree for the Alphaproteobacteria. *Journal of Bacteriology* 189, 4578–4586.
- Wu, M., and Scott, A.J.** (2012). Phylogenomic analysis of bacterial and archaeal sequences with AMPHORA2. *Bioinformatics* 28, 1033–1034.

Wulff, N.A., Zhang, S., Setubal, J.C., Almeida, N.F., Martins, E.C., Harakava, R., et al. (2014). The complete genome sequence of Candidatus *Liberibacter americanus*,

associated with citrus huanglongbing. *Molecular Plant-Microbe Interactions* 27, 163–176.

SUPPLEMENTARY DATA

A



B

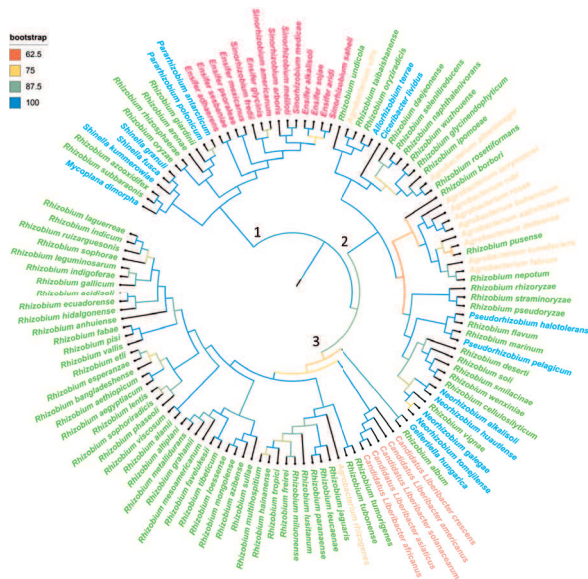


Figure S1: Phylogenetic analysis using the AMPHORA gene set

Phylogenetic analysis using the AMPHORA gene set places *Liberibacter* spp. within *Rhizobium*. The section within the red box has been enlarged in Figure B.

A: Evolutionary relation of Proteobacteria based on the alignment of 31 concatenated AMPHORA Proteins. AMPHORA genes have been extracted from the sequenced genomes of 1,603 Proteobacteria species. The inner ring indicates the taxonomic order to which the branch belongs. The outer ring indicates the genus to which species of the genera *Agrobacterium*, *Bradyrhizobium*, *Liberibacter*, *Rhizobium*, and *Sinorhizobium* of the order Rhizobiales. Bootstrap value is marked using colors on the nodes.

B: Zoom in of tree A

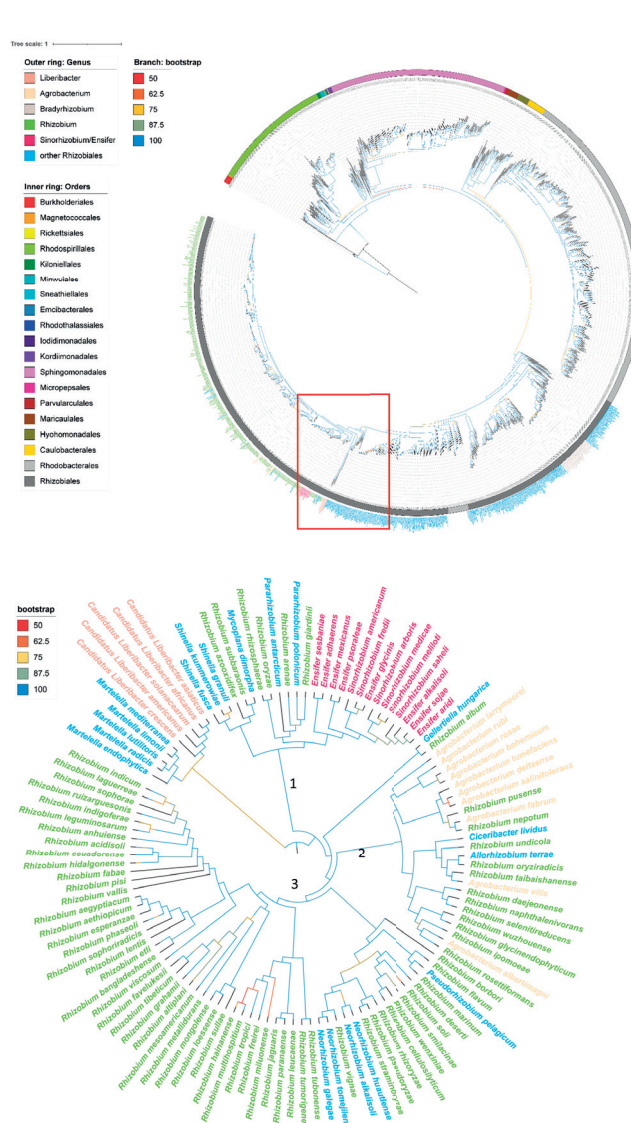


Figure S2: Phylogenetic analysis using the BUSCO gene set

Phylogenetic analysis using the BUSCO gene set positions *Liberibacter* spp. as an early-branching lineage within Rhizobiaceae (a separate genus).

A: An evolutionary tree based on 54 BUSCO Proteins. BUSCO genes have been extracted from the sequenced genomes of 1370 Proteobacteria species (Talbe S2). The inner ring indicates the taxonomic order to which the branch belongs. The outer ring indicates the genus to which species of the genera *Agrobacterium*, *Bradyrhizobium*, *Liberibacter*, *Rhizobium*, and *Sinorhizobium* of the order Rhizobiales. Bootstrap value is marked using colors on the nodes. The section within the red box has been enlarged in Figure B.

B: Zoom in of tree A

Phylogenetic analysis based on most conserved AMPHORA proteins shows that *Liberibacter* is nested in the *Rhizobium* genus

Table S1: In single AMPHORA protein RAxML gene trees, Liberibacter follows either pattern A or pattern B.

Follows pattern A	Follows pattern B
DnaG	Pgk
Frr	rplB
infC	rplK
nusA	rplL
pyrG	rplN
rplA	rplT
rplC	rpmA
rplD	rpsC
rplE	rpsI
rplF	rpsJ
rplM	rpsK
rplS	rpsM
rplP	rpsS
rpoB	smpB
rpsB	tsf
rpsE	

6

General Discussion

Jing Wang

Laboratory of Molecular Biology, Department of Plant Sciences, Graduate School
Experimental Plant Sciences, Wageningen University & Research, Wageningen
6708 PB, the Netherlands.

The molecular mechanisms underlying the interaction between citrus phloem tissue and Candidatus *Liberibacter asiaticus* (CLAs) remain poorly understood. To address this knowledge gap, it is critical to gain a deeper understanding of CLAs pathogenesis and host–pathogen interactions. This thesis aims to contribute to this by developing and applying novel methodologies tailored to these research needs.

This thesis integrates a rapid citrus infection system with phloem-specific transcriptomics. It employs a rhizobial model for in planta drug screening and a comparison of gene regulation. Further, it redefines the evolutionary position of CLAs to guide future research strategies.

1. *LIBERIBACTER*: NOT SO LIBERATED AFTER ALL

1.1. Phylogeny of *Liberibacter* species

Previous phylogenetic studies have consistently placed Candidatus *Liberibacter* species within the order Rhizobiales, based on analyses of 16S rRNA sequences and shared protein-coding sequences. These studies included both genome-wide and single-copy ortholog-based approaches (Duan et al., 2009; Thapa et al., 2020; Batarseh et al., 2023). They indicated the monophyly of the *Liberibacter* clade—i.e., the species currently classified as Candidatus *Liberibacter* are thought to have originated from a single common ancestor and to form a distinct lineage within Rhizobiaceae or in proximity to it. Phylogenomic reconstructions such as those by Thapa et al. (2020) and Batarseh et al. (2023) reported high bootstrap support for this grouping, suggesting an evolutionary trajectory shared among the known *Liberibacter* species, including *L. asiaticus*, *L. americanus*, *L. africanus*, and *L. solanacearum*. As a result, *Liberibacter* has generally been treated as an independent genus within the α -proteobacteria, distinct from other genera such as *Rhizobium*, *Agrobacterium*, and *Sinorhizobium*.

While previous studies have consistently treated Candidatus *Liberibacter* as a distinct genus within the Rhizobiaceae or near its base, our phylogenomic analyses described in chapter 4 challenge this classification by demonstrating that *Liberibacter* spp. are nested within the *Rhizobium* genus. This difference arises from the methodology used for phylogenetic analysis. We expanded the taxon sampling to include ~1600 α -Proteobacteria genomes, including >500 species from Rhizobiales, and adopted a gene set composed of the 31 most conserved AMPHORA housekeeping proteins. These genes have slower evolutionary rates, making them ideal for mitigating long-branch attraction effects. In our tree, *Liberibacter* spp. no longer form an isolated clade; instead, they fall within a broader lineage that corresponds to the *Rhizobium* genus, indicating that *Liberibacter* represents an internal branch of *Rhizobium* rather than a separate genus.

In contrast, when we used a broader BUSCO gene set (including more diverged proteins), *Liberibacter* spp. were misplaced outside the *Rhizobium* core, consistent with the topology seen

in previous studies (Duan et al., 2009; Thapa et al., 2020; Batarseh et al., 2023). We confirmed that this discrepancy is caused by gene conservation levels: genes with less evolutionary divergence between *Liberibacter* and *Rhizobium* yielded trees that placed *Liberibacter* within *Rhizobium*. In contrast, trees built with faster-evolving genes consistently assigned *Liberibacter* outside *Rhizobium*. This pattern was validated through Ka/Ks analyses and subset-based reconstructions, showing that only highly conserved genes can resolve the true evolutionary position of *Liberibacter*. Our phylogenetic analysis places *Liberibacter* species within the genus *Rhizobium*. However, in this discussion, we use “*Rhizobium*” to refer to plant-associated bacteria that can also have a free-living lifestyle. We reserve the name “*Liberibacter*” for those *Rhizobium* bacteria that have evolved a sieve element–limited lifestyle and, due to a loss of a major part of their genome, have a phloem restriction in their host plants.

To further clarify the evolutionary origins of *Liberibacter* species, future studies should employ a systematic microsynteny analysis across a broad sampling of α -proteobacterial genomes. While previous comparisons have often focused on pairwise alignments between *Liberibacter* species and individual *Rhizobium* or *Sinorhizobium* species, such limited sampling is prone to artifacts and may obscure phylogenetic relationships across the Rhizobiaceae. As demonstrated in this thesis, incorporating a wide range of α -proteobacterial representatives, including early-branching and environmentally independent (free-living) species, provides a more reliable basis for determining the evolutionary position of *Liberibacter*.

1.2. Single or multiple origin of *Liberibacter* species?

Although all currently known *Liberibacter* species cluster together in our phylogenetic analysis, this may not necessarily reflect a single common evolutionary origin (Chapter 5). The grouping could be an artifact of long-branch attraction, driven by the high evolutionary rate and genome reduction. So further analyses are required to determine whether there is a single origin or there might be multiple origins of *Liberibacter* species.

The extant *Liberibacter* species exhibit strong host specificity: *L. asiaticus*, *africanus*, and *americanus* are restricted to citrus; *L. crescens* was isolated from mountain papaya (*Vasconcellea pubescens*); *L. solanacearum* infects solanaceous crops such as tomato and potato; and *L. europaeus* colonizes pear (*Pyrus communis*) (Bové, 2006; Teixeira et al., 2005; da Graca et al., 2022; Leonard et al., 2012; Jain et al., 2019; Liefting et al., 2009; Secor et al., 2009; Sumner-Kalkun et al., 2020; Raddadi et al., 2011).

These plant hosts are phylogenetically distant, having diverged tens of millions of years ago. The Rutaceae family (citrus) diverged from the Rosaceae (pyrus) approximately 107 million years ago, while the Solanaceae (e.g., *Solanum* spp.) split from both Rutaceae and Rosaceae even earlier, around 110–120 million years ago (Magallón et al., 2015; Li et al., 2019). Mountain papaya belongs to the Caricaceae, which diverged from the asterid lineage (containing Solanaceae) over 90 million years ago (Antunes et al., 2015). Further, the psyllid vectors

are only feeding on plant species of a single plant family (e.g., *Diaphorina citri* and *Trioza erytreae* on Rutaceae; *Cacopsylla pyri* on Rosaceae; *Bactericera trigonica* and *Trioza apicalis* on Apiaceae). The large phylogenetic distance of the host plants, as well as the involvement of different psyllid species, makes a scenario of a single *Liberibacter* origin in the youngest common ancestor of these hosts—followed by widespread loss of compatibility across innumerable species—less parsimonious than multiple origins. So most likely, the current *Liberibacter* species evolved at least four times independently from different ancestral *Rhizobium* lineages. This suggests that the clustering of the *Liberibacter* species is caused by long-branch attraction. However, the ancestors are in all cases *Rhizobium* species because phylogenetic trees based on the most conserved genes place them within the genus *Rhizobium*.

To understand the divergence of three *Liberibacter* species related to HLB, it is important to consider the evolutionary history and biogeography of citrus itself. Modern cultivated citrus originated primarily in Southeast Asia, where the ancestral gene pools of mandarins (*Citrus reticulata*), pummelos (*C. maxima*), and citrons (*C. medica*) diversified (Wu et al., 2018).

In contrast, citrus cultivation in Africa and the Americas is the result of much more recent human-mediated introductions. Citrus species were brought to Africa via Arab trade routes (a segment of the Maritime Silk Road) in antiquity, and were subsequently introduced to the Americas during the Columbian Exchange (ca. 15th–16th centuries), likely originating from European or North African stocks (Langgut et al., 2017; McNeill J R, 2019). Thus, the global spread of citrus is a historically recent event, with non-Asian citrus populations derived from limited founder genotypes.

Given this geographic and evolutionary background, one plausible scenario is that *Liberibacter* evolved in Asia from a phloem-colonizing *Rhizobium* ancestor. Since the Asian psyllid feeds on all citrus species, this will have resulted in spreading among them. When citrus was introduced in the Middle East and Africa, this was in regions where the Asian citrus psyllid (*D. citri*) did not occur. However, it has been shown that the African citrus psyllid (*T. erytreae*), although belonging to a different genus, can transmit *L. asiaticus* as well as *L. africanus* (Reynaud et al., 2022). Therefore, it seems plausible that the *Liberibacter* that was present in citrus, which was introduced in the Middle East, was spread by the African psyllid. Subsequent geographic separation most likely resulted in the evolution of *L. africanus*. As the citrus that was introduced in the Americas originated from the Middle East (via Europe), it was most likely contaminated with a *Liberibacter* that evolved from the Asian ancestor. In the Americas, the only psyllid that currently feeds on citrus is *D. citri*, most likely introduced last century (Hall D G, 2008). Therefore, the original insect vector before *D. citri*'s arrival remains unknown.

Overall, *Liberibacter* causing HLB likely originated in Asia and then spread to Africa and the Americas, where geographic isolation resulted in the evolution of the new species.

Taken together, this historical reconstruction raises a question: through what ecological and molecular steps could a plant-associated *Rhizobium* endophyte gain durable access to sieve element cells and, over time, become phloem-restricted?

2. ENDOPHYTIC ANCESTORS AND THE PHLOEM NICHE: A PRECURSOR TO *LIBERIBACTER* SPECIATION

Rhizobium spp. and related species like *Bradyrhizobium* spp. are frequently detected in the root and leaf microbiomes of legumes and non-legumes, including citrus (Xu et al., 2018). Strains from non-legume hosts in general lack *nod* and *nif* genes (Cheng et al., 2018; Jones et al., 2016; Wasai-Hara et al., 2020). So their ancestral function is related to that of the plant-associated microbiomes, whereas the ability to form nitrogen-fixing root nodules is first acquired by horizontal gene transfer (Schneijderberg et al., 2018; Masson-Boivin et al., 2009; Remigi et al., 2016). Citrus hosts taxonomically distinct communities in the rhizosphere, root endosphere, and phyllosphere. In all these compartment *Rhizobium* species have been identified (Xu et al., 2018). This is most likely also the case in the other host plants in which *Liberibacter* evolved. I hypothesise that *Liberibacter* species evolved from *Rhizobium* species present in leaf endospheres, as these are already adapted to a lifestyle inside plants.

The microbiome of the citrus phloem has been studied by analysing midribs of leaves (Sagaram et al., 2009; Zhang et al., 2013; Yang et al., 2016). However, such studies cannot reveal whether bacteria are inter- or intracellularly hosted. This would require, for example, the isolation of phloem sap. Recently, Killiny and Jones (2024) introduced a centrifugation method to obtain phloem sap from excised stems; the sap enabled acquisition of CLAs by psyllids. Applying high-throughput sequencing to such sap could clarify whether sieve elements harbor unique endophytic communities.

Studies on rhizobia forming root nodules have shown that the bacteria can only enter living cells by a very sophisticated mechanism, infection thread formation that requires the *nod* genes (Roche et al., 1996; Tsyganova et al., 2021). As endophytic rhizobia lack these genes, it seems unlikely that they can have an intracellular lifestyle by which they enter living (sieve element) cells without the help of, for example, insects.

The barriers formed by the plant cell wall and the sieve element plasma membrane, together with pattern-triggered immunity. Despite its nutrient richness, the phloem is hypothesized to be largely sterile—analogueous to the human bloodstream (Cheng et al., 2023). Although CLAs DNA can be detected in citrus seeds, vertical transmission has not been observed, which is consistent with the idea that bacteria cannot enter plant cells without a specialized mechanism: Morelli et al. (2020). Therefore, an insect-mediated route is the most plausible way bacteria can enter sieve elements: a sap-feeding hemipterans could introduce phyl-

losphere/leaf endospheres-resident *Rhizobium* into sieve elements via contaminated stylets—consistent with documented vectoring of non-obligate endophytes and other bacteria by piercing–sucking insects (Nadarasah & Stavrinides, 2011; Perilla-Henao & Casteel, 2016; López-Fernández et al., 2017).

Whether any bacterium can persist inside a phloem sieve element depends on the defense mechanism that can be induced in the sieve element–companion cell complex. Anucleate sieve elements depend on companion/parenchyma cells that likely serve as immune gatekeepers, triggering rapid defenses such as callose deposition at sieve plates, phloem protein (P-protein) aggregation, and reactive oxygen species (ROS) bursts (Eckardt NA, 2001; Will et al., 2013; Liu et al., 2021); yet phloem immunity remains poorly resolved. Thus, successful intracellular colonization of sieve elements by any bacterium, including a possible *Rhizobium*-like ancestor of *Liberibacter*, would have required the ability to suppress or evade these SE/CC-mediated defense responses. (Jiang et al., 2019).

When such insect-mediated entry occurred, a *Rhizobium*-like ancestor would first need to persist and replicate inside sieve elements to reach transmissible titers, and move systemically enough that feeding insects can reacquire it. A plausible evolutionary improvement for the bacterium is a shift from mere stylet contamination to a stage when it becomes hosted within the vector. This would markedly increase transmission efficiency. Hosted intracellular in phloem, will result in relaxed selection on nutrient acquisition, motility, competition, and other free-living traits likely driving progressive genome reduction and speciation.

This evolutionary hypothesis raises a central question: if the endophytic *Rhizobium* ancestor was likely symbiotic/commensal rather than pathogenic, how did *Liberibacter* evolve pathogenicity?

3. PATHOGENICITY OF *LIBERIBACTER*

A recent study on a *MYC2*-centered jasmonate (JA) module showed that the inefficient functioning of this causes HLB. Comparative transcriptomics of susceptible and tolerant species within the Rutaceae family identified *PUB21* (a U-box E3 ligase) as an HLB susceptibility gene (Zhao et al., 2025). *PUB21* targets the transcription factor *MYC2*, and upon ubiquitination, *MYC2* is degraded by the proteasome. Tolerance or susceptibility to HLB depends on the level of *PUB21* activity. This is controlled at two levels. HLB-tolerant species have a dominant-negative *PUB21* paralog (*PUB21DN*). This dimerizes with *PUB21* by which it titrates out *PUB21*. Further, susceptible citrus species have a helitron insertion in the *PUB21* promoter. This forms several *MYC2*-binding motifs, creating a stronger negative feedback loop in which *MYC2*, upon activation, transcriptionally up-regulates *PUB21*. In this way, it accelerates its own degradation. and weakens JA-mediated defense. CLas might accelerate

the *MYC2* proteolysis as its effector *SDE5* has shown in vitro to stimulate *PUB21-MYC2* interaction, and overexpression in transgenic citrus increases CLas sensitivity. Genetic perturbations confirm causality. *MYC2* overexpression lowers CLas titers, whereas *MYC2* silencing raises them. Further, *PUB21DN* expression in a susceptible citrus species reduces CLas titers as well as HLB symptoms. So, turning a susceptible species into a tolerant one. In contrast, overexpression of *PUB21* leads to a higher CLas titer.

So in susceptible citrus, HLB symptom development is correlated with a high titer of CLas. However, a high titer of CLas does not always lead to HLB symptoms. For example, in periwinkle, CLas reaches high titers, but without HLB-like symptoms (Teixeira et al., 2008; Ya et al., 2018).

Several studies have shown that in citrus with high titers of CLas, callose is deposited at sieve plates (often with *PP2* co-plugging), which reduces pore diameter and causes phloem transport failure (Achor et al., 2010; Folimonova & Achor, 2010; Koh et al., 2012; Brodersen et al., 2014; Deng et al., 2019; Achor et al., 2020; Granato et al., 2019). In contrast, a high titer of CLas does not induce callose deposition in periwinkle, with a corresponding absence of sieve-plate plugging and little impairment of phloem transport (Ding et al., 2015; Zheng et al., 2023). Why callose deposition is not induced in periwinkle is not clear, but it might be plant species dependent.

Building on the citrus-periwinkle difference with respect to callose deposition, I propose that sieve-plate callose deposition is an initial driver of HLB symptom development. In citrus with high CLas titers, callose-biosynthesis genes are induced. These are members of the *CalS/GSL* family-including *CalS7*. This expression coincides with callose deposition. A mechanistic bridge from immunity to callose is provided by salicylic acid (SA) signaling. In Arabidopsis, SA triggers expression of *GSL/CalS* genes, and this results in plasmodesma closure by callose deposition (Wang et al., 2013; Wu & Ma, 2018). In citrus with HLB symptoms, SA pathway activation is observed. This positions SA as a very probable trigger for callose deposition in later infection stages (Pang et al., 2020), while the absence of callose deposition in periwinkle could mean that a high titer of CLas does not trigger SA signaling.

How upon callose deposition other responses are induced and symptoms develop requires more studies in a time series in an experimental system in which a synchronized infection can be induced (see Section, “Early phloem response genes: a critical frontier for HLB research”)

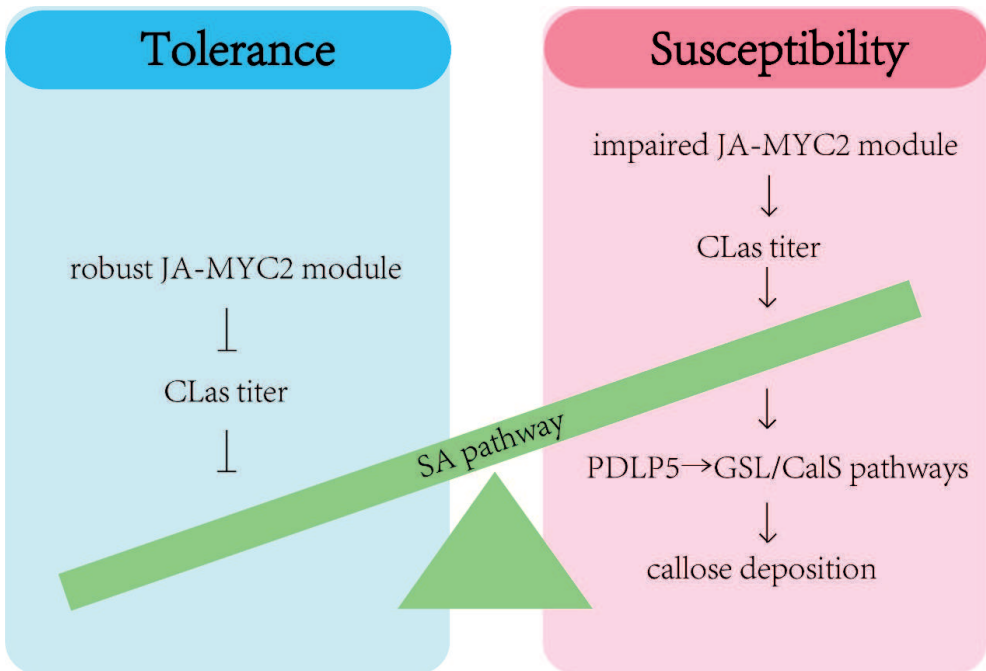


Figure 1: Proposed model linking *JA-MYC2* immunity, CLas titer, and callose deposition in HLB development. In tolerant Rutaceae species, a robust *JA-MYC2* module restricts CLas accumulation, preventing downstream phloem responses. In susceptible citrus, *PUB21*-mediated degradation of *MYC2* weakens JA defense, allowing higher CLas titers. Elevated bacterial loads activate SA signaling, inducing *PDLP5-GSL/CalS* pathways and callose deposition at sieve plates, which contributes to phloem blockage and HLB symptom development.

4. RECYCLING OLD FRIENDS: RHIZOBIUM-LEGUME SYMBIOSIS AS A SURROGATE PLATFORM TO STUDY CLAS GENES

Because CLas remains unculturable, a meaningful surrogate is important. Preferably, it must be a close relative and support plant-based assays. Our phylogenetic analyses placed *Liberibacter* within the *Rhizobium* genus. This makes *Rhizobium spp*-based root nodules an attractive in planta system to study the function of CLas genes. However, at the time I started the research described in Chapter 3, CLas was considered to be an independent genus within the rhizobiaceae (Duan et al., 2009; Thapa et al., 2020). Therefore, we selected the *Sinorhizobium-Medicago* root nodule symbiosis as an experimental system, as it has very advanced molecular and genetic toolkits for both host and bacterium.

The research described in Chapters 2 and 3 provides 2 cases that can be used to evaluate the usefulness of the nodule symbiosis to study CLas genes. Further, the functionality of CLas genes in free-living *Sinorhizobium* was studied in a previous study (Barnett et al., 2019).

Insight into the function of a CLas gene can be obtained by complementing a *Sinorhizobium* mutant in the orthologous gene. It requires that this mutation result in a detectable phenotype. For the free-living bacteria, this can, for example, be the (dis)ability to grow at different environmental conditions or an altered gene expression pattern. In case root nodules are used, the mutation in the *Sinorhizobium* gene should result in a nodule phenotype. These can be parameters like nodule number and size, or cytology, or expression of a few nodule marker genes, or even spatial transcriptomics.

Firstly, free-living *Sinorhizobium* was used to evaluate complementation efficiency and confirm the basic functionality of CLas genes. Notably, Barnett et al. (PNAS, 2019) expressed several CLas transcription factors (TFs) in *Sinorhizobium meliloti* to establish a high-throughput screening system for inhibitors of these TFs. Their study showed that more than half of the tested CLas TFs could complement the mutation phenotype and activate their target promoters. So they are active and have a similar function as the *S. meliloti* orthologue in this heterologous background. This relatively high level of functional complementation indicates that many CLas transcription factors can operate within the *Sinorhizobium* regulatory environment, which supports the feasibility of using *Rhizobium* species as heterologous hosts for testing CLas gene function. Considering our phylogenetic evidence that CLas is nested within the *Rhizobium* genus, this functional compatibility is consistent with the evolutionary proximity of these bacteria within the order Rhizobiales and supports the use of *Rhizobium* species as suitable surrogates for validating CLas gene function. In case the CLas gene, to be studied, has an orthologue in *Rhizobium*, two experimental routes can be followed. If the function of the *Rhizobium* orthologue is already known, the role of the CLas gene can be inferred accordingly. It can then be tested whether the CLas gene is able to complement the corresponding *Rhizobium* mutant phenotype in free-living bacteria and in nodules. Successful complementation would demonstrate that the CLas gene retains a conserved function.

If the function of the *Rhizobium* orthologue is unknown, a targeted mutant can be generated to disrupt the orthologous locus. In case this leads to a phenotype, a first insight into the *Rhizobium* gene is obtained and the procedure as described above can be followed. This approach not only reveals the function of the CLas gene, but also provides novel insight into the uncharacterized *Rhizobium* gene itself.

As described in Chapter 3, when a CLas gene successfully complements a nodule phenotype, the same system can subsequently identify compounds that inhibit its activity. This dual strategy—functional complementation followed by inhibitor screening—enables both functional characterization of CLas genes and the establishment of a biologically relevant, plant-based platform to discover potential therapeutic agents against HLB.

Among the available rhizobial-legume symbiosis models, I favor the pea (*Pisum sativum*)–*Rhizobium leguminosarum* bv. *viciae* system. Pea offers several key advantages as a host. Its

genome and genetic architecture have been comprehensively resolved, as shown by recent large-scale sequencing (600 varieties) studies (Feng et al., 2025, Nature). These resources provide a robust foundation for molecular and comparative analyses, including the identification of key developmental and symbiotic regulatory pathways. In addition, *Pisum sativum* has well-established transformation and mutagenesis protocols, enabling precise genetic manipulation and functional assays for the host genes.

In the past, pea was a major research system for nodulation. It was ideal for genetic studies, and the first early nodulin genes were cloned from pea. Research on pea nodulation was strongly reduced when *Medicago* was selected as the model system. Now genetic tools and technologies have advanced, and pea can be as efficiently used as e.g. *Medicago*.

Furthermore, the pea-*R. leguminosarum* *bv viciae* symbiosis is a well-characterized, efficient nodulation system that allows quantitative assessment of nodule formation, structure, and function. Recent advances in spatial transcriptomics have further enhanced the power of this model: single-cell and spatially resolved transcriptomic methods now make it possible to simultaneously capture the expression profiles of both bacterial and plant cells within intact nodules, together with their spatial organization. Integrating these datasets provides unprecedented insights into the molecular dialogue and metabolic exchange between symbiont and host at cellular resolution.

As mutagenesis of the bacterial partner is of key importance, it is worth emphasizing that *Rhizobium leguminosarum* *bv viciae* has long been one of the genetically best-characterized rhizobia. A few decades ago, major research groups—such as those at Leiden University and the John Innes Centre—established this species as a central model for symbiotic nitrogen fixation. During that period, highly efficient mutagenesis methods were developed, including chemical mutagenesis, transposon insertion libraries, and *sacB*-based allelic exchange systems for targeted gene disruption and complementation. These classical tools enabled the systematic identification of *nod*, *nif*, and *fix* loci, laying the foundation for modern functional genomics in rhizobia.

Today, the development of efficient CRISPR-based editing systems has made *R. leguminosarum* *bv viciae* one of the most easily manipulated plant-associated bacteria. CRISPR/Cas technologies now enable precise and rapid generation of targeted knockouts, point mutations, and promoter replacements, with minimal off-target effects. For researchers studying CLas, this mature CRISPR platform provides a ready-to-use and reliable surrogate system: CLas orthologues can be precisely edited, expressed, or complemented in *R. leguminosarum*, enabling rapid evaluation of their effects on host-microbe interactions in a well-established intracellular context.

So, the pea-*R. leguminosarum* model combines a genetically tractable plant host, a well-studied microsymbiont, and cutting-edge spatial omics tools, making it an ideal system for identifying bacterial gene function and extrapolating insights to phloem-limited pathogens such as CLAs.

In addition to nodule-based systems, root- or leaf-associated *Rhizobium* endophytes can also serve as valuable surrogate models. Although these endophytes do not reside within phloem sieve elements, they occupy intercellular niches that still enable intimate plant-microbe interactions. Notably, *Rhizobium* and *Bradyrhizobium*—two close relatives of CLAs—have been extensively studied, and large-scale mutant libraries, including transposon and CRISPR-based collections, are already available (Pobigaylo et al., 2008; Wheatley et al., 2020). The functions of many genes involved in their endophytic lifestyles, such as those mediating host colonization, immune evasion, and nutrient exchange, have been systematically characterized (Flores-Tinoco et al., 2020; Knights et al., 2024).

These existing resources provide a powerful foundation for CLAs-related research. For many molecular mechanisms that are not strictly specific to phloem localization—such as immune recognition, hormone signaling, or metabolite exchange, these endophytic systems offer an experimentally tractable platform for functional analysis of CLAs orthologues. Transgenic *Rhizobium* endophytes can be readily reintroduced into their natural habitats within plant roots or leaves, allowing in situ assessment of colonization, persistence, and host responses. When coupled with spatial transcriptomics or single-cell resolution approaches, these systems can yield high-dimensional insights into the molecular dialogue between *Rhizobium* and host tissues, including local immune modulation and metabolic adaptation. Together, such well-developed genetic resources and experimental flexibility make *Rhizobium* endophyte–plant associations an attractive and complementary model for dissecting the molecular basis of bacterial–host interactions relevant to CLAs and other intracellular or endophytic pathogens.

5. EARLY PHLOEM RESPONSE GENES: A CRITICAL COMPONENT FOR HLB RESEARCH

A central obstacle in deciphering CLAs–citrus interactions is that the decisive biology unfolds in the sieve element–companion cell (SE/CC) complex at low bacterial loads and on short timescales. In naturally infected trees, colonization is patchy and asynchronous; by the time symptoms are obvious, the phloem has already undergone secondary changes, for example, starch hyperaccumulation, massive callose deposition, oxidative damage, and this obscures the initial responses.

By using our synchronized infection system (chapter 2), it became possible to capture the earliest local responses to HLB with temporal precision. This method allows stable and reproducible infection of citrus seedlings and, importantly, enables accurate prediction of which leaves will become infected. As a result, phloem tissues can be sampled at defined early stages before systemic spread or secondary degeneration occurs.

In Chapter 2, we used an approach that combines this synchronized infection system with laser capture microdissection (LCM) to obtain phloem-specific transcriptomes at the early stage of HLB infection (40 days post inoculation, dpi). In this dataset, 187 of the top 300 differentially expressed (DE) genes (40 dpi vs uninoculated) were absent from previously published late, symptomatic leaf datasets, and 72 genes were significantly upregulated at 40 dpi (FDR < 0.01). These findings indicate that earlier studies from symptom-expressing trees overlooked a substantial fraction of early HLB-responsive genes. Among the newly identified transcripts are two branched-chain amino acid (BCAA) metabolism genes—the only two BCAA-related genes annotated in the citrus genome—suggesting that activation of BCAA pathways may play a critical role in the initial phloem response to CLAs. Such genes provide new mechanistic insights into how CLAs triggers localized metabolic reprogramming and early defense signaling within the phloem.

In a comparison of the 40 dpi dataset with field-derived phloem samples representing later infection stages, we observed that secondary responses, such as down-regulation of photosynthesis-related genes and enhanced expression of programmed cell death marker were induced in the field samples. These were weaker in the early-stage samples, but still detectable. Future research should therefore move further upstream in time, establishing a detailed temporal series to capture the induction and subsequent attenuation of early HLB-responsive genes immediately following CLAs entry into the phloem.

In selecting an appropriate method to obtain phloem-specific transcriptomes, LCM represented a suitable choice at the time of this study. However, with the rapid advancement of spatial transcriptomic technologies (Marx V, 2021; Ståhl et al., 2016), applying spatial transcriptomics to analyze cross-sections of citrus veins at defined early time points of CLAs infection has become the most promising next step. This approach would allow the generation of transcriptomic maps of individual cells within infected veins at precisely known infection stages. Such high-resolution data would enable us to dissect how distinct phloem cell types—sieve element cells, companion cells, and phloem parenchyma cells—differ in their responses to CLAs, thereby providing deeper insight into the cellular mechanisms of infection. Moreover, spatial transcriptomics could reveal how infection-derived signals move from the phloem into surrounding mesophyll tissues, illuminating the transition from localized to systemic responses. If spatial transcriptomic methods for prokaryotes continue to mature (Sarfatis et al., 2025), it may even become feasible to simultaneously capture the bacterial transcriptome of CLAs within infected tissues. This would markedly enhance our

understanding of the host–pathogen interplay by revealing which CLas genes are expressed and essential during early infection, and which remain suppressed.

Studies on early phloem-specific promoters can also contribute to applications to create tolerance to HLB. We currently lack promoters that are rapidly and specifically activated in phloem tissues upon CLas infection. Such promoters could be used to drive the expression of antimicrobial peptides (AMP). Currently, most AMP studies rely on constitutive, high-expression promoters (Coca et al., 2004; Sun et al., 2025; Zou et al., 2023). As a result, every cell in the citrus plant continuously produces large amounts of AMPs, even in the absence of infection, leading to unnecessary energy expenditure. Moreover, such excessive and ubiquitous AMP expression may also pose a potential threat to beneficial microorganisms. In our transcriptome dataset, we identified several genes that are highly expressed at 40 dpi, but show little or no expression in orchard samples representing late-stage HLB infection. Comparative analysis against whole-leaf transcriptome datasets can help verify that these genes are enriched in phloem tissues rather than broadly expressed across the leaf. The promoters of these early, phloem-specific genes therefore represent promising candidates for driving AMP expression in a targeted and inducible manner. Further validation will require spatial transcriptomic data from even earlier stages of CLas infection. In addition, I think that exclusive early inducibility may not be essential, as promoters that remain active throughout the infection period—or even until CLas is fully cleared—could also be acceptable. If strong, localized expression of AMPs is sufficient to eliminate CLas at the initial infection site, systemic spread would be prevented. In such a scenario, the high-level AMP expression would remain confined to the local phloem region where psyllid feeding and CLas transmission had initially occurred.

In conclusion, a synchronized infection system, combined with appropriately designed transcriptomic analyses, provides a powerful means to capture the molecular events underlying the early interaction between CLas and citrus. This approach not only offers a more comprehensive view of the initial host–pathogen dynamics within the phloem but also enables the identification of practical tools, such as CLas-inducible promoters, that could be applied to drive AMPs for targeted disease control.

Concluding remarks

HLB research is challenged by the unculturable nature of CLas and the inaccessibility of early phloem infection events. This thesis addresses these barriers by integrating evolutionary genomics, synchronized infection biology, and surrogate functional systems.

First, by expanding phylogenomic sampling and focusing on conserved markers, this work refines the evolutionary placement of CLas and supports that is derived from an *Rhizobium*-like ancestor. This re-framing aligns CLas with well-studied plant-associated bacteria and

provides an evolutionary rationale for leveraging *Rhizobium* models to study phloem-restricted pathogens.

Second, a synchronized micrografting system coupled with phloem-specific transcriptomics captures early citrus responses to CLas before secondary tissue degeneration occurs. These data reveal previously unrecognized early-stage phloem genes, offering insights into the initial molecular dialogue between host and pathogen and identifying inducible, phloem-preferential promoters that may support targeted HLB control strategies. This system is well-suited to use other citrus varieties that are more susceptible or more tolerant to HLB. However, the best novel insights can most likely be obtained by using mutants. A good example is the work on the *MYC2* module. This study showed that overexpression of a dominant negative form of *PUB21* can confer a susceptible variety in a tolerant one, whereas overexpression of wild-type *PUB21* has the opposite effect. When such approaches can make use of cell type (e.g. phloem) specific promoters, it has a high potential to provide major novel mechanistic insights.

Finally, rhizobia-based symbiosis systems are demonstrated as useful surrogates for testing CLas gene function and screening inhibitors in planta, bridging the gap created by the organism's unculturable nature.

Together, these approaches advance both conceptual and practical understanding of CLas biology: redefining its evolutionary context, exposing early phloem responses critical for disease establishment, and enabling functional investigation through tractable symbiotic systems. Continued development of early-stage, phloem-resolved analytical tools and surrogate platforms will further support mechanistic discovery and the rational design of interventions against HLB.

REFERENCES

- Barnett, M. J., Solow-Cordero, D. E., & Long, S. R. (2019). A high-throughput system to identify inhibitors of *Candidatus Liberibacter asiaticus* transcription regulators. *Proceedings of the National Academy of Sciences*, 116(36), 18009–18014.
- Batarseh, T. N., Batarseh, S. N., Morales-Cruz, A., et al. (2023). Comparative genomics of the *Liberibacter* genus reveals widespread diversity in genomic content and positive selection history. *Frontiers in Microbiology*, 14, 1206094.
- Bové, J. M. (2006). Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Journal of Plant Pathology*, 88(1), 7–37.
- Cheng, X., Polman, S., Franken, C., et al. (2018). A genetically and functionally diverse group of non-diazotrophic *Bradyrhizobium* spp. colonizes the root endophytic compartment of *Arabidopsis thaliana*. *BMC Plant Biology*, 18(1), 61.
- Cheng, X., Yan, Z., Li, Q., et al. (2025). Chinese chestnut did not induce negative plant–soil feedback during centuries of growth. *Science of the Total Environment*, 970, 178883.
- Clark, K., Franco, J. Y., Schwizer, S., et al. (2018). An effector from the Huanglongbing-associated pathogen targets citrus proteases. *Nature Communications*, 9(1), 1718.
- Coca, M., Bortolotti, C., Rufat, M., Penas, G., Eritja, R., Tharreau, D., ... & San Segundo, B. (2004). Transgenic rice plants expressing the antifungal AFP protein from *Aspergillus giganteus* show enhanced resistance to the rice blast fungus *Magnaporthe grisea*. *Plant Molecular Biology*, 54(2), 245–259.
- da Graça, J. V., Cook, G., Ajene, I. J., et al. (2022). A review of the ‘*Candidatus Liberibacter africanus*’ citrus pathosystem in Africa. *Phytopathology*, 112(1), 44–54.
- Du, P., Zhang, C., Zou, X., et al. (2021). ‘*Candidatus Liberibacter asiaticus*’ secretes non-classically secreted proteins that suppress host hypersensitive cell death and induce expression of plant pathogenesis-related proteins. *Applied and Environmental Microbiology*, 87(8), e00019–21.
- Duan, Y., Zhou, L., Hall, D. G., et al. (2009). Complete genome sequence of citrus Huanglongbing bacterium, ‘*Candidatus Liberibacter asiaticus*’, obtained through metagenomics. *Molecular Plant-Microbe Interactions*, 22(8), 1011–1020.
- Feng, C., Chen, B., Hofer, J., Shi, Y., Jiang, M., Song, B., ... & Cheng, S. (2025). Genomic and genetic insights into Mendel’s pea genes. *Nature*, 1–10.
- Flores-Tinoco, C. E., Tschan, F., Fuhrer, T., Margot, C., Sauer, U., Christen, M., & Christen, B. (2020). Co-catabolism of arginine and succinate drives symbiotic nitrogen fixation. *Molecular Systems Biology*, 16(6), e9419.
- Hiruma, K., Gerlach, N., Sacristán, S., et al. (2016). Root endophyte *Colletotrichum tofieldiae* confers plant fitness benefits that are phosphate status dependent. *Cell*, 165(2), 464–474.
- Huang, G., Chang, X., Hu, Y., et al. (2024). SDE19, a SEC-dependent effector from ‘*Candidatus Liberibacter asiaticus*’ suppresses plant immunity and targets *Citrus sinensis* Sec12 to interfere with vesicle trafficking. *PLoS Pathogens*, 20(9), e1012542.
- Jain, M., Cai, L., Fleites, L. A., et al. (2019). *Liberibacter crescens* is a cultured surrogate for functional genomics of uncultured pathogenic ‘*Candidatus Liberibacter*’ spp. and is naturally competent for transformation. *Phytopathology*, 109(10), 1811–1819.
- Jones, F. P., Clark, I. M., King, R., et al. (2016). Novel European free-living, non-diazotrophic *Bradyrhizobium* isolates from contrasting soils that lack nodulation and nitrogen fixation genes. *Scientific Reports*, 6, 25858.
- Knights, H. E., Ramachandran, V. K., Jorrin, B., et al. (2024). *Rhizobium* determinants of

- rhizosphere persistence and root colonization. *The ISME Journal*, 18(1), wrae072.
- Leonard, M. T., Fagen, J. R., Davis-Richardson, A. G., et al.** (2012). Complete genome sequence of *Liberibacter crescens* BT-1. *Standards in Genomic Sciences*, 7(2), 271–283.
- Liefting, L. W., Sutherland, P. W., Ward, L. I., et al.** (2009). A new ‘Candidatus *Liberibacter*’ species associated with diseases of solanaceous crops. *Plant Disease*, 93(3), 208–214.
- López-Fernández, S., Mazzoni, V., Pedrazzoli, F., et al.** (2017). A phloem-feeding insect transfers bacterial endophytic communities between grapevine plants. *Frontiers in Microbiology*, 8, 834.
- Marx, V.** (2021). Method of the Year: Spatially resolved transcriptomics. *Nature Methods*, 18(1), 9–14.
- Nadarasah, G., & Stavrinides, J.** (2011). Insects as alternative hosts for phytopathogenic bacteria. *FEMS Microbiology Reviews*, 35(3), 555–575.
- Perilla-Henao, L. M., & Casteel, C. L.** (2016). Vector-borne bacterial plant pathogens: interactions with hemipteran insects and plants. *Frontiers in Plant Science*, 7, 1163.
- Pobigaylo, N., Szymczak, S., Nattkemper, T. W., & Becker, A.** (2008). Identification of genes relevant to symbiosis and competitiveness in *Sinorhizobium meliloti* using signature-tagged mutants. *Molecular Plant-Microbe Interactions*, 21(2), 219–231.
- Raddadi, N., Gonella, E., Camerota, C., et al.** (2011). ‘Candidatus *Liberibacter europaeus*’ sp. nov. behaves as an endophyte rather than a pathogen. *Environmental Microbiology*, 13(2), 414–426.
- Sarfatis, A., Wang, Y., Twumasi-Ankrah, N., & Moffitt, J. R.** (2025). Highly multiplexed spatial transcriptomics in bacteria. *Science*, 387(6732), eadr0932.
- Schneiderberg, M., Schmitz, L., Cheng, X., et al.** (2018). A genetically and functionally diverse group of non-diazotrophic *Bradyrhizobium* spp. colonizes the root endophytic compartment of *Arabidopsis thaliana*. *BMC Plant Biology*, 18(1), 61.
- Secor, G. A., Rivera, V. V., Abad, J. A., et al.** (2009). Association of ‘Candidatus *Liberibacter solanacearum*’ with zebra chip disease of potato. *Plant Disease*, 93(6), 574–583.
- Ståhl, P. L., Salmén, F., Vickovic, S., et al.** (2016). Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. *Science*, 353(6294), 78–82.
- Sumner-Kalkun, J. C., Highet, F., Arnsdorf, Y. M., et al.** (2020). ‘Candidatus *Liberibacter solanacearum*’ distribution and diversity in Scotland. *Scientific Reports*, 10(1), 16567.
- Sun, D., Jia, Z., Zhu, J., et al.** (2025). Antimicrobial peptides and their potential applications in plant protection. *Agronomy*, 15(5), 1113.
- Teixeira, D. D. C., Saillard, C., Eveillard, S., et al.** (2005). ‘Candidatus *Liberibacter americanus*’ associated with citrus Huanglongbing in Brazil. *International Journal of Systematic and Evolutionary Microbiology*, 55(5), 1857–1862.
- Thapa, S. P., De Francesco, A., Trinh, J., et al.** (2020). Genome-wide analyses of *Liberibacter* species provide insights into evolution, phylogeny, and virulence factors. *Molecular Plant Pathology*, 21(5), 716–731.
- Wasai-Hara, S., Hara, S., Itakura, M., et al.** (2020). Diversity of *Bradyrhizobium* in non-leguminous sorghum plants. *Microbes and Environments*, 35(1), ME19102.
- Wheatley, R. M., Ford, B. L., Li, L., et al.** (2020). Lifestyle adaptations of *Rhizobium* from rhizosphere to symbiosis. *Proceedings of the National Academy of Sciences*, 117(38), 23823–23834.
- Zhang, S., Lu, Y., He, J., et al.** (2025). A ‘Candidatus *Liberibacter asiaticus*’ effector controls sugar metabolism and endocytosis in *Citrus sinensis*. *BMC Genomics*, 26(1), 717.
- Zhang, S., Wang, X., He, J., et al.** (2023). A Sec-dependent effector contributes to viru-

lence in 'Candidatus *Liberibacter asiaticus*'.
Frontiers in Plant Science, 14, 1224736.

Zhao, P., Yang, H., Sun, Y., et al. (2025). Targeted
MYC2 stabilization confers citrus Huan-
glongbing resistance. *Science*, 388(6743),
191–198.

Zou, F., Tan, C., Shinali, T. S., et al. (2023). Plant
antimicrobial peptides: classification,
production, and applications. *Food &
Function*, 14(12), 5492–5515.

S

Summary

SUMMARY

Huanglongbing (HLB), or citrus greening disease, is one of the most devastating threats to global citrus production. It is caused by the phloem-limited bacterium *Candidatus Liberibacter asiaticus* (CLAs), transmitted by the Asian citrus psyllid (*Diaphorina citri*). The disease leads to phloem blockage, leaf chlorosis, fruit deformation, and eventually tree death. Despite decades of research, progress in understanding the molecular basis of CLAs–host interactions has been limited by three major constraints: the unculturable nature of CLAs, the lack of synchronized infection systems for early-stage studies, and the absence of tractable in planta models for gene function or drug validation. In this thesis, I aimed to address these challenges by developing new experimental methods and proposing new mechanistic perspectives that together advance our understanding of CLAs biology and HLB pathogenesis.

In **Chapter 1**, I introduce the historical background, epidemiology, and biological characteristics of *Candidatus Liberibacter asiaticus* and HLB, summarizing why progress in this field has been slow. I outline the three major bottlenecks in HLB research—the lack of a culturable bacterium, the absence of synchronized early-stage infection systems, and the challenges of studying phloem-specific responses in whole-leaf samples—as well as the limitations of current control strategies. This chapter establishes the conceptual and technical gaps that motivate the development of new methodologies throughout the thesis.

In **Chapter 2**, we developed a modified micrografting (MiG) system that allows rapid and synchronized infection of citrus seedlings using buds from HLB-symptomatic scions. This system achieved systemic CLAs invasion within 40 days—significantly faster than conventional grafting or psyllid-mediated inoculation. Using laser-capture microdissection (LCM) of phloem tissues followed by RNA sequencing, we generated the first phloem-specific early-stage transcriptome of citrus responding to CLAs. Through this approach, we identified 75 previously unreported early-responsive genes. These genes not only provide insight into early host responses but also represent promising infection-inducible promoters for targeted antimicrobial applications.

In **Chapter 3**, I analyzed 79 pairs of homologous genes shared between CLAs and the symbiotic megaplasmid pSymA of *Sinorhizobium meliloti* (Sme) to explore how CLAs has evolved its gene regulation compared to its free-living relatives. By constructing dual-promoter reporter fusions and expressing them in the Sme–*Medicago* nodule system, I visualized promoter activities in planta. I found that none of the homologous pairs displayed an identical expression pattern. CLAs promoters were often active in infection threads and symbiosomes, while their pSymA counterparts were weak or inactive. These results indicate that the regulatory networks of CLAs have diverged profoundly from those of rhizobia, likely reflecting adaptation to a stable intracellular phloem niche.

In **Chapter 4**, I used the *Sme-Medicago* root nodule symbiosis as a heterologous model to study the CLas transcription factor *RpoH*, a key regulator of stress responses, to enable in planta functional testing of CLas genes. Deletion of *RpoH* in *Sme* caused severe defects in nodule development. Complementation with CLas *RpoH* partially restored nodule formation, demonstrating functional conservation between the two species. Importantly, the previously identified inhibitor rosiglitazone maleate reversed this complementation effect and also suppressed CLas titers in infected citrus seedlings. I thus established a tractable in planta screening platform for CLas functional assays and drug evaluation, providing a valuable bridge between molecular studies and applied disease control.

In **Chapter 5**, I performed large-scale phylogenomic analyses of over 1,600 α -proteobacterial genomes using the most conserved AMPHORA housekeeping genes. My results showed that *Liberibacter* species are nested within the genus *Rhizobium*, rather than forming a separate lineage. This contrasts with earlier studies, which used faster-evolving gene sets and were likely affected by long-branch attraction. The refined phylogenetic placement implies that *Liberibacter* represents a specialized, genome-reduced *Rhizobium* lineage that independently adapted to intracellular life in phloem and psyllid hosts. This evolutionary insight not only clarifies the taxonomic position of CLas, but also supports the use of *Rhizobium*-based systems as surrogates for its study.

Together, these studies provide a framework linking molecular mechanisms, experimental models, and evolutionary context. By establishing a rapid and synchronized infection system, developing in planta surrogate models, and redefining the evolutionary roots of CLas, I have contributed both practical tools and conceptual advances to the study of HLB. The MiG-LCM approach enables discovery of early defense markers and inducible promoters; the rhizobium-based systems allow gene functional testing and inhibitor screening; and the phylogenetic reconstruction places CLas within the *Rhizobium* genus.

Collectively, this work opens new directions for functional genomics, molecular pathology, and targeted engineering of HLB resistance in citrus, paving the way toward sustainable management of HLB and other phloem-limited bacterial diseases.

A

Acknowledgements

ACKNOWLEDGEMENTS

My life has drifted with the wind, carried onward by the quiet hands of fate.

For a long time, I did not know where the road would lead. I simply followed the current, through seasons of uncertainty, through departures and arrivals, through days of quiet perseverance and nights of silent doubt.

Yet when I look back, I see that after every long winter, spring has found its way to bloom; behind the heaviest clouds and deepest mist, the sky has always been waiting to clear.

Along this journey, I have beheld many landscapes: dawn breaking over unfamiliar places, the gentle beauty of changing seasons, and a world far wider than the one I once imagined.

But the most beautiful scenery was never only the mountains, rivers, or distant horizons. In many corners of the world, and especially here in this lowland country, the most beautiful scenery has always been the people — kind and generous souls who lit my path and made this wandering life feel less alone.

My deepest gratitude goes first to my promotor, Ton Bisseling. Thank you for giving me the freedom to wander in science, while always keeping a light on the path ahead. Your wisdom, patience, and trust have shaped not only this thesis, but also the way I have learned to think, to question, and to stand again after uncertainty. In moments when the road seemed blurred, your guidance was like a steady compass, quiet but certain, reminding me to look further, think deeper, and keep moving forward.

Sincere gratitude also goes to René Geurts and Xu Cheng, who together managed and cared for our Rhizosphere group with dedication and warmth. Thank you for creating a place where ideas could grow and questions were welcomed. Your kindness, clarity, and generous help made the daily journey of research lighter, steadier, and more meaningful. Under your guidance and care, the Rhizosphere group became not only a scientific environment, but also a place of encouragement, trust, and quiet belonging.

To all members of the Rhizosphere group, thank you for the companionship, discussions, encouragement, and small acts of kindness that filled these years. A research group is more than benches, meetings, and experiments; it is made of people who share the long days, the failed attempts, the small victories, and the quiet understanding that comes from walking similar paths. Having spent this chapter of my life among you has been a true fortune.

Deep gratitude goes to those who accompanied and supported me along the path of Huanglongbing research. My heartfelt thanks go to Feng Zhang and Xianfeng Ma, who worked

with me over many years and offered generous support to my experiments. Thank you for the warm hospitality you extended to me in Changsha. Because of you, studying Huanglongbing from the Netherlands felt less distant, less solitary, and more closely connected to the land where citrus trees grow and where this research truly matters. Sincere thanks also go to Melanie Barnett and Sharon Long. At moments when this research needed ideas, materials, and direction, you offered thoughtful answers and careful help with remarkable generosity. Your support reached me across distance and became a quiet but important light on this scientific path.

In some places, small matters can quietly consume a great deal of strength. Fortunately, I found myself in MolBi, a place that seems to lift and hold everyone, allowing us to keep our minds on science and our hearts on the questions we came here to pursue. My sincere thanks go to Maria Augustijn and Marie-José van Iersel, who made so many things run smoothly, often before we even realized how much we needed them. Your swiftness in booking the defence slot was especially remarkable. Warm thanks also go to Carolien Franken, who has always seemed like a magical Doraemon, ready with every small item, practical solution, and piece of guidance we might need.

My gratitude also extends to all colleagues in MolBi, who together created an office environment where work could be enjoyed, and where laughter often filled the days. Of course, heartfelt thanks also go to Olga Kulikova, who, when needed, helped us protect a quiet and concentrated space for writing.

Inevitably, my deepest gratitude turns to my parents. Thank you for your unconditional love and selfless support, for giving me not only the strength to move forward, but also the freedom to choose my own path.

Finally, a few simple words to my wife and my daughter. My gratitude to you does not truly need to be written at the end of this thesis, for it is already written into every ordinary day of my life. The road ahead is still long, and life will continue to unfold in ways we cannot yet know. But with you beside me, even the simplest days feel full, warm, and deeply blessed. Thank you for being my home, my tenderness, and my greatest happiness.

Jing

20th April 2026, Suzhou, China

