



Engineering of citrus to obtain huanglongbing resistance

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Huanglongbing (HLB) disease is threatening the sustainability of citriculture in affected regions because of its rapid spread and the severity of the symptoms it induces. Herein, we summarise the main research findings that can be exploited to develop HLB-resistant cultivars. A major bottleneck has been the lack of a system for the *ex vivo* cultivation of HLB-associated bacteria (CLs) in true plant hosts, which precludes the evaluation of target genes/metabolites in reliable plant/pathogen/vector environments. With regard to HLB vectors, several biotechnologies which have been proven in laboratory settings to be effective for insect control are presented. Finally, new genotypes that are resistant to CLs or their insect vectors are described, and the most relevant strategies for fighting HLB are highlighted.

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Introduction

Huanglongbing (HLB), formerly known as citrus greening disease, has become a devastating problem in the last decade, threatening citriculture worldwide. The disease is associated with phloem-limited intracellular *Candidatus Liberibacter* species (CLs) which are transmitted by the psyllid insects *Diaphorina citri* and *Trioza erytreae*, with the most aggressive CL–vector combination being formed by CL asiaticus (CLAs) and *D. citri* (Figure 1). Symptoms include phloem dysfunction and root decline, altering the plant source–sink relationships [1^{*}]. Despite extensive research efforts, HLB is still incurable owing to the

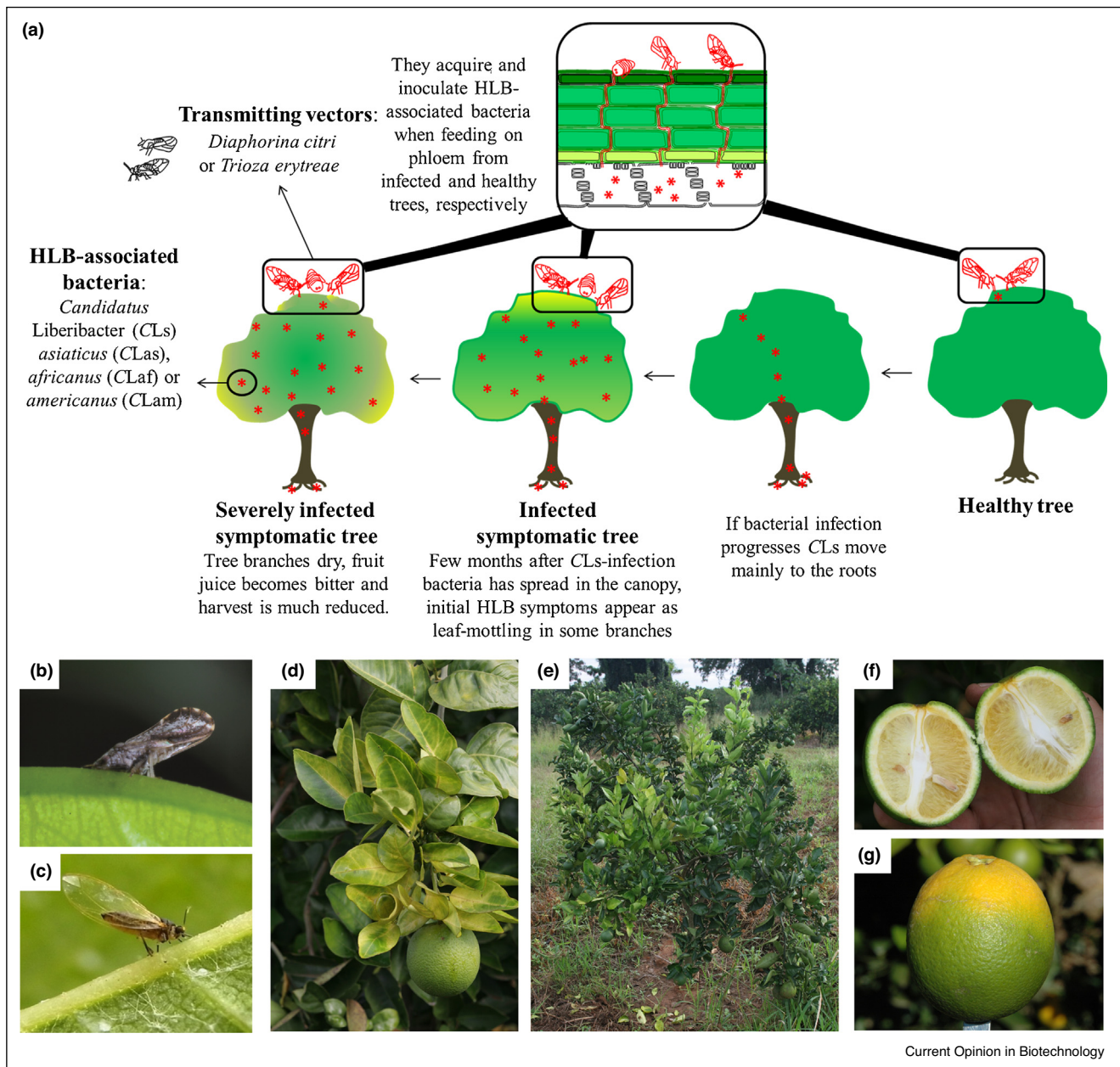
complexity of plant–vector–bacterium interactions [2^{*}]. Currently, the best HLB management strategies are vector control with insecticides, the elimination of affected trees, and improved cultural practices [3^{**}]. As HLB hampers the existence of an environmentally and economically sustainable citriculture, there is a pressing need to find long-term sustainable solutions for controlling the disease. Although many research studies related to HLB have been published over the last two years (Figure S1), this present review focuses only on those that had searched for HLB-resistant *Citrus* relatives and investigated the biology of HLB-associated bacteria and transmission vectors with the potential to develop biotechnological solutions to fight the spread of the disease.

HLB-associated bacteria (CLs)

Despite multiple attempts, axenic cultures of CLs have not yet been established [4^{*}], and most of the information about the biology of these species has been gleaned from their genome sequences [5]. The manually curated analysis of genomes from six CLs strains has led to the annotation of hundreds of genes related to their metabolism and transport as well as expanded the list of CLs auxotrophic for different amino acids, vitamins, and steroids [6], which may be acquired from their hosts. The combination of genome-derived metabolic data with RNA-Seq data from CLs-infected psyllids and *Citrus* hosts has revealed specific metabolic traits operated by the bacteria in each *Citrus* host type and hinted at target genes whose inhibition may block bacterial growth. Six CLs transcriptional regulators (RpoH, VisNR, LdtR, LsrB, PhrR, and CtrA) have been suggested to be essential for bacterial survival and multiplication [7]. Additionally, it has been proposed that this kind of obligate parasite with reduced genome size may also rely on the associated microbiota to acquire and synthesise essential metabolites [8^{**}]. Thus, research on phloem-associated microbiota may uncover new target genes or organisms for controlling CLs.

The inability to culture CLs axenically has also hampered research on their pathogenic mechanisms. Most studies are based on genome mining for identifying potential virulence factors. Up to 121 putative general secretory pathway (Sec)-dependent effectors (SDEs) have been described for CLs and 30 for CL americanus and CL africanus [9–11]. Some of their roles have been investigated by comparing gene expression levels in different

Figure 1



Process of Huanglongbing (HLB) spread, transmitting psyllid vectors and characteristic symptoms of the disease.

Representative scheme of vector-mediated transmission of *Candidatus Liberibacter* bacteria associated to HLB from infected to healthy trees **(a)**. The name of the species refers to the continent where they were first found and identified. CLAs is currently spread in Asia and America predominantly, CLaf in Africa and Arabian Peninsula and CLam in Sao Paulo (Brazil). Representative photographs of HLB-associated bacteria transmitting vectors **(b,c)** *Diaphorina citri*, also known as Asian citrus psyllid (spread in Asia and America) **(b)** and *Trioza erytreae* or African citrus psyllid (spread in Africa and Arabian Peninsula mainly) **(c)**. Characteristic HLB symptoms **(d)–(f)**: Leaves with typical asymmetrical mottling **(d)**, advanced stage of HLB infection, in which complete yellowed shoots are already visible **(e)**, internal **(f)** and external **(g)** aspect of fruits from trees affected by HLB in which deformation, seeds abortion and inversion of degreening are observed. ©Pre-HLB, A. Fereres and Fundecitrus.

CLAs-infected samples or by using bacterial and plant surrogate model systems [7,9–14]. However, differences in the host responses and the pathogenic mechanisms of these surrogate systems and those of CLs have yet to be verified in their natural hosts and so the results are not

entirely reliable [8**]. To the best of our knowledge, only three putative SDEs have been investigated through the stable transformation of *Citrus* hosts. Sec-delivered effector 1 (*SDE1*) and *SDE15* were found to enhance CLAs infection when overexpressed in transgenic grapefruit,

the former by reducing both the overall papain-like cysteine protease (PLCP) activity and the plant defence responses [15,16^{*}] and the latter by acting on accelerated cell death 2 (*ACD2*), a proposed susceptibility target gene for CLs [17]. Similar to *SDE1* and *SDE15*, the overexpression of CLas P_{235} induced HLB-like deficiencies in transgenic *Citrus* plants [18]. These types of studies involving CLs and true host plants are time-consuming because of the difficulties in obtaining transgenic lines and performing consistent CL challenge inoculations, rendering high-throughput screening impossible. Recently, a high-throughput antimicrobial testing method for *Citrus* plants and CLs has been developed [19^{**}]. It involves the *Rhizobium rhizogenes*-mediated induction of hairy root formation by *Citrus* explants infected, or not, with CLs. The roots are then transferred to plates containing the growth medium supplemented with the molecules to be assayed. By measuring the variations in CLas multiplication in these *in vitro* cultures, the authors identified six antimicrobial peptides and eight growth inhibitors. Hairy root-based assays can be completed in a little more than a month, being much faster than conventional methods, and they mimic the host-pathogen environment. Moreover, as *Citrus* plants are suited to *R. rhizogenes*-mediated genetic transformation, this hairy root methodology would also allow the performance of genetic tests for identifying or corroborating — in a straightforward manner — the potential host target genes which lead to CL resistance, as reported for potato and CL solanacearum [19^{**}].

Transmission vectors of HLB-associated CLs

The Asian citrus psyllid *D. citri* is well known at the entomological and biological levels [20^{**}]. Studies over the last decade have indicated that odour blends, rather than specific volatile compounds (VOCs), are cues to host seeking by *D. citri*, which is able to discriminate between different host blends and even between the different qualitative conditions of host plants. Electrophysiological and behavioural assays have allowed the identification of *D. citri*-repellent VOCs from guava [21,22]. Recently, five Chinese plants were reported to be even more repellent than guava to *D. citri* in olfactometry assays [23]. The VOCs responsible for the repellent effect could be exploited biotechnologically to turn attractive *Citrus* plants into repellent ones. Alternatively, by modifying the synthesis of the volatiles responsible for the host attraction response, cultivars that are less attractive to *D. citri* can be obtained.

Psyllid insects also rely on visual cues for host selection and are highly attracted to bright yellow and green colours which resemble new flushes. The attraction to these colours may explain the presence of *D. citri* in non-host plants, which are unable to support their long-term survival [24]. It has been demonstrated through electropenetration graphs that although *D. citri* is able to access the

xylem of some non-host plants, it is unable to reach the phloem despite multiple attempts [25]. This is probably because HLB-carrying psyllids may require a response from plants to gain compatible access to the phloem, as occurs for other phloem feeders. Thus, the outcome of plant–insect interactions stems from the interplay between the host defence machinery and the effector molecules secreted in the insect saliva. Available genomic data and new proteomic data from *D. citri* saliva and salivary glands are now being used to identify potential effectors [26–28].

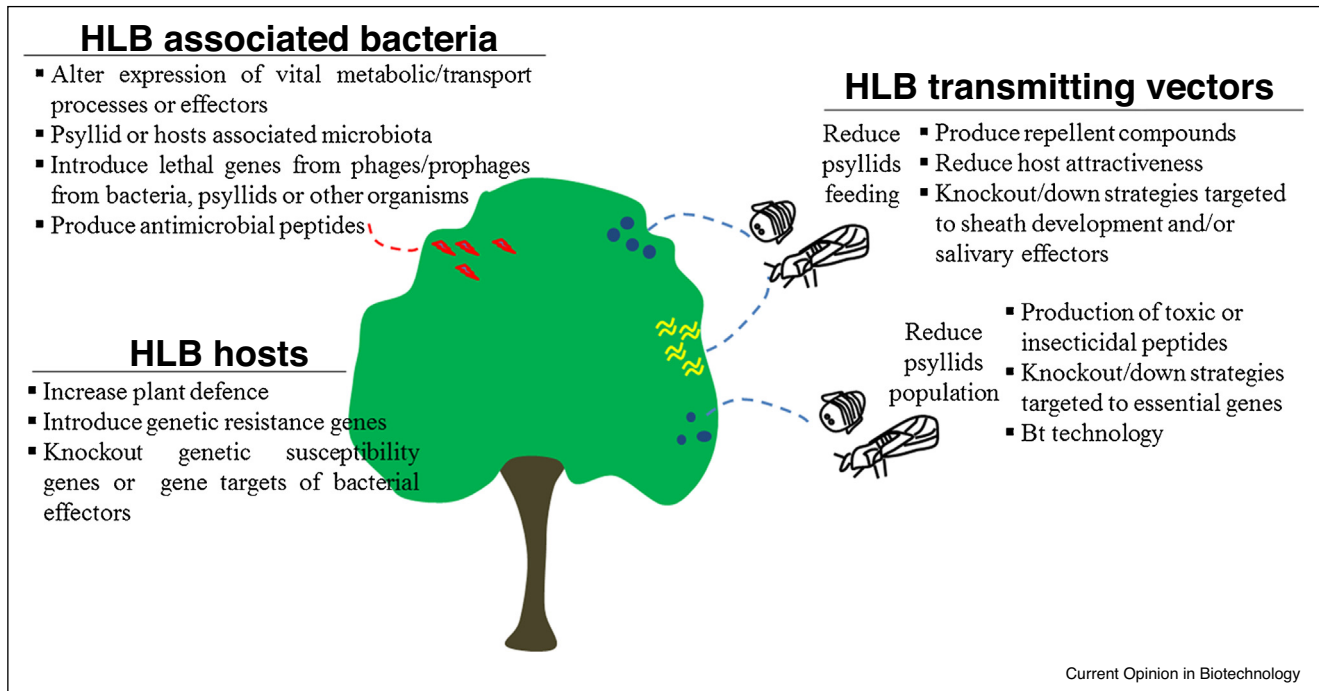
The use of RNA interference (RNAi) to target the vital genes of *D. citri* through the exogenous delivery of dsRNAs has proven to be effective [29,30], suggesting that such delivery via transgenesis could be a feasible strategy for limiting psyllid populations. An attempt to test this was performed using the vector *Citrus tristeza virus* (CTV), which has three main advantages over stable *Citrus* transformation: the technique is faster, can be applied to CLas-infected trees, and is directed to the phloem and associated cells. Of the nymphs resulting from parents that had been fed on plants inoculated with CTV virions targeting the *D. citri* abnormal wing development (*awd*) gene, 45% displayed wing malformation and approximately 20% less psyllids reached the adult stage [31]. The targeting of multiple psyllid genes could increase the mortality of the insects, thereby limiting the spread of CLs.

Another potential biotechnological process for controlling *D. citri* is based on *Bacillus thuringiensis*, some strains of which can induce high psyllid mortality. Different Cry toxins have been identified as being responsible for this effect, with Cry1Ab being highlighted in different studies [32,33]. Optimisation of the Cry1Ab peptide, its use in combination with other Cry toxins with synergistic or additive effects, and/or the addition of gut-binding anchors to toxins (as previously reported to control other pests) may lead to higher psyllid mortality. Moreover, the transgenic overexpression of such toxins directed to the phloem could result in the generation of psyllid-killing plants.

Hosts of HLB-associated CLs and transmission vectors

The resistance of host plants to HLB could be derived from their resistance to the CLs or their transmission vectors or both. HLB-associated bacteria (CLs) and transmission vectors have a wide host range, covering most species of the Aurantioideae, a subfamily of the Rutaceae. This includes all *Citrus* genotypes as well as *Poncirus* and *Fortunella* relatives and their hybrids with *Citrus*, which is relevant because these relatives are widely used as rootstock for citriculture and for ornamental purposes, respectively. Although *Citrus* cultivars show different degrees of sensitivity to CL infection, all of them are susceptible,

Figure 3



Proposed biotechnological strategies to obtain commercial citrus cultivars resistant to HLB.

Strategies can be directed to limit the population of psyllid transmitting vectors (by engineering plants producing toxic or insecticidal peptides, Bt toxins, dsRNAs to knockdown insect's vital genes or knockouts of these genes by genome edition) or to reduce their feeding on host plants (by limiting their attractiveness or turning them deterrent to the psyllids, by knockdown/knockout psyllids genes involved in sheath development or responsible for effectors synthesis). Alternative strategies can be directed to limit HLB-associated bacteria multiplication, for example by targeting essential metabolic/transport genes, bacterial effectors, by overexpressing antimicrobial peptides or lethal genes from phages or prophages from bacteria, psyllids or other organisms. Decipher if these bacteria rely on associated microbiota to survive and target it worth further research.

Finally, avoiding plant infection can also be addressed by increasing plant defence mechanisms, by introducing genetic resistance genes or by knocking out susceptibility genes or targets of bacterial effectors. Because HLB symptoms develop at a wide-range of bacterial titers, the goal would be to avoid bacterial infection/multiplication and stacking diverse biotechnological strategies would be likely required to achieve it durably.

allowing bacterial multiplication and displaying symptoms which affect production. For example, the degrees of CLAs-induced damage in Eureka lemons and Tahiti limes are milder than those in most orange and mandarin cultivars. *Citrus* is surmised to be a relatively new alternative host for CLs, and the short period of co-evolution of both organisms has not yet resulted in the emergence of resistance in these plants [34]. The clonal propagation of most commercial *Citrus* genotypes may also be responsible for this lack of resistance. Recently, some CLAs-resistant Aurantioideae species have been described [35^{••},36,37]. For example, some Oceanian *Citrus* relatives from the *Eremocitrus* and *Microcitrus* genera as well as their hybrids and hybrids with *Citrus* showed full resistance to CLAs infection, as characterised by the lack of bacterial multiplication in aggressively challenge-inoculated plants (Figure 2). It has been speculated that the acquisition of resistance to CLAs by the *Eremocitrus* and *Microcitrus* spp. may be related to the loss or inactivation of susceptibility genes which took place after the Asian and Oceanian

species diverged [35^{••}]. Some other species from the Citrinae subtribe (sexually incompatible with *Citrus*) show partial resistance to CLAs. Aurantioideae species that are highly attractive to *D. citri* — but are CLAs resistant — can be used in trap-and-kill approaches [37], by applying chemicals or engineering them to be lethal to psyllids. Investigations into how these CLAs-resistant genotypes respond to *CL americanus* or *CL africanus* infections would be interesting for unravelling the pathogenic mechanisms of CLs.

Despite that HLB-transmitting psyllids show variations in their preference to different *Citrus* species and *Citrus* relatives, to the best of our knowledge, none of these plant genotypes have been described as being fully resistant to HLB-transmitting vectors. Nevertheless, some *Poncirus trifoliata* accessions, described as non-preferred hosts, present variable degrees of antibiotic (affecting nymphal development and adult longevity) and anti-xenotic (less oviposition, higher egg mortality, and less

adult emergence) effects on *D. citri* [38–40]. However, whether these effects are related to biochemical or physical causes remains to be uncovered.

The *Eremocitrus* and *Microcitrus* resistance to CLAs and the detrimental effects of some *Poncirus* cultivars on *D. citri* development have promoted breeding programs aimed at obtaining HLB-resistant varieties. Although such efforts take decades to complete, they could be sped up if segregation progenies allow the identification of hitherto unknown molecular markers associated with HLB resistance [41,42]. Alternatively, the identification

of genes related to HLB resistance or susceptibility could be transferred to relevant commercial cultivars via transgenic approaches, which despite being a slow process with *Citrus* plants is still faster than conventional breeding. Currently, only a few transgenic strategies against HLB have been tested. *Citrus* cultivars overexpressing genes encoding antimicrobial peptides with proven effects against other pathogenic bacteria (e.g. synthetic D2A21, a modified plant thionin, a codon-optimised version of the cecropin B gene, or certain attacins) did not show durable CLAs resistance, only some titer reduction [43–46]. When *NPR1*, a gene related to systemic

Figure 2

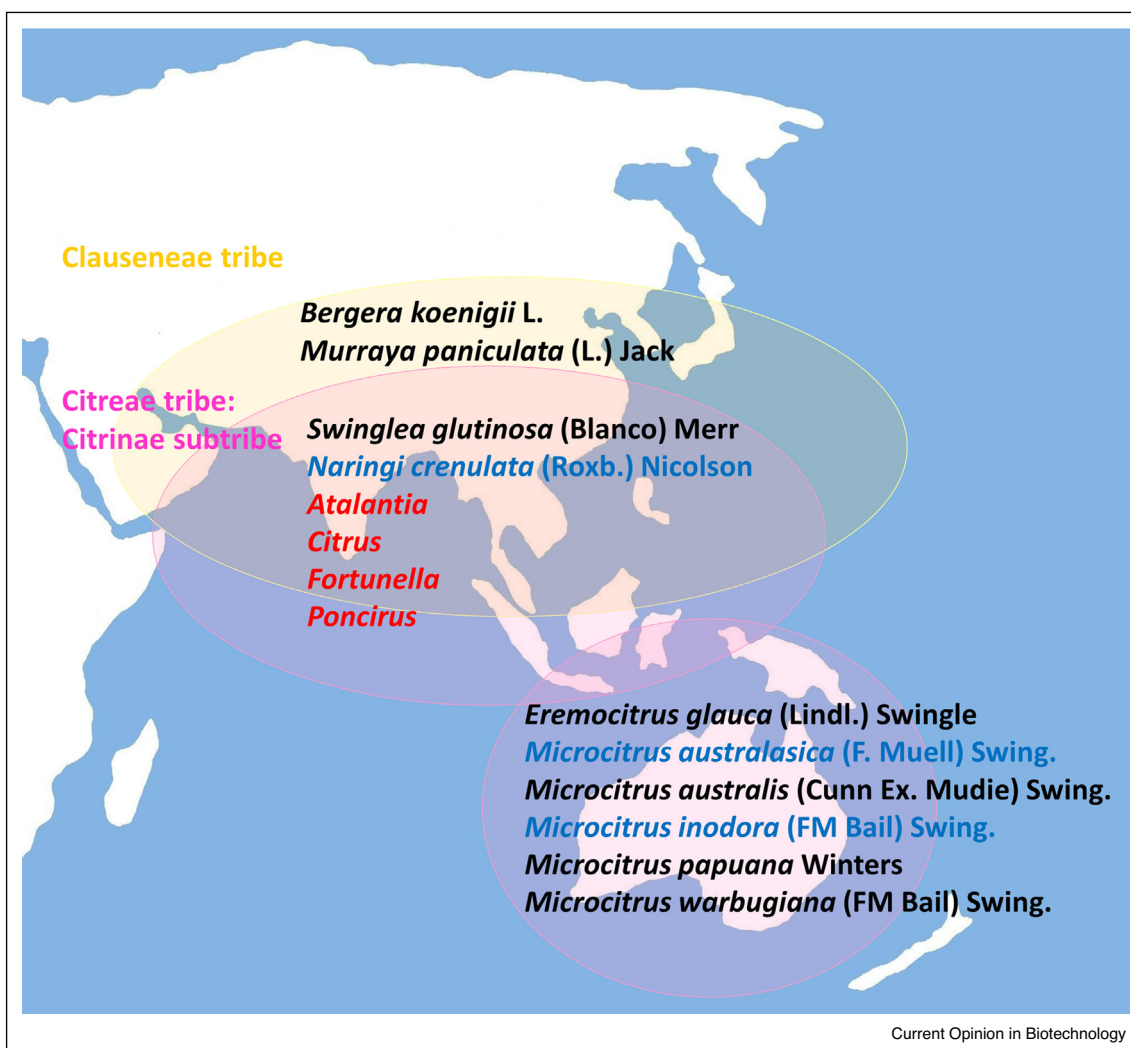


Illustration showing CLAs resistant/susceptible representative genotypes and their geographical origin.

Geographical origin of genotypes from Clauseneae tribe is marked by a yellow area, while that of Citreae tribe, Citrinae subtribe is delimited by pink circles. CLAs-resistant genotypes are indicated in black letters. CLAs partially resistant and susceptible genotypes are blue and red-lettered, respectively. Clauseneae tribe species are generally resistant to CLAs. Within Citreae, partial resistance and susceptibility to CLAs starts to appear phylogenetically in the Citrinae subtribe, being all *Citrus* species and cross-compatible genotypes of Asian origin susceptible to the bacterium. Intriguingly, all *Citrus* relatives (sexually compatible with *Citrus*) of Oceanian origin are partially or full resistant to CLAs. Alternatively, they may lack susceptibility traits as they were never exposed to CLAs and to its vector. Adapted from Alves *et al.* [35*].

acquired resistance, was overexpressed in grapefruits and oranges, the *CL*s titers were also reduced [47,48]. Moreover, transgenic plants expressing chimeric constructs of *NPR1*, thionin, and antimicrobial peptides did not exhibit typical leaf mottling [49]. Recently, engineered oranges deterrent to *D. citri* in laboratory experiments have been generated [50]. The effects of these strategies on the incidence and spread of HLB in the field have yet to be reported.

Conclusions and future prospects

After almost two decades of research on HLB, there is still an urgent need for satisfactory and lasting solutions to control its spread and incidence. Although many scientific reports have been published, the challenges of working with woody plants and uncultivable bacteria have limited the acquisition of critical knowledge about this pathosystem. The unravelling of the interactions between *CL*s and plant hosts — which is vital to the design of biotechnological strategies for acquiring plant resistance to bacteria — is now more feasible thanks to the recently developed hairy root system [19**]. This will likely allow the high-throughput screening of potential anti-*CL*s molecules (antimicrobial peptides, effectors, etc.), genetic modification strategies, and unequivocal identification of *CL* virulence factors. The uniform distribution of *CL*s in roots [51] will also help to solve another important problem encountered in this pathosystem: the highly variable titers of *CL*s detected, even within the same plant, depending on the inoculation and sampling methods used owing to the uneven distribution of bacteria in the plant [52,53], which may also lead to the equivocal classification of some plants or genotypes as being resistant. Furthermore, the different environmental conditions in diverse citrus regions can cause differences in bacterial titers of 1–2 orders of magnitude in naturally infected orchards, and this does not preclude the immense damage induced by HLB. Additionally, research on HLB-resistant genotypes is expected to generate new information related to bacterial susceptibility or resistance genes.

To date, there has been no effective treatment (conventional or biotechnological) to limit infections by any intracellular prokaryotic bacteria. Thus, in order to limit HLB spread, the faster strategies would be those focused on generating resistance to the transmission vectors. Like other phloem-sucking insects, HLB psyllids inject effectors and enzymes through their salivary sheath into the plant to facilitate their feeding and suppress the plant defences. Therefore, targeting of the molecules involved in these processes may limit/prevent the feeding activity and thereby the inoculation of *CL*s into the host plant. Reducing the massive reproduction rate of the psyllids or disrupting their attraction to host plants would also help to reduce HLB spread. Furthermore, increasing our knowledge about psyllid–host interactions, such as on

the effectors required to establish a compatible interaction between the two organisms, would reveal additional targets to be addressed.

Because of the complex genetic and reproductive features of *Citrus* plants and the difficulty in creating new elite commercial *Citrus* varieties after conventional and time-consuming breeding programs, especially considering the available sources of full resistance, it is our opinion that the development of genetically modified plants is the most durable way to obtain HLB-resistant cultivars of economic relevance. The difficulty in fighting both HLB-associated pathogens and their vectors leads us to consider that the best strategy would be to stack target genes to limit both the multiplication of *CL*s and the attraction of psyllids to hosts and their subsequent feeding and reproduction (Figure 3). Consumer rejection of genetically modified organisms, especially in Europe, points to cisgenic or gene-editing developments as being more convenient, albeit CRISPR/Cas technology without T-DNA insertion is yet to be established in *Citrus*. Until resistance to HLB is achieved, the combination of genetically modified repellent cultivars with cultural strategies directed at limiting the spread of psyllids (e.g. by using traps, killer plants, or insecticide peptides) could help to reduce the impact of this disease. Alternatively, to reduce bacterial infections, antimicrobial peptides with the potential to reduce bacterial titers could also be used, such as those identified from *CL*s-resistant Aurantioideae plants [54*].

Conflict of interest statement

Nothing declared.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.copbio.2021.06.003>.

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