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Can CRISPR Win the Battle against Huanglongbing

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Review Article

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ABSTRACT

Huanglongbing (HLB) is the most destructive disease of citrus. The causal agent is Candidatus Liberibacter spp. The pathogen is phloem-limited gram-negative bacterium. HLB has caused huge loss in citrus fruit production. All commercial citrus cultivars can be infected by this disease and no cure for it. In this review, we discuss disease detection, host responses upon infection. Because conventional breeding can't confer citrus resistance to HLB, transgenic approach is the alternative to produce disease resistant citrus facing the urgent need. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is the most recent and advanced genome editing technology that has been successfully applied to develop canker resistant citrus. In this review, the potential application of CRISPR technology in citrus resistance to HLB is discussed.

INTRODUCTION

Huanglongbing (HLB) is the most destructive disease of citrus. The typical symptoms are yellowing of the leaf veins and adjacent tissues, premature defoliation, dieback of twigs, ultimately leading to death of infected citrus trees [1]. The affected trees show retarded growth, off season flowering, bearing unattractive, smaller fruits that are bitter in taste. Recently, outbreak of HLB has been a major problem in citrus that resulted in declining fruit quality and quantity [2]. The causal agent is phloem-limited uncultured gram-negative bacterium [3,4]. All commercial citrus cultivars are susceptible to HLB regardless of its root stock.

HLB infected citrus worldwide, including Asia, Africa and Americas. In USA, it was first reported in south Florida (2005). The causal agent *Candidatus* Liberibacter asiaticus (CLas) spread rapidly to Louisiana, Georgia, Texas *etc.* HLB is transmitted and vectored by Asian citrus psyllid (ACP) [5]. Grafting can transmit HLB too [6]. Till now, there is no effective management for HLB. Normally, insecticide is used to control vector to suppress spreading of HLB [7]. To prevent the HLB spreading, the infected trees need be removed and destroyed. Heat treatment can reduce the symptoms of infected trees by elimination of CLas [8]. However, heat treatment can't prevent the treated trees from secondary infection and it is impractical to treat big trees in field. The topics of how to detect the disease, responses of host plant to HLB, and the perspective of CRISPR in controlling HLB are discussed in this review.

LITERATURE REVIEW

Disease Detection

HLB has long latent period, and it is difficult to distinguish HLB infected trees from zinc deficient trees due to similar appearance of leaves [9]. Therefore, it is necessary to confirm disease infection by molecular tools. Southern blot was used

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to detect bacterium from plant tissue at first [10]. Southern blot is time consuming and the sensitivity is lower as compared to Polymerase Chain Reaction (PCR)-based methods. Due to the low titer and uneven distribution of Clas in citrus, PCR was used to distinguish Candidatus Liberobacter asiaticum and *Candidatus Liberobacter africanum* combined with XhaI enzyme digestion [11]. Quantitative real-time PCR was used to detect pathogen based on 16S rDNA [12]. To increase the sensitivity, primers from nrdB which has five copies in genome were used to reduce Ct value of 1.68 (SYBR Green PCR) and 1.77 (TaqMan PCR) [13]. Now, a rapid field test system has been developed to detect Clas from psyllid as well as plant [14]. PCR based methods are rapid and sensitive, but it is difficult to be used in screening at larger scale. Therefore, imaging techniques were developed for the purpose of large screening [15,16].

Citrus Responses to HLB Infection

Based on plant symptoms and ability to grow post infection with HLB, 30 different genotypes of citrus were divided into four classes: 1) sensitive, which showed severe chlorosis on leaves, growth was inhibited greatly, and the infected plants were dead, such as Valencia sweet orange, Ducan grapefruit etc.; 2) moderately tolerant, plants showed distinguished symptoms but grew normally, however died, such as Sour orange and Mexican lime etc.; 3) tolerant, which showed minimal symptoms such as Eureka lemon, Persian lime, Carrizo citrange, and *Severinia buxifolia*; 4) seven genotypes showed variable symptom. The titers of Clas were not related with the severity of the symptoms [17].

Since CLas is restricted in phloem, different parts of sweet orange were observed under microscope to detect the cellular structural change due to HLB infection. HLB infected sweet orange leaves showed phloem damage and plugging of sieve pores [18]. The HLB infected stem exhibited cell wall thickness and collapse were observed in phloem, and the symptoms were more severe in stem than in root [19]. The phloem were damaged in HLB tolerant rough lemon and susceptible sweet orange at the similar level, but phloem transportation was maintained much better in rough lemon [20]. The damage of cell wall and cell membrane in phloem by Clas was caused by movement of pathogen between cells [21].

Comparative metabolomics between HLB infected, zinc-deficient, and healthy 'Valencia', showed difference among the different samples [22]. In the HLB infected leaves, proline accumulated at the highest level. Beta-elemene, (-) trans-caryophyllene, and alpha-humulene exclusively accumulated at lower level in HLB infected leaves. But, the concentrations of proline and arginine were lower in Las positive fruit than in healthy fruit of 'Valencia' [23]. Metabolomic changes were found in HLB infected Hamlin as well [24]. Phenylaninine, tryptophan, lysine and asparagine accumulated more in phloem sap of HLB tolerant varieties than in HLB sensitive varieties [25]. Volatile organic compounds (VOC) that protect plants from attack of insect and pathogen were analyzed among HLB sensitive varieties and tolerant varieties. The HLB tolerant cultivars contained more VOC as well as compound with antimicrobial activities [26].

Gene expression changes at transcriptional level upon HLB infection in leaf, stem and root of sweet orange were analyzed to compare the gene differential expression from microarray analysis [18,19]. RNA-Seq was employed to compare gene transcriptional profiling of leaf and fruit or fruit [27,28]. Microarray study revealed that the genes significantly regulated in leaf, stem, and root were 624, 885 and 111, respectively [18,19]. Among the differentially expressed genes, the numbers of upregulated genes were 307, 551 and 56 in leaf, stem and root, respectively. The differentially expressed genes covered many aspects of cellular functions, including response to disease, cell wall biogenesis, signal transduction, carbohydrate metabolism, protein degradation, phytohormones, metal transportation etc. Due to the advantages of RNA-Seq over microarray, RNA-Seq was performed to determine the disease mechanisms of HLB [27-29]. Source-sink disruption and weak plant immunity response were the main mechanisms of disease. But, it showed that HLB symptoms development depended on host response rather than on carbohydrate starvation from the microarray data by comparing fruits from HLB infected sweet orange and girdled fruit [30]. Transcriptional responses to Clas infection between HLB susceptible and tolerant citrus were analyzed to study the mechanisms of disease tolerance. It could be hypothesized that tolerance in Rough lemon to HLB may be from the minimized influence in phloem transportation and fast response to CLas by comparing transcriptional profiling with susceptible sweet orange at 5, 17 and 27 WAI (weeks after inoculation) [20]. Another microarray study showed that the HLB tolerant US-897 had more genes involved in pathogen defense expression at higher level without CLas infection compared to susceptible 'Cleopatra mandarin'. Constitutive disease resistance protein (CDR1), 2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase may contribute to tolerance of US-897 to HLB [31]. The citrus cultivars in the above two studies had different genetic background. It is difficult to draw a conclusion on the mechanisms of tolerance to HLB. Recently, RNA-Seq was conducted using two genetic close related cultivars HLB tolerant "Jackson" grapefruit hybrid and HLB susceptible "Marsh" grapefruit. The results showed that basal defense played an important role in resistance to HLB [32]. Because small RNAs participate in disease resistance, small RNA profiling was determined in HLB infected sweet orange. MiR399 was upregulated by HLB infection and resulted in phosphorus deficiency of infected plant [33,34] **(Table 1)**.

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Table 1. Host response to HLB infection.

Host response		Citrus cultivar	Plant organ	Highlight	Ref.
Phenotype		30 citrus cultivars	--	HLB sensitive, moderate tolerant, tolerant	[17]
Metabolonmic analysis		Valencia orange	Las^+ fruit, Las^- fruit	Metabolomic profiles changed, Las infection lead to concentration decrease in proline, arginine, adenosine, and sugars. But, Las infection leaded to increased concentration of citrate.	[23]
Metabolonmic analysis		HLB infected, zinc-deficient, healthy Valencia	Leaves	Develop biomarkers to distinguish HLB infected plant from zinc deficiency plant.	[22]
Transcriptomic analysis	Microarray	Citrus sinensis	Leaf	624 genes were regulated, 307 genes were up-regulated and 317 genes were down-regulated.	[18]
Transcriptomic analysis	Microarray	Citrus sinensis	Stem	551 genes were up-regulated, 334 genes were down-regulated	[19]
Transcriptomic analysis	Microarray	Citrus sinensis	Root	56 genes were up-regulated, 55 genes were down-regulated.	[19]
Transcriptomic analysis	Microarray	Rough lemon and sweet orange	Leaf	Clas induced fast and stronger disease resistant response in tough lemon	[20]
Transcriptomic analysis	Microarray	US-897, 'Cleopatra' mandarin	--	Disease resistant genes were expressed at high level in US-897 without Clas infection	[31]
Transcriptomic analysis	Microarray	Hamlin and Valencia	Leaf, fruit	HLB disease symptoms development in host may lie in host response rather than carbohydrate starvation.	[30]
Transcriptomic analysis	RNA-seq	Valencia	Fruit, leaf	Disturb source-sink metabolic function insufficient bacterial clearance to cause HLB.	[27]
Transcriptomic analysis	RNA-seq	Valencia	Fruits	Up-regulated genes: photosynthesis, ATP synthesis, protein degradation and misfolding. Down-regulated genes: heat shock proteins,	[28]
Transcriptomic analysis	RNA-seq	"Jackson" grapefruit "Marsh" grapefruit	Leaf	Basal defense is the main mechanism of disease resistance to HLB based on two close relatives responded to HLB at transcriptional level.	[32]
Proteomics analysis		Sweet orange	Leaf	HLB upregulated proteins: chitinase, lipoxygenase and miraculin-like proteins	[35]
Proteomics analysis		Grapefruit	Leaf	13 proteins were up-regulated and 56 proteins were down-regulated by HLB.	[36]
Proteomics analysis		Lemon	Leaf	Protein up-regulated: starch synthase Proteins down-regulated: photosynthesis-related proteins and defense-related proteins.	[37]
Proteomics analysis		Volkameriana and Navel	Leaf	Protein, glutathione-S-transferase, involved in radical in detoxification was up-regulated in Volkameriana by HLB.	[38]
Proteomics analysis		Grapefruit	Leaf	Heat treatment can be induced proteins repressed by HLB infection, such as photosynthesis-related proteins, chaperones *etc.* This study can give clue to select disease resistant genes.	[39]
Small RNA profile		Citrus sinensis	Leaves and barks	Mir393 was up-regulated by Las to contribute sypmtom development	[34]
Nutritional status		Grapefruit Lemon	Leaf	HLB infection resulted in reducing concentrations of Ca, Mg, Fe, Zn, Mn, Cu, and increasing concentration of K.	[36, 37]
Cell structure		Sweet orange, rough lemon, *Citrus reticulate* cv. Limau Madu,	Leaf, stem, root	HLB infection can result in phloem cell collapse in HLB susceptible cultivar as well as tolerant cultivar. But, the tolerant cultivar can maintain transportation well.	[18-21]

The genes differentially expressed at translational level were much less than at transcriptional level in 'Madam Vinous' sweet orange [35]. Only 10 and 20 proteins were differentially expressed in non-HLB symptomatic sample and HLB symptomatic sample when compared with mock-inoculated controls. Among the 20 differential expressed proteins, 13 were up-regulated and 7 were down-regulated. The down-regulated proteins were annotated as unknown function. Seven proteins were induced in the two LAS+ samples, including chitinase, four miraculin-like proteins, lipoxygenase and Cu/Zn superoxide dismutase. ATPase alpha subunit was only induced in leaves with HLB symptoms. HLB infected grapefruit showed the similar results regarding to the up-regulated proteins [36]. There were 69 proteins that showed differential expression in Las positive leaves compared with healthy leaves. Only 13 proteins were up-regulated. Besides chitinase, miraculin-like proteins and Cu/Zn superoxide dismutase induced in sweet orange, lectin-related proteins, peroxiredoxins, CAP 160 and granule-bound starch synthase were induced in grapefruit. The 56 down-regulated proteins were associated with protein synthesis, protein folding and photosynthesis. On the contrary, the defense-related proteins were down-regulated in lemon that is tolerant to HLB, such as lectin-related proteins, chitinase, and miraculin-like proteins [37]. Upon HLB infection, chaperones HSP 70 and an isoflavone reductase-related protein were up-regulated in lemon. Proteins associated with photosynthesis and starch synthesis were similar with grapefruit and sweet orange. The differences in responses between HLB susceptible and tolerant cultivars were confirmed by proteomes between Navel orange (susceptible) and Volkameriana (moderately tolerant) [38]. This study highlighted the detoxification pathways in Volkameriana tolerance to

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HLB infection. Since heat treatment can reduce HLB symptoms [8], proteomics analysis was conducted between four grapefruit samples -Las-heat, -Las+heat, +Las-heat, and +Las+heat. Compared with -Las-heat sample, only Las infection caused 23 proteins down-regulated and 31 proteins up-regulated. Heat treatment resulted in induction of 74 proteins and suppression of 9 proteins. Combined heat treatment and Las infection together, 83 proteins were up-regulated and 10 proteins were down-regulated. Two proteins that belong to chaperones, a HSP70-like protein and a RuBisCO-binding 60 KDa chaperonin, were down-regulated in Las infected samples, but they were highly up-regulated by heat treatment. Las infection could repress photosynthesis. But, heat treatment could increase photosynthesis by up regulation chlorophyll. Four proteins, a ferritin-like protein, a putative lipoxygenase protein, glucosidase II beta subunit-like protein, and a glutathione S-transferase that are related to disease resistance were up regulated with heat treatment but down regulated in HLB infected sample without heat treatment. Proteins related with redox homeostasis were down regulated in +Las-heat plants, but all identified differentially expressed proteins associated with redox homeostasis were induced in +Las+heat plants [39].

Transgenic Approach is the Only Way to Confer Citrus HLB Resistance at Present

Plants resistance can be divided into two categories: PTI (pathogen-associated molecular patterns triggered immunity) and ETI (effector-triggered immunity). Plant transmembrane pattern recognition receptors (PRRs) recognize conserved microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs) to trigger plant disease resistant response. ETI is triggered by interaction of plant R protein and pathogen Avr protein directly or indirectly, typical reaction is HR (hypersensitive response) [40]. PTI can confer plant broad spectrum disease resistance. But, the PTI immunity may be subverted by pathogen effectors and the extent of PTI varies from one species to another species and is controlled by multi-genes. ETI can enhance PTI and confer plant completely resistance to a specific pathogen. PTI and ETI pathways are overlapping [41]. Conventional breeding of HLB resistance citrus can't be achieved due to the unavailability of HLB resistant citrus in nature. It is extremely difficult to make mutant library of citrus to screen HLB resistant plant or susceptible plant due to long growing time and large number of plants required. Therefore, transgenic approach is the feasible choice of breeding HLB resistant citrus.

DISCUSSION

Recently, two reports described that citrus enhanced HLB resistance by transgenic approach [42,43]. NPR1 is a core factor in plant systemic acquired resistance that has been widely used to enhance plant resistance to pathogens, such as wheat, strawberry, cotton, carrot, rice, etc. The transgenic citrus over expression NPR1 showed enhanced resistance to HLB in field trial [42]. Thionins are low molecular weight proteins solely presented in higher plants, and they are toxic to bacteria and fungi *in vitro* [44-51]. Thoinins that belong to PR13 family are considered as plant antimicrobial peptides (AMPs) [52]. A modified thoinin was transformed into citrus and the transgenic citrus showed stronger resistant to citrus canker and HLB [43].

To improve plant disease resistance by overexpression protein, PRR such as FLS2 and EFR were used to enhance resistance to bacteria disease [53,54]. R gene also can be transformed into plant to confer plant disease resistance. The third group of candidates are plant AMPs. Manipulating disease susceptible genes can confer plant disease resistance by knock down method. There are many question need be answered in order to confer citrus HLB resistance. What are the virulence mechanisms of Clas? How does CLas take advantage of citrus? Are there any genes in citrus that are manipulated by CLas to develop HLB symptoms? The research on the virulence mechanisms of CLas was hampered because CLas can't be cultured. After releasing CLas genome, two reports shed light on the virulence mechanisms of CLas [55,56]. The second prophage of CLas encode a peroxidase that can scavenge Reactive oxygen species (ROS) in plant demonstrated by transient expression in *N. benthaiana* leaves [55]. ROS play a central role in plant disease response via programmed cell death (PCD) and serve as the signal molecules in systemic acquired resistance (SAR) [57]. Another signal molecule, salicylic acid, the plant defense hormone, is degraded by an enzyme SahA encoded by CLas [56]. The SahA transgenic tobacco suppressed HR caused by infiltration of non-host pathogen Xanthomonas citri Subsp. citri A306. An effector Las5315 was transient Expressed in *N. benthamiana* and caused cell death in infiltrated leaves. The effector subcellular localization is in chloroplast [58,59]. But, the role of Las5315 in HLB symptom development is unknown. However, the virulence mechanisms of CLas will get more knowledge with more publications on effectors in the future.

CRISPR is the third generation of genome editing tool after ZFN (Zinc-finger nuclease) and TALEN (transcription activator-like effector nuclease) [60]. CRISPR has advantages over ZFN and TELEN, such as simple, able to use at large scale and targeting multi-loci simultaneously [61, 62]. Genome editing tools were used to confer crop disease resistance successfully. TALEN was used to mutant three MILDEWRESISTANCE LOCUS (MLO) alleles in bread wheat to confer resistance to powdery mildew [63]. CRISPR was used to edit LOB1 that is the susceptible gene of citrus canker at coding region and promoter region to confer citrus canker resistance, respectively [64,65]. CRISPR has one advantage over all other technologies: the modified plant may not contain foreign DNA, so the edited plant can be released to market as commercial production for human consumption [66]. Due to the vague virulence mechanisms of HLB, CRISPR can't achieve the goal of conferring citrus HLB resistance at present, however, CRISPR is a power tool with great potential in breeding HLB resistant citrus after discovery HLB susceptible gene from citrus [67,68].

CONCLUSION

HLB is the biggest threat to citrus industry. Many efforts have been put into understanding the mechanisms on how CLas

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causes disease symptoms. But, there are large gaps in understanding the disease development. The time cause study did not give clue on how disease symptoms developed with time. It needs more time points to draw the fine map of gene regulation. Moreover, no specific phloem cell type transcriptome is available to understand how phloem response to CLas infection. Hence, the susceptible gene of HLB is not identified yet. Therefore, CRISPR is only a promising tool to control HLB at present. CRISPR requires to identify susceptible genes. It needs more works to identify pathogen effectors and elucidate the virulence mechanisms. Regarding to citrus HLB resistance, it mainly depends on plant basal defense. Heat treatment reduce HLB symptoms may be due to the activation of PTI at higher temperature [67]. To enhance citrus HLB resistance, more genes need be transformed into plant targeted to different pathways [68]. A system from Arabidopsis, uORF-mediated translation was used to express AtNPR1 without fitness costs of rice, may benefit transgenic approach for citrus HLB resistance. Genes can enhance PTI will help plant fight HLB as well as more powerful AMPs. It needs more research on HLB from different disciplines and integration efforts from scientists, growers and governments to win the battle against HLB.

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