Spotlight

Dual Functions of a Stable Peptide against Citrus Huanglongbing Disease

Xu Wang [1,4] Jian Chen [2,4]
Na Liu [1,*] and
Zheng Qing Fu [3,*]

The most prominent problem in the current citrus industry worldwide is the epidemic of citrus Huanglongbing (HLB), also known as greening disease. Huang *et al.* identified a stable peptide, which has antimicrobial activities and induces systemic immune response against HLB, from Australian finger lime. This peptide effectively suppresses disease symptoms in citrus and protects healthy trees against this disease.

While the world is hopefully recovering from the coronavirus disease 2019 (COVID-19) pandemic through vaccinations at an unprecedented pace, HLB poses the greatest threat to the global citrus industry. Once a citrus tree is infected with HLB, there is no cure. Although many different approaches have been tried in the field, the situation is still not improving. HLB has spread to over 50 countries [1]. It has been reported that >80% of citrus trees in Florida are infected with HLB, which has caused a 74% reduction in citrus production [2]. HLB is present in >300 counties in 10 provinces of mainland China and has destroyed >50 million citrus trees [3].

HLB is extremely difficult to control for several reasons [4]. It is transmitted by sap-sucking psyllid insects which spread the disease very quickly. Inability to grow host-free culture of the bacterial pathogen causing this disease, *Candidatus* Liberibacter asiaticus (*C*Las), in the past has made it difficult to conduct experiments [5]. In addition, HLB has a very long asymptomatic incubation time, which makes it challenging to detect the disease at an early stage. Once HLB is diagnosed, it is already too late. The whole field has probably been infected. The disease affects and eventually kills the entire citrus tree.

Recently, an exciting new paper reported that a stable antimicrobial peptide is effective in treating and preventing HLB [6]. The authors isolated a heat-stable antimicrobial peptide *Microcitrus australasica* stable antimicrobial peptide (MaSAMP) from the HLB-tolerant Australian finger lime *M. australasica* (Figure 1). This peptide not only has antimicrobial activity but also induces systemic defense responses. Consequently, this peptide can effectively reduce pathogen growth and suppress disease symptoms in HLB-positive trees and protect healthy citrus trees against new infection.

One of the most effective ways to control plant diseases is to utilize the natural plant resistance and tolerance that have developed during millions of years of coevolution with plant pathogens. Although *most* commercial citrus varieties are susceptible to HLB, HLB tolerance has been found in some hybrids and close citrus relatives including Australian finger lime. In an attempt to determine the underlying mechanisms of HLB tolerance, Huang *et al.* performed comparative expression analysis of small RNAs and their target genes between susceptible citrus cultivars and tolerant citrus and microcitrus hybrids [6] (Figure 1). A list of candidate genes, which could be responsible for the HLB-tolerant phenotype, has been identified [7]. Among them, one candidate gene encodes a 67 amino acid (aa) peptide which shares homology with the 109 aa *Arabidopsis* heat-stable protein HS1 with antimicrobial activity [6]. This 67 aa peptide was named SAMP. The authors found that all HLB-tolerant citrus relatives express a long (109 aa) and at least one short (67 aa) version of SAMP, whereas HLB-susceptible citrus varieties only have long SAMPs (LSAMPs) of 109 and 118 aa.

Further study demonstrated that the expression of short *SAMP* mRNA in HLB-tolerant citrus hybrids was significantly higher than that of long *LSAMP* in HLB-susceptible citrus trees. In addition, western blot analysis using an anti-SAMP antibody showed that the 6.7 kDa short version of SAMP was present in the HLB-tolerant Australian finger lime and seven trifoliate oranges, but not in the HLB-susceptible citrus variety *Citrus sinensis* (Figure 1). Taken together, the expression of the 67 aa SAMP peptide correlates with HLB tolerance. To identify the most effective SAMP variant, the authors screened SAMPs from several citrus relatives and found that MaSAMP from Australian finger lime has the strongest effect in suppressing disease development and pathogen growth in plants [6].

Liberibacter crescens (*Lcr*), a close relative of *C*Las, can be cultured on BM7 or modified BM7 medium in the laboratory [8]. Therefore, *Lcr* strain BT-1 was used to test the antimicrobial activity of MaSAMP [6]. The authors found that 10 μM of MaSAMP is sufficient to kill bacterial cells and induce aggregation as early as 30 minutes after treatment. Lower concentrations of MaSAMP, 1 μM or even 100 nM, can kill bacteria more efficiently than 172 μM of the antibiotic streptomycin 5 h after treatment.

It is well known that high temperature can potentially denature proteins or peptides, rendering them dysfunctional. Heat sensitivity is also a major drawback of using antibiotics to control plant bacterial diseases. Surprisingly, MaSAMP remains functional after exposure to 60°C for 20 h, whereas streptomycin completely lost its bacteria-killing activity under the same conditions (Figure 1). Using transmission electron

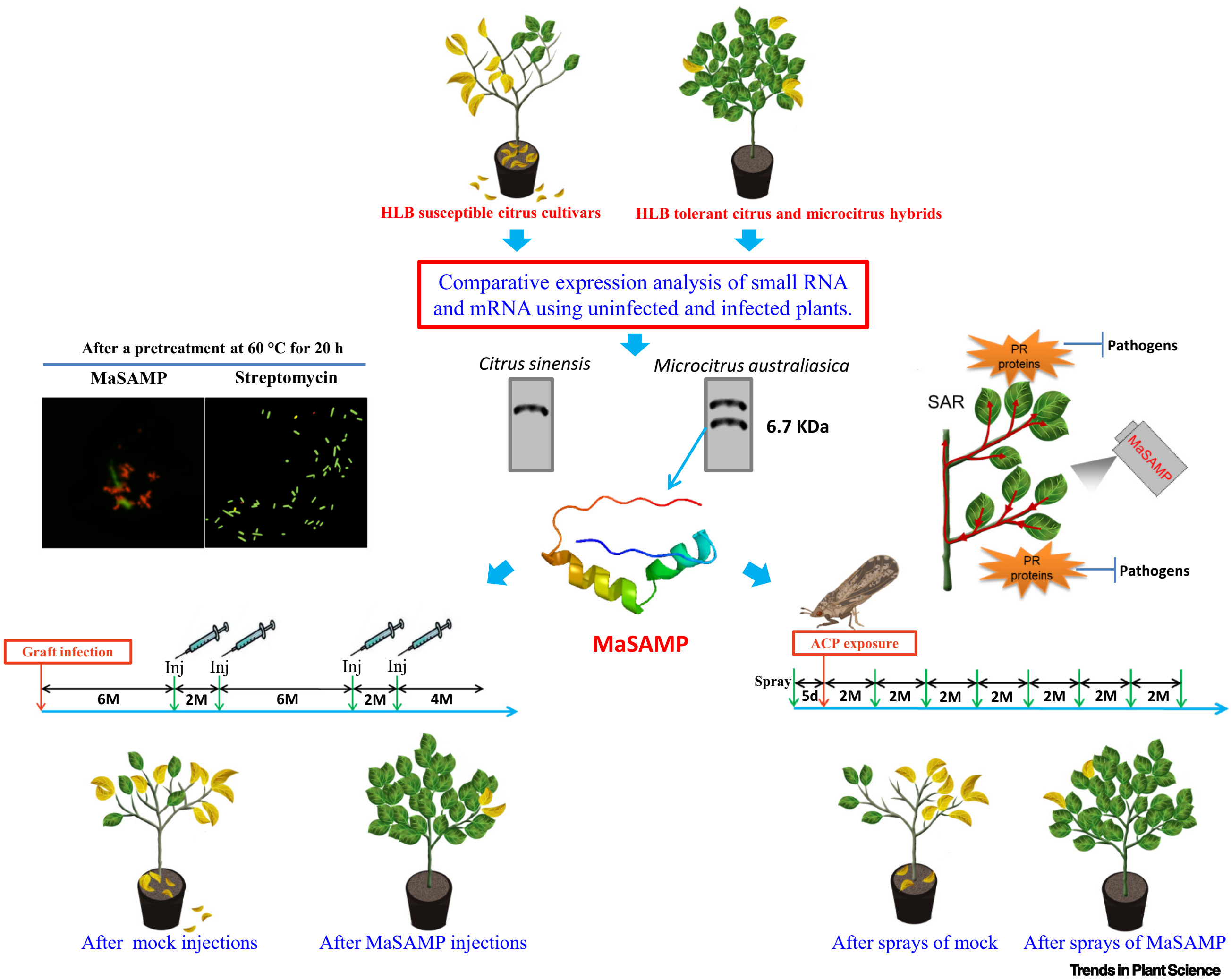

Figure 1. Identification of the Heat-Stable MaSAMP (*Microcitrus australasica* Stable Antimicrobial Peptide) and Its Dual Functions in Citrus Greening Disease (Also Known as Huanglongbing, HLB). Through comparative analysis of the expression of small RNAs and their target genes between HLB-susceptible commercial citrus cultivars and HLB-tolerant citrus and *Microcitrus* hybrids, candidate genes were identified that are potentially involved in HLB tolerance. One candidate gene encodes a 67 amino acid (aa) peptide with two predicted α-helices and this peptide shares homology with an *Arabidopsis* antimicrobial protein. This 67 aa peptide was named SAMP. All susceptible citrus varieties have only the long (LSAMP) peptides of 118 and 109 aa, whereas the HLB-tolerant citrus relatives have an additional short SAMP of 67 aa. Subsequent studies showed that the MaSAMP from Australian finger lime (*M. australasica*) had the strongest effect on suppressing HLB disease and pathogen growth. In contrast to the antibiotic streptomycin, which lost its bactericidal activity after incubation at 60°C, the MaSAMP peptide exhibited strong bacteria-killing activity against *Liberibacter crescens* (*Lcr*), a culturable close relative of the HLB bacterial pathogen *Candidatus* Liberibacter asiaticus (*C*Las), even after pretreatment at 60°C for 20 h. Multiple injections of MaSAMP into citrus plants effectively reduced the titer of *C*Las and suppressed disease development. MaSAMP delivered by foliar spraying is taken up by citrus plants and moves systemically in the vascular system, making it a perfect choice for controlling phloem-limited *C*Las. Importantly, MaSAMP induces the expression of plant defense genes, including pathogenesis-related (*PR*) genes encoding antimicrobial proteins, and activates systemic acquired resistance (SAR). Consequently, MaSAMP protects healthy citrus plants against HLB infection. The predicted 3D structure of MaSAMP was generated using the Phyre2 web portal. Abbreviations: ACP, Asian citrus psyllid; d, days; Inj, injection; M, months.

microscopy, the authors found that application of MaSAMP to *Lcr* caused rapid cytosol leakage and cell lysis. MaSAMP was found to be enriched in the outer membrane of *Lcr*, suggesting that MaSAMP disrupts the outer membrane to cause cell lysis. Further studies showed that MaSAMP is most effective against α-proteobacteria, whereas higher concentrations are needed for MaSAMP to inhibit γ-proteobacteria. Through structural modeling it was predicted that the MaSAMP peptide has two α-helices that are connected via a proline-rich hinge and flanked by flexible N- and C-termini [6] (Figure 1). To determine which region in MaSAMP is essential for its antimicrobial activity, the authors tested the bactericidal activity of different truncated

MaSAMPs, including the double helix, α-helix1 and α-helix2. The results indicate that the α-helix2 is mainly responsible for its bacteria-killing activity.

To test whether MaSAMP suppresses HLB disease development, the authors used pneumatic truck injection to deliver MaSAMP into HLB-infected citrus trees [6] (Figure 1). The authors performed three independent experiments using three different sets of similarly HLB-infected *Citrus macrophylla*, Madam Vinous sweet orange (*Citrus sinensis*), and Lisbon lemon trees. After three to four injections of 10 μM of MaSAMP at intervals of 2 months, the titer of *C*Las and the extent of HLB symptoms in the infected trees were significantly reduced compared with mock-treated trees. Typically, the MaSAMP-treated citrus trees developed new healthy flushes and exhibited a rapid decrease in bacterial titer, whereas most mock-treated plants developed severe disease symptoms and later died.

Priming is a physiological response in which plants are prepared to initiate a much stronger and quicker defense response upon pathogen attack [9]. The authors found that MaSAMP has strong priming activity [6] (Figure 1). MaSAMP induced the expression of plant defense genes and activated systemic defense responses after it was sprayed onto *Nicotiana benthamiana* and tomato plants. *Nonexpressor* of pathogenesis-related genes 1 (NPR1) and suppressor of G2 allele of skp1 (SGT1) are indispensable for MaSAMP-induced plant defense responses, whereas BAK1 is not required for MaSAMP recognition and/or signaling [6].

What makes this peptide very promising is that MaSAMP is actually taken up by citrus plants. After foliar-spray application, the MaSAMP remains stable inside citrus plants for at least 1 week and moves systemically through the citrus vascular system, which makes MaSAMP ideal for treating the phloem-colonizing HLB bacterial pathogen *C*Las (Figure 1). The authors also showed that multiple foliar spraying of MaSAMP at intervals of 2 months effectively protects healthy citrus trees against HLB infection [6] (Figure 1).

Future studies focusing the identification of the MaSAMP receptor may help to better understand how MaSAMP is perceived by plants to activate systemic defense responses. It has been shown that multiple applications of benzothiadiazole (BTH), an active analog of the plant defense hormone salicylic acid, at 3 or 4 day intervals dramatically compromise plant growth [10]. It would be helpful to determine whether multiple applications of MaSAMP have a negative impact on plant growth, although MaSAMP is less likely to compromise fitness when it is applied at 2 month intervals. Of course, this peptide must undergo large-scale field trials before it can be commercialized. Alternatively, we can also introduce the *MaSAMP* gene into commercial citrus trees through crossing and genetic engineering, including knock-in modification using CRISPR/Cas technology, to protect them from HLB [11,12].

Acknowledgments

We thank Kalan Ferate and Mark Fu for editing this manuscript. Z.Q.F. was supported by the National Science Foundation (IOS-1758994).

Declaration of Interests

The authors declare no conflicts of interest.

[1]College of Life Sciences, Henan Agricultural University, Zhengzhou 450002, China
[2]International Genome Center, Jiangsu University, Zhenjiang 212013, China
[3]Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA
[4]These authors contributed equally to this work

*Correspondence:
naliu@henau.edu.cn (N. Liu) and zfu@mailbox.sc.edu (Z.Q. Fu).

https://doi.org/10.1016/j.tplants.2021.04.006

Published by Elsevier Ltd.

References

1. Dala-Paula, B.M. *et al.* (2018) Effect of Huanglongbing or greening disease on orange juice quality, a review. *Front. Plant Sci.* 9, 1976
2. Singerman, A. and Rogers, M.E. (2020) The economic challenges of dealing with citrus greening: the case of Florida. *J. Integrat. Pest Manag.* 11, 3
3. Zhou, C. (2020) The status of citrus Huanglongbing in China. *Trop. Plant Pathol.* 45, 279–284
4. Gottwald, T.R. (2010) Current epidemiological understanding of citrus Huanglongbing. *Annu. Rev. Phytopathol.* 48, 119–139
5. Ha, P.T. *et al.* (2019) Host-free biofilm culture of '*Candidatus* Liberibacter asiaticus', the bacterium associated with Huanglongbing. *Biofilm* 1, 100005
6. Huang, C.Y. *et al.* (2021) A stable antimicrobial peptide with dual functions of treating and preventing citrus Huanglongbing. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2019628118
7. Huang, C.Y. *et al.* (2020) Identification of citrus immune regulators involved in defence against Huanglongbing using a new functional screening system. *Plant Biotechnol. J.* 19, 757–766
8. Fagen, J.R. *et al.* (2014) *Liberibacter crescens* gen. nov., sp. nov., the first cultured member of the genus *Liberibacter*. *Int. J. Syst. Evol. Microbiol.* 64, 2461–2466
9. Fu, Z.Q. and Dong, X. (2013) Systemic acquired resistance: turning local infection into global defense. *Annu. Rev. Plant Biol.* 64, 839–863
10. Canet, J.V. *et al.* (2010) Resistance and biomass in *Arabidopsis*: a new model for salicylic acid perception. *Plant Biotechnol. J.* 8, 126–141
11. Wang, N. (2021) A promising plant defense peptide against citrus Huanglongbing disease. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2026483118
12. Jia, H. and Wang, N. (2014) Targeted genome editing of sweet orange using Cas9/sgRNA. *PLoS One* 9, e93806