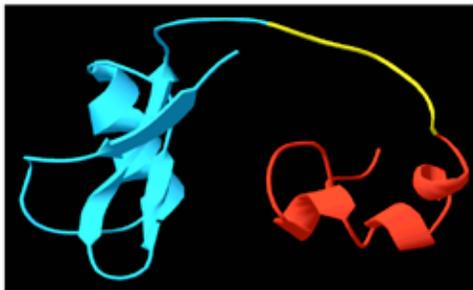


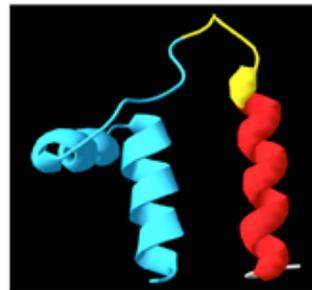
Chimera	Recognition Domain	Lysis Domain
A	Thionin-1*	D4E1
B	LBP*	Thionin-2
C	Serine Protease*	Thionin

*Different isoforms can be used.

Figure 1. Design of the therapeutic chimeras with recognition and lysis domains. (A) A schematic representation of the cell envelope of Gram-negative bacteria such as "Candidatus Liberibacter," *Xanthomonas citri* subsp. *citri* (Xcc) and *Xylella fastidiosa* (Xf) illustrates shared structural components (lipopolysaccharide [LPS] and outer-membrane protein [OMP]) where protein chimeras bind and break the bacterial cells into small pieces. (B) Listing of chimeras designed in this project is shown. The Thionin1-D4E1 and LBP-Thionin2, listed as chimera A and B, respectively, are currently being evaluated in expression and efficacy studies. Models of (C) chimera A (Thionin1-D4E1) and (D) chimera B (LBP-Thionin2) show recognition (magenta) and cell lysis or rupturing (red) regions or domains. In both of these models, an amino acid linker, GSTAPPA (yellow), is used to join the two domains.



C Chimera A



D Chimera B

NOVEL THERAPY OF HIGH-PRIORITY CITRUS DISEASES

Hau Nguyen, Guixia Hao, Ed Stover and Goutam Gupta

SUMMARY

Huanglongbing (HLB), citrus canker and citrus variegated chlorosis (CVC) are three high-priority citrus diseases, all caused by a single category (Gram-negative) of bacteria. Any approach that could kill the causative bacteria when they first infect the tree would be very useful, and the same approach may be able to prevent all three diseases.

In this project, we lay the foundation of one such approach. We show that the design and delivery of a protein chimera clears both HLB-associated 'Candidatus *Liberibacter asiaticus*' (CLas) and canker-causing *Xanthomonas citri* subsp. *citri* (Xcc) and blocks disease symptom development. This chimera has two functional domains derived from citrus. One domain (called the recognition domain) targets the specific membrane features conserved in these three bacteria, whereas the other domain (called the lysis domain) creates membrane pores

in these bacteria. These two domains are connected by a flexible linker, which allows synergy in function of the two domains, thereby facilitating rapid death of the bacteria. Two routes are chosen for the delivery of the in planta protein chimeras via transgenic citrus and nanocapsules. The transgenic citrus lines express the chimeric protein and provide long-term protection against bacterial diseases, whereas the nanocapsules containing the protein chimera potentially can be applied to the infected trees to clear the bacteria and block the disease development.

We already have demonstrated the efficacy of a transgenic citrus expressing a chimera in blocking HLB and canker disease development. Efficacy studies with transgenic citrus expressing other chimeras (expected to possess improved anti-bacterial activity) are currently in progress. Finally, construction of the nanocapsules is also underway to deliver the anti-bacteria protein chimeras.

INTRODUCTION

HLB, canker and CVC are three high-priority bacterial diseases in citrus¹. Although known for a century in East Asia, it was only recently that HLB was introduced into the Americas, including the United States and Brazil, two of the top citrus-producing nations². Typical HLB symptoms are leaves with blotchy mottling. HLB shortens the lifespan of the trees and reduces the yield and quality of the fruit. HLB is now widespread in most citrus-producing areas of Asia, Africa and most of the Americas. Most citrus cultivars are susceptible to HLB. However, the disease severity appears to be different in different cultivars. HLB is associated with an insect-vectored bacterium. 'Candidatus Liberibacter asiaticus' is a phloem-limited bacterium associated with HLB and is carried by an insect vector called Asian citrus psyllid (ACP).

In the United States, ACPs carrying CLAs have been encountered in all states where citrus is grown. More than 80 percent of citrus trees in Florida already are infected with CLAs. A small number of citrus trees in Texas also are infected. Only a few citrus trees with CLAs have been found and removed in California, although ACPs are encountered in a number of the state's citrus growing areas. There currently is no cure for HLB, nor is there any naturally occurring citrus cultivar that is resistant to HLB.

Like HLB, citrus canker is an old disease, which originated in Southeast Asia, but has spread in several citrus-producing countries. In the United States, citrus canker has been an

issue for some time in Florida³. Citrus canker symptoms include brown spots with yellow haloes on the leaves. The disease causes citrus trees to prematurely drop leaves and fruit. *Xanthomonas citri* subsp. *citri*, the bacterium responsible for the disease, is spread from infected trees to healthy trees by wind-driven rain or on contaminated tools, clothing and equipment.

Citrus variegated chlorosis (CVC) first appeared in Brazil in 1987 and has rapidly become one of the most economically important diseases affecting sweet orange production in Brazil⁴. *Xylella fastidiosa* (Xf-CVC strain), the causal agent of CVC, is a xylem-limited bacterium vectored by several species of sharpshooter leafhoppers in the order Hemiptera. Some of these sharpshooters already are established in Florida. Since all citrus cultivars in Florida are susceptible to CVC, this disease can pose a threat to the Sunshine State's citrus industry. There is currently no cure for CVC.

THE PROJECT GOAL AND HYPOTHESIS

The main goal of this project is to develop the same therapy that can be applied to HLB, canker and CVC. Although the mechanisms of disease development by these three gram-negative bacteria are different^{2,4}, it is our hypothesis that there are common and conserved features that can be targeted through a common therapy. In this project, the protein chimera or fusion proteins are created in such a way that they have two different functional regions: one for recognizing the bacterial cells and binding to them and the other for breaking them down into small pieces. The two regions or domains (recognition and lysis) target the outer membrane features that are present in all three bacterial types.

PROGRESS TO DATE

Figure 1 lists different chimeric proteins that we have designed. The regions that recognize the bacterial cells are either derived from a citrus protein (called lipid-binding peptide or LBP) that binds to the lipids (lipopolysaccharide) on the surface of bacterial outer-membrane⁵ or a citrus protease that cleaves the bacterial outer-membrane proteins (OMP)⁶. The other regions or domains of the chimeric proteins that cause destruction or lysis of bacterial cells are derived from citrus antimicrobial peptides (AMPs). The latter include citrus Thionins, which are small peptides with dual functions in that they recognize bacterial outer membrane (LPS) and bind to it, as well as create numerous holes (pores) in the membrane and kill the bacterium⁷.

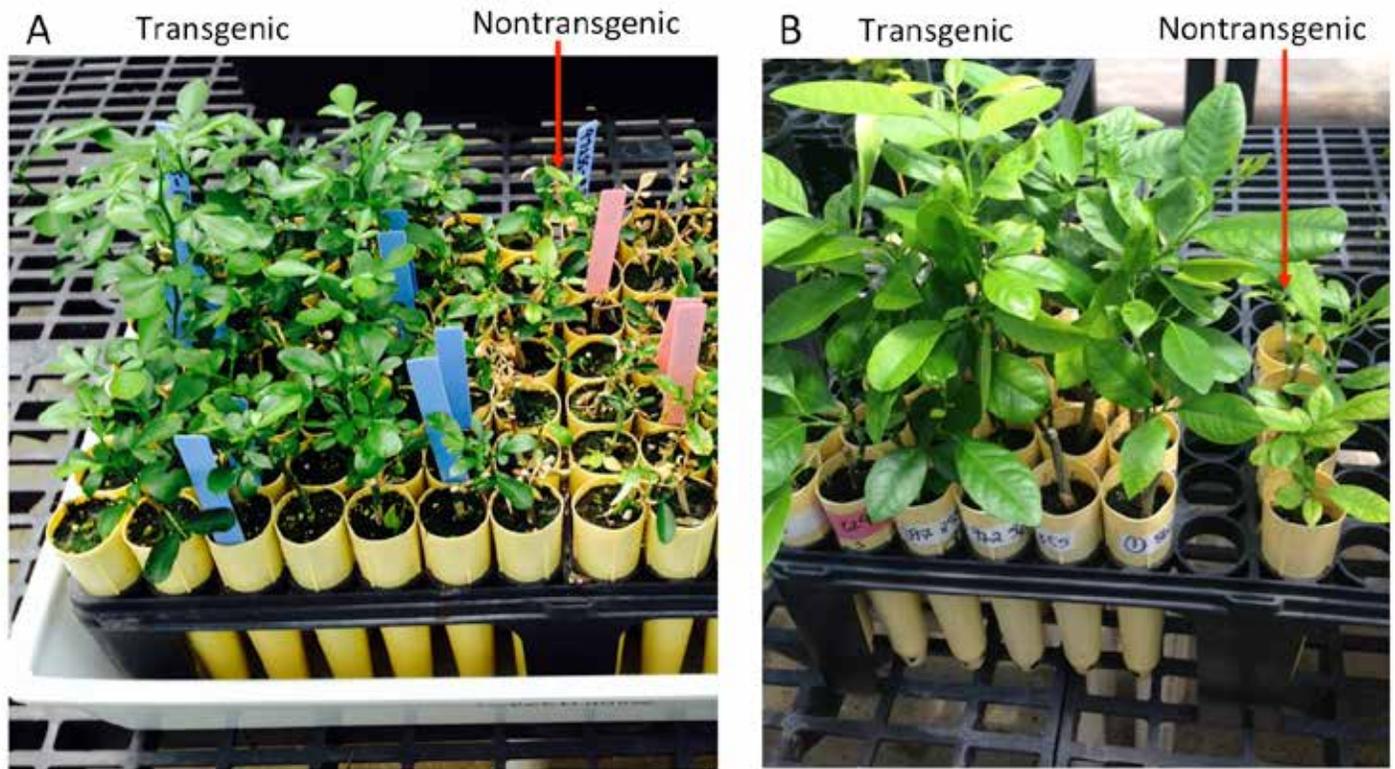


Figure 2. Transgenic citrus (Carrizo) expressing the Thionin1-D4E1 chimera shows protection against both citrus canker and HLB (Collaborator: Ed Stover, USDA-ARS, Ft. Pierce, Florida). Transgenic citrus expressing the Thionin1-D4E1 chimera is protected after Xcc challenge (A, left panel), whereas nontransgenic citrus is not (A, right panel). Transgenic citrus shows protection against HLB, (B, left panel) whereas nontransgenic citrus does not (B, right panel). All the citrus trees were grafted with Liberibacter-infected rough lemon.

In this study, we created three types of chimeric proteins as follows:

- A. Thionin1-D4E1 (D4E1 - a synthetic but citrus-friendly AMP designed by Ed Stover)
- B. LBP-Thionin2
- C. Serine protease-Thionin

Note that there are several citrus variants of Thionin, LBP and protease. Thus, different chimeras can be constructed within each category, thereby allowing us to choose the chimera with the highest anti-bacterial activity.

Thionin1-D4E1 chimera was the first one that we started working on three years ago. At that time, the sequence of the citrus genome was not completed, and hence complete screening of citrus Thionin was not possible. Therefore, we chose a tobacco Thionin called Thionin1, which has 70 percent identity with a citrus Thionin (as observed after the completion of the citrus genome sequencing). After the availability of the completed citrus genome sequencing^{8,9}, we designed the LBP-Thionin2 chimera in which both Thionin and LBP were chosen from the citrus proteome.

Our first objective was to develop transgenic citrus (Carrizo and Hamlin) that shows resistance to all three citrus diseases – HLB, citrus canker and CVC. This was achieved by transforming citrus plants with chimeric protein genes such as Thionin1-D4E1 and LBP-Thionin2. Transgenic plants expressing Thionin1-D4E1 chimeric protein showed a significant increase in resistance to both citrus canker and HLB in greenhouse studies (Figure 2). These results validated our

hypothesis that the same chimeric proteins can protect citrus plants against multiple bacterial diseases. Testing of transgenic plants expressing LBP-Thionin2 chimeric protein for disease resistance is currently underway in greenhouse.

The second goal of our project was to design nanocapsules (Figure 3^{10,11}) containing the AMPs listed above and deliver them into citrus plants to combat HLB infection.

We have determined that *in planta* delivery of nanocapsules will require a large-scale amount of CTV capsid protein, which we will obtain by isolating and purifying the protein from the CTV virions.

CONCLUSION

Progress made so far suggests that protein chimera of recognition and lysis domains provide effective and broad-spectrum protection against multiple bacterial diseases in citrus. *In planta* delivery of the protein chimera enhances the immune defense in citrus.

Our future work will follow the success with the protein chimera, which will include efficacy testing of other chimeras designed to show a better activity against citrus diseases. 🍊

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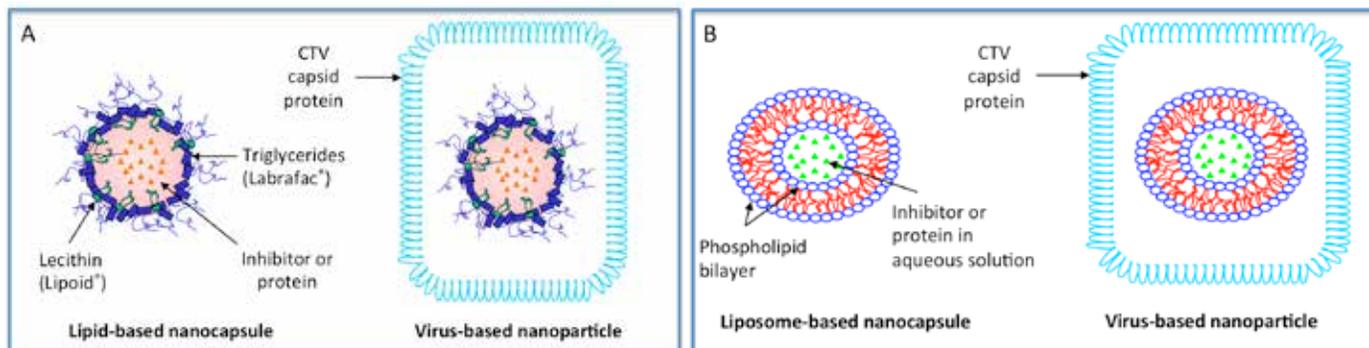


Figure 3. Design of two types of lipid-based nanocapsules carrying a small molecule or protein therapeutic agent. A lipid-based inner shell (hydrophobic interior) contains inhibitory therapeutic agents (A, left panel). An outer shell of Citrus tristeza virus (CTV) capsid protein is used to specifically deliver the therapeutic agents into the citrus phloem tissue (A, right panel). Inner shell with liposome (hydrophilic interior) contains inhibitory therapeutic agents (B, left panel). An outer shell of CTV capsid protein is used to specifically deliver the inhibitory therapeutic agents into the citrus phloem tissue (B, right panel).

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Glossary

Gram-negative bacteria: Bacteria are divided into two distinct groups of Gram-negative and Gram-positive based on their cell wall architecture. Gram-negative bacteria have an outer membrane with thinner cell walls and do not retain the crystal violet stain as opposed to Gram-positive bacteria.

Protein chimera: Chimeric or fusion proteins are engineered through the joining of two or more specific genes. The latter are eventually translated into corresponding proteins, each having a different functional property or domain to serve a particular purpose.

In planta: Inside plant cell or tissue

Transgenic plant: A plant that is genetically modified by adding and expressing a foreign or native gene(s) to its genome for the purpose of making it resistant or tolerant to pests, diseases, drought, salt, etc. or improving its yield or horticultural quality.

Nanocapsule: Nanoscale shells (10-1000 nanometers) are made out of a nontoxic polymeric membrane that encapsulates a therapeutic drug for delivery.