Photos courtesy of Cecile Robertson

Figure 1. Sweet orange citrus seedlings protected against HLB infection by CTV expressing antimicrobial peptides (left) vs. controls infected with a CTV empty vector (right)

Identifying HLB therapeutics with CTV-based vectors

By Choaa A. El Mohtar

Projected citrus production in Florida for the 2022–23 season is less than 20 million boxes. This is more than a 90% decrease compared to the 2003–04 season, which was around 292 million boxes. The main reason for the decrease is huanglongbing (HLB) disease. The University of Florida Institute of Food and Agricultural Sciences (UF/IFAS) citrus tristeza virus (CTV) research team has been focused on using CTV-T36 infectious cDNA clone-based vectors to identify therapeutics for the management of HLB.

CTV-T36 cDNA vectors were engineered to study the biology of the CTV-T36 genotype endemic in Florida. Bioassays using it revealed a very mild phenotype on the different citrus genotypes tested. Further, aphid transmission assays from the CTV-T36 infectious clone-initiated infection are sporadically transmitted between seedlings at less than 1% under optimal greenhouse conditions by the brown citrus aphid.

KNOCKING OUT GENES

Vectors based on CTV-T36 are engineered by adding an extra gene to the T36 cDNA infectious clone, enabling the overexpression or knocking out of a gene of interest for many years. Tagging the virus with fluorescent proteins enabled researchers to visualize the infection under the microscope. This revealed continuous fluorescence in infected cells of citrus seedlings for many years. Later, a truncated version of citrus genes introduced into CTV enabled the manipulation of the RNA interference (RNAi, a gene regulation mechanism and a defense mechanism against invading nucleic acids in eukaryotes) pathway in citrus to knock out its own genes.

Targeting the phytoene desaturase gene (responsible for chlorophyll protection) for silencing via CTV revealed a photobleaching phenotype. The phenotype is still clear many years after the original initiated infection. Hence, CTV-T36 based virus vectors would be a tool to overexpress or knock out genes from the phloem tissue of citrus.

Why use the CTV-T36 cDNA infectious clone-based vectors to identify therapeutics to mitigate HLB disease? Despite the technical challenges, engineering the CTV-T36 infectious clone vectors is much faster and easier to carry to citrus than producing a CRISPR or transgenic plant for every therapeutic sequence. Thus, multiple therapeutics could be screened simultaneously by producing different CTV vectors independently. This results in a much faster and more efficient in vivo screening than producing engineered plant for each therapeutic.

CTV virus is limited to the phloem tissue of citrus where Candidatus *Liberibacter asiaticus* (CLas) bacteria, the suspected causal agent of HLB, reside. Therefore, potential therapeutics expressed from CTV-T36 vectors will be delivered directly to the phloem tissue the CLas bacteria is inhabiting. Further, despite colonizing the same tissue, CTV and HLB have no synergistic interaction in mixed infections in citrus.

PEPTIDES TARGET BACTERIA

The first approach was to target the bacteria directly via small antimicrobial peptides (AMPs). AMPs are part of defense strategies in many organisms against invading pathogens. In the case of HLB, the AMP would kill the CLas bacteria when coming together in the citrus phloem, which should provide resistance/tolerance to citrus. AMPs are relatively small, ranging in size from 10 to 70 amino acids. Thus, CTV vectors are expected to express them for more than a decade.

AMP sequences to target the CLas bacteria via CTV vectors were gathered from scientific publications and via collaborations. A major collaboration is with Kranthi Mandadi at Texas A&M University, where recently, a new technology was developed called hairy root assay (Irigoyen et al., 2020). The technology was deployed as a preliminary screening tool for AMPs against CLas before expressing from the CTV vector.

Researchers have screened more than 100 peptides expressed from the CTV vector. All CTV-AMPs did not provide immunity to protected plants screened. However, there was a range of HLB phenotypes from very severe to tolerant depending on the expressed peptide.

Recently, researchers identified via CTV a promising peptide among those preliminarily screened through the hairy root technology. Most sweet orange seedlings protected with the CTV vector expressing the promising peptide revealed HLB symptoms in some branches a few months after inoculation with the high HLB-positive ACP inoculum pressure. However, unlike control plants, the HLB phenotype did not progress, and the plants grew healthy-looking flush despite being confirmed CLas positive by qPCR assays (Figure 1, page 12).

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WORKING TOWARD RESISTANCE

The ultimate solution for HLB is the development of resistant plants. Engineering resistance into commercial lines via CRISPR (targeted editing of the citrus genome like natural mutations) by knocking out genes that allow infection by CLas or knocking out genes that reduce defense would provide non-GMO resistance/tolerance. The problem is identifying the genes to knock out that would give this response.

However, researchers used the CTV-RNAi technology to identify such targets. They have built 60 CTV-RNAi vectors targeting citrus genes, 44 of which target defense genes. The rest target citrus genes that enhance the infection. Among the different sets of CTV-RNAi protected sweet orange seedlings screened, only two sets revealed HLB tolerance phenotype. The first target identified is a negative regulator of plant defense. The second target enhances the CLas infection. Collaborators are using these targets to produce permanent genetic mutations through CRISPR technology.

Citrus breeding is limited by the extensive time required for new crosses to become mature enough to enable another round of crosses. In rootstock development, time is required for new rootstocks to produce seed for production. Similarly, CRISPR editing and transgenic production is limited by the long juvenile phases. Thus, inducing the transition to maturity by enabling early flowering will decrease the time required to produce HLB resistant/tolerant genotypes.

Flowering is the result of a complex set of reactions involving physiological and environmental cues in which multiple pathways and many transcription factors are involved. To assist in fast-forwarding the generation of tolerant/resistant citrus genotypes, researchers are using the CTV vector to induce early flowering in citrus.

A prototype of a CTV vector expressing a gene to induce flowering was made. It efficiently induced flowers in a few months in citrus, but the construct was unstable and difficult for breeders to use and did not induce flowers in all varieties. The second year is beginning of a three-year funded project to develop better CTV vectors that retain the flowering genes more stably and induce flowering in most citrus lines. After inducing flowering, CTV virus vectors will be successfully eliminated from the infected tissue.

CONCLUSION

In conclusion, CTV-T36 vectors are benign in most citrus scion/rootstock combinations and have the ability to express a sequence of interest for many years. If enabled, their deployment in two pioneering ways can identify AMPs that would target CLas directly as well as knocking out citrus genes that induced tolerance in sweet orange seedlings. The ultimate solution for HLB is a resistant citrus tree. Research is helping accomplish this by generating a system that efficiently transitions plants to maturity by inducing early flowering in citrus.

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