Tools for temporary gene expression in the HLB battle

By Amit Levy and Choaa El-Mohtar

When thinking about genetic engineering, the first thing that comes to mind is usually GMOs (genetically modified organisms) In a GMO, a plant genome is engineered to include additional genetic sequences, usually genes with some useful activity that give the plant an advantage. This method is called a stable method because genes will be inherited to the next generation. It is usually very slow, especially with tree crops such as citrus, which have a long juvenile stage that ranges from four to six years. It makes introducing and evaluating any genetic trait a two-decade process. However, this is only one possibility.

In theory, it is not necessary to add anything to the genome as long as we can find other ways to express desired genes. In these other ways, genetic sequences will not be incorporated into the plant genome and will not be inherited to the next-generation. This type of gene expression is called non-stable (or transient). Although it comes with this important limitation, non-stable gene expression still holds some key advantages. First, since there is no change to the plant genome, it will not be considered a GMO and can therefore escape some of the lengthy and expensive regulatory processes. Second, these methods are usually much faster to carry out than generating new transgenic plants. The major advantage of a non-stable plant transformation is that it results in a much faster change. Given this advantage (fast) and the limitation (non-heritable), non-stable expression methods are usually considered short- to medium-term solutions, until a more stable solution can be found.

Since making transgenic plants is such a slow process in citrus, the biggest advantages transient expression

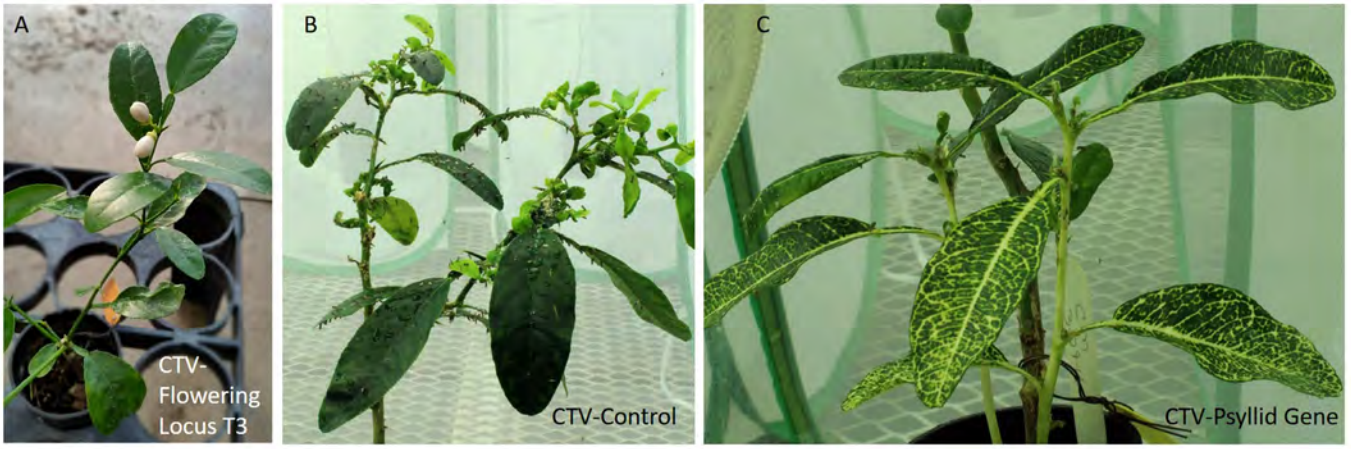

Figure 1. Expression of the flowering locus by CTV makes plants flower earlier (A). The effect of CTV RNAi on the establishment of psyllid feeding on the plants is shown (B and C). There are high levels of psyllid feeding on control plants (B) versus very little feeding on CTV plants (C).

methods offer is that they are efficient and easy ways to screen candidates and identify new ones. These systems can speed up the search for new gene candidates that can provide solutions to citrus diseases. Here, we will briefly describe two methods for efficient, non-stable gene expression in citrus and address their potential applications. These methods are the citrus tristeza virus vector and a handheld gene gun.

CITRUS TRISTEZA VIRUS VECTOR

Citrus tristeza virus (CTV) is a member of the family Closteroviridae. The virus was thoroughly studied because it can be used to express foreign sequences of interest. The result is a set of vectors that can express a differential level of a gene of interest. CTV expression of a therapeutic sequence gives a higher level of expression than a transgenic plant. A big advantage is that this expression is happening in the phloem tissue, where the HLB bacterium is present and the psyllid insect feeds.

Working with CTV is relatively easier and faster to design, build and carry to plants than CRISPR (see page 12) and transgenic plants. Another advantage of the CTV vector is that it is available for the current generation of plants, especially if a new peptide is discovered with the ability to heal/recover

HLB-infected plants. CTV can be used to express multiple genes simultaneously, and there is potential to target different aspects of the disease triangle.

Another use of CTV expression vectors is to rapidly convert HLB-resistant juvenile citrus to maturity. This is made possible by expressing the FT3 flowering locus that induces flowering in citrus seedlings (Figure 1, page 8). Lastly, CTV can be used for either expression or elimination of expression, in a process called RNAi.

In order to fight HLB using the CTV virus vectors, a cooperative network among researchers was set up to target the three components of the disease triangle, which includes the citrus plant, the HLB bacteria and the insect vector. With the plant, the first strategy being pursued aims to boost the defense response of the plant by using CTV vectors that individually express short sequences from the HLB bacteria in citrus. These sequences should trigger the citrus plant defense mechanism/s against the HLB bacteria before the infection takes place. Upon entering the citrus plant, the bacteria will face an activated plant defense resulting in tolerance/resistance to HLB. In addition, researchers are also trying to decrease expression of citrus genes that are necessary for the bacteria to cause disease by using RNAi.

The second target in the disease triangle is the bacteria itself. Directly screening therapeutic peptides against HLB bacteria is not possible because it is not cultured yet. We are using the plant phloem tissue, where both CTV and HLB bacteria are present, to target it directly in the plant. The first strategy is to target the HLB bacteria through small natural and engineered antimicrobial peptides (AMP) that are directed at killing or reducing the level of the bacteria in the phloem tissue. Researchers are currently screening more than 100 AMPs for activity against HLB. The second strategy is to target the bacteria through breaking its cell wall. A third strategy to target HLB is via the cell-to-cell communication signals called "quorum sensing." Changes in these signals will affect the bacterial movement in the plant and transmission by the insect.

The third and final component of the disease triangle is the psyllid insect vector. Psyllid adults and juvenile instars suck the citrus phloem sap in order to get nutrients. When doing so, they will also suck whatever is added into the phloem sap with CTV. In this way, researchers are targeting the psyllids by CTV-based expression/RNAi vectors via two different strategies. The first strategy is dependent on eliminating the expression of psyllid genes necessary for their life cycle. We have selected 15 genes for RNAi targeting. The second strategy is dependent on using the CTV expression vector to add toxins to the psyllid diet. Upon sucking the phloem sap from the citrus plant, these toxins will move to the psyllid and kill it. In summary, CTV expression/RNAi vectors are promising tools, which will fast-forward citrus research in the fight against HLB.

HANDHELD GENE GUN

The second method for gene delivery into citrus uses a gun. But this is not the kind of gun you will be able to purchase at the next gun show. The only thing this gun shoots is DNA. Its

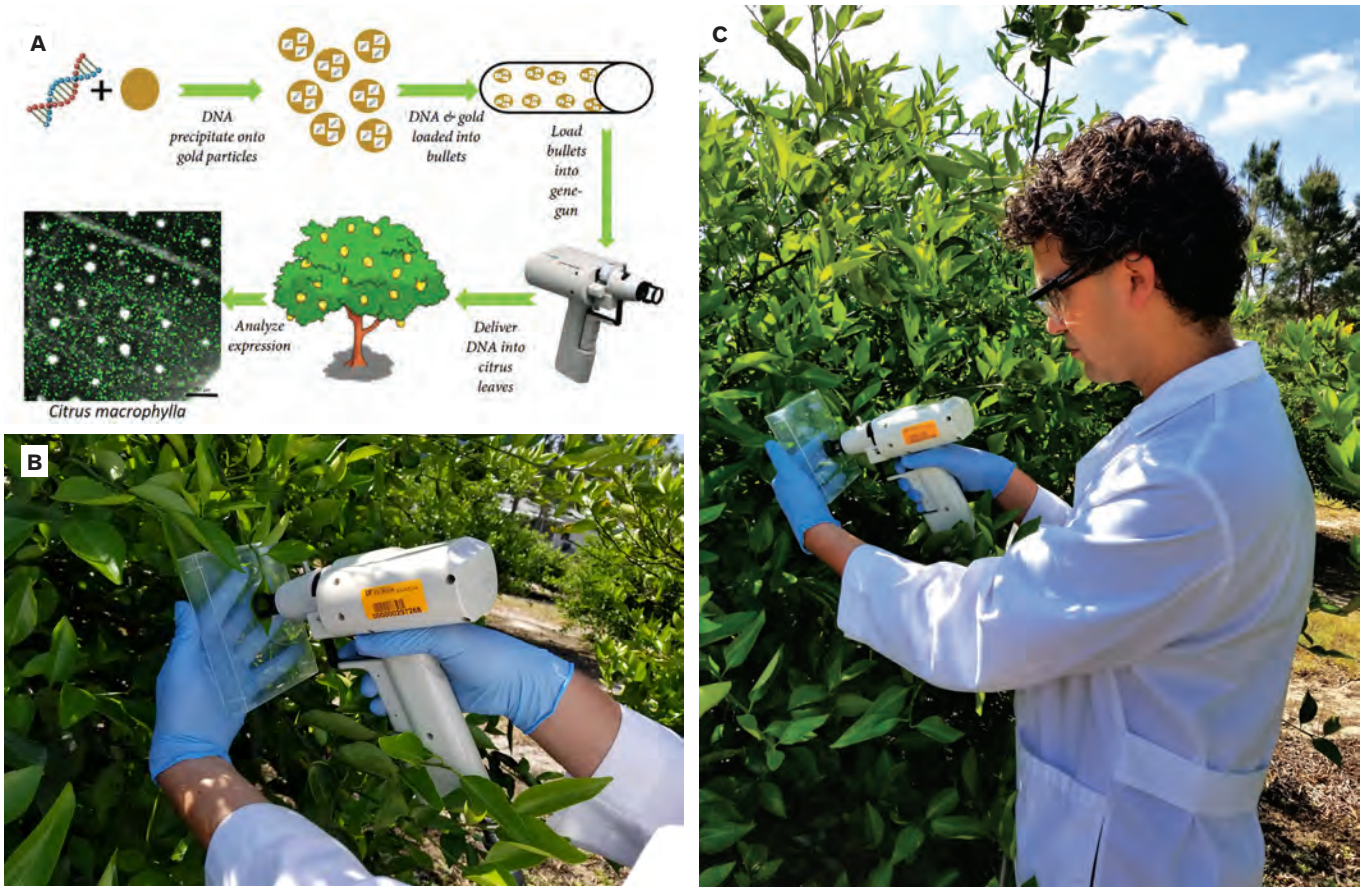

Figure 2. A diagram shows how the gene gun works (A). The gene gun is very portable and can be easily used in any desired setting, including the grove (B and C).

bullets are made from plastic tubes that contain many tiny gold particles (0.6-micrometer diameter) attached to the DNA to be delivered into the plant. The gas pressure does not come from gunpowder combustion, but instead uses helium. Release of the helium pressure will propel the gold particles at a very high speed and deliver them into the target plant cells (Figure 2).

Working with CTV is relatively easier and faster to design, build and carry to plants than CRISPR.

Unlike CTV, researchers are only beginning to explore the use of this gun with citrus. But they already have found that in each bombardment event DNA can be delivered to thousands of cells in the citrus leaves, and that these genes are expressing active proteins. This method works efficiently with every citrus variety that has been examined (*Citrus macrophylla*, Carrizo, Duncan grapefruit and Madam Vinous), and therefore seems to be highly suitable for incorporating DNA within these plants.

Using a gun to introduce DNA and transform citrus plants has many advantages. First, its ease of use allows efficient testing of gene candidates before moving forward to generating transgenic plants. Researchers can achieve a high level of gene expression in citrus leaves very quickly, as short as three days. In addition, researchers can easily scale up the expression levels by shooting a leaf at different locations or by bombarding many different places in the tree.

This system's efficiency can also enable use of CRISPR-Cas9 in the plant and generate mutations in target genes without making GMOs. Like any good gun, the gene gun allows the ability to aim carefully and target any particular area of choice, in order to control exactly where the genes will be expressed in the plant.

For example, researchers are currently testing the use of the gene gun in order to directly bombard phloem-rich tissues (such as the bark) to deliver therapies into the place where the HLB bacterium is present. The gene gun is highly portable and can be easily used in any setting. This can allow the delivery of therapies directly into trees in the grove, if needed (Figure 2).

There are still many things to explore with the gene gun. One of the main limitations of the system is that it is costly. Therefore, studies will be needed in order to determine its economic feasibility to resolve threats such as greening. Researchers are currently exploring the possibilities of this system to deliver therapies for HLB.

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