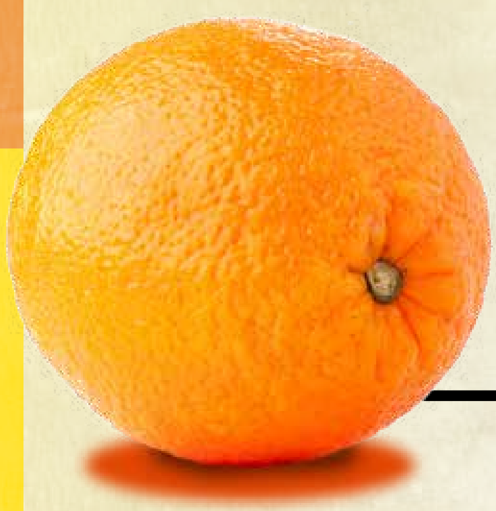

COVER STORY

Using genetically modified biotechnology to improve citrus

By Manjul Dutt and Jude Grosser

Florida has a large and vibrant citrus industry, mainly producing grapefruits and sweet oranges. This industry has faced numerous challenges in the past, in the form of diseases such as citrus canker and the tristeza virus or damage due to hurricanes and freezes. Growers have lost hundreds of thousands of trees to these challenges, but have always bounced back by planting new groves and resets in their existing groves.

The recent huanglongbing (HLB) outbreak has led the industry to a crossroads. This bacterial disease caused by a non-indigenous pathogen has resulted in substantial economic losses and threatens the survival of our industry. Resistance to HLB is not present in the commercial grapefruit and sweet orange cultivars grown in Florida. Citrus canker disease remains a problem, and a relatively new introduced fungal disease, citrus black spot, also has the potential to be a problem. Improved commercially acceptable cultivars that can effectively control these diseases are urgently needed to maintain a vibrant industry.

CONVENTIONAL AND MUTATION BREEDING

Citrus genetic improvement efforts utilize different techniques to produce the same outcome. In conventional citrus breeding, scientists mix two sets of DNA — one from the mother and the other from the father — to produce hybrid embryos through a process called meiosis. These embryos give rise to hybrid seeds that nature alone would be unlikely to ever produce. A large population of seeds (containing the DNA from both mother and father) have to be subsequently germinated and rigorously evaluated in order to identify progeny exhibiting industry-favorable characteristics.

Improved citrus cultivars have also been produced through natural as well as induced mutations. Natural mutations have occurred spontaneously over time. Tree limbs containing a natural mutant and fruits observed to be very different from the parent have been selected, evaluated and mass-propagated. Tissue culture-induced cell mutations (somaclonal variation), or zapping budwood with radiation to induce mutations, can also result in the development of improved cultivars. Both of these are shotgun approaches requiring large plant populations to find improved selections. These processes can cause bits and pieces of DNA to break and rearrange themselves differently, or a point mutation that is a single nucleotide change in the DNA's sequence, producing changes that can result in an improved cultivar.

Natural mutations have resulted in most of our sweet oranges and grapefruits, and artificial mutations have produced seedless mandarins such as N40W-6-3 (Seedless Snack), Tango, Fairchild LS and Kinnow LS as well as the scab-tolerant mandarin US Furr ST. Several of the new sweet oranges recently released by University of Florida/Institute of Food and Agricultural Sciences (UF/IFAS), including Valquarius and OLL-8, are products of somaclonal variation.

Citrus has a long juvenile period, and most cultivars take at least six years from seed germination to flower. Rapid genetic improvement to combat emerging abiotic and biotic conditions is usually hampered by this long juvenile phase. Although every single improved citrus cultivar grown in the world has arisen from the above-mentioned techniques, these techniques cannot always guarantee the genetic integrity of the new cultivar in relation to the existing parental cultivar(s). In these cases, planted trees may contain thousands of unknown changes. Such methods have been used for almost a century. There is minimal risk, nobody worries about regulating such arrangements, and the products are perfectly acceptable for use in organic production. These radical transformations work, but nobody worries too much about them.

WHAT IS GM PLANT IMPROVEMENT?

In contrast to conventional plant improvement using the above mentioned techniques, production of genetically modified (GM) plants (also called transgenic or genetically engineered plants) can result in rapid improvement of an existing variety. The most commonly used process of introducing a gene to a plant is called "*Agrobacterium*-mediated genetic engineering." With this process, a specific gene (DNA sequence) of interest is inserted into the host plant's genome with the help of a common pathogenic soil bacterium called *Agrobacterium*, often referred to as "nature's own genetic engineer." This bacterium can enter and infect the plant cell through a wound. The bacterium inserts a special sequence of its own DNA into the plant cell's DNA. This original sequence of inserted wild-type DNA can be altered, removing bad DNA (deleterious genes) and replacing it with disease-resistant genes scientists want inserted.

After the new beneficial DNA is delivered by the *Agrobacterium*, scientists use an antibiotic to kill the *Agrobacterium*, so the engineered cells are no longer infected. Any engineered cell containing the small extra piece of inserted beneficial DNA can then be made to produce a whole plant using tissue culture techniques. This plant will therefore contain all the original DNA of the cultivar, with the addition of a small, "foreign" DNA imparting some physiological or biochemical advantage to the new plant. In citrus, the new plant could now have a new gene that imparts tolerance to diseases without changing the DNA of the plant or the integrity of the cultivar. This is often referred to as "precision breeding."

WHAT ARE WE DOING?

The citrus improvement team at UF utilizes GM technologies to create new tools to facilitate conventional plant breeding. The major problem facing the industry is the various diseases affecting citrus. Biotechnology-mediated strategies are being developed to combat these diseases. Just as animals contain white blood cells that are involved in protecting the body against infections, plants also have the ability to produce an immune response following infection. This immune response [known as systemic acquired resistance (SAR)] occurs after plants are infected and can provide strong resistance to subsequent infections.

An SAR gene called NPR1 isolated from *Arabidopsis*, a mustard plant relative, is being evaluated for its ability to control HLB. Inserting this gene in citrus using *Agrobacterium* keeps it switched on at all times and results in the tree's immune system being constantly turned on and ready to fight against infection. All genes are controlled by small sequences of DNA called promoters. Simply put, a promoter is a switch

Figure 1: University of Florida graduate student Ethan Neilsen stands next to a 4-year-old, NPR1-expressing, transgenic sweet orange tree, which has consistently tested negative for HLB.

that tells the gene to express at specific locations in the plant. A constitutive promoter or switch turns the gene on throughout the entire plant.

We have produced several transgenic sweet orange trees containing this gene — being driven by either a constitutive promoter or a phloem-targeted promoter (HLB resides in the phloem) — that have demonstrated enhanced tolerance to HLB. Engineered sweet oranges with this gene have remained HLB resistant after two and a half years in a greenhouse harboring the disease and insects, as well as after four years in the field at a site with high HLB pressure (Figure 1).

If the SAR-induced immunization signal is mobile and can be transmitted from one part of the plant to the other, it is possible that a transgenic rootstock expressing the *Arabidopsis* NPR1 gene and grafted onto a non-transgenic scion can adequately protect the top part against infection. We have produced transgenic rootstocks that are in the process of being evaluated to test this hypothesis. Efforts are also currently underway to incorporate this gene into commercial scions and rootstocks (including the new HLB-tolerant cultivars being released by the UF citrus improvement team) to provide them with an additional boost.

In addition, a number of genes derived from other plants such as grapes, beans, pepper, soybean, tobacco and tomato are being evaluated for their ability to provide durable resistance toward HLB. Transgenic plants (both scion cultivars and rootstocks) are also being produced by a process called "gene stacking." In this process, genes with different modes of action are being inserted into citrus. Growing citrus is a long-term investment, and it is possible that HLB or other pathogens could mutate to break down the resistance provided by a single gene. Adding one or more genes with differing modes of action provides a backup that should prevent this from happening, resulting in stable, long-term resistance.

DEVELOPING CONSUMER-FRIENDLY GM CITRUS

Citrus is considered a wholesome part of the daily diet, and it is possible that consumers could be leery of accepting GM orange juice or fresh citrus containing viral and bacterial DNA sequences or the required antibiotic resistance gene needed for *Agrobacterium*-mediated engineering — even if the introduced DNA helps impart durable disease resistance and has been proven to be safe. Development of GM citrus where all the introduced DNA comes from edible plants should eliminate this concern.

We have developed a unique protoplast (a naked cell that had its cell wall completely removed using digestive enzymes) genetic engineering (transformation) system that can potentially allow the addition of only edible plant-derived transgenes without any additional bacterial/viral DNA sequences. In this system, a plant-derived visual reporter gene is inserted along with the gene of interest directly into an isolated citrus protoplast with the help of polyethylene glycol or electroporation.

Why is the visual reporter gene needed? In the process of protoplast transformation, millions of cells are exposed to the foreign DNA, with many of those cells taking up the DNA. However, only a few cells are able to successfully incorporate the introduced beneficial DNA into their nucleus and make it part of their own genome. We need a system that allows the scientist to select these few transgenic cells from the large number of untransformed cells, as necessary to regenerate engineered plants.

We have isolated a reporter gene called Ruby (derived from the blood orange) that results in the production of purple anthocyanin. Using a citrus-derived, embryo-specific promoter (a switch that turns a gene on only in a developing embryo), we have developed a DNA transformation construct that allows this anthocyanin to be expressed in the initial stages of plant regeneration (purple embryos), but is subsequently switched off in the developing/mature plant. Utilizing the Ruby gene, we can now recover phenotypically normal engineered citrus plants containing introduced beneficial new DNA only from edible plants, thereby increasing their potential consumer acceptance.

The Florida citrus industry is at a crossroads … GM biotechnology … offers great hope.

GM TECHNOLOGY TO REDUCE JUVENILITY

In addition to using biotechnology to directly solve pressing disease or abiotic problems, our research is also being used to aid conventional breeding. Conventional citrus breeding is a long-term process mainly due to the time it takes plants to reach flowering maturity. This long time makes evaluation of new hybrids time-consuming and costly. A number of factors influence the length of the tree's juvenile stage, and if that could be sped up, the tree could flower earlier.

There is a key gene called FT that produces a protein that drives the floral induction process. This protein is naturally present in low levels in juvenile plants. The quantity increases as the plant reaches maturity. We have isolated this floral induction gene (producing the Citrus FT or CFT protein)

Figure 2: A purple-leaved juvenile, flowering, transgenic Carrizo citrange rootstock transformed with two genes: the Clementine-derived FT gene and the grape-derived VvMybA1 anthocyanin gene. Plants were transformed with these two genes for visual transgenic plant selection and for proof of concept of functionality of the FT gene.

from Clementine, and using GM technology, incorporated it into the Carrizo rootstock genome. Instead of turning on when the plant matures, we have engineered the gene to turn on at a high level all the time. This pattern leads to an increase in FT protein levels and flowering of 3-month-old Carrizo plantlets (Figure 2).

There are scientific reports claiming that this protein is mobile and able to traverse up to the scion through the graft union. We are in the process of evaluating this in citrus. If true, these CFT Carrizo plants could be used as rootstocks, letting the FT protein travel from the rootstock to the scion to reduce the juvenile phase in promising non-transgenic hybrids, as well as in new GM citrus plants produced from juvenile explants being produced by the UF/IFAS Citrus Research and Education Center citrus improvement team. This could significantly reduce the time and field space required for evaluating newly created germplasm.

WHAT'S IN THE FUTURE?

GM biotechnologies are not new. These safe and established techniques have been used for almost 20 years to help farmers maintain yields and profits, while farming with less environmental impact. These tools have been confined to basically two traits on large-acreage field crops for many reasons, but their use in papaya saved a dying Hawaiian industry. A similar parallel is poised to happen in citrus, as promising public solutions stand to cure a seemingly relentless disease. The Florida citrus industry is at a crossroads, and utilization of GM biotechnology to grow transgenic trees producing safe-to-eat fruit that is resistant to HLB and other diseases offers great hope.

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