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Reducing Breeding Time in Citrus Through Biotechnology

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Project Summary

Economically important traits for the improvement of citrus such as resistance or tolerance to huanglongbing (HLB) have been identified in several citrus types and relatives, and markers for these traits are being identified. In addition, early flowering citrus types for use in citrus variety breeding are urgently needed. Early flowering has been achieved in many plant species; however, this trait has been unexpectedly difficult to reproduce in citrus. This project is exploring alternative strategies for producing early flowering citrus types.

Citrus variety improvement using conventional methods (i.e., hybridizing two citrus types with desirable traits, growing out the resultant hybrid seedlings, evaluating the hybrids for improved tree and fruit characteristics and using selected hybrids in further crosses) is very time consuming. Despite several biological constraints, it is possible to produce many hybrids; both through the crossing of closely related citrus types and by crossing citrus varieties with more distantly related relatives (i.e. wide hybridization). However, many diverse traits in the citrus gene pool will take decades to exploit using only conventional breeding. The major constraint in citrus breeding is the lengthy period of juvenility (the time between planting hybrid seed and their development into flowering, fruiting trees), which can be eight years or longer.

The overall goal of our research is to develop and use early flowering citrus types to speed up the breeding process, making it possible to rapidly introduce valuable traits from citrus relatives or non-commercial citrus types into high-quality commercial varieties. Our original goal was to use transgenic methods[1] to produce transgenic hybrids that flowered in one year or less. This method has been developed in annual plants and is being implemented in other perennial plants with long juvenile periods, including plums, apple and poplar (Tränkner et al. 2010). A rapid cycling method would make it possible to incorporate priority traits (such as HLB resistance) and should allow breeding to occur much more rapidly than is possible with conventional breeding alone.

We have conducted many experiments with citrus involving the use of molecular methods and genetic transformation. Most of the experiments were conducted with two genes that facilitate the identification of transgenic plants during the transformation process: the selectable kanamycin-resistance gene (*nptII*) which allows selection of transgenic plants since only they survive in the presence of the kanamycin antibiotic; and the β-glucuronidase (*GUS*) gene as a scorable marker, which produces a visible blue color in transgenic tissue when the appropriate substrate is present. In addition, a gene promoter[2] that was expected to induce constitutive (always on) expression was used to control the flower-inducing transgene. The flowering gene that we have tested most extensively is called *FLOWERING LOCUS T (FT)*. This gene is believed to encode the "florigen" flower inducer (the hypothesized hormone-like molecule responsible for controlling and/or triggering flowering in plants). Over-expression (expression of a transgene copy of an already present gene) of *FT*s in many different species has led to early flowering. There are several slightly different copies of this gene in citrus.

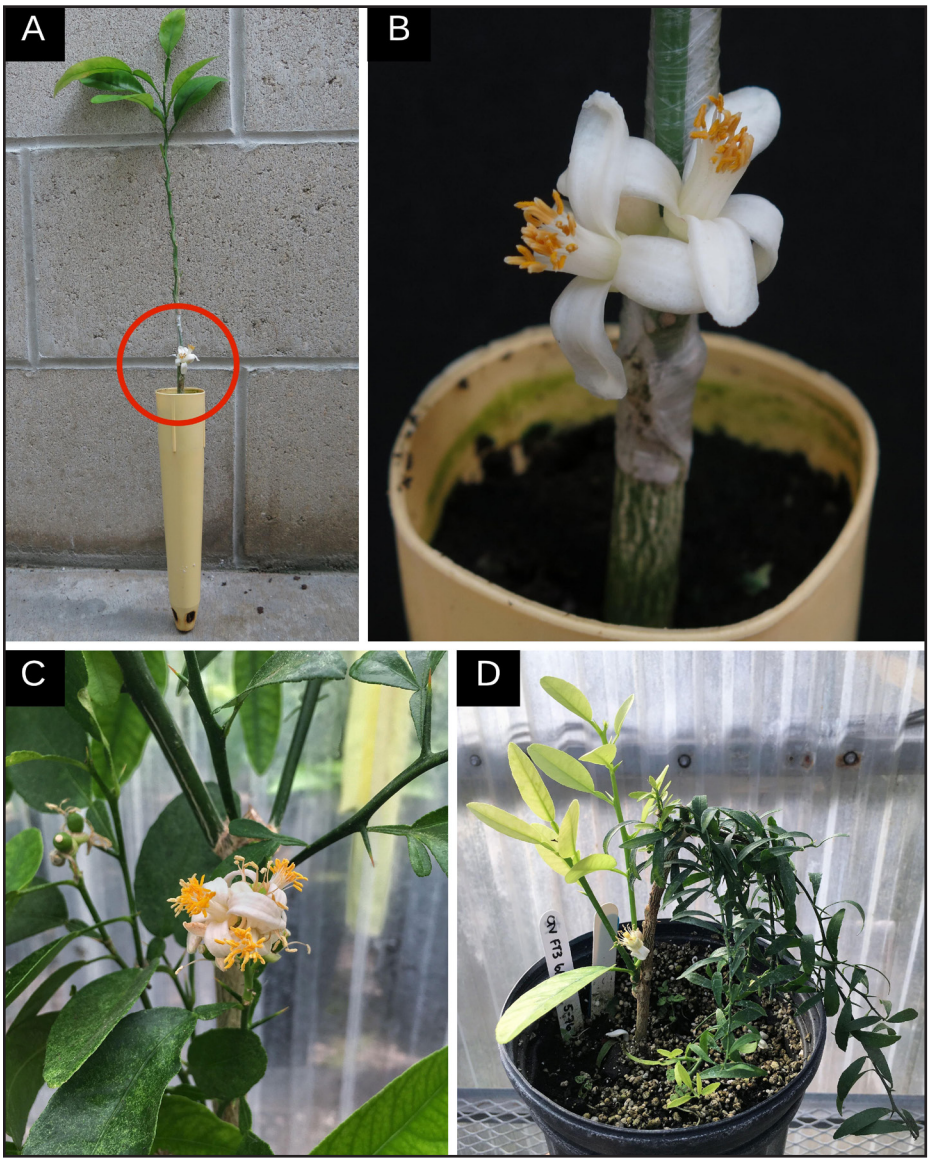

Figure 1: Alemow grafts infected with* CTV-FT3 *flowering in March 2017 in Fort Pierce, Florida (A and detail in B), and in June 2017 in Gainesville, Florida (C-D). Alemow grafted on finger lime (*Eremocitrus*) is shown in panel D.

We cloned three citrus genomic sequences and characterized them. They have been named *FT1*, *FT2* and *FT3*. *FT1* and *FT2* may be alleles (two forms of the same gene) at a single locus (location in the DNA). *FT3* is clearly different and appears to be the *FT* gene whose expression is most closely associated with flowering. We have used all three of these to transform both tobacco, as a model species, with which we can get rapid results, and also some citrus types. In tobacco, transformation with any of the sequences leads to early flowering. When the citrus *FT3* gene

was overexpressed in citrus ('Carrizo' citrange, 'Hamlin' sweet orange and 'Duncan' grapefruit) with a constitutive promoter, we obtained flowering too soon, and transgenic plants were difficult to regenerate.

We are conducting a number of experiments to overcome our problem of too much *FT* gene expression in citrus, by concentrating on *FT3*. One general strategy is to try to "dial down" the expression of the transgene. We are testing other, non-constitutive (less intense) promoters. Some of these promoters limit gene expression to specific plant tissues – for example, only in the phloem (part of the plant vascular system), where *FT* is thought to be naturally expressed. Such localized expression may avoid production of *FT* that affects important plant processes other than flowering. For example, we have 13 transgenic 'Carrizo' plants with the phloem-specific citrus sucrose synthase (*CitSS*) promoter driving *FT3* expression (*CitSS::FT3*) and 43 'Carrizo' plants with *396SS::FT3* (another citrus phloem-specific promoter) that will be validated soon.

In addition, we are testing inducible promoters. We have 20 transgenic 'Carrizo' plants that have a *PR1::CiFT3* (pathogenesis-related protein 1 gene promoter) that can be induced by application of salicylic acid. Finally, we have made a construct wherein *FT3* is controlled by a promoter that should be induced only when the transgenic plants are exposed to a specific inducing agent, in this case, a pesticide that is already registered for use in citrus. The construct has been tested carefully to determine that it is correctly cloned. Both tobacco and citrus explants have been transformed with this construct, and we are awaiting further results. These were described previously (Moore et al. 2016).

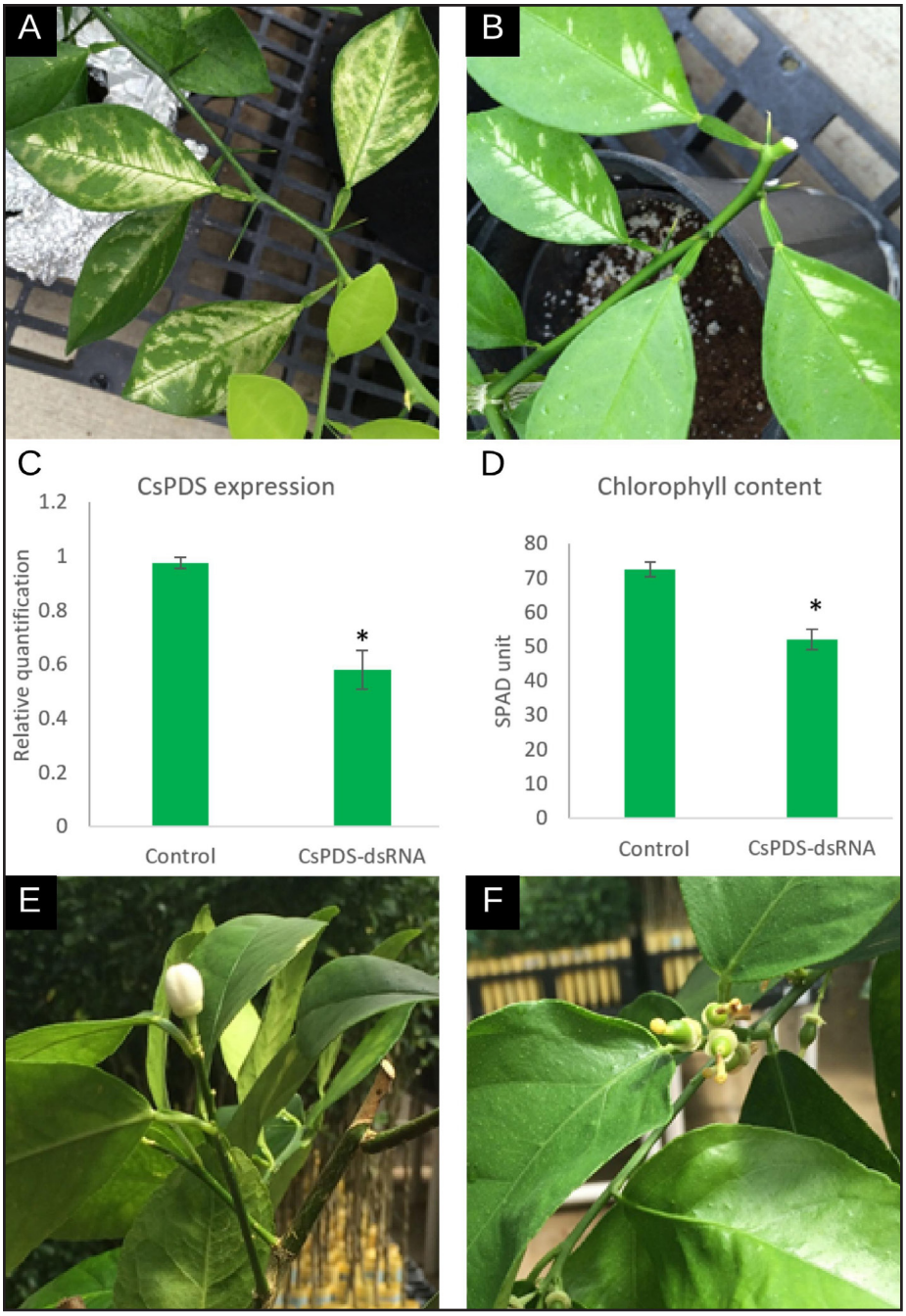

Figure 2: Soil applications of dsRNA targeting the PDS gene caused a photo-bleaching phenomenon (A and B), reduced CsPDS gene expression (C) and chlorophyll content (D) (an asterisk indicates values are statistically different from the control). Soil applications of dsRNA targeting the TFL1 gene induced flowering (E) and fruit development (F).

A different approach uses a citrus tristeza virus (CTV) vector (which was partially constructed by others and generously provided to us) containing the *FT3* gene for *in planta* expression. The virus can be delivered to already established plants, and the *FT* gene sequence is expressed in the citrus plant, but not incorporated into the genome; hence, this method does not require production and regeneration of transgenic plants via tissue culture. CTV inhabits the phloem, which is also the site of *FT* action. One *CTV-FT3* vector was produced by Siddarame Gowda, Ph.D. (University of Florida, Citrus Research and Education Center) in collaboration with Ed Stover, Ph.D., and used to inoculate an alemow plant (*Citrus macrophylla*) (Folimonova et al. 2007). This plant was confirmed to have *CTV-FT3* in all newly grown branches. Using budwood from this plant, a group of 90 seedlings (largely pomelo hybrids) were graft-inoculated from July to August 2016 in Fort Pierce, Florida (**Figure 1 A-B**). From December 2016 to January 2017, two plants showed flowering at the grafting site. Thirty additional trees, mainly 'Pineapple' sweet

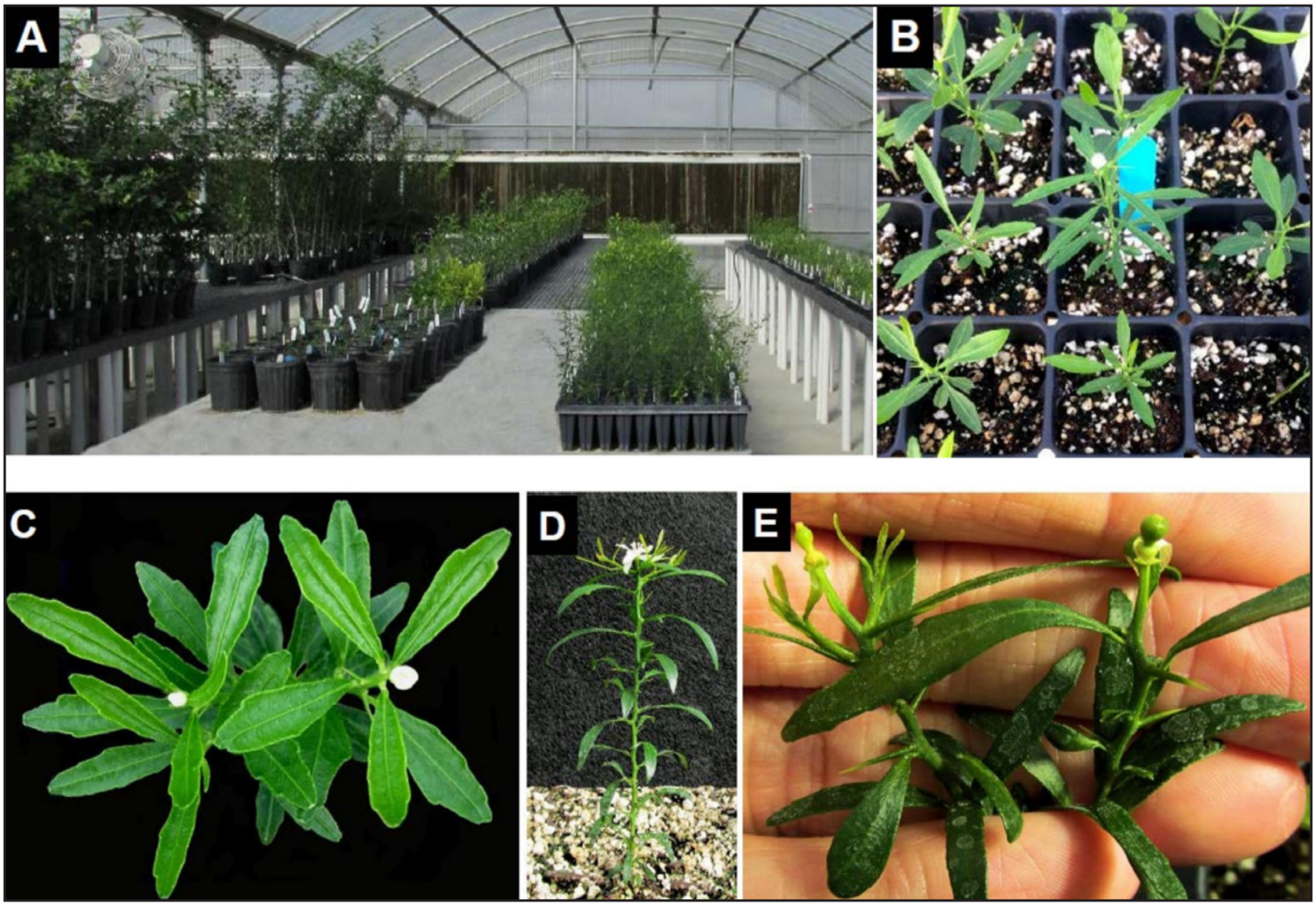

Figure 3: Early flowering individuals were identified from a two-month-old F2 mapping population derived from crosses between 'Pineapple' sweet orange and* Eremocitrus glauca *planted from seed in an insect proof greenhouse (A). Photographs were taken in November 2015, with a greenhouse temperature setting of 80°F. Two-month-old seedling flowering in seedling tray (B). Appearance of early flowering seedlings (C and D). Fruit development (E).

orange, 'Duncan' grapefruit, sour orange and 'Kuharski' rootstock have been graft-inoculated in Gainesville, Florida, using a different CTV vector construct (provided by Svetlana Folimonova, Ph.D., University of Florida, Plant Pathology Department) and confirmed to be *CTV-FT3* positive (**Figure 1 C-D**). So far, early flowering has not been observed on varieties other than alemow.

FT is only one gene of many involved in the process. There are other naturally occurring genes that also promote flowering and some genes whose expression delays flowering. Therefore, we are testing methods to reduce the production of an important naturally occurring citrus "flower delaying" gene using RNA interference technology, where the RNA product of a transgene reduces the expression of a target gene by inducing degradation of its RNA. Two hair-pin RNA[3] constructs of different lengths [156 base pairs (bp) and 313 bp] were used to silence the terminal flower gene (*TFL1*), which delays flowering. Transformation with these constructs has been completed, and 46 'Carrizo' transgenic plants were obtained. These plants are being tested for early flowering.

In addition, we are using a non-transgenic approach through the direct application of chemically synthesized double-stranded RNA (dsRNA) to induce the degradation and inhibit the expression of citrus genes. In one test, as proof of concept, application of a dsRNA designed to target *CsPDS* (involved in chlorophyll synthesis) resulted in a photo-bleached appearance (**Figure 2 A-B**), along with reduced *CsPDS* expression and chlorophyll content (**Figure 2 C-D**). This suggests that dsRNA could be applied to plants to effectively regulate citrus genes. To test this, a 150-bp dsRNA targeting *TFL1* was applied to Persian lime plants as a soil drench (200 µg dsRNA per pot weekly). The lime plants, under greenhouse conditions, flowered after 11 treatments (**Figure 2 E-F**).

Future Directions

In future experiments, we will explore two additional approaches. One is the use of a genome editing method called CRISPR/Cas9. This technology allows very precise editing or addition of citrus genes without needing to insert sequences from other organisms (e.g., bacterial or viral DNA), a step

necessary with *Agrobacterium*-mediated transformation. This is not only more precise, but also avoids the presence of foreign DNA that is subject to federal regulation. The other approach is to hybridize valuable varieties with non-transgenic early flowering mutants that we have identified. Again, such hybrids would not be regulated for field planting.

We hope with these additional strategies, we will be able to rapidly produce and test genes of interest that may induce the early flowering characteristic that is needed for shorter breeding times. Then we can concentrate on the rapid production of improved citrus types, using crosses between citrus varieties and with citrus relatives, to provide new alternatives to citrus breeders and producers, especially HLB tolerance.

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Glossary

[1]Transgenic Methods: The process of introducing foreign DNA into a host organism's genome (its complete set of genetic material). The DNA that is transferred to the recipient can be from other individuals of the same species or even from unrelated species.

[2]Gene Promoter: a DNA sequence that determines how much, where and under what conditions a gene product is made.

[3]Hairpin RNA: A short fragment of RNA that can bind to itself due to a complementary sequence making a "hairpin" type of structure; this type of RNA fragment can be used to silence target gene expression via RNA interference mechanisms.

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