Engineering Citrus Using Recent Advances in Gene Editing Technologies

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Year 2 of 2 (80% Complete)

Objectives

1. Induction of rapid flowering
2. Multiplex gene editing of three potential disease susceptibility genes
3. Optimize gene editing approaches

Problem and Significance

The overall goal of this project is to develop a suite of approaches to rapidly and effectively generate citrus varieties that show increased tolerance to huanglongbing (HLB) disease using Crispr-Cas9 gene editing approaches. We have developed strategies to simultaneously knock out multiple genes at once in citrus (multiplex gene editing) which now affords the possibility of stacking multiple desirable genetically engineered traits together. Citrus species with stacked traits are more effective at meeting the needs of growers since traits such as yield, disease tolerance, fruit quality, flowering time, and other traits can be modified concurrently, providing for the rapid and precise engineering of a desirable citrus cultivar in a known genetic background.

Benefit to Industry

The outcomes of this project have multiple benefits to industry; the overall goal of improvements in the use of Crispr-Cas9 gene targeting and multiplex gene targeting provides a resource and methodology to engineer a wide range of different traits into specific varieties. The specifics of these improvements will be used to engineer citrus for early flowering, which can be used to accelerate breeding programs. The engineering of citrus with loss of function for three *PP2* genes that are candidates for being involved in disease susceptibility could provide a new paradigm for how to combat HLB, and knockouts of these PP2 genes can be generated in many citrus varieties. Together, these approaches should provide better tools for accelerating genetic engineering of citrus, and may define specific mechanisms to confer tolerance to HLB.

Progress Summary

Objective 1: Induction of early flowering: We have been manipulating several different genes in an effort to induce early flowering. The goal is to identify a mechanism by which early flowering is achieved in ~1-2 years, so that plants are robust and can provide seed, but not so accelerated that plant growth and fruit development is compromised. We have overexpressed the *FT* gene and have recovered multiple Carrizo Citrange plants that display precocious flowering (Figure 1). We have also shown that rooted cuttings maintain the early flowering phenotype. We are currently testing whether early flowering is graft transmissible, and preliminary results suggest that scions grafted on to FT-overexpressing rootstock can induce early flowering. We have also explored the possibility that two other flowering regulators, the genes *TFL* and *CEN*, can be mutated to produce accelerated flowering. We have generated multiple Crispr-Cas9 engineered Carrizo Citrange lines that we have confirmed via sequencing are either homozygous or biallelic for the *TFL* or *CEN* mutations. We are currently growing these plants to determine if they exhibit any early flowering phenotypes.

Objective 2: Multiplex gene editing: We have shown that multiplex gene editing is effective at mutating at least three genes simultaneously (Zhang et al., 2020). Using this multiplex gene editing approach, we have 30 confirmed lines in which the *PP2B10, PP2B14,* and *PP2B15* genes are mutated alone or in combination. These *PP2* genes encode phloem proteins and are prime candidates for conferring susceptibility to HLB. Thus we now have multiple plant lines that can be used as a platform to further investigate whether elimination of *PP2* gene activity is sufficient to confer tolerance to HLB.

Objective 3: optimizing gene editing approaches: We have taken a variety of approaches to both improve *Agrobacterium*-mediated transformation of various citrus varieties, as well as explored methods for non-GMO gene editing approaches in citrus. We have improved methods for *Agrobacterium*-mediated transformation and regeneration in several citrus varieties, including Pineapple sweet orange, Valencia sweet orange, and Jackson grapefruit. These improvements have involved refining the media used for transformation and regeneration, as well as testing out a variety of light regimes to enhance regeneration.

We have also been testing protoplast regeneration protocols, as we can introduce non-integrative constructs into protoplasts which in turn allows for non-GMO gene editing. To date, we have not made headway on this goal of protoplast regeneration, in part due to COVID-related delays.

We now have a suite of protocols that allow us to successfully genetically engineer a number of citrus varieties. This will allow us to rapidly generate gene-edited lines for a variety of value-added traits of interest to citrus producers and consumers. The next steps in these processes are to partner with other researchers to test our gene-edited lines for tolerance to HLB, as well as develop a pipeline to allow researchers to obtain their own specific gene-edited lines of interest.

Figure 1 Overexpression of FT induces early flowering in Carrizo Citrange. Left, precocious flowering in an 8 month old plant; right, fruit (arrow) being produced on a 1 year old plant.

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Publications and Presentations

Zhang, F, Rossignol, P, Huang, T, Wang, Y, May, A, Dupont, C, Orbovic, V, and Irish V.F. 2020. Reprogramming of Stem Cell Activity to Convert Thorns into Branches. *Current Biology.* 15: 2951-2961