Development of mature citrus tissue transformation technology

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Year 1 of 3 (10% Complete)

Objectives

1) Disarming the *Agrobacterium* 1416 and creation of the 1416 and EHA105 GAANTRY strains for transformation of citrus tissue.

2) Transformation comparison of Agrobacterium 1416, 1416 GAANTRY, EHA105 GAANTRY and EHA105 (control) strains.

3) Combine the wounding, surfactants, hormone type/timing, novel media additives for mature citrus transformation evaluation.

Problem and Significance

Transformation technology in citrus is difficult and time consuming this is limited to a few non-elite cultivars. Without improvements to the citrus transformation process when a cure for HLB is discovered a majority of the existing citrus cultivars will lose commercial viability. Previously, though a CRB funded proposal (5200-165), we studied budwood transformation as a means of increasing citrus transformation efficiency. During this project we discovered techniques such wounding, surfactants, hormone type/timing, novel media additives, genes that induce tissue growth and those that inhibit cell death. From this data we produced an enhanced transformation vector and improved existing citrus transformation efficiency. We also discovered a novel wild *Agrobacterium* (1416) that appeared effective for mature citrus transformation. We propose developing this novel wild agrobacterium specifically for its mature citrus transformation capacity. Further, the *Agrobacterium* 1416 would also be converted into a GAANTRY system that is effective and stable system for stacking multiple genes within the *Agrobacterium* for plant transformation. The stacking process was previously demonstrated to successfully assemble a large 28.5 kb T-DNA construct containing ten cargo sequences (10-stack) in transgenic Arabidopsis and potato plants. Finally, we plan to combine the enhanced transformation techniques discovered during the 5200-165 research and analyze for additive effects using both the traditional *Agrobacterium* EHA105 and the novel 1416 strain for mature tissue transformation.

Benefit to Industry

The proposed research will investigate the use of a novel *Agrobacterium* (1416) for enhanced mature citrus tissue transformation. If successful, we will produce a novel transformation protocol for mature citrus tissue around this strain (1416). Constructs have been designed to enhance the plant cell survival during transformation and to provide an early 'readout' of transformation success. The vectors used during this study will be complementary with the RMCE 'Lilac Lime founder line (5200-155) research project. If successful, the RMCE EXCH testing will be used to remove the selectable marker genes nptII and codA during the assays and allow regenerationm of shoots for production of plants. The *Agrobacterium* (1416) strain will be made competent for both traditional transformation with a binary vector and for the newly GA*A*NTRY system (Collier et al., 2018) and tested for efficiency against the traditional EHA105 specifically on mature citrus tissue.

GA*A*NTRY (Gene Assembly in *Agrobacterium* by Nucleic acid Transfer using Recombinase technologY) is an effective and stable system for stacking multiple genes within an *Agrobacterium*. The gene stacking system utilizes easy-to-handle cloning vectors for the insertion of sequences of interest. The system enables the precise integration of cargo sequences into the GA*A*NTRY strain using a simple *in vivo* stacking procedure. The resulting *Agrobacterium* strain carries a T-DNA containing the sequences of interest and can then be directly used for citrus transformation. The system appears to have an effective transfer capacity of 40-50kb (effectively 20-25 genes). In other words, entire metabolic or disease resistant pathways can be moved into citrus if needed.

The novel *Agrobacterium* (1416) proposed for development will be designed specifically for the citrus transformation and a CRADA (contract) will be established between the USDA and CRB providing access to the IP developed for this strain and protocol. Successful techniques, constructs and *Agrobacterium*

strains will be made available to the citrus community by presentations at scientific conferences and by publication in a peer-reviewed journal and be disseminated for research upon request. As this project involves *Agrobacterium*, biotechnology and genetic engineering, federal regulatory review and approval will be needed prior to commercialization of citrus cultivars exhibiting desirable traits.

Funding received for this project will leverage existing USDA-ARS funding (which supports the project leaders' salaries and research facilities). This project works synergistically with previous California Citrus Research Board funded projects (Awards #5200-140, 5200-142 and 5200-165).

Plans and Procedures

Currently, HLB is considered to be one of the most serious citrus diseases in the world and currently there is no cure. While need for disease resistance genes is obvious the ability to get the resistance into the citrus is also a bottleneck to saving the industry. Current transformation technology is not amenable with many elite citrus cultivars.

Previously, under project 5200-165 our group investigated budwood transformation as a means of increasing citrus transformation efficiency. From this research we found a number of independent techniques that improved citrus transformation. These include wounding the tissue prior to transformation, use of surfactants, hormone type and timing applied, novel media additives that stimulate plant growth and health, genes that induce tissue growth and those that inhibit cell death. As a related side-project we also investigated a large collect of wild *Agrobacterium* and tested them directly on citrus tissue. A novel wild *Agrobacterium* (1416) was found that appeared effective for wide range of citrus cultivars for both juvenile and mature citrus transformation. However, these were preliminary results and require validation. The *Agrobacterium* 1416 is a wild strain so requires disarming (removing genes that produce gall). The proposed research will disarm and retest the *Agrobacterium* 1416 for mature tissue transformation. During the disarming process we will also convert 1416 into a GAANTRY competent strain. For evaluation we will compare the 1416, 1416 GAANTRY, EHA105 and EHA105 GAANTRY strains for mature citrus transformation capacity. In an attempt to enhance citrus transformation further we will combine and evaluate the techniques of wounding, surfactants, hormone type/timing, novel media, novel media additives and transiently expressed growth enhancing genes. Each one of these techniques has previously improved our citrus transformation on an individual basis, thus by combining we predict an additive effect and overall gain in efficiency. The transformation protocol will concentrate on mature tissue from Carrizo and Mexican Lime (due to its use in the original Founder lines) but mature Washington Navel, Tango Gold, Lisbon Lemon and Persian Lime will also be evaluated.

Progress Summary

COVID-19 related delays provide
1. The date any institutional closures or restrictions began March 18th 2020;
2. Research efforts suspended only essential personnel allowed to keep project alive.
3. Closure was lifted October 13th 2020.

Currently this project has been delayed due to contracting services at the USDA, issuing of a visa to hire a postdoc and of course COVID-19 delaying everything. No money has been spent at this time and a No Cost Extension (NCE) has been requested in effect pushing the entire first year of the project into year 2. With that said and postdoc has been identified and is waiting for visa and travel restrictions to lift. Also, portions of this project were started at the before the COVID-19 shutdown and were allowed to continue. These include disarming the novel 1416 agro bacterium along with the novel media trials. The preliminary media trials showed promising results where Washington Navel and Grapefruit responded to 5 out of 20 recipes, while Mexican Lime showed increased growth and overall health in 2. The novel agro 1416 used to for transformation with vector pCTAG-KCN3. Shoot regeneration seen from mature tissue of Washington Navel (7.8%), Mexican Lime (8.2%), Lisbon Lemon 8A (10%) and Cocktail Grapefruit (22%). Figures 1-4.

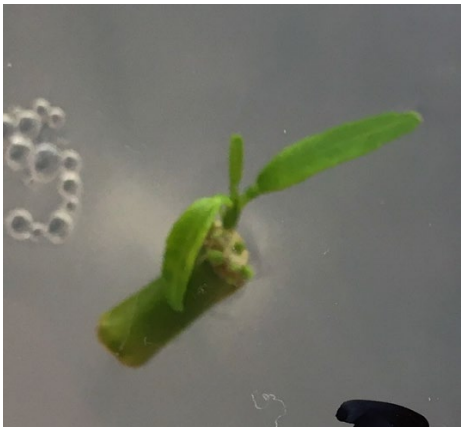

Figure 1. Mature Cocktail Grapefruit; 22% shoot forming efficiency.

Figure 3. Mature Mexican Lime; 8.2% shoot forming efficiency.

Figure 2. Mature Lisbon Lemon 8A; 10% shoot forming efficiency.

Figure 4. Mature Washington Navel 8A; 7.8% shoot forming efficiency.

Conclusions

Due to COVID-19 and contraction events this begun in earnest. Howerver, a pilot project run by Min Shao (project 5200-155) and Jennie Huynh (5200-165) examining the effects of various media recipes on different cultivars using mature tissue was begun. In this pilot project 20 different basal medias recipes were explored. Mature tissue from Washington Navel and Cocktail Grapefruit responded favorable to 7 out of the 20. Mature Eureka Lemon and Mexican Lime responded favorable to 3 recipes while the mature Clementine Mandarin tissue shown the least and

responded to only a single media with enhanced growth. By combining this information with the use of the novel agrobacterium 1416 allowed transformation of mature tissue from Washington Navel (7.8%), Mexican Lime (8.2%), Lisbon Lemon 8A (10%) and Cocktail Grapefruit (22%). While this data is preliminary it is the best transformation with successful regeneration results we have achieved.

CRB Project # 5200-170

Publications and Presentations

Nothing to report from 02/25/20 to 10/30/20 due to COVID-19 related delays.