PROJECT CONCLUDED-FINAL REPORT

Genetic Engineering of Citrus

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The main goal of this project has been to develop genetic transformation methods for the improvement of citrus cultivars important to the California industry. The two major scion cultivars of lemon (Lisbon and Eureka) were selected to serve as the primary focus for these experiments during the initial years of the program. As a result, two functional transformation systems based on organogenic regeneration were developed. One began with three- to five-week-old nucellar seedlings, whereas the other started with flush growth from mature trees. The seedling system applied only to the Eureka cultivar due to the fact that Lisbon epicotyl tissue did not regenerate.

Based on the results of others, we had emphasized the work with mature cuttings in an attempt to reduce or eliminate juvenility in recovered plants, at the expense of lower overall efficiencies. Observations of the plants recovered from mature cuttings during FYs '02-'03 and '03-'04 indicated that they indeed retained some mature characteristics such as reduced thorn size and branching. Some of the trees flowered for the first time during the spring of 2004 in Riverside, California, and a few more did the same in 2005. However, the reduction in juvenility, if any, was too small to offer a distinct advantage relative to the seedling-based system.

Establishing embryogenic regeneration systems from mature tissues of lemon for the purposes of transformation was also investigated during the initial years of the program. However, concerns with induced genetic variations of embryogenic cells due to extended periods in culture as undifferentiated callus, and the fact that some of the world's leading experts on citrus embryogenesis do not use this technique to produce transgenic citrus plants in their own laboratories, led us to question the practical utility of this approach with respect to applications where a seedling-based organogenic system was possible.

Consequently, the focus of the transformation program shifted away from any work on embryogenesis and instead evolved to developing methods that begin solely with seedling explants. This was true not only for lemon, but all of the other citrus cultivars that are part of the program (please see below).

Progress on the project during the '07-'08 FY was slowed significantly relative to previous budget cycles. Funding shortages due to the freeze in January of '07 led to a reduction in available human resources by over 70%, including a 40% cut to the PI and the complete loss of one full-time scientist and four part-time lab assistants. Consequently, the first several months of the current reporting period were devoted to resuming previous activity levels by hiring and training competent laboratory personnel and replenishing consumables such as citrus seeds that were acquired seasonally through the time intensive process of juicing individual fruits, many of which are considered seedless.

Finally, because of the loss one of the PIs (Dr. Fisk) working full-time on the project in April of '08, it was decided to initiate a change in emphasis upon his departure that required additional training for the laboratory staff that was in place (please see below).

Finish improving organogenic regeneration and transformation protocols for lemon, Washington navel and valencia sweet oranges, sour orange, lime, and Kinnow and owari mandarins: Investigations during the two most recent funding cycles prior to this one with different genetically engineered strains of *Agrobacterium*, selected antioxidants in the co-cultivation medium and various co-cultivation temperatures and times led to substantial increases in post co-cultivation regeneration. Regeneration efficiencies were dramatically improved from a base of 48% to a reproducible level of greater than 90% (Table 1).

This result was one of the most significant accomplishments of recent funding cycles and represented an overall enhancement in regeneration efficiency of nearly ten fold since the work began midway through FY '03-'04.

Table 1. Summary of Citrus Variety Responses Following Transformation Methods Optimization.

	Eureka	W. Navel	Valencia	M. Lime	Kinnow	Owari	S. Orange
Post co-cultivation Regeneration	>90%	>90%	>90%	73%	12%	55%	37%
GUS Positive Regeneration	57%	59%	47%	46%	50%	–	40%
PCR Positive	93%	71%	79%	100%	50%	–	87%

Table 2. Pooled Summary of Transformation Efficiencies over the Past Five Funding Cycles.

Fiscal Years	Regen events/10 Epicotyl Segs	Rooting Efficiency of Regener Shoots	GUS Positive[1]	PCR Positive[2]
'03 - '04 & '04 - '05	35.2 ± 3.2	57.7% ± 4.7	21.5% ± 2.8	60.3% ± 3.7
'05 - '06 & '06 - '07	28.5 ±1.7	52.4% ± 2.3	56.7% ±2.1	92.8% ± 2.8

1 Only shoots that regenerated under selection and subsequently rooted and survived under greenhouse conditions were analyzed for GUS gene expression.
2 Only plants testing positive for GUS expression were analyzed by PCR to confirm the physical presence of the GUS gene within DNA extracts of those plants.

Approximately 180 plants were recovered and transitioned to the greenhouse during FY '05-'06. Just over half (99) were screened for transgenic activity and, of those, an astonishing 57% tested positive for the GUS transgene. Many of these plants were subjected to more rigorous molecular methods that analyze for the physical presence of the introduced genes during FY '06-07. Polymerase chain reaction (PCR) analyses demonstrated that greater than 90% of the plants that tested positive at the level of gene expression also tested positive for the presence of the gene (Table 1).

The change in project emphasis noted above involved a shift away from experiments intended to optimize and/or finish methods development with varieties currently part of the program to a strategy focused on more carefully evaluating the efficacy of the protocols already established.

Work over the past several years resulted in the regeneration of hundreds of trees. However, only a relatively small fraction of them were analyzed for transgene expression or by PCR, which is a more rigorous molecular test for transformation. Now that many of these trees have reached a size sufficient to support various types of analyses, the primary effort over the latter half of the current funding cycle was to more thoroughly characterize the current inventory using PCR.

With an increase in sample size, it was anticipated that the results reported in Tables 1 and 2 would decline to numbers more in line with those established for transformation systems involving unrelated plant genera. Results from this work were being used to assemble a more complete evaluation of the methods currently in place and to help determine which should be considered complete or in need of more optimization.

Not withstanding a severe decline in efficiency numbers and with the exception of Owari Satsuma, the current data suggested that the methods are complete and represent the first fully functional transformation system available for citrus varieties important to the California industry. As such, a goal for future funding cycles was to seamlessly transfer this technology to centralized plant transformation facilities.

Begin experiments to establish organogenic and/or embryogenic regeneration systems for Mandarin varieties that are currently important in California or have the potential of becoming so: Other than the identification of relevant varieties to include in the investigation and potential sources from which to obtain them, work towards this objective was not conducted during FY '07-'08 due in large part to the reasons already cited above.

Begin integrating an *Agrobacterium*-mediated gene transfer step with the developed regeneration systems in selected mandarin varieties through the expression of a reporter gene encoding green fluorescent protein (GFP) and/or ß-glucuronidase (GUS): This objective was not addressed during the most recent funding cycle because no new mandarin varieties were introduced into the program (see above).

Screen and conduct molecular analyses of transformed citrus plants expressing the introduced transgenes: As stated above, this was the primary emphasis during the latter half of the current funding cycle. The work was initiated in an effort to more completely assemble an accurate assessment of the transformation methods currently in place. During that time, leaf samples were harvested from approximately 110 trees to prepare genomic DNA samples, with many more extractions completed the remainder of FY '07-'08. PCR analyses of these samples were in progress. Preliminary results suggested that indeed the numbers shown in Tables 1 and 2 would need to be adjusted downwards as the sample size increases. However, it must be emphasized that this work was at an early stage.

Begin investigating alternatives to commercially protected or publicly sensitive features of the transformation method in a citrus-specific context: The first two proposed areas of focus were the testing of recently identified bacteria capable of moving genetic sequences into plant cells and the use of maker genes that do not encode bacterial-derived antibiotic resistance. Investigation into these areas was planned for possible future funding cycles.

As resources and time allow, continue transforming citrus varieties with genetic sequences encoding potentially useful or experimentally relevant traits developed by others and us within the citrus improvement community: Several transformation experiments with genetic sequences that may be important for seedlessness were initiated and with plants in the pipeline currently in progress. Work in this area was more limited for two reasons-one was the termination of the seedlessness project in recent years, and the second was due to poor seed germination.

Table 2 summarizes the overall improvement trend in transformation and regeneration efficiencies for all of the varieties under investigation over the past several funding cycles. Of particular note is the rather dramatic increase in positive transgenic regeneration (compare GUS and PCR analyses results between funding cycles) that were a direct result of the enhanced selection systems in combination with improvements to the other variables described above.