

Production of a New Generation of Avocado Rootstocks by Somatic Hybridization and Genetic Engineering

Continuing Project: Year 6 of 6

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- **Cryopreservation**

Cryopreservation of embryogenic avocado cultures is now routine. Two procedures have been developed: rapid cooling to -196°C aka “vitrification” and slow or step wise cooling to -196°C . The latter procedure involves cooling from room temperature to -80° at $-1^{\circ}\text{ min}^{-1}$, followed by rapid cooling to -196°C . These two procedures are described in the accompanying manuscripts (Efendi and Litz, 2003a,b). All of the avocado genotypes that are currently being utilized and any genotypes that have potential for being utilized are currently in storage in liquid nitrogen. We have attempted to introduce as many of the genetically modified lines into cryostorage as possible; however, there are still remain several that are unprotected. Darda Efendi, whose doctoral dissertation research described the development of cryogenic storage of avocado, has been awarded the Ph.D. for this work.

These protocols are not only essential tools for storage of experimental materials, but are also critical as back up repositories for the long term maintenance of avocado genetic resources. **We have entered into discussions with the curator and research leader of the USDA National Germplasm Repository about introducing the national avocado germplasm collection into cryostorage** under the terms of a USDA Plant Germplasm Evaluation Cooperative Agreement. This will follow the precedent of mango that we are establishing.

- **Genetic manipulation**

Earlier in this research study, protocols for genetic transformation and somatic hybridization of avocado were described; however, problems associated with the recovery of large numbers of plants from independently manipulated cell lines affected the full exploitation of the procedures. The most important impediments have been 1) failure of an apical meristem to develop in most of the somatic embryos; and 2) apical necrosis which affects many of the somatic embryos that develop otherwise normally. We have been able to overcome these limitations of the procedure by developing a micrografting protocol. Accordingly, as shoots emerge from the somatic embryos, they are excised and grafted onto in vitro-grown seedling rootstocks. Using this procedure, we have been able to rescue between 70 and 100% of the regenerants. **Micrografting represents a very significant advance in our ability to handle these materials.**

Genetic transformation (This subject and the related Somatic Hybridization work will be presented as an invited talk at the World Avocado Congress.)

Embryogenic cultures have been transformed with several gene constructs using *Agrobacterium tumefaciens* strains EHA101 and hypervirulent EHA105.

Genes implicated in host defense response

1. Antifungal protein gene

Although the first transgenic somatic embryos began to germinate in late May, 2001, more synchronous germination began to occur in December-January (2001 and 2002). In order to rescue these regenerants, we utilized the micrografting protocol outlines above.

As indicated in the report of April, 2003 the first shipment of transgenic avocado plants to Dr. John Menge occurred on February 21, and consisted of 38 plants of five separately transformed lines. This was done under the aegis of 1) a Material Transfer Agreement between the University of Florida and the University of California-Riverside and 2) authorization of the USDA APHIS for interstate movement of transgenic material. We are prepared for another shipment of plants.

We have characterized three of these lines using molecular tools, i.e., Southern hybridization and PCR analysis. The first publication describing the production and recovery of transgenic avocado plants will be submitted for journal publication during this year.

2. Chitinase and Glucanase genes

We have successfully transformed embryogenic avocado cultures with a construct containing chitinase and glucanase genes on several occasions during the past two years; however, the recovery of somatic embryos from these cultures is very low. Consequently, we have utilized another approach to recover regenerants (see Somatic hybridization section below).

3. Chalcone synthase gene

Embryogenic cultures that have been transformed with the chalcone synthase gene (isolated from avocado fruit with anthracnose symptoms) have been recovered at low frequency; somatic embryos and plants have recovered at low frequency, and shoots are being micrografting onto *in vitro*-grown seedlings; however, grafting efficiency is poor.

4. Magainin

Embryogenic avocado cultures have been genetically transformed with a construct containing the magainin gene, which has been demonstrated to have strong antimicrobial properties. There is no regeneration yet.

5. *pac1* ribonuclease gene

This study has been undertaken in order to test the validity of transformation with the yeast *pac1* ribonuclease gene for controlling the avocado sunblotch viroid. This is not part of the CAC-supported project; however, the embryogenic lines were generated as part of the program. Embryogenic avocado cultures have been transformed with the *pac1* gene,

although somatic embryos have not been recovered. To date, movement of the *pac1* ribonuclease into the chloroplasts with the agency of the transit peptide has not been observed.

Genes implicated in control of fruit ripening

This study has been completed. Although it is not a part of the CAC-supported research project, the embryogenic lines that were utilized were generated as part of this program. Embryogenic avocado cultures were genetically transformed with the bacterial gene SAMase (S adenosylmethionine hydrolase), which degrades a precursor of ethylene. Transformed somatic embryos have been recovered and shoots have been micrografted onto in vitro-grown seedling rootstocks. Transformation has been confirmed by PCR analysis and Southern hybridization.

Somatic hybridization

1) The transformed embryogenic cultures containing chitinase, glucanase and chalcone synthase genes have shown poor regeneration potential. In order to exploit the antimicrobial properties of these genes, we have combined these transformed lines with the highly embryogenic transformed cultures containing the antifungal protein gene by means of somatic hybridization. **We have fused ‘Hass’ protoplasts that contain the antifungal protein gene, the *bar* gene (Basta resistance) and the *gus* gene with ‘Gwen’ protoplasts that contain the chitinase and glucanase genes and the *nptII* gene (kanamycin resistance).** Somatic hybrids have been selected on medium containing Basta and kanamycin. Somatic embryos were recovered, and shoots from these somatic embryos have been micrografted onto in vitro-grown avocado seedlings. These regenerants harbor three antimicrobial genes and are tetraploid. **These regenerants are slow growing but should be ready for transfer to John Menge in a few months.**

2) **Somatic hybridization between avocado and distantly related *Persea* spp.** was shown to be feasible earlier; however, the procedure was very tedious and expensive in terms of materials and manpower. Only a single regenerant was recovered that could be verified as a somatic hybrid and it was unstable over time. Using a strategy similar to that described above, we have utilized genetically transformed embryogenic avocado cultures and non morphogenic *Persea* sp. callus. The conditions for somatic hybridization are thus: ‘Hass’ protoplasts that contain the antifungal protein gene, the *bar* gene (Basta resistance) and the *gus* gene have been hybridized with *Persea* sp. protoplasts containing the chalcone synthase gene and the *nptII* gene (kanamycin resistance). We have selected for somatic hybrids in medium containing kanamycin and Basta, and have recovered somatic embryos. We have been able to regenerate plants by micrografting the transgenic somatic hybrid shoots. The somatic hybrids are tetraploid and contain the complete genomes of their donor parents together with the transgenes of interest. **These plants are ready for transfer to John Menge. This can be done under the aegis of the same Material Transfer Agreement between the University of Florida and the University of California-Riverside issued earlier. Authorization has been requested from USDA APHIS for interstate movement of this transgenic material.**

Other

Isidro Suarez, a Fulbright scholar and candidate for the Ph.D. degree (December, 2003) has been examining the interaction of avocado cell culture and ASBVd. **Although it is not a part of the CAC-supported research project, the embryogenic lines that were utilized were generated as part of this program.** This study has found that standard methods for eliminating viroids from infected plants (micrografting and nucellar culture) are ineffective for ASBVd in avocado. Mr. Suarez has demonstrated, however, that ASBVd mutates very rapidly in embryogenic cultures, and has developed a very interesting system for studying the host: viroid interaction.