Genetic Transformation of Avocado (*Persea americana* Mill.): A Coordinated Strategy for Crop Improvement

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**Abstract**

The primary breeding objectives for avocado include production problems associated with clonal rootstocks and with scion varieties. As part of an integrated approach for improving avocado with biotechnology tools, rootstock and scion selections are being genetically transformed in order to address some breeding objectives. The objectives include: 1) control of Phytophthora root rot disease of avocado rootstocks; 2) suppression of ethylene biosynthesis to control fruit ripening and to stimulate on-the-tree fruit storage of West Indian avocados; 3) control of fruit and foliage fungal disease, e.g., anthracnose; 4) control of avocado sunblotch disease. These strategies complement somatic hybridization, in vitro mutation induction and conventional breeding studies for avocado cultivar improvement.

**INTRODUCTION**

The avocado is one of the major fruit crops of tropical and subtropical regions of the world. The major producing countries include Mexico (1,040,390 Mt), Indonesia (270,000 Mt), USA (200,000 Mt), Brazil (173,000 Mt), Colombia (158,000 Mt), Dominican Republic (140,000 Mt), Chile (135,000 Mt), Peru (95,000 MT), China (85,000 Mt) and South Africa (66,500 Mt) (FAO, 2005). Chile and Mexico are the largest exporters of fruit. There are three geographic races of avocado according to Scora and Bergh (1990) who based their classification on isozymes, leaf terpenes, morphology, physiology, field observations and molecular markers: 1) *var. americana* Mill. (Mexican); 2) *var. guatemalensis* Williams (Guatemalan); 3) *var. drymifolia* (Select. and Cham.) Blake (West Indian).

The avocado tree is very heterogeneous with complex flowering that assures outcrossing. The juvenile period is relatively long, and has been a deterrent to establishment of breeding programs. The evaluation of progeny from either controlled or open pollinations requires approx. 7 years to flowering and then several more years in order to properly evaluate the seedling trees. Marker-assisted selection could allow seedlings to be screened at an early stage of development, but only fruit color (Mhameed et al., 1995) and flesh fiber (Sharon et al., 1998) have been linked with molecular markers. Lavi et al. (1993a, b) suggested that horticultural interesting traits in avocado are probably coded by several loci, each having several alleles in each locus. Using the tools of biotechnology, which include somatic embryogenesis, protoplast culture, somatic hybridization, in vitro mutagenesis followed by selection and genetic transformation, we are attempting to address specific breeding objectives of this crop.

**Fruit Ripening**

Production of avocados in subtropical regions is restricted to relatively few cultivars, including 'Hass' and 'Fuerte' among others. According to Whiley (1992), mature fruit of Mexican and Mexican ×Guatemalan selections, can be stored on-the-tree for up to three months. Ripening is triggered when mature fruit are picked. This has facilitated avocado production in subtropical regions because: 1) production can be focused on one or a few outstanding cultivar(s); 2) nearly year-round production is possible; 3) post harvest storage costs are reduced if fruit are stored on-the-tree; and 4) marketing of a standard product is possible. In the humid lowland tropics, year-round avocado
production is based on West Indian or West Indian × Guatemalan cultivars, which characteristically develop to maturity and ripen on the tree. According to Crane et al. (1992), >20 avocado cultivars are grown in south Florida in order to provide fruit year-round. This results in significantly greater problems with respect to: 1) post harvest storage; 2) lack of uniformity among cultivars; and 3) marketing of a uniform product.

**Phytophthora Root Rot**

The most significant production problem of avocados is a root disease, Phytophthora root rot (PRR), caused by the soil-borne pathogen *Phytophthora cinnamomi* Rands (Zentmyer, 1980). Resistance to PRR is unknown within *Persea americana* Mill., although levels of tolerance have been identified in some avocado seedlings, i.e., 'Duke 7', 'Toro Canyon', etc. High levels of resistance to PRR occur within closely related species within *Persea* subgenus *Eridaphne*; however, these species, including *P. borbonia*, *P. pachypoda*, etc., are sexually and graft-incompatible with avocado (Bergh and Elstrand, 1986). Pliego-Alfaro and Bergh (1992) suggested that forced hybridization of avocado with PRR-resistant species might be feasible by means of somatic hybridization. Although Witjaksono et al. (1998; 1999) have described procedures for regeneration from avocado protoplasts, somatic hybrids have been unstable in vitro (Witjaksono, pers. commun.).

**Avocado Sunblotch Disease**

Avocado sunblotch disease is caused by the avocado sunblotch viroid (ASBvd), which is seed-borne, transmitted by pollen and natural root grafting (Horne et al., 1941; Whitsell, 1952) and by vegetative propagation. Eradication of plants with symptoms and use of non-infected plants provide the only strategies for controlling the spread of this disease. Molecular indexing techniques for detecting infected plants have been developed, but their practical application is limited due to their expense (Dale, 1985; Bonfiglioli et al., 1994; Schnell et al., 2001).

**Anthracnose**

Avocado fruit, flowers and foliage are susceptible to infection by *Colletotrichum gloeosporioides* Penz., the cause of anthracnose. This disease is a limiting production factor in the lowland, humid tropics. Resistant selections have not been identified, and control is currently effected by the regular and periodic application of fungicides.

**MATERIALS AND METHODS**

**Embryogenic Cultures**

Embryogenic avocado cultures for genetic transformation studies are established according to previously published protocols (Witjaksono and Litz, 1999a). Cultures are induced from explanted immature zygotic embryos on semisolid medium containing B5 major salts (Gamborg et al., 1968), MS (Murashige and Skoog, 1962) minor salts, 0.4 g liter\(^{-1}\) thiamine HCl, 100 mg liter\(^{-1}\) myo-inositol, 30 g liter\(^{-1}\) sucrose, and 0.1 mg liter\(^{-1}\) picloram (MSP).

Embryogenic suspension cultures are established by subculture of 0.5 or 1 g proembryonic masses (PEMs) into 40 ml or 80 ml medium in 125 ml or 250 ml flasks, respectively. Newly initiated SE-type suspension cultures (Witjaksono and Litz, 1999a, b), which is characteristic of most avocado genotypes, develop cotyledonomary somatic embryos under induction conditions, but proliferate by formation of secondary embryos; this type of culture requires regular sieving in order to recover the smallest fraction. PEM-type cultures consist of proembryonic masses (PEMs) of various sizes, are easier to maintain and can be genetically transformed with greater ease. PEMs <0.8 mm are used as inocula. Maintenance medium consists of MSP with 2 mg liter\(^{-1}\) NH\(_4\)NO\(_3\) and 30.3 mg liter\(^{-1}\) KNO\(_3\) (MS3:1P medium).
Genetic Transformation

Embryogenic suspension cultures are genetically transformed using disarmed strains of *Agrobacterium tumefaciens* (EHA 101 and EHA 105) following a slight modification of the two-step procedure of Cruz Hernandez et al. (1998). The genetically engineered constructs that have been utilized are indicated in Table 1.

Avocado PEMs are being transformed using the two-step procedure described by Cruz-Hernandez et al. (1998). Embryogenic cultures are grown on semi solid maintenance MS3:1P medium and then abraded with a soft camel hair brush. The abraded cultures are incubated with acetosyringone-activated *Agrobacterium tumefaciens* containing a genetically engineered plasmid with the gene(s) of interest, a selectable marker (*nptII* or *bar*) and either with or without *gus* (β-glucuronidase) in liquid maintenance medium, and co cultured at 100 rpm. *Agrobacterium tumefaciens* is eliminated after 3 days of co culture by incubating the cultures in maintenance medium supplemented with either kanamycin sulfate or hygromycin and 200 mg liter\(^{-1}\) cefotaxime. Initial selection for resistance to the selection agent(s) for 2-4 months occurred in liquid maintenance medium containing antibiotic, followed by more intensive selection for 2 months with double strength antibiotic. PEMs were cultured in double strength antibiotic in order to eliminate chimaeras followed by subculture onto maturation medium supplemented with selection agent.

Somatic embryo development is initiated according to the procedure of Witjaksono and Litz (1999b; 2002) by subculture of transformed PEMs onto semi solid maturation medium, consisting of MS basal medium, without selection agent. Normal somatic embryos are opaque and white (Witjaksono and Litz, 1999b), and various factors, including increased gellan gum and sucrose concentrations, can affect development of good quality somatic embryos. Recent studies have demonstrated that filter-sterilized coconut water (20% v/v) can improve the development of bipolarity and germination frequency of avocado somatic embryos (Witjaksono and Litz, 2002).

Avocado somatic embryo maturation resembles zygotic embryo development, and consists of globular and heart stages, without a distinguishable torpedo stage, and a cotyledonary stage (Witjaksono and Litz, 1999b). Transformed somatic embryos, and leaves and roots of plantlets derived from somatic embryos have stained positively for *gus* according to the XGLUC reaction (Jefferson, 1987).

RESULTS AND DISCUSSION

Avocado Diseases

Genetic transformation is an environmentally sustainable alternative way to produce improved avocado rootstocks and scions with resistance to pathogens, particularly *Phytophthora cinnamomi*, the cause of Phytophthora root rot and *Colletotrichum gloeosporioides*, the cause of anthracnose. Various antifungal proteins have been utilized to confer resistance against different plant pathogens (Lamb et al., 1992; Cornelissen and Melchers, 1993).

Transgenic plants that constitutively express different pathogenesis-related (PR) proteins, i.e., chitinase and β-1,3-glucanase, show resistance to fungal infection (Broglie et al., 1991); however, higher protection is achieved when more than one PR protein is employed (Zhu et al., 1994). The mycelial cell walls of the pathogen *Phytophthora cinnamomi* are composed primarily of glucan, and embryogenic cultures have been genetically transformed with a construct that contains the chitinase and glucanase genes. Transgenic somatic embryos have been recovered; however, germination has not occurred from these somatic embryos.

In order to address the problem of anthracnose of avocado, embryogenic cultures have been genetically transformed separately with the antifungal protein (AFP) and chalcone synthase genes and with the construct containing both chitinase and glucanase. Transgenic regenerants containing the AFP gene have been recovered. Transgenic somatic embryos containing the glucanase and chitinase genes together have been
recovered, although regeneration has not occurred. Embryogenic cultures that express the chalcone synthase gene have been recovered; however, somatic embryos have not developed from these cultures.

Although it has been shown that resistance of *Solanum tuberosum* to the potato spindle tuber viroid can be engineered by genetic transformation of the nuclear genome with the yeast ribonuclease pac1 gene (Sano et al., 1997), the avocado sunblotch viroid replicates in the chloroplasts. Consequently, we are attempting to transform the avocado chloroplasts with pac1 in order to block replication of ASBvd.

**Avocado Fruit Ripening**

Embryogenic cultures have been separately transformed with two gene constructs: ACC deaminase (Klee et al., 1991) and SAMase (Good et al., 1994). Somatic embryos have not been recovered from these cultures to date.

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**Literature Cited**


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Table 1. Genes that are being utilized for avocado transformation.

<table>
<thead>
<tr>
<th>Targeted character</th>
<th>Gene</th>
<th>Promoter</th>
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<tr>
<td>fruit ripening</td>
<td>SAM hydrolase</td>
<td>avocado fruit specific cellulose</td>
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<tr>
<td></td>
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