Avocado Rootstock Development by Somatic Hybridization and Genetic Engineering

Continuing Project; Year 4 of 5

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Benefits to the Industry

A new generation of avocado rootstocks with a high level of resistance to Phytophthora root rot (PRR) and with other good horticultural characteristics.

Objectives

This project has 3 major objectives:

- somatic hybridization of avocado with PRR-resistant *Persea* species and *Nectandria* sp. by means of interspecific protoplast fusion;
- genetic transformation of existing avocado rootstock and scion cultivars;
- developing an efficient in vitro protocol for propagating existing and newly developed rootstocks.

All of these objectives are dependent upon an efficient system for regenerating avocado from cell and tissue cultures, i.e., somatic embryogenesis, together with standard micropropagation.

Summary

Somatic Hybridization

Several years ago, high levels of resistance to PRR were identified in many small-seeded *Persea* spp. within the subgenus *Eriodaphne* (Bergh & Lahav, 1996). These *Persea* species, including *P. borbonia*, *P. cinerascens* and *P. pachypoda*, are sexually and graft-incompatible with avocado. An in vitro procedure, i.e., somatic hybridization, which involves the fusion of avocado protoplasts with protoplasts of PRR-resistant *Persea* spp. can be used to overcome the problem of sexual incompatibility. The protocol involves the controlled fusion of leaf protoplasts of PRR-resistant species with protoplasts derived from embryogenic cultures of avocado (Witjaksono *et al.*, 1998; 1999a). Although putative somatic hybrids have been recovered using this approach, the frequency of successful fusion events was very low, usually less than 0.001%. In order to increase genetic recombination between avocado and the PRR-resistant species, we developed a

modified procedure for somatic hybridization. This has involved the use of callus cultures that have been initiated from stem segments of *P. borbonia*, *P. cinerascens* and *P. pachypoda* micropropagated plantlets. Protoplasts can be isolated from these cells for fusion with embryogenic avocado protoplasts.

Nonmorphogenic callus is initiated from stem pieces of 3 PRR-resistant *Persea* species on semisolid Murashige & Skoog (1962) medium that has been modified to optimize growth. NH4NO3 and KNO3 concentrations and ratio (2:1) have been altered and the plant growth regulators, benzyladenine (BA) and naphthaleneacetic acid (NAA) are used at 5.0 and 0.5 mg liter-1 respectively. Nonmorphogenic calluses are routinely grown as suspension cultures in liquid initiation formulation. The growth rate of nonmorphogenic *P. pachypoda* callus in suspension exceeds that of *P. cinerascens*, whereas *P. borbonia* callus do not grow well under these conditions. These cell cultures are good sources of PRR-resistant protoplasts for fusion studies. Most importantly, they are unable to grow and divide in medium that supports growth of embryogenic avocado cultures.

Interspecific hybridizations between embryogenic avocado and *P. pachypoda* protoplasts and between embryogenic avocado and *P. cinerascens* protoplasts are being carried out. We believe that somatic embryos from the interspecific hybridizations involving avocado and *P. cinerascens* were first successfully recovered in late 1999. The identification of these somatic hybrids has been based upon morphological markers, and the embryos have characteristics that are typical of both parents. A few of these putative hybrids have begun to germinate in vitro. This strategy is resulting in recovery of putative somatic hybrids with much greater efficiency. A similar procedure has been followed with somatic hybridization between avocado and *P. pachypoda*, although somatic embryos have not been recovered at this time.

Genetic Transformation

Following infection of plant tissues by plant pathogens, certain proteins are activated in resistant host tissues that restrict the spread of the pathogen. Such proteins have been referred to as pathogenesis-related (PR) proteins. Many genes that code for PR proteins have been cloned, including chitinase, glucanase, defensin, the antifungal protein, etc. The mycelium walls of *Phytophthora* species consist of a polyglucan macromolecule, which is degraded by the enzyme glucanase. We have established a procedure for genetically transforming embryogenic avocado cultures (Cruz-Hernandez *et al.*, 1998), and recovery of transgenic avocado cultures expressing the PR gene, chitinase, under the control of the 35S constitutive promoter has been reported.

Embryogenic avocado cultures have been genetically transformed with constructs that contain a PR, i.e., chitinase, glucanase, and the antifungal protein (AFP), alone and together with one other gene. Constructs containing the following PR genes have been used: glucanase, glucanase + AFP, AFP and chitinase. The genes have been cloned in the plasmid pBI121, and have been transferred into avocado using the avirulent *Agrobacterium tumefaciens* strains LBA4404 and EHA101. Embryogenic 'Thomas', 'Hass' and 'Fuerte' are being transformed. In addition, avocado cultures have been genetically transformed with a PR gene, chalcone synthase, isolated from avocado fruit by Dr. D. Prusky (Volcani Research Center) and believe to confer resistance to avocado fruit diseases, e.g., anthracnose.

Since 1999, Dr. Miguel A. Gomez Lim of CINVESTAV, Irapuato, Mexico, has been working to identify and clone root-specific promoters from avocado that can be used instead of the 35S constitutive promoter. This will enable us to transform scion avocado selections with one or more PR gene(s) that would be expressed in the root system only.

Using the avocado fruit-specific cellulase promoter, we have made a construct using pPZP200 with the bacterial gene S-adenosylmethionine (SAMase). SAMase degrades a precursor of ethylene, and effectively blocks ethylene production. Another construct is based upon pMON10117, and includes the bacterial gene ACC deaminase driven by the CaMV 35S promoter. ACC deaminase also degrades a precursor of ethylene. By transforming avocado with these genes it should be possible to block avocado fruit ripening.

Somatic Embryogenesis

The regeneration pathway that is fundamental to the success of the recovery of somatic hybrids and transgenic plants (see above) involves the *in vitro* induction of clonal avocado embryos. Conditions for optimizing this response have now been well characterized (Witjaksono & Litz, 1999a & b; Witjaksono *et al.*, 1999a). The recovery of plants from individual somatic embryos is generally low (<1.0%); however, it has been posible to increase plant production by a) micropropagating shoots emerging from germinating somatic embryos, and b) pulsing somatic embryos with 2p to 20% (v/v) coconut water during their later stages of maturation. Using the latter approach, it is possible to increase plant recovery from certain clonal lines to approximately 25%. Germinated somatic embryos and rooted plantlets derived from somatic embryos and growing on minimal plant growth medium are acclimated by exposure to an artificial atmosphere consisting of 20,000 ppm CO2 in a nitrogen gas carrier under 160-180 µmol m^{-2} s^{-1} illumination provided by cool white fluorescent bulbs. These conditions prior to transplantation into potting mixture increase the survival of avocado somatic embryo regenerants (Witjaksono *et al.*, 1999b); however, the rate of survival is still too low for this strategy to be utilized on a commercial scale. Improving the rate of recovery of plants in the greenhouse is a high priority.

Relevant Publications

1997
Witjaksono & R.E. Litz. 1997. Somatic embryogenesis from avocado (*Persea americana* Mill.) protoplasts. In Vitro Cell. Dev. Biol. 33: 48A (abstract).

1998
Cruz-Hernandez, A., Witjaksono, R. E. Litz & M. A. Gomez-Lim. 1998. *Agrobacterium tumefaciens* mediated transformation of embryogenic avocado cultures and regeneration of somatic embryos. Plant Cell Rep. 17: 493-503

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1999

Witjaksono & R. E. Litz. 1999a. Induction and growth characteristics of embryogenic avocado (*Persea americana* Mill.) cultures. Plant Cell Tiss. Org. Cult. 58: 19-29.

Witjaksono & R. E. Litz. 1999b. Maturation and germination of avocado (*Persea americana* Mill.) somatic embryos. Plant Cell Tiss. Org. Cult. 58: 141-148.

Witjaksono, R. E. Litz & F. Pliego-Alfaro. 1999a. Somatic embryogenesis in avocado (*Persea americana* Mill.) In Somatic Embryogenesis in Woody Plants, Vol. 5, ed. by S. M. Jain, P. K. Gupta & R. J. Newton. Kluwer Academic Publishers, Dordrecht, pp. 197-214.

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2001

Witjaksono & R. E. Litz. 2001a. Somatic embryogenesis of avocado (*Persea americana* Mill.) and its application for plant improvement. Acta Hortic. (in press)

Witjaksono & R. E. Litz. 2001b. Genetic transformation and regeneration of avocado. In: Plant Genetic Engineering Vol 5. Improvement of Major Vegetables and Fruit Crops, ed. by P. K. Jaiwal & R. P. Singh (in press)