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Avocado Rootstock Development by Somatic Hybridization and Genetic Engineering

Continuing Project; Year 4 of 5

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Benefit 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:

1. somatic hybridization of avocado with PRR-resistant *Persea* species and *Nectandria* sp. by means of interspecific protoplast fusion;
2. genetic transformation of existing avocado rootstock and scion cultivars;
3. 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

1) Somatic Hybridization

High levels of resistance to PRR have been identified in many *Persea* spp. within the subgenus *Eriodaphne* (Bergh & Lahav, 1996). Unfortunately, these species, which include *P. borbonia*, *P. cinerascens* and *P. pachypoda*, are sexually and graft-incompatible with avocado. Somatic hybridization, involving the fusion of avocado protoplasts with protoplasts of PRR-resistant *Persea* spp. is one strategy that can be used to overcome the problem of incompatibility. The protocol that has been developed has involved the controlled fusion of protoplasts from leaves of PRR-resistant species with protoplasts derived from embryogenic cultures of avocado (Witjaksono *et al.*, 1998; 1999a). Although putative somatic hybrids can be recovered using this approach, the frequency of successful fusion events has been very low, usually less than 0.001%. In order to increase genetic recombination between avocado and the PRR-resistant species, we have been developing a modified somatic hybridization procedure. This has involved the use of callus cultures that have been initiated from stem segments of *P. borbonia*, *P. cinerascens* and *P. pachypoda* *in vitro* micropropagated plantlets. Protoplasts that have been isolated from these cells are being used for fusion with embryogenic avocado protoplasts.

Nonmorphogenic callus is routinely initiated from stem pieces of 3 PRR-resistant *Persea* species on semisolid Murashige & Skoog (1962) medium that has been modified to optimize their growth. NH_4NO_3 and KNO_3 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. After their establishment on semisolid plant growth medium, nonmorphogenic calluses are inoculated into liquid medium of the same formulation, and are grown as suspension cultures. 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 the plant growth medium (containing picloram) that supports growth of embryogenic avocado cultures.

Interspecific hybridizations between embryogenic avocado and nonmorphogenic *P. pachypoda* protoplasts and between embryogenic avocado and nonmorphogenic *P. cinerascens* protoplasts are being carried out. In a parallel set of experiments, we are genetically transforming cell suspensions of the PRR-resistant *Persea* spp. with the gene conferring resistance to kanamycin. Our strategy in the coming months will be to stimulate fusions between embryogenic avocado protoplasts and the kanamycin-resistant nonmorphogenic *Persea* spp. protoplasts. It will then be possible to directly select for somatic hybrid cells in embryogenic avocado medium (containing picloram) that has been supplemented with kanamycin, because cells without kanamycin resistance and the ability to divide in the presence of picloram cannot survive in the selection medium. In this way, the number of somatic hybrids resulting from protoplast fusions will be increased. The efficiency of recovery of interspecific somatic hybrids that have resulted from these strategies will be compared with our traditional approach.

2) Genetic Transformation

Following invasion of plant tissues by pathogens, certain proteins are activated in

resistant host tissues that effectively restrict the spread of the pathogen. Such proteins have been referred to as pathogenesis-related (PR) proteins. Many of the genes that code for PR proteins have been cloned. These include chitinase, glucanase, defensin, the antifungal protein, etc. The cell walls of *Phytophthora* spp. 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 last year reported preliminary recovery of transgenic avocado expressing the PR gene, chitinase, under the control of the 35S constitutive promoter.

During the past year, we have altered our protocol for genetic transformation of avocado. Instead of utilizing constructs that contain a gene for a single PR protein, we have utilized gene constructs that contain a PR, i.e., chitinase, glucanase, and the antifungal protein (AFP), alone and together with one other PR 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* strain LBA4404. Embryogenic 'Thomas', 'Hass' and 'Fuerte' are being transformed. (It has not been possible to obtain 'Duke 7' material for initiating embryogenic cultures.)

In late 1998, a subcontract was put into place with Dr. Miguel A. Gomez Lim of CINVESTAV, Irapuato, Mexico, who will identify and clone a root-specific promoter from avocado that can be used instead of the 35S constitutive promoter. This will enable us to transform embryogenic cultures of scion avocado selections with one or more PR gene(s) that would be expressed in the root system only. This should enable the growth of 'Hass', for example, on its own roots in PRR-affected areas.

3) 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). According to our earlier report, the recovery of plants from individual somatic embryos is low (<1.0%). Therefore, in order to increase the number of plants that can be regenerated, it has been essential to micropropagate the shoots emerging from germinating somatic embryos using a standard shoot culture strategy. In this manner, a large number of shoots can be regenerated from a single somatic embryo; rooting of individual shoots *in vitro* has been demonstrated with a high level of efficiency. The acclimatization of *in vitro* rooted plants is critical for survival of *in vitro*-derived plantlets. To date, we have addressed this problem empirically. Germinated somatic embryos and rooted plantlets derived from somatic embryos and growing on minimal plant growth medium are exposed to an artificial atmosphere consisting of 20,000 ppm CO₂ in a nitrogen gas carrier under 160-180 (mol 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 germination frequency of somatic embryos and improving the rate of recovery of

plants in the greenhouse remain a high research priority, and will continue to be addressed as part of this study.

Relevant Publications

- 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
- Witjaksono, R. E. Litz & J. W. Grosser. 1998. Isolation, culture and regeneration of avocado (*Persea americana* Mill.) protoplasts. Plant Cell Rep. 18: 235-242.
- Witjaksono & R. E. Litz. 1999a. Induction and growth characteristics of embryogenic avocado (*Persea americana* Mill.) cultures. Plant Cell Tiss. Org. Cult, (in press)
- Witjaksono & R. E. Litz. 1999b. Maturation and germination of avocado (*Persea americana* Mill.) somatic embryos. Plant Cell Tiss. Org. Cult, (in press).
- 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.
- Witjaksono, B. Schaffer, A. Colls, R. E. Litz, & P. A. Moon. 1999b. Avocado shoot culture, plantlet development and net CO₂ assimilation in an ambient and enhanced CO₂ environment. In Vitro Cell. Dev. Biol. (in press).