Avocado Rootstock Development by Somatic Hybridization and Genetic Engineering

Continuing Project; Year 5 of 6

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

Summary

1. *In Vitro* Regeneration

improved plant recovery. All of the objectives of this project are dependent upon efficient regeneration of avocado from cell and tissue cultures, i.e., somatic embryogenesis, together with standard micropropagation. During the course of this project, we have optimized these procedures so that they are reliable and adaptable to different genetic engineering approaches (Witjaksono & Litz, 1999a & b; Witjaksono *et al*., 1999a; Witjaksono & Litz, 2001a, c). A persistent development problem, however, has been the abnormal development of most somatic embryos - failure of the shoot meristem to develop. Consequently, only 0.001 - 1% of avocado somatic embryos can form plants. A major accomplishment during the past year has been to increase very significantly the frequency of shoot meristem organization. This has been achieved by pulsing developing somatic embryos with coconut water, and has resulted in plant recovery rates of 10-25%, depending on the cultivar (Witjaksono & Litz, 2001c).

Impact: This has had a major impact on genetic transformation and somatic hybridization experiments, and it is now possible to rescue a large proportion of the genetic diversity that is produced.

Storage of embryogenic avocado cultures. Annually, during the course of this project, it has been necessary to re-establish cell and tissue cultures from avocado trees. We have estimated that the time required for this procedure each year is at least one month. During the past year, we have developed a procedure for the long term storage of embryogenic avocado cultures in liquid nitrogen (Efendi *et al*., 2001). Embryogenic cultures are immersed in a cryoprotectant solution of DMSO and glycerol for 15 min, cooled to -80^{o}C at a rate of 1^{o}C per min, and plunged into liquid nitrogen (-196^{o}C). In order to restore growth to cryopreserved cultures, they are warmed to room temperature, washed thoroughly with avocado plant growth medium and re-plated on plant growth medium (Figs. 1 & 2). The following cultivars have been successfully cryopreserved 'Booth 7', 'Fuerte', 'Gwen', 'Hass', 'Lula', 'Reed' and 'T362'.

Impact. Cryopreservation of avocado cultures will improve the efficiency of our program, ensure continuity of germplasm and ultimately can be used to back up field plantings of avocado genetic resources.

2. Genetic Transformation

When plant tissues are infected 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. The procedure for genetically transforming embryogenic avocado cultures was first defined in our group by Cruz-Hernandez *et al*. (1998).

During the past two years, embryogenic avocado cultures were genetically transformed with constructs that contain a PR, i.e., chitinase, glucanase, chalcone synthase and the antifungal protein (AFP), alone and together with one other PR gene. Because of the limitation imposed by poor somatic embryo development and germination, it has not been possible until the past year to recover large numbers of transgenic plants. Constructs containing the following PR gene(s) have been used: glucanase, glucanase + AFP, AFP, glucanase + chitinase, chalcone synthase and chitinase. The genes have been cloned in the plasmid pBI121, and have been transferred into avocado using avirulent *Agrobacterium tumefaciens* strains EHA101 and EHA105. Embryogenic 'Hass' and 'Fuerte' have have been transformed with several of the constructs, and transgenic somatic embryos are germinating (Figs. 3-7). We anticipate that transgenic avocado plants containing chitinase, AFP and chitinase + glucanase genes will be available for the UC Riverside PRR rootstock evaluation in the next few months.

Impact. We anticipate that selections made among the transgenic regenerants will have resistance to PRR.

3. Somatic Hybridization

High levels of resistance to PRR have been identified in many small-seeded *Persea* spp. within the subgenus *Eriodaphne* (Bergh & Lahav, 1996). The PRR resistant species, including *P. borbonia*, *P. cinerascens* and *P. pachypoda*, are sexually and graft-incompatible with avocado. Somatic hybridization, which involves the fusion of avocado protoplasts with protoplasts of PRR-resistant *Persea* spp. , is one approach for overcoming sexual incompatibility. Although putative somatic hybrids have been recovered, the frequency of successful fusion events is very low, usually less than 0.001%. This, together with the low frequency of normal somatic embryo development noted above, has limited the application of this procedure. In the previous year, a procedure, involving fusion of callus derived protoplasts of *P. borbonia*, *P. cinerascens* and *P. pachypoda* with embryogenic avocado protoplasts was adopted. This procedure, used in conjunction with the more efficient method for recovery of normal somatic embryos is now being used on a large scale.

Impact. We anticipate that selections made among the somatic hybrids will have resistance to PRR.

4. Other studies

Because it is now possible to regenerate relatively large numbers of plants from somatic embryos, embryogenic avocado cultures have been irradiated with 25Gy (LD_{50}) provided by a ^{60}Co irradiation source. Somatic embryos have been recovered from irradiated cultures, and are germinating (Witjaksono & Litz, 2001d). We anticipate that plants from irradiated avocado cultures will be available for the UC Riverside PRR rootstock evaluation in the next few months.

Impact. We anticipate that randomly induced mutations in avocado may result in enhanced resistance to PRR.

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

Litz, R. E. 1998. *In vitro* conservation of *Persea*. In Avocado Genetic Resoures: Global Status of Conservation Programs. ed. by M. L. Arpaia & C. A. Qualset. University of California Press (in press)

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. 1998. Biotechnology strategies for improving avocado. Cal. Avo. Soc. Yrb. 82: 101-118.

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.

Witjaksono, B. Schaffer, A. Colls, R. E. Litz, & P. A. Moon. 1999b. Avocado shoot culture, plantlet development and net CO_2 assimilation in an ambient and enhanced CO_2 environment. In Vitro Cell. Dev. Biol.35: 238-244.

2000

Witjaksono & R. E. Litz. 2000.Cell suspension culture of *Persea pachypoda* and *P. cinerascens*. In Vitro Cell. Dev. Biol. 36: 69A (Abstract).

Litz, R. E. & Witjaksono. 2000. Avocado transformation. In: Handbook of Transgenic Food Plants, ed. by Y. H. Hui, G. Khachatourians, D. Lydiate, A. McHughern, W. K. Nip & R. Scorza. Marcel Dekker, New York (in press)

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)

Witjaksono & R. E. Litz. 2001c. Enhanced plant recovery from avocado somatic embryos. Plant Cell Tiss. Org. Cult. (in press)

Witjaksono & R. E. Litz. 2001d. Effect of ionizing irradiation on somatic embryogenesis of avocado. Plant Cell Rep. (in press)

Efendi, D., R. E. Litz & F. Al Oraini. 2001. Cryopreservation of embryogenic avocado (*Persea americana* Mill.) cultures. In Vitro Cell. Dev. Biol. 37, 39A (Abstract).

Witjaksono, D. Efendi & R. E. Litz. 2001. Liquid coconut endosperm improves somatic embryo recovery and conversion of avocado (*Persea americana* Mill.) Proc. Plant Growth Reg. Soc. (Abstract)

Figures

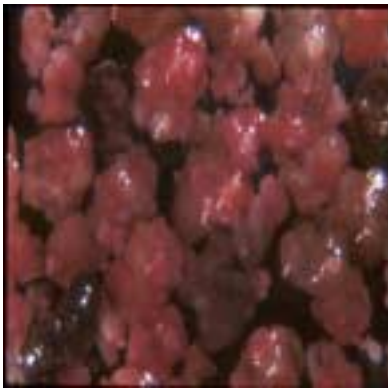

Figure 1. Embryogenic 'Hass' culture following cryopreservation at -196°C. Culture has been stained with TTC for viability.

Figure 2. Recovery of 'Hass' somatic embryos following cryopreservation.

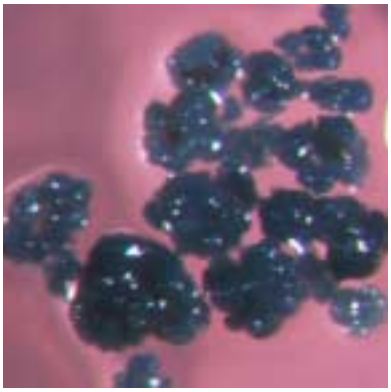

Figure 3. Embryogenic 'Fuerte' culture genetically transformed with chitinase + glucanase genes. Blue stain (GUS) is a marker for transformation.

Figure 4. 'Hass' somatic embryo genetically transformed with the antifungal protein (AFP) gene.

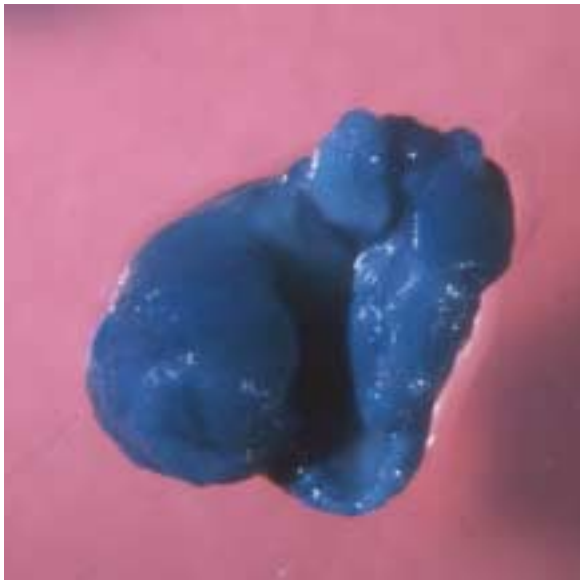

Figure 5. Genetically transformed 'Hass' somatic embryos.

Figure 6. Germination of genetically transformed 'Hass' somatic embryo.

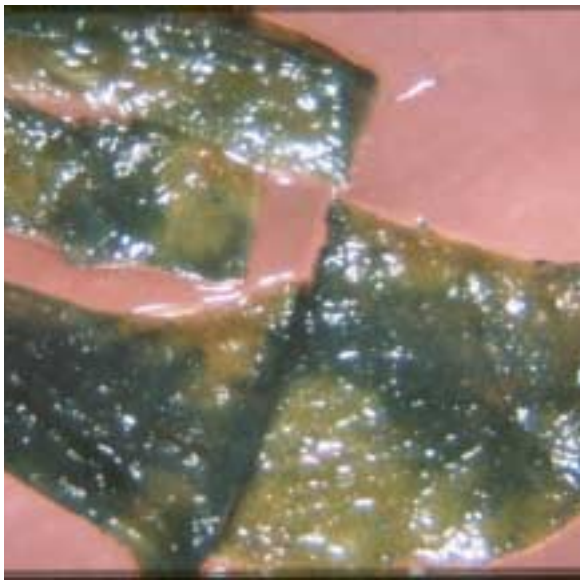

Figure 7. Leaf of genetically transformed (chitinase + glucanase) 'Hass' showing GUS reaction.