In Vitro Cell.Dev.Biol.-Plant (2008) 44:254–262
DOI 10.1007/s11627-008-9117-2

GENETIC TRANSFORMATION

Recovery of avocado (*Persea americana* Mill.) plants transformed with the antifungal plant defensin gene PDF1.2

Simon H. T. Raharjo · N. F. N. Witjaksono · Miguel A. Gomez-Lim · Guillermo Padilla · Richard E. Litz

Received: 20 March 2007 / Accepted: 10 March 2008 / Published online: 21 June 2008 / Editor: Jude W. Grosser

Abstract Embryogenic avocado cultures derived from 'Hass' protoplasts were genetically transformed with the plant defensin gene (*pdf1.2*) driven by the CaMV 35S promoter in pGPTV with *uidA* as a reporter gene and *bar*, the gene for resistance to phosphinothricin, the active ingredient of the herbicide Finale® (Basta) (Bayer Environmental Science, Research Triangle Park, Durham, NC). Transformation was mediated by *Agrobacterium tumefaciens* strain EHA105. Transformed cultures were selected in the presence of 3.0 mg l^{-1} phosphinothricin in liquid maintenance medium for 3–4 mo. Liquid maintenance medium consisted of modified MS medium containing (per liter) 12 mg NH_4NO_3 and 30.3 mg KNO_3 and supplemented with 0.1 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose, 3.0 mg l^{-1} phosphinothricin, and 0.41 μM picloram. Somatic embryo development from transformed cultures was initiated on MS medium supplemented with 45 g l^{-1} sucrose, 4 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 10% (*v*/*v*) filter-sterilized coconut water, 3.0 mg l^{-1} phosphinothricin, and 6.0 g l^{-1} gellan gum. Limited plant recovery occurred from somatic embryos on semi-solid MS medium supplemented with 3.0 mg l^{-1} phosphinothricin, 4.44 μM 6-benzylaminopurine (BA), and 2.89 μM GA_3; transformed shoots were micrografted on *in vitro*-grown seedling rootstocks. Approximately 1 yr after acclimatization in the greenhouse, transformed shoots were air-layered to recover transformed roots. Genetic transformation of embryogenic cultures, somatic embryos, and regenerated plants was confirmed by polymerase chain reaction (PCR), Southern blot hybridization, the XGLUC reaction for *uidA*, and application of the herbicide Finale® to regenerated plants.

Keywords Avocado · Genetic transformation · Somatic embryo · Phosphinothricin

S. H. T. Raharjo · N. F. N. Witjaksono · G. Padilla · R. E. Litz (✉)
Tropical Research & Education Center, University of Florida,
18905 SW 280 Street,
Homestead, FL 33031-3314, USA
e-mail: relitz@ufl.edu

S. H. T. Raharjo
Faculty of Agriculture, University of Pattimura,
Kampus Poka, Jl. Ir. Putuhena,
Poka Ambon 97233, Indonesia

N. F. N. Witjaksono
Bogor Botanical Garden, Plant Tissue Culture Laboratory,
Jl. Ir. H. Juand 13,
Bogor 16003, Indonesia

M. A. Gomez-Lim
Unidad Irapuato, CINVESTAV,
Irapuato, GTO, Mexico

Introduction

The avocado, *Persea americana* Mill., is one of the most important fruit crops of the world, with an annual production estimated to be 3,271,962 MT in 2005 (FAOSTAT 2006). There are three subspecies within *P. americana*, also referred to as the Mexican, Guatemalan, and Antillean races, of which the Mexican race is subtropical, the Guatemalan race is of the tropical highlands, and the Antillean race is of the tropical lowlands (Knight 2002). The greatest production occurs in subtropical regions, and the most important cultivar is 'Hass', a complex hybrid involving Mexican and Guatemalan ancestry. 'Hass' is also the major export avocado cultivar (Newett et al. 2002).

The avocado tree is heterogeneous with a complex pollination mechanism that favors outcrossing (Gazit and

Degani 2002). The juvenile period is lengthy, 6–8 yr, and conventional breeding is difficult and time-consuming. With the exception of a few recently released cultivars, such as 'Lamb Hass', the major avocado cultivars have not been derived from breeding programs (Lahav and Lavi 2002). Despite the importance of the avocado, little is known about the genetics of the species. Advances in avocado breeding have been impeded by its long juvenile period, heterogeneity, the time required for evaluation of the progeny of controlled pollinations, and the absence of molecular markers for important horticultural traits.

The major factors that limit avocado production include diseases such as Phytophthora root rot, caused by the oomycete *Phytophthora cinnamomi* Rands; anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz.; and avocado sunblotch disease caused by the avocado sunblotch viroid (ASBVd). The diseases affecting avocado fruit have become the greatest hindrances affecting international trade. There is little or no immunity to these and other diseases within the species (Litz et al. 2005). For example, immunity to phytophthora root rot occurs within *Persea* species in the subgenus *Eriodaphne*; however, *Eriodaphne* species are sexually and graft-incompatible with the avocado (subgenus *Persea*) (Litz et al. 2005). The rootstock cultivars 'Duke 7', 'Martin Grande', and 'Thomas' are moderately tolerant of phytophthora root rot.

Genetic transformation is considered to be an important option for protecting avocado from infection by ASBVd, *C. gloeosporioides*, and *P. cinnamomi*, and for addressing specific horticultural problems, control of fruit ripening in the Antillean cultivars (Litz et al. 2005). The embryogenic regeneration pathway of avocado has been described from elite (mature phase) nucellar explants (Witjaksono et al. 1999; Suarez et al. 2006) and from non-elite (zygotic embryo) explants (Mooney and Van Staden 1987; Pliego Alfaro and Murashige 1988; Raviv et al. 1998; Witjaksono and Litz 1999a, b). Although the production of somatic embryos occurs with high efficiency, the incidence of good quality, bipolar embryos is unacceptably low, and has been reported to be <2% or lower, depending on the genotype (Pliego Alfaro and Murashige 1988; Raharjo and Litz 2005). Nonetheless, Cruz Hernandez et al. (1998) demonstrated that embryogenic cultures could be transformed and somatic embryos could be recovered, although plant regeneration was not reported.

The objective of current research was to define the complete protocol for transformation of embryogenic cultures of avocado and recovery of transgenic plants with expression of the *pdf1.2* gene in leaves and roots. The *pdf1.2* gene codes for a defensin from *Arabidopsis thaliana*, which is induced by exposure to different phytopathogenic fungi, including *Alternaria raphans*, *A. brassicola*, *Fusarium oxysporum f.* sp. *matthiolae*, and *F. oxysporum f.* sp. *raphans* (Epple et al. 1997). This suggests a role for the gene in the plant defense response to fungal infection. The ultimate goal of this work is to engineer resistance in avocado against pathogenic fungi.

Materials and Methods

Induction of embryogenic avocado cultures. Embryogenic cultures consisting of proembryonic cells and masses were induced from zygotic embryos extracted from immature fruit (<1.0 cm long) of 'Hass' avocado obtained from the University of California germplasm repository in Riverside (Witjaksono and Litz 1999a). Immature fruits were surface-disinfested for 20 min in 2% (*w*/*v*) sodium hypochlorite with two to three drops of Tween-20® per liter, and sterilant was removed by rinsing two times with sterile deionized water. Immature embryos were removed from a sterile fruit under axenic conditions, and explanted on sterile induction medium. Induction medium consisted of semi-solid B5 (Gamborg et al. 1968) major salts, MS (Murashige and Skoog 1962) minor salts and supplemented with 0.1 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose, 0.41 μM picloram, and 8.0 g l^{-1} TC agar (Sigma-Aldrich, St. Louis, MO) (Witjaksono and Litz 1999a) in sterile plastic Petri dishes (100×20 mm) that were sealed with Parafilm®. Cultures were incubated in darkness at 27°C.

Maintenance of embryogenic cultures. Embryogenic cultures were transferred onto semi-solid maintenance medium, which consisted of MS basal medium, and supplemented with 0.1 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose, 0.41 μM picloram and 8 g l^{-1} TC agar. For growth of suspension cultures, the nitrogen composition of MS major salts composition of semi-solid maintenance was modified to include (per liter) 12 mg NH_4NO_3 and 30.3 mg KNO_3. The embryogenic line utilized in this study was derived from protoplasts that had been isolated from embryogenic cultures (Witjaksono et al. 1998), and was designated 'pHass'. Approximately 200 mg of 'pHass' proembryonic masses was inoculated into 40 ml of liquid maintenance medium in 125-ml Erlenmeyer flasks. The flasks were sealed with aluminum foil and Parafilm®, and maintained on a rotary shaker at 100 rpm and 27°C and under subdued light. Embryogenic 'pHass' cultures were transferred to fresh liquid maintenance medium at 2-wk intervals.

Genetic transformation. The antifungal defensin gene (*pdf1.2*) used in this study was driven by the CaMV 35S promoter and cloned in binary vector pGPTV, which also contained the *uidA* reporter gene (*gus*) and the *bar* gene for resistance to phosphinothricin, the active ingredient of the

herbicide Finale® (Fig. 1). The *pdf1.2* gene was isolated from *A. thaliana*, and is known to be induced by different phytopathogenic fungi (Epple et al. 1997). The vector was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation. *Agrobacterium* strain EHA105 containing pGPTV-BPDF1.2 was grown in 1.0 ml Luria-Bertani (LB) broth at 27°C for 48 h. A single colony was plated on fresh LB medium containing filter-sterilized 50 mg l^{-1} kanamycin sulfate and 20 mg l^{-1} rifampicin for 48 h at 27°C. Three colonies of EHA105/pGPTV-BPDF1.2 were inoculated into 5-ml LB medium and incubated at 125 rpm at 27°C for 16-h. The culture was diluted to 20 ml with modified LB medium containing filter-sterilized 50 mg l^{-1} kanamycin sulfate and 20 mg l^{-1} rifampicin to which 100 µl of 3 µM acetosyringone was added, and further incubated for 6 h at 125 rpm at 27°C.

Inhibition of PEM growth by phosphinothricin. The concentration of phosphinothricin that inhibited growth of embryogenic suspension cultures was determined. The inoculum consisted of embryogenic 'pHass' cultures from five flasks (80 ml liquid maintenance media in 250-ml Erlenmeyer flasks). Five concentrations of phosphinothricin were tested: 0 (control), 0.5, 1.0, 2.0, 3.0, and 4.0 mg l^{-1} with five replications for each treatment and the control. Filter-sterilized phosphinothricin was added to 40 ml liquid maintenance medium in 125-ml Erlenmeyer flasks, and 1.0 ml of settled cell volume (SCV) of proembryonic masses was inoculated into each flask. The flasks were capped with aluminum foil and sealed with Parafilm®. Cultures were maintained on a rotary shaker at 125 rpm at 27°C under diffuse light. The SCV of proembryonic masses was measured after 5 wk. Proembryonic masses were decanted into sterile graduated plastic centrifuge tubes and the SCV was measured after approximately 1 min. Data were analyzed by ANOVA at 1% confidence level (SAS Institute, Cary, NC).

Co-cultivation with Agrobacterium. Avocado 'pHass' proembryonic masses were transformed according to a modification of the protocol of Cruz Hernandez et al. (1998). Ten days after subculture, suspension cultures were sieved through sterile nylon filtration fabric (mesh size 1.8 mm), and approx. 3.0 g of the small fraction was plated on semi-solid maintenance medium in Petri dishes (100×20 mm), and gently abraded with a sterile camel hair brush. Approximately 400 mg of abraded proembryonic masses was inoculated into 80-ml liquid maintenance medium in 250-ml Erlenmeyer flasks, and a 1.0-ml aliquot of log phase acetosyringone-activated EHA105/pGPTV-BPDF1.2 was added to each flask.

Proembryonic masses and acetosyringone-activated EHA105/pGPTV-BPDF1.2 were co-cultivated at 27°C for 3 d at 100 rpm. The proembryonic masses were then transferred to liquid maintenance medium supplemented with filter-sterilized 200 mg l^{-1} cefotaxime and 500 mg l^{-1}carbenicillin to eliminate *A. tumefaciens*. After 2 wk, the proembryonic masses were transferred into fresh liquid maintenance medium supplemented with filter-sterilized 200 mg l^{-1} cefotaxime, 500 mg l^{-1}carbenicillin and 3.0 g l^{-1} phosphinothricin, and subcultured at 2-wk intervals for 3 mo.

Somatic embryo development. The putatively transformed avocado embryogenic cultures were subcultured onto somatic embryo development medium (Witjaksono and Litz 1999b, 2002), which consisted of MS medium supplemented with 4 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 45 g l^{-1} sucrose, filter-sterilized 3.0 mg l^{-1} phosphinothricin and 6.0 g l^{-1} gellan gum. Autoclaved medium was also supplemented with 0, 10, or 20% (*v*/*v*) filter-sterilized coconut water extracted from freshly harvested immature nuts. The medium was decanted into sterile plastic Petri dishes (100×20 mm). The inoculum size was approximately 10 mg proembryonic masses, and was spread evenly over the surface of the somatic embryo development medium. There were six to eight Petri dishes for each treatment. The Petri dishes were sealed with Parafilm®, and were maintained in darkness at 27°C. The total number of mature, white-opaque somatic embryos (>0.5 cm diameter) on each Petri dish was recorded. Data were analyzed by analysis of variance (ANOVA) at 1% confidence level (SAS Institute).

Recovery of transformed avocado plants. Mature, white-opaque somatic embryos (>0.5 cm diameter) were plated on semi-solid plant recovery medium, MS basal medium supple-

Figure 1. Map showing the Ti region of plasmid pGPTV-BPDF1.2 utilized in this study to generate transformed avocado plants. The antifungal defensin gene *pdf1.2* is flanked by the CaMV 35S promoter and terminator. Right and left borders are indicated as RB and LB, respectively. The reporter *uidA* intron and selective *bar* genes employed are also indicated. Further details on the vector are described in Becker et al. (1992).

mented with 4 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose, filter-sterilized 3.0 mg l^{-1} phosphinothricin, 4.44 μM BA, 2.89 μM GA_3, and 2.0 g l^{-1} gellan gum in Petri dishes (100×20 mm). There were nine somatic embryos in each Petri dish and Petri dishes were sealed with Parafilm and were incubated at 27°C under cool white fluorescent bulbs (40 μmol m^{-2} s^{-1}) with a 16-h photoperiod.

Plant recovery from avocado somatic embryos is very low, as most somatic embryos fail to develop either a shoot and/or a root meristem (Pliego Alfaro and Murashige 1988). As a result, 'pHass' shoots derived from transformed and control somatic embryos were excised and micrografted on *in vitro*-grown 'Peterson' avocado rootstocks, according to the procedure of Raharjo and Litz (2005). Following surface-sterilization of intact seeds from mature 'Peterson' fruit, the embryo axis from each seed, together with a cube of cotyledonary tissue (ca. 15×15×8 mm), was explanted onto semi-solid MS basal medium in Petri dishes (100×20 mm) and incubated at 27°C and a 16-h photoperiod (60–80 μmol s^{-1} m^{-2}). Germinating seedlings 7–12 d after shoot emergence, or approximately 3 wk after explanting, and which had at least one root, were used as rootstocks. The seedling shoots were decapitated and the somatic embryo shoots were grafted *in vitro* onto 'Peterson' rootstock by the top slit method. After the micrograft unions had formed, the rapidly growing transgenic and control 'pHass' shoots (scions) were removed and side-grafted onto 'Peterson' seedling rootstocks in the greenhouse (Raharjo and Litz 2005).

As transgenic 'pHass' regenerants were not on their own roots, the scions were air-layered after approximately 1 yr. A strip of bark was carefully removed from the scion above the graft union. The exposed area was covered with moist sphagnum, and sealed within polyethylene film. After adventitious roots had formed from the transgenic 'pHass' scions within the sphagnum ball, the air-layered scion was removed from the rootstock, and the rooted plant was potted in a peat–perlite mixture.

General in vitro conditions. The pH of semi-solid plant growth media was adjusted to 5.7 with either 0.1 N HCl or 0.1N KOH before autoclaving at 1.1 kg cm^{-2} for 20 min at 121°C.

Confirmation of transformation. Different developmental stages of putatively transformed 'pHass' tissue were assayed for *uidA*. Embryogenic cultures, somatic embryos, leaves, and roots were washed with sterile deionized water and treated with X-Gluc (5-bromo-4-chloro-3-indolyl B-D glucuronide) (Sigma-Aldrich) according to Jefferson (1987). The tissues were washed with methanol/acetone (3:1) and stored in 10% (*v*/*v*) glycerol.

To verify the presence of the *bar* gene in regenerated transgenic 'pHass' plants, the herbicide Finale® was applied as a spray to leaves of transformed and non-transformed avocado plants in the nursery at the rate of 31.2 ml Finale® l^{-1}. In addition, transformed and non-transformed plants were sprayed with the same volume of distilled water.

Total DNA was extracted from leaves of putatively transformed avocado plants (Doyle and Doyle 1990) to amplify a partial segment of the inserted *pdf1.2* and *uidA* genes in transformed plants. The polymerase chain reaction (PCR) Master Mix kit (Promega, Madison, WI) was used following the instructions from the manufacturer. Primers AFP-F003 (5′-GGCTAAGTCTGCTACCATCGTTAC-3′) and AFP-R351 (5′-AGCAACATAACATATCTGGTAC-3′) were used to produce a 349-bp amplification band for *pdf1.2*, and primers 5′-CAGCGAAGAGGCAGTCAACGGGGAA-3′ and 5′-CATTGTTTGCCTCCCTGCTGCGGTT-3′ were used to produce a 683-bp amplification band for *uidA* from leaf DNA of transformed plants. A PTC-100™ Programmable Thermal Controller (MJ Research, Waltham, MA) was used, and the temperature cycle program consisted of 3 min at 94°C (preheating), 35 cycles of 30 s at 94°C (denaturation), 30 s at 60°C (annealing), 60 s at 72°C (elongation), and 5 min at 72°C (final elongation). In addition to plant DNAs, pGPTV-BPDF1.2 DNA was included as a positive control. The PCR products were separated on a 1.0% agarose gel, stained with ethidium bromide using Tris, Phosphate, and ethylenediaminetetraacetic acid (EDTA) (TPE) as a running buffer (Sambrook and Russell 2001), and visualized using UV light.

Total DNA extracted from leaves from greenhouse-grown transformed plants was used for Southern analysis. Leaves of non-transformed plants were used as the control. Genomic DNA was isolated from 1 g of leaf tissue using

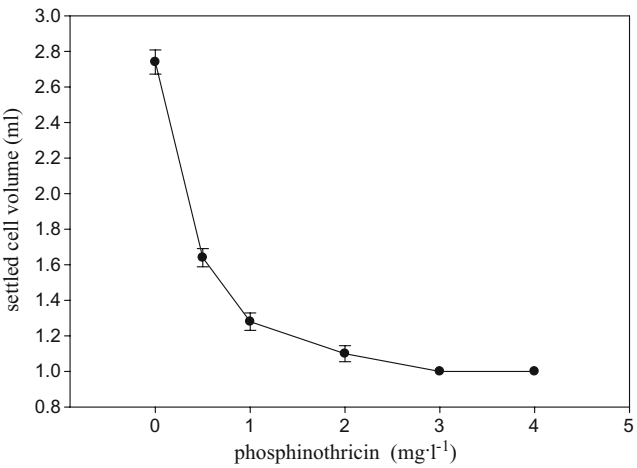

Figure 2. Growth of embryogenic avocado suspension cultures in response to different phosphinothricin concentrations. Data represent the means of five replications for each concentration ± standard error. PR>*F* 0.0001; r^2=0.978545; $y^{-1} = a + be^{-x}$.

Table 1. Efficiency of recovery of transformed somatic embryos (SE) from proembryonic masses (PEMs), transformed SE shoots, micrografts, *ex vitro* grafts, and air-layered shoots

No. of somatic embryos (SE) from 10 mg PEMs[y,z]						
0% coconut H_2O	10% (*v*/*v*) coconut H_2O	20% (*v*/*v*) coconut H_2O	% shoot recovery from SEs	% survival micrografts	% survival *ex vitro* grafts	% air layering success
3.8±0.36	9.2±0.8	8.2±1.06	0.0016	83.6	74.5	94.6

[y] PR > F 0.0001; r^2 =0.998777; $y = a + b/\ln x$.
[z] Data represent means ± SE

the Plant DNAzol® Reagent (Invitrogen) according to the manufacturer's instructions. Ten micrograms of DNA were digested with *Hin*dIII, resolved by agarose gel electrophoresis (1% gel) and blotted onto Hybond–N+ nylon membrane (Amersham Pharmacia Biotech) as described by Sambrook and Russell (2001). The membrane was hybridized to a radioactively labeled probe synthesized with the Rediprime II kit (Amersham Pharmacia Biotech) using as template the whole coding region of the *pdf1.2* gene. Hybridization was performed at 65°C in the Rapid-hyb buffer (Amersham Pharmacia Biotech) with washes at the same temperature and the membrane was exposed to Kodak Biomax Film at −70°C. The probe was labeled to a specific activity of approximately 110 cpm/mg DNA by random priming (Feinberg and Vogelstein 1983).

Results

Embryogenic 'pHass' avocado cultures were transformed with *uid*A, the *pdf1.2* defensin gene from *A. thaliana* and the *bar* gene, and transformation was confirmed in embryogenic cultures, somatic embryos, regenerated shoots, and adventitious roots of transgenic plants.

The growth of embryogenic 'pHass' avocado suspension cultures was completely suppressed by 3.0 and 4.0 mg l^{-1} phosphinothricin (Fig. 2), and the effect was highly significant ($p<0.01$; PR>F 0.0001). Therefore, 3.0 mg l^{-1} phosphinothricin was used to select for embryogenic cultures that had been genetically transformed and contained the bar gene.

Three months after co-culturing 'pHass' embryogenic cultures with genetically modified *A. tumefaciens* and with continuous selection in the presence of 3.0 mg l^{-1} phosphinothricin, the proembryonic masses were completely transformed on the basis of X-Gluc staining for *uidA* (Fig. 3*a*). The inclusion of filter-sterilized coconut water at either 10 or 20% in the maturation medium containing 3.0 mg l^{-1} phosphinothricin significantly improved the recovery of opaque-white somatic embryos (>0.5 cm diameter) from embryogenic cultures (Table 1) ($p<0.01$; PR>*F* 0.0001; r^2=0.998777; $y = a + b/\ln x$). In each Petri dish that had been inoculated with 10 mg of proembryonic masses, an average of 9.2±0.80 (10% coconut water) and 8.2±1.06 (20% coconut water) opaque mature transformed somatic embryos were recovered. In contrast, on development medium without coconut water, only 3.8±0.36 opaque mature transformed somatic embryos were recovered from 10 mg of proembryonic masses. Somatic embryos that developed from transformed embryogenic cultures were positive for the presence of *uidA* based on the XGLUC histochemical reaction (Fig. 3*b*).

The development of transformed somatic embryos that were either bipolar or that had a shoot apex was <0.001% (Table 1); however, shoots from transformed 'pHass' somatic embryos were successfully rescued by micrografting onto 'Peterson' rootstock *in vitro* (83.6% success). Shoots from micrografted plants were successfully grafted on 'Peterson' rootstocks in the nursery (74.5% success), and vigorously growing transgenic transformed 'pHass' scions were established (Fig. 4*a*,*b*). Rooting of transformed 'pHass' shoots was induced by air layering (Fig. 4*c*,*d*) and

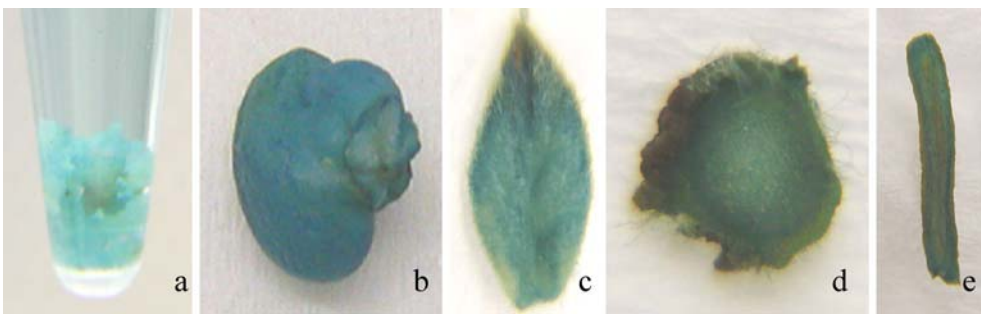

Figure 3. GUS expression in various tissues of transformed 'pHass' avocado with pGPTV-BPDF1.2 construct. (*a*) embryogenic culture; (*b*) mature, opaque somatic embryo; (*c*) leaf, (*d*) stem cross section and (*e*) root longitudinal section from a single regenerated plant.

Figure 4. Avocado plant recovery following transformation with the antifungal defensin gene *pdf1.2*. (*a*) transformed scion grafted *ex vitro* onto 'Peterson' rootstock; (*b*) 6-wk-old plant recovered by *ex vitro* grafting; (*c*) and (*d*) air-layering of transgenic 'pHass'; (*e*) 18-mo-old self-rooted transgenic 'pHass' avocado.

transformed 'pHass' plants on their own roots were recovered with 94% success (Fig. 4*e*). The presence of *uidA* was confirmed in leaves and stems of regenerated transformed 'pHass' somatic embryo-derived shoots (Fig. 3*c*,*d*). The adventitious roots formed by air layering transformed grafted 'pHass' shoots were also positive for *uidA* (Fig. 3*e*).

The presence of the *pdf1.2* and *uidA* transgenes in avocado leaves of regenerated plants was confirmed by PCR (Fig. 5*a*,*b*). Amplification of *uidA* was observed at approximately 683 bp (Fig. 5*a*) and amplification of *pdf1.2* occurred at approximately 349 bp (Fig. 5*b*) in DNA from leaves and from plasmid DNA as the positive control. These amplified bands corresponded with the expected sizes between the *npt*II primers. The negative control, consisting of leaves of nontransformed plants, did not show any amplification product. These results indicated the presence of the *uidA* and *pdf1.2* genes in the recovered avocado plants.

The integration of *pdf1.2* into the avocado genome was demonstrated by Southern blot analysis (Fig. 6). The *pdf1.2* gene failed to hybridize with digested DNA from leaves of nontransformed 'pHass' avocado plants, whereas DNA from leaves of the assayed transformed plants yielded a single band of approximately 2 kb in all lines examined. The Southern blot data confirmed the results of the PCR, and indicate that the *pdf1.2* gene has been integrated into the avocado genome.

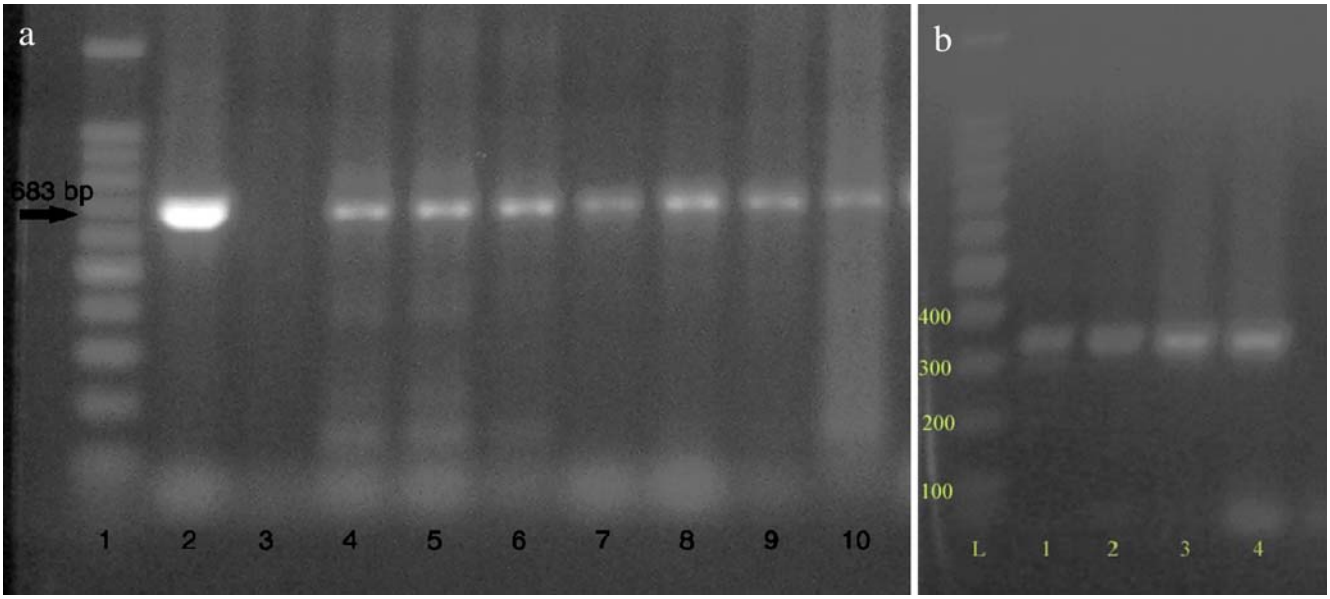

Figure 5. PCR assays of 'pHass' with *pdf1.2* gene. (*a*) Amplification of *uidA* gene segment, plasmid DNA (+control, *lane 2*), non-transformed plant (− control, *lane 3*), different transformed plant lines (*lanes 4–10*); (*b*) amplification of *pdf1.2* gene segment, different transformed plant lines (*lanes 1–3*), plasmid DNA (+ control, *lane 4*). Samples in this figure and in Fig. 6 were from the same selected lines.

Application of Finale® as a foliar spray to the leaves of transformed avocado plants did not result in injury, whereas the leaves of the control plants were dead approximately 4 d after foliar application of Finale® (Fig. 7). These results also confirmed the presence and expression of the *bar* gene in transformed avocado plants.

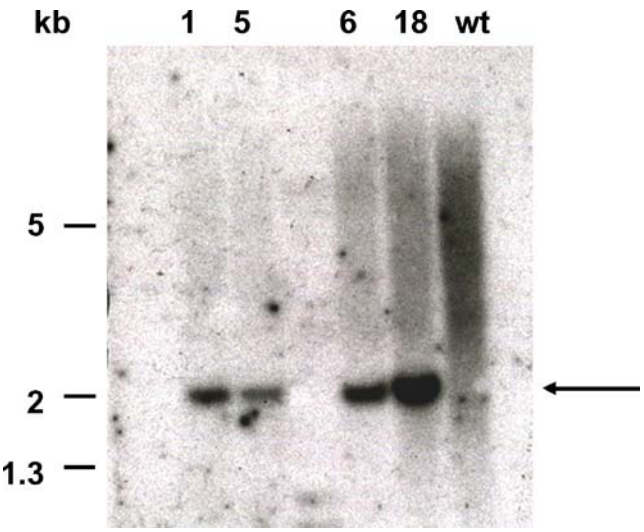

Figure 6. Southern blot analysis of the *pdf1.2* gene in leaves of transformed 'pHass' avocado plants. Genomic DNA isolated from the lines indicated (numbers at the top) was digested with *Hin*dIII, fractionated by gel electrophoresis, transferred on to a nylon membrane and hybridized with the radiolabeled probe (the *pdf1.2* gene coding region). *WT* non-transformed avocado plant. Molecular weight markers are indicated on the left. The *arrow* indicates the bands detected. Samples in Fig. 5 and this figure were from the same selected lines.

Discussion

The recovery of avocado plants by somatic embryogenesis is a complex process that involves a sequence of steps that are necessary to overcome limitations of this regeneration pathway. The development from proembyos through mature somatic embryos occurs quite efficiently (Witjaksono and Litz 1999a, b); however, root formation and shoot development occur in very few somatic embryos. Pliego-Alfaro and Murashige (1988) demonstrated that the meristems fail to organize in the majority of avocado somatic embryos. The low recovery of shoots from transformed somatic embryos was also observed in this study. As a result, micrografting followed by *ex vitro* grafting was essential for the recovery

Figure 7. Application of Finale® to non-transformed avocado plant (*left*) and genetically transformed avocado plant (*right*) containing the *bar* gene.

of vigorously growing plants (Raharjo and Litz 2005). As rootstock development is a major objective of avocado improvement, it is important that self-rooted plants could also be recovered from genetic transformation studies; we have successfully air-layered transgenic avocado shoots, although rooting of cuttings has been reported to be extremely difficult with this species (Bender and Whiley 2002; Crane et al. 2006).

Because of the problems that have been encountered with regeneration of plants from avocado somatic embryos, Cruz Hernandez et al. (1998) could only confirm transformation in embryogenic cultures and early cotyledonary stage somatic embryos. The current report describes the successful transformation of embryogenic avocado cultures with *uidA*, *bar*, and the defensin gene *pdf1.2,* and the recovery of transformed plants. Transformation was confirmed by X-GLUC staining of embryogenic cultures, somatic embryos, leaves, stems, and roots for the presence of *uidA*, PCR reaction for the presence of *uidA* and *pdf1.2*, Southern blot hybridization for the presence of *pdf1.2*, and resistance of regenerated plants to foliar application of the herbicide Finale®. The Southern data indicate that a single insertion occurred in all transgenic lines that were tested. The fact that the detected bands are of the same size also suggests that the lines that were selected for testing must have been derived from a single transformation event. This may be caused by the very low rate of shoot and plant recovery from the avocado somatic embryogenesis regeneration pathway.

The *pdf1.2* defensin gene from *A. thaliana* employed in this work is probably involved in plant defense against fungal infection, and in fact, overexpression of the *pdf1.2* gene in banana plants, protects plants against *Fusarium* spp. and *M. fijiensis* infection (Gonzalez Rodriguez, J.A., Perea-Arango, I. and Gómez Lim M.A., personal communication). Hence, overexpression of this gene in avocado may provide a defense against anthracnose and other fungal pathogens.

The results of this study demonstrate that transgenes can be expressed throughout the 'pHass' avocado plant; however, this could only be achieved by rescuing the shoots of somatic embryos by micrografting followed by *ex vitro* grafting and subsequent air-layering of shoots to stimulate adventitious root formation. Genetic transformation of avocado to address scion and rootstock cultivar improvement objectives is therefore achievable. Transgenic avocado plants are under scrutiny to observe the development of disease symptoms.

Acknowledgements The assistance of Pamela A. Moon and John Menge and the support of the California Avocado Commission are gratefully acknowledged.

References

Becker D.; Kemper E.; Schell J.; Masterson R. New plant binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol. Biol.* 20: 1195–1197; 1992.

Bender G. S.; Whiley A. W. Propagation. In: Whiley A. W.; Schaffer B.; Wolstenholme B. N. (eds) *Avocado botany production and uses*. CAB International, Wallingford Oxon, pp 189–211; 2002.

Crane, J. H.; Balerdi, C. F.; Maguire, I. Avocado growing in the Florida home landscape. http://edis.ifas.ufl.edu/MG213. Cited 9 Dec, 2006.

Cruz-Hernandez A.; Witjaksono; Litz R. E.; Gomez-Lim M. *Agrobacterium tumefaciens*-mediated transformation of embryogenic avocado cultures and regeneration of somatic embryos. *Plant Cell Rep* 17: 497–503; 1998.

Doyle, J. J.; Doyle J. L. Isolation of plant DNA from fresh tissue. *Focus* 12: 12–14; 1990.

Epple P.; Apel K.; Bohlmann H. ESTs reveal a multigene family for plant defensins in *Arabidopsis thaliana*. *FEBS Letters* 400: 168–172; 1997.

FAOSTAT. http://apps.fao.org. Cited 9 Dec, 2006.

Feinberg A. P.; Vogelstein B. A. A technique of radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem*. 132: 6–13; 1983.

Gamborg O. L.; Miller R. A.; Ojima K. Plant cell cultures. I. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell. Res*. 50: 151–158; 1968.

Gazit S.; Degani C. Reproductive biology. In: Whiley A. W.; Schaffer B.; Wolstenholme B. N. (eds) *Avocado botany production and uses*. CAB International, Wallingford Oxon, pp 101–134; 2002.

Jefferson R. A. Assaying chimeric genes in plants: the gus fusion system. *Plant Mol. Biol. Rep* 5: 387–405; 1987.

Knight R. J. Jr. History, distribution and uses. In: Whiley A. W.; Schaffer B.; Wolstenholme B. N. (eds) *Avocado botany production and uses*. CAB International, Wallingford Oxon, pp 1–14; 2002.

Lahav E.; Lavi U. Genetics and breeding. In: Whiley A. W.; Schaffer B.; Wolstenholme B. N. (eds) *Avocado botany production and uses*. CAB International, Wallingford Oxon, pp 39–69; 2002.

Litz R. E.; Witjaksono; Raharjo S.; Efendi D.; Pliego-Alfaro F.; Barcelo-Munoz A. *Persea americana* Avocado. In: Litz R. E. (ed) *Biotechnology of fruit and nut crops*. CAB International, Wallingford Oxon, pp 326–349; 2005.

Mooney P. A.; van Staden J. Induction of embryogenesis in callus from immature embryos of *Persea americana*. *Can. J. Bot.* 65: 622–626; 1987.

Murashige T.; Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497; 1962.

Newett S. D. E.; Crane J. H.; Balerdi C. F. Cultivars and rootstocks. In: Whiley A. W.; Schaffer B.; Wolstenholme B. N. (eds) *Avocado botany production and uses*. CAB International, Wallingford Oxon, pp 161–188; 2002.

Pliego Alfaro F.; Murashige T. Somatic embryogenesis in avocado (*Persea americana* Mill.) *in vitro*. *Plant Cell Tiss. Org. Cult.* 12: 61–66; 1988.

Raharjo S. H. T.; Litz R. E. *In vitro* and *ex vivo* grafting for somatic embryo rescue and plant recovery in avocado (*Persea americana* Mill.). *Plant Cell Tiss. Org. Cult.* 82: 1–9; 2005.

Raviv A.; Avenido R. A.; Tisalona L. F.; Damasco O. P.; Mendoza E. M. T.; Pinkas Y.; Zilkah S. Callus and somatic embryogenesis of *Persea* species. *Plant Tiss. Cult. Biotechnol.* 4: 196–206; 1998.

Sambrook J.; Russell D. W. *Molecular cloning: A laboratory manual*. 3rd ed. Cold Spring Harbor Press, Cold Spring Harbor2001.

Suarez I.; Schnell R. J.; Kuhn D. A.; Litz R. E. Recovery of avocado plants (*Persea americana* Mill.) from embryogenic nucellar cultures from an avocado sunblotch viroid-infected tree. *Plant Cell Tissue Org. Cult.* 84: 27–37; 2006.

Witjaksono; Litz R. E. Induction and growth characteristics of embryogenic avocado (*Persea americana* Mill.) cultures. *Plant Cell Tissue Organ Cult*. 58: 19–29; 1999a.

Witjaksono; Litz R. E. Maturation and germination of avocado (*Persea americana* Mill.) somatic embryos. *Plant Cell Tissue Organ Cult*. 58: 141–148; 1999b.

Witjaksono; Litz R. E. Somatic embryogenesis of avocado (*Persea americana* Mill.) and its application for plant improvement. *Acta. Hortic*. 575: 133–138; 2002.

Witjaksono; Litz R. E.; Grosser J. W. Isolation, culture and regeneration of avocado (*Persea americana* Mill.) protoplasts. *Plant Cell Rep* 18: 235–242; 1998.

Witjaksono; Litz R. E.; Pliego-Alfaro F. Somatic embryogenesis in avocado (*Persea americana* Mill.).. In: Jain S. M.; Gupta P. K.; Newton R. J. (eds) *Somatic embryogenesis in woody plants vol 5*. Kluwer Academic Publishers, Dordrecht, pp 197–214; 1999.