Development of an efficient transient transformation protocol for avocado (*Persea americana* Mill) embryogenic callus

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Running head: Transient transformation in avocado embryogenic callus

Abstract

An efficient protocol for transient transformation of avocado embryogenic callus has been established, using the PDS-1000/He system and the reporter *gus* gene driven by the sunflower polyubiquitin promoter. Best physical parameters for transient transformation were 900 psi helium pressure and 6 cm target distance. The level of transient *gus* expression was slightly higher when the amount of DNA per shot was increased from 0.6 to 1.8 µg, but it was not significantly modified by the type of microprojectile used (tungsten vs. gold particles). The transient transformation assay developed in this research was used to test the strength of different promoters and the expression of fluorescent reporter genes. Four constitutive promoters, sunflower polyubiquitin, CaMV35S, CaMV35S with enhancer, and rice actin 1, as well as a trichome specific promoter, ATP, were analyzed. Polyubiquitin and ATP promoters yielded the highest number of *gus* expressing foci, while no expression was detected with the Act1 promoter from rice. Embryogenic callus was also bombarded with plasmids pXK7S*NF2 and pXK7RNR2, harboring the enhanced green fluorescent gene, *EGFP*, and the red fluorescent gene DsRed, respectively. Both fluorescent proteins were detected 24 and 72 h after bombardment, but the observed transformation efficiency was slightly higher in GFP bombarded cells. The transient transformation system described here can be used as a fast way to select suitable promoters and/or fluorescent genes, needed to undertake stable transformation studies in avocado using currently available protocols.

Keywords. Biolistic · Embryogenic cultures · Fluorescent reporter genes · Particle bombardment · Promoter analysis · Subtropical fruit

Introduction

Avocado (*Persea americana* Mill.) is a subtropical tree cultivated for its fruits, which are a well-balanced source of nutrients and vitamins, but also a rich source of oil (Chanderbali *et al.* 2008). This species is highly heterozygous, with a long juvenile phase and a high rate of flower abscission and immature fruit drop. Due to these limitations, breeding programs have been relatively unsuccessful, and most important cultivars have been derived from open pollination and dooryard tree selection (Litz *et al.* 2007). One of the main problems of avocado cultivation is the lack of improved rootstocks tolerant to soil borne pathogens, mainly *Phytophthora cinnamomi* Rands and *Rosellinia necatrix*. Genetic transformation could be a useful tool to enhance disease tolerance in this species. Different strategies have been assessed to generate disease resistant plants, including the expression of pathogenesis related proteins and/or the overexpression of components of the plant pathways

involved in pathogen resistance (Punja 2001). The success of these biotechnological approaches depends on the gene used, but also on the strength and tissue localization of transgene expression, both factors depending on the regulatory sequences driving the transgene. Moreover, even in the case of frequently used constitutive promoters, such as CaMV35S or ubiquitin, promoter strength varies significantly with the species (Peremarti *et al.* 2010). Thus, prior to undertaking a transformation program, it is generally advisable to evaluate different promoters to identify a strong regulatory sequence for each particular species.

Several factors influencing the production of stable transgenic avocado plants via *Agrobacterium tumefaciens* infection have recently been evaluated (Raharjo *et al.* 2008; Palomo-Ríos *et al.* 2012). Although several transgenic lines have been obtained, this process is labor-intensive, time-consuming and shows low efficiency. Thus, the generation of stable transformed plants in this species is unsuitable for some biotechnological assays, such as the evaluation of transgene expression driven by different promoters or the screening of fluorescent proteins. Transient transformation via biolistic DNA delivery appears as a suitable tool for these kinds of analyses, providing a fast and convenient alternative to stable transformation. Prior to use, physical and biological parameters for particle bombardment need to be optimized for each particular tissue. The aim of this investigation was to characterize the best conditions for transient biolistic transformation of avocado embryogenic cultures. The protocol developed in this research has been used to analyze the strength of several constitutive and tissue-specific promoters, as well as the activity of the fluorescent proteins GFP and DsRed.

Materials and Methods

Plant material and culture conditions. Embryogenic avocado cultures, cv. 'Duke 7', were established from immature zygotic embryos on MS medium supplemented with 0.41 µM picloram and solidified with 6 g l^{-1}agar (Sigma A-1296) (MSP medium), as previously described (Pliego-Alfaro and Murashige 1988). Cultures were incubated in the dark at 25±1 ºC. Embryogenic cultures were maintained on MSP medium and subcultured at monthly intervals.

Optimization of biolistic parameters. The PDS-1000/He system (Bio-Rad) was used for particle bombardment of avocado embryogenic cultures. Initially, standard conditions for bombardment were a 680 mm Hg vacuum pressure, a gap distance from the rupture disc to the macrocarrier of 0.6 cm, and a macrocarrier flight distance of 1.1 cm. In all experiments, explants for transformation consisted of 0.5 g of small globular structures, 1-3 mm size, placed at the centre of a Petri dish containing MSP medium. These explants were bombarded with 6 µl of

the DNA-coated microprojectile suspension, i.e. tungsten m-20 particles, 1.3 µm average size, coated with a plasmid DNA solution at 1 µg/µl as previously described (Sanford *et al.* 1993). After bombardment, explants were cultured for 48 h in the dark and then, the histological GUS assay (Jefferson 1987) was performed to determine the transformation efficiency as the number of blue spots per bombarded plate.

In the first experiment, the effects of helium pressure (450, 900, 1500 and 1800 psi) and target distance (6 and 9 cm) on transient transformation were assessed following a 4x2 factorial design. Following optimization of transformation conditions, the effects of particle type, tungsten m-20 particle vs. 1 µm diameter gold particle, and amount of DNA per shot, 0.6 µg vs. 1.8 µg, on transient transformation efficiency were tested. A single factor per experiment was tested in these assays. In both experiments, pCGUΔ1 plasmid (Binet *et al.* 1991) was used. Afterwards, the efficiency of five different plasmids was evaluated, using the biolistic parameters previously optimized. Plasmids evaluated containing the *gus* gene under the control of different promoters were as follows: pCGUΔ1 harboring the sunflower polyubiquitin promoter *ubB1* (Binet *et al.* 1991), pGUSINT including the *CaMV35S* promoter (Vancanneyt *et al.* 1990), pJGUS5 with the *CaMV35S* promoter with an enhancer (Day *et al.* 1991), pAct1-F with the rice *actin-1* gene promoter (McElroy *et al.* 1991), and pATP-GUS containing the trichome specific promoter *ATP* from *A. thaliana* (Gutiérrez-Alcalá *et al.* 2005). Four bombardments per treatment were performed and all the experiments were conducted twice.

Transformation with fluorescent proteins. For the transformation with fluorescent proteins, two plasmid constructs were used: pXK7S*NF2, harboring the enhanced green fluorescent, *EGFP*, and *gus* genes in tandem, and pXK7RNR2 containing a double red fluorescent gene, *DsRed* (Karimi *et al.* 2007). In both plasmids, a nuclear localization signal sequence of a putative transcription factor from tobacco preceded the fluorescent genes. These reporter genes were driven by *CaMV35S* promoter with an enhancer.

Transient expression of fluorescence reporter genes in bombarded embryogenic callus was observed in an epi-fluorescence microscope (Nikon), equipped with GFP-B (460-500 nm excitation filter and 505 nm barrier filter) and G-2A (510-560 nm excitation filter and 575 nm barrier filter) filters for GFP and DsRed visualization, respectively. The number of cell clusters showing green or red fluorescence was reported 24 and 72 hours after bombardment, using non-bombarded embryogenic callus as control. Eight bombardments per fluorescent protein were performed.

Statistical analysis. Data from the experiment to determine best helium pressure and target distance conditions were subjected to a model I two-way analysis of variance (Sokal and Rohlf 1995). In the rest of experiments, one-way ANOVA test was employed. Levene´s test for homogeneity of variances was performed prior to ANOVA and the LSD test at P=0.05 was used to determine differences among means. All statistical analyses were performed with the SPSS software.

Results

In a preliminary experiment using the bombardment parameters reported to be optimal for transient transformation of olive embryogenic callus (Pérez-Barranco *et al.* 2009), 6 cm target distance and 900 psi helium pressure, we observed that the pCGUΔ1 plasmid was efficiently expressed in avocado embryogenic callus, yielding an average number of blue spots per bombardment of 91.6±33.5 (mean±SD). Thus, this plasmid was used for optimization of physical parameters of the particle bombardment system in avocado. Firstly, the effect of helium pressure, 450, 900, 1500 and 1800 psi, and target distance, 6 and 9 cm, were tested, in an experiment with a 4x2 factorial design. Statistical analysis of the data by two-way ANOVA showed a significant effect of helium pressure ($P<0.05$), but neither target distance nor interaction between both factors were significant. Best results were obtained with 900 and 1500 psi (Fig. 1). A slightly lower number of *gus* expressing foci was obtained at 450 psi, whereas the number of blue spots decreased significantly at 1800 psi. Regarding target distance, in general, a slightly higher number of blue spots was obtained at 6 cm target distance, but the differences were not statistically significant. Most *gus* expressing foci appeared as well defined spots with a strong blue coloration; however, some diffused areas with a pale blue colour were also visible. Using the best bombardment conditions, 6 cm target distance and 900 psi, several parameters, e.g. type of microprojectile and DNA per shot, were evaluated to increase transient transformation rates. The increase of DNA concentration to deliver 1.8 µg per shot instead of the standard amount of 0.6 µg slightly increased the transformation rate, but the differences were not statistically significant (64.7±14.3 (mean ± SD) blue spots per bombarded plate for the control vs. 76.5± 21.6 for the 1.8 µg DNA treatment, respectively). Similarly, the change of tungsten microprojectiles for gold particles of similar size did not increase the number of blue spots per shot (74.5±12.2 blue spots per bombarded plate for tungsten vs. 70.7±3.6 for gold microprojectiles, respectively).

The protocol developed for transient transformation of avocado embryogenic callus was used to test the strength of different constitutive and tissue specific promoters. A large variability in the expression of *gus* gene among the different plasmids used for biolistic bombardment was observed (Fig. 2). The plasmid pCGUΔ1

containing the *gus* gene under the control of *ubB1* promoter yielded a number of *gus* expression foci significantly higher than plasmids pGUSINT or pJGUS5, harboring the *gus* gene under the control of *CaMV35S* or *CaMV35S* with enhancer, respectively (Fig. 3). Interestingly, the plasmid pATP-GUS carrying the trichome specific promoter *ATP* yielded a number of blue spots similar to the one observed with the pCGUΔ1, and significantly higher than the number of blue foci obtained with the other two constitutive promoters (Fig. 3). Finally, no transient transformation was detected when using the plasmid pAct1-F harboring the monocotyledonous promoter from rice *actin-1* gene.

Control avocado embryogenic callus showed high levels of background fluorescence when visualized with epi-fluorescence microscope equipped with GFP or DsRed fluorescent specific filters (Fig. 4A, C). In spite of this, the adjustment of the exposure conditions allowed the detection of some spots showing fluorescent signals above the background level in callus bombarded with plasmids pXK7S*NF2 or pXK7RNR2 (Fig. 4B, D). Using the first plasmid, the mean number of GFP spots per bombarded callus was 32±11, 24 h after bombardment. This value was slightly lower, 26±6, when samples were analyzed 72 h after bombardment. Regarding the transient transformation with the DsRed reporter gene, bombardment with the plasmid pXK7RNR2 yielded a lower number of spots showing red fluorescence, 20±3, than the one observed with the EGFP. Transient expression was maintained after 72 h, although the number of red fluorescent spots was reduced. To assess the utility of DsRed in avocado transformation, embryogenic callus was inoculated with *Agrobacterium tumefaciens* harboring the pXK7RNR2 plasmid following the protocol described by Palomo-Ríos *et al*. (2012). Interestingly, a heterogeneous red fluorescent signal was detected in some inoculated callus after one month of culture (results not shown). DsRed expression on avocado callus was maintained during several subcultures in MSP medium. Experiments are in progress to recover transgenic plants from these embryogenic cultures.

Discussion

In addition to its use in the stable transformation of species recalcitrant to *Agrobacterium* infection, particle bombardment system is also useful for transient expression studies. Microprojectile velocity is the most important physical factor in the success of biolistic transformation. This parameter is generally controlled by the helium pressure and the distance travelled by the microprojectiles, and should be optimized for every tissue type (Birch and Franks 1991). In the case of avocado embryogenic cultures, helium pressure was more determining than target distance in the transformation rate. The best transient transformation rate was obtained at a medium helium pressure, 900 psi, while cells were excessively damaged at the highest pressure, reducing significantly

the number of blue spots per bombarded callus. Similar results have been obtained in olive embryogenic callus (Pérez-Barranco *et al.* 2009). In general, the increase of the amount of DNA delivered per shot improves the transformation efficiency (Lowe *et al.* 2009). As expected, in avocado embryogenic callus, the number of blue spots per bombarded explant was slightly increased when higher amounts of DNA per shot were used, although the differences with the standard DNA concentration were low and non-statistically significant. The use of gold particles instead of tungsten microprojectiles did not improve the avocado transformation rates, despite their lower toxicity to plant cells (Sanford *et al.* 1993). An alternative approach to enhance the rate of success transient transformation could be the use of osmotic treatments. In maize embryogenic suspension cells, the culture of cells in an osmoticum-containing medium increased 2.7 fold the transient *gus* expression rate (Vain *et al*. 1993). This treatment however, was not successful in other systems more similar to avocado cultures, such as olive embryogenic calli (Pérez-Barranco *et al*. 2009).

The transient transformation protocol developed in this research was used to test the strength of different promoters and the expression of fluorescent reporter genes in avocado embryogenic callus. Among the constitutive promoters analyzed, sunflower polyubiquitin *ubB1* was better than *CaMV35S*, with or without enhancer, to conduct *gus* expression in avocado cultures. Similar results have been obtained in other embryogenic cultures, e.g. Indica rice (Li *et al.* 1997), olive (Pérez-Barranco *et al.* 2009), and sorghum (Tadesse *et al.* 2003). By contrast, *CaMV35S* was more effective than ubiquitin promoter in oil palm embryogenic callus (Parveez and Majid 2008). The superior behavior of ubiquitin promoter could be explained by the enhanced expression of this promoter under stress situations (Christensen and Quail 1996), as it occurs under in vitro conditions. The promoter from the rice housekeeping gene *actin-1* drives strong activity in most tissues of transgenic rice plants (Zang *et al.* 1991). However, no transient expression was observed in bombarded avocado cultures. In general, monocot promoters are not well expressed in dicot plants, likely due to different requirements for mRNA processing in dicot and monocot plants (Keith and Chua 1986). McElroy *et al.* (1991) observed that *act-1* promoter was not expressed in bombarded tobacco suspension cells. In potato, by contrast, a strong GUS expression was observed in multi-copy transgenic plants where *gus* gene was under the control of *act-1* promoter (Nadolska-Orczyk *et al.* 2007).

As epidermis is the first barrier controlling the entrance of pathogens in plant tissues, these cells are a good target for transgenic expression of pathogen defense proteins. The tissue specific promoter *ATP* was isolated from the gene *OASA1* which encoded a O-acetylserine(thiol)lyase from *A. thaliana*. This promoter is highly expressed in trichomes (Gutiérrez-Alcalá *et al.* 2005), specialized structures derived from epidermal cells, but

also in epidermal tissue (Martín-Pizarro, Plant Biology Department, University of Málaga, personal communication, June 2013). Arabidopsis plants expressing a *Trichoderma* α-1,3-glucanase gene under the control of this promoter showed enhanced tolerance to *Botrytis cinerea* (Calo *et al.* 2006). In avocado embryogenic cultures, *ATP* promoter was expressed at high levels, and the pATP-GUS plasmid yielded a number of *gus* expression foci similar to the one obtained with the pCGUΔ1 carrying the sunflower ubiquitin promoter.

Fluorescent reporter genes have been shown to be an excellent tool for the evaluation of transformation parameters and early selection of transgenic tissues, being GFP the most commonly employed (Stewart, 2001; Hraška *et al.* 2006). However, the expression of GFP fluorescence differs depending on the GFP variant, the target tissue and the promoter used (Hraška *et al.* 2006). Using a transient transformation assay, we have shown that EGFP and DsRed reporter genes can be successfully expressed in avocado embryogenic callus. As observed in other embryogenic calli (Leclercq *et al.* 2010), avocado callus showed auto-florescence but this did not prevent the visualization of cell clusters expressing fluorescent reporter genes. Furthermore, both proteins could be detected with an epifluorescence microscope, avoiding the use of more sophisticated equipment, e.g. a confocal laser scanning microscope. The level of DsRed expression was slightly lower than the one detected with EGFP. However, DsRed is of special interest in plants, since this fluorescence protein is more stable than GFP (Verkhusha *et al.* 2003) and its spectral fluorescence does not interfere with chlorophylls, as shown with GFP (Stewart 2006). The utility of DsRed in avocado transformation was also confirmed in preliminary transformation experiments using embryogenic callus inoculated with *Agrobacterium tumefaciens*. DsRed expression was detected in some calluses after a short time, one month, from infection.

In conclusion, an efficient protocol for transient transformation of avocado embryogenic cells has been developed. Using this method, we have shown that *ubB1* and *ATP* promoters can be excellent candidates for conducting trangene expression studies in this species. Furthermore, we have shown that EGFP and DsRed fluorescence reporter genes can be expressed and detected in this tissue. Using a protocol developed by our group (Palomo-Ríos *et al.* 2012), experiments are currently in progress to obtain stable transgenic avocado cultures carrying these fluorescence genes for early selection of transgenic tissues.

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Figure legends

Figure 1. Effect of helium pressure on the transient expression of *gus* gene in avocado embryogenic callus. Data correspond to mean±SD. Means with different letters indicate significant differences by LSD at P=0.05.

Figure 2. Expression of the *gus* gene in avocado embryogenic callus bombarded with pCGUΔ1, pGUSINT or pATP plasmids. Control corresponds to non-bombarded tissue.

Figure 3. Effect of plasmid type on the transient expression of *gus* gene in avocado embryogenic callus. Data correspond to mean±SD. Means with different letter indicate significant differences by LSD at P=0.05.

Figure 4. Expression of the GFP and DsRed reporter genes in avocado embryogenic callus bombarded with pXK7S*NF2 (B) or pXK7RNR2 (D) plasmids, respectively. A, C corresponds to non-bombarded tissue visualized under GFP or DsRed filters, respectively. Bar corresponds to 1 mm.