Agrobacterium-mediated transformation of avocado (*Persea americana* Mill.) somatic embryos with fluorescent marker genes and optimization of transgenic plant recovery

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Key message

Novel method to recover avocado plants from somatic embryos transformed with fluorescence marker genes. Use of these genes could allow reduction of selection pressure improving the efficacy of transformation process.

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30 **Abstract**

31 Avocado globular somatic embryos were transformed with three binary vectors, pK7FNF2, pK7RNR2 and
32 pK7S*NF2, harboring the marker genes *gfp*, *DsRed* and a *gfp-gus* fusion gene, respectively. GFP and DsRed
33 fluorescence was detected in embryogenic lines growing in selection medium two months after *Agrobacterium*
34 inoculation. The fluorescence signal was maintained thereafter in transgenic calli, as well as in maturated
35 somatic embryos. Red fluorescence in pK7RNR2 transgenic lines was higher and more easily observable than
36 GFP fluorescence. Furthermore, calli transformed with pK7S*NF2, harboring *gfp-gus*, showed higher level of
37 fluorescence than those transformed with pK7FNF2, two *gfp*. To improve plant recovery, maturated transgenic
38 embryos that failed to germinate or showed an underdeveloped shoot were cultured for 4 weeks in a medium
39 with 1 mg l^{-1} TDZ and 1 mg l^{-1} BA after partial removal of cotyledons. A 50% of embryos developed one or
40 several shoots on the cut surface. These embryos were cultured for 4 additional weeks in a medium with 1 mg
41 l^{-1} BA for shoot elongation and then, shoots were grafted *in vitro* onto seedling rootstocks. Culture of
42 micrografts in solid MS medium supplemented with 1 mg l^{-1} BA allowed a 60-80 % success rate. Young leaves
43 from transgenic plants showed GFP or DsRed fluorescence located in the nucleus. The results obtained indicate
44 that fluorescent marker genes, especially *DsRed*, could be useful for early selection of transgenic material and
45 optimization of the transformation parameters in avocado. Furthermore, the protocol established allowed the
46 successful recovery of transgenic plants, one of the main limiting steps in avocado transformation.

47

48 **Keywords** Avocado · fluorescent markers · plant regeneration · transgenic plants

49 **Introduction**

50 Avocado (*Persea americana* Mill.) is one of the most important tropical fruit. The biotechnological
51 improvement of this species through genetic transformation is hampered by the recalcitrant nature of avocado
52 explants to regenerate *in vitro*. Embryogenic cultures derived from immature zygotic embryos are the most
53 common explant source for avocado regeneration and transformation (Pliego-Alfaro et al. 2013). These cultures
54 are easily obtained but the conversion of somatic embryos (SE) to plants occurs at very low rates, apparently
55 due to the disorganization of apical shoot meristem (Sánchez-Romero et al. 2005; Palomo-Ríos et al. 2013). An
56 additional factor that hinders the recovery of transgenic avocado plants is the high selection pressure used in
57 most *Agrobacterium*-mediated transformation protocols (Raharjo et al. 2008; Palomo-Ríos et al. 2012). Due to
58 these problems, few researches have successfully obtained transgenic plants in this species (Cruz-Hernandez et
59 al. 1998; Raharjo et al. 2008; Palomo-Ríos et al. 2012). In a recent report, Palomo-Ríos et al. (2012) evaluated
60 several factors influencing the efficiency of the *Agrobacterium*-mediated transformation of avocado
61 embryogenic cultures. The culture of transgenic SE in liquid germination medium supplemented with
62 benzyladenine (BA) enhanced the recovery of transgenic plants, but some transgenic lines still failed to
63 germinate or produced weak shoots that could not be micropropagated.

64 Fluorescent proteins, e.g. GFP or DsRED, have been used as visual selection markers in other tree species
65 (Escobar et al. 2000; Zhu et al. 2004; Pérez-Clemente et al. 2005; Yancheva et al. 2006; Leclercq et al. 2010;
66 Mishra et al. 2010). These proteins are vital markers and allow the monitoring of transgene expression along
67 the whole regeneration process from early stages of the transformation protocol (Hraška et al. 2006). In *Hevea*
68 *brasilensis* and *Coffea canephora* embryogenic cultures, the combined use of a fluorescent marker gene and a
69 selectable gene allowed reducing the antibiotic selection pressure and/or the time required for selection
70 (Lecrercq et al. 2010; Mishra et al. 2010). Fluorescent marker genes have not been used for the stable
71 transformation of avocado. However, Chaparro-Pulido et al. (2014) demonstrated that both *gfp* and *DsRED*
72 genes could be successfully expressed in avocado embryogenic callus when transiently transformed via
73 biolistic.

74 The main goal of this research was to improve the procedure for the *Agrobacterium*-mediated
75 transformation of avocado embryogenic cultures and the recovery of transgenic plants. Then, the usefulness of
76 fluorescent marker genes in the genetic transformation of avocado has been assessed. For this purpose, avocado
77 SE have been transformed with several constructs harboring two different fluorescent marker genes, *gfp* and
78 *DsRed*, and the expression of these genes has been monitored along the transformation and plant regeneration

79 processes. Secondly, a procedure for the conversion of transgenic SE to plants has been established. The main
80 features of this protocol are the culture of maturated somatic embryos in the presence of thidiazuron (TDZ) and
81 the *in vitro* grafting of transgenic shoots onto seedling rootstocks.
82
83
84 **Materials and methods**
85
86 **Plant material**
87 Globular somatic embryos from D2.3 avocado embryogenic line were used as explants for genetic
88 transformation. D2.3 embryogenic line was established from an immature zygotic embryo, cv. 'Duke 7',
89 according to Pliego-Alfaro and Murashige (1988) on Murashige and Skoog medium (Murashige and Skoog
90 1962) supplemented with 0.41 µM picloram (MSP medium) and solidified with 6 g l^{-1} agar (Sigma A-1296).
91
92 **Transformation vectors and *Agrobacterium* strain**
93 Three binary vectors harboring fluorescent proteins were used: pK7FNF2, pK7S*NF2 and pK7RNR2. These
94 vectors were developed using Multisite Gateway technology (Karimi et al. 2007). All vectors harbored the
95 *neomycin phosphotransferase II* (*nptII*) under the control of the *nopaline synthase* promoter, and the marker
96 genes were preceded by *CaMV35S* promoter. The stop codon of the fluorescent proteins was eliminated and
97 alternative stop codons were located downstream to fuse two markers genes (two *gfp* genes in pK7FNF2; *gfp*
98 and *β-glucuronidase* in pK7S*NF2; two *DsRed* genes in pK7RNR2) (Karimi et al. 2007). In all vectors, the
99 first marker gene is preceded in frame by a nuclear localization signal (NLS) from a tobacco transcription factor
100 (Grebenok et al. 1997). The *gfp* gene used was an enhanced *gfp* (*egfp*), containing more than 190 silent base
101 mutations, which allows mRNA to be efficiently translated and increases its expression (Yang et al. 1996). The
102 *gfp* gene in pK7S*NF2 and the first *gfp* in pK7FNF2 contained the PIV2 intron of the ST-L1 gene from *Solanum*
103 *tuberosum* within its coding sequence, preventing its expression in *Agrobacterium*.
104 The binary vectors were introduced into the disarmed *A. tumefaciens* strain AGL1 (Lazo et al. 1991) by
105 the freeze-thaw method (Höfgen and Willmitzer 1988). For avocado transformation, *Agrobacterium* cultures
106 were grown for 24 h at 28ºC in LB medium at 250 rpm. Then, bacterial suspensions were centrifuged at 4,000
107 *g*, the pellets were washed with 10 mM $MgSO_4$ and finally diluted in liquid MSP medium at OD_{600nm} of 0.5.
108

109 **Genetic transformation and plant regeneration**

110 Genetic transformation of avocado somatic embryos was performed as described by Palomo-Ríos et al. (2012). 111 Globular embryos were immersed for 20 min in the diluted *Agrobacterium* suspension under mild agitation. 112 Then, explants were blotted dry on sterile filter paper and cultured in solid MSP medium for 48 h. Afterwards, 113 explants were washed for 2 h with liquid MSP medium supplemented with 300 mg l^{-1} timentin, blotted dry and 114 transferred in groups of three SE to selection medium, i.e. solid MSP medium supplemented with 250 mg l^{-1} 115 timentin and 50 mg l^{-1} kanamycin. The embryos were transferred to fresh medium monthly and kanamycin 116 concentration was gradually increased from 50 to 75 mg l^{-1} and finally to 100 mg l^{-1}. A single transformation 117 experiment with each vector were carried out, including 12 plates with 10 groups of three embryos each. 118 Transformation efficiency was estimated as the percentage of inoculated explants actively growing on selection 119 medium with 100 mg l^{-1} kanamycin. After 3-5 months of culture in the selection medium, SE showing new 120 embryogenic callus growth were maturated following the protocol of Palomo-Ríos et al (2013). Briefly, 0.4 g 121 friable callus was used to inoculate 40 mL liquid MSP medium in 100 mL flasks. The suspensions were 122 incubated in an orbital shaker at 120 rpm under low irradiance with a 16 h photoperiod at 25ºC. After 9 days, 123 the cultures were sieved through two meshes of 2 and 1 mm pore size respectively and the fraction retained 124 over the 1-mm mesh was cultivated onto B5m10A maturation medium (MS formulation with macronutrients 125 from Gamborg et al. (1968) gelled with 10 g l^{-1} agar) over cellulose acetate membranes for 5 weeks (Palomo-126 Ríos et al. 2012). Then, the SE that turned white and opaque (WOSE) were cultured on B5m10A without 127 cellulose acetate membranes for 5 weeks, followed by another 5 weeks on B5m10A supplemented with 45 g l^{-} 128 ^{1} sucrose and 20% (v/v) filter sterilized coconut water. Finally, maturated WOSE larger than 5 mm were 129 germinated as described by Palomo-Ríos et al. (2012). Plants were micropropagated and acclimatized 130 following the protocol of Barceló-Muñoz et al. (1999).

131 To increase plant recovery, germinated embryos showing visible buds and that had failed to grow using 132 the above described protocol, were subjected to the following treatments: Following partial removal of the 133 cotyledons, embryos were cultured for 4 weeks in MS medium supplemented with either 1 mg l^{-1} BA, 1 mg l^{-} 134 ^{1} TDZ or 1 mg l^{-1} BA and 1 mg l^{-1} TDZ, exposing the buds on the medium surface. More than 60 WOSE from 135 embryogenic calli from 7 independent transgenic lines were used as explants in each treatment. Afterwards, 136 the WOSE showing shoot growth were transferred to MS medium supplemented with 1 mg l^{-1} BA for 4 137 additional weeks for further shoot elongation. Finally, the shoots that reached 3-5 mm in length were grafted 138 onto *in vitro* seedlings rootstocks, cv. Topa-Topa, as described by Pliego-Alfaro and Murashige (1987) with

139 some modifications. For seed germination, MS basal medium gelled with 6 g l^{-1} agar was used. Micrografted
140 plants were cultured in MS medium supplemented with 1 mg l^{-1} BA for 2-4 months. Afterwards, elongated
141 scions were excised and micropropagated as indicated by Barceló-Muñoz et al. (1999).
142

143 **Visualizing fluorescent expression**

144 The *gfp* or *DsRed* expression in embryogenic callus growing on selection medium was initially analyzed after
145 15 days from *Agrobacterium* inoculation and monthly thereafter, at the moment of each transfer of transgenic
146 SE onto fresh medium, for 5 months. The fluorescent signal was visualized with a Nikon AZ-100 epifluorescence
147 microscope, through a 460-500 nm filter for GFP fluorescence and 510-560 nm filter for DsRED fluorescence.
148 After the selection period, the maturated embryos obtained were also analyzed with the epifluorescence
149 microscope. Fresh leaves from micropropagated transgenic plants were analyzed with a stereomicroscope Leica
150 SP5 II. Non-transformed callus, maturated embryos and leaves were used as negative controls to adjust the
151 parameters of exposure time to ensure the exclusion of auto fluorescence and to compare the fluorescent signal
152 level among the different transgenic lines.

153 GUS activity in putative transgenic SE from embryogenic lines transformed with pK7S*NF2 was
154 visualized using the histochemical GUS assay (Jefferson 1987).
155

156 **Molecular analysis of transgenic material**

157 The transgenic nature of avocado plants was confirmed by polymerase chain reaction (PCR) amplification.
158 Genomic DNA was isolated from leaves of control and putative transgenic lines using QUIAGEN DNeasy®
159 Plant Mini Kit. Aliquots of these DNA extracts were used to amplify by PCR a 432 bp fragment belonging to
160 the *gfp* gene or a 592 bp from *DsRed* gene. Primers used in PCR were F: 5´-catgaagcagcacgacttct-3´ and R: 5´-
161 agcaggaccatgtgatcg-3´ for the amplification of the *gfp* fragment and F: 5´-ggagttcatgcagttcaagg-3´ and R: 5´-
162 ctcgttgtggttggtgatgt-3´ for the *DsRed* fragment. Primers were designed using "Prime 3" software. PCR
163 conditions were as follows: 95ºC for 4 min followed for 30 cycles of 95ºC for 45 s, 60ºC for 45s and 72ºC for 1
164 min, and a final 10 min extension at 72ºC.

165 For Southern blot analysis, 1 µg of DNA isolated from embryogenic calli were digested overnight with *EcoRI*,
166 fractionated in a 0.8% agarose gel and transferred to a Hybond N^+ membrane. A PCR DIG Probe Synthesis Kit
167 from Roche was used to obtain a 700 bp probe by PCR amplification of the *nptII* gene from pK7S*NF2 plasmid.

168 Primers used were 5´-gaggctattcggctatgactg-3´ and 5´-atcgggagcggcgataccgta-3´ (Álvarez et al. 2004). The filter
169 was hybridized at 42ºC using a DIG High Prime DNA Labeling and Detection Starter Kit II from Roche.
170
171
172 **Results**
173
174 **Transformation and plant regeneration**
175 The efficiency of transformation was above 10% for each vector. SE from 5 putative transgenic lines obtained
176 with pK7S*NF2 and pK7RNR2 plasmids and 3 lines with pK7FNF were maturated following the protocol
177 described by Palomo-Ríos et al. (2013). The number of mature WOSE recovered varied in the range 10-15
178 WOSE/100 mg callus, depending on the transgenic line. Only one of the lines transformed with pK7S*NF2 did
179 not produce enough number of WOSE to continue with the procedure for plant recovery.
180 The germination frequency of maturated transgenic embryos varied greatly among the lines, from 4-15%,
181 being these values much lower than those obtained in control SE, higher than 30%. Furthermore, many of the
182 transgenic shoots obtained displayed poor growth and could not be micropropagated. Fig. 1A-F shows the aspect
183 of maturated WOSE prior to germination and the different stages of the process for plant recovery using the
184 standard germination protocol.
185 To improve plant recovery from germinated WOSE that showed a small underdeveloped shoot,
186 cotyledons were partially removed and the remaining part of the SE cultured in MS medium supplemented
187 with either BA, TDZ or a combination of both cytokinins at 1 mg l^{-1} (Fig. 1-H). After 4 weeks, media
188 supplemented with TDZ displayed higher percentages of WOSE with developed shoots than the BA
189 supplemented medium (Fig. 2). The percentage of shoot recovery was slightly higher, >50%, in the medium
190 supplemented with both TDZ and BA (Fig. 2). Resulting shoots were small with a high number of slim leaves.
191 The culture of these propagules on MS medium supplemented with 1 mg l^{-1} BA for 4 additional weeks allowed
192 stem elongation (Fig. 1-I).
193 To enhanced plant vigour, shoots that had reached 3-5 mm in length were grafted onto *in vitro* rootstocks.
194 After 4 months, elongated shoots were obtained from over 60-80% of the grafts; these shoots were excised,
195 micropropagated and finally acclimated according to Barceló-Muñoz et al. (1999). Fig. 1G-L shows the
196 different steps of the improved protocol for recovery of transgenic avocado plants. With this protocol, plants

197 from 7 independent transgenic lines (3 with pK7S*NF2, 2 with pK7RNR2 and 2 with pK7FNF plasmids,
198 respectively) could be recovered.

199 The transgenic nature of acclimated plants was confirmed by PCR analysis (Fig. 3A). All transgenic lines
200 analyzed showed the correct amplification of the *gfp* or *DsRed* gene fragment, while no amplification was
201 observed in the wild-type, non-transformed DNA. Southern blot analysis was carried out in the above mentioned
202 7 transgenic lines; number of transgene copies inserted ranged between 1 and 3 (Fig. 3B).

203

204 **Expression of fluorescent marker genes during SE transformation**

205 The expression of *gfp* and *DsRed* genes in *Agrobacterium* inoculated calli growing in selection medium was
206 analyzed by an epifluorescence microscope. At early stages of transformation, 15 days from *A. tumefaciens*
207 infection, calli did not show any fluorescent signal. However, after 2 months of culture on selection medium,
208 some sectors of actively growing calli showed a clear GFP or DsRED fluorescence signal (Fig. 4). Transgene
209 expression was maintained in embryogenic calli during the rest of the selection phase (Fig. 4). In general,
210 embryogenic lines transformed with pK7RNR2 exhibited a red fluorescent signal more easily detectable than
211 lines transformed with *gfp* gene. Furthermore, GFP fluorescence was higher in SE transformed with pK7S*NF2
212 when compared with those transformed with pK7FNF2, although both plasmids harbored the same *gfp* gene.
213 pK7S*NF2 transformed lines also displayed a strong GUS activity when subjected to histochemical GUS assay
214 (results not shown). In the other hand, the fluorescence level was stronger in calli in active proliferation than in
215 older cultures, being this effect of callus age more evident in lines transformed with the *gfp* marker gene than in
216 *DsRed* transformed lines. In all cases, the fluorescence level was higher when the explants were cultured in
217 medium without kanamycin, after finishing the selection phase (results not shown). After the maturation
218 procedure, WOSE from the different transgenic lines obtained showed a homogeneous green or red fluorescence
219 (Fig. 5). It is noteworthy that all transgenic lines recovered expressed the fluorescent marker genes, not observing
220 significant differences in the level of fluorescence among the different embryogenic lines within each construct.

221 Leaves from transgenic plants regenerated *in vitro* were also analyzed to detect marker genes. Regardless
222 of the construct used for transformation, the fluorescent signal was only observed in young developing leaves,
223 about 5 mm in length, located close to the apical meristem (Fig. 5). As expected, both GFP and DsRED signal
224 were detected in the nucleus.

225

226

Discussion

Most *Agrobacterium*-mediated transformation protocols include a selection phase to avoid the recovery of non-transgenic material or chimeras. In avocado, the high selection pressure needed to get transgenic embryogenic cultures exerts a strong stress in the material lowering the rates of plant recovery (Raharjo et al. 2008; Palomo-Ríos et al. 2012). Fluorescent marker genes have been used to improve transformation protocols by allowing the visual detection of transformed tissues. This may permit the manual selection of transgenic tissues in media with a lower concentration, or even the absence, of selective agents (Miki and McHugh 2004). To use this system, ideally, fluorescent marker genes should be highly expressed from early phases of transformation and the auto florescence of the tissue should not interfere with the detection of transgene expression. The GFP or DsRED fluorescence signal in transformed and control avocado embryogenic cultures was checked during the selection process. Although in other species auto fluorescence has been detected in non-transformed tissue (Molinier et al. 2000; Hraška et al. 2005; Robić et al. 2009), control avocado somatic embryos did not show fluorescence and the signal intensity level in embryos transformed with the three vectors was always clearly higher than control.

Frequently, GFP protein is detectable at high levels a few hours after co-culture phase and *gfp* expression decreases after some days (Mercuri et al. 2001; Jeoung et al. 2002; Maximova et al. 2003). Maximova et al. (1998) developed an exhaustive study of *gfp* expression in transgenic apple leaves. They found an increase on *gfp* expression 9 days after the *Agrobacterium* infection, followed by a reduction and stabilization of the fluorescent signal at 11-15 days, probably, as a consequence of non-integrated T-DNA degradation or gene silencing. Thereafter, the fluorescent signal was increased due to the proliferation and stabilization of transformed cells. Similarly, Jin et al. (2012) detected an early high transient *gfp* expression 3 days after the transformation of cotton, decreasing until 13 days, when the transformation events and *gfp* expression turned stable. In avocado SE, neither GFP nor DsRED fluorescence were detected at early phases of *Agrobacterium* transformation, starting to appear 2 months after the infection process. After this time, the expression of both marker genes remained stable during the selection culture. However, an increase in the fluorescence intensity was detected when the embryos were cultured without kanamycin (selection agent) in the medium, independently of the transgenic line, possibly due to a less stressful culture conditions. The long time required for the observation of fluorescence in avocado when compared with other species is likely due to the low growth rate of embryogenic callus after *Agrobacterium* infection.

256 The red fluorescent signal derived from DsRED protein in embryogenic lines transformed with pK7RNR2
257 construct was always higher and clearer than the green fluorescence observed in lines transformed with the g*fp*
258 marker gene. It has been shown that DsRED is more stable than GFP (Verkhusha et al. 2003) and its spectral
259 fluorescence does not interfere with chlorophylls in green tissues (Stewart 2006). These features make this
260 marker gene especially suitable for plant transformation. When the two constructs containing *gfp* gene were
261 compared, lines transformed with the pK7S*NF2 plasmid, harboring g*fp* and *uidA* marker genes, showed higher
262 fluorescence intensity than those transformed with pK7FNF2, double *gfp*. According to Karimi et al. (2007), the
263 use of double fluorescence marker genes within the same vector yields a higher signal than single genes;
264 however, our results do not support this statement. The reasons for the lower efficiency of pK7FNF2 plasmid
265 on *gfp* expression are unclear, since both plasmids harbor the same *gfp* variant, enhanced *gfp*, under the control
266 of the same constitutive promoter, *CAMV35S*. This different signal level between vectors harboring single and
267 double *gfp* was observed during embryogenic culture selection and SE maturation; although, in the first case,
268 the differences were lower in the absence of selection antibiotics.

269 The results obtained indicate that *gfp* and *DsRed* can be used as marker genes in avocado SE transformation,
270 helping in the detection of transformed tissue. However, the time required for the expression of these genes
271 suggests that it is necessary to keep the antibiotics in the medium through all the selection process of transgenic
272 cells. Without selection antibiotics, the growth of non-transgenic embryos would be uncontrolled, making more
273 difficult the selection of transformed material. The combined use of fluorescent and selection genes, mainly
274 *nptII*, has successfully been employed in the transformation of embryogenic cultures in other species. Leclercq
275 et al. (2010) obtained a decreased number of escapes, as well as a reduction of 2 months in the selection process,
276 when using *gfp* and *nptII* to select transgenic material in embryogenic callus of *Hevea brasilensis*. These authors
277 also tested the selection of transformed tissue using only *gfp* expression; the selection time and the number of
278 chimeras could be reduced although the number of transgenic lines was also reduced. A higher transformation
279 efficiency was also observed by Muβmann et al. (2011) in petunia plants using combined selection of GFP
280 expression and *nptII*. Finally, Corredoira et al. (2012) avoided the selection of chimeras by visually selecting
281 only completely fluorescent *European chestnut* embryos transformed with *gfp*, also combined with the use of
282 *nptII* selection.

283 The recovery of transgenic avocado plants from SE is extremely difficult due to the recalcitrant nature of
284 this material to mature and germinate *in vitro* (Pliego-Alfaro et al. 2013). The pre-maturation of SE on top of
285 cellulose acetate membranes followed by the treatment of mature embryos in liquid germination medium

286 improved shoot emergence in non-transformed SE, reaching germination values in the range 30-40% (Palomo-
287 Ríos et al. 2013). Lower germination rates have been obtained when using this improved protocol in SE
288 transformed with the fluorescent marker genes, and even more, some transgenic lines did not germinate. To
289 improve shoot emergence, germinated WOSE that showed small sprouts that failed to grow were cultured in
290 medium supplemented with cytokinins after partial removal of cotyledons. Media with TDZ yielded the best
291 response and a 40-50% of explants developed shoots after 4 weeks of culture. TDZ is a potent growth regulator
292 mimicking the *in vitro* morphogenetic effects of auxin and cytokinin (Murthy et al. 1998). This growth regulator
293 has been used to induce bud sprouting in some species. In peanut, *Quercus robur* and kodo millet, many new
294 buds were formed on the surface of embryos cultured in TDZ (Joshi et al. 2008; Martinez et al. 2008; Ceasar
295 and Ignacimuthu 2010).

296 Generally, elongated avocado shoots recovered through the use of TDZ showed low vigour and they
297 required a further transfer to a BA containing medium prior to grafting onto *in vitro* germinated seedlings. *In*
298 *vitro* grafting has previously been used to recover avocado transgenic plants (Raharjo et al. 2008; Palomo-Ríos
299 et al. 2012). This improved protocol allowed the recovery of plants transformed with the three different plasmids.
300 As expected, leaf tissue from *in vitro* avocado plants displayed a green or red nuclear fluorescent signal, being
301 this higher in young leaves close to the apical meristem.

302

303 In conclusion, the results obtained show that the fluorescent marker genes, *gfp* and *DsRed* are efficiently
304 expressed in avocado somatic embryos and could be used to select transgenic material. However, antibiotic
305 selection should be maintained since fluorescent signal was not detected at the early phases of transformation.
306 Moreover, DsRED yielded a higher fluorescent signal that was more easily visualized than GFP, making this
307 marker gene especially suitable for avocado transformation. The protocol for transgenic embryo conversion has
308 been improved by the culture of maturated SE in a medium with TDZ and the micrografting of developing shoots
309 onto *in vitro* germinated seedling rootstocks. Overall, these results will be helpful to get transgenic avocado
310 plants with genes of interest.

311

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315

316 **Data archiving statement**

317 The used vectors sequences are available in Plant Systems Biology
318 (https://gateway.psb.ugent.be/search/index/overexpression/any) and published in Karimi et al
319 (2007).

320

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430 **Fig.1** (A-F) Standard protocol for plant recovery from avocado somatic embryos. White opaque somatic
431 embryo (WOSE) (A), sprouted embryo (B), embryo with developed shoot (C), isolated plant in proliferation
432 medium (D), acclimated plant (E) and plant recovered in the greenhouse (F). (G-L) Improved protocol for
433 plant recovery from transgenic avocado somatic embryos. Transgenic WOSE (G), underdeveloped shoot
434 formed on a transgenic embryo (H), shoot developed after culture on MS medium supplemented with 1 mg
435 l^{-1} BA and 1 mg l^{-1} TDZ followed by culture on medium with 1 mg l^{-1} BA (I), transgenic micrografted shoot
436 (J), acclimated transgenic plant (K) and recovered transgenic plant growing in the greenhouse (L). Bar:
437 1cm

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440 **Fig.2** Percentage of transgenic WOSE developing shoots after 1 month culture in MS medium
441 supplemented with 1 mg l^{-1} BA, 1 mg l^{-1} TDZ or 1 mg l^{-1} BA and 1 mg l^{-1} TDZ. Data correspond to
442 percentages ± EE. Different *letters* indicate significant difference by χ^2 at P=0.05. A minimum of 60 WOSE
443 were used in each treatment.

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446 **Fig.3** A: PCR amplification of a 432pb fragment of *gfp* gene and 592 pb fragment of *DsRed* gene in DNA
447 isolated from transgenic lines transformed with: pK7S*NF2 (A), pK7FNF2 (B) and pK7RNR2 (C). MW:
448 molecular weight marker; P1: plasmid pK7FNF2; P2: plasmid pK7RNR2; NT1 and NT2: non-transformed
449 DNA; A, B and C: each lane correspond to DNA from independent transgenic lines. B: Southern blot
450 analysis from 7 independent lines transformed with: pK7S*NF2 (A), pK7FNF2 (B) and pK7RNR2 (C).
451 MW: molecular weight marker; NT: non-transformed DNA; P1: plasmid pK7S*NF2.

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454 **Fig.4** Expression of *gfp* and *DsRed* fluorescence genes in avocado callus at 2, 3 and 5 months after *A.*
455 *tumefaciens* infection with pK7FNF2 (A), pK7S*NF2 (B) and pK7RNR2 (C) plasmids. Right column
456 images correspond to non-transformed calli visualized under GFP (A,B) and DsRed (C) filters

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459 **Fig.5** Expression of *gfp* and *DsRed* fluorescence genes in avocado WOSE (upper row) and leaves (lower

460 row) of *in vitro* plants transformed with pK7FNF2 (A), pK7S*NF2 (B) and pK7RNR2 (C) plasmids. Bar:

461 100 μm

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