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Short communication

Production of transgenic apricot plants from hypocotyl segments of mature seeds

César Petri[a,1], Hong Wang[b,1], Lorenzo Burgos[c], Jesús Sánchez-Navarro[d], Nuria Alburquerque[c,*]

[a] Departamento de Producción Vegetal, Universidad Politécnica de Cartagena, Paseo Alfonso XIII, 48, 30203 Cartagena, Spain
[b] Institute of Fruit and Floriculture Research, Gansu Academy of Agricultural Sciences, Anning, Lanzhou 730070, China
[c] Grupo de Biotecnología de Frutales, Departamento de Mejora Vegetal, CEBAS-CSIC, Campus de Espinardo, Apartado de correos 164, 30100 Espinardo, Murcia, Spain
[d] Instituto de Biología Molecular y Celular de Plantas (IBMCP), CSIC-Universidad Politécnica de Valencia, CPI Ed. 8E, Camino de Vera s/n, 46022 Valencia, Spain

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ABSTRACT

Apricot (*Prunus armeniaca* L.) is one of the most recalcitrant species for genetic transformation. In this work; we report the regeneration of transgenic apricot plants from mature seed hypocotyl segments (cv. 'Canino' and 'Moniquí') for the first time. Moreover, this is the first report of the regeneration of apricot transgenic plants by means of the selective *bar* gene. *Agrobacterium tumefaciens* strain LB4404 harbouring a binary plasmid with *eyfp* and *nptII* genes (pMOG-*eyfp*-IV2) or a plasmid with *gus* and *bar* genes (pFGC5941-*gus*), was used in this study. A gradual selection strategy with aminoglycoside antibiotics made it possible to produce transgenic plants with $1.5 \pm 0.5\%$ efficiency using 10 μM paromomycin (PAR). When buds were isolated and placed in a meristem development medium, PAR was changed to 20 μM kanamycin (KAN). As the buds developed and elongated, the concentration was increased up to 140 μM KAN based on a 20 μM increase every 4 weeks. When *bar* was the marker gene, the regeneration-inhibitory PPT concentration (75.7 μM) was applied immediately after the co-culture. In this case, the PPT concentration was reduced afterwards to 10.1 μM in the micropropagation medium. This strategy allowed for the regeneration of transgenic shoots with $3.8 \pm 1.4\%$ efficiency. Transformation events were monitored by detection of the reporter gene (*eyfp* or *gus*) expression. PCR and Southern blot analysis confirmed the stable integration of the transgenes into the apricot genome. Transformed shoots were rooted on a selective medium, acclimatised, cultured and raised under greenhouse conditions. Our results confirm that the process used in this study is an efficient transformation system for the apricot species which could be used for functional genomics studies and the development of transgenic rootstocks.

1. Introduction

Apricot (*Prunus armeniaca* L.), like other species of fruit trees, is recalcitrant to regeneration and genetic transformation. As far as we know, 'Helena' remains the only commercial apricot cultivar that has been genetically modified (López-Noguera et al., 2009; Petri et al., 2008a, 2012, 2008b), but this methodology is highly genotype-dependent. In 1992, some transgenic apricot lines from immature cotyledons were produced by Laimer da Câmara Machado et al. (1992), but no further work has been published. In a previous work, we described an efficient adventitious shoot regeneration system from the mature seed hypocotyl segments of different apricot cultivars. Furthermore, we observed a chimeric shoot after preliminary *Agrobacterium*-mediated transformation experiments (Wang et al., 2011). However, transformed plants were not regenerated. In this report we describe the successful regeneration of transgenic apricot plants from hypocotyl segments of cv. 'Canino' seeds using our previously published regeneration procedure coupled with an aminoglycoside antibiotic selection strategy. Moreover, we report the regeneration of transgenic plants from hypocotyl segments of cv. 'Moniquí' using an herbicide selection strategy making use of the *bar* gene, encoding the enzyme phosphinothricin *N*-acetyltransferase (PAT) that confers tolerance to phosphinothricin (PPT) in transgenic plants (Miki and McHugh, 2004). PPT-tolerant plants containing the *bar* gene

* Corresponding author. Fax: +34 968 396213.
E-mail address: nalbur@cebas.csic.es (N. Alburquerque).
[1] These authors have contributed equally to this work.

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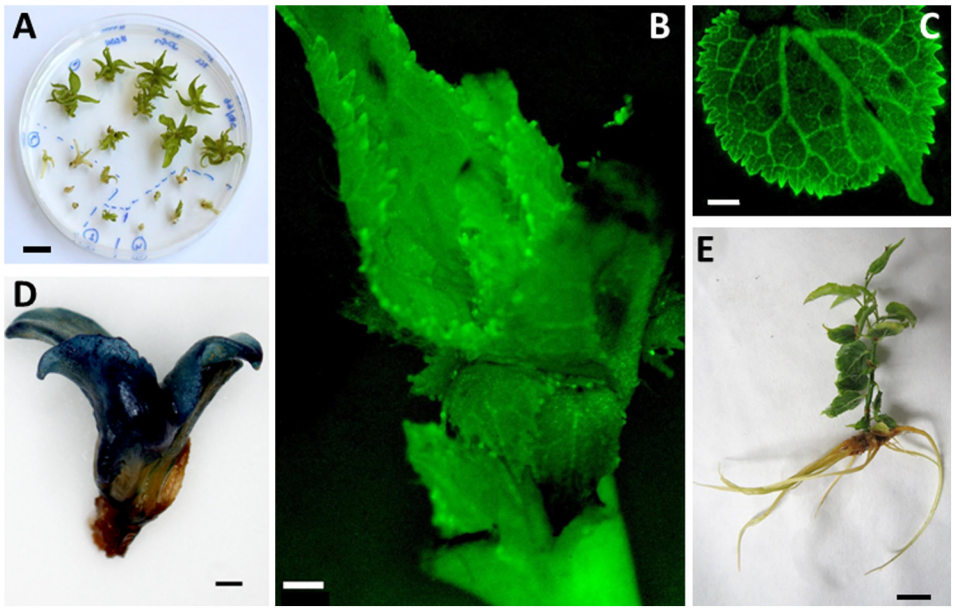

Fig. 1. Recovery, selection and rooting of transformed apricot buds. (A) Effect of kanamycin on regenerated buds placed on selective medium. GFP expression in shoot (B) and leaf (C) of a 'Canino' line transformed with the pMOG-*eyfp*-IV2 plasmid. (D) GUS detection on a 'Moniquí' bud regenerated after infection with the pFGC5941-*gus* plasmid. (E) Rooted transgenic 'Canino' shoot. Bars represent 10 mm in A, E and 1 mm in B–D.

have been deemed safe by various international government regulatory agencies for unconfined release and food and livestock feed use (AGBIOS, 2003: http://www.cera-gmc.org/GMCropDatabase accessed 27.05.15), and their use may facilitate new cultivars or rootstocks for commercialization.

The methodology described in this manuscript may be used not only to confirm the hypothesis of the study, but also for functional genomics studies, for the introduction of new traits that could be used further in conventional apricot breeding programs or for producing transgenic rootstocks, since most apricot cultivars are grafted onto apricot seedlings (Wang et al., 2011). Additionally, the methodology seems to be less genotype-dependent than the transformation of apricot leaves and might be transferable to several apricot genotypes.

2. Materials and methods

2.1. Plant material

Mature seed hypocotyl segments from the apricot cultivars 'Canino' and 'Moniquí' were used as the source of explants. Seed disinfection and explant preparation was performed according to previously published procedures (Wang et al., 2011). Briefly, seed kernels were immersed in a 1% sodium hypochlorite solution with 0.02% Tween-20 for 20 min and rinsed four times with sterile distilled water. Disinfected seeds were soaked in sterile water overnight at 4 °C and the seed coats removed with a scalpel. The radicle and the epicotyl were discarded, and the hypocotyl was sliced into three cross-sections (0.5–1 mm).

2.2. Agrobacterium tumefaciens *constructs*

The *A. tumefaciens* strain LB4404 (Hoekema et al., 1983), carrying the binary plasmids pMOG-*eyfp*-IV2 or pFGC5941-*gus*, was used in this study (Fig. 3B). Reporter genes in plasmids (*eyfp* or *gus*) contained an intron (IV2) derived from the potato (*Solanum tuberosum*) ST-LS1 gene (Eckes et al., 1986) in order to prevent bacterial expression.

A single colony of the engineered A. tumefaciens strains was inoculated into 10 ml of Luria–Bertani medium with 34.3 μM streptomycin sulfate and 83.3 μM KAN, and incubated overnight at 28 °C with constant agitation (175 rpm), reaching an $O.D._{600}$ of 0.2–1.0. The cultures were centrifuged at 3000 × *g* for 15 min and resuspended in 50 ml of bacterial infection medium [MS salts, 2% (w/v) sucrose, and 100 μM acetosyringone]. The culture in the bacterial infection medium was shaken (175 rpm) at 25 °C for 5 h before use.

2.3. Regeneration and transformation experiments

The shoot regeneration medium (SRM) consisted of 3/4-strength Murashige and Skoog (MS) salts, full-strength MS vitamins, 2% (w/v) sucrose, 0.7% (w/v) purified agar, 7.0 μM thidiazuron (TDZ) and 0.25 μM 3-indolebutyric acid (IBA), pH 5.8 (Wang et al., 2011). A direct regeneration pathway with no intermediary callus phase was observed.

The effect of aminoglycoside antibiotics on regeneration from apricot hypocotyls was previously studied for 'Canino' explants (Wang et al., 2011). The effect of PPT on adventitious regeneration from 'Moniquí' explants was determined by adding different concentrations (0, 5, 10.1, 15.1, 30.3, 50.5, 75.7, 100.9 or 151.4 μM) to SRM.

Hypocotyl segments from 'Canino' and 'Moniquí' seeds were cultured with the *Agrobacterium* suspension for 20 min. After infection, explants were positioned on SRM with 0.63 mM cefotaxime sodium (CEF) and 10 μM PAR or 75.7 μM PPT, and incubated in the dark at 23 ± 1 °C. After 1 week explants were transferred to a 16-h photoperiod (20–25 $\mu mol\ m^{-2}\ s^{-1}$, cool-white fluorescent lamp). Ten transformation experiments with a total of 461 explants of 'Canino' (aminoglycoside selection) were carried out. Transformation experiments with 'Moniquí' seeds applying 75.7 μM PPT for selection were repeated three times using a total of 185 explants.

2.4. Recovery of putative transgenic shoots

Bud clusters from 'Canino' and 'Moniquí' transformation experiments were isolated and placed on a specific medium for apricot meristem proliferation (MGM). Briefly, the medium consisted of