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Short communication

Gene stacking in 1-year-cycling *APETALA1* citrus plants for a rapid evaluation of transgenic traits in reproductive tissues

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ABSTRACT

Rapid flowering is crucial to perform functional genomic studies to investigate reproductive biology characteristics and fruit quality-related traits in fruit trees. However, long generation cycles of woody plants considerably delay this evaluation. Through genetic transformation, juvenile periods can be significantly shortened by overexpression of flower meristem-identity genes. Transgenic *APETALA1* (*AP1*) citrus plants behave as rapid-cycling trees, since 1-year-old seedlings promptly show precocious flowering and fruiting. By transgene stacking into these short-generation *AP1* and *nptII*/GUS-positive plants, expression of novel transgenes could theoretically be examined as quickly as 1 year after retransformation. Establishment of the selection and regeneration conditions for the production of retransformed individuals with marker genes is detailed in this communication. *Hpt* and *bar* genes were used as the second selectable marker genes. PCR and Southern blot analyses confirmed the recovery of retransformed shoots. *AP1* transcript accumulation and GUS and GFP expression were assessed in leaves, and flowers and fruit organs of rapid-cycling retransformed lines, respectively, as early as 1 year after plant generation and during three consecutive years, demonstrating that the principle of stable transgene stacking on early-fruiting transgenic trees is feasible.

1. Introduction

To gain full potential of genetic transformation as a tool to make reverse genetic studies on flower and fruit development and quality aspects in woody fruit plants with long juvenile periods, it would be necessary to be able to transform mature tissues from such species. However, transformation systems for mature tissues of fruit and forest trees are almost inexistent due to the low transformation and organogenic potential of the starting plant material, and when available, low transformation frequencies are generally reported (Peña and Séguin, 2001). Juvenile tissues are more amenable to tissue culture and gene transfer but transformed plants usually require from 4 to more than 20 years to start flowering, depending on the species.

It has been shown that over-expression in juvenile tissues of flowering meristem-identity genes such as *LEAFY* (*LFY*), *APETALA1* (*AP1*) or *FLOWERING LOCUS T* (*FT*) from *Arabidopsis thaliana* and homologues from other plants led to early flowering in hybrid aspen, citrange (*Citrus sinensis* L. Osb. × *Poncirus trifoliata* L. Raf.) and *P. trifoliata* (Weigel and Nilsson, 1995; Rottmann et al., 2000; Peña et al., 2001; Endo et al., 2005; Böhlenius et al., 2006). In some cases, ectopic transgene expression induced alterations in development and/or infertile flowers. Conversely, we showed that *AP1*-transgenic citrange plants were fully normal and produced fertile flowers that set fruits with seeds. These traits were transmitted to the apomictic progeny resulting in plants that kept a genetic background identical to that of the mother plant and had a generation time of about 1 year from seed to seed (Peña et al., 2001). Considering that citrange seedlings are transformable (Cervera et al., 1998), *AP1*-transgenic plants could be excellent materials for a rapid evaluation of flowering and/or fruit traits profiting the theoretical possibility of retransforming early-flowering genotypes with transgenes of interest.

With this objective, we attempted to establish a retransformation system for an *AP1*-citrange line. However, the choice of an inappropriate *AP1*-transformant as starting material and the use of non-fully fixed selection conditions for the second round of transformation resulted in recovery of retransformants at very low frequency in preliminary experiments (Cervera et al., 2006; our unpublished results). Given the potential outstanding possibilities of the strategy, we have decided to refine retransformation tools and conditions. Here we demonstrate that by: (1) selecting as source material a single-copy *AP1*-transformant exhibiting consistent *AP1* mRNA expression and conspicuous flowering every spring during at least five consecutive years, and (2) developing proper screening conditions to recover retransformants, the rapid-cycling

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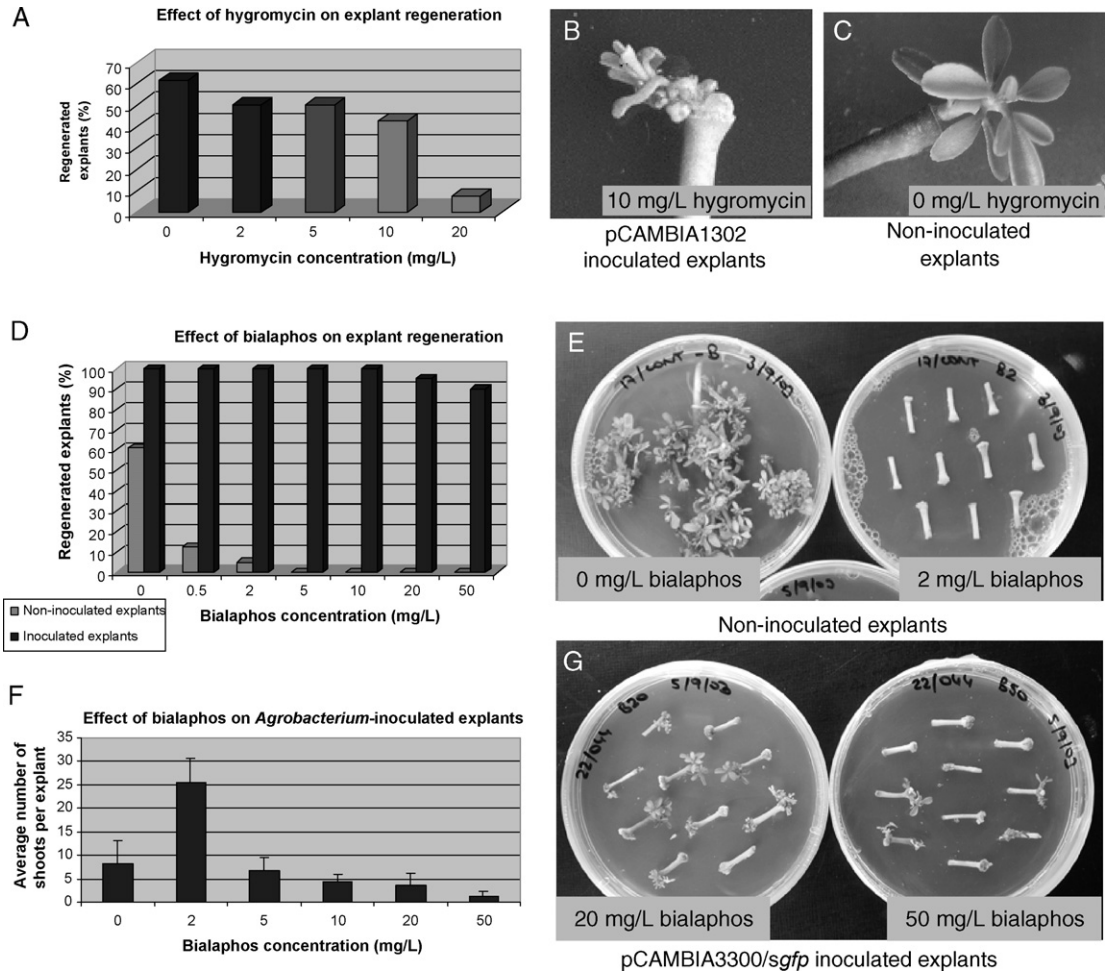

Fig. 1. Hygromycin and bialaphos regeneration tests. Forty Carrizo citrange epicotyl segments were cultured per treatment in cocultivation medium (CM) for 3 days, and then transferred to shoot regeneration medium (SRM) supplemented with 0, 2, 5, 10, or 20 mg/L of hygromycin (A) and 0, 0.5, 2, 5, 10, 20, or 50 mg/L of bialaphos (D) (Cervera et al., 1998; Peña et al., 2004). Shoot regeneration was examined 2–3 months after culture. The same data were taken after transformation of *AP1*-Carrizo citrange explants with *Agrobacterium tumefaciens* strain EHA105 carrying either pCAMBIA1302 or pCAMBIA3300/*sgfp*. Photographic records of shoot formation from non-inoculated (C and E) and inoculated (B and G) explants were captured. Effects of bialaphos on shoot regeneration (% regenerated explants (D) and average number of shoots per explant (F)) were also studied on *Agrobacterium*-inoculated explants. Inoculation was carried out by immersion of explants for 15 min in a suspension of *Agrobacterium* in inoculation medium and cultured on CM for 3 days and then on SRM plus 500 mg/L cefotaxime, 250 mg/L vancomycin and assayed concentrations of selective agent. Transgenic plant recovery was achieved by in vitro grafting and subsequent greenhouse grafting on vigorous rootstocks (Peña et al., 2004).

citrange tree becomes an excellent alternative to investigate flower and some fruit quality-related issues in citrus in a reasonably short period of time.

2. Results and discussion

2.1. Efficient stacking of transgenes in early-flowering citrange transformants

For retransformation experiments, *AP1*-transgenic line AP1-13.20 was selected, because it exhibited early and regular flowering each spring during five consecutive years, contained a single copy of the T-DNA (carrying *35S::uidA*, *Nos::nptII* and *35S::AP1* transgenes), and showed a good expression of the *AP1* transgene. T1 nucellar progeny was used as the source of seedlings for the new round of transformation and regeneration through organogenesis, and grafting in vitro of regenerated shoots served to recover whole plants (Cervera et al., 1998). Hygromycin phosphotransferase (*hpt*) and phosphinothricin acetyl transferase (*bar*) genes were chosen as selectable marker genes to be tested for transformation of AP1-13.20 seedlings, based on previous successful use of both marker genes in grapefruit and Ponkan mandarin transformation (Costa et al., 2002; Li et al., 2002, 2003).

In vitro regeneration experiments were performed to determine the minimal hygromycin and phosphinothricin concentrations needed to control organogenesis from citrange explants. In the case of hygromycin, regeneration was considerably restricted at a concentration of 20 mg/L (Fig. 1A). A slight toxicity was, however, noticed from a concentration higher than 10 mg/L, causing developmental anomalies in regenerated shoots (Fig. 1B and C). Due to this effect, concentrations of 10 mg/L hygromycin were initially tested for citrange transformation. Results from toxicity curve tests using bialaphos as a regeneration-controlling agent indicated that a concentration of 5 mg/L was the most appropriate for citrus transformation (Fig. 1D and E).

Retransformation experiments were performed using *Agrobacterium tumefaciens* strain EHA 105 carrying the binary plasmids pCAMBIA1302 (GenBank accession no. AF234298) or pCAMBIA3300/*sgfp* (Fig. 2A) and regeneration/selection media supplemented with 10 mg/L hygromycin or 5 mg/L bialaphos, respectively. Selection with hygromycin was effective although certain problems of toxicity were shown. Shoots arising from hygromycin-resistant explants were abnormal, showing thick leaves and reduced growth compared to control regenerants (Fig. 1B and C). After the first month of selection, antibiotic concentration was decreased to 5 mg/L in the following subcultures. Normal growth was restored