

High efficient transgenic plant regeneration from embryogenic calluses of *Citrus sinensis*

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Abstract

Transformation and high efficient regeneration of transgenic plants from embryogenic calluses of Bingtang sweet orange [*Citrus sinensis* (L.) Osbeck] was reported. Embryogenic calluses were inoculated with *Agrobacterium tumefaciens* strain EHA105, harboring the binary Ti plasmid pROK II and carrying a neomycin phosphotransferase II (NPTII) gene, an intron β -glucuronidase (GUS) gene and the *Arabidopsis APETALA1* (AP1) gene. Transformation treatment was with inoculation time of 30 min, co-culture of 3 d at 23 °C and supplementation of the co-culture medium with 2 mg dm⁻³ acetosyringone (AS). Kanamycin (50 mg dm⁻³) was effective to inhibit the growth of non-transformed calluses while it did not affect the transformed ones. The total number of transformed callus lines was 7 with 100 % embryo induction. High efficient regeneration of the transgenic embryos (88 % with 4 - 5 shoots per embryoid) was realized within 3 months. Integration of the transgene into the citrus genome was confirmed by histochemical GUS staining, polymerase chain reaction (PCR) analysis with AP1-specific primer and Southern blot hybridization with a 712 bp PCR fragment of AP1 as the probe.

Additional key words: *Agrobacterium tumefaciens*, Bingtang sweet orange, GUS, kanamycin, PCR, plantlet regeneration, Southern blot, transformation.

Introduction

Bingtang sweet orange [*Citrus sinensis* (L.) Osbeck] is one of the most favourite citrus cultivars in China. So far there is no report on genetic improvement of this cultivar. In the past decade, great progress has been made in citrus genetic transformation. Different kinds of explants including epicotyl segments (Moore *et al.* 1992, Gutiérrez *et al.* 1997, Pena *et al.* 2004), thin transversal epicotyl sections (Bespalkok Filho *et al.* 2003, Molinari *et al.* 2004), internodal segments (Pena *et al.* 1997, Perez-Molphe-Balch and Ochoa-Alejo 1998, Cervera *et al.* 2000, Ghorbel *et al.* 2000, Domínguez *et al.* 2004), protoplast (Fleming *et al.* 2000, Niedz *et al.* 2003, Guo *et al.* 2005) have been used for transformation and regeneration.

However, the efficiency of transformation and regene-

ration of transgenic citrus plants is still low. The genetically identical background of the embryogenic calluses and high efficient regeneration and rare occurrence of chimeras during regeneration are positive features of this transformation system (Li *et al.* 2002). Recently, the embryogenic callus of Bingtang sweet orange was established and conserved in our laboratory. The established embryogenic callus is a major potential advantage of transformation. Initial work with *Agrobacterium*-mediated transformation of various citrus cultivars suggests that transformation of such explant is possible (Li *et al.* 2002, 2003). In this paper, embryogenic callus of Bingtang sweet orange was used for genetic transformation for the first time and exhibited very well in both transformation and regeneration.

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Abbreviations: AS - acetosyringone; BA - 6-benzylaminopurine; Cef - cefotaxime; Cm - chloramphenicol; GUS - β -glucuronidase; Kin - kinetin; Km - kanamycin; LB medium - Luria-Bertani medium; MT - Murashige and Tucker; NAA - α -naphthaleneacetic acid; NPTII - neomycin phosphotransferase II; PCR - polymerase chain reaction.

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Materials and methods

Explant preparation: The embryogenic calluses of Bingtang sweet orange [*Citrus sinensis* (L.) Osbeck] were selected as explants in this study due to its short cycle of culture and high embryogenic potential. Embryogenic calluses were subcultured on solid Murashige and Tucker (1969, MT) basal medium with 40 g dm⁻³ sucrose (MTS). Before being inoculated with *Agrobacterium*, the calluses were cultured in liquid MT medium containing 0.5 g dm⁻³ malt extract, 1.5 g dm⁻³ L-glutamine, 50 g dm⁻³ sucrose, and 2 mg dm⁻³ acetosyringone (AS) for 4 d as previously reported (Li *et al.* 2002).

Bacterial strain and vector: *Agrobacterium tumefaciens* strain EHA105 harboring the binary plasmid pROKII kindly provided by Dr. Pena (Spain) was used as the

plasmid vector for transformation. Three gene cassettes in the T-DNA, 35s-API-ter, 35s-uidA (GUSINT)-ter and nos-NPTII-ter, served respectively as the target gene, the reporter gene and the selectable marker gene (Fig. 1). The intron inserted in the protein-coding region of the uidA gene prevents its expression in *A. tumefaciens* (Pena *et al.* 2001). A fresh single colony of disarmed *A. tumefaciens* strain EHA105 was selected and multiplied on solid Luria-Bertani (LB) medium with 30 mg dm⁻³ kanamycin (Km) and 50 mg dm⁻³ chloromycetin (Cm) for 48 h at 28 °C. Prior to infect the embryogenic calluses, the bacteria were transferred to liquid MT medium containing 2 mg dm⁻³ AS in an orbital shaker at 28 °C and 180 rpm for 2 h and then adjusted to an absorbance A₆₀₀ = 0.5 - 0.8.

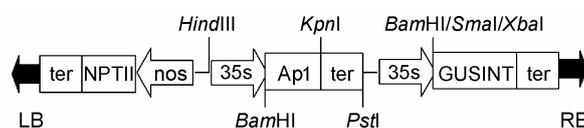


Fig. 1. Schematic representation of the T-DNA of pROK II with its position of different enzymes. RB - right border; LB - left border; nos - nopaline synthase promoter; NPTII - gene for neomycin phosphotransferase; ter - terminator of nopaline synthase; 35S - 35S promoter of cauliflower mosaic virus; AP1 - the *Arabidopsis* flowering regulating gene; GUSINT - GUS gene for β -glucuronidase with an intron.

Transformation and regeneration: Before transformation experiments, the effect of three concentrations of Km (0, 50, 100 mg dm⁻³) was evaluated on embryogenic calluses, embryo induction. The inoculation procedure of embryogenic calluses was according to Li *et al.* (2002) with minor modification. After a 3-d cocultivation period in the dark at 23 °C, the calluses were transferred to selection medium (MTS basal medium supplemented with 400 mg dm⁻³ cefotaxime (Cef) and 50 mg dm⁻³ Km), and subcultured every 4 weeks in darkness at 26 °C till resistant calluses formed. Then the resistant calluses were transferred to embryogenesis medium (EME500; MTS solid medium containing 500 mg dm⁻³ malt extract) containing both antibiotics to induce embryos under 14-h photoperiod at 26 °C and irradiance of 33 μ mol m⁻² s⁻¹. Resistant embryos were transferred to EME1500 medium (basal MTS solid medium containing 1500 mg dm⁻³ malt extract) containing both antibiotics to get enlargement and maturity. Then, the mature embryos were transferred to shoot regeneration medium [SRM; basal MT medium plus 0.5 mg dm⁻³ benzyladenine (BA), 0.5 mg dm⁻³ kinetin (Kin), 0.1 mg dm⁻³ α -naphthaleneacetic acid (NAA), and 30 g dm⁻³ sucrose] (Guo *et al.* 2002) with no antibiotics. For rooting, transgenic shoots were transferred on to root medium [RM; containing half strength MT medium supplemented with 0.5 mg dm⁻³ NAA, 0.1 mg dm⁻³ indolebutyric acid (IBA), 25 g dm⁻³

sucrose, 0.5 g dm⁻³ activated charcoal] containing 25 mg dm⁻³ Km or grafted onto rootstocks *in vitro* to produce whole transgenic plants.

Histochemical analysis by GUS assay: Transformed clones at different developmental stages including resistant calluses, globular embryos, shoots and leaves, were tested by histochemical staining for GUS activity. The tissues were immersed in the 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc) substrate solution based on Jefferson (1987) with minor modification (0.1 mg cm⁻³ Cm, 0.9 mg cm⁻³ X-gluc, 20 % methanol, 50 % H₃PO₄ buffer and 0.1 % Triton X-100, at pH 7.0) and incubated at 37 °C for 12 h. To select the putative transformants, histochemical analysis were performed at least three times at 30-d intervals.

PCR and Southern blot analysis: DNA was extracted from embryos originated from transformed and non-transformed calluses using the CTAB method. The 712 bp AP1 cDNA fragment primers were 5'-TAGA CAAGTGACATTCTCGA-3' and 5'-GAAGCAGCCAA GGTTCAGT-3'. Reactions were performed according to Pena *et al.* (2001), using 5 min of 94 °C and 35 cycles including 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C, plus a final step of 72 °C for 2 min. For Southern blot analysis, the DNA was extracted from the transformed shoots using CTAB method according to

Cheng *et al.* (2003). A total of 15 µg genomic DNA digested with *Hind* III (which has only one restriction enzyme site in the plasmid) was separated on a 0.8 % agarose gel, and transferred onto *Hybond-N⁺* blotting membrane under alkaline conditions. The membrane was

probed to a 712 bp specific PCR fragment of AP1 labelled with ³²P. To eliminate the endogenous gene disturbing, a 360 bp fragment homologous to AP1 cloned from citrus (not published) was used as another probe to hybridize with the same membrane.

Results and discussion

Three concentrations of Km (0, 50 and 100 mg dm⁻³) were used to determine the sensitivity of embryogenic calluses. Km in concentration 50 and 100 mg dm⁻³ could not inhibit the growth of embryogenic calluses completely for the first culture. However, the calluses could not grow further, turning brown and dying after 30 - 60 d of further culture on the same medium. Furthermore, the embryogenic calluses could not develop into embryos at 50 or 100 mg dm⁻³ Km (data not shown). Thus, the selection concentration of Km was set as 50 mg dm⁻³ for the whole process of regeneration.

Resistant calluses formed on selection medium after 1 to 2 months in darkness at 26 °C (Fig. 2A). Encouragingly, some little globular embryos also developed during this stage. Resistant calluses dedifferentiated into globular embryos and small embryos got enlarged on EME500 medium (Fig. 2B). Half to one month later, the total number of resistant embryos was 180, and globular embryos got matured when cultured on EME1500

medium in 1 to 3 months. Then, the mature embryos gradually grew into shoots on SRM without antibiotics (Fig. 2C), where the first shoot developed one month later and other shoots grew out within 3 - 6 months. The regeneration efficiency was 88 %. Elongated green shoots were subcultured on SRM containing 50 mg dm⁻³ Km and 200 mg dm⁻³ Cef to avoid the growth of escapes and persistence of *Agrobacterium tumefaciens*, respectively. Finally, transgenic shoots rooted on RM (Fig. 2D) or regenerated into plantlets by shoot - tip grafting onto rootstocks (Fig. 2E).

Positive detection of GUS gene was observed in resistant calluses, globular embryo, shoot and leaf (Fig. 3). Only those positive all the time in three repetitive GUS detections were considered as putative transformants, which were then proved by PCR analysis. By this method, 7 initial putative transformed callus clusters were produced, and 100 % of which formed embryos with 88 % conversion to shoots.

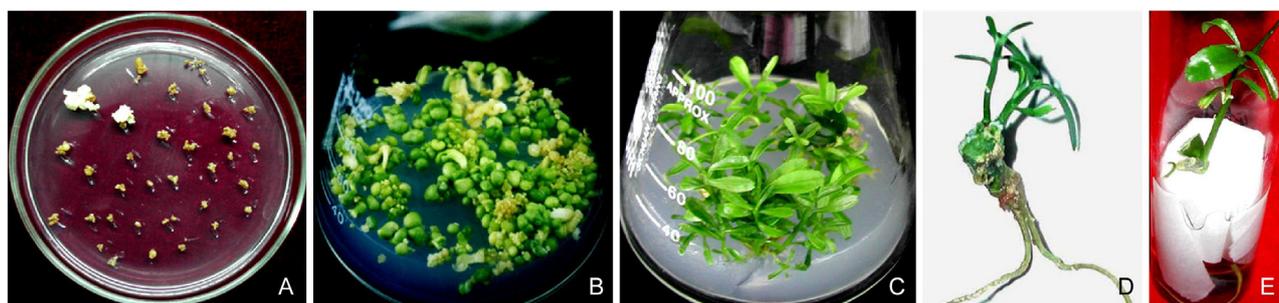


Fig. 2. Regeneration of transformed citrus embryogenic calluses: *A* - Resistant calluses grew out after being selected on selection medium for 6 weeks; *B* - Embryogenesis of transformed calluses on EME500 medium containing 50 mg dm⁻³ Km and 400 mg dm⁻³ Cef; *C* - Shoots regenerated on SRM medium free of Km; *D* - Root and shoot grew out of embryo on SRM medium containing 25 mg dm⁻³ Km and 200 mg dm⁻³ Cef; *E* - Shoot-tip grafting *in vitro* on trifoliolate orange rootstock (*Poncirus trifoliata*).

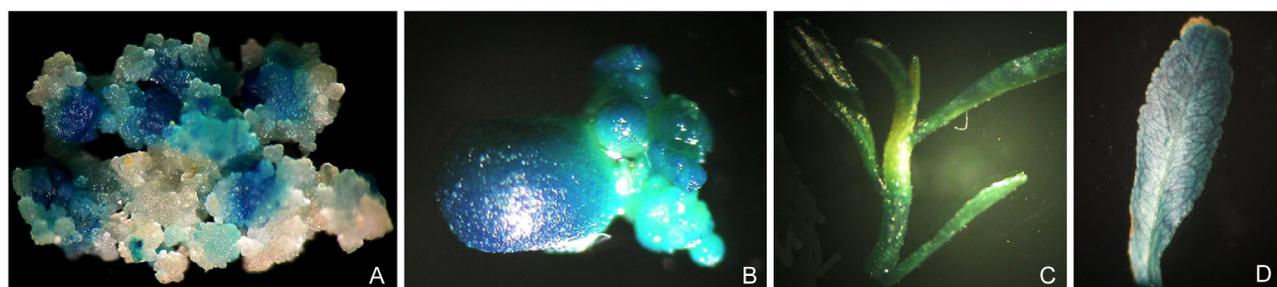


Fig. 3. GUS activity detection by histochemical analysis in different tissues of transformed citrus: *A* - Resistant calluses stained slightly; *B* - Globular embryos stained strongly; *C* - Shoot stained positively; *D* - Dark blue staining in leaf.

The 712 bp AP1 gene fragment was amplified from the embryos which were derived from the 7 initial putative calluses, and no band appeared from the negative control sample (Fig. 4), confirming our experience that explants positive all the time could be considered as putative transformants. For molecular analysis of stably transformed plants, Southern blot hybridization was conducted. DNA extracted from 4 PCR positive plants was digested with *Hind* III and hybridized to the probe specifically made from coding sequence of AP1 gene. One expected band (> 9 kb) appeared in all 4 transformants, while no band appeared in the non-transformed control when hybridization was performed under high stringent conditions (washing the hybridized membrane with $0.1\times$ SSC agitation for at least 2 h at 65 °C) (Fig. 5B). Bands in lane 5 and 6 were not strong probably due to the poor quality of DNA samples. It was reported that there is homologue of AP1 existing in citrus (Pillitter *et al.* 2003), thus a 360 bp fragment from citrus which has a high identity with *Arabidopsis* AP1 gene was cloned and used as a probe to hybridize with the same membrane as above mentioned. Two bands arose in the transformants and only one band in the negative control (Fig. 5A). One of the two bands, the same size (> 9 kb) as the first hybridization with the 712 bp AP1 probe, was the target gene AP1 transferred, and the other, comparatively small (< 6 kb), was the endogenous homologue to AP1, which meant there is homologue of AP1 in citrus indeed and the AP1 gene has been integrated into the citrus genome.

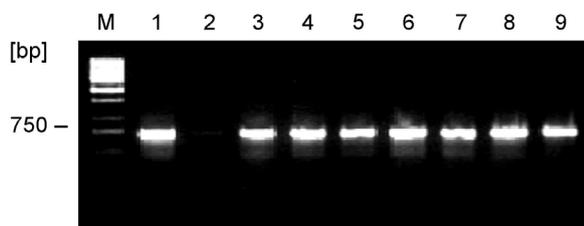


Fig. 4. PCR-amplified AP1 gene of 712 bp DNA from 7 transformed embryos. Lane M is 100 bp marker, lane 1 is plasmid, lane 2 is non-transformed control, lanes 3 - 9 are transformed embryos. The arrow indicates 750 bp fragment.

There are two difficulties existing in plant transformation. One is the high frequency regeneration of

escapes and chimeras under selection medium containing Km (Yu *et al.* 2002, Domínguez *et al.* 2004, Molinari *et al.* 2004), and the other is low efficiency of transgenic plant regeneration (Mitić *et al.* 2004, Molinari *et al.* 2004). Using an alternative selective agent or elongating the exposure time to Km, or enhancing the concentration of Km did not solve these two problems (Gutiérrez *et al.* 1997, Araújo *et al.* 2004). To get high efficient regeneration of transgenic plants as well as low frequency

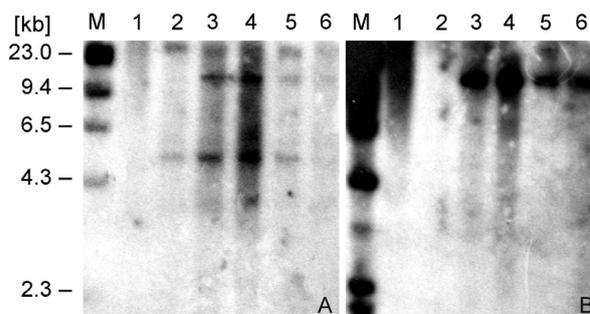


Fig. 5. Southern blot hybridization of one non-transformed control plant (lane 2) and four transformed plants (lanes 3 - 6). Genomic DNA extracted from leaves of transformed and untransformed plants was digested with *Hind* III. Lane 1 is plasmid pROKII digested with *Hind* III and lane M is Lambda DNA/*Hind* III marker. The probe was a 360 bp AP1-like gene DNA fragment cloned from citrus (A), and a 712 bp PCR fragment of AP1 gene (B).

of escapes and chimeras, the selection procedure such as suitable concentration and proper time of selective agent should be optimized (Mitić *et al.* 2004). In this study, Km at 50 mg dm^{-3} was found effective in regeneration of transgenic plants. Selection pressure was avoided during the transition from embryo to shoot, which significantly favours shoot formation and shortens the regeneration time. In conclusion, a large number of transgenic embryos and high efficient regeneration of transgenic shoots (88 % with 4 - 5 shoots per embryoid) was realized. It proved that citrus embryogenic calluses as transformation explants could convert high number of transformation events into regenerated plants efficiently. Right now, using the same system and explants, transgenic citrus plants are being obtained with other interest target genes in our laboratory (unpublished data).

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