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Multiple shoot induction from seedling epicotyls and transgenic citrus plant regeneration containing the green fluorescent protein gene

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ABSTRACT. This research aimed to optimize the organogenesis of epicotyl segments and to efficiently obtain transgenic plants of 'Bingtang' sweet orange (*Citrus sinensis* L. Osb.), an elite citrus cultivar in China. Organogenesis induction was induced in epicotyl segments of 2 weeks old seedlings of this cultivar. Two important factors influencing organogenesis in vitro viz. hormone combination (IAA and BA) and cut modes were characterized. IAA had a positive effect on bud formation only when BA was used at the concentration of 2.0 mg/l, and an inhibitive effect was observed with higher or lower concentration of BA. The number of regenerated buds reached up to 8.9 per explant with the combination of IAA 0.2 mg/l and BA 2.0 mg/l. Among cut modes, oblique cut performed the best for its effect on the number and quality of regenerated shoots and its convenience to manipulate. With the optimized hormone combination and oblique cut mode, citrus transformation with green fluorescent protein (GFP) gene was performed and twelve independently transformed plant lines were achieved. Southern blot hybridization confirmed the stable integration of GFP gene into the citrus genome. The successful transformation of this cultivar revealed that it is possible to introduce other genes with agronomic traits into it. Furthermore, these GFP expressing transgenic plants could serve as a visual marker material for citrus somatic fusion and sexual hybridization.

Keywords: Auxin; Citrus; Genetic transformation; Green fluorescent protein; Oblique cut; Shoot formation.

Abbreviations: **AS,** acetosyringone; **BA**, 6-benzyladenine; **Cef,** cefotaxime; ***GFP*,** green fluorescent protein gene; **Km,** kanamycin; **IAA**, indole-3-acetic acid; **LB**, (Luria-Bertani) medium; **MBI medium**, solid MT medium containing 2.0 mg/l BA, 0.2 mg/l IAA and 30 g/l sucrose; **MT medium,** Murashige and Tucker.

INTRODUCTION

Improvement of citrus by conventional method is hampered by polyembryony, sexual incompatibility and male or female sterility (Guo and Deng, 2001; Grosser and Gmitter, 2005). Genetic transformation is an alternative to overcome these difficulties. For successful transformation, regeneration of whole plants from the transformed cells is a prerequisite. Cut modes and hormones may affect in vitro citrus shoot regeneration. Transversal cut, the most popular cut mode (Moore et al., 1992; Pena et al., 2004), is simple to manipulate but produces the fewest adventitious buds. Longitudinal cut, a newly developed but infrequently used cut mode (Yu et al., 2002; Kayim et al., 2004), producing the most adventitious buds, is laborious and difficult to manipulate especially when the epicotyl explants are thin or weak. For hormones, the effect of auxin on shoot regeneration was rarely concerned, though the main hormone effect on bud formation was due to the addition of BA (Garcia-Luis et al., 1999). Till now, little was known on the effect of IAA and its interaction with BA in citrus regeneration.

The green fluorescent protein gene (*gfp*) from the jellyfish *Aequorea victoria* as a vital marker has attracted increasing interest and is considered to have several advantages over other visual marker genes (Ghorbel et al., 1999). Expression of GFP5 results in greatly improved levels of fluorescence and using the modified gene (mgfp5-ER) has potential to regenerate intensely fluorescent and fertile plantlets (Siemering et al., 1996; Haseloff et al., 1997). The goal of this study was: 1) to find a moderate and efficient cut mode and optimize a hormone combination for multiple shoot induction and regeneration of citrus seedling epicotyls, and 2) to rapidly achieve transgenic plants of an elite citrus cultivar with the GFP gene, which will be promising for further genetic transformation with agronomic traits.

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MATERIALS AND METHODS

Etiolated seedlings of 'Bingtang' sweet orange (*Citrus sinensis* L. Osb.) were used as explant sources. Seeds were decontaminated as described by Moreira-Dias et al. (2001) and germinated at 26°C in the dark in test tubes containing MT medium (Murashige and Tucker, 1969) with 25 g/l sucrose, 7.5 g/l agar, and pH 5.7. Epicotyl cuttings (1 cm long) from 2 weeks old seedlings were plated horizontally on the culture medium. Twenty-five explants were evenly distributed in 90 × 20 mm Petri plates containing approximately 20 ml medium. Hundred cuttings from 20 seedlings were measured per treatment with three repetitions. At the end of the incubation period (45 d), the number of visible buds and shoots were counted.

Effect of hormones on bud formation

The epicotyl explants were cut obliquely and incubated on solid MT medium (containing 30 g/l sucrose), supplemented with BA (1.0, 2.0, 3.0 mg/l) alone or combined with 0.2 mg/l IAA, in the dark/light condition (two weeks of darkness followed by 31 d in the light).

Effect of cut modes on the formation of buds and shoots

In addition to longitudinal and transversal cut modes, oblique cut was used in the present study. To manipulate the oblique cut mode, the epicotyls were cut obliquely with 3-4 mm end. Segment cuttings were incubated on MBI medium (solid MT medium containing 2.0 mg/l BA, 0.2 mg/l IAA and 30 g/l sucrose, PH 5.7) and cultured in the dark/light condition.

Transformation and regeneration

The disarmed *A. tumefaciens* strain EHA-105 harboring a binary vector plasmid pBin-mg*fp*5-ER (Haseloff et al., 1997) was used. Two experiments each with 60 epicotyl segments were performed. The transformation procedure was: a fresh single colony of disarmed *A. tumefaciens* strain EHA105 was selected and multiplied on solid LB (Luria-Bertani) medium with 50 mg/l kanamycin (Km) for 48 h at 28°C. The bacteria were collected, transferred to liquid MT medium in an orbital shaker at 28°C and 180 rpm for 2 h, and then adjusted to an absorbance A_{600} = 0.5-0.8. Epicotyl cuttings were infected with the adjusted *A. tumefaciens* for 30 min, and then dried by sterile filter paper and co-cultured on solid MT medium containing 2 mg/l AS (acetosyringone) for 3 days in the dark at 23 °C. After co-cultivation, the explants were screened on selective medium [MBI medium supplemented with cefotaxime (Cef) 400 mg/l and 50 mg/l Km] under dark/light condition. Explants were screened every four weeks on the same medium till resistant shoot regenerated. GFP expressing shoots were excised from the cut end and enlarged on the Km free (but containing Cef 200 mg/l) medium. Well-developed shoots were either induced to root as described by Guo et al. (2002) or grafted onto rootstocks to obtain whole transgenic plants as described by Deng et al. (1993).

Detection of GFP by fluorescence stereomicroscopy and molecular analysis

After co-cultivation, the explants were screened for GFP transient fluorescence and periodical examination was done under a fluorescent stereomicroscope equipped with a Leica fluorescence stereomicroscope (MZFLIII) comprising a 480/40 nm exciter filter, a 505 nm LP dichromatic beam splitter and a 510 nm LP barrier filter. Buds and shoots expressing green fluorescence were considered as putative transgenics.

Southern blot analysis was performed to confirm the stable integration of the *GFP* in the transgenic plants. DNA was extracted from leaves according to Cheng et al. (2003). Genomic DNA of three *GFP* expressing plants and one non-transformed plant was digested with *Eco*RI, separated on 0.8% (w/v) agarose gels and blotted onto nylon membranes (Hybond-N^+, Amersham). Films were probed with a P^{32}-labelled fragment of the *GFP* prepared by PCR. The PCR specific primers of *GFP* were PL: 5′-TGGCCAACACTTGTCACTAC-3′ and PR: 5′-AGGACCATGTGGTCTCTCTT-3′, which resulted in a 500 bp fragment amplified from the plasmid template. PCR reactions were the same as described by Shi et al. (2002).

RESULTS

Effect of growth regulators and cut modes on indirect bud and shoot organogenesis

Under dark/light condition, the buds differentiated from the callus formed at the cut end. Bud formation increased when BA concentration was enhanced (Figure 1). Meanwhile, the number of quiescent shoots regenerated increased. When combined with 0.2 mg/l IAA, the additive effect appeared at 2.0 mg/l of BA. The mean number of buds reached a maximum of 8.9 per explant, among which about five buds could elongate to shoots. Therefore, MBI medium was chosen as the optimal medium for use during transformation of 'Bingtang' sweet orange epicotyl explants.

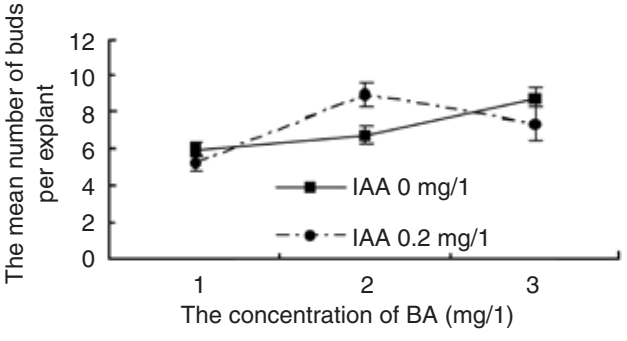

Figure 1. Influence of BA and IAA (mg/l) on adventitious bud formation in epicotyl segments of 'Bingtang' (*Citrus sinensis* L. Osb.) sweet orange.

Table 1. Effect of different cut modes on buds and shoots regeneration.

Different cut modes	Longitudinal cut	Oblique cut	Transversal cut
The mean number of buds per explant	19.5 a	8.9 b	2.5 c
The mean number of developed shoots with two leaves per explant	5.5 a	5.2 a	1.8 b

Data are the means of 100 epicotyl segments from three independent experiments. Numbers with same letters were not significantly different at α=0.05 according to Duncan's multiple range tests.

Concerning easiness of manipulation for different cut modes, oblique cut proved as simple as transversal cut but more efficient and convenient, longitudinal cut was difficult and laborious. Both oblique and longitudinal cuts could increase the wound area of epicotyl explants, resulting in more callus formation and shoot regeneration than transversal cut (Figure 2, A1-C3). Longitudinal cut gave the highest number of buds per explant followed by oblique cut and transversal cut (Table 1). However, most buds regenerated by longitudinal cut were weaker and smaller than those regenerated by oblique cut (Figure 2, C3). In addition, regarding shoots with at least two leaves regenerated, there was no significant difference between those produced by oblique and longitudinal cuts, while transversal cut still resulted in the least (Table 1). Taken together, oblique cut performed the best *in vitro*.

GFP as a visual marker to localize the sites of transgene expression at early stages after transformation

GFP expression could be detected transiently during co-cultivation. The cut end of the non-transformed and transformed epicotyl showed red autofluorescence (Figure 3A) and green fluorescence (Figure 3B), respectively. Four weeks later, that small green fluorescent buds sprouted and more than 2 transgenic buds expressing the GFP gene formed at one cut end were often observed (Figure 3C). Simultaneously, non-transformed shoots could also regenerate at the same cut end on selective medium (Figure 3E). Well-grown non-transformed shoots exhibited strong red fluorescence after one more month of growth on MBI medium (Figure 3D). GFP expressing shoots (longer than 0.5 cm) were physically separated in vitro and elongated on Km free medium (MBI medium containing 200 mg/l Cef) (Figure 3F). Transgenic plants recovered by rooting or by grafting onto trifoliate orange (*Poncirus trifoliata*) rootstock were transferred to soil in the greenhouse and showed normal growth like the controls after six months (Figure 4). Totally, twelve independent transgenic plant lines were obtained and transplanted; the ultimate transformation efficiency was 10%.

Different integration patterns of GFP

Genomic DNA from randomly selected *GFP* expressing

Figure 2. Comparison of three different cut modes on callus and bud formation with transversal cut (A1-A3), oblique cut (B1-B3) and longitudinal cut (C1-C3). A1, B1, C1, Callus formation after 2 weeks culture under darkness; A2, B2, C2, Bud formation one week after being transferred to light; A3, B3, C3, Shoot elongation under light conditions. Scale bars, 1.0 mm.

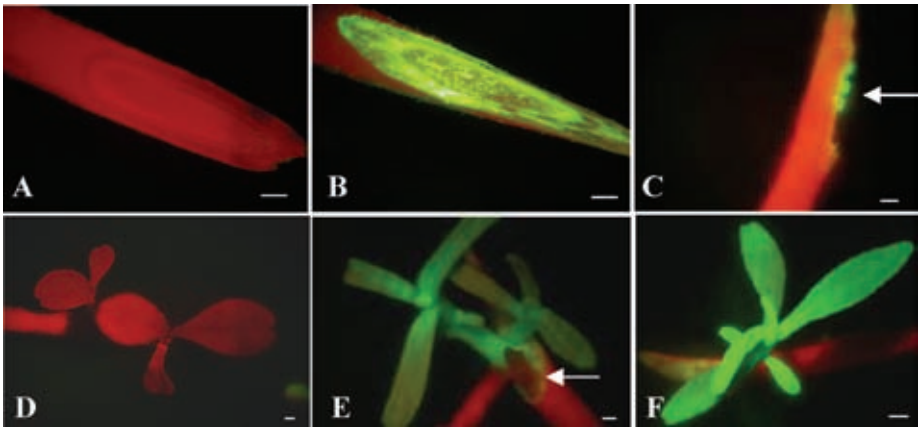

Figure 3. *GFP* expression in transformed and non-transformed epicotyl cut end, buds and shoots. A, Red fluorescence in non-transformed epicotyl cut end; B, Green fluorescence in transformed epicotyl cut end after co-cultivation; C, Three transformed buds sprouted along the cut end 4 weeks after cultivation on selective medium (arrow indicates the transformed buds); D, Non-transformed shoots with red fluorescence; E, Two transgenic shoots and one escape regenerated from the cut end 8 weeks after cultivation (arrow indicates the escape); F, Well-developed transgenic shoot with bright green fluorescence. Scale bars, 1.0 mm.

Figure 4. Transgenic citrus plant growing in the greenhouse.

plants and a non-transformed plant was analyzed by Southern blot. The GFP gene fragment (500 bp) was used as a probe to confirm the presence of the transgene. Different integration patterns in the transgenic plants, with one to 4 copies at different loci were identified when *Eco*RI (only one enzyme site in the T-DNA) was used. As was shown in Figure 5, all the three *GFP* expressing plants contained the target gene (Lanes G2, G3, and G5). No hybridization signal was detected in non-transformed control plant (Lane G28). No correlation between copy number and levels of *GFP* expression was revealed in this study.

DISCUSSION

Establishment of highly efficient regeneration protocol for existing cultivars will help to improve and broaden the usage of genetic transformation technique in citrus. In the present study, bud formation increased with the enhanced BA concentration, and similar results were previously demonstrated by several authors (Goh et al., 1995; Costa et al., 2004). However, IAA enhanced bud formation at 2.0 mg/l concentration of BA, and inhibited bud formation at 1.0 or 3.0 mg/l. This was different from previous report in which the contribution of auxins was marginal on bud formation (Garcia-Luis et al., 1999) and different from the result of Moreira-Dias et al. (2000). Using different citrus genotypes or different concentrations of hormones might be responsible for the different results. The results here show that an additive effect might have produced between BA and IAA when they were combined at the proper proportions. Such results have not been reported in citrus previously and need further validation. Though giving approximately same number of buds, elongated shoots produced by 3.0 mg/l BA were less than those produced by 2 mg/l of BAP + 0.2 mg/l of IAA (data not shown). This may be correlated with the concentration of BA: the lower concentration of BA, the larger the size of the shoots produced (Gutierrez-E et al., 1997).

Bud formation was significantly affected by different cut modes (i.e. transversal cut, oblique cut and longitudinal cut); and the number of regenerated buds increased with the enlarged cut area (Yu et al., 2002). In addition, transformation mostly occurred in dividing cells such as cambium callus formed at the cut ends of the explants (Cervera et al., 1998; Pena et al., 2004). Longitudinal and oblique cuts are more efficient than transversal cut in producing wound area and cambial callus, and should perform better in transgenic shoot regeneration. Several papers have clarified that longitudinal cut gave

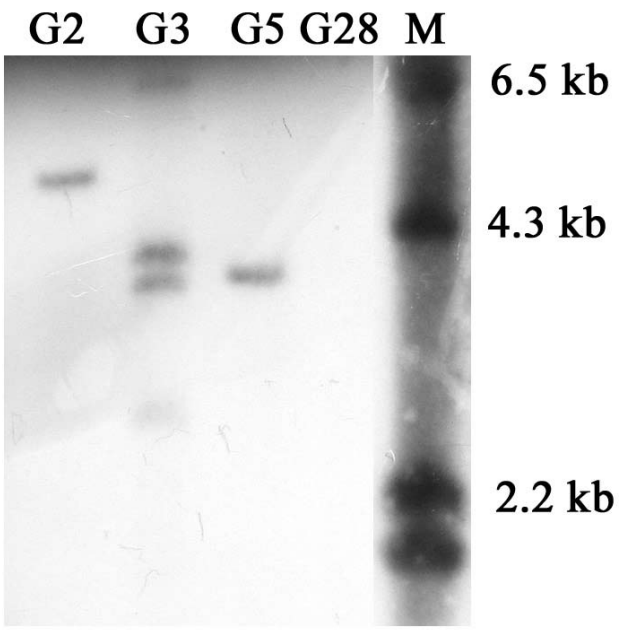

Figure 5. Southern-blot analysis of the *GFP* from three green fluorescent protein-expressing plants and one non-transformed plant. Genomic DNA digested with *Eco*RI, which has only one enzyme site in the plasmid. Lane G28, non-transformed plant; Lanes G2, G3, G5, *GFP* expressing plants; M, λ DNA/*Hind*III Molecular weight marker, Molecular weights are indicated in kilobases on the right.

higher transformation efficiency in Carrizo citrange than transversal cut (Yu et al., 2002; Kayim et al., 2004). However in this study, except for difficulty in manipulation, most buds regenerated by longitudinal cut remained quiescent and poor, which might be correlated with the nutrition competition between buds and shoots; the more buds formed, the fewer shoots elongated. Taken together, oblique cut performed the best in this study.

Citrus transformation based on the optimized regeneration protocol was successful, and totally twelve putative transgenic lines were obtained which grew normally in the greenhouse. The transformation efficiency of Bingtang sweet orange (*C. sinensis*) obtained in this study (10%) was much higher than that of 'Xuegan' (4.3%) (Yu et al., 2002) and Ridge pineapple (2%) (Gutierrez-E et al., 1997), lower than that of Hamlin (15%) (Mendes et al., 2002) and Valencia (23.8%) (Boscariol et al., 2003). These results revealed that the transformation efficiency was genotype-dependent, and the regeneration protocol in this study was suitable for transformation and it may be possible to introduce other agronomic traits into this elite cultivar. Using nptII as a selectable marker, more than 60% regenerated shoots were escapes (Costa et al., 2002; Almeida et al., 2003). Contrastingly, *GFP* as a non-destructive and vital marker gene could discriminate escapes and chimerical shoots at an early stage, thus saved time, significantly improved efficiency and expedited the molecular breeding process.

Transgenic citrus plants obtained in this study could serve as mesophyll parent (citrus mesophyll protoplasts are not regenerable) for protoplast isolation and then fused with protoplasts from embryogenic calluses of another parent using *GFP* as a visual marker. Somatic hybrid cells at an early developmental stage during somatic hybridization could be screened and monitored (Guo and Grosser, 2005). Furthermore, since most citrus species are polyembryonic, it is hard to obtain sexual hybrids by crossing; using transgenic citrus plants expressing the GFP gene as the pollen parent, sexual embryos will be easily detected and separated for further in vitro culture to recover sexual hybrid progenies facilitated by in vivo fluorescence of GFP expression. The transgenic plants expressing GFP gene produced herein are valuable materials for these studies in the future.

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柑橘實生苗上胚軸多叢芽誘導及轉綠色螢光蛋白基因植株之再生

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本研究的目的是對冰糖橙上胚軸再生芽的器官發生條件進行優化並且有效地獲得轉基因柑橘植株。採用 2 周苗齡的實生苗上胚軸進行器官誘導再生。通過對影響離體器官發生的兩個重要因素，即激素組合和切割方式進行分析，表明 IAA 在添加 BA 2.0 mg/l 時才促進芽的形成，低於或高於此濃度對芽的形成有抑制作用。在 IAA 0.2 和 BA 2.0 mg/l 組合下，再生芽數達到最多，平均每個外植體8.9個。在切割方式中，斜切方式在再生芽數和再生芽品質上都顯示了極好的效果。採用上面的激素組合結合斜切的方式，將綠色螢光蛋白（GFP）基因轉入冰糖橙上胚軸並獲得了 12 個株系的純合轉化植株。對轉基因植株進行 southern 雜交鑒定，表明 GFP 已經穩定整合到柑橘基因組中。該轉化的成功表明將具有經濟性狀的基因引入該品種是可行的，並且獲得的帶有 GFP 螢光的轉基因植株可以用於柑橘體細胞融合和有性雜交等基礎研究。

關鍵詞：生長素；柑橘；遺傳轉化；綠色螢光蛋白；斜切方式；芽的形成。