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MICROPROPAGATION

Improved protocol for the transformation of adult *Citrus sinensis* Osbeck 'Tarocco' blood orange tissues

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Abstract

The production of transgenic citrus plants from adult tissues is difficult because of low regeneration and transformation rates. To increase the transformation efficiency of adult citrus tissues, an improved protocol involving adult *Citrus sinensis* Osbeck 'Tarocco' blood orange tissues was developed. Explants were pre-incubated in a liquid medium prior to infection by *Agrobacterium tumefaciens*. Plant materials were also incubated on callus-induction medium supplemented with various combinations of cytokinin (Cyt) and kanamycin (Kan). An appropriate pre-incubation of the explants increased the transformation efficiency of adult tissues. During the callus-induction period, the Cyt type and Kan concentration had the largest and smallest effects on the transformation efficiency, respectively. The most effective combination of plant growth regulator and Kan for the transformation of 'Tarocco' blood orange tissues was 2 mg L^{-1} 2-isopentenyl adenine and 50 mg L^{-1} Kan. The transformation efficiency under the optimized conditions was 11.7%. A Southern blot analysis confirmed the integration of the transgene. These results indicated that the transformation efficiency of adult citrus tissues can be enhanced by optimizing the transformation conditions.

Keywords Citrus · Adult tissue · Transformation · 'Tarocco' blood orange

Introduction

Many explant sources have been used as target cells to incorporate foreign T-DNA into the citrus genome. These materials can be divided into two types, namely juvenile explants (Peña *et al*. 1997; Li *et al*. 2002; Peng *et al*. 2017) and adult explants (Cervera *et al*. 1998a, 2008; Marutani-Hert *et al*. 2012). The transformation of citrus species has been more successful when juvenile tissues are used as the starting material. However, transgenic citrus plants generated from juvenile explants have a long juvenile phase before they start to flower and produce fruits, which is detrimental for the rapid evaluation of horticulturally and/or commercially important traits introduced into transgenic citrus plants. Therefore, the use of adult tissues as the starting material is a more ideal option for the genetic improvement of citrus species. Unfortunately, because the regeneration and transformation potential is lower for adult citrus tissues than for juvenile tissues, the transformation of adult citrus material is problematic. Consequently, an efficient transformation procedure needs to be developed to enable researchers to introduce specific genes into adult citrus tissues and obtain a sufficient number of transgenic plants to evaluate the introduced traits. Over the past 2 decades, factors influencing the transformation of adult citrus material have been extensively investigated to enhance explant cell competence for the integration of T-DNA and organogenesis. For example, researchers have focused on the viability of re-invigorated adult materials (Cervera *et al*. 2008) as well as the effects of cultivating in darkness (Cervera *et al*. 2008; Marutani-Hert *et al*. 2012), various types of media (Cervera *et al*. 1998a, 2008; Marutani-Hert *et al*. 2012), the types and concentrations of plant growth regulators (Rodríguez *et al*. 2008; Fávero *et al*. 2012; Marutani-Hert *et al*. 2012), kanamycin (Kan) concentrations (Cervera *et al*. 2008), and citrus genotypes (Rodríguez *et al*. 2008). Although considerable efforts have gone into establishing an efficient transformation system for adult citrus tissues, the transformation efficiency remains relatively low.

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Methods for the transformation of adult citrus tissues generally include the following stages: invigoration of the starting materials, pre-incubation of explants, co-cultivation, callus induction, and shoot regeneration from the callus. Of these stages, the pre-incubation of explants is an optional step, but it helps increase the cell division rate (Peña *et al*. 2004) and transformation efficiency (Dutt and Grosser 2009). However, there is a lack of studies that have explored the effects of a pre-incubation step on the transformation efficiency of adult citrus tissues. During the callus-induction phase, plant growth regulators and Kan are used together to promote the proliferation of transformed cells. The application of 6-benzylaminopurine (6-BAP) alone or combined with 1-naphthaleneacetic acid (NAA) to regenerate shoots from adult tissues has resulted in various and even contradictory effects depending on the genotype used (Rodríguez *et al*. 2008). Marutani-Hert *et al*. (2012) reported that zeatin riboside (ZEA) is a more efficient alternative to 6-BAP and NAA for transforming sweet orange (*Citrus sinensis* Osbeck), grapefruit (*Citrus paradisi* Macf.), citron (*Citrus medica*), and citrange rootstock US-942 (*Citrus reticulata* 'Sunki' × *Poncirus trifoliata* 'Flying Dragon'). These results revealed the importance of using appropriate plant growth regulators during the transformation of adult citrus tissues. During the callus-induction period, Kan is required to prevent non-transgenic events, but high Kan concentrations restrict the regeneration of transgenic and non-transgenic cells. For the transformation of adult citrus tissues, 100 mg L^{-1} Kan is commonly used (Almeida *et al*. 2003; Fávero *et al*. 2012; Marutani-Hert *et al*. 2012). This concentration is also suitable for transforming juvenile citrus tissues (Cervera *et al*. 1998b; Fagoaga *et al*. 2001). However, Cervera *et al*. (2008) reported that Kan inhibits adventitious bud regeneration even at low concentrations in 'Clementine' mandarin (*Citrus clementina* 'Clemenules') adult tissues. On the basis of these results, it was speculated that pre-incubating explants prior to the infection by *Agrobacterium tumefaciens* and applying an appropriate cytokinin (Cyt) at a suitable concentration and an ideal Kan concentration during the callus-induction period may improve the transformation efficiency of adult citrus tissues. Therefore, in this study, we developed a method for enhancing the transformation potential of adult citrus tissues by investigating the effects of a pre-incubation period and various Cyt and Kan combinations on the transformation efficiency.

Materials and Methods

Plant materials Shoots from adult *C. sinensis* Osbeck 'Tarocco' blood orange trees were harvested in March at the National Citrus Germplasm Repository in Chongqing, China. Buds from the collected shoots were grafted onto greenhouse-grown juvenile citrange seedlings. In the following year, the newly sprouted first and second flushes (about 20 cm long) were used as the starting adult plant materials.

Bacterial strain and vector structure *Agrobacterium tumefaciens* strain EHA105A cells harboring the binary plasmid pGN-GUS:NPTII preserved in the laboratory (CRIC, Chongqing, China) were used for genetic transformations. From the right to left borders, the pGN-GUS:NPTII plasmid contained the fusion gene *GUS:NPTII* with a Cauliflower mosaic virus 35S promoter and a nopaline synthase terminator. The *GUS:NPTII* sequence served as the selectable marker and reporter gene.

Basic transformation protocol The flushes were stripped of their leaves, thorns, and tender apical parts. They were then disinfected with a 0.1% (*w/v*) HgCl solution for 10 min and rinsed four times with sterile water. All buds were removed from the flushes, after which the flushes were cut transversely into nodal segments (about 1 cm long). The nodal segments were subsequently immersed in an *A. tumefaciens* suspension (OD_{600} = 0.5) for 15 min and then dried with sterile tissue paper. The suspension was prepared as previously described by Dutt and Grosser (2009). The nodal segments were co-cultivated on solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 2 mg L^{-1} 2-isopentenyl adenine (2-IP), 2 mg L^{-1} indole-3-acetic acid (IAA), 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 100 μM acetosyringone, 30 g L^{-1} sucrose, and 8 g L^{-1} agar powder (TaKaRa, Dalian, China) for 3 d at 26°C in darkness. They were then transferred to a solid callus-induction medium containing either 1, 2, or 3 mg L^{-1} 6-BAP, 2-IP, or ZEA along with 25, 50, or 75 mg L^{-1} Kan, 0.5 mg L^{-1} NAA, 250 mg L^{-1} vancomycin (Van), 250 mg L^{-1} cefotaxime (Cef), 30 g L^{-1} sucrose, and 8 g L^{-1} agar powder. Samples were incubated for 14 d at 28°C in darkness. The explants were transferred to the bud-regeneration medium lacking plant growth regulators and Kan, but supplemented with 250 mg L^{-1} Van and 250 mg L^{-1} Cef. To regenerate shoots, the samples were incubated at 28°C in the MLR-350 growth chamber (Sanyo, Osaka, Japan) with a 16-h photoperiod and light intensity of 55 μmol m^{-2} s^{-1} white light (Philips, Amsterdam, Netherlands). Regenerants were recovered by *in vitro* grafting as described by He *et al*. (2011). In this study, the pH of the MS medium was adjusted to 5.8 with NaOH. Before use, the MS medium was sterilized at 121°C and 115 kPa for 20 min. The plant hormones and antibiotics were sterilized with a 0.22-μm Millipore Express® polyethersulfone membrane filter (Merck Millipore, Damstadt, Germany), and were added to the autoclaved MS medium. All chemicals used in this study were obtained from TaKaRa. Three independent experiments were conducted, with 300 to 400 explants per experiment.

Pre-incubation conditions Prior to the infection by *A. tumefaciens*, nodal segments were incubated in a liquid

MS medium containing 2 mg L^{-1} 2-IP and 2 mg L^{-1} IAA at 28°C on a rotary shaker (100 rpm) for 0, 3, 6, 9, or 12 h. The explants were then transformed as described above. The callus-induction medium consisted of solid MS medium containing 3 mg L^{-1} 6-BAP, 0.5 mg L^{-1} NAA, 50 mg L^{-1} Kan, 250 mg L^{-1} Van, 250 mg L^{-1} Cef, 30 g L^{-1} sucrose, and 8 g L^{-1} agar powder.

β-Glucuronidase activity in regenerants and explants The glucuronidase (GUS) activity was analyzed at 3–5 mo after the inoculation as described by Marutani-Hert *et al.* (2012). The basal ends of stems on the regenerated shoots were excised and treated with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid in a solution consisting of 100 mM NaH_2PO_4, 100 mM Na_2HPO_4, 0.5 mM $K_4[Fe(CN)_6]$, 0.5 mM $K_3[Fe(CN)_6]$, 10 mM EDTA-Na_2, and 1 g L^{-1} Triton™-X100. After an overnight incubation at 37°C, the samples were destained with anhydrous ethanol overnight at 37°C and then examined with a stereomicroscope (Leica Microsystems, Wetzlar, Germany). Shoots with blue-stained basal ends were considered transgenic and were grafted *in vitro* (He *et al.* 2011). After all regenerants were stained, the explant ends were cut and used to assess GUS activity. Each blue spot was considered to be an independent transgenic event. The mean number of blue spots per explant was calculated.

Molecular analysis of adult transgenic citrus plants A polymerase chain reaction (PCR) was completed to detect the transgene. Total DNA was extracted from the leaves of GUS-positive shoots with a plant DNA extraction kit (Aidlab, Beijing, China). The following primer pair was used to amplify the *GUS:NPTII* gene (predicted 1800-bp PCR product): 5′-AAGACGATCTACCCGAGCAATAA-3′ and 5′-CTAACCAAGAGCTTTAGCTTCAC-3′. The PCR solution comprised 1 μL total DNA, 10 μL 2× PCR mix (TaKaRa), 0.25 mM each primer, and double-distilled H_2O for a final volume of 20 μL. The PCR amplification was completed on a thermocycler (Biometra, Goettingen, Germany) with the following program: 94°C for 3 min; 30 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 1 min; 72°C for 7 min. The transformation efficiency was calculated by dividing the number of PCR-positive plants by the total number of inoculated explants.

For a Southern blot analysis, genomic DNA was digested with the *Eco*RI restriction enzyme (TaKaRa). A probe was amplified from the pGN-GUS:NPTII plasmid with the abovementioned primer pair to detect the *GUS:NPTII* insertion events. The Southern blot analysis was performed as described by Peng *et al.* (2015).

Data analysis Data were analyzed with the SPSS® 17.0 program (IBM, Armonk, NY) and are presented herein as the mean ± standard deviation. The significance of any differences was determined according to Tukey's test ($p < 0.05$).

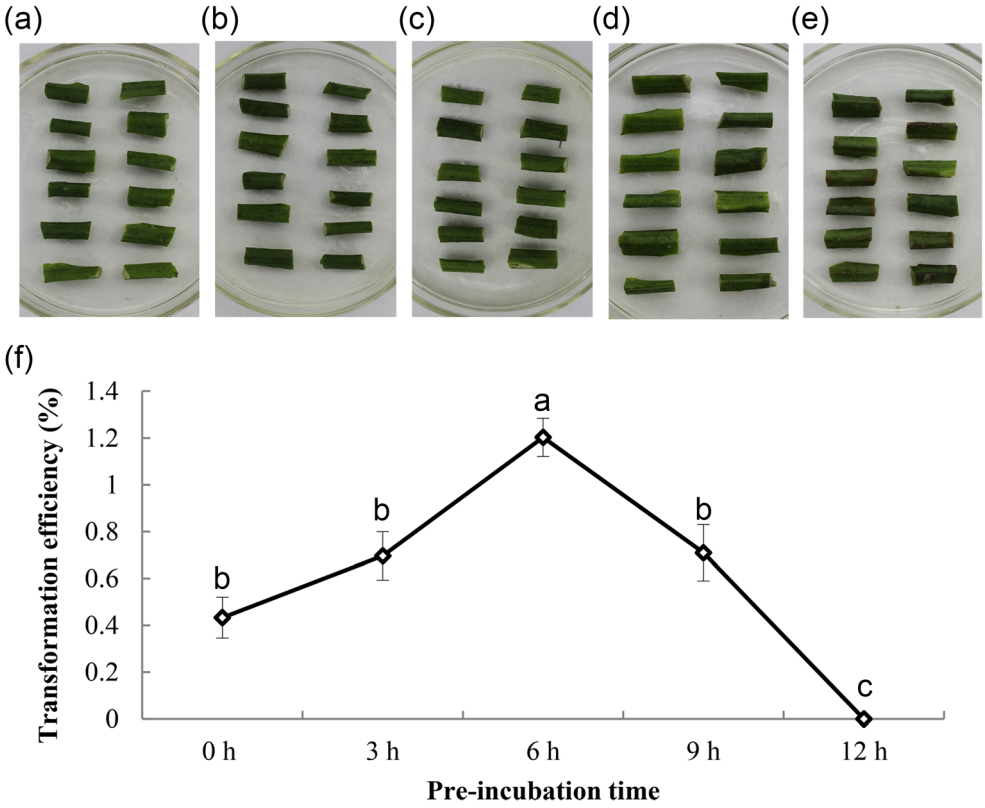

Figure 1. Effects of the pre-incubation time on the transformation efficiencies of adult *Citrus sinensis* Osbeck 'Tarocco' blood orange tissues. (*a*, *b*, *c*, *d*, and *e*) Explant performances with a pre-incubation time of 0, 3, 6, 9, or 12 h, respectively, at 3 d post pre-incubation. (*f*) Effects of the pre-incubation time on the transformation efficiencies of adult 'Tarocco' blood orange tissues. Data were collected at 3 to 5 mo after the pre-incubation of explants. Different letters represent significant differences among the tested pre-incubation times according to Tukey's test ($p < 0.05$).

Table 1. Various treatment combinations of cytokinin and kanamycin in the callus-induction medium for adult 'Tarocco' blood orange (*Citrus sinensis* Osbeck)

Treatments	RE (%)	TE (%)	BS
6-BAP + Kan (mg L^{-1})			
1 + 25	4.3 ± 1.2^{ij}	0.2 ± 0.2^{i}	0.2 ± 0.0^{jk}
1 + 50	1.3 ± 0.5^{j}	0.0 ± 0.0^{i}	0.1 ± 0.0^{k}
1 + 75	0.3 ± 0.5^{j}	0.0 ± 0.0^{i}	0.1 ± 0.0^{k}
2 + 25	3.3 ± 1.2^{j}	0.1 ± 0.1^{i}	0.4 ± 0.1^{fghijk}
2 + 50	3.0 ± 0.8^{j}	0.1 ± 0.1^{i}	0.3 ± 0.1^{ijk}
2 + 75	2.0 ± 0.5^{j}	0.0 ± 0.0^{i}	0.1 ± 0.1^{jk}
3 + 25	8.8 ± 0.1^{hij}	0.3 ± 0.2^{hi}	0.7 ± 0.2^{efgh}
3 + 50	11.3 ± 1.7^{hij}	0.4 ± 0.1^{hi}	0.4 ± 0.1^{ghijk}
3 + 75	3.6 ± 0.3^{j}	0.0 ± 0.0^{i}	0.1 ± 0.1^{k}
ZEA + Kan (mg L^{-1})			
1 + 25	30.2 ± 4.2^{efg}	1.6 ± 0.4^{fghi}	0.7 ± 0.2^{efgh}
1 + 50	24.9 ± 1.0^{defg}	1.0 ± 0.2^{ghi}	0.6 ± 0.2^{efghij}
1 + 75	18.0 ± 1.2^{efgh}	0.1 ± 0.1^{i}	0.3 ± 0.1^{hijk}
2 + 25	31.0 ± 1.2^{de}	3.9 ± 1.4^{cde}	1.4 ± 0.2^{bc}
2 + 50	17.1 ± 2.6^{fghi}	4.7 ± 0.4^{bc}	0.8 ± 0.1^{efg}
2 + 75	10.7 ± 2.9^{hij}	3.0 ± 0.8^{def}	0.4 ± 0.1^{fghijk}
3 + 25	4.2 ± 1.1^{ij}	1.8 ± 1.0^{fgh}	2.3 ± 0.4^{a}
3 + 50	3.6 ± 0.1^{j}	1.5 ± 0.4^{fghi}	1.2 ± 0.2^{cd}
3 + 75	0.9 ± 0.3^{j}	0.9 ± 0.3^{ghi}	0.5 ± 0.2^{efghijk}
2-IP + Kan (mg L^{-1})			
1 + 25	52.3 ± 0.8^{c}	4.1 ± 0.6^{cd}	0.6 ± 0.1^{efghi}
1 + 50	29.5 ± 3.6^{efg}	2.5 ± 0.6^{efg}	0.5 ± 0.1^{efghijk}
1 + 75	13.5 ± 2.6^{ghij}	0.6 ± 0.3^{hi}	0.2 ± 0.1^{jk}
2 + 25	123.5 ± 10.5^{a}	5.8 ± 0.8^{b}	1.2 ± 0.2^{cd}
2 + 50	74.7 ± 6.6^{b}	9.1 ± 1.5^{a}	0.8 ± 0.1^{ef}
2 + 75	64.4 ± 5.9^{bc}	6.1 ± 1.6^{b}	0.3 ± 0.1^{hijk}
3 + 25	35.6 ± 2.8^{e}	2.7 ± 1.0^{def}	1.7 ± 0.3^{b}
3 + 50	19.0 ± 4.5^{efgh}	3.5 ± 1.0^{cde}	0.9 ± 0.2^{de}
3 + 75	10.7 ± 6.0^{hij}	1.4 ± 0.9^{fghi}	0.6 ± 0.2^{efghij}

Data were calculated 3 to 5 mo after infection by *Agrobacterium tumefaciens*

Different *letters* represent significant differences among the tested conditions according to Tukey's test ($p < 0.05$)

RE, regeneration efficiency; *TE*, transformation efficiency; *BS*, number of blue spots per explant; *2-IP*, 2-isopentenyl adenine; *6-BAP*, 6-benzylaminopurine; *ZEA*, zeatin; *Kan*, kanamycin

Results

Effects of the pre-incubation time on the transformation efficiency of adult citrus tissues To increase the transformation efficiency of adult 'Tarocco' blood orange tissues, the explants were treated for various pre-incubation periods before infection by *A. tumefaciens*. The duration of the pre-incubation period (0, 3, 6, 9, or 12 h) in a hormone-rich liquid medium influenced the responses of adult citrus tissues. The 'Tarocco' blood orange explants tolerated a pre-incubation period of 0, 3, and 6 h (Fig. 1*a*, *b*, *c*). However, if the pre-incubation period exceeded 6 h, some of the 'Tarocco' blood orange explants turned brown and finally died during the following transformation (Fig. 1*d*, *e*). The data revealed the transformation efficiency following a 6-h pre-incubation period was significantly different from that after a 0-, 3-, or 9-h pre-incubation period (Fig. 1*f*). Thus, a 6-h pre-incubation step prior to the transformation was sufficient to enhance the transformation efficiency of adult 'Tarocco' blood orange tissues.

Optimized combinations of Cyt and Kan in the callus-induction medium To optimize the production of adult transgenic citrus plants, the effects of the following three factors were investigated: Cyt type, Cyt concentration, and Kan concentration. The Cyt and Kan combinations in the callus-induction medium are listed in Table 1. When the explants were incubated in darkness, callus tissue formed at the cut ends. The Cyt and Kan combinations affected the induction of the 'Tarocco' blood orange callus at 14 d after the co-cultivation. When the same Cyt and Kan concentrations were used, the callus formed better with the 2-IP (Fig. 2*a*) and ZEA (Fig. 2*b*) treatments than with the 6-BAP (Fig. 2*c*) treatment. Additionally, the shoots gradually regenerated from the callus tissue after the explants were exposed to a 16-h photoperiod (Fig. 2*d*, *e*, *f*).

The regeneration efficiencies were determined at 3 mo after the inoculation. The regeneration was relatively poor when explants were cultured on the callus-induction medium supplemented with 6-BAP or ZEA along with Kan (Table 1; Fig. 3*a*, *b*). However, adventitious buds were highly induced by the 2-IP and Kan combinations (Table 1; Fig. 3*c*). Moreover, each tested factor and the interaction between two or three factors had a significant effect on the regeneration efficiency (Table 2). The Cyt type and Kan concentration had the largest and smallest effects on the regeneration efficiency, respectively (Table 2). The highest regeneration efficiency was recorded for the explants cultured on the callus-induction medium supplemented with 2 mg L^{-1} 2-IP and 25 mg L^{-1} Kan (Table 1; Fig. 3*c*).

The transformation efficiency was calculated at 4 to 5 mo after the inoculation. Specifically, the effects of the three analyzed factors on the transformation efficiency were evaluated by an analysis of variance (ANOVA). Each of the three factors as well as the interactions between two of the three factors significantly influenced the transformation efficiency (Table 2). Of these three factors, the Cyt type and Kan concentration had the largest and smallest effects on the transformation efficiency, respectively (Table 2). Of the 6-BAP, ZEA, and 2-IP treatments during the callus induction step, 2-IP represented the best Cyt type for the transformation of 'Tarocco' blood orange adult tissues (Table 1; Fig. 3*d*, *e*, *f*).

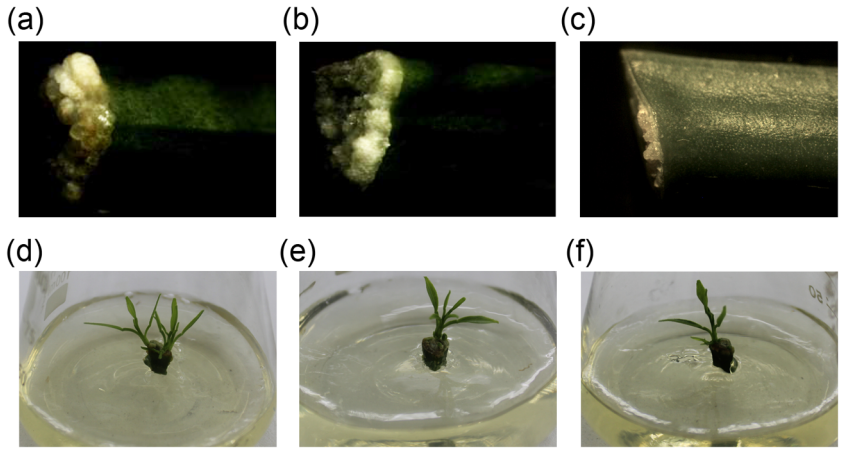

Figure 2. Callus development and adventitious growth at the cut ends of adult *Citrus sinensis* Osbeck 'Tarocco' blood orange tissues. (*a*, *b*, and *c*) The callus developed from the adult explants of 'Tarocco' blood orange at 14 d after the co-cultivation step. (*d*, *e*, and *f*) Adventitious growth of adult 'Tarocco' blood orange at 1 mo after the callus induction step. (*a* and *d*), (*b* and *e*), and (*c* and *f*) present the growth of 'Tarocco' blood orange cultured on 2 mg L^{-1} 2-isopentenyl adenine, zeatin, and 6-benzylaminopurine, respectively, along with 50 mg L^{-1} kanamycin.

When samples were treated with 2 mg L^{-1} 2-IP and 50 mg L^{-1} Kan, the transformation efficiency of the 'Tarocco' blood orange was 9.1, which was significantly higher than that for the other combinations of 6-BAP, ZEA, or 2-IP and Kan (Table 1; Fig. 3*d*, *e*, *f*). These results suggested that the compositions of Cyt and Kan in the callus-induction medium significantly influenced the transformation efficiency of adult citrus tissues.

Occurrence of transgenic events To investigate the transformation potential of adult 'Tarocco' blood orange tissues, the occurrence of transgenic events was investigated. The GUS activity assay revealed blue spots at the cut end of explants (Fig. 4*b*). The ANOVA indicated that the Kan concentration, Cyt concentration, Cyt type, and random two-factor interactions significantly affected the number of blue spots per explant. The Kan and Cyt concentrations had the greatest and least impacts, respectively (Table 2). The mean number of blue spots per explant varied from 0.1 ± 0.1 to 2.3 ± 0.4, depending on the Cyt and Kan combinations (Table 1; Fig. 3*g*, *h*, *i*). Compared with the transformation efficiency, the occurrence of transgenic events was significantly higher, which indicated the decreased ability of the callus to induce shoot production.

Transformation efficiency On the basis of the abovementioned results, the optimal pre-incubation period and callus-induction medium composition were combined to develop an efficient protocol for transforming adult 'Tarocco' blood orange tissues. This protocol included a 6-h pre-incubation step in liquid medium containing 2 mg L^{-1} 2-IP and 2 mg L^{-1} IAA. The pre-incubated explants were then transformed. During the callus-induction phase, the explants were incubated on solid MS medium supplemented with 2 mg L^{-1} 2-IP, 50 mg L^{-1} Kan, 0.5 mg L^{-1} NAA, 250 mg L^{-1} Van, 250 mg L^{-1} Cef, 30 g L^{-1} sucrose, and 8 g L^{-1} agar powder. Under these culture conditions, the average transformation efficiency for three independent experiments was 11.7% after the GUS activity analyses and PCR detection.

Molecular confirmation of adult transgenic citrus plants To confirm the transgene integration in PCR-positive plants, 10 PCR-positive plants were analyzed in a Southern blot (Fig. 4*c*). The *GUS:NPTII* signal was detected in all of the tested plants, whereas no signal was detected in the wild-type plants (Fig. 4*d*). These observations implied the *GUS:NPTII* fusion gene was stably integrated into the citrus genome.

Discussion

An effective transformation procedure is crucial for generating transgenic citrus plants. Although studies involving the transformation of adult citrus tissues have been conducted for nearly 2 decades, the transformation efficiency remains very low. To enhance the transformation competence of adult citrus materials, an improved protocol was developed by optimizing the pre-incubation time and the composition of the callus-induction medium. This improved protocol increased the transformation efficiency of adult 'Tarocco' blood orange tissues.

The success of T-DNA transfer and integration is likely dependent on the host cell division cycle, with only cells undergoing DNA replication able to integrate foreign T-DNA (Villemont *et al*. 1997). The pre-incubation of explants prior to the infection by *A. tumefaciens* can stimulate cell dedifferentiation and division (Ghorbel *et al*. 2000). These treated cells can then be used for transformation experiments, in which they exhibit an increased ability to integrate foreign T-DNA, thereby resulting in increased transformation efficiencies (Ainsley *et al*. 2001; Dutt and

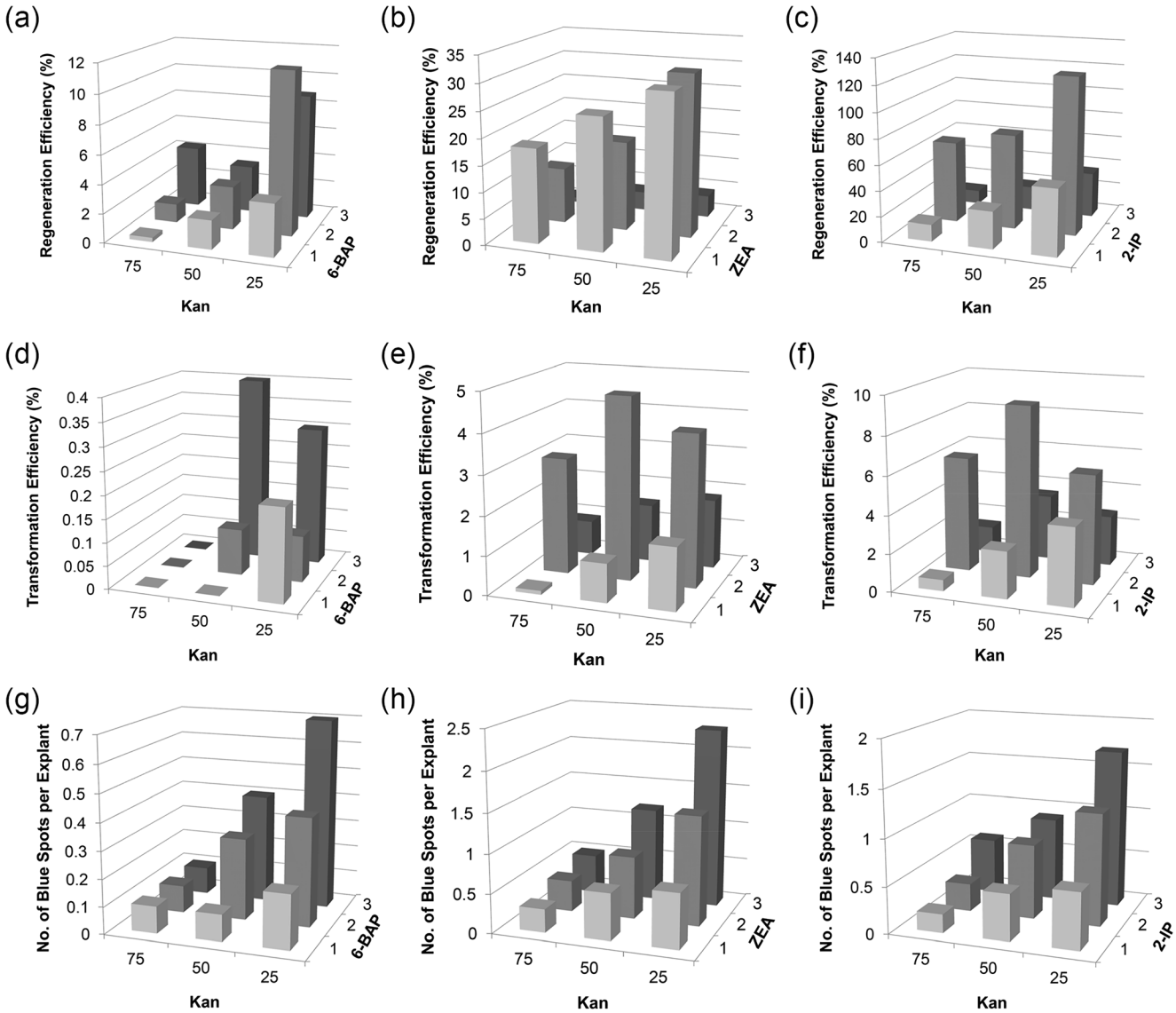

Figure 3. Effects of 1, 2, or 3 mg L^{-1} 2-isopentenyl adenine (2-IP), 6-benzylaminopurine (6-BAP), and zeatin (ZEA) as well as 75, 50, or 25 mg L^{-1} kanamycin (Kan) on the (*a*, *b*, and *c*) regeneration efficiency, (*d*, *e*, and *f*) transformation efficiency, and (*g*, *h*, and *i*) number of *blue spots* per explant of adult *Citrus sinensis* Osbeck 'Tarocco' blood orange tissues.

Grosser 2009). The transformation competence of many plants has been enhanced based on this strategy (Jacq *et al*. 1993; Ding *et al*. 2009; Yasmeen 2009). In this study, a limited explant pre-incubation period increased the transformation efficiency of adult 'Tarocco' blood orange tissues. However, a prolonged pre-incubation period adversely affected the physiology of the explants (Fig. 1). Dutt and Grosser (2009) reported that there are no significant differences among juvenile citrus explants pre-incubated in a hormone-rich liquid medium for 3 or 6 h, or even overnight. The adult cells at the cut ends of explants may produce more phenolic compounds than juvenile cells. These results suggest that the pre-incubation period for the transformation of adult citrus tissues should be optimized to enhance the transformation efficiency.

A balanced composition of plant growth regulators is important for inducing the proliferation of transformed cells, which is favorable for transgenic events. The presence of Cyt in the callus-induction medium promotes cell division and callus formation. Unlike previously described procedures in which 6-BAP (Cervera *et al*. 1998a, 2008; Fávero *et al*. 2012) was used to induce the proliferation and regeneration of transformed cells, we determined that among the three tested Cyt types (6-BAP, ZEA, and 2-IP), 2-IP had the greatest potential to induce the proliferation of transgenic cells during the callus-induction phase. A previous study concluded that the addition of 2 mg L^{-1} 2-IP to the co-cultivation medium improves the transformation of adult 'Bingtang' orange (*C. sinensis* Osbeck) tissues (Yang *et al*. 2009). Our results are consistent with these earlier findings. Specifically,

Table 2. An ANOVA analysis for the effects of kanamycin concentration, cytokinin concentration, and cytokinin type on the regeneration efficiency, transformation efficiency, and number of blue spots per explant of adult *Citrus sinensis* Osbeck 'Tarocco' blood orange tissues

Source	Dependent variable	Type III sum of squares	Degree of freedom	Mean square	*F* value	*p* value
Corrected model	RE	61798.361a	26	2376.86	133.014	0
	TE	426.285b	26	16.396	22.604	0
	BS	21.779c	26	0.838	20.029	0
A	RE	4877.735	2	2438.868	136.484	0
	TE	20.999	2	10.499	14.475	0
	BS	7.117	2	3.559	85.086	0
B	RE	9352.212	2	4676.106	261.684	0
	TE	104.204	2	52.102	71.833	0
	BS	4.331	2	2.165	51.773	0
C	RE	26506.344	2	13253.17	741.674	0
	TE	201.814	2	100.907	139.119	0
	BS	5.869	2	2.934	70.165	0
A × B	RE	778.506	4	194.627	10.892	0
	TE	11.395	4	2.849	3.927	0.007
	BS	1.877	4	0.469	11.218	0
A × C	RE	3840.137	4	960.034	53.725	0
	TE	11.451	4	2.863	3.947	0.007
	BS	1.3	4	0.325	7.77	0
B × C	RE	15717.116	4	3929.279	219.89	0
	TE	65.369	4	16.342	22.531	0
	BS	0.744	4	0.186	4.448	0.004
A × B × C	RE	726.31	8	90.789	5.081	0
	TE	11.055	8	1.382	1.905	0.078
	BS	0.541	8	0.068	1.618	0.141
Error	RE	964.94	54	17.869		
	TE	39.168	54	0.725		
	BS	2.258	54	0.042		
Total	RE	103012.009	81			
	TE	806.864	81			
	BS	57.244	81			

A, B, and C represent the kanamycin concentration, cytokinin concentration, and cytokinin type, respectively

RE, regeneration efficiency; *TE*, transformation efficiency; *BS*, number of blue spots per explant

2 mg L^{-1} was the optimal 2-IP concentration in the callus-induction medium for the transformation of adult 'Tarocco' blood orange tissues (Table 1). These observations have expanded the types of Cyt that can be used for callus induction and suggest the importance of using the appropriate Cyt at ideal concentrations to increase the transformation efficiency of adult citrus tissues.

Kan was used as the selective agent to inhibit non-transgenic events. However, high Kan concentrations also restrict the regeneration of transgenic cells, resulting in decreased transformation efficiencies. In this study, 50 mg L^{-1} Kan was sufficient to balance the growth of non-transgenic and transgenic cells during the transformation of adult 'Tarocco' blood orange tissues.

In the present study, most of the transgenic cells exhibiting GUS activity lacked morphogenetic ability and failed to generate a complete transgenic event. This suggested that the low transformation efficiency of adult citrus tissues may be primarily due to the low regeneration ability of the transformed cells. This phenomenon is consistent with the findings of Cervera *et al.* (2008), who reported a low transformation frequency relative to the mean number of blue spots per explant. Growth regulators and Kan were not used in the regeneration medium after a 14-d incubation of explants in darkness. This method can produce strong regenerated shoots and lead to increased survival rates during *in vitro* grafting. However, it may not facilitate the regeneration of transformed cells, which requires growth

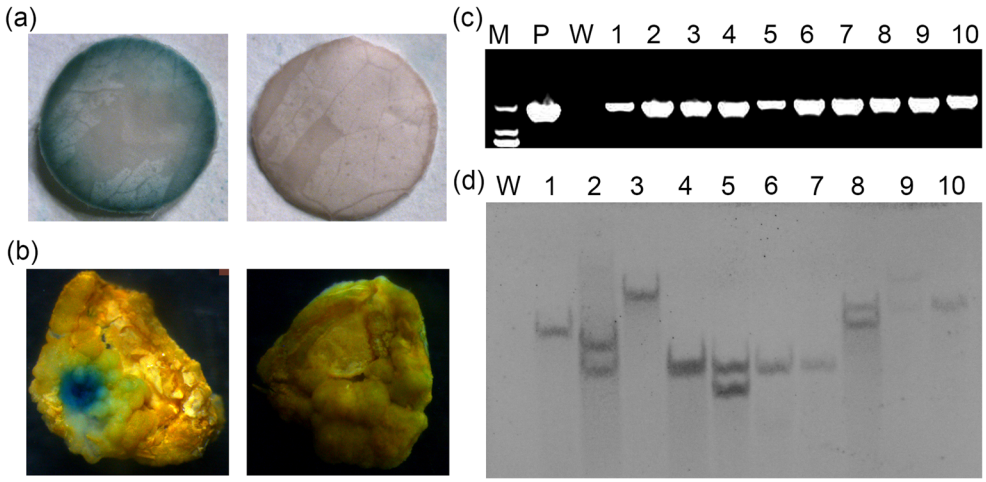

Figure 4. Analysis of *Citrus sinensis* Osbeck 'Tarocco' blood orange transgenic events. (*a*) GUS activity assay of a leaf disc from transgenic plants. The left and the right panels present the leaf discs from transgenic and wild-type plants, respectively. (*b*) GUS activity assay of the cut ends of explants. A *blue spot* represents a transgenic event (*left*). No transgenic events were detected for the wild-type plants (*right*). (*c*) PCR analysis of the *GUS-NPTII* fusion gene. M, marker; P, plasmid; W, wild-type; *lanes 1–10*, GUS-positive plants. (*d*) Southern blot analysis of transgenic plants. W, wild-type; *lanes 1–10*, corresponding PCR-positive plants in (*c*).

regulators. The application of low concentrations of auxin and Cyt in the regeneration medium may stimulate adventitious shoot formation (Almeida *et al*. 2003). Additionally, using Kan as the selective agent in the callus-induction medium does not completely prevent the replication of non-transformed cells at the cut end of explants (Domínguez *et al*. 2004). The early removal of Kan from the regeneration medium results in the competitive growth of transformed and non-transformed cells. Gradually decreasing the concentration of plant growth regulators and Kan until they are completely eliminated from the regeneration medium has been an effective method for producing transgenic shoots (Fávero *et al*. 2012). Therefore, additional research is required to determine the optimal concentrations of growth regulators and Kan in regeneration media, which may further increase the transformation efficiency.

Conclusions

The results of this study confirm that a pre-incubation step and the optimized combinations of Cyt and Kan in the callus-induction medium affect the efficiency of T-DNA transfer. Therefore, the transformation efficiency of adult citrus tissues can be improved by modifying the transformation protocol.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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<tr><td>联系人</td><td colspan="4">彭爱红（联系电话：13883578451）</td></tr>
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作者: Peng, Aihong; Zou, Xiuping; Xu, Lanzhen; 等.
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					小类	植物科学	3 区			
					小类	细胞生物学	3 区			

附件 2：SCI 检索结果记录

第 1 条，共 1 条

标题: Improved protocol for the transformation of adult Citrus sinensis Osbeck 'Tarocco' blood orange tissues

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