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ORIGINAL PAPER

Highly efficient transformation of the *GFP* and *MAC12.2* genes into precocious trifoliate orange (*Poncirus trifoliata* [L.] Raf), a potential model genotype for functional genomics studies in *Citrus*

Bin Tan · Ding-Li Li · Shi-Xiao Xu · Gai-En Fan · Jing Fan · Wen-Wu Guo

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Abstract Precocious trifoliate orange (*Poncirus trifoliata* [L.] Raf), an extremely early flowering mutant of *P. trifoliata*, is an attractive model for functional genomics research in *Citrus*. A procedure for efficient regeneration and transformation of this genotype was developed by using green fluorescent protein (GFP) gene as visual marker and etiolated stem segments as explants. In vivo monitoring of GFP expression permitted a rapid and easy discrimination of transgenic shoots and escapes. Transformation efficiency was 20.7% and the transformants were identified by polymerase chain reaction (PCR) and Southern blot analysis. Moreover, the transgenic lines expressed variable amounts of the *GFP* gene as revealed by real-time PCR analysis. Fifteen transgenic plants flowered 18 months after transfer to the greenhouse and six of them set fruits. GFP expression was also observed in the transgenic flowers and fruits. To test the utility of this system for functional genomics studies, an *Arabidopsis thaliana MAC12.2* gene with the potential to produce seedless fruits was introduced into this genotype, and the traits of the transgenic fruits were characterized. The successful transformation of this perennial woody genotype with extremely short juvenility will allow us to test the function of cloned genes in citrus, the improvement of which is hindered by a long juvenility period.

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B. Tan · D.-L. Li · S.-X. Xu · G.-E. Fan · J. Fan · W.-W. Guo (✉)
National Key Laboratory of Crop Genetic Improvement,
National Center of Crop Molecular Breeding,
Huazhong Agricultural University,
Wuhan 430070, China
e-mail: guoww@mail.hzau.edu.cn

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Abbreviations

AS	acetosyringone
BA	6-benzyladenine
GA_3	gibberellic acid
GFP	green fluorescent protein
IBA	indole butyric acid
KT	kinetin
MT medium	Murashige and Tucker (1969)
NAA	α-napthaleneacetic acid
NPT II	neomycin phosphotransferase II

Introduction

Citrus is a major fruit crop in the world. The market is demanding citrus fruits with improved quality, e.g., seedlessness, easy peeling with attractive size and color, etc. (Deng 2005). The improvement of fruit quality by traditional breeding techniques has been constrained by complex biology of citrus, especially the long juvenile period. Genetic transformation provides the means for adding a single agronomic trait in perennial plant cultivars without altering their phenotype (Deng and Duan 2006). Until now, various genes have been introduced into citrus species and its relatives to improve agronomical traits, including enhancing abiotic stress tolerance (Cervera et al. 2000; Fagoaga et al. 2007) and disease resistance (Ananthakrishnan et al. 2007; Gonzalez-Ramos et al. 2005; Omar et al. 2007; Zanek et al. 2008), shortening the juvenile phase (Duan et al. 2007a; Endo et al.

2005; Peña et al. 2001), rootstock (Gentile et al. 2004), and fruit improvement (Costa et al. 2002; Guo et al. 2005; Koltunow et al. 2000; Li et al. 2002; Wong et al. 2001). Among these, fruit improvement is of particular importance as it can be very attractive for consumers. However, the characteristics of transgenic fruits were not analyzed in these reports due to the long juvenile period.

Juvenile phase in citrus ranges between 2 and 20 years and has been one of the limiting factors for citrus genetic improvement. An alternative to resolve the problem of the long juvenility in genetic improvement of citrus fruits is to use mature tissues as the explants. This method has been proved successful for the transformation of adult sweet orange (Cervera et al. 1998, 2005), sour orange (Ghorbel et al. 2000), and some mandarin genotypes (Cervera et al. 2008). But the procedure has limited application because mature tissues are recalcitrant to *Agrobacterium tumefaciens* infection and transformation. Another method is to use a model genotype to test gene function prior to the application in genetic improvement of commercial citrus fruits. *Arabidopsis* and tobacco as test systems were successfully used to pretest genes to be used in citrus in efforts to obtain seedless fruits (Koltunow et al. 1998). Tomato can also be considered as a model plant to confirm the function of genes cloned from citrus. However, these systems have limitations due to the different genetic background, expression pattern, and regulating mechanism between citrus and the model species. The ideal strategy is to use a short juvenility citrus genotype as model transformation system. The efficient transformation system of West Indian lime (*Citrus aurantifolia*) and kumquat (*Fortunella* sp.), which have a short juvenility period, has been successfully established (Koltunow et al. 2000; Yang et al. 2007).

Precocious trifoliate orange (*Poncirus trifoliata* [L.] Raf), an extremely early flowering mutant from *P. trifoliata*, has a short juvenile period of 1–2 years. The mutant has significant differences from its wild-type: (1) high sprouting rate, short juvenile period, and early fruit set; (2) fast formation of floral bud; (3) flowering and bearing fruits several times in 1 year; (4) high fruit setting; (5) dwarf and short internode (Liang et al. 1999). These special traits make it a useful model plant for the evaluation of gene function related to the characteristics of flowers and fruits in citrus. So far, reporter genes have been widely used in citrus genetic transformation (Almeida et al. 2003; Cervera et al. 2008; Dominguez et al. 2004; Duan et al. 2007b; Ghorbel et al. 1999; Grosser et al. 2000; Guo et al. 2005; Omar and Grosser 2008; Zheng et al. 2006). The green fluorescent protein (GFP) from *Aequorea victoria* as a vital marker has several advantages over other visual reporter genes and has been proved extremely useful for reporting gene expression in transformed plants. In this study, we intend to establish an efficient regeneration and transformation procedure of precocious trifoliate orange by introducing a modified *GFP* gene (*mgfp5*-ER) using stem segments as explants for potential functional genomics studies in citrus specifically and long juvenile perennial woody plants generally. Furthermore, an *Arabidopsis thaliana MAC12.2* gene (Payne et al. 2004) with the potential to produce seedless fruits was introduced into this genotype to test the utility of this system for functional genomics studies.

Materials and methods

Plant materials and culture media

Etiolated seedlings of precocious trifoliate orange were used as explant sources. Well-developed precocious trifoliate orange seeds freshly removed from the fruits were treated in 1 M NaOH for 10 min and rinsed with tap water, followed by a 2% solution of sodium hypochlorite treatment for 15 min. The sterilized seeds were rinsed three times with sterile distilled water. Seed coats were removed and embryos were placed in test tubes containing 25 mL of MT (Murashige and Tucker 1969) medium with 25 g/L sucrose and 7.5 g/L agar and then maintained at 26±2°C in the dark for 5 weeks. Stem segments approximately 1.0-cm-long were collected. Coculture medium (CM) consisted of solid MT with 1.0 mg/L 6-benzyladenine (BA), 0.5 mg/L kinetin (KT), 0.1 mg/L α-napthaleneacetic acid (NAA), 100 mmol/L acetosyringone (AS), and 30 g/L sucrose. The primary shoot regeneration medium (SRM1) consisted of CM (without 100 mmol/L AS) with 50 mg/L kanamycin (Km) and 400 mg/L cefotaxime (Cef). The secondary shoot regeneration medium (SRM2) was mostly identical to the SRM1 with a minor modification that BA concentration was decreased to 0.5 mg/L. Shoot elongation medium (SEM) consisted of solid MT with 0.1 mg/L BA, 0.25 mg/L gibberellic acid (GA_3), 0.1 mg/L indole butyric acid (IBA), and 0.2 mg/L activated charcoal with 200 mg/L Cef. Root regeneration medium (RRM) consisted of solid 1/2 MT with 0.5 mg/L NAA, 0.1 mg/L IBA, and 0.5 g/L activated charcoal with 20 mg/L Km and 200 mg/L Cef. The antibiotics and hormones were filter sterilized and added to autoclaved medium.

Transformation and regeneration

The disarmed *A. tumefaciens* EHA105 harboring the binary plasmid pBIN-*mgfp*5-ER (Haseloff et al. 1997) was used as the vector system for transformation. The pBIN-*mgfp*5-ER T-DNA contained the Nospro-*npt*II-Noster cassette as a selectable marker and the 35Spro-*mgfp*5-ER-35Ster cassette as a vital marker. Three experiments were performed. The

transformation procedure was as described by Duan et al. (2007a, b) with several minor modifications. Briefly, the explants were blotted dry, placed horizontally on CM, and cultured at 21°C in the dark for 3 days after 20 min incubation. Stem segments were soaked in sterile water with 400 mg/L Cef for 5 min and rinsed three times with sterile distilled water. The explants were dried by sterile filter paper and transferred to SRM1. The cultures were maintained in the dark for 2 weeks at 28°C and then transferred to 16 h photoperiod (Peña et al. 1995). After 3 weeks, the explants producing shoots were transferred to the SRM2 medium and excised the latent buds in the explants.

Detection of GFP by fluorescence stereomicroscopy

Before transfer to SRM2, explants were examined in vivo periodically under a fluorescence 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. The red autofluorescence from chlorophyll was not blocked with any interference filter. Photographs were taken using Nikon E4500. Transformation frequency was evaluated as the total number of green fluorescent shoots per total number of *Agrobacterium*-inoculated explants.

PCR and Southern blot analyses

Standard polymerase chain reaction (PCR) techniques were used to detect target gene sequences in leaf samples from regenerated transgenic plants. The *GFP* gene primers were: 5′-AGGACCATGTGGTCTCTCTT-3′ and 5′-TGGCCAA CACTTGTCACTAC-3′, which produced a 500-bp fragment. Reactions were subjected to 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C.

DNA was isolated from leaves of 15 individual PCR-positive lines (lines 1–15) and control plants in the greenhouse according to Cheng et al. (2003). For Southern blot analysis, 15 μg DNA samples were digested with *Hin*dIII, separated on 0.8% (*w*/*v*) agarose gels, blotted to nylon membranes (Hybond-N^+, Amersham), and probed with P^{32}-labeled specific fragment.

Real-time PCR quantification

Total RNA was extracted from the fresh leaves of six individual transgenic lines (lines 1, 3, 7, 10, 13, and 15) and one nontransgenic plant according to Liu et al. (2007). To monitor GFP expression in transgenic plants, a primer set was designed with the Primer Express software (Applied Biosystems, Foster City, CA, USA) for real-time PCR (forward primer: TCCAAGGAGATATAACAATGAAGACTAATC; reverse primer: AATTGGGACAACTCCAGTGAAAA). The control primer set and real-time PCR procedure were as described by Liu et al. (2007). Output data generated by the instrument onboard software Sequence Detector Version 1.3.1 (PE Applied Biosystems) were transferred to a custom-designed Microsoft Excel macro for analysis.

p12.2GUS2-1 construct, Southern blot, and characteristics of transgenic fruits

In order to evaluate the efficiency of the precocious trifoliate orange transformation system for gene function test, a *MAC12.2* gene which was cloned from parthenocarpic *knuckles* mutant of *Arabidopsis* was used. The construction of p12.2GUS2-1 (kindly provided by Dr. Koltunow, Australia, CSIRO) T-DNA contained the Nospro-*npt*II-Noster and the KNUpro-*MAC12.2:GUS*-Noster cassettes as described in detail by Payne et al. (2004).

Southern blot analysis was performed to confirm the stable integration of the *MAC12.2:GUS* fusion gene in the transgenic plants. DNA samples (15 μg) were digested with *Hin*dIII, separated on 0.8% (*w*/*v*) agarose gels, blotted to nylon membranes (Hybond-N^+, Amersham), and probed with P^{32}-labeled specific fragment of the *MAC12.2:GUS* fusion gene by PCR. The *MAC12.2:GUS* fusion gene primers were 5′-CACACACATCCTTCACTCTTC-3′ and 5′-CATTACGCTGCGATGGATCCG-3′.

The fruits of *MAC12.2* lines and control were picked after full maturation and their polar and equatorial diameters were evaluated, respectively. Each fruit was cut transversely to evaluate total seed number.

Results

Optimization of transformation for precocious trifoliate orange

Stem segments from precocious trifoliate orange were cocultivated with *A. tumefaciens* EHA105 carrying the binary vector pBIN-*mgfp*5-ER. After cocultivation in the dark, the explants were examined in vivo under a stereomicroscope with 480 nm excited light. The cut end of the transformed stem segments showed green fluorescence (Fig. 2a). Then, the cultures were transferred to the SRM1 in darkness for 2 weeks and afterward under 16 h photoperiod. On this medium, a pale green and compact callus (Fig. 1a), which showed green fluorescence (Fig. 2b), appeared on the cutting end in about 10 days. And after selection for an additional 3 weeks, multiple adventitious buds differentiated on the surface of these calli through a process of indirect organogenesis. The escapes and non-transformed shoots appeared red due to the autofluorescence of the chlorophyll. The shoots which did not show

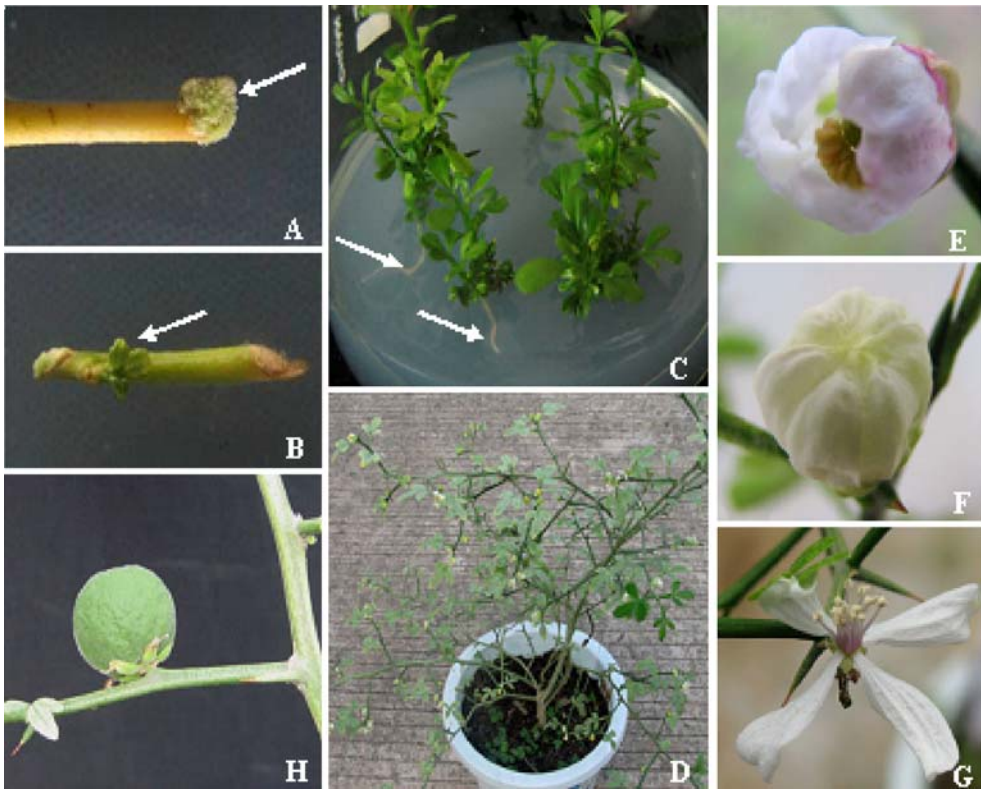

Fig. 1 Production of transformed precocious trifoliate plants. **a** Regenerated callus from the cut end formed after about 10 days in SRM1 (*white arrow*); **b** regenerated latent bud from the leaf axil of stem segment (*white arrow*); **c** transgenic shoots self-rooted in the SEM (*white arrow*); **d** transgenic plants flowering after 18 months in the greenhouse; **e** normal flower; **f** abnormal flower; **g** pistil-abortion flower; **h** transgenic plant bearing fruit

green fluorescence were excised. The explants with shoots showing bright green fluorescence (Fig. 2c) were transferred to SRM2 for subsequent molecular analyses. Meanwhile, the latent buds from the leaf axil of the explants (Fig. 1b) were excised to avoid competition of nutrition with the shoots from the cut end. It is a pivotal step in the transformation of precocious trifoliate orange because of its short internodes.

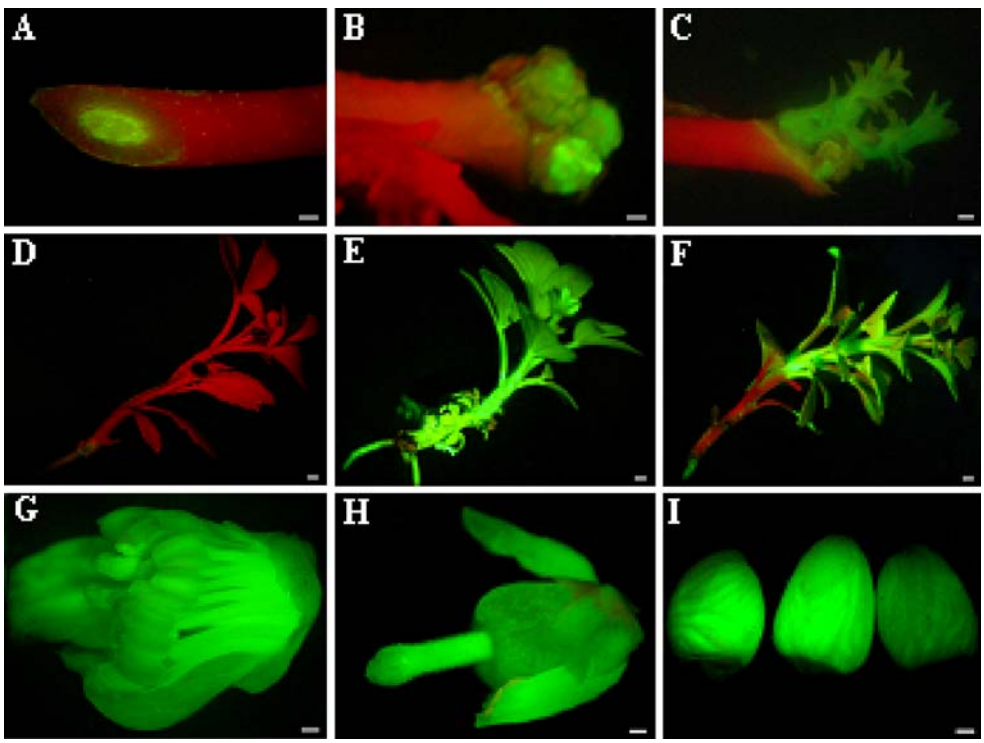

Fig. 2 GFP expression in stem segments cut ends, shoots, plantlets, flower, fruitlet, and seeds (**a**–**j**). Green fluorescence in transformed stem segments after cocultivation (**a**) and cultured on SRM1 for 10 days (**b**); green fluorescence in transformed shoots (**c**); red fluorescence in nontransformed plantlet (**d**); green fluorescence in different transformed plantlets (**e**, **f**); green fluorescence in flower (**g**), fruitlet (**h**), and seeds (**i**) of transgenic plants. Scale bars=1.0 mm

Defining regeneration efficiency as the percentage of stem segments with shoots obtained per stem segments inoculated and transformation efficiency as the percentage of GFP-positive shoots obtained per stem segments infected, the efficiency of regeneration and transformation was 91.8% and 20.7%, respectively (Table 1). GFP-expressing shoots (longer than 0.5 cm) were physically separated in vitro and elongated on SEM. Interestingly, several GFP-expressing shoots were rooted automatically after transfer into SEM for 3 weeks (Fig. 1c). Transgenic plantlets were recovered by rooting on RRM. When rooted shoots were approximately 3–4 cm long and had two to three well-developed roots, they were enclosed in a plastic bag, watered, and monitored. Totally, over 100 transgenic precocious trifoliate orange plantlets were obtained.

The transgenic plantlets were successfully acclimatized and hardened. Twenty-five well-developed plantlets were repotted to the greenhouse for subsequent analyses. Fifteen transgenic plants flowered 18 months after transfer to the greenhouse and showed normal growth (Fig. 1d). Normal flower (Fig. 1e), abnormal flower (petal is closed in the beginning of flowering and opened in the full bloom, as shown in Fig. 1f), and pistil-abortion flower (Fig. 1g) were observed among the 15 transgenic plants with flowers. Among the three flower phenotypes, normal flower is the main phenotype and was also observed in the control plants. Six of 25 transgenic plants were bearing fruits (Fig. 1h). Using the procedure for genetic transformation, plants with fruits were obtained within 2 years, significantly faster than with other citrus cultivars.

Different patterns of GFP expression in transgenic plantlets under the fluorescence stereomicroscope

About 80–100 days after cocultivation with *Agrobacterium*, the plantlets were examined in vivo under a stereomicroscope with 480 nm excited blue light. Different phenotypes over the expression of the *GFP* gene in regenerated transgenic plantlets were observed. Some transformants appeared full green fluorescence (Fig. 2e), others showed partial green fluorescence (Fig. 2f), and nontransformed plantlets showed red fluorescence (Fig. 2d). The fluorescence intensity varied among different parts of the plantlets, being higher in young tissues (shoot apices, young leaves) than in old ones (Fig. 2f). The roots of all transformants showed bright green fluorescence. In old tissues, low metabolism and chlorophyll accumulation partially masked the green fluorescence provided by GFP (Ghorbel et al. 1999).

The stability of GFP expression was maintained in the transgenic precocious trifoliate orange plants under greenhouse condition, in not only vegetative tissues but also reproductive organs, such as flowers (Fig. 2g) and resulting fruitlets (Fig. 2h) and seeds (Fig. 2i). GFP analysis seems to be a good choice for rapid visual selection of transformed tissues, but further and final confirmation of transformation by PCR and Southern blot is required.

Molecular analyses of the GFP-expressing plants

Putative transgenic plantlets were first observed to test if green fluorescence exists and then analyzed by PCR to verify the presence of the *GFP* gene in their genome. The 500-bp *GFP* gene fragment was amplified from 15 randomly selected plants (Fig. 3a). No amplificant was detected in the DNA samples from nontransgenic regenerated control plants (Fig. 3a, lane C), which emitted red autofluorescence under blue light (Fig. 2d).

Southern blot analyses were performed to confirm the stable integration of the *GFP* gene cassette. The *GFP* gene fragment (500 bp) was used as a probe to reveal the presence of the transgene. Different integration patterns in the transgenic plants from one to three copies at different loci were observed when *Hin*dIII was used. As shown in Fig. 3b, all 15 GFP-expressing plants contained the target gene (lanes 1–15). No hybridization signal was detected in a nontransformed control plant (lane C).

Six individual transgenic lines with full green fluorescence expression and varied copy numbers as confirmed by

Table 1 Regeneration frequency and transformation frequency of shoots from precocious trifoliate orange stem segments inoculated with *A. tumefaciens*

Experiments	Stem segments evaluated	Stem segments producing shoots	Total no. of shoots	GFP-positive shoots	Transformation efficiency (%)
1	194	179 (94.8)	577	41	21.1
2	190	173 (91.1)	450	37	19.5
3	221	198 (89.6)	551	45	20.4
Mean	–	– (91.8)	–	–	20.7

Regeneration frequency was calculated dividing the number of stem segments producing shoots×100 by the total number of evaluated stem segments. Transformation efficiency was calculated dividing the number of GFP-positive shoots×100 by the total number of evaluated stem segments.

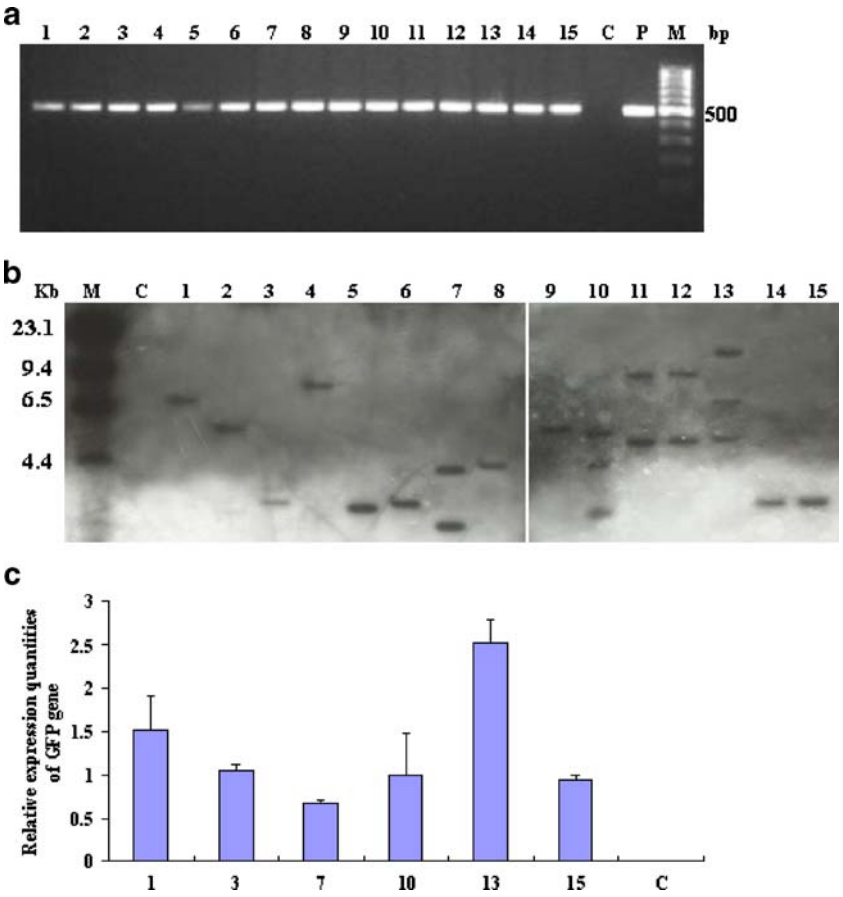

Fig. 3 Molecular analyses of GFP-expressing plants. **a** PCR detection of the *GFP* gene in putative GFP-expressing plants. *Lanes 1–15* GFP-expressing plants, *lane C* nontransformed plant, *lane P* plasmid pBIN-*mgfp5*-ER, *lane M* 100 bp DNA ladder. **b** Southern blot analysis of the *GFP* gene from PCR-positive plants. Genomic DNA was digested with *Hin*dIII, which has only one enzyme site in the plasmid. *Lanes 1–15* PCR-positive plants (lines 1–15), *C* nontransformed plant, *M* λ DNA/*Hin*dIII molecular weight marker; molecular weights are indicated on the *left*. **c** Real-time PCR analysis of the relative expression levels of the *GFP* gene in transgenic precocious trifoliate orange lines. *1*, *3*, *7*, *10*, *13*, and *15* transgenic lines of precocious trifoliate orange, *C* nontransgenic plant

Southern blot were selected for real-time PCR analysis. The results indicated that the GFP transgenes expressed in the leaves of the transgenic precocious trifoliate orange plants with variable transcript accumulation among individual transgenic lines. As shown in Fig. 3c, the GFP expression level of line 13 (three copies) was significantly higher than that of line 10 (three copies) and that of lines 1, 3, and 15 (one copy) were obviously different and slightly higher than line 7 (two copies). This result suggested that there was no correlation between copy number and transgene expression.

Production of transgenic lines with *MAC12.2* gene

A total of 35 plant lines were obtained from cocultivated etiolated stem segments. Control and 35 putative transgenic plants were repotted into the greenhouse for subsequent analyses. Southern blot analysis performed on selected transgenic plants using a gene-specific probe confirmed the presence of *MAC12.2* and *GUS* sequence in precocious trifoliate orange genomes (Fig. 4). The hybridized bands indicated the integration of one to four copies of the target gene in transgenic plants (lanes 1–10). Thirty-three transgenic plants flowered 15 months after being transferred to the greenhouse and nine of them set fruitlets. The nontransformed plants also flowered after being transferred to the greenhouse and set fruitlets.

Five ripe fruits from five different transgenic lines (P8, P5, P2, P16, and P6-2) were eventually obtained, as each transgenic line yielded only one fruit due to severe physiological drop. Fruit characteristics of five lines were analyzed and compared to the control. The fruit size of transgenic lines was almost the same as that of the control

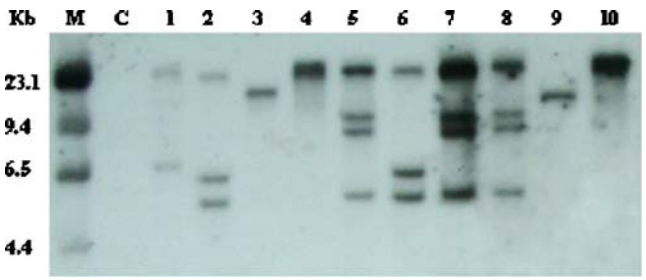

Fig. 4 Southern blot analysis of the *MAC12.2:GUS* fusion gene from PCR-positive plants. Genomic DNA was digested with *Hin*dIII, which has only one restriction site in the plasmid. *Lanes 1–10* PCR-positive plants (lines 1–10), *C* nontransformed plant, *M* λ DNA/*Hin*dIII molecular weight marker; molecular weights are indicated on the *left*

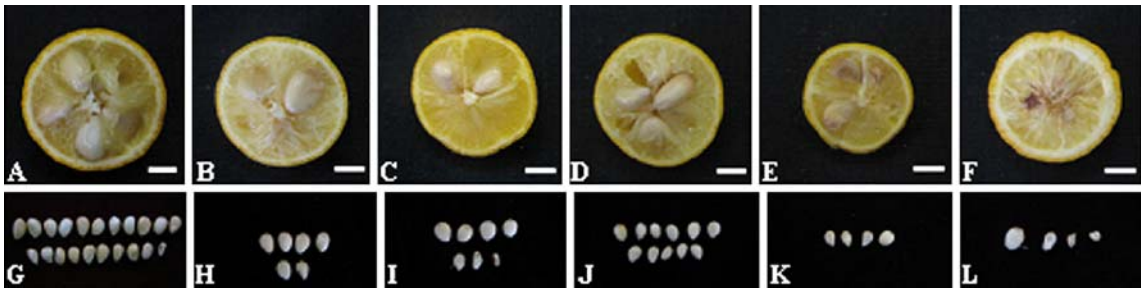

Fig. 5 Characteristics of *MAC12.2* transgenic fruits. **a** Fruit of control; **b–f** fruits of different transgenic lines (P8, P5, P2, P16, and P6-2); **g** seeds of the control fruit; **h–l** seeds of the transgenic fruits derived from different transgenic lines (P8, P5, P2, P16, and P6-2). Scale bars=5.0 mm

except for P16 (Fig. 5a–f; Table 2). Fruits of the control were very seedy and have 22.8 seeds on average (Fig. 5g), while the seed number of transgenic lines was much less and possessed only 11, seven, six, or as few as four seeds per fruit (Fig. 5h–l; Table 2). Moreover, some seeds in the transgenic lines were flattened and reduced in size.

Discussion

This study described successful *Agrobacterium*-mediated transformation of the precocious trifoliate orange with the plasmid pBIN-*mgfp5*-ER, encoding for the *GFP* gene. Over 100 transgenic lines containing the *GFP* gene were obtained and 15 transgenic plants were analyzed by PCR and Southern blot analyses. The GFP expression was also observed in six individual transgenic lines by real-time PCR analysis.

One advantage of precocious trifoliate orange transformation is the high regeneration efficiency (91.8%) and transformation efficiency (20.7%). In the indirect regeneration pathway, BA is essential for callus development and bud formation (Moreira-Dias et al. 2000). And as Peña et al. (2003) reported, cambial cells on the cut surfaces of the explants under specific culture conditions producing callus cells are the major target for *Agrobacterium* action. When explants were transferred to SRM1 (BA 1.0 mg/L) in darkness for 2 weeks, the regeneration process was similar to that described by Peña et al. (2004) and callus was observed 10 days later. SRM1 was successful in promoting the regeneration of more transgenic adventitious buds or shoots.

Table 2 Fruit characteristics of *MAC12.2* transgenic precocious trifoliate orange plants

Lines	Seed number/fruit	Average diameter (cm)	
		Polar	Equatorial
P8	6.0 (n=1)	1.92	1.94
P5	7.0 (n=1)	1.89	2.02
P2	11.0 (n=1)	2.06	2.04
P16	4.0 (n=1)	1.44	1.55
P6-2	4.0 (n=1)	2.04	2.01
Control	21.7 (17–25, n=3)	2.27	2.26

The numbers in parenthesis specify the range of number of seeds per fruit and n=number of fruits

Transformation efficiency depends considerably on plant genotype. In comparison with published data, the efficiency of transformation reported in the present investigation is much higher than that of "Xuegan" (4.3%) (Yu et al. 2002), Ridge pineapple (2%) (Gutiérrez-E et al. 1997), and Bingtang sweet orange (10%) (Duan et al. 2007b), lower than that of Valencia (23.8%) (Boscariol et al. 2003) and similar to the Carrizo citrange (20.6%) (Peña et al. 1995). Previous studies in trifoliate orange (Endo et al. 2005; Iwanami et al. 2004; Kaneyoshi et al. 1994; Wong et al. 2001) were also very successful. But transformation efficiency is different among previous reports. Kaneyoshi et al. (1994) has shown that the frequency of shoot formation was 35.6% in the segments inoculated with pBI121 and 44.6% in pBI101-O12-p1, and histochemical GUS assay showed that 55.4% (pBI121) and 87.7% (pBI101-12-p1) of shoots expressed the GUS gene. In the present investigation, the ability of shoot regeneration in precocious trifoliate orange mutant is much higher than that of the wild trifoliate orange, while transformation efficiency is lower in the mutant. However, transformation efficiency of the wild-type was only 2.1–4.6% in another report (Iwanami et al. 2004). These differences were probably due to the factors that affect the transformation and regeneration process. However, the protocol reported in the present investigation is suitable for the transformation system of fruit-specific gene function tests.

The primary advantage of transforming precocious trifoliate orange is that transgenic plants could flower and set fruits within 1–2 years. Production of transgenic plants is fundamental to investigate plant gene function as well as to improve agronomic traits in citrus. However, the long juvenility phase is a serious limiting factor in genetic modification, especially for developmental evaluation of

flowers and fruits. Direct transformation of mature tissue is ideal for early evaluation of genetically modified characteristics. Cervera et al. (1998, 2005) produced sweet orange transformants that flowered and set fruits in about 1 year after in vitro regeneration by using this method. However, direct transformation of mature material is not easily achieved because of low transformation competence and regeneration potential of mature tissues (Cervera et al. 2008). Koltunow et al. (2000) introduced the *SDSL-1* gene, a decreased seed number gene, into West Indian lime which had a shorter juvenile period of 2–3 years and ultimately obtained fruits with reduced seeds. But the transformation efficiency was very low (3.2%). In addition, Yang et al. (2007) established the transformation procedure of kumquat, which is very close to *Citrus* and had a short juvenile period of 2 years. The final transformation efficiency was only 3.6%. Compared with West Indian lime and kumquat, transformation efficiency in precocious trifoliate orange could be up to 20.7%. Furthermore, transgenic precocious trifoliate orange plants could flower and set fruits within 18 months after transfer to the greenhouse. In other fruit crops, Petri et al. (2008) established a high transformation efficiency system in plum which could be a new tool for functional genomics studies in *Prunus* spp.

In summary, we showed that the transformation procedure as presented herein is a simple and highly productive system for developing transgenic precocious trifoliate orange plants. Establishment of an efficient transformation system is important for functional genomics analyses. Based on the model precocious trifoliate orange transformation system, the *Arabidopsis MAC12.2* gene, which has the potential to induce fruit seedlessness in precocious trifoliate orange, was introduced to test the gene function. Thirty-five independent transgenic lines were obtained and five transgenic lines set fruits. The characteristics of the transgenic fruits were analyzed, which indicated that they have significantly less seeds than the control.

The system is ideal for the study of gene function. We expect to screen more functional genes relating to economically important traits which could be further introduced into commercial citrus cultivars for creating new transgenic lines with improved fruit quality.

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